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Structural and organisational conditions for the appearance of a functionally integrated organisation in the transition from prokaryotic to eukaryotic cell

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Dans ses écrits un sage Italien

Dit que le mieux est l'ennemi du bien

Voltaire, La Béguele (1772)

To my teachers

CONTENTS

INTRODUCTION

The concept of functional (or physiological) integration is at the core of most of definitions of organism and biological individual, thus being explanatorily relevant to both biology and philosophy of biology. However, it suffers from two main related problems: first, it is a very general notion encompassing any causal interdependence of functions, thus being unsuitable for characterising biological organisations as physiological units; secondly, it lacks a *theoretical framework* to understand this concept. This PhD thesis aims to investigate the relationship between functional integration and biological individuality by studying the nature and the role of physiological integration in one of the major evolutionary transitions: the origin of the eukaryotic cell from the prokaryotic one. In this introductory section, I am going to review how functional integration is currently employed in biology and philosophy, underlining the limitations and open questions of such a concept. Then, I present the scope and the methodology of this thesis and I conclude by summarising the content and the main findings of each of the chapters.

FUNCTIONAL INTEGRATION IN BIOLOGY AND PHILOSOPHY: AN INTRODUCTORY CRITICAL REVIEW

Most of the definitions of functional (or physiological) integration provided by textbooks in cell biology, (human) physiology and pathophysiology intuitively assume that a) functional integration is nothing but a *causal interdependence* of biological functions; and b) this causal interdependence explains the *physiology* (as well as the pathophysiology) of organisms. Such a characterisation makes functional integration an *umbrella term* encompassing *any* form of functional coordination and functional interdependence in *whatever* biological system. In philosophy of biology, functional integration is at the core of any definition of *organism*, since the organism appears as a *physiological unit* exhibiting a *coordinated behaviour* and *integrated systemic capacities*. Both in biomedical sciences and philosophy, these characterisations of functional integration are pretty general and loose and they do not often examine which physiological dimensions make an organism a functionally integrated whole. As a result, current definitions of functional integration do not provide a criterion (or norm) to distinguish different biological organisations on the basis of their

internal physiological integration. Let me examine in this section how functional integration is used and explained in biomedical sciences and philosophy of biology.

The concept of functional integration is central to medicine, as physiology and pathophysiology are grounded on the idea that human beings (and in general multicellular organisms) are physiologically integrated wholes. Indeed, textbooks in medical physiology explain the physiology of the whole organism in terms of the functional integration *within* an organ system and *among* different organ systems (Hall 2016). This physiological integration can be lost during a pathological state: for example, the heart failure is characterised by the loss of the *normal* physiological integration between heart contraction, cardiac output, and their regulation made by the endocrine system and the autonomic nervous system. However, it is worth noting that pathological states may lead to a new kind of functional integration that takes the form of *physiological compensations*: for example, the heart failure determines a physiological compensation of the functions performed by the heart, the kidney, the endocrine system, and the autonomic nervous system (Jameson et al. 2018). This new (*pathologic*) integration is what physicians usually call "disease".

However, the definition of functional integration in medicine is a very complex issue, because it entails an in-depth analysis of how a multicellular organism (like plants, animals, most of fungi, and some algae) is functionally integrated. In this thesis, I prefer to follow a bottom-up strategy by addressing the issue of functional integration in minimal, yet paradigmatic and sufficiently complex, forms of life. Accordingly, this thesis will focus on the concept of functional integration at the unicellular level and on its role in one of the earlier evolutionary transitions (i.e. eukaryogenesis), thus addressing disciplines such as molecular biology, biochemistry, genetics, cell biology, and evolutionary cell biology.

In classical molecular biology, genetic and phenotypic aspects of the cell were explained by studying the circularity between DNA replication, transcription, and translation (Crick 1958). The central dogma of molecular biology was a reductionist stance that did not leave much room for the problem of how a cell is physiologically integrated. By contrast, current approaches in molecular biology (notably in systems biology) tend to study genetic mechanisms in terms of networks that interact with other cellular networks (e.g. the metabolic network or the signalling network) (Medina 2013). In this thesis, I consider the link between DNA replication, transcription, and translation in the light of more systemic conditions of the cell (e.g. metabolic needs, developmental and reproductive processes, sensorimotor capacities) that reflect a functionally integrated organisation between the molecular levels and systemic cellular processes. The functional integration between

molecular functions and cellular systemic conditions also entails the study of genetic functions in the context of their regulation (i.e. the gene regulatory pathways studied by genetics) and their relationship with metabolic pathways (studied by biochemistry).

Molecular biology, genetics, and biochemistry are fundamental parts of cell biology, which studies the whole of cellular processes: *metabolism*, intra- and inter-cellular *communication*, and the *cell cycle* (including growth, DNA replication, transcription and translation, and reproduction)[1]. These processes are often defined as integrated for three basic reasons (Alberts et al. 2015): first, metabolism provides the cell with the energy for all its activities, thus sustaining each phase of the cell cycle; secondly, both metabolism and cell cycle hinge on a huge number of intra- and inter-cellular signals that collectively constitute cellular communication; finally, each cell produces chemical signals that respond to the internal conditions (e.g. metabolic and developmental) of the cell, so that the cell-cell communication is related to the metabolic and developmental conditions of each cell. Nevertheless, the concept of functional integration is not conceptualised (and problematised) in cell biology, thus not explaining how a cell is a functionally integrated whole. What is at stake is, first, which are the *cellular processes* that represent the main actors of the integration of a cell; secondly, how they are *concretely integrated* and how we can describe them in a unified framework. Thirdly, whether or not it is possible to find similarities and differences in the kind and degree of functional integration in *unicellular organisms*. These three points represent the core of the theoretical questions of this thesis, as I will show a bit later.

Another aspect that is worthy of note is the role played by functional integration in *evolutionary biology*. Apparently, this field seems to have nothing to do with physiological integration, because the main object of evolutionary biology is the study of evolutionary transitions through the help of (comparative) phylogenetics, computational phylogenetics, comparative anatomy, etc. In fact, *evolutionary transitions* can be interpreted as *global modifications* in the *functional integration* of a certain biological organisation. Indeed, what evolves is not only the genes and the phenotypic traits, but also the functions performed by these traits and the way in which they are integrated to perform systemic properties (Margulis 1970; Buss 1987; Bonner 1988; Maynard-Smith and Szathmáry 1995).

[1] Actually, the concept of metabolism, broadly understood, encompasses all these aspects, since metabolism is the ongoing and cyclic set of (both material and energetic) processes of (self) construction, reconstruction, repair, growth and re-production of the cell (Morowitz 1968; 1992; Moreno and Ruiz-Mirazo 1999; Ruiz-Mirazo et al. 2004; Barandiaran and Moreno 2008). Even the interactive operations of the cell depend ultimately on the metabolic organisation. Hence, to achieve all these processes, the cell needs to viably organise a host of local functions.

As an example, we can consider the transition from prokaryotic to eukaryotic cell: the global reorganisation of the proto-eukaryotic cell entailed a radical morphological and functional change that included the appearance of the organelles, the increase in cell size, the achievement of new functions or the transformation of previous ones, thus leading to a completely new *functional integrated organisation*.

After having addressed how functional integration is currently employed in biological and medical sciences, let me review how this notion is used in *philosophy* and which domains are related to it. I introduce two important concepts (i.e. biological function and biological mechanism) that are the *conceptual tools* of the thesis; then, I present the *philosophical debate* within which the problem of functional integration will be addressed and on which this thesis seeks to provide a new theoretical perspective.

The concept of functional integration, as the name suggests, makes explicit reference to the term "function", which has sparked off a lively debate in philosophy of biology about the nature of biological functions. We can identify three main positions: the *etiological* approach, the *dispositional* view, and the *systemic* account.

The *etiological* approach explains the nature of biological function in terms of their evolutionary history (Wright 1973; Millikan 1984, 1989; Neander 1991; Griffiths 1993). The *dispositional* account (Bigelow and Pargetter 1987) considers functions as the disposition of a biological trait to perform a certain activity. While the etiological account is *backward-looking* and explains functions in the light of their evolutionary history, the dispositional approach is *forward-looking* and defines functions in terms of their future effects, so as to increase the overall fitness of an organism (Mitchell 2003). A third account is the Cummins' (1975) one that has stressed the importance of the current *systemic* role (i.e. the physiological role) played by a function within a biological system. Finally, a synthesis between the etiological and systemic account is provided by the *organisational* view of functions (Mossio et al. 2009), which defines biological functions as causal relations subject to closure (i.e. mutual dependence) in living systems. The origin of biological functions is explained in evolutionary terms.

Whereas the first two accounts leave no room for the concept of functional integration, the systemic and organisational accounts stress the theoretical importance of the functional interdependence of biological functions in the systemic context of a living being. For this reason, although I do not analyse in detail the debate on functions in the chapters of this thesis, I implicitly

adopt an organisational view of biological functions, considering them in the context of their mutual interdependence in the physiology of an organism.

A significant contribution to the understanding of how a material structure can achieve a form of functional integration has been made by the new-mechanistic [2] debate on the concepts of mechanism and machine. This theoretical account, originated in the 90's (Bechtel and Richardson 1993), interprets mechanisms as "entities and activities organised such that they are productive of regular changes from start or set-up to finish or termination conditions" (Machamer et al. 2000, p. 3). Interestingly, a mechanism is understood as an organisation of functional parts that, depending on how they are arranged, achieve a new integrated functional result (Wimsatt 1986; Bechtel and Abrahamsen 2005; Levy 2014; Militello and Moreno 2018). The new-mechanistic debate has mostly focused on the mechanisms of cell biology and molecular biology (Bechtel and Richardson 2010; Machamer et al. 2000; Darden 2008, 2009) and neuroscience (Craver 2007) and has stressed two fundamental levels of mechanistic integration: first, a mechanism is an *integrated process* inasmuch as it is the result of the orchestrating functioning of its component operations (Bechtel and Abrahamsen 2005); secondly, mechanisms are *integrated among each other*, in the sense that mechanisms are parts of other mechanisms and are composed of other mechanisms, giving rise to distinct mechanistic levels (here, integration is understood in the philosophical sense of "constitution" or "parts-whole relationship") (Craver 2001). As such, the concept of functional integration is present in the mechanistic vocabulary, but always in an implicit way, *without* a *clear conceptualisation* of this notion in the mechanistic explanations of life sciences.

Biological functions and mechanisms are *regulated* so as to meet the physiological needs of an organism. Following the cybernetic tradition (Wiener 1948), the biological literature often identifies biological regulation with feedback loops (Heinrich and Schuster 1996; Wolkenhauer and Mesarovic 2005; Tsokolov 2010; Konieczny et al. 2014), which are circuits in which an output returns to its input either by opposing it (negative feedback) or by enhancing it (positive feedback). From an organisational and organicist perspective [3], biological regulation is performed by *regulatory subsystems* that are endogenously synthesised and functionally decoupled from the functions and

[2] In the philosophical literature, we often find the label "new-mechanistic" to distinguish the contemporary reflection on (biological) mechanisms from the classical mechanistic philosophy that dates back to the philosophical thinking of the XVII century (e.g. Descartes', Hobbes', and Newton's philosophies).

[3] Both the organisational and the organicist views share the idea that biological functions and properties need to be studied in the systemic context provided by a biological organisation or an organism. In this sense, these theoretical frameworks are intrinsically holistic and systemic. For recent reviews on the history of organicism, I refer the reader to Gilbert and Sarkar (2000), Etxeberria and Umerez (2006), Wolfe (2014).

mechanisms that they control (Bich et al. 2016, 2020), thus leading to a *hierarchical regulation* that characterises living beings (Pattee 1991; Winning and Bechtel 2018).

Functional integration requires *biological regulation*, inasmuch as "all current living systems employ forms of hierarchical control to modulate the relations between their constitutive subsystems in such a way that they are capable to coordinate their basic functions and achieve integration" (Bich 2018, p. 138). The ontological status of functional integration, as I will argue all along the thesis, is intimately connected with the problem of biological regulation, inasmuch as the different functional dimensions of an organism (e.g. metabolic, developmental, reproductive, sensorimotor processes) *control* and *regulate one another*, thus fostering (hierarchical) *dependency relationships* among them.

In the biological and philosophical literature, the concept of functional integration is not separated from that of *organism*, basically because an organism is usually considered as an "integrated system of interdependent structures and functions" (Lwoff 1966). In many theoretical accounts, the interdependence of the parts of an organism entails "functional and structural cohesion" (Collier 2004, p. 13), the "maintenance of boundary between individual and environment" (Godfrey-Smith 2011, p. 71), "high cooperation and very low conflict" (Queller and Strassmann 2009, p. 3144), and being "capable of reproduction, so has a life cycle, and whose parts work (mainly) for the good of the whole" (Okasha 2011, p. 59)[4]. By these criteria, unicellular forms of life (i.e. bacteria, archaea, and unicellular eukaryotes) and eukaryotic multicellular systems are considered as paradigmatic organisms. In most of the above-mentioned definitions, the main explanatory purpose is to define an organism and, in this context, functional integration is an *explanatory tool* for characterising an organism, rather than being the object itself of a theoretical investigation.

Let me address now the issue of *individuality*. According to some authors, the concept of biological individual can be equated to that of organism (Queller and Strassmann 2009; Folse and Roughgarden 2010; Clarke 2010). However, increasing criticisms have been levelled at the ontological status of collective life forms (e.g. symbiotic associations, colonies of bacteria or of insects) that exhibit very specific forms of collective (integrated) behaviour, despite *not* having the features of functional integration that are typical of an organism (e.g. clear-cut boundaries, a cohesive structure, system-level reproduction). Accordingly, some philosophers (Dupré and O'Malley 2009; Nicholson 2014; Pradeu 2016) have underlined that the category of biological

[4] Similar criteria for the relationship between functional integration and organismality can also be found in Wolvekamp (1966); Bock (1989); Sober (1991); Lewontin (2000); Ruiz-Mirazo et al. (2000); Godfrey-Smith (2013).

individual is *superordinated* to that of organism, thus raising the problem of its ontological status and, more importantly, the place and the role of *functional integration* in the definition of a biological individual. For this reason, the relationship between functional integration and biological individuality is the very core of this thesis and I shall summarise now the most important aspects of the large (and very sophisticated) debate on biological individuality and its connection to functional integration.

According to Lidgard and Nyhart (2017), the difficulty of defining biological individuals was already encountered by Thomas Huxley (1852) and Herbert Spencer (1864) in the 19th century. Over the last two centuries, 24 different definitional criteria for biological individuality were provided, ranging from propagation to life cycles and from causal integration to fitness maximisation (Lidgard and Nyhart 2017, pp. 19-21), thus giving rise to a multifaceted landscape of concepts and theories. These definitional criteria can be divided into five main categories that could eventually overlap: first, *developmental* and *reproductive individuality*; secondly, *evolutionary individuality*; thirdly, *genetic individuality*; fourthly, *structural individuality*; finally, *functional* (physiological) *individuality*.

The importance of *reproduction* in biological organisations was firstly conceptualised by Dawkins (1976, 1982), who employed the term "replicator" to designate any *individual entity* (i.e. a unit of replication) capable of transmitting its biological features to descendants. According to Dawkins, genes are the most paradigmatic case of replicators; yet, more inclusive entities than genes could also work as replicators, thus leaving open the question of which biological entities are replicators. Griffiths and Gray (1994) argued that a unit of replication entails *developmental* systems and processes, because "the developmental process or life cycle is a series of developmental events which forms a unit of repetition in a lineage. […] The developmental system is the structured set of resources from which the life cycle is reconstructed in each generation" (Griffiths and Gray 1994, p. 304).

In the same vein, Griesemer (2000, 2016) underlined the *continuity* between *development* and *reproduction*, arguing that a biological individual is characterised by the achievement of the capacity to reproduce through development and to generate entities (i.e. the offspring) that proliferate by means of reproduction (Griesemer 2000, p. S362). The intimate relationship between development and reproduction has led philosophers and biologists (de Sousa 2005; Wilson 2005; Rainey and Kerr 2010) to emphasise the importance of life cycles (i.e. a set of processes for development and reproduction that takes the form of a cycle) for explaining the nature of biological individuals. Life cycles pose two interesting questions about the *functional integration* of an individual: first, how

development and reproduction are *functionally interdependent* in terms of biological mechanisms; secondly, how development and reproduction depend on *global physiological capacities* (e.g. metabolism) of an individual and which functional contribution they make to the physiology of an individual.

Another important criterion to define individuality is the *evolutionary* one, which considers an individual as unit that that can evolve across time under the action of natural selection, thus being a unit (or level) of selection. The idea of evolutionary units (or *Darwinian individuals* in Godfrey-Smith's (2009, 2013) terms) was firstly introduced by Lewontin (1970), who argued that a *level of selection* (i.e. an organism or a set of organisms upon which natural selection acts) is characterised by *variation* (i.e. the capacity to undergo genetic and phenotypic changes), *heritability* (i.e. the transmission of genetic and phenotypic features to the offspring), and a *differential fitness* produced by variation. Some authors have pointed out that differential fitness is the outcome of *adaptation*, which is the capacity of an individual to adapt to a specific environment (niche) in order to produce a maximisation of the fitness (Gardner and Grafen 2009; Folse and Roughgarden 2010; West et al. 2015).

Lewontin's criteria has encouraged the contemporary debate on evolutionary individuality where it has been argued that several (non-organismic) biological organisations, such as species (Ghiselin 1974; Gould and Lloyd 1999), holobionts[5] (Bordenstein and Theis 2015), and colonies of bacteria (Ereshefsky and Pedroso 2013), are levels of selection. Apparently, evolutionary individuality does not entail functional integration; in fact, as Militello et al. (2020) have recently argued, a necessary condition for an individual to be an evolutionary unit is the ability to reproduce as a whole (i.e. a system-level reproduction) in such a way as to recreate the same biological organisation[6]. This capacity requires that a number of physiological capacities of an evolutionary individual are *integrated* among one another.

A third criterion for defining a biological individual is based on genetic aspects that collectively make an individual a *genetic unit*. Santelices (1999) has proposed that there are degrees of individuality that can be characterised in terms of *genetic uniqueness* (i.e. the presence or absence

[5] A holobiont is a kind of symbiotic relationship between a multicellular eukaryote (e.g. plants or animals) -the host- and a variety of microorganisms (viruses, bacteria, archaea, unicellular fungi and protists) that live within it.

[6] This means that there is a continuity between the developmental and evolutionary dimension of a biological individual: if an individual is able to grow and reproduce, it should also generate an offspring that can evolve across time under the action of natural selection, thus being a unit of selection.

of a unique genome) and *homogeneity* (i.e. the number of genetic changes during ontogeny)[7]. Some biologists (Doolittle 2013; Martins and Locke 2015) have pointed out that the absence of genetic uniqueness and genetic homogeneity can enhance a very *coordinated* behaviour and *collective* functions, as it occurs in colonies of bacteria: they share a high number of genes through lateral gene transfer[8] and they often exhibit highly coordinated behaviour, like in the case of biofilms. These theoretical accounts suggest that the *functional integration* of an individual also depends on a specific *genetic configuration* that in turn hinges on a very specific *biological organisation* that could constrain the behaviour of genes and genetic expression.

The philosophical literature has highlighted a fourth essential aspect of individuality: the *functional integration* of an individual depends on how the *parts* and the *whole* are related one to another. Some authors (Zylstra 1992; Korn 2002) have underlined that individuals exhibit a *hierarchy* of entities, ranging from atoms to species, that determine levels of organisation that are *mutually related*: each level of organisation contributes to the constitution of higher levels; in turn, higher levels constrain the functions of lower levels (Korn 2002). It has also been emphasised that the parts of an individual exhibit *clear spatial boundaries* and work in a *temporally coordinated manner* with the other parts (Haber 2013; Hamilton and Fewell 2013). Queller and Strassmann (2009) have argued that the parts of an individual must exhibit a high degree of *cooperation* and a low level of *conflict* in such a way that the individual, as a whole, can exhibit a functionally integrated organisation. A fourth structural aspect, stressed by Godfrey-Smith (2009, 2013) and Folse and Roughgarden (2010), is that the parts of an individual must exhibit some *functional differentiation* in order to make the overall system functionally viable.

A fifth dimension for defining a biological individual is represented by the physiological capacities that make a biological organisation a *functional unit*. Dupré and O'Malley (2009) stressed the importance of metabolism, which is "typically a collaborative activity" (Dupré and O'Malley 2009, p. 13) that involves different organisms in symbiotic organisations. The authors suggest that metabolic relationships among different organisms leave open the possibility of characterising biological individuals not in terms of organismic features, but rather in terms of metabolic relationships. Pradeu (2010, 2016) has argued that the immune system plays a fundamental role in constituting

[7] Some individuals (e.g. some plants and algae) may not have a unique genome because of "a variable number of replicas through clonal propagation" (Santelices 1999, p. 152), whereas others (e.g. tumour cells) may have genetic uniqueness, but not genetic homogeneity, because their "genotypes change markedly during ontogeny" (Santelices 1999, p. 153).
[8] Lateral (or horizontal) gene transfer is the passage of genetic material from an organism to another. It distinguishes from vertical gene transfer, which is the transfer of genetic material from parent to offspring through reproduction.

the individual as a functional unit, as immune interactions "are systemic (as opposed to local) and […] responsible for the acceptance or rejection of constituents in the organism" (Pradeu 2010, p. 258). In spite of stressing the importance of two fundamental aspects of physiological individuality, the above-mentioned accounts focus on *single* functional dimensions without considering them in a more systemic and organisational context.

Conversely, a more systemic approach to physiological individuality is provided by a number of organisational accounts that interpret it in terms of *biological autonomy*. Being autonomous does not mean being independent from the surroundings, but rather that the internal behaviour and the actions of an individual are not (rigidly) determined by the surroundings (Varela 1979; Maturana and Varela 1980; Rosen 1991; Collier 2000; Kauffman 2000; Rosslenbroich 2014; Moreno and Mossio 2015). We can distinguish two fundamental dimensions of autonomy: the *constitutive* processes (e.g. metabolism and gene transcription and translation) that allow a biological organisation to self-maintain, and the *interactive* processes (e.g. sensorimotor capacities and inter-organism communication) that enables an organism to interact with the environment according to its own internal norms (Mossio and Moreno 2010; Moreno and Mossio 2015). These two dimensions are *functionally integrated* among each other, because the constitutive dimension requires that the system be able to interact with its surroundings so as to find nutrients; at the same time, the interactive capacities require constitutive processes, such as the energy provided by metabolism (Di Paolo 2005; Moreno and Etxeberria 2005; Barandiaran and Moreno 2008; Moreno et al. 2008; Arnellos and Moreno 2015; Moreno and Mossio 2015).

To conclude, the concept of functional integration occupies an essential role both in biology and philosophy. In biology, it is intuitively understood as a causal interdependence of *biological mechanisms* that give rise to systemic capacities (e.g. the physiology of a cell or a multicellular system). In philosophy, functional integration is closely connected with the issues of *organismality* and *individuality*. I have stressed the dialectics between organismality, broadly understood as a strongly integrated system of interdependent parts and functions, and the five main definitional categories for individuality that provide some important clues as to the developmental, evolutionary, genetic, structural and functional dimensions of functional integration.

Both the biological and philosophical literature raise two important issues about the concept of functional integration. First, in most cases, physiological integration is an *explanans*, rather than an *explanandum*, without a solid theoretical foundation. In other words, functional integration is currently employed to explain what a biological system, organism, or individual *is* or *does*, but very

few works have clearly established a theoretical framework for functional integration. Secondly, the lack of conceptual clarity makes it a *vague concept* that "would not define the degrees of biological individuality" (Pradeu 2010, p. 252) especially in all those biological organisations (e.g. symbiotic associations) that exhibit coordinated behaviour without being full-fledged organisms.

SCOPE OF THE THESIS

This thesis seeks to fill the theoretical gap that has been identified above and to contribute to the current debate about biological individuality and autonomy in *philosophy of biology*. In this section, I present the objectives, the theoretical questions, the case-study and the reasons for its choice, the epistemological and ontological position adopted, and the relevance of the thesis in the context of the contemporary biological and philosophical debate.

The *first objective* of the thesis is to understand *which structures* and *functions* need to be integrated for making a *cell* a physiologically integrated unit. I shall elucidate, the organisational conditions enabling a cell to exhibit *systemic capacities* (e.g. metabolism, regulation, signalling, development, reproduction, and sensorimotor capacities). The focus of this thesis is on a very specific, but already sufficiently wide (and complex), group of *unicellular* organisms that includes bacteria, archaea, and unicellular eukaryotes (i.e. protozoa, unicellular algae, and unicellular fungi). Furthermore, following Pattee's dictum, it is highly fruitful in science and in philosophy to study all those case-studies which show *the minimal degree of complexity* and the *maximal conceptual interest* in relation to a specific theoretical issue.

The second –and fundamental-- objective is to evaluate the *similarities* and *differences* in how prokaryotes and eukaryotes are functionally integrated. This implies to evaluate the contribution made by *functional integration* to the understanding of *biological individuality*, by studying the kind of functional integration required for prokaryotic and eukaryotic unicellular organisations to be physiological and evolutionary units. Furthermore, I shall address how functional integration affects the constitutive and interactive processes (i.e. their *autonomy*) of prokaryotic and eukaryotic unicellular organisations.

The third and last purpose is the formulation of a *theoretical proposal* for functional integration in the transition from the prokaryotic to eukaryotic cell in eukaryogenesis, which encompasses both biology and philosophy. I shall provide a qualitative characterisation of physiological integration that

could be helpful for both a biological theory of cell organisation and a philosophical understanding of the ontological status of a biological individual.

Thus, the key questions of this thesis can be framed as follows:

1. How can simple functional structures constitute more complex functional structures in a cell?
2. Which organisational mechanisms and processes of a cell need to be integrated, so as to make it a physiological and evolutionary autonomous unity?
3. What are the similarities and differences between eukaryotic and prokaryotic cells in terms of functional integration?

The first question will be addressed by examining how simple molecular components assemble to generate a new complex function in molecular machines (chapter 1). This issue will be deepened in chapter 2, where I will study the functional relationship between constitutive (notably metabolic), regulatory, and signalling mechanisms. These two chapters provide a conceptual basis to address the second and the third questions.

Most of the thesis (chapters 3-6) is devoted to the questions 2 and 3 and, in order to explore them, I have chosen as a case-study, the *transition* from the *prokaryotic* to *eukaryotic cell*, because it is an outstanding example of appearance of a new *functionally integrated organisation* from a previous one. This specific period of the evolutionary history of life, which approximately occurred 1.6-2.2 billion years ago, is extremely relevant for two main reasons. First, it is the outcome of a long and very complex process of *endosymbiosis between prokaryotes* that gave rise to some of the current eukaryotic organelles, such as mitochondria, chloroplasts, and perhaps also the nucleus (Sagan 1967; Margulis 1970; Lane 2015; Martin et al. 2015). What is at stake is therefore to investigate how a symbiotic association of different organisms can achieve such a high degree of physiological integration that it exhibits collective physiological behaviours, a common life cycle, and common reproductive capacities. Secondly, the transformation of the endosymbionts into eukaryotic organelles *directly* determined, or at least *indirectly* contributed to, structural (e.g. the appearance of the endomembrane system and the increase in genome and cell size) and functional (e.g. new forms of gene regulation, mitosis and meiosis) modifications of the proto-eukaryotic cell. This entailed new levels of *functional differentiation* and *integration* among different *organelles* (see Margulis and Fester 1991; Sapp 1994; Moran 2006; Martin and Müller 2007; Gilbert 2014).

The analysis of this case-study has two aims: first, to characterise and compare the types and degrees of functional integration in bacteria, archaea, and unicellular eukaryotes, so as to set out a

theoretical framework for *functional integration* in *prokaryotic and (unicellular) eukaryotic organisations*. Secondly, to understand the *constitution of functional integration* in the transition from one kind of individuality (the prokaryotic one) to another (the eukaryotic cell) by means of essential biological processes such as endosymbiosis, endosymbiotic gene transfer, invagination of internal membranes, etc. Even though there are *other* interesting *case-studies* (e.g. the achievement of new degrees and forms of functional integration in the origin of multicellularity or the appearance of functional integration in some cases of holobionts), there are some practical reasons that justify my choice. The appearance of functional integration in the transition from prokaryotes to eukaryotes is not only highly complex and scientifically relevant but also very rich in scientific details, because many studies on eukaryogenesis have so far been conducted.

Finally, the study of functional integration in the context of eukaryogenesis will be carried out by adopting an *organisational approach* [9] that address biological phenomena and properties by considering the specific organisation in which they are embedded (Mossio et al. 2009, 2016). More specifically, biological organisations exhibit a circularity: "they generate and maintain a set of *structures* acting as *constraints* which, by harnessing and channelling the *processes* and *reactions* occurring in the system, contribute to sustain each other and then the system itself" (italics mine) (Moreno and Mossio 2015, p. xxix)[10]. As a result, the organisational approach considers both the structural features and the physicochemical aspects for understanding biological systems.

From a biological point of view, the thesis will study three main aspects of the transition from prokaryotic to eukaryotic cell: first, the changes in the molecular composition and the appearance of new macromolecules (e.g. some molecular motors such as dynein, kinesin, and myosin) that globally affected the physiology of the proto-eukaryotic cell; secondly, systemic changes in metabolism, life cycle, and sensorimotor capacities that were connected to the appearance of eukaryotic organelles; thirdly, the evolutionary hypotheses behind eukaryogenesis.

It is worth noting that this thesis does *not* aim to formulate new *phylogenetic* hypotheses about eukaryogenesis. In fact, it will *discuss* the current hypotheses and theories about eukaryogenesis, often using phylogenetic analysis, for characterising functional integration in unicellular organisations and making new *plausible hypotheses* about the most important milestones in eukaryogenesis, always comparing the current prokaryotic and (unicellular) eukaryotic

[9] It is not possible here to present in detail all the works that have used an organisational approach to the study of biological systems. I just mention some of the most influential authors: Ganti (2003), Kauffman (2000), Maturana and Varela (1980), Pattee (1972, 1973), Piaget (1967), Rosen (1970, 1991), Waddington (1968-1972).

[10] For a detailed discussion of the concept of *constraint* and its role in the organisational framework, see section 2.2.

organisations. As such, this thesis seeks to make a contribution not only to the current studies about the origin of eukaryotes in *evolutionary biology*, but also to the theoretical characterisation of physiological integration in *cell biology*.

METHODOLOGY

In order to establish a theoretical framework for functional integration, this thesis combines the descriptive approach of the *methodological naturalism* with the *normative evaluation* of the epistemic and practical consequences of the theoretical frameworks of life sciences.

By methodological naturalism, I mean the study of the ontology of natural phenomena and properties through the analytical and conceptual tools provided by natural sciences. In this thesis, *evolutionary* and *cell biology* provide me with an important set of empirical data and theories that turn out to be extremely helpful to characterise the concept of functional integration in unicellular organisms. All along the thesis, I study key organisational aspects of unicellular organisms that form a common and coherent *theoretical core* that is common to both prokaryotic and eukaryotic cells. This will also permit me to illuminate the differences between prokaryotes and eukaryotes in terms of their physiological integration, and to put forward hypotheses about the origin of eukaryotes.

More specifically, the chapters of the thesis explore the concept of functional integration by testing the following *hypotheses*. First, the biochemical network of a cell requires the *functional integration* of a set of macromolecules exhibiting the features of *molecular machines* (or motors). Secondly, a basic level of functional integration in a cell is represented by a specific *interdependence between metabolic and genetic processes*, *signalling* and *regulatory mechanisms*. This level of integration represents the fundamental pillar upon which other functional (and more complex) levels of integration can be achieved. Thirdly, symbiotic associations can give rise to different forms of functional integration because of different structural organisations that exert very specific constraints on the behaviour of the individual components. Fourthly, the internal division of the space through membranes produces a better control over the flow of molecules and metabolites within the cell; this requires a new form of systemic regulation and *physiological integration* of *intracellular communication*. Fifthly, prokaryotic cytoskeletal-like proteins and eukaryotic cytoskeleton provide the cell with *coordinated sensorimotor capacities*, an *integrated organisation of the intracellular space*, and an overall coordination between *developmental* and *reproductive* phases. Sixthly, functional integration is a necessary condition for a *collective reproduction*.

The normative side of the project, linked to the naturalist one, aims to determine to what extent naturalistic descriptions provide norms for the conceptualisation of functional integration and the normative consequences stemming from it. This is crucial in evaluating the theoretical implications of a conceptual framework of functional integration in organisational terms. The normative aspect of the project consists of two main parts.

First, I review the concept of functional integration in the philosophical debate about biological individuality and biological autonomy, underlining the conceptual *potentials* and the theoretical *weaknesses* of both perspectives. I will evaluate the current definitions of biological individuality and biological autonomy in the light of the results obtained from the case-studies. This part of the work is thought not only as a critical examination of the current state of the art, but also as a way to provide philosophy of biology with a new theoretical framework for functional integration.

Secondly, I will discuss the results of the case-studies through the lens of *philosophical discussions* so as to create a unique frame of reference encompassing both biology and philosophy.

MAIN FINDINGS AND LAYOUT OF THE THESIS

The order of the chapters of this thesis clearly reflects the six working hypotheses, representing the gradual progression from the *simplest* to the more *complex* levels of functional integration in unicellular organisms. This succession allows us to show the structural and functional changes that determined a new functionally integrated organisation in the transition from the prokaryotic to eukaryotic cell. The six chapters are similarly structured: an *introduction* to the key topic of the chapter; a *critical review* of the scientific and philosophical literature on the subject matter; a number of *biological case-studies*, usually taken from the prokaryotic and eukaryotic domains, which provide us with empirical investigations about the researched topic; a *theoretical* and *philosophical discussion* of the results of the case-studies, often comparing prokaryotic and eukaryotic organisms and relating them to the issues presented in the critical review; finally, a *conclusion* about the researched topic.

I would like to put into evidence three aspects of the architecture of the thesis. First, since each chapter has its own state of the art, there is no chapter uniquely devoted to it. I strongly believe that, since the problem of functional integration in unicellular organisms entails many different related issues, the best way to deal with it is to decompose and analyse them into different related chapters. Secondly, a theoretical proposal for functional integration, which merges the results of

each of the six chapters, will be provided in the "Conclusions" of this thesis. Thirdly, each chapter may eventually be read as a single paper; however, its overall significance can be grasped only in the global context of the thesis and in the ordered succession of the six chapters. I therefore present now the content of the six chapters of the thesis.

The *first chapter* investigates the concept of machine at the macroscopic and microscopic level and provides a definition of *machine* as a device consisting of a variable number of component parts and channelling a flow of energy and matter so as to make work. I make a comparison between the properties of machines at the macroscopic level (e.g. computers or gear trains) and those of *molecular machines* in the artificial domain of nanotechnology and in the natural domain of molecular and cell biology. I show that the *biochemical network* of a cell is based upon a *functional integration* of *macromolecular machines* that perform important biological functions. As such, I consider the functional integration of macromolecular machines as the first step for a characterisation of functional integration at the cellular level.

The *second chapter* explores the relationship between metabolic and genetic processes of a cells, the intra- and extracellular signals, and the cellular regulatory mechanisms. I argue that there is a mutual functional dependence between them, inasmuch as metabolic and genetic processes are regulated by proteins acting on genes or on proteins. In turn, regulatory proteins are triggered by intra- or extracellular signals that depend on metabolic processes, as they can be intermediate products of metabolic processes. Together with the integration of macromolecular machines, the *functional integration* between *metabolic*, *genetic*, *regulatory*, and *signalling processes* is the other fundamental level of the functional integration of a cell. They represent the theoretical basis for understanding the process of structural and functional complexification that occurred in eukaryogenesis.

The *third chapter* examines the kind and degree of functional integration exhibited by two fundamental types of prokaryotic collective organisations: bacterial and archaeal colonies (biofilms) and the endosymbiotic relationship between two bacteria. I defend the thesis that the engulfment of one prokaryote within another determines a *stronger degree* of *physiological integration*, compared to biofilms, with the potential to evolve into a new full-fledged individual with collective reproductive capacities and the ability of generating a parent-offspring lineage. Thus, this chapter provides not only a plausible hypothesis for the *origin* of *mitochondria* and *chloroplasts*, but also a global examination of the biological dimensions of functional integration in the *prokaryotic* domain.

The *fourth chapter* analyses how the *division* of the *intracellular space* through membranes affects the overall *functional integration* of a cell. Although intracellular membranes are often considered as a distinguishing feature of eukaryotic cells, some species of bacteria also have a primitive system of endomembranes. Thus, I compare the role played by endomembranes in bacteria and in eukaryotes by focusing on their similarities and differences. I argue that the appearance of the nuclear envelope and of the endomembranous system represented a fundamental step in eukaryogenesis that entailed new regulatory and signalling pathways for controlling the coordination among the functions performed by these organelles. Furthermore, I suggest that the appearance of internal membranes was a very demanding (energetic) organisational change that paved the way for an important functional specialisation in the eukaryotic cell.

The *fifth chapter* studies the role played by the *cytoskeleton* and *cytoskeletal-like protein*s in the achievement of *functional integration* in *symbiotic organisations*. I compare the physiological role played by the cytoskeletal-like proteins in some bacterial endosymbionts of eukaryotes and the role played by the eukaryotic cytoskeleton in the control of mitochondria and chloroplasts. I argue that the prokaryotic cytoskeletal-like proteins and the eukaryotic cytoskeleton play a pivotal role in the acquisition and coordination of sensorimotor capacities and that the emergence and maintenance of collective biological identities involves a strict control of the motile abilities of their constituting members. This entails a restriction, but not necessarily a complete loss, of the agential capacities of the individual parts. As a result, eukaryogenesis entailed a strong control of the sensorimotor capacities of mitochondria, plastids, and also other organelles by developing a highly efficient cytoskeletal system that regulates and coordinates their displacement within the cell. Therefore, the cytoskeleton can be considered as a fundamental aspect of the functionally integrated organisation of eukaryotic cells.

The *sixth chapter* evaluates the relationship between a *system-level coordinated reproduction* and *functional integration*. The main question that I address is the type of physiological integration required for a cell to reproduce as a whole, leading to a parent-offspring lineage. I analyse two clear examples of system-level coordinated reproduction: the binary fission in bacteria and the mitosis in eukaryotes. I argue that system-level coordinated reproduction is mutually dependent on developmental processes so as to generate a life cycle that is sustained by and also sustains metabolic processes. Moreover, the functional interdependence between system-level reproduction, growth, and metabolism requires three levels of mechanisms that are functionally integrated: the regulatory proteins controlling life cycle, the cytoskeletal proteins and motor

proteins controlling the spatial coordination during cellular fission, and the nutrient-dependent signals coordinating the life cycle with metabolism. In the last part of the chapter, I discuss the relationship between system-level coordinated reproduction and biological individuality.

Finally, the "Conclusions" section gives an overview of the results achieved in each chapter and addresses the theoretical implications of the thesis in the current philosophical and biological debate. In a nutshell, I put forward a theoretical proposal for functional integration consisting in the global capacity, enabled by specific spatial constraints, of a biological organisation to perform system-level regulation, spatio-temporal coordination of the parts, and system-level reproduction.

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CHAPTER 1 STRUCTURAL AND ORGANISATIONAL CONDITIONS FOR BEING A MACHINE[1]

1.1 INTRODUCTION

Cell biology, synthetic biology, and nanotechonology have been conducting pioneering research into nanomachines, which are a subset of macromolecules (usually proteins) that perform functions by chanelling a flow of energy and matter. Nanomachines perform many important cellular functions, thus playing a fundamental role in cell physiology. Nevertheless, some criticisms, raised by some philosophers and biologists, have recently been levelled at the use of the term "machine" in the context of cells and, more generally, living beings. The detractors argue that "machine" usually refers to the artificial devices of the macroscopic world, and therefore it cannot be applied to the microscopic domain of macromolecules. Thus, this chapter aims at studying the *conditions* that enable an artificial or biological organisation to be considered a machine and at evaluating the role played by biological nanomachines in the *cellular physiological network*.

In philosophy of biology, the concept of 'machine-like system' has been extensively employed in the neo-mechanistic framework to describe biological mechanisms, since said mechanisms have been regarded as the functional components of a system which behaves like a machine. Neo-mechanistic accounts have so far focused on the epistemological aspect of mechanistic explanations in the life sciences, with a rough analogy often being drawn between (biological) mechanisms and machines. Nevertheless, as pointed out by Moore (2012), Skillings (2015) and Nicholson (2013), there are some relevant differences (mainly due to different size scales) in the physicochemical behaviour of macroscopic machines, on the one hand, and microscopic devices, on the other, that make this analogy rather dangerous. As a result, these authors have argued that the analogy between macroscopic machines and microscopic devices (such as synthetic nano-machines or certain biological macromolecules) should be taken with a grain of salt and, in most cases, completely dismissed. Yet the issue is far from simple, since the conceptual framework of contemporary nanotechnology is based on the idea that some biological macromolecules are indeed machines,

[1] The ideas presented in this chapter and most parts of this chapter have already been published in Militello and Moreno (2018).

and can therefore be artificially reproduced using a bottom-up approach, according to which a supramolecular structure may be built by assembling smaller molecular components.

No comprehensive ontological analysis of the concept of machine and, particularly, the status of machine of certain kinds of microscopic devices (synthetic as well as biological) has yet been carried out by either neo-mechanistic accounts or the philosophy of (nano)technology. In an attempt to fill this void, this chapter aims to establish the conceptual boundaries of the concept 'machine' and to understand to what extent some molecular devices may be defined as such. It is worth stressing that this chapter is *not* aimed at claiming that organisms are machines, but rather at evaluating whether or not molecular synthetic devices and some biological macromolecular structures share common properties that make all of them 'machines'. In order to understand whether some molecular devices are machines, it will be necessary to analyse the structural and physicochemical conditions of not only nanoscale devices, but also macroscopic machines, since the term 'machine' was originally coined to refer to macroscopic man-made devices (e.g. Archimedean simple machines), and only later, during the 20th century, was it applied to the domain of biological macromolecules.

In light of the above, the research questions to which this chapter seeks to respond can be summarised as follows:

1. What are the structural and organisational features of artificial macroscopic machines, synthetic molecular machines and biological molecular machines?
2. To what degree does the 'machine-like' analogy fit a class of molecular devices operating at the nanoscale?
3. Is the 'machine-like' analogy appropriate for describing the operation of certain kinds of macromolecules in living cells?

An understanding of the ontological status of (nano)machines has two important explanatory consequences for the neo-mechanistic debate and nanotechnology. First, the clarification of the term 'machine' may shed some light on the biological mechanisms that are based on them[2]. Second,

[2] The term 'mechanism' is currently used in neo-mechanistic literature for designating both the (epistemological) problem of the explanatory power of mechanistic explanations (among others, Bechtel and Richardson 2010; Glennan 1996; Bechtel and Abrahamsen 2005) and the (ontological) organisation of –namely biological- mechanisms (among others, Machamer et al. 2000; Craver 2001). When I state that I focus on the mechanisms "based on" (or performed by) machines, I merely claim that I describe, from an ontological perspective, the configuration of the mechanisms that are

since the cornerstone of nanotechnology is the possibility of artificially reproducing certain biological macromolecules, the differences between biological and artificial molecular machines highlight the limits of its theoretical framework.

The structure of the chapter is as follows. Section 1.2 presents and discusses neo-mechanistic accounts of 'machine-likeness'. Then, Section 1.3 analyses the features of artificial macroscopic machines. Section 1.4 offers a critical exploration of the structure and functioning of synthetic and biological molecular machines, and Section 1.5 focuses on the specific case of biological molecular devices, taking into account the criticisms and arguments put forward by Moore (2012), Skillings (2015), and Nicholson (2013) against the machine-likeness of nanoscale devices. Finally, Section 1.6 offers some concluding remarks.

1.2 THE CONCEPTS OF MACHINE AND MECHANISM IN NEO-MECHANISTIC ACCOUNTS IN BIOLOGY

The idea that organisms can be explained through an analogy with machines is rooted in Descartes' thinking, as laid out in *Discourse on the Method* (1637 (1999)) and *Treatise on Man* (1664 (1972)). Since the publication of these seminal works, it has been widely assumed[3] that each anatomical part performs a distinct and specific biological function in the same (or at least, similar) way as the different parts of a machine make up a mechanism. The concepts of 'machine' and 'mechanism' are at the core of many biological descriptions (from genetics to evolutionary biology), and play a pivotal role in the neo-mechanistic view.

However, until recently, no precise definition of the term 'mechanism' had been developed. The first *basic mechanistic account* was clearly provided by Machamer, Darden and Craver[4] (2000), and has significantly influenced subsequent debates on not only the nature of biological mechanisms, but also the nature of machines. The MDC account defines biochemical mechanisms (e.g. neurotransmission and the mechanisms of DNA and RNA replication, transcription and translation) in terms of entities performing regular activities from start to finish conditions. Implicitly, this concept of mechanism is based on the way man-made machines work, since mechanisms have long been considered the functional parts of a machine-like system (Glennan 1996, pp. 51-52; Bechtel

performed by the component parts of a specific kind of system (i.e. a machine). Hence, I do not address the issue of the explanatory power of mechanistic explanations.

[3] This is not to say that this view has not met with strong opposition (i.e. vitalism and, later, organicism).

[4] Hereinafter, I shall refer to Machamer, Darden and Craver's account as the MDC definition.

and Richardson 2010, p. 17). Thus, as Nicholson points out (2012), one of the meanings sometimes carried by the concept of 'mechanism' is that of 'machine'.

Although these authors have developed a set of precise definitions for the concept of mechanism, they have not convincingly justified its relationship with the concept of machine. There are two main reasons for this. First, the development of a theory of machines has been essentially ignored by the advocates of mechanistic accounts in biology, who use the concept of mechanism in an epistemological-explanatory sense rather than an ontological one[5]. Consequently, the use of the machine analogy to explain biological systems has generally been supported by rather intuitive ideas about what a machine actually is. Second, some neo-mechanistic accounts have provided a very broad definition of 'mechanism' that encompasses both mechanisms which are based on machines and mechanisms which are not. Thus, the relationship between mechanisms and machines appears vague and unclear. I shall explain these two claims in more detail below.

In relation to the first aspect, the definitions of mechanisms offered by Bechtel and Richardson (2010) and Glennan (1996), while emphasising the fact that mechanisms behave *like* the functional components of a machine, fail to provide a detailed analysis and description of the ontological status of a machine, or indeed the machine-like behaviour of some biological macromolecules. Rather, they focus on the *epistemological* nature of mechanistic explanations and, collaterally at least, the epistemological aspect of *machine-likeness* (i.e. the fact that a machine may be explained through mechanistic accounts). In the same vein, Levy (2014) links the concept of 'machine-likeness' to decompositional strategies[6], since a machine can be decomposed by virtue of two features: first, the differentiation of parts (Levy 2014, p. 5); and second, the local relations among the component parts (Levy 2014, pp. 5-6). In other words, modularity and internal interactions among the local functions of a system provide it with a certain degree of order, as well as decomposability, which in turn allow it to be defined as a 'machine'. In spite of their importance, however, these aspects do not shed any light on the ontology of a machine.

As regards the second claim, the MDC definition of mechanism in terms of 'entities and activities organised such that they are productive of regular changes from start to termination conditions' (MDC 2000, p. 3) is much broader and encompassing than the conceptual core of the operation of

[5] Although Illari (2013) stresses that Bechtel's view is epistemic whereas Craver's account is ontic, I will not address this issue here. Instead, I will examine why a number of (mainly epistemological) accounts of (biological) mechanisms have not so far focused on the ontology of (nano)machines.

[6] By decompositional strategies I mean an epistemological account of the behaviour of a system in terms of the local behaviour of its subsystems (component parts) and their causal interrelations (compare Bechtel and Richardson 2010).

a machine. Here again there are two main reasons for this. First, because the component parts of a machine (the 'entities') are not only organised, but also held together in a (meta)stable structure, nearly in thermodynamic equilibrium[7]. Second, because 'the activities' of the components of a machine take place only when an input of energy occurs and are aimed at displacing a force, doing work or performing a function. Accordingly, the mechanism of a machine needs a thermodynamically-stable structure, and this requirement is not included in the MDC definition. An MDC mechanism could be either the result of the activities of parts organised in a thermodynamically-stable structure (and would therefore coincide with my concept of the mechanism of a machine), or the result of a far-from-equilibrium organised set of coupled processes. Many biochemical pathways indeed produce a functional activity (that which the MDC account defines as 'mechanism'), which may be explained as resulting from clearly distinguished 'parts' (i.e. the chain of reactions catalysed by specific enzymes), understood as processes. However, as shown in the following sections, this kind of mechanism is not compatible with my concept of the mechanism of a machine, because a biochemical pathway fails to exhibit some important features of machines, such as a thermodynamically-stable structure or an energy input to do work. For these reasons, the basic mechanistic account provided by MDC does not clarify the difference between those mechanisms which are based on machines and those which are not.

Usually neo-mechanistic accounts (notably MDC 2000; Craver 2001; Bechtel and Abrahamsen 2005; Bechtel and Richardson 2010) use the term 'organisation' to refer to the specific way the different parts of a machine are arranged so as to perform a given function. The use of this term is however a bit ambiguous. For, on the one hand, strictly speaking, the component parts and the operations of a machine may be said to be (structurally, spatially, and temporally) *ordered*. Yet, on the other hand, in order to perform a function, they should contribute to the maintenance of a system to which they belong (i.e., they are generated in this system, and they contribute to its maintenance). More precisely, I say that a machine performs a function insofar as it is embedded in a context (for example a specific social organisation) where certain material structures (i.e. machines) are produced. If machines are rightly designed and fabricated, they can also contribute to the maintenance of the context itself (for example the life of society to some extent depends on the existence of machines). And in a similar vein, certain macromolecular devices in the cell perform

[7] By this, I mean that the structure of a machine (i.e. the specific assemblage of its component parts) would be preserved even if the exchange of matter and energy with its surroundings were almost zero (i.e. thermodynamic equilibrium). The *stability* of a structure is different from the *functionality* of a machine, because functionality requires an exchange of matter and energy between the machine and its surroundings.

a function because they are embedded in the cellular 'organisation', which they contribute to maintain and where they are produced. It is a human 'organisation' that produces an artificial machine and provides them with a specific function; and it is a biological 'organisation' that produces a molecular machine and provides them with a specific function. In both cases the term 'organisation' is what justifies that a given composite material structure, constraining a flow of energy, achieves a function (see for details Mossio et al 2009 and Moreno and Mossio 2015). Derivatively, it would be sensible to say that the *ordered* structure of the functional parts constituting a machine is also "*organised*" in order to fulfil the global function performed by the machine as a whole.

In sum, the (neo)mechanistic use of the term 'mechanistic explanation' is much more liberal than mine, as I focus *only* on the mechanisms performed by machines. Since the purpose of this chapter is to conduct an *ontological* examination of the concept of 'machine', I will not enter here into current (and important) debates about the *explanatory* validity or limits of the (neo)mechanistic accounts, particularly in light of the challenges raised by the success of network-like explanations, which are usually incompatible with the idea of functional decomposition (Zednik 2011; Kaplan 2015; Bechtel 2017).

Although the neo-mechanistic debate has so far devoted most of its attention to the epistemology of biological mechanisms, there is still a long tradition of studies on the structure and functioning of man-made machines. Serious attempts to define what a machine actually is can be traced back to the second half of the 19th century, when the German engineer Franz Reuleaux developed a theory which posited that a machine is a kinematic chain of elementary links called 'kinematic pairs'. In his book 'The Kinematics of Machinery', the term 'machine' refers to a system that converts an energy input into an energy output by exploiting the mechanism(s) of its component parts that displace an applied force and, therefore, do work (Reuleaux 1876)[8]. The functional components of a machine exhibit a specific *design* that allows them to harness the physicochemical processes underlying the behaviour of said machine. In other words, a machine is a set of *functional constraints* that are *interlocked* so as to harness the action of physical laws in order to achieve a new (*composite-integrated*) function, as pointed out by Polanyi (1968). To do so, a functional hierarchy must be established, and a spatial and temporal order must be imposed on the functional constraints. As Wimsatt (1986) highlighted, the functional components of many machines can be *partially*

[8] This author defined a machine as 'a combination of resistant bodies so arranged that by their means the mechanical forces of nature can be compelled to do work accompanied by certain determinate motion' (Reuleaux 1876).

intersubstituted within a certain range of configurations and without changing systemic properties. As a result, the nature of a composite-integrated function of a machine is determined by the structure and functions of its constituents (principle of compositionality). A crucial feature of machines is that they consist of a number of modular parts that are assembled according to a specific design so as to assume a distinctive shape. Accordingly, the pieces of a machine can be isolated due to modularity, and are gathered in a very specific way in order to perform a certain function. Another essential feature of a machine is its compactness, namely the structural co-dependence of its component parts, which is a result of the design of the machine. Compactness allows a machine to exhibit clear boundaries that distinctly distinguish it from its surroundings.

In the light of the above, it is apparent that there is a tension between the concepts of 'machine' and 'mechanism' in the current neo-mechanistic framework. I propose to resolve this ambiguity as follows: I define a *machine* a meta-stable structure, which can persist in thermodynamic equilibrium, consisting of a number of functional interdependent parts that constrain an energy flow to do work and perform a systemic function. I characterise a *mechanism performed by a machine* as the set of all functions carried out by the component parts of the machine that allow it to harness a flow of energy and matter and to do work. In other words, 'machine' designates a certain kind of a *structure*, whereas a 'mechanism based on a machine' refers to its *systemic functionality*. The mechanism of a machine is the result not only of the specific structure of a machine, but also of a human or biological context that provides a machine with a specific (structural) order of its component parts and a particular mechanism. Indeed, to a certain degree, one can abstract the functioning of a machine from its material and organisational embodiment. Yet, although features such as design, structural stability, shape, compactness, modularity and compositionality pertain to the structure (i.e. to machine), but not its functionality (i.e. its mechanism), they should indirectly inform our understanding of a mechanism also. As a matter of fact, the mechanisms of each machine constrain a flow of energy by virtue of the specific shape of the component parts of a machine and the way in which they are ordered.

For these reasons, in this chapter I will focus on the nature of machines (what they are and what aspects define their operations) and analyse to what extent the machine-analogy can be applied to the core of all living organisations, i.e. the cell. It is true that biological machines are microscopic and their physicochemical properties are very different from those of macroscopic machines. But before analysing the implications of the nanoscale, I shall first clarify what a machine is by analysing the example of artificial macroscopic machines.

1.3 ARTIFICIAL MACROSCOPIC MACHINES[9]

The oldest and simplest macroscopic machines can be traced back to Archimedean simple machines (e.g. levers, screws and pulleys, etc.), which are devices that modify the direction or magnitude of a force in order to do work against a single load force. Simple machines are often considered the building blocks of more complex 'compound machines'. Power sources are exploited to transmit power[10] or transform motion and, therefore, perform a mechanism[11]. Both simple and compound machines do work by harnessing a flow of energy into an ordered process so as to achieve a pre-specified function[12]. This is made possible by a set of specific material *structures, which act as constraints,* functionally harnessing the flow of energy so as to produce a forward motion. When a macroscopic machine is at work, the summation of all external forces and torque is *not* zero (the machine is *far* from *mechanical* equilibrium). Since the movement and the work of a macroscopic machine are the outcome of the relative internal motion of its component parts, they must be assembled in an ordered way (following specific design rules) in order to achieve a functionally-integrated operation. This is commonly referred to as the 'structure' of a machine.

The design of a macroscopic machine is closely linked to its functionality, insofar as *shape*, *form*, and *size scale* determine certain kinds of mechanisms and not others. According to Reuleaux (1876), a machine consists of an assemblage of resistant bodies (*links*), which are connected together (the so-called 'kinematic pairs') by movable joints so as to form a *kinematic chain* with one link fixed and having the purpose of transforming motion. Reuleaux's characterisation of machines primarily encompasses mechanical devices and, therefore, considers component parts as rigid structures. However, many contemporary machines exhibit constituents which are not rigid, but rather flexible, such as magnetic parts (e.g. in an electromagnetic coil), fluidic components (e.g. in a refrigerator), and so forth. The links of a machine are structures that move in the air or in a vacuum by exhibiting *relative motion* that is constrained by the number of links, the type of joint used to connect them

[9] In this section, I mainly refer to *mechanical machines* such as steam machines, cars, pumps, etc. Of course, there are many other kinds of non-mechanical machines (e.g. computers) which I have not described here, because all of them share the same basic features of what I have called 'machine'. Hence, for the sake of simplicity, I focus on mechanical machines as paradigmatic examples of artificial macroscopic machines.

[10] Power is the transmission of energy from the place where it is generated to another place, so as to perform useful work.

[11] In machine theory, when one link is chosen as the framework of reference for the movement of all other links, it is called the 'frame link'. Once a frame link is set out in a kinetic chain, and it is possible to generate an output motion in response to an input motion, the kinetic chain is called a 'mechanism'.

[12] Needless to say, the function of an artificial macroscopic machine is specified by its designer.

and the shape of the mating surfaces. Each link is connected to the other links through joints that transmit movement from the input link ('driver') to the output link ('follower'). Since each link is aimed at maintaining constant spatial relationships between the elements of its pairs (Dicker et al. 2003, p. 6), the way in which the pieces of a machine are assembled together is crucial to defining the mechanism, the work, and the kind of function performed. Indeed, the overall function of a machine hinges on the *compositionality* of the local functions performed by its parts. A good example of a macroscopic machine design is a gear pump, which exploits the rotation of gears to displace fluids. A gear pump consists of two gears (links) that are connected through a contact zone (movable joint) which allows two gears to pivot with respect to each other in such a way that they form a kinematic chain. In order to work properly, each gear must maintain a specific angle with respect to the other one (constant spatial relationship). It is important to underline that a key requirement for macroscopic machines is that the parts be structurally co-dependent, so that the overall organisation is *stable* and, at the same time, *compact,* with *clear spatial boundaries*.

The structure of a macroscopic machine (i.e. the structural interdependence among its parts) may be said 'stable', because it is maintained regardless of whether or not the device is actually doing work (and performing a function). For example, the structure of a refrigerator or a car is stable, since it is maintained regardless of whether or not these machines are switched on or off (i.e. if they actually work or not). Then, macroscopic machines may be defined as 'compact', because they exhibit a specific design and their component parts are assembled in such a way to be closely and firmly united in a distinct pattern. For example, the component parts of a refrigerator or a car are closely interlocked in such a way that they have a compact aspect. Finally, the component parts of a macroscopic machine show clear spatial boundaries, because their different pieces are assembled in a specific way so as to build a macroscopic device. For instance, a refrigerator is composed of clear distinct assembled parts such as a thermally insulated compartment and a heat pump that transfers the heat from the inside to the outside of the refrigerator.

Thus, the component parts of an artificial macroscopic machine perform a mechanism because of the ordered structure *of their constraints*. This ordered structure of constraints is evident in the way in which the links are assembled (*design*) so as to channel the motion of each part in a certain direction. The structure of constraints is designed so as to minimise the inertial and friction forces acting on the parts (i.e. the links) of the machine. *Friction forces*, which act on the mating surfaces between two links, affect the motion of the parts of a macroscopic machine, because friction forces (i.e. dry friction) determine the tractive force between a body and a tangential force. Much the same

occurs with *inertial forces*, which oppose any change in the velocity of motion or the torque of a rigid body. Since friction and inertia influence the sliding velocity of the mating surfaces of the links and any changes in their velocity (respectively), the overall movement, and thus the mechanism, of a macroscopic machine is inevitably affected by these physical forces.

All in all, to constitute a mechanism in a macroscopic machine, each link of a kinematic chain must exhibit a specific shape and dimension, as well as a distinct connection with the other links in such a way as to ensure a certain degree of freedom (DOF)[13] and, therefore, perform a relative motion. Since design is crucial to enabling the component parts to work and to perform a certain function, the links of a machine (e.g. wheels, gears, cams and pistons, etc.) must be assembled in a particular way so as to perform a certain kind of mechanism and a specific function. For example, a four-bar linkage (see Fig. 1.1) is a mechanism that can perform a wide variety of movements depending on *how* the four links are assembled and connected together: it can be employed in a pumpjack to draw oil from the subsoil by using a planar quadrilateral linkage; or alternatively, it can be used in a train suspension mechanism to allow the wheel to rotate through a slider-crank linkage. In short, the concepts 'mechanism', 'function' and 'work' in a macroscopic machine should be understood in terms of how the component parts are assembled so as to achieve a functionally-integrated action.

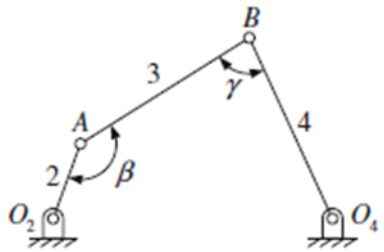

Figure 1.1 a four-bar linkage: o_2, a, b, and o_4 are the joints that allow links (2, 3, 4) to move with a specific angle (β and γ). the link 2 is the input link and the link 4 the output link (simón 2016, p. 15).

1.4 MOLECULAR MACHINES

Having clarified the core concept of 'machine', I will now turn to what are often referred to as 'molecular machines'. Here we find two very different systems: 'molecular machines' and biological

[13] The degree of freedom (DOF) of a mechanical system is defined as 'the number of independent parameters that unambiguously define its position in space at every instant' (Simón et al. 2016, p. 2).

'molecular machines', which while sharing many features, also diverge in many other important ways. For this reason, I shall divide the analysis into two parts. Firstly, I shall argue why, despite the specific differences generated by the nanoscale, it is still correct to talk about machines at the molecular scale. And secondly, and perhaps more importantly, I shall explore why it also makes sense to classify certain macromolecular structures operating in cells as machines.

Let me begin by considering, from a generic perspective, the current view regarding what a 'molecular machine' (MM) actually is. First of all, an MM is defined as any discrete number of molecular components that produce quasi-mechanical movements (output) in response to specific stimuli (input) (Ballardini et al. 2001). Unlike macroscopic machines, the configuration space of MMs is not defined by their six degrees of freedom[14], but rather by their free-energy landscapes[15] (i.e. Gibbs free energy[16] of interacting molecules) (Astumian and Hänggi 2002; Astumian et al. 2016).

More specifically, MMs are characterised by three important elements: firstly, thermal noise; secondly, structural anisotropy; and, thirdly, an energy input (Astumian 2002). Thermal noise[17] acts as 'thermal activator' of MMs, since it provides them with an amount of energy to overcome energy barriers[18]. If the noise intensity is low, molecules are pinned at a potential minimum and they cannot diffuse; on the contrary, if the noise intensity is high, molecules overcome the potential barrier and begin to diffuse (Astumian 2002; Astumian and Hänggi 2002). Thermal noise randomly 'pushes' an MM back and forth without a specific direction. Nevertheless, MMs exhibit a directional movement by combining structural anisotropy with an energy input (Astumian and Hänggi 2002). Structural anisotropy is the asymmetric distribution of reaction products around an MM and it acts as an asymmetric kinetic barrier. When an energy input (chemical, photochemical, etc.) is provided, structural anisotropy generates a concentration gradient of chemical potential that *constrains* Brownian motion and *generates* a directed motion of an MM. Thus, as a result of the interplay between thermal noise, structural anisotropy, and an energy input, an MM is able to functionally harness an energy source, constrain Brownian motion and perform a (biological) task[19].

[14] The six degrees of freedom of a macroscopic rigid body are defined by three rotatory movements (roll, pitch, yaw) and three translational movements (surge, heave, sway).

[15] The energy landscape is the mapping of all possible spatial conformations of a molecule. The energy landscape is a continuous function that associates each physical state of the molecule with the corresponding energy.

[16] Gibbs free energy is a thermodynamic potential used by a thermodynamic system to do work at a constant temperature and pressure. The simple equation for Gibbs free energy is: $\Delta G = \Delta H - T\Delta S$, where ΔH is the enthalpy change and ΔS is the change in entropy of the process.

[17] Thermal noise is the electronic noise determined by the thermal agitation of the charge carriers.

[18] Energy barrier (or activation energy) is the least amount of energy required to trigger a chemical reaction.

[19] Feynman (1963) pointed out that it is impossible to have a molecular device (the so-called 'Brownian ratchet') that is able to extract work from thermal noise because of the inviolability of the second law of thermodynamics. However, a

It is crucial to emphasise that MMs usually operate in aqueous solutions where they are subject not only to important thermal fluctuations, but also to viscous forces that render inertial ones negligible. Since the role played by viscous forces is completely different at the macroscopic and microscopic levels, the Reynolds number[20] (i.e. a dimensionless parameter comparing the effect of inertial and viscous forces) is different for macroscopic and microscopic devices. Macroscopic machines have a high Reynolds number, and inertial forces are important whereas viscous ones are negligible. Microscopic machines, on the other hand, have a low Reynolds number, meaning that viscous forces are fundamental and inertial forces negligible within the system.

Unlike macroscopic machines, MMs operate very near to mechanical equilibrium because the viscous drag force[21] is equal and opposite the net mechanical force. The 'mechanical equilibrium' of a molecular system is a dynamic condition in which every forward motion of a particle is cancelled by its microscopic reverse (i.e. a backward motion) (Astumian 2012), and it is therefore different from the concept of thermodynamic equilibrium. Accordingly, the presence of a ratchet mechanism in an MM allows it not only to direct movement but also to keep the system very near to, but not at, mechanical equilibrium. Although MMs are *close* to *mechanical* equilibrium, they are *far* from *thermodynamic* equilibrium, since they dissipate energy to their environment.

Unlike macroscopic machines, which exploit many different energy sources (mechanical, thermal, chemical, electrical, etc.), MMs consume chemical, photochemical, and electrochemical energy. Chemically-driven MMs are subjected not only to thermal noise but also to the principle of microscopic reversibility, according to which *at equilibrium* the forward and backward paths of a reversible reaction are equally likely to occur. In order to overcome microscopic reversibility, chemically-driven MMs cyclically switch between different mechanical states, a process known as 'chemical gating', during which the selective binding/unbinding of a catalyst allows the device to increase its chemical potential and modify the reaction rate constant in such a way that the reaction can follow only one path (forward or backward). As a result, the mechanochemical cycle of binding/unbinding a catalyst is the way in which chemically-driven MMs constrain a chemical energy input in order to carry out directional movement, do work, and bypass microscopic reversibility (Astumian 2012; Astumian et al. 2016).

molecular machine does combine thermal noise with structural anisotropy and energy (chemical, photochemical, and electrochemical) sources to do work. For this reason, MMs are also called 'Brownian ratchets' (Astumian 2002).

[20] Reynolds number is expressed by the ratio between $a\nu\rho$ and η ($R = a\nu\rho/\eta$); where a is the acceleration, v the velocity, ρ the density of the fluid, and η the fluid's viscosity (Astumian and Hänggi 2002, p. 33).

[21] Viscous drag force is the force exerted by a fluid on an obstacle around which it flows.

Unlike chemically-driven MMs, light-driven ones exploit the allosteric conformational change generated by exergonic reactions (known as 'power stroke') to allow light energy to maintain a non-equilibrium steady state, thereby permitting molecules to move between two separate energy surfaces (Astumian et al. 2016). Another significant difference between chemically and light-driven machines is microscopic reversibility, since the former are subject to microscopic reversibility whereas the latter are not (Astumian et al. 2016).

In light of all these factors, it is sensible to avoid a hasty analogy between MMs and all types of macroscopic machines. A careful analysis is therefore required to assess the question. In the following two subsections I will analyse the structural and physicochemical organisation of both artificial (Section 1.4.1) and biological (Section 1.4.2) MMs.

1.4.1 ARTIFICIAL MMS

Since the beginning of the 21st century, a host of molecular devices have been artificially developed for technological use in different domains (nano-medicine, green nanotechnology, etc.) and with very different purposes. Nanotechnology can be considered an extension of supramolecular chemistry, a new avenue opened up during the 1970s (Lehn 1995). Artificial MMs (also called 'supramolecular structures') are built by assembling a discrete number of molecular components with the aim of performing a function through the mechanical movement of their parts. Energy sources are provided by photochemical and electrochemical energy inputs that cause exergonic reactions[22], which in turn power these artificial nano-devices. Photochemical and electrochemical energy is transformed into mechanical work through a 'motor-like' part.

Unlike macroscopic machines, MMs are built by harnessing the intrinsic self-assembly capacities of certain molecular components, according to which these components bind together through non-covalent interactions in such a way that the final assembled structure is able to perform mechanical movements (linear, rotatory, oscillatory, etc.), thus enabling a specific function to be carried out. This method for building an MM is called bottom-up assembly[23]. Artificial nano-machines are based on rotaxanes, catenanes and other related structures (Sauvage and Dietrich-Buchecker 1999; Balzani et al. 2005) which are assembled by employing *non-covalent interactions* such as hydrogen

[22] Endergonic reactions can also occur, but they have to be thermodynamically coupled with exergonic reactions in such a way that exergonic reactions drive or power endergonic ones.

[23] By a 'bottom-up' approach to molecular machines, I mean the construction of nanoscale devices and machines using a molecule-by-molecule method (Balzani et al. 2005).

bonding, coulombic forces and metal-ligand bonding, among others. Rotaxanes are dumbbell-shaped molecules surrounded by a macrocyclic compound with a ball at each end; catenanes consist of two interlocked rings (macrocycles) (Balzani et al. 2005) (see Fig. 1.2).

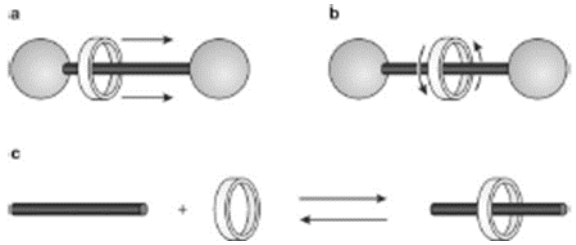

Figure 1.2 Interactions between a rotaxane and a macrocycle: a) ring shuttling, b) ring rotation, c) threading/dethreading equilibrium between a macrocycle and the axle of a pseudorotaxane (Credi et al. 2014, p. 6).

Like macroscopic machines, synthetic nano-devices carry out work and perform a function by virtue of the way certain molecular parts have been shaped and located so as to affect the relative motion of other component parts and, all together, harness the energy flow in a specific way. It is the *interlocked architecture* of the components (i.e. their design and structural co-dependence) that permits the overall system to *transform an energy input into work*, in order to perform a desired *function*. Like the links of a macroscopic machine, rotaxanes and catenanes generate *relative motion*[24] as the result of an energy input. Both mechanical movements and a variety of different functions of the molecular components of rotaxanes and catenanes are induced by external stimulation. For example, acid-base chemical inputs may strengthen or weaken the hydrogen bonding interactions that are responsible for assembly and spatial organisation. Another important physical constraint on the behaviour of rotaxanes and catenanes is represented by non-covalent interactions, since these interactions allow them to bind to one another reversibly. Since non-covalent interactions easily bind (and unbind) the component parts of a synthetic nano-device, supramolecular stability hinges on the control of these weak interactions. Thus, the basic principle underlying the construction of artificial MMs is the control of the non-covalent interactions that govern the relative mechanical movement of the building blocks so as to create a *functionally-*

[24] Rotaxanes and catenanes usually perform relative motion through the movements of rings, such as shuttling along the axle of the rotaxane dumbbell or rotation around another ring in a catenane structure.

integrated structure that is able to perform work, transport cargoes or signal molecules through molecular shuttles, etc. (Valero et al. 2017).

One example of artificial MMs is DNA nanotechnology (see Fig. 1.3), which combines rotaxanes, catenanes and related structures to create interlocked DNA structures that can be generated from both double-stranded and single-stranded DNA (Ackermann et al. 2010; Valero et al. 2017).

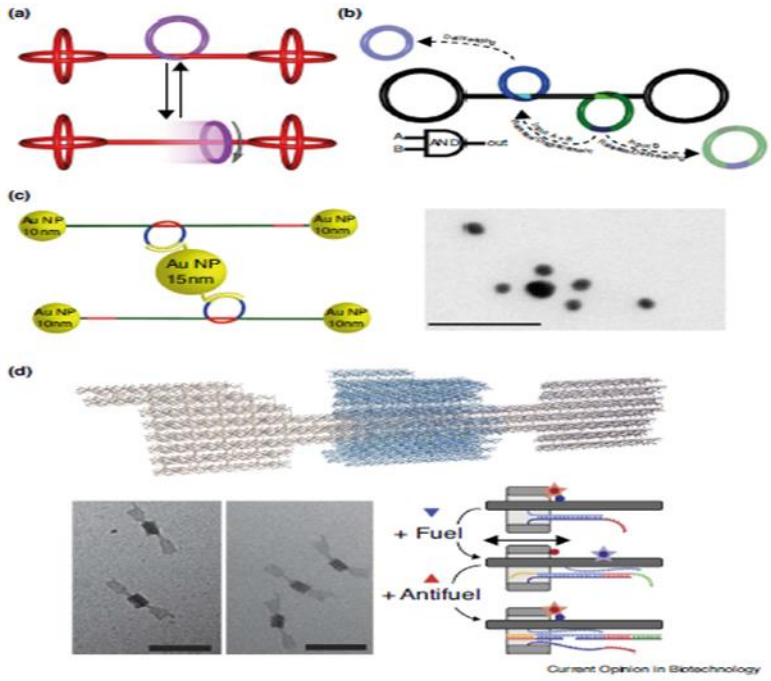

Figure 1.3 Representation of a DNA architecture a) double strand DNA rotaxane with spherical stoppers; b) controlled release of the rings; c) a gold (Au) nanoparticle hybridises two DNA rotaxanes; d) DNA origami rotaxane (Valero et al. 2017, p. 161).

1.4.2 Biological MMs

Biological MMs are a *subgroup of macromolecules* (mainly proteins) that are commonly found in both prokaryotic and eukaryotic cells. Noteworthy examples include molecular motors (such as dynein, myosin and kinesin), molecular pumps (such as transmembrane ATPases), molecular tweezers (such as DNA) and molecular switches (like rhodopsin)[25]. In the cellular environment, proteins are the molecular structures best suited to acting like 'machines', because their structure

[25] Whereas molecular motors are able to displace unidirectionally when powered by an external energy input, molecular tweezers hold items between their two arms. A molecular switch reversibly shifts between two or more stable states.

allows them to perform a wide variety of biochemical functions (from catalysis to cell signalling and signal transduction, and from cellular motility to ligand binding).

Here, I will analyse only *biomolecular motors* and *pumps*, since they are the best candidates to be considered MMs. There are two crucial features of biomolecular motors and pumps to take into consideration. First, like synthetic nano-devices, biomolecular motors and pumps emerge from self-assembly processes by harnessing the entropic effect generated by the translational displacement of the water molecules in the cytoplasm. Self-assembly occurs spontaneously if Gibbs free energy is negative (O'Mahoni et al. 2011). Since an increase in the entropy of the water molecules decreases their Gibbs free energy, the self-assembly process is stimulated within the cytoplasmic environment (Kinoshita 2016). And, second, since modularity lies in the fact that biomolecular motors and pumps are proteins, and proteins consist of modular parts (Trifonov and Frenkel 2009; Rorick and Wagner 2011), another important feature of most of MMs is modularity. As a matter of fact, they consist of a number of subunits, each with a specific size and form, which are integrated in order to keep the global structure stable and transform chemical energy into mechanical work by means of a mechanochemical cycle. Three examples of biomolecular motors are myosin, kinesin and dynein, on the one hand, and an example of pump is the F_0F_1ATPase, on the other.

Kinesin, myosin and dynein[26] are polymers generated by the self-assembly of their respective monomers. The movement of these biomolecular motors is due to a series of mechanochemical cycles during which a phosphoryl group, removed by ATP hydrolysis, causes a rearrangement of the elements of the ATP-binding site in the globular head, which in turn triggers structural changes in the track binding site. Next, the electrochemical energy generated by the motor domain is transduced by the neck domain into mechanical work by producing movement. When a phosphoryl group is released, a conformational change occurs in the globular head and the mechanochemical cycle ends.

F_0F_1ATPase (see Fig. 1.4) is a protein located in the inner mitochondrial membrane, which is synthesised by assembling a number of monomers into eight subunits and two functional regions (F_0 and F_1). Since the function of regions F_0 and F_1 is likened to that of the *stator* and *rotor* (respectively) of an electric motor, F_0F_1ATPase is considered a vivid illustration of a biomolecular motor. The F_0 subunits channel a proton flux, determined by an electrochemical gradient, which is

[26] Dynein is a protein that transports cargoes along microtubules in a cell by exploiting retrograde transport. Myosin is a protein that allows muscle contraction by interacting with actin. Kinesin is a protein that transports cargoes by sliding down microtubule filaments (anterograde transport).

exploited to allow F_1 to rotate. The rotatory movement is not random (but rather directed by subunits a and c of F_0) and determines the conformational change of subunit β of F_1, thus enabling the synthesis of ATP molecules (see Fig. 1.5).

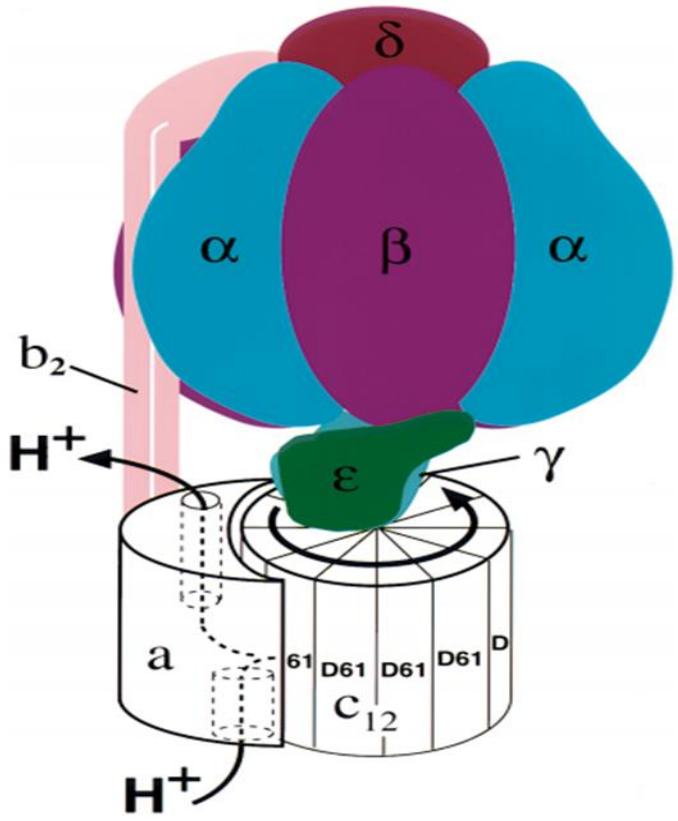

Figure 1.4 Regions, subunits, and rotatory movement of F_0F_1ATPase: F_0 region (subunits a and c), F_1 region (the other subunits) (Wilkens 2000, p. 338).

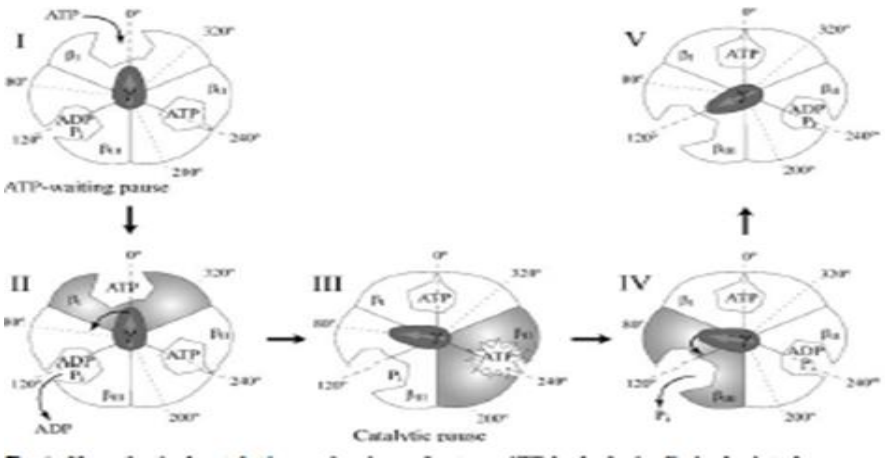

Figure 1.5 Conformational changes of the subunit β of F1 in order to synthesise ATP molecules (Feniouk and Yoshida 2008, p. 283).

A biological MM exists and performs work not only because of self-assembly and modularity, but also due to three structural principles. First, reactions occurring in the different subunits are *sequentially ordered* so as to form a clear-cut biochemical pathway. Second, macromolecular conformational change, which is allosterically regulated, is *temporally coordinated* with the reactions occurring in the other subunits of the protein complex. And finally, and this is the most important point, the overall function of a biomolecular machine depends on its *relationship with other biological molecules* that are present in the biochemical network of the cell. These structural principles underlie the behaviour of all biomolecular machines. By way of example, let me again consider F_0F_1ATPase. The rotation of the γ subunit of the F_1 region may occur only if the subunits of the F_0 region have previously constrained the proton flux towards the F_1 region (*sequential order*). In order to produce three ATP molecules, the rotatory movement of F_1 must be coupled with the conformational change (three states) of subunit β, in such a way that the phosphorylation of ADP generates an ATP molecule (*temporal coordination*). It is important to stress that the rotation of F_1 must be coupled with ATP hydrolysis, otherwise, a futile cycle occurs without ATP production. Finally, since the electrochemical gradient proton flux through the ATP synthase depends on the electron flux produced by the electron transport chain, the overall function of F_0F_1ATPase hinges on the biochemical pathways established in the protein complexes of the electron transport chain (*relationship with other biological molecules*).

The *interdependence* between a biological MM and the cell network is a key aspect that distinguishes MMs from artificial nano-devices. The *functional integration* of a biomolecular machine into the cellular network is a crucial organisational feature that makes it difficult to separate a biomolecular machine from its biochemical network, while at the same time explaining why artificial molecular machines are still a long way from being similar to biological ones. Biological MMs are embedded in a biochemical network in such a way that they appear functionally integrated into other biomolecular machines or biological macromolecules. This third characteristic is a key difference between artificial nano-devices and biomolecular machines, because synthetic nano-machines have not so far been incorporated into artificial biochemical networks. Consequently, whereas the energy input of biomolecular motors is constantly provided by the biochemical network in such a way that biological machines regenerate, synthetic nano-machines cannot do this, and therefore need an opposite input to reset (Balzani et al. 2005; Credi et al. 2014).

1.5 MACHINE-LIKENESS AT THE NANOSCALE

In the previous sections, the analysis of the structural and physicochemical conditions required by macroscopic and molecular machines has revealed that both types share a fundamental similarity in their organisation, since both are meta-stable structures consisting of functional parts that constrain an energy input so as to perform work and, therefore, fulfil a systemic function. This similarity is the main reason why a machine-based terminology is so widely used in the specialist literature to characterise these types of artificial and biological molecular systems.

Admittedly, this is not a sufficient reason to dismiss the importance of the differences which exist between classic macroscopic machines and their molecular analogues. As a matter of fact, several critical voices have recently raised fundamental objections to the consideration of molecular devices as machines. To be fair, however, these criticisms are directed mainly at biological molecular machines, and fail to address (explicitly, at least) the case of their artificial counterparts. Yet, since many of these criticisms discuss aspects linked purely to scale differences, I believe they implicitly include a rejection of the adequacy of a machine-based terminology to describe artificial molecular devices also. In this section, I will discuss the criticisms levelled by three authors: Moore, Skillings and Nicholson, before presenting my own view of the question. Whereas the arguments espoused by the first two authors focus exclusively on scale differences (and therefore, even though they only explicitly discuss the case of biological MMs, their arguments encompass artificial MMs also), Nicholson's criticism raises questions which pertain only to biological MMs. Thus, in my own analysis, I shall attempt to distinguish which part of the discussion specifically concerns only the biological case.

In a paper published in 2012, Peter Moore argues that macromolecules cannot be considered molecular machines because they are subject to physicochemical forces that are different from those of macroscopic machines, a circumstance which makes the analogy between macroscopic machines and macromolecules inappropriate. Moreover, he adds that 'the use of the word "machine" is pernicious because of its implication that the functional properties of macromolecules can be explained mechanically, which is simply not true' (Moore 2012, pp. 7-8). Moore is certainly right in claiming that the physicochemical laws underlying macroscopic machines are different from those of microscopic macromolecules, because a different size scale entails a great difference both in the structure and in the functions performed by these two kinds of device. As seen in Section 1.4, the behaviour of both biological and synthetic macromolecular devices is influenced by viscous

forces, thermal noise and potential energy differences in the free-energy landscape of macromolecules, etc. Together, these factors make the behaviour of these macromolecular structures *probabilistic,* not deterministic, because the laws of quantum mechanics replace Newton's laws of mechanics. In this sense, Moore is right in saying that the expression 'Brownian ratchet' should not be read in the deterministic sense of Newtonian mechanics (Moore 2012, p. 10), but rather as a linguistic label to simplify the interplay between structural anisotropy and an energy input to harness thermal noise. The criticism levelled by Moore (2012, p. 7) at 'structure-based movies'[27] of macromolecules is also fair, insofar as they are indeed an oversimplification of how real macromolecules (e.g. ribosomes, myosin, dynein, F_0F_1ATPase and so forth) generate motion and carry out work. In other words, Moore is right in claiming that the directional movement of macromolecules is not the same as that of a macroscopic machine (a car, for instance), because motion at the nanoscale is stochastic, not deterministic.

However, I do not agree with Moore's argument that these differences preclude the possibility of talking about (certain types of) macromolecular systems in terms of machines. Although they are indeed different from macroscopic machines due to the action of diverse physicochemical forces, they nevertheless share a common organisation. As we have seen, both macroscopic machines and the microscopic (biological as well as synthetic) devices studied so far are characterised by a number of *functionally-ordered component parts* that act as *constraints* on an energy input in order to do (useful) *work*. Moore (2012, p. 9) maintains that the operation of the component parts of a macromolecular 'machine' (e.g. the two subunits of a ribosome) are not directly related to their function because thermal fluctuations 'separate one functionally significant event from the next' (Moore 2012, p. 9). Thermal noise indeed distinguishes between macroscopic and microscopic causal sequence (which is *deterministic* in the former and *probabilistic* in the latter), but this does not prevent the global result of the device from being explained in terms of a specific sequence of functional operations. Hence, the specific way in which a macromolecular device behaves (e.g. the ribosome function of synthesising peptides) is due to the sequential organisation of a number of functions that are locally performed by the component parts of that same macromolecular device (e.g. the two subunits of a ribosome). Like macroscopic machines, microscopic ones carry out *systemic functions* by virtue of the *organisation of the local functions* fulfilled by their component parts.

[27] By this term Moore means all those pictures that depict the motion of macromolecules as a linear movement produced solely from their component parts.

The aim of Skillings' (2015) paper is to show the limits of the basic mechanistic account in explaining molecular processes and to propose a larger mechanistic framework in terms of multidimensional gradient. He does not openly criticise the idea of machine-likeness at nano-scale. However, he makes a comparison between macroscopic mechanical machines (such as a watch) and macromolecules (such as a ribosome) and he claims, in line with Moore (2012), that "the movements and the interactions of the parts of the watch explain how the watch works. The parts of a protein, like a ribosome, do not stand in the same relations as the parts of a mechanical clock" (Skillings 2015, p. 1145). Although this is undoubtedly correct, I find that it may lead to a misleading idea of 'machine' which is based on a (macroscopic) mechanical machine (like a watch). As I have already emphasised in section 1.2, a machine is a meta-stable structure consisting of interdependent parts which constrain a flow of energy and matter in order to do work and perform a systemic function. Accordingly, a machine is a kind of structure that encompasses different types of macroscopic and microscopic systems and, therefore, cannot be reduced to a (macroscopic) mechanical machine. In other words, both Moore's (2012) and Skillings' (2015) papers correctly criticise a rough analogy between (macroscopic) mechanical machines and MMs. However, these papers give the impression (Moore more explicitly, whereas Skillings implicitly) that it is *wrong* to consider artificial nano-devices, biomolecular motors and pumps, and ribosomes as machines at all. I argue that a broader, but at the same time more precise, definition of 'machine' does not prevent us to regard this subset of macromolecules as machines.

In addition to Moore's and Skillings' arguments, Nicholson (2013) also maintains that, if biological macromolecules were machines, they should have an organisation created by an intelligent designer, since "confronted with a machine, one is justified in inferring the existence of an external creator responsible for producing it in accordance with a preconceived plan or *design*" (Nicholson 2013, p. 671). Nicholson's claim can be dismissed by arguing that the existence of an intelligent designer is a necessary condition for achieving functional organisation in *man-made machines* (and for defining what is a useful task), but neither the existence of functional tasks nor the origin of the order of the (sub)functions involved in such tasks require an intelligent designer in *biological systems*. These two aspects may be explained by bearing in mind that biological systems are a very special form of *self-sustaining organisation*, capable of harbouring functional differentiation and undergoing an evolutionary history.

In a recent paper, Nicholson (2018) criticises the analogy between machines and organisms by offering some arguments taken from thermodynamics. Even though the paper focuses on organisms

as wholes, it is possible to apply some criticisms of the machine-likeness of living beings to biological macromolecules. Nicholson argues that there are three important differences between machines and biological organisations. First, "organisms have to constantly exchange energy and matter with their surroundings in order to maintain themselves *far from thermodynamic equilibrium*. Machines, on the other hand, exist in equilibrium or near-equilibrium conditions, and consequently do not have to constantly exchange energy and matter with their surroundings" (Nicholson 2018, p. 144). Second, machines are characterised by static stability (i.e. they do not need an energy input to preserve their structure), whereas biological organisations "exhibit a *dynamical stability*, which is based on their capacity to actively maintained a low-entropic 'steady-state'" (Nicholson 2018, p. 144). And third, the activity of a machine is temporary because of its switching on/off, while "the actively-maintained steady-state of an organism is *fixed and irreversible*" (Nicholson 2018, p. 144). Despite being correct, these remarks do not preclude the fact that, within a biological system as a whole, there are parts which exhibit a certain degree of stability in near-to-equilibrium conditions (i.e. self-assembling complex structures) and that, in particular, some biological macromolecules – notably *biomolecular motors* and *pumps*- have features (i.e. being near thermodynamic equilibrium, exhibiting static stability and temporary activity, etc.[28]) that allow them to be talked about in terms of machines. Thus, I believe that, despite all the aforementioned differences, these features (being near thermodynamic equilibrium, exhibiting static stability and temporary activity) allow us to subsume both macroscopic and microscopic man-made machines and a subset of macromolecules into the concept of 'machine'.

Nicholson is right to point out that biomachines exist within and hinge on a dissipative and autonomous organisation. If biological MMs exist, it is because they contribute to creating and maintaining a network of dependencies, namely a true 'closure of (macromolecular) machines', and this global network (i.e. the cell) exists in far-from-equilibrium conditions (Winning and Bechtel 2018). As a result, in spite of being precarious dynamical macromolecular structures, biomachines are relatively stable, since they are produced, regenerated and repaired within a network that they in turn create and support. Furthermore, as I acknowledge in Section 1.4.2, biomachines also

[28] One good example of this is how F_0F_1ATPase behaves in *brown adipose tissue*. The presence of an *uncoupling protein* (UCP) within the inner mitochondrial membrane dissipates the proton gradient generated by the complexes of the electron transport chain. When the UCP channel is open, no proton flux goes through the F_0F_1ATPase and, therefore, there is no production of ATP molecules, but rather heat production. In this case, the structure of the F_0F_1ATPase biomolecular machine is maintained, even if the machine does not work and performs no function (i.e. the production of ATP molecules).

perform their functions in so far as they coordinate their operations with many other biological processes.

1.6 CONCLUDING REMARKS

I have argued that, despite important differences derived from the change of scale, large molecular structures (sometimes, in the form of modules (Raanan et al. 2018)) may be either artificially or naturally assembled into an ordered whole, so as to perform a potentially useful activity. At the microscopic scale, the building blocks that need to be assembled to form the global functional device (i.e. 'the machine') are not inert parts, but *intrinsically-active entities*, which either human engineers or cellular machinery harness so as to achieve a suitable arrangement. In synthetic bio-engineering, different intrinsically-active macromolecular structures are harnessed to (once assembled) produce certain desired patterns of activity. Moreover, many of these patterns of activity are similar to those of biomolecular motors (myosin, kinesin and dynein) and pumps (ATPases), such as myosin, kinesin and dynein. For all these reasons, I conclude that scale-related differences do not justify dismissing the status of these devices as machines, and that both synthetic and some natural molecular devices can rightly be characterised as such since, ultimately, they are functionally-ordered sets of functional parts that, together, constrain a flow of energy so as to produce a new, more complex and integrated function. Moreover, as in macroscopic machines, in both synthetic and biological macromolecular devices, the combination of functional parts to produce new ordered wholes results in an open domain of functions.

However, here is where the specificities of biological macromolecular machines emerge. As I have stressed, whereas synthetic molecular machines exhibit a pattern of activity that is defined by an external intelligent agent, natural ones define their patterns of activity as a result of the organisation of cell's biochemical network. Since biological MMs perform a function by cooperating with many other similar devices within the biochemical network of the cell, they either support the maintenance of the global cellular organisation or, sooner or later, disappear. As has been pointed out by Arnellos and Moreno (2012), the functionality of cellular macromolecules is maintained by a set of mutually-dependent functional structures. Moreover, since biological MMs are highly vulnerable and constantly need to be supplied with energy, they can be maintained only through operations of repair and reproduction (Collier and Hooker 1999). The activity generated by macromolecular structures must be harnessed to produce and continuously repair the system in

which they are in turn built (Winning and Bechtel 2018). Recently, Bechtel (personal communication) has pointed out that one crucial difference between *synthetic* and *biological* molecular machines is that the former display a pattern of activity which becomes functional only through the external action of human beings who put them into a socially-defined system, whereas biological machines, which are intrinsically autopoietic, become functional by virtue of being produced by (and contributing to the maintenance of) a metabolic organisation.

The reason for this co-dependence between natural molecular machines and the cellular metabolic organisation is that, in a natural context, their respective origins can only be explained in terms of *co-evolution*. On the one hand, the functionality of biological molecular machines evolved because they were incorporated into a self-maintaining (SM) system; and on the other, the evolution of the overall dynamics of a SM system is intrinsically linked to the increase in structural and functional complexity of its biological molecular machines. Although geological or other types of abiotic processes played a pivotal role, biological molecular devices only began to perform functional activities within SM systems. Moreover, an SM organisation of mutually-dependent constraints ensured the self-maintenance of biological molecular machines.

Biological machines are highly precarious and their maintenance depends on the maintenance of other cellular mechanisms (i.e. the degradation and replacement of proteins). As I will further show in chapters 2, 4, 5, and 6 of this thesis, molecular machines (e.g. cytoskeletal motor proteins and rotary ATPases) play a fundamental role in the *functional integration* of cells. On the one hand, the cell's biochemical network is maintained by the specific contributions of each machine; and on the other, each biological MM is maintained by its participation in a largely distributed, far-from-equilibrium network (the set of processes and machine activities that constitute the cellular metabolism). The core organisation of biological systems (the living cell) is constituted by a host of molecular machines that participate reciprocally in their respective processes of fabrication, maintenance and operation. I will call this organisation a *functional integration of macromolecular machines*.

The fact that, as pointed out in section 1.2, artificial and biological machines are embedded, respectively, in a social and in a biological context is at the root of their *functional complexity*: even though each component part of a machine plays a *functional role* in constraining a flow of energy and matter, the *systemic function* (or *mechanism*) of a machine is something *new* and *not reducible* to the singular operations of the parts of the machine. The interesting role of machines is that they allow an increase of the functional complexity of the organisation where they are produced and to

which they contribute to maintain. The *organised* disposition of the components in a meta-stable structure produces *a new systemic function* that is different from the underlying sub-functional actions of these components.

Mossio and Moreno (2010) and Moreno and Mossio (2015) have developed the idea that the specific causal regime of living systems is a closure of constraints. Ultimately, this is an extremely difficult task, since the coordination of a complex set of constraints requires regulatory control of the biochemical network of the cell which is established by different molecular mechanisms and biological MMs (Bich et al. 2016, Winning and Bechtel 2018). Here I have argued that a machine is a complex, functionally-ordered set of constraints that together act as a whole, generating a new functional activity.

In sum, the appearance of machines was of paramount importance in prebiotic and biological evolution, because it opened up a new domain of *functional diversification*: new forms of mechanistically-complex functions could be achieved through different combinations of parts. Without the concept of machine, we could not understand how primitive self-sustaining chemical networks progressively achieved higher degrees of complexity, generating new domains of integrated functions on the basis of an ordered combination of functional molecular modules.

CHAPTER 2 FUNCTIONAL INTEGRATION BETWEEN REGULATORY SUBSYSTEMS, THE CONSTITUTIVE REGIME, AND SIGNALLING SUBSYSTEMS

2.1 INTRODUCTION

The first chapter has shown that *biological nanomachines* are mutually dependent on the mechanisms of the cellular *metabolic organisation*. Protein synthesis and metabolism are key components of the constitutive regime (C)[1] of a cell, insofar as they contribute to, respectively, the regeneration of cellular components and the control of the flux of energy and matter between the cell and the environment, thus enabling the cell to self-maintain. The activity of the constitutive regime is strictly *regulated* by several molecules (notably proteins) in such a way that the metabolic system satisfies the cellular physiological requirements in relation to the extracellular concentrations of nutrients or changes in environmental conditions. More specifically, gene expression (i.e. transcription and translation) and enzymatic activity are tightly regulated in order to modulate *metabolic* and *developmental* processes as a response to extracellular and intracellular signals. It has been argued that the set of the entities performing regulatory mechanisms (R) is a *subsystem*[2] acting upon and modulating the entities of the constitutive regime, giving rise to a *hierarchy of mechanisms* (Bich et al. 2016). Indeed, the functional relationship between the regulatory mechanisms and the constitutive processes is *asymmetrical*, because the former *directly constrain* the action of the latter, but the reverse is not true (Bich et al. 2016).

Despite this asymmetry, the regulatory subsystem and the constitutive regime exhibit a kind of *functional integration*, inasmuch as "the regulatory subsystem R is produced and maintained by the activity of the constitutive organisation C, whose dynamics is, in turn, modulated by R" (Bich et al. 2016, p. 255, footnote 21). Nevertheless, the relationship between C and R is mediated by a *third*

[1] All along the thesis, when I use the term "constitutive process", I refer to a biochemical process that differs from an interactive one. As such, constitutive processes include metabolic, genetic, regulatory, developmental, and reproductive processes. Nonetheless, in chapter 2, I employ the expression "constitutive regime" to designate the most basic dimensions of the constitutive processes of a cell: *metabolic* and *genetic* (i.e. DNA replication and gene expression) *functions*. I therefore use "constitutive regime" in the same sense as Bich et al. (2016).

[2] For the sake of simplicity, when I use the term "subsystem", I do *not* designate a part of the cell that is spatially separate from the rest, but rather a set of entities that perform the *same kind of mechanisms* (e.g. the subsystem of regulatory mechanisms or the subsystem of signalling mechanisms).

subsystem, the *signalling* one (S), the organisational contribution of which has not yet been fully investigated, thus leading to a *conceptual gap* in how C and R are functionally integrated. Thus, the *aim* of this chapter is *threefold*: first, to examine how R, C, and S are functionally integrated; secondly, to evaluate the physiological systemic consequences of the functional integration between R, C, and S for the cell; thirdly, to explore the evolutionary effects of the functional interdependence between R, C, and S especially with regard to eukaryogenesis.

In order to accomplish this purpose, I will examine how the regulation of transcription and enzyme activity are functionally linked to *metabolism, DNA replication, transcription, and translation* (i.e. the constitutive regime) and to the signalling subsystem in *bacteria*. I will also explore the systemic and evolutionary effects produced by this functional interdependence. The choice of this case-study is due to a basic reason: bacteria exhibit a minimal –but sufficiently complex- functional integration between R, C, and S, which represents the common core of functional integration in the prokaryotic and eukaryotic cell.

In the light of the above, the research questions of this chapter can be framed as follows:

1) How are the entities performing the mechanisms of the constitutive regime *functionally integrated* with those of the regulatory and signalling subsystems?

2) How does the functional integration between these three organisational levels affect the systemic capacities of bacterial cells?

3) What are the evolutionary effects of this functional integration, especially as regards eukaryogenesis?

I argue that the functional integration between the regulatory subsystem and the constitutive regime consists in a regime of *organisational closure* in which R directly constrains (i.e. modulates) C, and C can constrain R by means of the synthesis of the components of R and through the activity of S. Indeed, R is endogenously synthesised and regenerated by C and triggered by S, which is usually produced by C. An understanding of the functional integration between R, C, and S has two far-reaching explanatory consequences: first, it clarifies a fundamental organisational dimension of *functional integration* that is common to prokaryotes and eukaryotes; second, it provides a theoretical basis for understanding the evolution of complexity and the achievement of *new forms* of *functional integration* in eukaryogenesis.

This chapter is organised as follows. In section 2.1, I critically review some accounts that have explored the relationship between regulatory subsystems and the constitutive regime, devoting special attention to the core concepts of ‘constraint’ and ‘closure of constraints’. Then, in section

2.2, I analyse how regulatory subsystems and the constitutive regime are functionally integrated by studying the regulation of transcription and chemotaxis in bacteria. In section 2.3, I discuss the organisational role of the signalling system in the achievement of functional integration between R and C. Finally, section 2.4 offers some concluding remarks.

2.2 THE CONSTITUTIVE REGIME AND BIOLOGICAL REGULATION

Over the last decade, a number of works –usually grouped as the 'organisational account'[3]- have explored some basic biological properties that allow living beings to self-sustain and regenerate their constitutive components (Mossio et al. 2009; Mossio and Moreno 2010; Moreno and Mossio 2015). In particular, it has been argued that the processes that allow a biological organisation to self-sustain (i.e. the constutive processes) are constrained by regulatory subsystems that are under the influence of extracellular and intracellular signals (Bich and Moreno 2016; Bich et al. 2016). This section is aimed at critically reviewing the functional relationship between the *constitutive regime*, the *regulatory subsystems*, and the *signalling subsystem* by examining some crucial organisational features of each of them.

First of all, let me begin by defining what is a biological constraint and its role in the organisation of living beings. Basically, a constraint[4] is a structure that exert a *causal* role upon a *process* (i.e. a set of physicochemical changes) occurring in a biological system, at the *time scale* of the process[5], without being affected by it. This means that the action of a constraint is not directly affected by the dynamics of the process that it regulates (Mossio and Moreno 2010; Mossio et al. 2013; Moreno and Mossio 2015). Constraints harness a flow of energy and matter so as to keep living beings far from thermodynamic equilibrium and allow organisms to self-maintain. Constraints can be *externally* or *endogenously* produced: in the former case (e.g. when constraints are boundary conditions or restrictions in the configuration space), their existence does not depend on the

[3] The organisational framework is closely connected with the autopoietic tradition (Varela 1979; Maturana and Varela 1980, 1984; Rosen 1991; Kauffmann 2000) that considers living beings as organisations that produce their own components according to their own (biological) norms.

[4] The concept of 'constraint' was introduced in analytical mechanics "to characterise whatever auxiliary conditions must be appended to the fundamental equations of motion in order to predict more easily how a system will behave. […] [It] is simply some additional regularity or order which is not explicitly found in the initial conditions" (Pattee 1971, p. 161). These "auxiliary conditions" are exerted by specific structures that *reduce the degrees of freedom* of the system upon which they act (Pattee 1972).

[5] As pointed out by Moreno and Mossio (2015), the temporal scale of a constraint must be consistent with the processes upon which it acts. For example, the transformation of a substrate into a product and the enzyme catalysing this reaction must be at the same time scale (i.e. nanoseconds).

dynamics upon which they act; in the latter case (e.g. when constraints are enzymes or intermembrane proteins), the constraint is generated within the system upon which it is placed and "may possibly play a role in generating another constraint in the system, although no mutual dependence is realized" (Moreno and Mossio 2015, p. 5).

The expression 'constitutive organisation' denotes the set of entities and physic-chemical mechanisms that are involved in the *production, transformation and reparation* of the system, therefore determining its *identity* over time (Moreno and Mossio 2015). The constitutive organisation relies on (at least) three kinds of constraints: first, the *kinetic constraints* (e.g. enzymes) that modulate the rate of anabolic and catabolic reactions; second, the *spatial constraints* (e.g. membranes and intermembrane proteins) that selectively control the flow of nutrients and waste products through the cellular boundary; finally, the *template constraints* (i.e. DNA and mRNA) that control the transformation of sequences of amino acids into proteins (Mossio et al. 2016; Bich 2018). Each of these constraints depends on the others, thus leading to a *functional interdependence* among them: indeed, the synthesis and turnover of the proteins exercising a kinetic and spatial control hinge on the activity of DNA and RNA; likewise, the mechanisms of gene expression are constrained by kinetic constraints (e.g. DNA polymerase, RNA polymerase, etc.) and are sustained by a metabolic organisation that depends on the action of kinetic constraints. Metabolic processes, in turn, can occur by virtue of the selective control on the fluxes made by membranes and membrane machinery (Moreno and Mossio 2015).

In biological systems, the functional interdependence between a number of constraints is organised in a circular way (i.e. a '*closure*') which is aimed at channelling a flow of energy and matter (i.e. a thermodynamic flow) and permitting the self-organisation and self-maintenance of the overall system (Mossio and Moreno 2010; Moreno and Mossio 2015; Montévil and Mossio 2015). In very general terms, a set of constraints C realises closure if, for each constraint C_i belonging to C: 1) C_i depends on at least another constraint of C; and 2) there is at least another constraint C_j, belonging to C, that depends on C_i (Moreno and Mossio 2015, p. 20; Montévil and Mossio 2015, p. 186). The actions of different interdependent constraints occur at different *time scales*, in the sense that each constraint is conserved at the time-scale of the process upon which it acts, but it is temporally independent from other constraints related to it. As an example, the action performed by an enzyme on a metabolic process and its synthesis and turnover, which is performed by ribosomes and mRNA (*template constraints*), occur at different time scales (see Moreno and Mossio 2015, p. 19).

In order to cope with metabolic needs and respond to changes in environmental conditions, the constitutive regime is *regulated* by a number of molecules (particularly proteins) that collectively form the *regulatory subsystem*. Although biological literature usually identifies biological regulation with feedback mechanisms (Heinrich and Schuster 1996; Wolkenhauer and Mesarovic 2005; Tsokolov 2010; Konieczny et al. 2014), Bich et al. (2016) have argued that feedback mechanisms, in spite of playing a regulatory role, cannot be considered a regulatory subsystem in itself, because they are not functionally separated from the structures (e.g. enzymes) that they control. While feedback mechanisms depend on the processes that they control (e.g. an enzyme depending on the stoichiometric concentrations of the metabolic substrates and products the metabolic catalysis of which it constrains), a *regulatory* subsystem controls the activity of another, *regulated*, subsystem without being directly affecting by it. As such, the regulatory subsystem can be considered a second-order constraint directly acting on the first-order constraints of the constitutive regime (Bich et al. 2016). In line with this account, I will consider as 'regulatory subsystems' only those that do *not directly* depend on the constitutive subsystem and that exhibit a hierarchical organisation with regard to it.

In order for a system to be regulatory, it must satisfy five organisational requirements (Bich et al. 2016). First, the entities performing regulatory mechanisms R must be *endogenously produced* through gene expression by the constitutive regime C. Indeed, the proteins performing regulatory functions are intracellularly synthesised by genetic transcription and translation that are controlled by template constraints. Second, R must be *decoupled* from C, in the sense that "one or more variables in the regulatory subsystem are not directly dependent on the constitutive regime"[6] (Bich et al. 2016, p. 255). As a result, the regulatory subsystems exhibit a *high degree of freedom* with respect to the constitutive regime, in such a way that regulatory subsystems and the constitutive regime work at different rates and are not "directly dependent on each other" (Bich et al. 2016, p. 254)[7]. Third, R is activated by specific *signals* or *perturbations* occurring in either internal or external conditions, "rather than by a change in the concentration of the components in R" (Bich et al. 2016, p. 256). Fourth, regulatory subsystems must play a *functional role*, insofar as their goal is "to shift (either reversibly or irreversibly) between distinct constitutive/metabolic regimes C, C', C''…

[6] More specifically, this means that the activities of the regulatory subsystems do not depend, stoichiometrically, on the production of the regulatory subsystems made by the constitutive regime, although this latter is "responsible for the presence and amount of R [the regulatory subsystems] in the system" (Bich et al. 2016, p. 254).

[7] The decoupling between R and C is a necessary condition for defining regulation and this is the reason why negative feedback mechanisms, which are usually considered as examples of regulation, are discarded by Bich et al. (2016).

available to the system" (Bich et al. 2016, p. 256) and, consequently, contribute to the maintenance of the system. Fifth, the influence of R on C allows C to respond to a range of perturbations and cope with *new biological conditions*.

A key aspect of biological regulation is that it ultimately depends on a wide set of *extracellular* and *intracellular signals* that trigger a response in the entities performing regulatory mechanisms[8] In order to be perceived as a signal, a molecule (first messenger) needs to be recognised by another molecule (the receptor) so as to be transduced and to produce an output by means of a series of second messengers (a 'signalling cascade'). Signals exhibit some important organisational properties: first, in order to be identified as a signal, a molecule (*first messenger*) needs to be recognised by a cellular *receptor*. Second, when signals have to cover long distances (e.g. extracellular signals), they need to be encoded and decoded by a *signal transduction machinery*, which involves kinase cascades and positive feedback loops, in order to have a sufficient specificity and therefore overcome local distortions and random effects. Third, extracellular signals may be *amplified* when a single signalling molecule elicits a response involving a huge number of molecules. Fourth, a single receptor cannot respond to contradictory signals, simultaneously up-regulating and down-regulating a process (Konieczny et al. 2014). Hereinafter, when I use the term 'signalling system', I will refer to the subsystem consisting of *extracellular* and *intracellular signals* and the *signal transduction machineries*.

In the light of the above, I can draw three important conclusions. First, the current philosophical (i.e. organisational) literature has carefully studied the relationship between the constraints of the constitutive regime and those of the regulatory subsystems: the constitutive regime is involved in the production of the (constitutive) components of the regulatory subsystems; the latter modulates the rate of or activates/inactivates the activities of the constitutive processes. Second, the organisational role of the *signalling system* and its relationship with the regulatory subsystems and the constitutive regime has not been conceptualised, thus leaving a theoretical gap between these three fundamental dimensions of a cell. Finally, it seems apparent that R, C, and S somehow work in an *integrated* way, in order to allow the overall cell to self-maintain and respond to environmental perturbations. Nonetheless, the features of this functional integration have not yet been

[8] In the case of unicellular organisms (i.e. bacteria, archaea, and protists), signals can be both by-products (i.e. metabolites) produced by intracellular or extracellular metabolic processes and chemical elements (e.g. oxygen, minerals, etc.) that interact with a receptor that transduces the signal, thus triggering a regulatory response. In the case of multicellular eukaryotes, extracellular signals mostly consist in hormones, neurotransmitters, and cytokines which are produced in specific cells and released into the extracellular space.

investigated. Accordingly, in the following section, I will analyse how signals, the regulatory subsystems, and the constitutive regime are functionally connected in bacterial cells.

2.3 FUNCTIONAL INTEGRATION BETWEEN REGULATORY SUBSYSTEMS AND THE CONSTITUTIVE REGIME

In order to cope with environmental variations and sustain metabolic and growth processes, bacteria use two important regulatory strategies: first, they regulate the *biosynthesis* of their constitutive components (i.e. proteins) through *gene regulation* (notably *transcriptional* and *translational regulation)*; secondly, they activate or inhibit their constitutive components (i.e. enzymes) through *enzyme phosphorylation.*

Both the regulation of gene expression and enzyme phosphorylation are triggered by *intra* and *extracellular signals*. Intracellular signals include metabolites [9] and some proteins [10] that are endogenously synthesised by cytosolic enzymes and that indicate the status of the intracellular environment. Endogenous signals, which directly bind to regulatory proteins (e.g. the allosteric site of transcriptional factors), can be induced in response to external signals and are often involved in the regulation of metabolic pathways. Exogenous signals, which provide the cell with information on environmental conditions (e.g. the presence or absence of nutrients), include *metabolites* and *by-products*, which are produced by other cells [11]. These signals are carried to the bacterial cytoplasm through a number of *outer transporters* (notably porins) and, then, they are transmitted to the regulatory proteins through *signal transduction machineries* (Martínez-Antonio et al. 2003).

Since gram-negative bacteria (like E. coli) have two membranes (external and internal) separated by the periplasm, extracellular signals can penetrate in the periplasm only by passing through outer membrane proteins which contains selective channel proteins, the most numerous and important of which is formed by the porin proteins. Porins are substrate-specific, ion-selective, or also nonspecific channels that regulate the influx of small hydrophilic nutrient molecules and the efflux

[9] The term 'metabolite' refers a wide range of small molecules that are the end intermediate products of metabolism. The metabolite effectors affecting the transcriptional activity of E. coli include sugars (e.g., galactose or pyruvate), nucleotides (e.g., ATP or cAMP), amino acids (e.g., alanine or tyrosine), vitamins (e.g., biotin-5'-AMP or vitamin B12), ions (e.g., sodium (Na^+) or magnesium (Mn^+)), and others (e.g., formate or sulfur) (Martínez-Antonio tal. 2003, p. 745).

[10] An example is provided by some proteins (FNR SoxR, and OxyR) that sense changes in the oxide-reduction state of the cell (Martínez-Antonio et al. 2006).

[11] A cell can also receive many important informations on the environment through the so-called "environmental cues", which are not produced by other cells, but rather by the environment. Examples of environmental cues are chemical elements, changes in temperature, pH, and osmotic pressure.

of waste products, therefore playing a pivotal role in the passage of metabolites (i.e. extracellular signals). For example, glucose is transported by diffusion within the bacterial cell through porins as a passive process when the concentration of extracellular glucose is high (Nikaido 2003; Shimizu 2016).

In order to pass from the periplasm to the cytoplasm, extracellular signals need some proteins that concomitantly *transport* and *transduce metabolites*. A common type of bacterial transduction machinery is a two-component system[12] in which one protein, a *sensor kinase*[13], phosphorylates a *second protein* (i.e. a response *regulator*) that *triggers a regulatory response*. Phosphorelays (or phosphotransferase systems) are more complex versions of two component systems, since they have, besides the sensor kinase and the terminal response regulator, an intermediate response regulator lacing an output domain and a histidine-containing phosphotransfer protein (Mitrophanov and Groisman 2008).

2.3.1 THE REGULATION OF GENE EXPRESSION

Since not all proteins are needed simultaneously, bacteria can activate or inhibit the transcription and the translation of proteins in relation to their physiological requirements. Although the bacterial regulation of gene expression occurs both in transcription and in translation, the most important (and most controlled) part is the initiation of transcription. In spite of being performed by a variety of molecules[14], a primary role in the transcriptional regulation of bacteria and archaea is played by two kinds of proteins, *sigma* and *transcription factors*. Sigma factors are proteins that enable RNA

[12] Bacteria can exhibit also one-component systems which consist of proteins containing input and output domains and lacking histidine kinase and receiver domains (Ulrich et al. 2005).

[13] Sensor kinases are usually integral membrane proteins that can be either a permanent part of a cell membrane (transmembrane integral proteins) or a protein associated with one side of the membrane (monotopic integral proteins).

[14] A variety of proteins, small ligands and mRNA molecules are involved in the regulation of bacterial gene expression. For example, nucleoid associated proteins (NAPs) create DNA bridges by means of histone-like nucleoid-structuring protein H-NS that interfere with transcription, leading to gene silencing and anti-gene silencing activities (Bervoets and Charlier 2019, p. 319). Then, a class of proteins, the ribonucleases, control transcription by catalysing the degradation of the mRNA into smaller components so as to control gene expression. Other proteins, the RNA-binding proteins, bind specific RNA sequences or structures in order to regulate bacterial transcription and translation. Then, small ligands (e.g. the alarmone ppGpp or the catabolite activator protein (CAP)) can *directly* interact with the bacterial RNAP, enhancing or inhibiting transcription so as to respond swiftly and efficiently to environmental changes. Furthermore, some mRNA molecules –the so-called 'riboswitches'- can recognise small molecules that affect transcription termination and translation initiation. Finally, some epigenetic mechanisms (e.g. DNA methylation, especially of adenine) produce reversible changes in the structure of the DNA sequence that can repress gene transcription.

polymerase (RNAP) to bind to gene promoters[15], thus allowing for the initiation of transcription[16]. Sigma factors control global switches in the gene expression profile in response to stress conditions, and they also coordinate gene expression in time and space (Bervoets and Charlier 2019, p. 309). Transcription factors (TFs) are 'two-headed proteins' consisting in a *DNA-binding domain* and an *allosteric site* to which metabolites bind non-covalently or which enzymes covalently modify. The DNA-binding domain of TFs binds to the DNA promoter (generally near to or overlapping the binding site for RNAP) and performs a variety of mechanisms in order to promote (*activators*) or inhibit (*repressors*) the initiation of transcription. Activators generally stimulate the transcription of promoters by making protein-protein interactions with the transcription machinery (i.e. RNAP, DNA, and general transcription factors). Repressors inhibit transcription initiation by attaching to a DNA sequence (the operator), thus preventing the bond between the RNAP and the promoter (Collado-Vides et al. 1991).

The allosteric site of TFs binds to metabolites or chemical signalling molecules (i.e. environmental and intracellular signals) which have been transmitted and transduced by the *signal transduction machinery*. An interesting example of the relationship between the transcriptional regulation and the transduction machinery is provided by the phosphotransferase system EI (Enzyme I)- EII (Enzyme II) system in E. coli: when glucose is present in the periplasmic space, the EI-EII system transports and phosphorylates it (Fig. 2.1). The unphosphorylated domain of EII binds and inactivates the protein Mlc, which is a transcriptional repressor of the operator region of pts[17] genes. As a result, the transcription of pts genes, which synthesise proteins for glucose transport, is triggered. Furthermore, when glucose is present, the unphosphorylated domain EIIA can bind and inactivate LacY (the lactose permease) in such a way as to preclude the transport of lactose (Winkler and Wilson 1967). By contrast, when glucose is absent, the phosphorylated EII domain phosphorylates the adenylate cyclase, producing cAMP[18]. This molecule, which binds to Crp (also called 'Cap'), directly binds the DNA so as to activate the transcription of the lac operon (genes for the transport and metabolism of lactose in bacteria). Moreover, the EII domain does not bind the protein Mlc in such a way that it represses the transcription of pts genes (Plumbridge 1998).

[15] Gene promoters are DNA sequences where gene transcription begins.
[16] Another class of proteins, anti-sigma factors, bind to sigma-factors and inhibit transcriptional activity.
[17] pts is a group of genes involved in the synthesis of the phosphoenolpyruvate-dependent sugar phosphotransferase system (sugar PTS) that catalyses the phosphorylation of sugar substrates concomitantly with their translocation across the cell membrane.
[18] The cyclic adenosine monophosphate (cAMP) is a second messenger involved in different biological processes both in bacteria and eukaryotes.

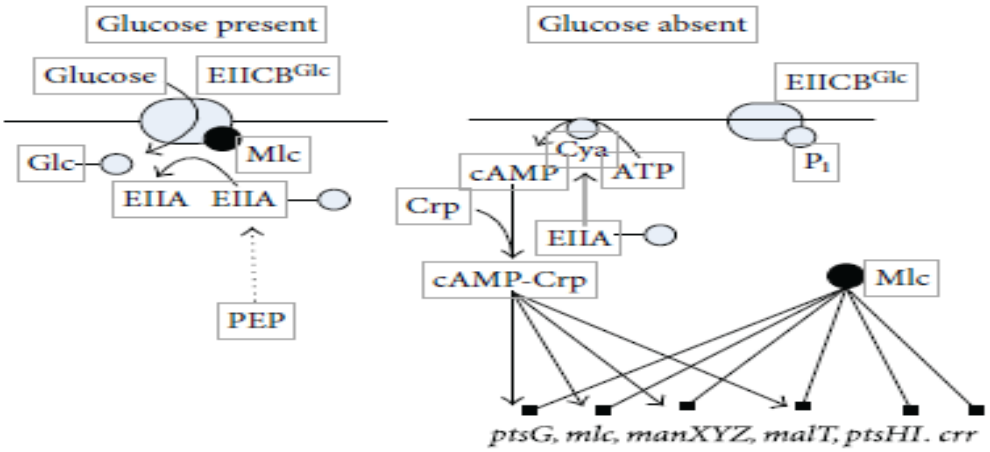

Figure 2.1 Control of transcriptional regulation in presence or absence of glucose in E. coli (Shimizu 2013, p. 9).

Transcriptional regulation controls protein synthesis and it is therefore involved in the *modulation* of a fundamental aspect of the constitutive regime: the *metabolism* (i.e. the increase and decrease of enzyme production). The way in which metabolism is genetically controlled is well exemplified by the genetic control of carbohydrate metabolism in E. coli. Glycolysis and gluconeogenesis are controlled by the combined action of the cAMP-Crp complex and transcriptional factors (the Cra[19]) which are triggered by extracellular glucose signals. When the extracellular concentration of glucose is high, the transcription of some of the genes synthesizing the enzymes of carbon uptake and glycolysis (e.g. ptsHI, pfkA, etc.) is activated, whereas the transcription of some of the genes producing enzymes involved in gluconeogenesis (e.g. pps and pck), tricarboxylic acid cycle, the two glyoxylate-shunt enzymes, and some electron transport carriers is repressed (Saier and Ramseier 1996; Saier et al. 1997; Shimizu 2016). The opposite process occurs when there is a low concentration of glucose in the extracellular environment.

Another important physiological dimension, which is involved in the self-maintenance of the bacterial cell and which is controlled by transcriptional regulation, is *bacterial growth*. Indeed, depending on the type and amount of nutrients in the surroundings, the growth rate of bacteria can considerably vary, being modulated by the regulation of gene expression. For example, when there is a low concentration of amino acids in the environment, the ppgPP (guanosine pentaphosphate) – an alarmone- directly binds to the RNAP, repressing the transcription of the genes for amino acid

[19] Cra stands for catabolite repressor/activator protein.

synthesis and inhibiting the autocatalytic activity of ribosomes which is a requirement for bacterial growth (Potrykus et al. 2011; Klumpp and Hwa 2014).

2.3.2 REGULATION BY MEANS OF ENZYME PHOSPHORYLATION

Bacteria can regulate the constitutive regime and interactive capacities not only through the regulation of gene expression, but also by means of *post-translational modification*, such as *enzyme phosphorylation*. This second regulatory strategy is faster than the first, because it involves a momentary activation/inactivation of the activity of the constitutive components and not an activation/inhibition of the production of *their structure.* In this subsection, I examine a classic example of regulation made by enzyme phosphorylation: the control of bacterial *chemotaxis* that consists in the modulation of *the rotatory movement of* bacterial flagellum. Indeed, the flagellum can perform either a clockwise (corresponding to tumble) or a counter-clockwise rotation (corresponding to run) in relation to the concentrations of metabolites in the surroundings. Globally, the regulation of chemotaxis plays a pivotal role in the control of bacterial motility, thus allowing favourable conditions for bacterial metabolism.

Like gene expression regulation, post-translational regulation is triggered by environmental and intracellular signals. In the case of chemotaxis, enzyme phosphorylation is triggered by the increase in repellent concentrations or also by the decrease in attractant concentrations in the extracellular environment. The *transduction* of extracellular signals is performed by a two-component system consisting of a sensor kinase (a histidine protein kinase) and a response regulator. The former catalyses the transfer of phosphoryl groups from ATP to one of their own histidine residues; the latter transfers phosphoryl groups from the kinase phosphohistidines to one of their own aspartic acid residues (Webre et al. 2003). Since most of histidine protein kinases are transmembrane proteins, they bind to extracellular signals, thus phosphorylating the response regulator which in turn diffuses around the cytoplasm and binds to the motor proteins that regulate flagella movement.

The histidine protein kinase involved in chemotaxis is the CheA[20] that phosphorylates CheY (the response regulator) which, in turn, binds to a flagellar protein (FliM), thus inducing a conformational change in FliM that modifies the sense of flagellar rotation (from counter-clockwise to clockwise) (Fig. 2.2). When CheY is dephosphorylated by CheZ, FliM changes its conformational change and the

[20] Since CheA is not an integral transmembrane protein, it receives signals and it is also regulated by different transmembrane chemotaxis receptors.

reverse movement of flagellar rotation (from clockwise to counter-clockwise) is performed. It is worth noting that the regulatory action of the CheA-CheY two component system depends on a set of five receptor transmembrane proteins that can be methylated, thus inhibiting CheA, or demethylated, thus activating CheA[21] (Webre et al. 2003; Bich and Moreno 2016).

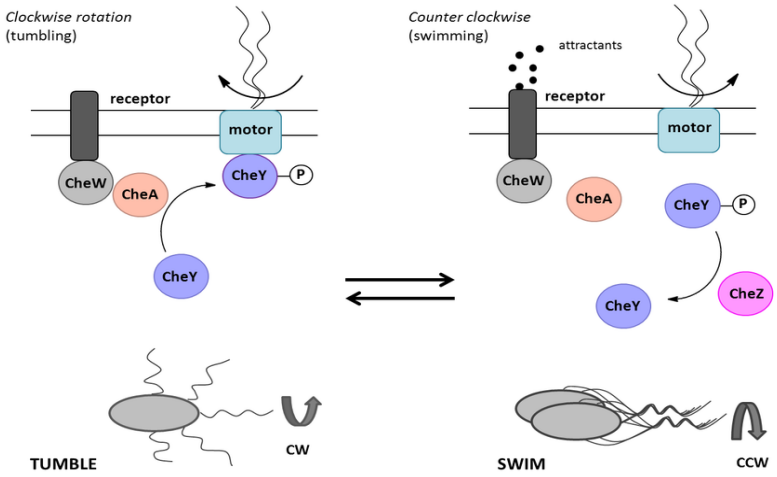

Figure 2.2 Regulation through enzyme phosphorylation in chemotaxis (Robinson et al. 2014).

2.4 THE FUNCTIONAL INTERDEPENDENCE BETWEEN CONSTITUTIVE CONSTRAINTS AND REGULATORY SUBSYSTEMS

The previous section has shown that prokaryotes control their metabolism (and growth) as well as their motility by regulating gene expression or by phosphorylating enzymes. The case-studies also show that regulatory mechanisms are triggered by signals (e.g. the metabolites produced by a bacterium) and environmental cues (e.g. the glucose present in the environment). It therefore seems that the constitutive processes, as already pointed out by Bich et al. (2016), are directly controlled by regulatory mechanisms and, indirectly, by signals acting on regulatory mechanisms. In this section, I shall discuss whether the constraints of the constitutive regime (i.e. *template*, *kinetic*, and *spatial* constraints) may affect the behaviour of signals and regulatory mechanisms.

First of all, there is a reciprocal causal loop between template constraints (i.e. DNA and mRNA) and regulatory proteins: regulatory proteins modulate gene expression and template constraints synthesise and regenerate the second-order constraints. It is worth stressing three important

[21] The five proteins are: Tar, Tsr, Trg, Tap, and Aer.

aspects of this causal loop: first, the structure (and its replacement), but *not* the *functionality*, of the regulatory subsystem is controlled by template constraints. For example, the regulatory mechanisms performed by transcription factors do not depend on the genes that synthesise them. Secondly, most of transcriptional regulatory proteins regulate genes that are *not involved in their production*, in such a way that there is *not* a feedback loop between a transcriptional regulatory protein and the gene that synthesises it. Let us consider, as an example, the relationship between the global regulator Cra and the genes regulated by it. The Cra protein of E. coli is a global TF that is synthesised by the cra gene; however, the Cra protein does not constrain the transcription mechanism of the cra gene, but rather that of the genes encoding biosynthetic and oxidative enzymes and sugar catabolism (e.g. ppsA, fbp, fruB, etc.). Thirdly, transcriptional regulatory proteins are *not* regulated by first order constraints, since they are able to self-regulate and regulate among each other, giving rise to a hierarchical network in which we can distinguish *global*[22] and *local regulators*[23].

Kinetic constraints (i.e. enzymes) play an important role in the activity of regulatory subsystems for three basic reasons. First, some enzymes can act either as coactivators (e.g. histone acetyltransferase) or corepressors (e.g. histone deacetylase), thus assisting the regulatory proteins involved in transcriptional regulation. Second, enzymes catalyse metabolic processes that produce the metabolites that activate (as intracellular or extracellular signals) regulatory subsystems[24]. Third, enzymes (e.g. kinases) are directly involved in in the signal transduction systems which are an essential aspect of the phenomenon of regulation.

Spatial constraints (i.e. porins, membrane receptors, and the signal transduction systems) affect regulatory mechanisms by selectively controlling the extracellular signals passing through the (outer and internal) bacterial membraneare. Moreover, signal transduction systems (i.e. two component systems and phophotransferase systems) trigger kinase cascades that result in the phosphorylation of response regulator proteins which play a fundamental role both in the regulation of gene expression and in the regulation through enzyme phosphorylation.

[22] Global regulators not only regulate themselves but also other TFs through feedback loops and complex relationships. Global regulators, which tend to be transcribed independently from the genes they regulate as a result of regulating many genes, can work with other global and local regulators in order to co-regulate the same promoters (e.g. the *melAB* promoter is regulated by both the global regulator CRP and the local regulator MelR) (Martínez-Antonio and Collado-Vides 2003).

[23] Local regulators are regulated by global regulators and regulate a single or very few operons (Martínez-Antonio and Collado-Vides 2003, p. 484).

[24] For example, the enzymes involved in glycolysis (e.g. hexokinase, phosphofructokinase, etc.) transform the glucose into pyruvate, producing a number of metabolites (e.g. fructose-1,6-bisphosphate and pyruvate) that affect the conformation and regulatory activity of TFs in E. coli (Martínez-Antonio et al. 2003).

In sum, the regulation of bacterial transcription and the control of bacterial chemotaxis show an interesting relationship between the constraints of constitutive processes (1st order constraints) and regulatory mechanisms (2nd order constraints). On the one hand, regulatory mechanisms control, without being controlled by, the action of first-order constraints, regulating gene expression and phosphorylating enzymes. On the other hand, template, kinetic, and spatial constraints affect the functionality of regulatory proteins in three ways: first, template constraints are involved in the *synthesis* and *turnover of* the regulatory proteins by means of protein synthesis. Secondly, spatial constraints (e.g. transmembrane proteins) may act as regulatory mechanisms on the flow of molecules (and signals) passing through them. Thirdly, kinetic constraints contribute to *produce* the metabolites that, acting as signals, trigger or inhibit regulatory proteins (Figg. 2.4 and 2.5).

Signals appear as a crucial *connecting point* between the functions performed by the constitutive constraints and those carried out by the regulatory subsystems. Indeed, in spite of being produced by metabolic processes, they do not directly constrain them, but rather regulatory proteins, giving rise to a functional integration between S, R, and C (Fig. 2.3). The organisational features and the systemic physiological consequences of this type of functional integration are discussed in the next section.

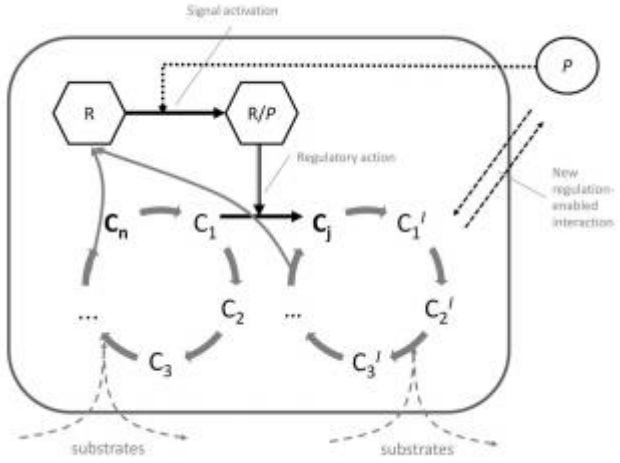

Figure 2.3 In this scheme, taken from Bich et al. (2016), the regulatory subsystem (R) constrains the constitutive regime (C) and the constitutive one produces the components of R. I accept this scheme, but I emphasise the role played by the signalling subsystem (in this scheme represented by "P") for understanding the functional connection between C and R.

2.5 THE ORGANISATIONAL ROLE OF THE SIGNALLING SUBSYSTEM IN THE ACHIEVEMENT OF FUNCTIONAL INTEGRATION BETWEEN THE CONSTITUTIVE REGIME AND THE REGULATORY SUBSYSTEMS

The regulation of gene expression and chemotaxis have shown that the constitutive regime, the regulatory subsystems and the signalling systems are functionally interdependent. We may wonder whether or not their underlying constraints give rise to *organisational closure* and, if the answer is positive, what this implies for the overall bacterial organisation.

As pointed out by Montevil and Mossio (2015, p. 186), a system is subject to closure if the following conditions are satisfied: first, each constraint C_i of the system *depends directly* on at least another constraint belonging to the system (C_i is dependent); second, each constraint C_i *generates* at least another constraint C_j belonging to the system (C_i is generative); third, usually the system cannot be *split* into two closed sets. Let us analyse whether the constraints of the three subsystems R, C, and S satisfy these three requirements.

Regulatory proteins constrain the behaviour of both spatial, template, and kinetic constraints. Regulatory constraints act on template constraints which in turn modulate the synthesis of proteins, whereas kinetic constraints control metabolic processes and the production of metabolites which, in turn, act as signals that trigger, and therefore constrain, regulatory proteins. A further role is played by spatial constraints (i.e. transmembrane proteins) which constrain the flow of extracellular metabolites from the exterior to the interior and back. As such, the constraints of each subsystem are dependent and generative at the same time, and moreover the whole system cannot be split into two closed sets, since the regulatory subsystem modulates the constitutive regime in relation to the environmental variations and internal physiological (metabolic) needs, and the constitutive regime *synthetises*, *regenerates*, *maintains* the structure, and *triggers* the functionality of the regulatory entities. As a result, the constraints of the regulatory, constitutive, and signalling subsystems satisfy Montevil and Mossio's requirements and are subject to an organisational closure.

The fact that regulatory entities are subject to a regime of closure with the constitutive constraints does not disprove the thesis that regulatory constraints must be dynamically decoupled from -i.e. not *directly* dependent on- the constitutive constraints (Bich and Moreno 2016; Bich et al. 2016; Bich 2018). Indeed, as previously emphasised, although regulatory proteins are *synthesised* by template constraints, their *functionality* does not hinge on template constraints, but rather on signals. Likewise, the mechanisms performed by regulatory proteins do not depend on the

concentrations of metabolic substrates and products (Bich et al. 2016), but rather on how specific metabolites bind to receptors (i.e. allosteric modification), thus triggering a regulatory response.

Accordingly, it seems apparent that *signals* and *signal transduction machineries* (i.e. the signalling subsystem) play a fundamental organisational role in the *functional integration* between the regulatory subsystems and the constitutive regime. In particular, four properties of the signalling subsystem allows it to globally act as an *interface* between the constitutive regime and the regulatory subsystems: first, the signal transduction machinery is able to *recognise* a wide set of molecules (notably metabolites) as signals so as to *amplify* them and generate an output that activates a regulatory response; second, the signal transduction machinery is highly *specific* in recognising signals in such a way that not all metabolic products activate a regulatory response; third, the signal transduction machinery *integrates* different signal transduction pathways and regulatory responses; finally, the signal transduction machinery is a fundamental source of *temporal coordination* in the regulatory response. Let us address each one of these four features.

The bacterial signal transduction machinery (e.g. the two-component system and the phosphotransferase system) usually consists of a transmembrane receptor protein that binds to a ligand so as to modify a response regulator and produce an output. In this basic mechanism, the ligand (e.g. a metabolite) is *interpreted* as *signal* in the sense that it binds only to specific classes of receptors, thus inducing a conformational change in receptors that, in turn, usually determines the phosphorylation of the response regulator. When a metabolite is *interpreted* as a signal by a receptor, the signal transduction machinery *transforms* a direct *product* of metabolism into a *message* that is capable of activating a regulatory response. In order to avoid the distortion of the information (especially through long distances), a signal needs to be *amplified* by means of signalling cascades.

Faithful transmission of information requires a *specificity* of the interaction between histidine kinases and the response regulators in the two-component system, in order to avoid *cross-talk* (i.e. the overlapping of signalling pathways). More specifically, the *specificity* of two-component signalling systems relies on the ability of a histidine kinase to discriminate its response regulator among many possible substrates. This is made possible by two intrinsic properties of histidine kinases: first, their bifunctionality; second, the generation of positive and negative feedback responses. As regards the first aspect, histidine kinases can play a twofold role: on the one hand, they can phosphorylate their response regulators; on the other, they can remove a phosphoryl group from the same response regulators in the absence of a stimulus (Russo and Silhavy 1993;

Salazar and Laub 2015). As such, histidine kinases balance the phosphorylation level of response regulators and they prevent cross-talk between different signalling pathways, because they eliminate phosphoryl groups which have been impropriately given by non-cognate histidine kinases to their regulators (Groban et al. 2009; Salazar and Laub 2015). Second, the phosphorylation of response regulators may positively activate (positive feedback) or negatively inhibit (negative feedback) the activity of the histidine kinase in such a way as to control the level of their target genes over time.

The activity of signal transduction machineries is *modulated* by a number of proteins, called 'two-component system *connectors*' (or simply 'connectors') which play an essential role in the *coordination* and *fine-tuning* of cellular processes (Mitrophanov and Groisman 2009). Indeed, connectors phosphorylate or dephosphorylate response regulators, thus modulating the activity of sensor kinases.

Connector proteins play a fundamental role in the *integration* between *signal transduction pathways* and (genetic) *regulatory subsystems*, because they allow for the *temporal coordination* between signal transduction pathways and regulatory responses. For example, some connectors (e.g. Rap A, RapE, and RapH) prevent two alternative genes (late competence genes and sporulation genes) from transcribing contemporarily, thus allowing the *time coordination* of the two distinct regulatory responses (Smits et al. 2007; Mitrophanov and Groisman 2008; Gao and Stock 2015).

In the case of transcriptional regulation, an important role in its temporal coordination is played by the joint action of intracellular and extracellular signals. Indeed, intracellular and extracellular signals *activate* different TFs, leading to a diverse (and sequential) regulatory response in transcription. *Internal* signals have been shown to trigger *global* TFs that control the transcription of *local* TFs which are mostly based on external signals (Martínez-Antonio and Collado-Vides 2003; Martínez-Antonio et al. 2006; Changa Janga et al. 2007). This suggests that extracellular signals must be combined with internal signals and that *endogenously* synthesised metabolites form the *core* of the transcriptional regulatory network, thus *coordinating* the response of TFs to both intracellular and extracellular signals (Seshasayee et al. 2006). As such, the bacterial cell can establish a *sequential activation* of TFs as a response to both internal metabolic needs and changes in environmental conditions.

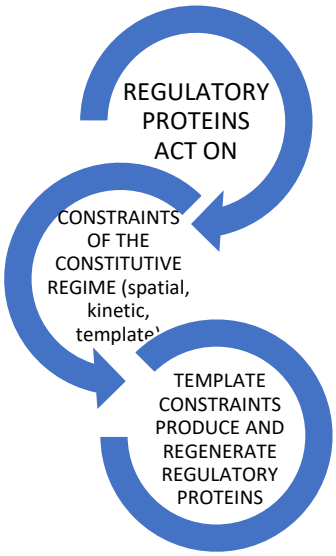

Figure 2.4 The Functional Relation between regulatory subsystems and the constitutive regime.

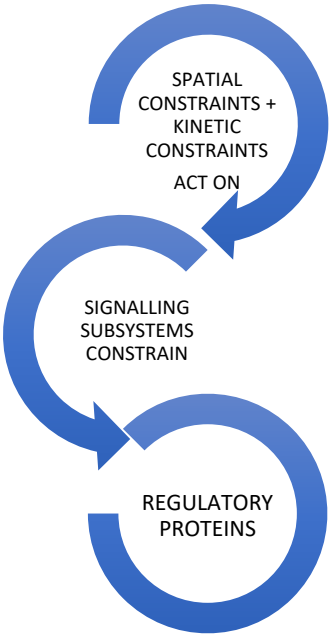

Figure 2.5 Signalling subsystems as an interface between the constitutive regime and the regulatory subsystems

The relationship between the regulatory, the constitutive, and the signalling subsystems shows a biological architecture in which control appears at the same time *hierarchical* and *heterarchical*, because there is a hierarchy of control in which each subsystem controls another subsystem without being *directly* constrained by it. Indeed, the regulatory subsystems constrain metabolism, but their activity is not directly constrained by the metabolic concentrations of enzymes; enzymes constrain the synthesis of many endogenous signals (e.g. metabolites), but their catalytic activity is not directly affected by the ligand-receptor interactions of signals; signals affect regulatory gene expression and

enzyme phosphorylation, but kinase cascades are not directly constrained by them. It is important to stress that in the cell, considered as a whole, there is not a *privileged* level of control that establishes rigid top-down mechanisms for one subsystem to another. In fact, this kind of biological organisation has been well characterised by Winning and Bechtel (2018), who have argued that the subsystems of biological organisations often exhibit *local* hierarchies in which, rather than a strict hierarchy, each subsystem can establish control "over mechanisms at the top of the local hierarchy" (Winning and Bechtel 2018, p. 299), thus realizing a heterarchical control model (Pattee 1991). Therefore, although regulatory subsystems control metabolic processes, they are in turn controlled by the signalling system, in such a way that it is not possible to consider the regulatory subsystem as the top-level, which controls without being controlled, of a threefold control hierarchy.

The closure between the regulatory subsystem, the constitutive regime, and the signalling subsystems has far-reaching systemic consequences for the bacterial cell: first, it allows the cell to *sense changes* in the concentrations of metabolites and other molecules in the internal and external environment so as to trigger a gene *regulation* or enzyme phosphorylation, in order to control those proteins involved in *metabolic* and *developmental processes*. Accordingly, the signalling system triggers a regulatory response that switches between different regimes of the metabolic and growth processes, so as to regulate them in relation to physiological requirements, thus substantially contributing to the self-maintenance of the overall cell. Second, the functionality of the signal transduction machinery clearly shows how the regulation of the *intracellular* environment depends on the information about the *extracellular* one and, in turn, on the flow of this information from the exterior to the interior is made possible by the signal transduction machinery. Finally, since signal transduction machineries permit a *temporal coordination* between the variation in the concentrations of metabolites and regulatory response, they help cells to temporally synchronize metabolic needs with the activation of regulatory subsystems, thus leading to a system capable of facing with internal and external perturbations and changing its mode of behaviour flexibly (Barkai and Leibler 1997; Alon et al. 1999; Kitano 2004; Klosik et al. 2017).

2.6 CONCLUDING REMARKS

In this chapter, I have discussed whether and how the regulatory subsystems and the constitutive regime are functionally integrated by examining two paradigmatic cases of biological regulation in bacteria: the regulation of gene expression and the regulation by means of enzyme phosphorylation.

These two case-studies have shown that regulatory subsystems directly modulate the action of the constraints (i.e. template and enzymatic constraints) by activating or deactivating protein synthesis and by triggering or inhibiting the activity of proteins. In turn, the constraints of the constitutive regime affect the behaviour of regulatory subsystems essentially in two ways: first, they (i.e. the template constraints) *synthesise* the proteins performing regulatory functions; secondly, they *trigger* the functionality of regulatory subsystems by producing and controlling the flow of the metabolites that act as signals.

I have argued that the signalling system (i.e. signals plus signal transduction machineries) plays a fundamental organisational role in the functional integration between the regulatory subsystems and the constitutive regime, since it allows the metabolic products to indirectly affect (in the form of signals) the behaviour of regulatory proteins. As a result, the constraints of the regulatory, signalling, and the constitutive subsystems are organisationally closed, inasmuch as they are dependent on each other. It has been shown that intracellular signals directly modulate regulatory response, thus informing regulatory subsystems on the intracellular metabolic status; extracellular signals, instead, must be 'read' by the *signal transduction machinery* which exhibit four important properties: interpretation and amplification of the ligand, specificity of the ligand-receptor bound, integration of signal transduction pathways and regulatory response, temporal coordination in the regulatory response. As such, intracellular signals act as an *interface* between the constitutive regime and the regulatory subsystem, whereas intercellular signals are a *connecting point* between the extracellular environment and the intracellular *milieu*.

The closure between the signalling, the regulatory, and the constitutive subsystems has important physiological consequences for the bacterial cell. In particular, it allows the regulation of *metabolic* and *growth* processes on the basis of the information on the conditions of the intracellular and extracellular environment. Then, the actions performed by the three subsystems require a temporal coordination that is facilitated by the signal transduction machinery (Gao and Stock 2015). Finally, the integration of the three subsystems enables the cell to cope with perturbations, thus achieving biological robustness.

I may try now to answer to the third and last question of this chapter: why has this kind of functional organisation been *evolutionarily* successful and why does it represent the organisational core for the achievement of a strong form of functional integration in eukaryogenesis? In order to respond to this question, we have to consider three key aspects: the maintenance of internal *dynamical stability* of C, the capability for *(co)evolving*, and the possibility of including *new levels of*

functional complexity. First, the integration between R, C, and S enables the cell to cope with internal and external perturbations by providing an adequate gene regulation and enzymatic regulation of the constitutive processes (i.e. metabolism and development). As such, the cell keeps a dynamical stability, inasmuch as it compensates the effects of a perturbation by means of adjustments of tightly coupled constitutive constraints (Weiss 1968; Rosen 1970). Accordingly, this kind of biological organisation has a higher chance to adapt to an impressive variety of environmental niches, to survive, and to differentially reproduce, thus undergoing natural selection. Secondly, in order to be evolutionarily successful, R, C, and S have likely co-evolved, in such a way that the structural and functional modifications of each of these three subsystems are intimately connected with the changes in the other two subsystems. As an example, the evolution of the signal transduction machinery (notably the two-component system) opened up a new domain of cellular functions, including the evolution and the emergence of new regulatory and metabolic capacities (McAdams et al. 2004; Perez and Groisman 2009; Capra and Laub 2012). Thirdly, R, C, and S must have been able to include new levels of *complexity* in the transition from prokaryotic to eukaryotic cell. The evolution towards more complex and larger signalling system has a significant cost: first, it is more difficult to keep signals straight and avoid unwanted cross-talk; second, it is more difficult to keep the fidelity of information flow inside the cell (Laub 2016). We may therefore conclude that the evolution from the prokaryotic to the eukaryotic cell entailed the achievement of new forms of signal specificity and recognition.

The closure between R, C, and S has left open the possibility of increasing the complexity of this basic architecture not only by *adding new structures and functions*, but also by *achieving more complex forms of functional integration between these three subsystems*. Indeed, the transition from the prokaryotic to the eukaryotic cell was characterised, as will be explained in the next chapters, by the achievement of new forms of gene regulation (e.g. epigenetic, post-transcriptional, post-translational modifications), new signal transduction pathways (e.g. the development of complex intracellular signals enabled by the eukaryotic cytoskeleton), and the modification of constitutive processes (e.g. oxidative phosphorylation and electron transport chain) that still have kept the organisational closure between R, C, and S found in the prokaryotic world. The achievement of more complex forms of functional integration between R, C, and S enabled the proto-eukaryotic cell to acquire a new global physiological viability.

CHAPTER 3 FUNCTIONAL INTEGRATION AND INDIVIDUALITY IN PROKARYOTIC COLLECTIVE ORGANISATIONS[1]

3.1 INTRODUCTION

The previous two chapters have examined two important dimensions of the functional integration of cells consisting in the closure between biological machines and in the interdependence between constitutive, regulatory and signalling processes in prokaryotic and eukaryotic cells. This chapter shifts our sight from individual cells to collective associations of cells, seeking to understand the structural constraints and the physiological mechanisms that enable symbiotic associations of prokaryotes to achieve a physiologically integrated organisation. More specifically, this chapter goes to the root of eukaryogenesis by examining the enabling conditions for the transformation of an endosymbiont into an organelle as it occurred in mitochondria and chloroplasts. The appearance of mitochondria and chloroplasts enabled the proto-eukaryotic cell not only to perform more complex functions but also to undergo the evolutionary changes that I will address in the following three chapters. Furthermore, the transformation of an endosymbiont into an organelle furnishes some important clues as to the origin of a new biological individual, thus shedding light on the philosophical issue of biological individuality, which will also be examined in chapters 5 and 6.

Collective associations are widespread in the biological world and give rise to very different organisations ranging from associations of bacteria and archaea to societies of multicellular organisms (e.g. social insects), yet only in certain cases these associations become an integrated individual. Thus, an intense debate about when collective associations constitute a new individuality has been taking place during the last decades. The central question underlying this debate can be summarised with Wilson's words: "at what point does a society become so well integrated that it is no longer a society?" (Wilson 1974, p. 54). Although Wilson's question refers to *animal* societies, it is so general that it can apply to any kind of biological association. Indeed, it clearly emphasises three fundamental aspects of the transition from a biological association to an individual: first, there are some specific *conditions* (summarised by "at what point") that permit an association to become a more cohesive whole; secondly, the transformation of an association into a more cohesive whole

[1] The ideas and most of the parts of chapter 3 have already been published in Militello et al. (2020).

involves the achievement of a certain degree of (functional) *integration* among the constituent organisms (indeed, they must be "well integrated"); finally, the process of integration among the parts of the association leads to something "that is no longer a society", therefore a (new) *individual* (i.e. an organismic-like- associative entity).

In the contemporary debate about composite biological individuality (e.g. biofilms, holobionts, colonies of insects), the two main approaches usually adopted – i.e. evolutionary and physiological – rely on the idea that a biological individual is an integrated whole whose functions are strongly interconnected. Yet little has been said about the *conditions* that may enable an association to become a functionally integrated individual and what mechanisms are involved. The reason lies in the fact that the very concept of functional integration, often considered as a synonym for 'physiological integration' (Pradeu 2010), has not been characterised in detail. To further complicate matters, functional integration is an especially multifaceted and complex aspect of biological organisations that includes several important dimensions of a biological system such as metabolic, regulatory and sensorimotor abilities, development, immunological responses, reproduction, etc. As a consequence of the unclear character of the notion of integration, not only current general definitions of biological individuality that appeal to it are somehow undermined, but also the mechanisms allowing an association to become a more integrated whole are mostly unexplored.

In an attempt to develop the notion of integration in more detail with the help of biological examples, I first analyse the fundamental *physiological mechanisms* that could explain the transition from an association of bacteria towards a new full-fledged, functionally integrated individuality; second, I examine the different *types* and *degrees* of *functional integration* enabled by different mechanisms, by taking into consideration their limits and potentials (understood as enabling conditions) to bring about further forms of integration. In particular, by adopting an organisational approach, I aim to connect the physiological dimension of the process of individuation with the evolutionary one through an analysis of the role of the different forms of physiological integration in the reproduction of a collective entity[2]. Finally, I provide a *more precise characterisation* of the notion of 'functional integration'. It is worth pointing out that this chapter is *not* aimed at drawing up a list of properties (or criteria) that sharply distinguish a loose association of organisms from an individual, but rather at exploring the *conditions* that can potentially permit the transition from the former to the latter and, on this basis, at contributing to a better understanding of what a 'functionally integrated individual' is.

[2] This aspect will further be analysed in chapter 6.

Despite the huge variety of biological collective associations, I have chosen to focus on *two case-studies* from the bacterial and archaeal domains: biofilms (i.e. colonies of single- and multispecies bacteria or also archaea) and the endosymbiotic relationship between two species of bacteria[3]. These case-studies have been chosen for three reasons: first, they are *minimal forms* of composite biological systems; second, they exhibit different *physiological mechanisms* that allow understanding why the collective association achieves in each case a very different degree of functional integration among its parts; finally, the endosymbiotic relationship between two species of bacteria may provide important *clues* as to the origin of a paradigmatic example of a new functionally integrated individual, the *eukaryotic cell*[4]*,* by evaluating the role played by different forms of collective spatial constraints in enabling functional integration.

The connection between the case-studies lies in three main aspects: first, a *common spatial constraint* (i.e. the ECM and the membrane of *Tremblaya*) that surrounds a set of prokaryotic cells; secondly, the *systemic control* of parts enabled by the common boundary which affects the type and degree of physiological integration achieved by the parts; thirdly, the *evolutionary potential* opened up by different kinds of common boundaries. As I shall argue in the detail, biofilms and the endosymbiosis between bacteria have diverse *spatial organisations* that constrain their constituting organisms differently, providing different *mechanisms* of *collective control*[5]. This provides them with a distinct type of stability over time and opens up different evolutionary possibilities for an association to give rise to an integrated individual. This thesis has two important explanatory consequences: first, it sheds light on the connection between spatial constraints and physiological mechanisms for explaining the functional integration of collective associations; secondly, it clarifies some important physiological dimensions of the idea of 'functional integration', thereby helping to better define the meaning of a 'functionally integrated individuality'.

The chapter is structured as follows. Section 3.2 examines some philosophical accounts that appeal to the notion of functional integration to ascribe individuality to biological associations.

[3] Other collective associations of prokaryotes include *colonies* stemming from the clones of single species bacteria/archaea (e.g. *Lactococcus lactis* or *Streptococcus thermophylus*) or intracellular parasites (e.g. *Vampirococcus* and *Bdellovibrio*). I will not analyse these cases here as they do not exhibit the features of a stable functionally integrated collective organisation. In the first case, they do not exhibit a common spatial constraint such as the EPS matrix. In the second case, intracellular parasitism is a transient not functionally integrated relationship where the host is killed.

[4] The focus on current forms of endosymbiosis as a possible way to provide a valuable clue as to the role played by endosymbiosis in the achievement of a "strong" physiological integration in eukaryogenesis moves in a similar direction to the one explored by Reyes-Prieto et al. (2014), which focuses on non-autonomous endosymbionts with extremely reduced genomes (also called symbionelles) to shed light on the origin of eukaryotic organelles.

[5] This idea is in line with the thesis that all multicellular association need to solve the issue of spatial control, and that different ways of doing so result in different types of organisations and different degrees of integration (Bich et al. 2019).

Section 3.3 analyses the organisation of biofilms and discusses the role of the extracellular polymeric substance (EPS) matrix in constraining and integrating the activity of the prokaryotic cells that compose them. Section 3.4 examines the organisational role played by engulfment and cross-control in the *Tremblaya-Moranella* association, the only well-studied case of endosymbiotic relationship between prokaryotes[6]. Section 3.5 discusses the organisational differences between these two forms of association (biofilms and endosymbiosis) by focusing on the roles of the EPS matrix and engulfment. Hence, it examines the main organisational issues raised by endosymbiosis and how they may have been solved during eukaryogenesis. Finally, the last section draws some conclusions on functional integration and biological individuality.

3.2 ASSOCIATION AND INTEGRATION IN BIOLOGICAL COLLECTIVE ORGANISATIONS

The concept of 'functional integration' is often invoked in the debate on biological individuality as a necessary element in developing an understanding of how living systems constitute, and can be identified as, coherent wholes both in evolution and physiology. In this context, this concept is usually employed as an *explanans*, rather than the main object of investigation, as the aim is to build general accounts of individuality. In the context of the debate on evolutionary individuality, for example, Hull pointed out (1980) that in order to be an object of natural selection a biological system must be able not only to undergo genetic variation and transmit it to the offspring, but also to interact with the environment as a cohesive physiological whole. Moreover, integration underlies reproductive capabilities. As Sober (1991) observes, all groups (e.g. colonies of insects, groups of cells, parasitic relationships) exhibit a certain kind of functional interdependence that consists of "parts of different sorts and these parts interacts so as to sustain the organism and allow it to reproduce" (p. 275). Sober suggests that all those functions involved in *self-maintenance* (e.g. metabolism) and *reproduction* somehow exhibit interdependence. Integration is also used to account for the absence of conflict and the presence of a high cooperativity among the component parts in such a way that they work as "bundles of adaptation", where all elements work toward a common evolutionary goal (Queller and Strassman 2009, 2016). In the same vein, Dupré and O'Malley (2009) emphasise that the functional integration of living beings, including collective

[6] Although the term "prokaryote" is nowadays substituted by Bacteria and Archaea, for the sake of simplicity, I continue to employ this word in the chapter to intend the unicellular organisms belonging to the two domains of Bacteria and Archaea.

associations, is characterised by the interconnection of *metabolic pathways* and *reproduction*. Metabolic processes are described generically as collaborative activities that entail a certain degree of functional interdependence. In the case of symbiotic associations, this usually takes the form of co-metabolism and synthrophy. Reproduction is a more complex issue in collective associations because vertical transmission and parent-offspring lineages do not always occur (See Skillings, 2016).

It is important to point out that the fundamental aim of this debate is to address the notion of individuality and not so much to clarify in the detail which types of *mechanisms* are required for self-maintenance, reproduction and cooperation. Functional integration, therefore, is used as a general notion in this context.

Hence, biological individuality understood in terms of physiology has received much less attention in the literature than evolutionary accounts. In this domain, the general notion of functional integration is expected to play an even more important role. In fact, it is still employed generically. It has been emphasised, for example, that the *immune system* plays a key role in explaining the interdependence of the functional parts of organisms and collective associations, because immune interactions "are systemic (as opposed to local) and […] responsible for the acceptance or rejection of constituents in the organism" (Pradeu 2010, p. 258; see also Howes 1998). This view, however, does not take into consideration the complexity of biological integration and the fact that a systemic control is performed not only by the immune system, but also by other regulatory subsystems and mechanisms that modulate and coordinate the functions of the components of the system. Moreover, the immune system depends on and is maintained by a more comprehensive physiological regime, which provides the energy for its functioning and that coordinates immune activity with those of the other functional subsystems.

Whereas the philosophical approaches mentioned above rely on the notion of integration as a generic concept (mostly as an *explanans*) to develop general accounts of individuality, this chapter aims to make this notion the main focus of the chapter and to address it as an *explanandum*. The objective is to analyse in the details how functional integration is achieved in biological associations by focusing on specific case studies. It is important to clarify two points. The first concerns the approach adopted. The chapter focuses on the *organisational aspects* underlying integration in biological associations, with the aim to identify what mechanisms enable transitions from loose collective associations to cohesive physiological wholes that are also capable to reproduce and evolve as units of selection. Focusing on current organisations (Moreno and Mossio 2015) means in

the first place identifying the physiological mechanisms that make possible different degrees and types of integration. Yet it also implies identifying the possibilities and bottlenecks of different types of organisation on the evolutionary scale.

The second point is the choice of the case study. To address the problem of how functional integration is achieved by biological associations and to explore how it can lead to a fully integrated individual, I focus on associations of prokaryotes (Bacteria and Archaea). The advantages are two. It is a minimal case, whose organisational features are expected to be less complex than in associations of eukaryotic cells, colonies of insects etc. In addition, it is widely accepted that it was from associations of prokaryotes that the eukaryotic cell originated. Therefore, the eukaryotic cell, which is widely accepted as a case of full-fledged biological individual, can work as a term of reference to discuss how far functional integration can in principle develop from the association of different prokaryotic organisms. This strategy allows me to identify how distinct mechanisms can lead to different types and degree of integration.

Several works have emphasised the importance of *control* and *regulatory mechanisms* in realising integration in different types of associations, from the development of multicellular systems (Arnellos et al. 2014; Griesemer 2016) to the physiology of symbiotic relationships (Catania et al. 2017; Bich 2019)[7]. Queller and Strassmann (2009, 2016), for example, have called into question the importance of spatial contiguity, the indivisibility of the parts, the development from a single lineage and the genetic uniformity among the members, to point out that it is the *control of conflict* and a *high cooperation* among the members of a society that are necessary for achieving a sufficient degree of functional integration. It is worth noting that most of these accounts have focused on associations of eukaryotic organisms, which exhibit forms and degrees of integration that are different from those of the prokaryotic world. Indeed, whereas the former give rise to multicellular integrated individuals, it is doubtful whether also the latter do so.

The discussion of these ideas in the specific context of prokaryotic associations has generated a debate regarding the status of biofilms; in particular, whether or not (and why) they can be considered integrated individuals. Ereshefsky and Pedroso (2013, 2015) and Doolittle (2013) have recently argued that multispecies biofilms can be considered individuals, because their extracellular

[7] As pointed out by Catania et al. (2017), *regulatory networks* play a pivotal role in defining the functional integration of symbiotic partners. These interdependent networks may be co-inherited (via vertical gene transfer) or re-established in a new generation (via horizontal gene transfer). This argument is also in line with Bich et al. (2016), who have argued that a functionally integrated organisation hinges on a complex set of regulatory mechanisms that allow it to coordinate the contributions of its functional parts and to handle perturbations.

matrix allows for a unitary interaction with their environment and because they are capable of reproduction, although they lack a high degree of germ-soma specialisation. In response, Clarke (2016) has argued that a biofilm does not actually interact as a whole, because "most interactions take place across spatial scales that are much smaller than an entire biofilm" (p. 205). In addition, since they do not have a collective reproductive system and bacteria can enter or exit the biofilm, biofilms cannot reproduce as wholes and they cannot vertically transmit genetic variations to future generations. Therefore, they cannot undergo group selection (Clarke 2016). According to Clarke (2016), in spite of exhibiting a certain degree of functional cohesion resulting from metabolic co-dependence and a certain form of collective border, biofilms do not perform collective mechanisms of interaction and reproduction, and therefore they do not evolve as individuals. One may suspect that these features depend on the fact that the bacterial components still keep a sufficiently high degree of autonomy and the biofilm as a system lacks more comprehensive (global) ways to control the behaviour of the bacteria.

To explore this different hypothesis, I will analyse the organisational role played by the extracellular polymeric substance (EPS) matrix and other control mechanisms in biofilms. The aim is to understand how such structures and mechanisms enable a certain type and degree of functional integration in biofilms, and to compare it with another type of organisation deriving from the endosymbiosis between bacteria, which has the potential for a different and stronger type of integration.

3.3 COLLECTIVE INTEGRATION IN BIOFILMS: DISTRIBUTED CONTROL AND THE ROLE OF THE EPS MATRIX

Biofilms are biological systems realised by ecological communities of (*single*- or *multispecies*) bacteria and archaea and by the extracellular polymeric matrix they produce. The development of a biofilm includes three sequential steps: first, the *attachment* of bacteria (or archaea) to a surface and the formation of a monolayer structure (that binds the bacteria together and to the surface); second, cell division and the production and deposition of the *EPS matrix,* which gives rise to a multilayer organisation; third, the *disassembly* of the matrix and the dispersion of cells.

In the first stage of biofilm life cycle, individual cells attach to a biotic or abiotic surface by means of *adhesins*[8] and give rise to a *monolayer biofilm* (Karatan and Watnick 2009). The production of

[8] Adhesins are cell-surface structures of bacteria that mediate transient or permanent surface attachment.

adhesins is triggered by the concentration of specific substances (e.g. oxygen or sugars) in the environment. The second stage begins when the spatial proximity of cells triggers the emission of several *extracellular signals* (e.g. mechanical, metabolic, inorganic, etc.) and the activation of *quorum-sensing* (QS) *mechanisms,* that collectively promote the synthesis and deposition of extracellular matrix components. At this stage, cells may attach to one another and to the EPS matrix, thus realising a *multilayer biofilm* where they undergo proliferation and differentiation into several cell types (Lopez et al. 2009). In the third stage, the EPS matrix disassembles and causes biofilm dispersion. This occurs in presence of a massive accumulation of toxic waste products, or when the system grows beyond the transport and distribution capabilities of EPS channels and the innermost layers of cells cannot receive enough nutrients. Biofilms employ several regulatory mechanisms that trigger dispersion in response to different stimuli (e.g. variations in concentrations of nutritional cues, oxygen and nitric oxide, presence of death bacteria) (Karatan and Watnik 2009).

Thanks to (at least) three types of *extra- and inter-cellular control mechanisms* – QS, EPS matrix and bacterial conjugation – a biofilm becomes a cohesive functional unit whose parts act and are maintained together. Bacterial conjugation is somehow favoured by the close spatial proximity of cells in specific areas of the biofilm, but it works locally at *short* (cell-to-cell) *ranges*. Therefore, I will focus on the former two, which act at *medium ranges*[9]*.* They constitute the main factors of integration of the whole system, because they are responsible for the *overall* development and functioning of the biofilm. QS is a distributed control system that relies on the *concentrations of a* set of signalling molecules that allow bacteria to coordinate their gene expression and trigger many of the changes in the biofilm through gradients of inter cellular activation. QS is triggered when the autoinducer concentration reaches a critical threshold because of cell density (Antunes and Ferreira 2009; Elias and Banin 2012). It functions as a feed-forward mechanism: the bond between signalling molecules and their bacterial receptors activates the expression of several genes, including those involved in the synthesis of these same signal molecules (Saxena et al. 2018). QS plays a pivotal role in the co-aggregation of different species of bacteria in multispecies biofilm[10], in the increase of

[9] Short range control relies on local cell-to-cell direct interactions. Medium range control is achieved when an ensemble of cells is constrained for example by the ECM. In multicellular organisms, such as animals, it happens at the level of tissues. QS relies on signals and can be considered as a distributed medium range control mechanism because it can affect a large number of cells by generating self-organised gradients. Long range control, instead, has a systemic reach and has the potential to constrain the activity of all the parts of the system. An example of long range control mechanisms from animals is the release of hormones, distributed throughout the system through vascularisation (see Bich et al. 2019 for more details).

[10] Let us consider, for example, colonisation of the human oral cavity by the bacterial species *Veillonella atypica* and *Streptococcus gordonii*. In order to colonise dental surfaces, *V. atypica* requires the presence of *S. gordonii*, because *S. gordonii* ferments sugars and releases lactic acid, which constitutes the preferred carbon source for *V. atypica*. The co-

biomass during the formation of a monolayer and a multilayer structure, and it activates a large number of genes involved in the synthesis of matrix components (Karatan and Watnik 2009). Finally, many bacterial species employ QS to coordinate the disassembly of the EPS matrix by promoting the inhibition of matrix components synthesis, the degradation of the matrix, and the synthesis of surfactants (Solano et al. 2014).

The EPS matrix (see fig. 3.1.) is a dynamic structure that consists of a variety of molecules (i.e. polysaccharides, proteins, lipids, extracellular DNA (eDNA), metal ions and water), which are bound together by weak physicochemical interactions (Flemming and Wingender 2010). The many functions of the EPS matrix – from the retention of water to enzymatic activity, from the organisation of space to protective barrier – are at the origin of the common developmental dynamics, the metabolic co-dependence, and the enhanced immunological response of biofilms.

Through mechanical forces and concentrations of eDNA, extracellular signals and enzymes, the EPS matrix places several functional constraints on the cells of the biofilm and it actively contributes to the realisation and functioning of the overall organisation of the system (Bich et al. 2019). It makes the association of cells much more cohesive and coordinated than in the planktonic state, leading to a three-dimensional architecture (Steinberg and Kolodkin-Gal 2015). During biofilm development, the presence of the EPS matrix mechanically inhibits the rotation of the flagella of the cells, and triggers intracellular signal cascades that increase the production and deposition of matrix molecules (Cairns et al. 2014). The 'activated matrix' (Flemming et al. 2007) – characterised by the presence of digestive enzymes, signal molecules, eDNA, lytic enzymes, etc. – is involved in the exchange of genetic material, in the control of cell behaviour, in the differentiation of cells into persister cells, spores, protease cells (Cairns et al. 2014; Steinberg and Kolodkin-Gal 2015), and in the control of the mobility of bacteria (Steinberg and Kolodkin-Gal 2015).

The EPS matrix promotes also the spatial proximity of cells and it is responsible for the presence of extracellular enzymes that give rise to an external digestive system, thus favouring integrated co-metabolism[11] and synthrophy[12] among symbiotic partners (Dragoš and Kovács 2017). Moreover, fluids can flow throughout the biofilm by virtue of channels, realised by the EPS matrix, that allow the diffusion of nutrients to the cells of the innermost layers, and the distribution and removal of

aggregation of bacteria from the two species is made possible by the fact that *V. atypica* produces a soluble chemical signal that triggers amylase expression in *S. gordonii*, thereby increasing the degradation of complex carbohydrates and lactic-acid production (Keller and Surette 2006).

[11] By 'co-metabolism', I mean the simultaneous degradation of two compounds: the degradation of the second compound hinges on the presence of the first compound.

[12] By 'syntrophy', I refer to the phenomenon by which one species feeds on the by-products of another species.

metabolic products (Sutherland 2001), also enabling medium range interaction and communication within the system.

The EPS matrix allows for the formation of different biochemical environments in such a way that otherwise incompatible bacteria (e.g. aerobic and anaerobic) may co-exist in the same biofilm. EPS matrix also reduces diffusion rates of the compounds within the biofilm matrix itself, modulates gene expression patterns and decreases growth rates of the biofilm cells, making the biofilm robust with respect to external sources of perturbations and pathogens. In addition, the EPS matrix allows for the interconnection of *innate* and *induced* resistance factors that make the overall biofilm more resistant to external agents (Andersson and O'Toole 2008), and it favours *multicellular strategies* and a *multilayer structure* that inhibit the diffusion of antimicrobial agents within the biofilm (Stewart and Costerton 2001). As a result, biofilms achieve a certain form of collective *immunological* capability and gain a fitness advantage over their planktonic state (Burmølle et al. 2014).

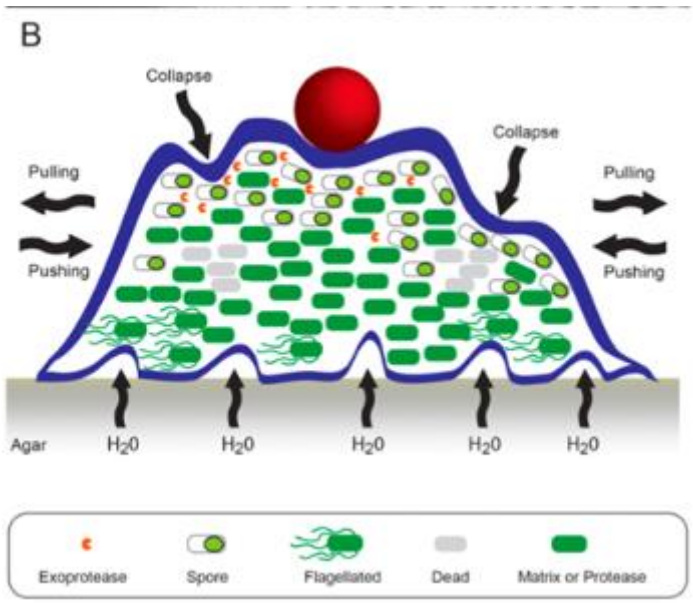

Figure 3.1 The biofilm matrix in B. Subtilis (Cairns et al. 2014, p. 588).

In nature, multispecies biofilms tend to be more common than single-species biofilms. However, these latter are present in a variety of infections and on the surface of medical implants (O'Toole et al. 2000). Single- and multispecies biofilms are essentially similar both in the stages of the extracellular matrix deposition and degradation (attachment, maturation, and dispersion) and in the mechanisms involved in bacterial communication (quorum sensing) and bacterial conjugation.

Nevertheless, they exhibit some differences in the interactions among bacterial partners. Multispecies biofilms exhibit much more variety of exchanges of nutrients and electrons than single-species biofilms, and thus they "gain energy from a series of reactions that a single species might lack" (Lohse et al. 2018, p. 27). Furthermore, it has been observed that the inclusion of other bacterial species in a single-species community may provide their members with numerous *physiological advantages* (e.g. passive resistance, metabolic cooperation, more efficient DNA sharing) (Wolcott et al. 2012). It is worth noting that, compared to single-species biofilms, multispecies biofilms can develop both *cooperative* relationships leading to increased biomass of the bacterial members and *competitive* relationships producing a decreased biomass of all members (Liu et al. 2016). It therefore seems that the life cycle of multispecies biofilms is subject to sharper fluctuations and, therefore, variability than that of single-species biofilms, thus potentially providing multispecies biofilms with increased capacities to invade surfaces, proliferate, and develop drug resistance. In the light of all these characteristics, it seems reasonable to suggest that both single- and multispecies biofilms exhibit the *same kind* of physiological integration enabled by the extracellular matrix; nonetheless, internal differentiation and functional diversity seems to be higher in some types of multispecies biofilms because of the higher variety of metabolic exchanges between bacterial partners.

In the light of the above, what *type* (and degree) of integration – and therefore, of individuality – does this form of association achieve? Integration in biofilms is achieved by *means of collective control* exerted by QS mechanisms and EPS matrix at longer ranges than those that characterise basic cell-to-cell interactions alone. This is also a *coarse-grained type control*, based on differences in concentrations (of signals, control macromolecules, etc.), which is exerted gradually in space, at short and medium ranges, through gradients of signalling interactions (QS) and of distributions of EPS molecules. Although this form of control is *not specific* – i.e. does not rely on single interactions for a certain effect – it can give rise to a high variety of behaviours within the collective system.

From the organisational point of view, the EPS matrix structures are higher-level control subsystems (exerted over the individual cells) that contribute at medium ranges to the structural and functional cohesiveness and cooperation within biofilms. This is the reason why some authors have regarded biofilms as interactively and evolutionarily cohesive biological integrated individuals (Ereshefsky and Pedroso 2013, 2015; Doolittle 2013), and even as full-fledged multicellular *organisms* (Shapiro 1988). However, due care should be exercised with regard to the *type* and *degree* of functional integration and individuality of biofilms, inasmuch as the capability of the EPS

matrix to give rise to a fully integrated system is *limited* by several factors. Firstly, the EPS matrix does control the activity and fate of their cells only at short and medium ranges, but not at long ranges (relative to the scale of the system), due to the lack of full-fledged vascularisation, among other things (Bich et al. 2019). It is worth noting that there is no specific constraint that has the capability of exerting a long-range type of control with a systemic reach. Indeed, global effects are achieved through self-organisation, expanding usually by means of gradients, which is the result of coarse-grained distributed control. Secondly, since the EPS matrix enables some degree of spatial segregation and functional differentiation only by means of gradients of concentrations, the internal modularity of the system is limited. Thirdly, the EPS matrix lacks components that make possible modularity and the construction of a global interface with the environment[13].

In sum, while providing cohesiveness, the EPS does not establish clear-cut global boundaries or interfaces, nor long range control mechanisms. Therefore, if compared with specialised membrane mechanisms found in unicellular systems or interfaces such as the epithelium in eukaryotic multicellular systems, the EPS exerts a weaker and less specific control upon the permeability and selectivity of the system as a whole. Furthermore, the modulation of fluid transport by EPS channels is limited. This in turn limits the overall capability of the collective system to grow and to control cells at longer ranges.

Finally, as a consequence of the distinctive organisation realised by biofilms and the kind of control exerted within them, the type of (*collective*) *reproduction* carried out by biofilms is affected by the fact that the cells may keep their autonomy and revert cell-differentiation. Although some specialised cells may play the function of spores (e.g. in *B. subtilis* biofilm) (Claessen et al. 2014) and some cheats (e.g. in *P. aeruginosa* biofilm) are considered as primitive forms of a germ cells (Rainey and Kerr 2010; Hammerschmidt et al. 2014), this type of differentiation – which is the result of self-organisation starting from local interaction rather than specific control mechanisms – does not satisfy the requirements for units of selection. Moreover, each germ cell has its own history of mutation as a soma cell before randomly differentiating into a reproductive spore. In addition, most biofilms are characterised by the entrance and dispersion of cells, so that it can be claimed that biofilms do not exhibit a type of reproduction coordinated at the level of the whole system. The lack of a unified reproduction is even more evident in multispecies biofilms, where different genetic pools are represented without a reproductive bottleneck.

[13] For example, it lacks collagen IV, which promotes the realisation of interfaces and organ formation in eukaryotic multicellular systems, due to the role it plays in the basement membranes (Fidler et al. 2017).

The fact that biofilms lack standard reproductive criteria for individuality (e.g. high levels of germ-soma specialisation, unified reproductive lineages, reproductive bottlenecks) poses some challenging questions about whether or not they can be regarded as units of selection, and thus evolutionary individuals. This issue forms the core of the debate between Clarke's (2016) and Ereshefsky and Pedroso's accounts (2013, 2015). According to Ereshefsky and Pedroso (2013, 2015), the criterion of evolutionary individuality based on the transference of genes from parents to offspring (vertical transmission) within the same lineage is too narrow. Thus, they propose a more open-ended approach according to which the members of a prokaryotic association (e.g. the prokaryotes of multispecies biofilms and consortia) share genes that provide them with mechanisms for trait transmission and reproduction. Thus, they "achieve evolutionary individuality but do not transmit their traits through single-species lineages. […] Trait transmission in such consortia is accomplished through both lateral and vertical gene transfer, and the reproduction (or production) of such consortia is typically accomplished by aggregation" (Ereshefsky and Pedroso 2015, p. 10131). By contrast, Clarke (2016), who defends a view of heritage and evolutionary individuality based on parent-offspring lineage, has argued that the EPS matrix cannot give rise to a common lineage, and therefore it is not possible to regard biofilms as units of selection, since heritable variation occurs at the level of the single bacterial components rather than at the level of the biofilm as a whole multispecies.

3.4 ENDOSYMBIOSIS IN PROKARYOTES: ENGULFMENT AND ITS IMPLICATIONS

In this section, I analyse a very different form of prokaryotic association, based on (asymmetric) engulfment of a species of bacteria within another (i.e. endosymbiosis). I am interested in this form of association because it is presumably of the type that led to a paradigmatic case of highly functionally integrated system: the eukaryotic cell and its organelles of endosymbiotic origin. Nevertheless, it is essential to underline that the endosymbiotic events that led to the origin of mitochondria and plastids in the eukaryotic cell were extremely rare in the prokaryotic world (Lane 2005; Booth and Doolittle 2015), probably because of the difficulty of overcoming conflicts between two prokaryotes fostering an endosymbiotic relationship.

Indeed, only *one* case of evolutionary stable [14] endosymbiotic relationship between two prokaryotes has been discovered so far[15]: a γ-proteobacterium (*Candidatus Moranella endobia*) that lives inside a ß-proteobacterium (*Candidatus Tremblaya princeps*). This association is very peculiar because it is not capable of an independent form of life; indeed, it exists only enclosed in specialised cells (the bacteriocytes) of a specific organ (the bacteriome) of the mealybug insects[16] (Figg. 3.2 and 3.3). Phylogenetically, the symbionts entered the mealybug at different times, the *Tremblaya* first and the *Moranella* later, so that their endosymbiotic relationship originated within the insect. In this sense, this endosymbiotic association shares some organisational features with the prokaryotic endosymbionts of sap-feeding insects, although in these latter cases the prokaryotic organisms live in eukaryotic cells (von Dohlen et al. 2001; McCutcheon and von Dohlen 2011). Like many endosymbionts of sap-feeding insects (e.g. Hodgkinia, Carsonella, Sulcia, etc.), both *Tremblaya* and *Moranella* have extremely reduced genomes that affect their metabolic (i.e. anabolic and catabolic pathways), genomic (i.e. DNA replication, transcription and translation), and regulatory functions (i.e. metabolic regulation and gene regulation). *Moranella*'s genome, which is four times larger than *Tremblaya's*[17], codes for RNA molecules and proteins that cannot be expressed by *Tremblaya* genome; but *Moranella*'s genome is far from being self-sufficient and the functioning of this organism depends, in turn, on some of the few gene products from *Tremblaya*.

In spite of these specificities and of the impossibility to consider the consortium as a living fossil of an earlier step in the eukaryogenesis, the *Moranella-Tremblaya* association deserves a careful analysis for my purposes, precisely because it shows the early-stage implications of engulfment among two types of prokaryotic cells. Thus, it can shed light on the possibilities opened by this

[14] By "*evolutionary* stable relationship", I mean a relationship that persist across several generations and that undergoes natural selection as a whole.

[15] Intracellular bacteria have been identified in some blue-green algae of the species *Pleurocapsa minor* in the seventies (Wujeck 1979), but the physiology of this association has not been investigated. Other cases of intracellular bacteria invading the periplasm (e.g. Bdellovibrio) or the cytoplasm (e.g. Daptobacter) of other bacteria have been found (Corsaro and Venditti 2006). However, these cases represent *transient* symbiotic relationships (i.e. parasites) that do not give rise to *an evolutionary stable* relationship.

[16] For the sake of the argument, I just focus on the endosymbiotic relationship between the two bacteria (*Tremblaya* and *Moranella*), leaving aside the functional contribution of the insect. Indeed, this chapter studies the functional integration of associations of *prokaryotes* and not the functional interdependence between prokaryotes and (multicellular) eukaryotes. Therefore, for clarity, hereinafter I will use the term 'host' to refer to *Tremblaya*, whereas the term 'endosymbiont' refers to *Moranella*. I will use "mealybug cells" for those eukaryotic cells that contain the *Tremblaya-Moranella* association.

[17] *Tremblaya*'s genome is 138,927 bp in length, whereas *Moranella*'s is 538, 924 bp (McCutcheon and von Dohlen 2011). The difference in genome size between *Tremblaya* and *Moranella* is consistent with the hypothesis that *Moranella* penetrated *Tremblaya* as a secondary endosymbiotic event (López-Madrigal et al. 2013a).

relationship and on the organisational problems it needs to overcome in order to maintain viability. Let us examine how engulfment affects this symbiotic relationship.

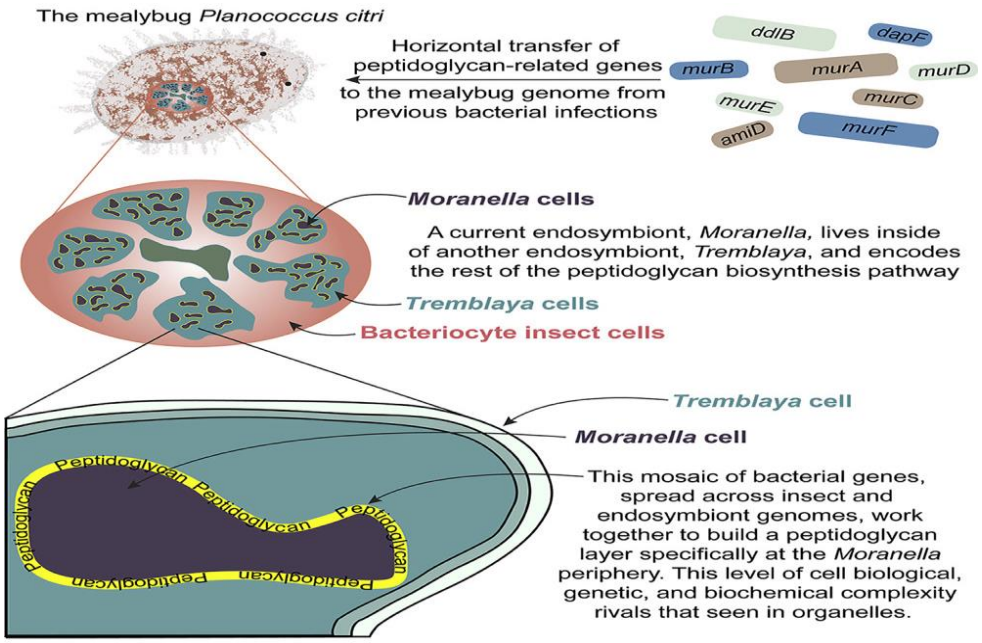

Figure 3.2 The endosymbiotic relationship between Tremblaya and Moranella (Bublitz et al. 2019).

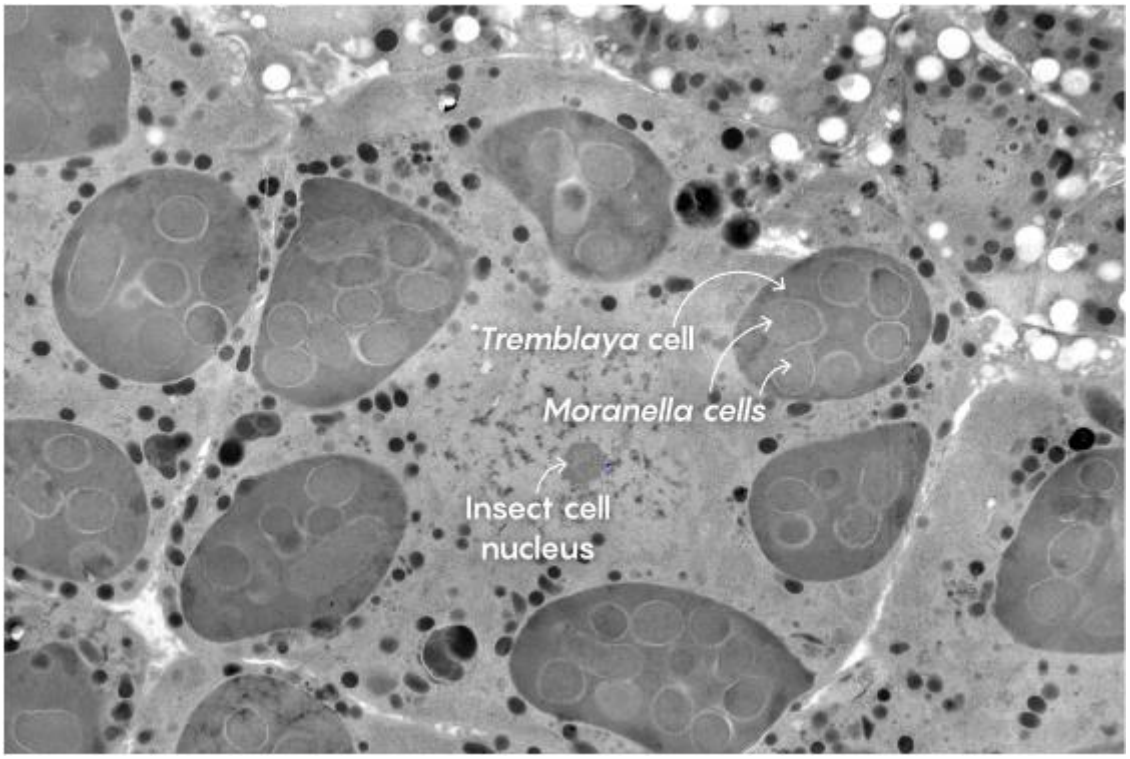

Figure 3.3. An electron micrograph image of the endosymbiotic relationship between Tremblaya and Moranella within the Mealybug (Callier 2019).

Engulfment creates a situation that is very different from the one brought about by the EPS matrix in biofilms, because now the different metabolic organisations (of the host, *Tremblaya*, and the endosymbiont, *Moranella*) share a common *selective control boundary,* i.e. the *Tremblaya'*s membrane, a global constraint which enables a systemic long range control on all parts, and determines the type and degree of physiological integration between them. Moreover, since one of the organisms is located within the cytoplasm of the other, global viability requires a different type of functional coordination. Like many endosymbiotic relationships, *Tremblaya* and *Moranella* exhibit *metabolic complementation*, since the symbiotic partners partially contribute to the same metabolic pathways. A good example is provided by the metabolism of carbohydrates. *Tremblaya* has the genes encoding for only two enzymes of the pentose phosphate pathways (transaldolase B and transketolase). The rest of the enzymes for the pentose phosphate pathways, glycolysis, the phosphotransferase system, and the pyruvate dehydrogenase complex are expressed by *Moranella's* genome (López-Madrigal et al. 2013a). Amino acid biosynthesis constitutes another clear example of metabolic complementation: *Tremblaya* contains genes encoding for ten essential amino acids, but none of the amino acid pathways is complete in either *Tremblaya* or *Moranella,* so that these pathways need to be complemented by a patchwork of metabolites and enzymes from both partners (McCutcheon and von Dohlen 2011). Other metabolic pathways are incomplete (e.g. the tricarboxylic acid cycle) or absent (e.g. the nucleotide synthesis de novo or the synthesis of vitamins and cofactors) in the consortium.

The functional interdependence exhibited by the *Tremblaya-Moranella* association, however, is much deeper than metabolic complementarity and complementation – i.e. the exchange of metabolites or intermediate substrates, respectively – which are widespread in nature and characterise biofilms as well. Importantly, *Tremblaya* and *Moranella* jointly realise the [Fe-S] cluster[18], which is usually not fully preserved in endosymbionts with reduced genomes (López-Madrigal et al. 2013a). The synthesis and assembly of this cluster requires a complex molecular machinery, and both members of the consortium are involved in the synthesis and maintenance of it, thus exhibiting a high degree of coordination. Another important aspect is that *Tremblaya* is totally dependent on *Moranella* for ATP synthesis, a feature that probably makes this consortium, according to Lopez-Madrigal et al. (2013a), the only known case in which all energy sources appear

[18] The [Fe-S]-cluster is a prosthetic group mainly involved in oxidation-reduction reactions. It plays several important functions related to energy metabolism and regulation. In particular, it plays a role in bacterial (and mitochondrial) respiratory complexes, in enzyme catalysis and in the sensing environmental or intracellular conditions to regulate gene expression (Lill 2009).

to be provided by only one of the partners. This is somehow analogous to what happens in the eukaryotic cell, where the mitochondria perform this function. Additionally, the cell-envelope structure is simplified in both bacteria, because both *Tremblaya* and *Moranella* have lost most of the genes for the synthesis of murein and lipopolysaccharides.

The high degree of functional coordination between the endosymbiont and the host can be seen in the entangled way their *genetic functions* are realised[19]. Transcription requires the contribution of both organisms. *Tremblaya* encodes all the essential subunits of RNA polymerase and a single sigma factor, but it lacks the genes responsible for the basic transcription machinery, and for RNA processing and degradation. By contrast, *Moranella* has a minimal but complete transcription machinery and a number of genes encoding proteins that assist transcription. Furthermore, several transcriptional regulators, the functions of which are not yet fully known, and which are usually absent in endosymbionts with reduced genomes, have been retained by the genome of *Moranella* and they may play a role in the control of the transcription in this organism. Regarding translation, the consortium performs a very complex *functional complementation*[20] and, according to López-Madrigal et al. (2013a), it may constitute the only known case for this specific function. While *Moranella* encodes more than 80% of the tRNA genes for the consortium, *Tremblaya* has retained tRNA genes for the most frequently used codons for alanine and, importantly, those for lysine, which are missing from *Moranella's* genome. Both *Tremblaya* and *Moranella* code for a high number of ribosomal proteins, giving rise to a ribosomal redundancy that could play a (not yet known) functional role for both symbiotic partners. However, only *Moranella* encodes ribosome maturation proteins and translational factors. In sum, the consortium shows a high degree of genetic complementarity: *Tremblaya* has lost most of the genes not only for metabolic but also for genomic functions, whereas *Moranella* has retained different genes for metabolism and genomic functions that *complement* those of *Tremblaya*, giving rise to a highly co-dependent relationship in which each partner partly contributes to the control mechanisms of the consortium.

In the light of the above, a fundamental question arises: how can this high degree of functional complementation happen? In order to answer to this question, I shall examine how *Tremblaya* and *Moranella* share functional constituents such as proteins through *Moranella*'s membrane. In order

[19] See López-Madrigal et al. (2013a) for the details.

[20] By 'functional complementation', I mean the exchange of components that perform, or contribute to, specific functions (such as proteins, tRNA, parts of ribosomal machinery etc.) between the members of the association. It is different from 'metabolic complementation' which, instead, consists in the exchange of intermediate metabolic substrates.

to perform its essential cellular functions, *Tremblaya* needs to import from *Moranella*'s cytoplasm not only metabolites, amino acids or carbon sources – as it is typical in common cases of endosymbiosis – but also functional control components such as proteins, tRNAs, ATP and molecular complexes (McCutcheon and von Dohlen 2011; López-Madrigal et al. 2011; López-Madrigal et al. 2013a)[21]. The consortium, therefore, requires a special transport system for the exchange of big molecules between the two partners[22]. *Moranella*'s genome encodes a limited set of active transporters (e.g. the phosphotransferase system for the transport of hexoses) and two channels (MscL and YbaL) associated with osmotic stress, which play an important role in the excretion of low molecular weight molecules (e.g. ions and metabolites) and small cytoplasmic proteins. The Sec translocon[23] machinery of *Moranella* exhibits a very reduced protein permeability and, therefore, it does not seem to be responsible for the provision of proteins and RNAs to *Tremblaya*. Hence, it has been hypothesised that the protein translocation from *Moranella* to *Tremblaya* may be due to a very primitive mechanism, yet effective in this case. It would consist in a transient perforation of the *Moranella* plasma membrane and of the osmotic channel MscL, controlled by osmotic stress[24]. It is made possible by two factors: 1) the peculiar composition of *Moranella*'s membrane, more subject to perforation; and 2) the unequal distribution of metabolic products in the two partners. In this way, the cell wall of *Moranella* is transiently damaged and proteins would be able to reach *Tremblaya* cytoplasm (López-Madrigal et al. 2013b), thus allowing the two partners to exchange control components such as proteins and operate in an integrated way.

From the above, I can make some important remarks. The establishment of an *endosymbiotic relationship* between two bacteria is a condition that has rarely been observed in the biological world, because engulfment creates an intimate relationship within the cytoplasm of one of the partners that is difficult to maintain. Indeed, the absence of a compartmentalised nucleus makes the genome of the prokaryotic host, not protected by the nucleus membrane, more susceptible to a bombardment from pieces of DNA of endosymbiotic origin, in such a way that the host would be genetically unstable and, therefore, not adaptive (Lane 2015). This is one of the reasons why

[21] The same happens, in lesser degree, in the opposite direction from *Tremblaya* to *Moranella.*

[22] Exchanging small molecules such as amino acids and metabolites is deeply different than exchanging proteins, tRNA or other big molecules, and the two cases depend on distinct mechanisms of transport. Unlike the case study, the endosymbiotic relationship between a eukaryotic host and prokaryotic endosymbionts usually relies only on the exchange of amino acids between the two partners. The import of amino acids into the endosymbionts and the export of other amino acids to the host is usually mediated by transporters provided by the host (Duncan et al. 2014).

[23] A translocon is a complex of secretory ('sec') proteins involved in the translocation of polypeptides across the membranes.

[24] Osmotic stress (or shock) is a sudden change in the solute concentration around a cell causing a change in the movement of water across the cell membrane.

bacterial engulfment usually takes the form of a *transient* and *ephemeral* parasitic relationship, which ends when the prey (the "host") is killed (e.g. *Vampirococcus* and *Bdellovibrio*) or the predator leaves the prey (e.g. *Daptobacter*).

The endosymbiotic relationship between *Tremblaya* and *Moranella* shows the exceptional conditions required nowadays for a viable mutualistic relation. The massive loss of genes[25], as demonstrated by *Tremblaya*'s genome, implies that many metabolic pathways are absent or incomplete (e.g. those for amino acid biosynthesis). More importantly, some genetic functions (e.g. DNA replication, recombination, repair, and transcription), and regulatory processes (e.g. transcriptional regulators and translational factors) are severally undermined. As we have seen, most of these functions are either complemented or supplied by *Moranella* and thus require the joint action of both partners, thus suggesting a *highly functional interconnected organisation* in which each bacterium requires the other one. For these reasons, Lopez-Madrigal et al. (2011; 2013a) make the strong claim that "'Ca. Tremblaya princeps' cannot be considered an independent organism, but that the consortium with its gammaproteobacterial symbiotic associate represents a new composite living being" (Lopez-Madrigal et al. 2011, p. 5587).

This case study is not aimed at putting the consortium in the same category as, for example, a eukaryotic cell, characterised by a number of specialised mechanisms that control the interaction between the cell and its organelles of symbiotic origin. I rather suggest that the *engulfment* between prokaryotes opens a series of challenges that need to be overcome in order to maintain a viable association. Unlike in bacterial biofilms, the establishment of a common boundary (the membrane of *Tremblaya*) makes possible the control of the global boundary conditions of the *Tremblaya-Moranella* system. While in biofilms the EPS matrix allows medium range control upon ensembles of cells, the endosymbiosis realized by the *Tremblaya-Moranella* association depends on a global spatial constraint, the membrane of *Tremblaya,* which has a (long-range) systemic reach upon all the components (the molecules and the endosymbionts in the cytoplasm of *Tremblaya*). Another important type of spatial constraint is the membrane of the *Moranella* endosymbionts. The

[25] The loss of genes is an interesting feature of many commensal and mutualistic (symbiotic) relationships and it has been hypothesised that it increases the fitness of the overall associations. Morris et al. (2012) have coined the expression of "Black Queen hypothesis" to posit that "certain genes, or more broadly, biological functions, are analogous to the queen of spades. Such functions are costly and therefore undesirable, leading to a selective advantage for organisms that stop performing them. At the same time, the function must provide an indispensable public good, necessitating its retention by at least a subset of the individuals in the community" (Morris et al. 2012). In most cases what is shared is metabolic products, giving rise to forms of "syntrophic" integration by forming ecological networks (Skillings, 2019). In other cases, the members of the association share functional components under collective constraints such as EPS matrix or a common boundary, thus giving rise to forms of cross-control that allow for forms of physiological integration (Bich, 2019).

presence of an internal spatial constraint, the membrane of *Moranella* symbionts, allows for a further compartmentalisation and modularity with the potential for the evolution of specific controllers capable to modulate the permeability of the internal compartment, like it happened in the case of organelles during eukaryogenesis. In this context, while exerting a systemic constraint at the global level, the endosymbiotic association exhibits more specific forms of (molecular) control, not achieved by means of self-organisation only, but exerted by functional components from either *Tremblaya* or *Moranella*, or by functional components which are assembled from parts synthesized by both symbionts.

Moreover, the engulfment favours the genetic reduction and functional reorganisation of the two symbionts, thus leading to symbiotic partners that are necessarily less autonomous than those of a biofilm. A fundamental aspect of the integration between *Tremblaya* and *Moranella* is that they exchange not only metabolites and amino acids, but also their main functional components. In the stable context of a nested endosymbiosis within the mealybug, this consortium has provided primitive, yet effective responses to the aforementioned challenges (e.g. the presumed passage of proteins, tRNA, etc.) through mechanisms related to osmotic stress.

3.5 ORGANISATIONAL IMPLICATIONS OF THE EPS MATRIX AND ENGULFMENT: A COMPARATIVE DISCUSSION

The previous two sections have highlighted the distinctive organisational features of biofilms and of the endosymbiosis between bacteria: their different types of collective borders that spatially constrain at different ranges the members of these associations and the forms of controls they enable, from coarse-grained distributed control in biofilms to more specific fine-grained ones shared by both partners in the endosymbiotic association. In this section, I compare their systemic implications. On the basis of this comparative discussion, I investigate the conceptual links between the engulfment of the *Tremblaya-Moranella* association and that of mitochondria and chloroplasts in the eukaryotic cell.

In the case of biofilms, the role of the collective spatial constraint is played by the EPS matrix: a dynamic extracellular structure that provides global cohesion, controls the activity of whole groups of bacteria (and archaea) at short and medium ranges, and maintains the cells adjacent to one another while differentiating gradients of space characterised by different boundary conditions, thus enabling functional differentiation and co-metabolism, syntrophy, common development and

an enhanced immune response of the overall biofilm. In the case of the endosymbiosis between two bacteria, instead, engulfment provides the two organisms with a common global membrane (i.e. the membrane of *Tremblaya*). This is a global spatial constraint that favours an asymmetric relation between the host and the endosymbiont with a much more intimate and demanding collaboration between the members of the association, which forces the establishment of systemic long-range control (relative to the size of the whole system) and enables the realisation of fine-grained specific control mechanisms to coordinate the activities of the members.

The EPS matrix of biofilms and the membranes of the endosymbiotic associations impose on the symbiotic partners *different types of spatial constraints* that affect their collective physiological functions. Although both EPS matrix and engulfment allow for metabolic coordination, they lead to *different* forms of collective *metabolic* organisations. In the case of biofilms, the spatial proximity of bacteria within the EPS matrix favours co-metabolism and synthrophy, and the release of enzymes in the EPS gives rise to an external digestive system. Nevertheless, bacteria keep their autonomy and can in principle develop new metabolic relationships or leave the biofilm altogether. In the case of endosymbiosis, by contrast, *engulfment* implies a very specific set of selective pressures that pave the way for a symbiotic "rabbit hole" in which incomplete metabolic pathways of the host are complemented by those of the endosymbiont and vice versa (Bennet and Moran 2015). Accordingly, in this inescapable association, metabolic interdependences are not facultative nor easily realisable by interchangeable partners, and they are also much more stable across time (i.e. over generations) if compared to the case of biofilms.

Yet the difference between bacterial endosymbiosis and biofilm is not limited to the stability of metabolic interdependencies, but includes two other aspects that have deeper organisational implications. In the first place, the bacterial EPS represents a collective and fuzzy spatial constraint, with little and *unspecific control* upon the passage of molecules or organisms through it. In the case of engulfment, instead, the membrane of the host provides the system with a common selective border characterised by *global and mo*re *precise mechanisms* of control of permeability and transport (i.e. the capability to modulate the internal pH, concentrations of metabolites, osmotic pressure, spatial and temporal distribution of specific chemicals). With regard to the internal organisation of space, the EPS allows for a *coarse-grained* spatial differentiation, while the membrane of the endosymbionts provides the endosymbiotic association with much more modularity due to the presence of internal compartments, opening up the possibility for a *fine-grained* control of permeability of the endosymbiont membrane and targeted transport of proteins.

In the second place, fundamental differences between the two types of associations arise also in the different ways of controlling *intercellular relations*. In biofilms, QS and the EPS matrix exert a *distributed control* upon the cells at short and medium ranges, by realising gradients of signalling interactions (QS) and of distributions of EPS molecules. In engulfed symbiosis, instead, functional coordination requires *specific control* at all ranges and avoidance of conflict. In the case of the *Moranella-Tremblaya* association, for instance, proteins, tRNA and other control molecules of *Moranella* need to pass across its membrane to enter the cytoplasm of *Tremblaya* (where they can directly control different biosynthetic processes or regulate basic functions). To a lesser degree, the same happens to some control molecules from *Tremblaya*, so that the whole consortium maintains viability through a very basic form of cross-control[26] and interlocked regulation[27]. Thus, engulfment cannot succeed unless a tight control of the most fundamental functions of both the host and the endosymbiont is established. This requires a deep functional re-organisation with an irreversible loss of autonomy of the former partners.

For these reasons, I suggest that the engulfment between two prokaryotes constitutes the fundamental requirement for the appearance of a strong, and non-facultative, functional integration between different symbiotic partners. This type of relationship is very demanding in organisational terms, insofar as such a specific control requires: (1) the presence of the right components in the right place at a given time; and (2) the implementation of mechanisms for transporting proteins and other complex control macromolecules across the membrane of the endosymbiont, not only basic building blocks such as metabolites and amino acids[28].

These different types of physiological integration pose some difficult questions about the relationship between functional integration, system-level coordinated reproduction, and heredity. It is not my purpose to find a solution to this complex issue; however, I suggest that in (prokaryotic) collective organisations a certain level of physiological integration is required to gain the capability of reproducing as a unit[29], because parts need to be functionally differentiated (e.g. between germ

[26] By 'cross-control', I mean one partner producing the components that control processes in the other.

[27] By 'interlocked' regulation, I mean the activity of regulatory mechanisms which rely on the components produced by both partners.

[28] The *Moranella-Tremblaya* consortium realises transport through a very basic mechanism based on osmosis in presence of a weakened membrane. In spite of being unspecific and inefficient, it can guarantee the viability of the consortium in the very stable environment of mealybug cells. In fact, this particular mechanism lacks the presence of complex channels and mechanisms for protein targeting that would allow much more specific control upon the localisation of functional components. A more stable and robust solution to this problem would instead require a much deeper re-organisation of the systems involved, which is indeed what it is supposed to have happened during the process of eukaryogenesis.

[29] The relation between functional integration and system-level reproduction will further be studied in chapter 6.

and soma) and their activities coordinated for this purpose. For example, under starvation conditions, some single-species biofilms (e.g. *B. subtilis* or *M. xanthus*) can produce collective forms of reproduction (e.g. the spores of *B. subtilis* or the fruiting bodies of *M. xanthus*) by means of (local) *contact mediated signals,* that are enabled by the spatial proximity of cells, and the resulting formation of gradients through self-organisation (Julien et al. 2000; Muñoz-Dorado et al. 2016). Thus, the existence of an *extracellular matrix*, which keeps bacteria close to one another, may play an organisational role not only in establishing a certain kind of physiological integration, but also a *diffused* and *transient*, *context-dependent* (i.e. dependent on starvation conditions), collective reproductive system, realised through coarse-grained control, based on local interactions and the resulting formation of gradients, rather than coordinated by means of global specific control mechanisms. Similarly, some 'multi-cellular prokaryotes' (Claessen et al. 2014; Lyons and Kolter 2015) such as filamentous bacteria (e.g. *N. punctiforme*), actinomycetes (e.g. *A. Israeli*) or beggiatoa (e.g. *B. leptomitoformis*) exhibit distinct kinds of collective reproduction that seem to be enabled by their spatial contiguity (e.g. through proteinaceous complexes) and a minimal degree of functional integration (e.g. intercellular signals, metabolic co-dependence) (see Claessen et al. 2014). In the case of endosymbiosis, *engulfed symbionts* are so tightly integrated that they cannot survive autonomously, and the endosymbionts can only be transmitted vertically due to the role of the host's membrane as global constraint. Consequently, the genes of both the host and the endosymbionts jointly change, and these variations can be selectively transmitted to the new generations, *making the whole system a unit of selection*. Moreover, engulfment allows for the implementation of further fine-tuned regulatory mechanisms during the evolution of the symbiosis to synchronise the processes of growth and division more precisely.

A basic form of coarse-grained physiological integration is therefore a necessary, but not sufficient, condition for a collective reproduction and a vertical transmission of genes, as shown by multi-species biofilms. Although they have an EPS matrix that allows distributed mechanisms of regulatory control and signalling for synchronising the members of the association, neither a global (long-range) control upon the reproduction of the components, nor unified mechanisms for the differential variation of the gene pool of a biofilm have been reported in the current literature so far (see, for example, Lopez et al. 2009; Liu et al. 2016). As a result, the cells of single and multispecies biofilms may evolve *independently from one another* rather than undergo *co-selection*, as *evolutionary* individuals instead do. By contrast, the type of integration enabled by engulfment, as a common boundary and control constraint, paves the way not only for a higher degree of

integration and functional differentiation, but also for a collective reproduction, despite the fact that the species involved are different.

The engulfed association among prokaryotes imposes a specific set of constraints on the symbiotic partners, which determine a dramatic reduction in the endosymbiont genome, and lead to an irreversible functional dependence between the symbiotic partners. Despite its limits, the relevance of the relationship between *Tremblaya* and *Moranella* consists in the fact that it involves not only a sophisticated complementation of metabolic and genomic functions between the host and the endosymbiont, but also control upon protein localisation and the reproduction and development of the endosymbionts. These dynamics, which are hardly sketched in the consortium *Tremblaya-Moranella*, achieve the highest expression in *the eukaryotic organelles* of *endosymbiotic origin*, such as mitochondria and chloroplasts, to give rise to full-fledged functionally integrated systems: the eukaryotic cells. Since most of the proteins controlling and regulating the internal processes of proto-mitochondria and proto-chloroplasts were progressively encoded in the nucleus and synthesised in the cytoplasm of the (host) cell, the appearance, among other things, of a *protein import and targeting machinery* played a major role in the conversion of endosymbionts into organelles. It allowed the host to directly control and regulate the functions of the future organelle and of the overall consortium (Martin 2010; Cavalier-Smith 2007).

In sum, a viable engulfment establishes several constraints on the organisations of both the host and the endosymbionts that are much tighter and demanding than those placed by the EPS matrix on the bacteria of a biofilm. It is not a coincidence, therefore, that only very few cases of prokaryotic endosymbiosis have been discovered so far. The evolutionary stable internalisation of a prokaryotic organism within another one raises a series of issues whose solution leads to a deeper reproductive, developmental and metabolic integration, based on a more precise form of central control. Ultimately, they may give rise to the appearance of a strong integrated identity. The EPS matrix, instead, leads to more ephemeral, although highly successful, organisations, in which symbiotic partners retain a basic autonomy and are kept together by distributed forms of control, without a fine-tuned control of the overall development and reproduction.

3.6 CONCLUDING REMARKS

Our case study shows that in the prokaryotic world, a process of association of individuals may lead to different forms of collective units, with *different types and degrees of integration*. In this context,

functional integration can be broadly understood as a phenomenon that originates when a set of different and initially autonomous organisations (each one with its own functional parts) begins to functionally cooperate and share their local functions. It leads to the establishment of a wider collective organisation where some functional constraints of the constituent organisations are interlocked and control one another's processes in such a way that the whole system achieves viability. In this context I have identified a series of crucial elements that enable different types and degrees of functional integration, specifically: (1) different types and ranges of collective spatial constraints exerted by the EPS in biofilms and the global membrane in the endosymbiotic association; (2) different forms of control exerted by both the spatial and the intercellular control mechanisms, i.e. the distributed coarse grained control characteristic of biofilms and the specific fine-tuned control realised in the case of endosymbiosis; and (3) the different degrees of cross-control and interlocked regulation that are required in order to modulate and coordinate intercellular interactions in the different associations, with especially strong requirements for the endosymbiotic case of *Tremblaya* and *Moranella*, where many of these mechanisms are assembled from functional components produced by both partners.

However, the necessity of the associated parts to achieve a global viability implies that there could not be an indefinite number of integration possibilities. On the contrary, given a specific set of entities, only a discrete number of collective organisations are stable on the physiological and evolutionary scales. In prokaryotic collective organisations, we can find either biofilm-type forms of association or endosymbiotic associations of prokaryotic cells; but the latter seem, for all the reasons that I have analysed, quite rare and fragile until a completely new organisation – the eukaryotic cell – is realised.

As we have seen, the appearance of a strong form of functional integration in the prokaryotic domain is a process characterised by an initial step where different individualities (autonomous entities) enter in a process of irreversible association. Most symbiotic associations of prokaryotes, like biofilms, cannot be considered as full-fledged functionally integrated individuals, but rather as communities of (sometimes highly) coordinated organisms, kept together by means of distributed control mechanisms. Among these associations, the key for the achievement of a *strong functional integration* is the creation of an *asymmetric compartmentalisation*: a spatial border, a global constraint that functionally acts as a selective frontier between the associated system and the external environment. At the same time, engulfment is a much more difficult way of achieving a stable association, and it was at the origin of a long evolutionary travel full of conflicts.

Engulfment triggers a cascade of events that opens up (and forces) several possibilities for structural and functional reorganisation and biological novelty in both symbiotic partners. Although we have no traces or examples of the presumably long process that led to the eukaryotic cell, the case study suggests that this process might have likely involved the modification or loss of old functions and the appearance of new capacities, reaching more or less viable intermediate stages until a global robust viability was reached. Viable internalisation could only have been achieved and maintained by increasing functional integration through new forms of global control, starting from the modulation of the permeability of the common boundary, to different systems of transport and targeting of functional components between the partners through the endosymbionts' membranes, which in turn made it possible to implement precise mechanisms of cross-control and interlocked regulation. All of them contributed towards the generation of a new and stronger form of individuality with a regulatory machinery in charge of all the internal functions, exemplified by the composite organisation of a eukaryotic cell, where the original endosymbiotic cells lost their former autonomy and became organelles. The emergence of a new functionally integrated organisation, therefore, requires a functional redefinition of both the original organisms and of the symbiotic consortium as a whole

In sum, functional integration can generally be defined as the degree to which the different components of a biological dynamic regime of self-maintenance depend on one another for their production, maintenance, activity and reproduction. If we take the eukaryotic cell as the reference example of new forms of full-fledged biological individuality resulting from association between prokaryotes, individuality can be understood in terms of the degree, scale and precision of the control and coordination of the parts that collectively make the system a viable functional whole (i.e. an integrated unit). To do so, even the minimal forms of biological (and, likely, proto-biological) organisation require, in the first place, some internal functional differentiation (Mossio et al. 2009). A cohesive integration between different functional tasks is achieved, then, when the differentiation of functions is coordinated at the system level by control and regulatory mechanisms that (1) act across the different entities participating in the association, and (2) are exerted in such a way that the differentiated components can contribute through their activity to the maintenance of the system. As we have seen, biological systems can give rise to different forms of functional associations, exhibiting different degrees of integration. In this process of integration, the deeper the co-dependency between the original organisations, the higher is their progressive loss of

autonomy, accompanied by the appearance of new forms of control upon the members of the association.

CHAPTER 4 DIVIDE ET IMPERA: HOW CELLULAR INTEGRATION AND CELLULAR CONTROL ARE ESTABLISHED THROUGH THE DIVISION OF THE INTRACELLULAR SPACE

4.1 INTRODUCTION

Although it has been claimed for a long time that intracellular membrane-bound compartments are a unique feature of eukaryotic cells, it has been shown that also some species of bacteria and archaea possess them (Shively 2006; Murat et al. 2010). Both prokaryotic and eukaryotic microcompartments play an important role in cellular physiological processes, such as metabolism, genetic functions, cell cycle, etc. However, key differences between prokaryotic and eukaryotic organelles lie in their morphology and functionality, basically because eukaryotic organelles exhibit a larger variety of structures and functions than prokaryotic ones.

Whereas the previous chapter has studied how a common physical boundary surrounding prokaryotic collective organisations enables them to achieve a certain type and degree of functional integration, this chapter examines the role played by prokaryotic and eukaryotic membrane-bound compartments in the internal division of the cytoplasmic space and its consequences for the cellular physiological integration. This issue will be addressed by studying the main physiological mechanisms that allow bacterial and archaeal microcompartments and eukaryotic endomembranes [1] to be functionally integrated within the cellular network. Furthermore, the evolutionary origin of and evolutionary possibilities opened up by these intracellular structures will be addressed.

Thus, the research questions of this chapter can be framed as follows:

1. What are the *organisational features* of prokaryotic and eukaryotic endomembranes and organelles?

[1] It is worth noting that this chapter studies, as examples of eukaryotic endomembranes, the nuclear envelope and the membranes of the organelles of the endomembrane system. The membranes of the organelles of endosymbiotic origin (mitochondria, chloroplasts, and hydrogenosomes) will not be analysed for two main reasons: first, their role has conceptually been addressed in chapter 3; secondly, I think that my argument is well justified by the nucleus and the endomembrane system, and thus, for reasons of space, I prefer to avoid a further section on the membranes of the organelles of endosymbiotic origin.

2. What types and degrees of *functional integration* are enabled by prokaryotic and eukaryotic microcompartments?

3. What is the origin of, and which evolutionary possibilities are opened up by, prokaryotic and eukaryotic membrane-bound compartments?

It will be argued that prokaryotic and eukaryotic organelles differently divide the intracellular space and perform different functions. In both cases, the internal division of the intracellular space allows for a general improvement of cellular functions. This entails distinct forms of control over the constitutive processes of the cell: in prokaryotes, constitutive processes mostly occur in the cytoplasm, which also exercises an almost complete control over the microcompartments; in eukaryotes, instead, the control over the constitutive processes is shared among the cytoplasm and the eukaryotic organelles, so that the control over the global physiology of the cell is quite uniformly distributed among the them. These distinct kinds of control imply different forms of integration between cytoplasm and microcompartments.

The chapter is structured as follows. Section 4.2 reviews the main organisational properties of biological membranes. Sections 4.3, 4.4, and 4.5 analyse the organisational features of prokaryotic microcompartments (4.3) and eukaryotic endomembranes (4.4 and 4.5). Section 4.6 makes a comparison between prokaryotic and eukaryotic organelles and examines their different contributions to the physiological integration of cells. Finally, section 4.7 makes some concluding remarks.

4.2 A CRITICAL VIEW ON (INTRA)CELLULAR MEMBRANES

Biological membranes play a pivotal role in cellular organisation, basically because their physicochemical properties are an enabling condition for the internal chemical network and more generally the cellular life. Much of the conceptual effort, both in biology (see Edidin 2003; Goñi 2016; Bernardino de la Serna et al. 2016) and in philosophy of biology (see Maturana and Varela 1973; Varela 1979; Moreno and Mossio 2015), has been devoted to the understanding of the organisational properties of the plasma membrane. However, biological membranes include not only the plasma membrane, but also the set of *internal membrane*s that divide the cytoplasmic space of not only eukaryotic cells but also of a number of prokaryotes (bacteria and archaea) into cytoplasmic subregions. In an effort to characterise the organisational features of intracellular

membranes, this section reviews the main biological properties of the cellular membranes and seeks to see to what extent these characteristics can be applied to membrane-bound compartments.

A fundamental tenet of the autopoietic and organisational framework is that a physical boundary allows the distinction between the *interior* and the *exterior* of the cell, thus permitting a clear separation between the living system and its surroundings (Maturana and Varela 1973; Varela 1979; Moreno and Mossio 2015). In the case of intracellular membranes, this aspect is more problematic, because they do not separate an inside from an outside of the cell. Nevertheless, to a certain extent, the analogy with membrane cell holds true, because the internal membranes divide the inner cellular space so as to create specific cellular subregions that allow for the coexistence of different, sometimes incompatible, biochemical pathways and the enhancement of the efficiency of metabolic processes, thus improving the overall cell physiology (Helle et al. 2013; Gabaldón and Pittis 2015). Furthermore, the membranes of some eukaryotic organelles (e.g. the membranes of the endoplasmic reticulum or the Golgi apparatus) increase the organelle surface area and the volume in such a way as to permit a larger number of molecules into the membranes, thus increasing metabolic fluxes (Marshall 2012).

Since membranes are interfaces between different environments, they can undergo *remodelling* (i.e. changes in membrane composition and shape) in response to environmental cues. Changes in membrane composition (e.g. the change of loosely packing unsaturated lipids with tightly packing saturated ones) are aimed at preserving the physicochemical properties of membranes (e.g. viscosity, surface change density, thickness), which must be homeostatically maintained within a narrow range that is compatible with cellular physiology (Ernst et al. 2018). Many cells, ranging from prokaryotes to ectothermic animals, adapt the physicochemical properties of membranes to changes in ambient temperature to keep membrane physical properties. For example, *Bacillus subtilis* can change the thickness of its plasma membrane in response to cold (Cybulski et al. 2010). The membranes of organelles also undergo remodelling in response to internal (metabolic) perturbations. For example, the membrane of the endoplasmic reticulum can be remodelled as a result of changes in lipid synthesis (Ernst et al. 2018). This plasticity of biological membranes allows cells and membrane-bound compartments to adequately respond to external and/or internal variations and represents a fundamental contribution to cell physiology.

Cell membranes act as *spatial constraints* that selectively control the flow of molecules and metabolites that enter into and exit from the cell. Indeed, molecules can pass through the

membrane in a passive or active way [2] and the passage of molecules is constrained by transmembrane proteins (e.g. channels, transporters) that mediate the transport. Likewise, both prokaryotic and eukaryotic endomembranes act as spatial constraints, inasmuch as they act as selective barrier that allow for both a passive and active transport of substances from and to the organelles. For example, some bacterial microcompartments consist of protein shells with pores that act as selective permeability barriers that control the movement of enzyme cofactors, substrates, and products between the interior of the microcompartment and the cytoplasm of the cell by employing both passive and dynamic gated mechanisms (Chowdhury et al. 2014; Bobik et al. 2015). Eukaryotic membrane-bound compartments also have transmembrane proteins that control the movement of macromolecules from one side to another of the membrane; for instance, the nuclear pores of the nuclear envelope represent a selective barrier between the nucleoplasm and the cytoplasm (Lamond and Sleeman 2015).

Cell membranes are functionally interdependent with metabolism: the former constrains the flow of (macro)molecules so as to provide the metabolic network with the proper concentrations of substrates; the latter maintains the physical boundary insofar as it synthesizes its constitutive components (e.g. lipids, proteins, glycoproteins, and glycolipids) (Maturana and Varela 1973; Moreno and Mossio 2015). Interesting questions arise about the functional relationship between the intracellular membranes and the cellular metabolism: if it is true that organelles perform metabolic functions, are they involved in the biogenesis of their membranes or, instead, do they depend on metabolic reactions occurring in the cytoplasm? The answer depends on the site(s) where lipids, proteins, glycoproteins, and glycolipids are synthesized. In the case of prokaryotic microcompartments, these macromolecules are produced in the cytoplasm and the origin of internal membranes is likely due to self-assembly and invagination mechanisms employing cytoplasmic proteins (Murat et al. 2010). As regards eukaryotic internal membranes, the situation is much more complex: proteins are synthesized partly in the ribosomes of the cytoplasm, partly in those of the rough endoplasmic reticulum; most of membrane phospholipids are produced in the Golgi complex, whereas others (e.g. cholesterol) in the endoplasmic reticulum (Simons and Sampaio 2011). As a result, the biogenesis of internal membranes is entangled with metabolic processes

[2] In passive transport, molecules can move by exploiting the differences in their concentrations between the two sides of the membrane either by means of simple diffusion or through transmembrane proteins; in active transport, molecules move against the concentration gradient and, therefore, they need additional energy provided by different sources (e.g. the ATP hydrolysis) to overcome the energetic barrier.

occurring both in the cytoplasm and in the organelles and new forms of intracellular transports (e.g. vesicles) are required.

Cell membranes allow cells to interact with the surroundings through the exchange of signalling molecules. Indeed, cell membranes have many transmembrane receptors that detect extracellular cues and signals (e.g. nutrients, preys, or signals from other cells) and produce signalling cascades that activate or inhibit cellular processes. In turn, transmembrane receptors can send signals to other cells in such a way as to promote intercellular communication. If we consider intracellular membranes, their receptors are involved in intracellular signalling pathways that allow the functional coordination between the organelles and the cytoplasm or also among the different organelles. For example, the gated pores of prokaryotic intracellular membranes exchange signals with the cytoplasmic membranes in order to coordinate their metabolic reactions with those occurring in the cytoplasm (Chowdhury et al. 2014). Eukaryotic intracellular membranes also exhibit an elaborate system of inter-organelle communication that allows for a functional coordination among different organisms (Hieda 2019).

As pointed out in the third chapter, physical boundaries (and cytoplasmic membranes) allow for a certain degree of physiological integration of the overall organisation, because they constrain physiological mechanisms (e.g. metabolic, signalling, immunologic) and favour their coordination. If the analogy between cellular and intracellular membranes holds true, we can suppose that somehow intracellular membranes play a role in the functional integration of the cell and, notably, in the functional integration between the organelles surrounded by the membranes and the cytoplasmic space. Furthermore, since both some prokaryotes and all eukaryotes exhibit intracellular membranes and organelles, we may also ask whether their membranes are enabling conditions for the same kind of physiological integration or if there are some organisational differences. Thus, in order to address these issues, I examine in the following sections the main physiological mechanisms involved in the functional integration between membrane-bound compartments and cytoplasm.

4.3 PROKARYOTIC MICROCOMPARTMENTS

For a few decades, bacteria and archaea have been considered cells without internal compartments, often relying on the assumption that only eukaryotic cells exhibit an endomembrane system. In fact, it has been shown that many bacteria (e.g. cyanobacteria, magnetotactic bacteria, planctomycetes)

and archaea (e.g. *Ignicoccus hospitalis*) are characterised by protein- or lipid-bounded compartments[3] (Shively 2006; Murat et al. 2010; Diekmann and Pereira-Leal 2013) that favour the formation of intracellular metabolic niches. This section aims at exploring the organisational features of *protein*- and *lipid*-bounded *microcompartments* and the mechanisms by which they are integrated in the prokaryotic cell.

Two kinds of organelles are bounded by a *proteinaceous* membrane: the carboxysomes of cyanobacteria and chemoautolithotrophs (Yeates et al. 2008; Kerfeld et al. 2018), and the gas vesicles of some bacteria and archaea mostly living in aqueous environments (Pfeifer 2015). Carboxysomes serve as the site for the first step of the Calvin cycle, since they host the reactions between RuBisCo[4] and carbon anhydrase[5], thus increasing the efficiency of the productive carbon fixation reaction (Yeates et al. 2008) (Fig. 4.1). *Gas vesicles* control the buoyancy of cells (Pfeifer 2015), in such a way that water molecules, in spite of entering the gas vesicle, cannot form droplets on the inner surface because of its hydrophobic nature. Thus, gas vesicles control the movement of bacteria and archaea in the water column and, also, their exposition to light, salt, and different environmental stimuli.

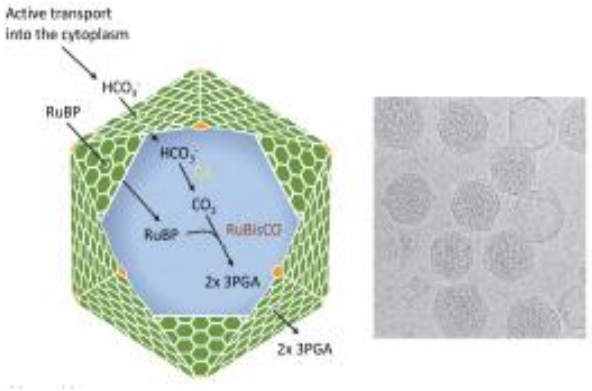

Figure 4.1 A carboxysome and the main reactions occurring within it (Bobik et al. 2015, p. 194).

Lipid-bounded organelles can be divided into three main categories: first, the magnetosomes of magnetotactic bacteria; second, the photosynthetic membranes of cyanobacteria (i.e. chromatophores and chlorosomes); finally, the internal membranes of Planctomycetes.

[3] These organelles stem from the inward folding (tubulation, invagination, or vesiculation) of the prokaryote's plasma membrane. As such, these prokaryotic micro-compartments have an autogenous origin, and not an endosymbiotic one.
[4] Ribulose-1,5-bisphosphate carboxylase/oxygenase is an enzyme involved in one of the most important carbon fixation pathways.
[5] RuBisCo catalyses the reaction of CO_2 with ribulose bisphosphate to two molecules of 3-phosphoglyceric acid and carbon anhydrase catalyses the transformation of bicarbonate into CO_2 (Murat et al. 2010).

Magnetosomes are lipid-bound spherical compartments in the cytoplasm of magnetotactic bacteria that surround magnetic particles, helping bacteria to be aligned with their magnetic field (Grant et al. 2018). The magnetosome membrane, which is continuous with or also derived from the inner cell membrane, contains a set of proteins and it is surrounded by a network of actin-like filaments that control magnetosomes position (Scheffel et al. 2006; Grant et al. 2018). Photosynthetic membranes[6] represent another important group of prokaryotic compartments that can be found in photosynthetic bacteria and that aim at maximising photosynthesis.

Bacterial *Planctomycetes* have one of the most interesting cases of bacterial organelles, inasmuch as they exhibit a cytoplasm that is divided into two distinct compartments: the *riboplasm* that contains the nucleoid and ribosomes, and the *paryphoplasm* that is the region between the outer and the inner membranes lacking ribosomes and often containing vesicles[7] (Fig. 4.2). Although *Planctomycetes* are gram-negative bacteria, they have three distinct features that are not present in this group of bacteria: first, an outer membrane that is highly invaginated, thus resembling the mitochondrial membrane (Santarella-Mellwig et al. 2013); second, some *Planctomycetes* (e.g. *Gemmata obscuriglobus*) have an additional third compartment surrounded by a double membrane that contains the nucleoid (Lindsay et al. 2001). Third, some *Planctomycetes* contain an organelle (i.e. the anammoxosome) within their cytoplasm which is enclosed by a single lipid bilayer. Anammoxosomes are responsible for the anaerobic ammonium oxidation (anammox) metabolism and their membrane likely plays a role in sparing energy from the passive diffusion of protons during the slow anammox metabolism (Neumann et al. 2014; Grant et al. 2018). The membrane of anammoxosomes is a diffusion barrier that is thought to retain the intermediates of the slow anammox reactions within the cell (Murat et al. 2010). Like the cytoplasmic membrane of Planctomycetes, also the anammoxosome membrane is highly invaginated, probably to increase energy generation and conservation (Neumann et al. 2014). A number of enzymes, mostly involved in the control of ammonium oxidation, localise to the anammoxosome matrix and are targeted via signal peptides for the sec or tat translocation systems (de Almeida et al. 2015; Grant et al. 2018).

[6] Photosynthetic membranes can be divided into three main groups: chromatophores, thylakoids, and the chlorosomes (Murat et al. 2010). Chromatophores are the result of a set of proteins that assemble in the inner cell membrane at sites that invaginate; thylakoids consist on a number of layers that assemble in such a way as to form a circle; chlorosomes are likely due to a self-assembly process of lipids and proteins which is different from the mechanisms used to form other lipid-bounded organelles (Murat et al. 2010).

[7] In the light of these morphological features, Planctomycetes have been suggested to be an intermediate step between the prokaryotic and eukaryotic cell. However, this hypothesis has been criticized by McInerney et al. (2011), who consider Planctomycetes as a case of analogy, but not of homology, of eukaryotic organelles.

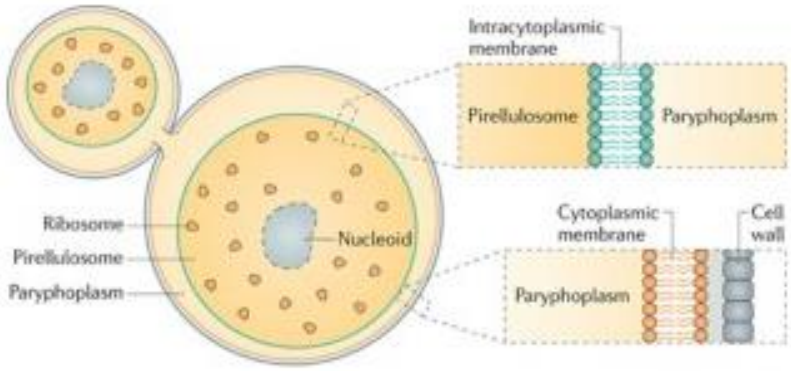

Figure 4.2 A planctomycete during binary fission (Fuerst and Sagulenko 2011, p. 404).

Membrane-bound compartments play a pivotal role in prokaryotic physiology, insofar as they encapsulate and concentrate enzymes and metabolic intermediates so as to *improve* specific *metabolic pathways*. For example, photosynthetic organelles maximise the efficiency of photosynthesis by increasing the number of available photosynthetic protein complexes and maximising the size of the membrane surface exposed to light (Murat et al. 2010). Prokaryotic organelles also play a pivotal role in isolating toxic or volatile metabolic intermediates from the rest of the cytoplasm in such a way that the toxic substances do not diffuse in the cytoplasm (e.g. the propionaldehyde and acetaldehyde of carboxysomes) and volatile elements (e.g. the CO_2 of carboxysomes or the intermediates of the slow anammox reactions within the anammoxosomes of Planctomycetes) do not rapidly diffuse across the cell envelope and into the environment (Chowdhury et al 2014, 2015; Kirst and Kerfeld 2019). Some organelles contribute to the global physiology of the cell by modifying global behaviors of the cell: this is the case, for example, of magnetosomes that orient magnetotactic bacteria within an external magnetic field through a network of actin-like filaments (Murat et al. 2010).

The functions of prokaryotic microcompartments not only *sustain* bacterial and archaeal metabolism, but they are also *produced* and *controlled* by the genetic and metabolic processes occurring in the cytoplasm. The protein components of bacterial microcompartments are synthesised and regulated by the genome of the prokaryote. For example, the proteins of the membranes of carboxysomes and gas vesicles are synthesised by a number of genes, generally clustered in one or more operons in the cytoplasm of bacteria and archaea (Murat et al. 2010). Moreover, the metabolic processes occurring within the microcompartments depend on the availability of metabolites, which need to be displaced from the cytoplasm to microcompartments.

In spite of not being fully understood, it is thought that metabolites could enter into microcompartments through gradient concentrations (Bobik et al. 2015), gated pores that open and close in response to cytoplasmic signals related to the metabolic status of the cell (Chowdhury et al. 2014; Bobik et al. 2015), or also through the colocalisation of sequentially acting enzymes that would allow metabolic intermediates to diffuse across the membrane (Bobik et al. 2015).

The position of prokaryotic organelles is not random and it depends on *cytoskeletal-like elements* and *cytoskeletal-like motor proteins* (Savage et al. 2010). As pointed out in chapter 1, motor proteins employ an energy input to do work and generate a force (e.g. a mechanical force). For example, some bacterial cytoskeleton-like proteins (e.g. mreB or parA) employ the energy provided by ATP or GTP to generate a mechanical force that allow *carboxysomes* to be linearly disposed within bacteria and functionally linked with other cellular components, and also equally distributed in each daughter cell during cell division (Savage et al. 2010). Likewise, *magnetosomes* form chains because of some actin-like proteins (MamB, MamM, and MamE), which polymerise in order to form a protein complex that generates lateral pressure to induce membrane curvature (Raschdorf et al. 2016; Grant et al. 2018). Cytoskeletal-like proteins are also fundamental to the segregation of bacterial organelles, as demonstrated by the segregation of carboxysomes and magnetosomes, which is controlled by bacterial cytoskeletal proteins such as ParA (Cornejo et al. 2014) and MamK (Toro-Nahuelpan et al. 2016), respectively.

In the light of the above, I can draw some important conclusions. First, the main purpose of prokaryotic microcompartments is to isolate and enhance metabolic reactions, thus contributing to the *cellular metabolism*. Secondly, both the constitutive components and the functionality of microcompartments depend on the *genetic* and *metabolic* processes that occur in the *cytoplasm*. Microcompartments and cytoplasm are physiologically integrated through a number of physiological mechanisms, such as the displacement of metabolites between the cytoplasm and the lumen of the microcompartments and the bond between the microcompartments and cytoskeletal proteins.

4.4 THE NUCLEAR ENVELOPE AND THE COMPARTMENTALISATION OF THE GENETIC MATERIAL

The nuclear envelope (NE) is a highly specialised membrane consisting of two phospholipid bilayers: one of them (the inner nuclear membrane (INM)) is closer to the nucleoplasm, the other one (the

outer nuclear membrane (ONM)) is closer to the cytoplasm and contiguous with the rough ER (rER) (Fig. 4.3). The INM and the ONM are crossed by nuclear pore complexes (NPCs), which are multiprotein structures consisting of a number of nucleoporins. The inner face of the nuclear envelope consists of a network of filamentous proteins that is called 'nuclear lamina'[8]. On the one hand, the NE spatially separates the nucleoplasm from the cytoplasm; but on the other hand, since it is structurally and functionally connected with the cytoplasm and the endoplasmic reticulum, it enables the nucleoplasm to be seamlessly integrated into the cytoplasm and with other eukaryotic subregions. Accordingly, the focus of this section is on the structural and functional organisation of the nuclear envelope and its contribution to the functional integration of the eukaryotic cell.

The NE allows a physical separation between the cytoplasm and the nucleoplasm. This latter consists of a number of membraneless compartments (e.g. nucleoli, Cajal bodies, splicing speckles[9]) (Lamond and Sleeman 2015) that are involved not only in genetic transcription, but also in metabolic functions (e.g. modulation of the access of nuclear enzymes and receptors to their substrates) (Lamond and Sleeman 2015). A significant evolutionary innovation introduced by the boundary between the nucleoplasm and the cytoplasm has been the *spatial* and *temporal separation* between the *transcription* and the *translation*, because the former occurs in the nucleus, whereas the latter in the ribosomes of the cytoplasm or of the rough endoplasmic reticulum at different times. Such a separation has enabled eukaryotic cells to develop a more complex regulation of transcription (e.g. the splicing of primary transcripts before the beginning of translation[10], a selective access of transcriptional regulators to chromatin, and new epigenetic mechanisms such as histone modifications or RNA interference) as well as of translation (e.g. a large variety of eukaryotic initiation factors), compared to prokaryotic cells (Devos et al. 2014).

The NE plays a pivotal role in the organisation of *chromatin* and in certain aspects of *gene expression* (D'Angelo and Hetzer 2006*).* Both in protozoans and in metazoans, several proteins of the NE (e.g. nucleoporins, INM proteins, and lamin proteins) form a fibrous network of direct and

[8] Although nuclear lamina is widespread among eukaryotes, some protozoa (e.g. *Saccharomyces cerevisiae*) lack it, but have some proteins (e.g. Mplp1 and Esc1) that play the functional role of the lamina (Rout et al. 2017).

[9] Nucleoli are involved in the synthesis of rRNA and assemblage of ribosomal units. They exhibit a dynamic structure undergoing cycles of assembly and disassembly during each cell cycle. Cajal bodies, which do not contain rRNA or rRNA genes, are mostly involved in the maturation of nuclear ribonucleoprotein complexes, including snRNPs and snoRNPs. Splicing speckles are thought to act as reservoirs that supply factors for the splicing of nascent pre-mRNA at nearby genes. Other nuclear bodies (e.g. PML bodies, clastosomes, paraspeckles) have been discovered, but their functions are not clearly known (Lamond and Sleeman 2015).

[10] This kind of post-transcriptional regulation is very important physiologically, because the translation of unspliced pre-mRNA would produce proteins that are not only nonfunctional but also potentially negative inhibitors of translation (Görlich and Kutay 1999).

indirect protein-protein interactions that binds to chromatin domains, providing them with an anchorage site and spatial stability (D'Angelo and Hetzer 2006; Gavrilov and Razin 2015). The fact that chromatin is localised to the NE has important functional consequences for the regulation of gene expression. For example, some studies have suggested that chromatin-NE interactions may play an *inhibitory* regulatory role for *transcription*, because it has been found that negative regulators of transcription localise to the nuclear periphery[11] (D'Angelo and Hetzer 2006; Padeken and Heun 2014).

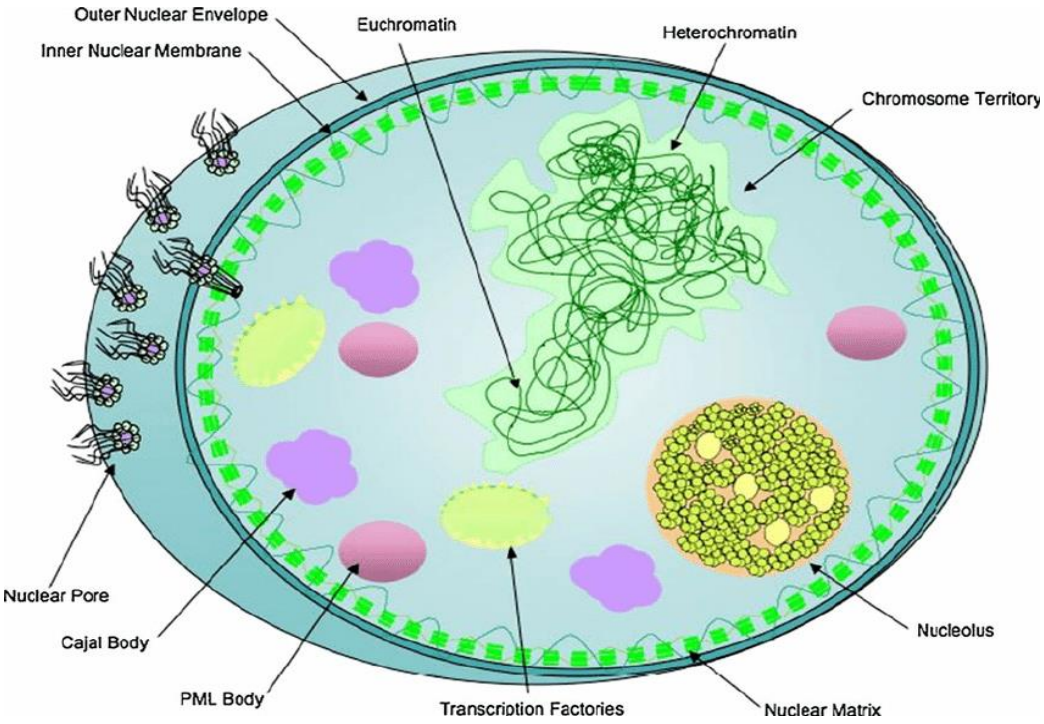

Figure 4.3. Structure of the eukaryotic nucleus and nuclear envelope (Rynearson and Sussman 2011, p. 113).

The NE is important not only for separating the nucleoplasm from the cytoplasm, but also for ensuring a *selective passage* of *macromolecules* from the nucleoplasm to the cytoplasm and the rough endoplasmic reticulum and back. Indeed, RNA molecules (e.g. tRNA, mRNA, rRNA) are transcribed in the nucleus and need to be *exported* to the ribosomes that are located in the cytoplasm or in the rough endoplasmic reticulum. Likewise, many proteins, which are synthesised in the ribosomes of the nucleoplasm or of the rough endoplasmic reticulum, are essential for nuclear functions (e.g. histones, transcriptional factors, splicing factors) and must be *imported* into the

[11] This holds true both in protozoa and in metazoans. For example, it has been shown that a protozoon such as Trypanosoma brucei has some nucleoporins (e.g. the NUP1) involved in the gene silencing (e.g. the silencing of the variant surface glycoprotein made by the nucleoporin NUP1) (Obado et al. 2016). Likewise, the NE protein MAN1 inhibits bone morphogenic protein signaling during *Xenopus* development (Osada et al. 2003).

nucleus (Lamond and Sleeman 2015). Nuclear import and export are enabled by the interaction between NPCs and a number of receptors (importins and exportins[12]). For nuclear import to occur, after the importin α/ß heterodimer has bound *protein cargos* and the RanGTPcomplex, importin ß mediates contact with NPCs in such a way that protein cargos and the importin α/ß complex can be released within the nucleus (Görlich and Kutai 1999). Furthermore, the proteins synthesised in the rough endoplasmic reticulum can pass through the nuclear envelope either by rapid passive diffusion (small proteins) or by an active transport through the NPCs (large proteins) (D'Angelo and Hetzer 2006). As regards nuclear export, RNA molecules bind to mobile export receptors and are exported into the cytoplasm through the NPCs. Small RNAs (e.g. tRNAs and microRNAs) directly bind to export receptors (the so-called 'exportins'), whereas large RNAs (e.g. mRNAs and rRNAs) are exported via specific adaptor proteins, after being assembled into ribonucleoprotein particles (Köhler and Hurt 2007).

A major organisational feature of the nucleus is its *interaction* with the *cytoskeleton* (i.e. microtubules, actin filaments, and intermediate filaments). This is enabled by a family of proteins (the Nesprins), mostly found in the ONM, and by the LINC complex (Linker of Nucleoskeleton and Cytoskeleton) consisting of SUN-domain proteins[13] and KASH proteins that span both membranes of the nuclear envelope. It is worth noting that Nesprins, the LINC complex, and KASH proteins bind to *molecular machines* (notably cytoskeletal motor proteins) (see chapter 1) that constrain a flow of energy and matter to do work and generate a mechanical force that can displace the nucleus along cytoskeletal filaments. As such, the bond between the NE and the cytoskeleton plays a fundamental role in the functional integration of the nucleus in the eukaryotic network and has at least three main physiological consequences.

First, the Nesprins are bound to the domains of the LINC complex and together they connect the nuclear envelope to the microtubules and actin filaments in such a way as to control *nuclear positioning* and *movement* (Tapley and Starr 2012). KASH proteins and Nesprins can use two mechanisms to move the nucleus: the former consists in recruiting motor proteins (dynein and/or kinesin) to the surface of the nucleus, in such a way as to provide the nucleus with the force to move along the microtubule. The latter consists in tethering the nucleus to a moving actin network (this mechanism is also promoted by the SUN proteins). It is thought that nuclear positioning and movement influence the organisation and mechanical properties of the cytoskeleton itself,

[12] Importins and exportins are proteins that mediate the import or export of macromolecules, respectively.
[13] SUN-domain proteins directly interact with KASH proteins.

cytoplasmic signalling, and accessibility of the nucleus to signalling pathways (Gundersen and Worman 2013). The NE is a cytoskeletal integrator that actively contributes to the *organisation of microtubules* by binding to some molecules (e.g. the MTOCs[14]) and *actin* through the action of nesprins (Gunderson and Worman 2013). Furthermore, the nuclear movement generates mechanical forces that could eventually *regulate* cytoplasmic *signalling pathways* (e.g. Rho GTPase and MAP kinase pathways) and the position of the nucleus may also alter its responsiveness to signalling pathways that regulate transcription and mRNA transport and localisation (Gunderson and Worman 2013). Finally, the position of the nucleus may modulate the access of transcription factors and second messengers, present in the cytoplasm, into the nucleoplasm (Gundersen and Worman 2013).

Secondly, the *cytoskeletal filaments* can be considered as a scaffold that spatially connects the *nuclear* and the *plasma membrane*, permitting the *propagation* of *mechanical stimuli* from the extracellular environment to the nucleus and back. In order to elicit a physiological response, mechanical stimuli need to be transduced[15] by two fundamental receptors that trigger *signalling cascades*. A first family of receptors, the *integrins*, is located in the plasma membrane and is critical for both the organisation of multiple nuclear components (e.g. chromatin and nucleoli) and the dynamics and intracellular localisation of heterochromatin (Ramdas and Shivashankar 2015; Hieda 2019). A second family, the *LINC complex* (Linker of Nucleoskeleton and Cytoskeleton), is placed in the NE and spans the INM and the ONM. Thus, signals can travel in two directions: from the cytoplasm to the nucleoplasm ("outside-in signals") and from the nucleoplasm to the cytoplasm ("inside-out signals") (Hieda 2019). *Outside-in signals* can be divided into two main pathways: in the former, mechanical stimuli are directly transferred to the nucleus via the *LINC complex*[16]; in the latter, transcription factors (e.g. Yes-associated proteins) shuttle between the cytoplasm and the nucleus through the NPCs and are regulated by their association/dissociation with actin filaments. *Inside-out signals* result in the transfer of signals from the nucleoplasm to the cytoplasm across the NE (Hieda 2019). It has been shown that the domain proteins of the LINC complex play a role in the

[14] The microtubule-organising center (MTOC) is a eukaryotic structure from which microtubules emerge. Particularly, the NE associates with the MTOC and determines where microtubule ends are anchored (Gundersen and Worman 2013, p. 1385).

[15] Mechanical transduction consists in the transformation of mechanical stimuli (e.g. changes in pressure) into chemical signaling cascades called "mechanosensors".

[16] The proteins of the LINC (i.e. the SUN-domain and the KASH-domain proteins) interact with lamins and chromatin in the nucleus, whereas nesprins associate with various elements of the cytoskeleton in the cytoplasm. Potential candidates for the regulation of the LINC complex include intraluminum calcium, ubiquitinylation, torsinA, the redox environment of the NE, and the chromatin (Hieda 2019).

inside-out signals: for example, the SUN-domain is involved in the activation/inhibition of the RhoA (a class of GTPases) and in the activation of the assembly of focal adhesions (macromolecular complexes that transmit mechanical force and regulatory signals between the extracellular matrix and the cell) (Hieda 2019).

Thirdly, the bond between the NE and microtubules plays an important role in *mitosis* (see also chapter 6), during which the replicated chromatin needs to be equally divided into the daughter cells by the mitotic spindle. Thus, microtubules, which are excluded from the nucleus in interphase, need to gain access to the chromatin. Through the action of *motor proteins* (e.g. dynein), microtubules exert mechanical forces on the NE and deform it, thus favouring the NE breakdown and the elimination of a membrane around chromatin at the beginning of mitosis. At the end of mitosis, the NE reassembles around the decondensing chromatin and microtubules remaining around the chromatin area after spindle disassembly (De Magistris and Antonin 2018).

To conclude, the NE is a fundamental *hinge* between the *nucleoplasm* and the *cytoplasm* that reveals some fundamental aspects of the physiological integration between them. First, the NE allows for the selective entrance and exit of molecules in and from the nucleus, in such a way that key components of transcriptional process can be imported and the transcripts exported. In this sense, the NE not only ensures the communication between different parts of the cell, but it also provides genetic and metabolic processes with a kind of *control* and *regulation* that is fundamental to the eukaryotic physiology. Secondly, the NE is strongly linked with the cytoskeletal filaments; this allows the nucleus not only to change *position* in the cell, but also to receive and generate *signals* that are constantly exchanged with the nucleoplasm in such a way to coordinate its functions with those of other eukaryotic subregions. Thirdly, the cytoskeleton constrains the movement of the nucleus and displace it in order to satisfy *physiological needs* of the cell. In some cases, as shown by mitosis, the cytoskeleton may transiently disrupt the NE so as to favour the generation of daughter cells.

4.5 THE EUKARYOTIC ENDOMEMBRANE SYSTEM AND THE INTER-ORGANELLE COMMUNICATION

The eukaryotic endomembrane system consists of a series of interconnected organelles, each one performing a fundamental role in the physiology of the eukaryotic cell: the endoplasmic reticulum

(ER), the Golgi apparatus, lysosomes, and endosomes [17] (Fig. 4.4). These microcompartments *communicate* among each other through both the direct contact of membrane contact sites and the vesicular transport over longer distances. This section aims at examining three organisational aspects of the eukaryotic endomembrane system: first, the structure of the ER, the Golgi apparatus, the lysosomes, and the endosomes and their functional contribution to the eukaryotic cell physiology; secondly, the role played by contact sites and vesicular transport in inter-organelles communication; finally, how the eukaryotic cell functionally sustain the organelles of the endomembrane system.

The ER is the largest eukaryotic organelle and it consists of two main parts: the rough sheets and the smooth tubules. The membrane of the rough sheets is *contiguous* with the outer nuclear membrane and is characterised by a high number of ribosomes which are the main sites for the biogenesis, folding and post-translational modifications of proteins (Braakman and Hebert 2013). The smooth tubules, instead, do not contain many ribosomes because of their highly curved and smooth surface (Schwarz and Blower 2016). As a result, they are not involved in the biogenesis of proteins, but rather in in the synthesis and transport of lipids[18] (Fagone and Jackowski 2009), in the calcium storage (Clapham 2007), in the carbohydrate metabolism (Hebert et al. 2015), and in the signalling between the ER and other organelles (Westrate et al. 2015).

The Golgi apparatus is adjacent to the ER and exhibits a series of membrane-enclosed disks that form a cisternal structure (the so-called "stack") with a distinct polarity: the *cis-face* that receives material from the ER, and the *trans-face* that excretes some material towards lysosomes, secretory vesicles and the cell membrane (Day et al. 2013). The Golgi apparatus serves two key functions: first, it performs some important post-translational modifications, such as the removal or addition of carbohydrates[19] to the proteins previously synthesised in the ER. Secondly, the Golgi complex, after having received many secretory proteins from the ER, packages them by means of a number of signals[20] and send them to specific subcellular destinations (Short and Barr 2000). Although the exact way in which vesicles are transported within the Golgi apparatus is not fully understood, two models have been proposed: the vesicle transport and the cisternal maturation. According to the

[17] It is worth noting that the membranes of chloroplasts and mitochondria, in spite of exhibiting some functional connections with the endomembrane system, are not included in it. Indeed, all the organelles of the eukaryotic endomembrane system are involved in secretory pathways, giving rise to a single functional unit.

[18] The synthesis of phospholipids occurs in a region between the ER and the Golgi apparatus which is rich in tubules and vesicles (Fagone and Jackowski 2009).

[19] The process of attaching a carbohydrate to a molecule is called 'glycosylation'.

[20] Usually, signals induced by GTPases.

former, cargo molecules pass through the pre-existing Golgi cisternae by fusing with the cis-face and releasing their content in the cisternae. Then, cargo molecules reach other places of the Golgi apparatus and are put into new vesicles. The cisternal maturation model proposes that the ER vesicles melt away to form the cis-face cisterna which progressively maturates by releasing enzymes and other components in the rest of Golgi cisternae (Jackson 2009).

Endosomes and lysosomes form a unique system consisting of spherical organelles having an acidic intracellular environment that break down fundamental macromolecules into their constituent components. Endosomes can be divided into three categories: early, recycling, and late endosomes. Early endosomes act as major sorting stations that receive the vesicles filled with proteins coming from the plasma membrane; recycling endosomes recycle coated vesicles back to the cell surface; finally, late endosomes receive coated vesicles and fuse with lysosomes so as to trigger the proteolytic process (Hu et al. 2015). Lysosomes contain a number of enzymes (i.e. hydrolases) that are responsible for the breakdown of proteins, lipids, and carbohydrates into their building blocks (i.e. amino acids, fatty acids, and monosaccharides, respectively). Lysosomes contain many ion channels (e.g. Ca^{2+}, Cl^{-}, H^{+}) and transporters (e.g. amino acid, lipid, sugar, and heavy metal exporters) that permit them to receive both extracellular cargos via endocytic pathways and intracellular components through autophagy.

The organelles of the endomembrane system perform different functions and produce different molecules that need to be exchanged among the different micro-compartments in a very coordinated and regulated way. One strategy for *inter-organelle communication* consists in *membrane contact sites* which are domains where the membranes of the ER and those of other organelles (i.e. mitochondria, lysosomes, endosomes, the Golgi apparatus, lipid droplets, and the plasma membrane) are adjacent to each other, thus facilitating the exchange of proteins, lipids, and calcium between them[21] (Helle et al. 2013). A key aspect of membrane contact sites is that they

[21] Membrane contact sites are characterized by four distinct types of proteins: structural, functional, regulator and sorter proteins. Structural proteins hold two distinct organelles together (the tethers) at a defined distance (the spacers). Functional proteins (e.g. ion channels and pumps, lipid transfer proteins, metabolite channels and transporters) allow for the passage of molecules from one side to the other of the membrane contact site. Regulator proteins modulate (e.g. by means of phosphorylation or the change in the redox state) the behaviour of the other proteins of membrane contact sites. The activity of these regulatory proteins of the membrane contact sites is controlled by cellular metabolism, thus establishing a fundamental functional dependence of membrane contact sites from the overall cell physiology. For example, the contact site between mitochondria and vacuoles (the vCLAMP) is regulated by the phosphorylation of Vps39 provided by cellular kinase cascades (Hönscher et al. 2014). Finally, sorter proteins recruit proteins into the contact site (Scorrano et al. 2019).

transmit Ca^{2+} to mitochondria and plasma membrane[22], thus acting as *signalling platforms* for the regulation of *organelle biogenesis*, *dynamics*, *inheritance*, *positioning*, *fission*, and *autophagy* (Wu et al. 2018; Scorrano et al. 2019).

Another fundamental strategy employed by organelles to communicate among each other is the use of *vesicles* for *transporting* lipids and proteins (Fig. 4.4). Vesicles are produced[23] by a donor organelle and fuse with an acceptor in another part of the cell, thus passing through membrane barriers without modifying the functional organisation of the organelles (Gomez-Navarro and Miller 2016). Vesicles can move in two different directions: either they flow from the ER to the Golgi apparatus to the plasma membrane (i.e. the *exocytic* pathway*)* or from the plasma membrane to the endosomes/lysosomes to the Golgi complex to the ER (i.e. the *endocytic* pathway). Vesicles can be divided into three main groups: COPII[24], COPI, and clathrins. COPII are the vesicles that transport lipids and proteins from the ER to the Golgi apparatus (anterograde transport), COPI from the Golgi to the ER (retrograde transport) and between Golgi cisternae, finally, clathrins are vesicles that form in the plasma membrane and in the Golgi apparatus and they fuse with endosomes or lysosomes. Vesicles are filled with proteins and lipids by means of a number of molecules (e.g. adaptors, receptors, and accessory factors) that, after having received the signals sent by cargo proteins or lipids, recruit cargo proteins into vesicles (Geva and Schuldiner 2014; Gomez-Navarro and Miller 2016).

[22] In the cells of eukaryotic multicellular organisms, ER membrane contact sites also trasmit apoptotic signals to mitochondria (Wu et al. 2018).

[23] Although the regulation of vesicle biogenesis is not clearly understood, it is supposed that cargos may allosterically regulate the synthesis of coated vesicles (Gomez-Navarro and Miller 2016).

[24] COP stands for Coat protein complex which consists of four different protein subunits that are involved in the budding process.

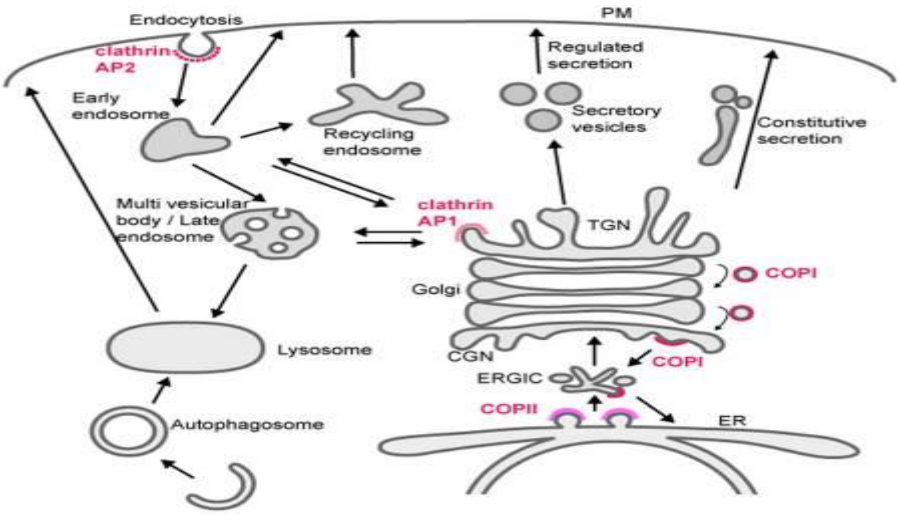

Figure 4.4. The endomembrane system and the vesicle transport (Sato et al. 2013).

Vesicular transport is tightly *regulated* in such a way that the transport of molecules among the different organelles is highly *coordinated* and *integrated*. A number of small GTPases[25] regulate the *maturation* of the Golgi and endosomes, the *coordination* of the steps of vesicular transport, and the *integration* of vesicular transport steps (Segev 2011). More particularly, although the exact mechanism is not known, small GTPases are thought to be involved in the maturation of Golgi cisternae and also endosomes by recruiting some protein effectors that change the protein composition of the compartments of the Golgi apparatus and endosomes. Small GTPases interact with protein effectors[26], thus ensuring that vesicles produced at a specific compartment (e.g. the ER) are targeted to and fuse with the right organelle (e.g. the Golgi apparatus) (Segev 2011). Finally, small GTPases play a pivotal role in the integration of individual transport steps into whole exocytic and endocytic pathways[27] (Segev 2011). Although the exact mechanisms underlying small GTPases activation are not fully understood, it is thought that they are activated by a number of *proteins* localised on the *membranes* of the *organelles*. For example, it has recently been shown that the

[25] GTPases are a family of hydrolases enzymes that activate by binding to guanosine triphosphate (GTP). Small GTPases are involved in signaling processes, playing the role of molecular switches and signal transducers for many cellular events. In humans, an important family of GTPases involved in the regulation of vesicle transport is the Rab family.
[26] For example, Rab1 interacts with two protein effectors, such as the p115 and the GM130 (Segev 2011).
[27] As an example, the two main steps of the yeast exocytic pathway, from ER to Golgi and from Golgi to the plasma membrane, are integrated into one pathway by means of the interaction between the Rab Ypt and the modular GEF complex TRAPP (Segev 2011, p. 37).

TRAPP protein complexes[28] and GEF[29] proteins, which are localised on the Golgi membrane, bind to small GTPases, thus triggering the regulation of vesicular transport (Lipatova and Segev 2019).

The overall functioning of the organelles of the endomembrane system is crucially dependent on *global* cellular conditions, such as cell growth, survival and homeostasis (Farhan and Rabouille 2011). Indeed, the binding between extracellular signals[30] and cell membrane receptors[31] triggers cytosolic signalling pathways[32] which, in turn, bind to organelle transmembrane receptors, thus producing signaling cascades that *remodel* the organelle architecture[33] and *regulate* the functions performed by the organelles of the secretory pathway.

For example, the protein export from the ER secretory pathway is modulated by signaling cascades (e.g. MAP kinases) that are triggered as a response to extracellular signals. Indeed, both the ER chaperones (e.g. calnexin) and COPII vesicles (e.g. Sec16) are phosphorylated by MAP kinases in such a way that the cell as a whole modulates the cargo flux through the ER. Likewise, the organisation of Golgi cisternae and the Golgi protein trafficking is modulated by signaling cascades (e.g. p38 MAP kinases, PKA[34]), activated by extracellular signals, which phosphorylate key Golgi proteins in such a way as to remodel Golgi structure and facilitate post-Golgi trafficking (Farhan and Rabouille 2011). As regards endosomes and lysosomes, they contain a number of proteins that sense cellular signals (i.e. small GTPases) related to the cellular nutrient availability so as to activate or inhibit lysosomal catabolic processes (Settembre et al. 2013). As such, the endo-lysosomal system plays a fundamental role in cellular homeostasis and also in the regulation of basic cellular functions, including cell growth and death (Xu and Ren 2015).

It is worth noting that the organelles of the endomembrane system not only receive but also *generate* and *modulate* cellular signals, thus producing systemic physiological responses for the eukaryotic cell. For example, the MAP kinases cascades bind to some proteins present in the ER (e.g.

[28] TRAPP, which stands for 'Transport Protein Particle', is a multi-subunit protein that binds to ER derived vesicles and put the vesicle closer to the membrane acceptor. Two families of TRAPP, TRAPP I and TRAPP II, have so far been discovered (Cai et al, 2008).

[29] GEF (Guanine Nucleotide Exchange Factor) is a protein that activates GTPases by promoting the bound between GTPases and GTP.

[30] For example, growth factors or cytokines that are involved in cell growth, survival, and homeostasis (Farhan and Rabouille 2011).

[31] For example, cytokine receptors, receptor tyrosine kinases, etc.

[32] The most important molecules for signaling cascades include kinases (e.g. the mitogen-activated protein (MAP) Kinase pathway), phosphatases, GTPases, nucleotides, and lipid mediators (Farhan and Rabouille 2011).

[33] The remodelling of organelle architecture (membrane biogenesis) refers to the regulation of the amount and composition of organelles in relation to the cellular needs (Nunnari and Walter 1996).

[34] PKA stands for Protein Kinase A.

Ras) and Golgi (e.g. Ras, Raf, dynamins) membranes, thus triggering signal cascades that are sent to the plasma membrane in order to activate cell growth, proliferation, secretion, etc.

In view of the foregoing, it is possible to distinguish *three* organisational dimensions of the functional integration of the organelles of the endomembrane system. First, the functions performed by the ER, the Golgi apparatus, the endosomes and lysosomes are linked among each other by means of *inter-organelle communication* that is integrated because of a number of signalling cascades (e.g. GTPases and MAP kinases), which are activated by proteins located on the organelle membranes. Second, the mechanisms performed by the organelles of the secretory pathway depend on the overall cell physiology, inasmuch as they are triggered by *environmental* and *cytosolic signals* that bind to organelle membrane receptors, thus informing organelles about the extracellular and intracellular conditions and producing an adequate physiological response. Finally, the eukaryotic cell physiology is functionally dependent on the organelles of the endomembrane system for two basic reason: first, their mechanisms directly affect *eukaryotic physiology*; second, the signals that are sent from the organelles of the secretory pathway to the plasma membrane *modulate* eukaryotic *cellular behaviours*.

4.6 ORGANISATIONAL DIMENSIONS OF FUNCTIONAL INTEGRATION IN PROKARYOTIC AND EUKARYOTIC MEMBRANE-BOUND COMPARTMENTS

This section aims at comparing prokaryotic and eukaryotic membrane bound compartments so as to appreciate their different contributions to the functional organisation and integration of unicellular organisms. The case-studies show that different organisations of the intraorganismic space (i.e. through distinct types of internal membranes) differently affect the overall physiology of the organism. Furthermore, they highlight that the organisation of the internal organismic space *constrains* the way how the functions of the component parts interact and are integrated among each other. This is in line with Bich et al. (2019), who have argued that the functional organisation of the intercellular space (enabled by the extracellular matrix) plays a pivotal role in the physiology of multicellular organisms. The extracellular matrix acts as a spatial constraint on the functions of the component parts of multicellular organisms, allowing for fundamental properties such as space differentiation, intercellular communication, and cell fate and behaviour.

Prokaryotic and eukaryotic endomembranes are similar in that they divide the intracellular space into different micro-regions, each of which is a biochemical micro-environment that, on the one

hand, is physically separated from the cytoplasm, but, on the other, is physiologically connected with it. In both cases, the isolation of biochemical reactions has far-reaching physiological consequences: first, it allows the occurrence of reactions otherwise impossible (e.g. the reactions with toxic metabolic intermediates in prokaryotic microcompartments); secondly, it allows for more complex forms of regulation (e.g. the regulation of transcription and translation in different compartments of cells); thirdly, it favours the appearance of new and more complex biochemical pathways (e.g. vesicles and the secretory pathway in the eukaryotic cell).

A first important difference between prokaryotic and eukaryotic microcompartments lies in the kinds of biological functions that they perform. In the case of prokaryotic lipid- or protein-bounded microcompartments, their main physiological role consists in *isolating* some metabolic intermediates, which would be toxic or too volatile in the cytoplasm, and carry out a variety of *metabolic* (notably catabolic) reactions. For example, carboxysomes perform important reactions of the Calvin cycle, magnetosomes allow bacteria to be aligned with their magnetic field[35], and the anammoxosomes of planctomycetes perform anaerobic ammonium metabolic relationships. In all these cases, prokaryotic microcompartments optimise metabolic reactions through the isolation of specific metabolic intermediates, most of them toxic or too volatile in the cytoplasm, and enzymes. It therefore seems that prokaryotic microcompartments play the role of an 'extension' (so to speak) of prokaryotic cytoplasm-based metabolism, without being involved in the production of the constitutive components of the cell (e.g. synthesis of RNA molecules, proteins and lipids) nor in the (genetic) control of them.

By contrast, the nucleus and the endomembrane system are involved not only in metabolic pathways (e.g. the synthesis of certain lipids and cholesterol in the smooth endoplasmic reticulum) but also (and very importantly) in the transcription (nucleus), the translation (the ribosomes of the rough endoplasmic reticulum), the post-transcriptional (endoplasmic reticulum) and post-translational (Golgi complex) control. This is a major organisational difference between prokaryotic and eukaryotic organelles, because it entails that eukaryotic organelles are actively involved in the *synthesis* and in the *regulation* of the constitutive components (proteins and lipids) of the cellular network. Indeed, all the metabolic pathways occurring in the cytoplasm can occur only if their constitutive proteins (e.g. enzymes, signalling molecules, receptors, transporters and pumps) are present. Both their synthesis and the genetic regulation is completely controlled by the nucleus and

[35] Although magnetosomes do not contribute directly to metabolism, they indirectly affect it because enable magnetotactic bacteria to search for oxygen.

the endomembrane system; in this sense, they exert a form of control, which is absent in prokaryotic microcompartments, on cellular processes.

The different functions performed and the distinct kind of control exerted by prokaryotic and eukaryotic organelles entail different ways of physiological integration within the cellular network. The functional integration between prokaryotic microcompartments and the prokaryotic cell has (at least) three organisational features. First, the biogenesis of the membranes of microcompartments depends on the genetic machinery placed in the cytoplasm, thus establishing a direct causal relationship between the structure of the organelle and the genetic machinery of the cell (Murat et al. 2010). Secondly, the metabolic functions of microcompartments can occur only if metabolites are correctly sent from the cytoplasm to the lumen of the microcompartment; this is enabled by the intrinsic physicochemical properties of the intracellular membranes and their transmembrane proteins (Bobik et al. 2015). Thirdly, the metabolic reactions of prokaryotic microcompartments provide the cell with important metabolic products that are fundamental to the global cellular metabolism, thus contributing to the self-maintenance/constitutive dimension of prokaryotic cells (Murat et al. 2010). Since prokaryotic microcompartments do not perform transcription and/or translation, they do not require a complex protein machinery for genetic regulation. Furthermore, due to the lack of several microcompartments within the same cell, the existence of a sophisticated system for the intracellular communication is unnecessary in the prokaryotic cell.

Compared to prokaryotic microcompartments, the functional integration between the nucleus and the cytoplasm is much more complex, including more complex signalling and regulatory pathways. One of the major obstacles to surmount to achieve a physiological integration between the nucleus and the rest of the cell is represented by the *control* and the *coordination* of genetic transcription with many other cellular processes. This is an arduous task that requires the interplay between several entities. First, the *cytoskeleton*, which not only displaces the nucleus but also transmits the mechanical stimuli from the nucleoplasm to the cytoplasm and back (Tapley and Starr 2012). Secondly, *transmembrane receptors* (of the NE and of the cellular membrane) that send cytoplasmic signals that trigger or inhibit genetic transcription in relation to cellular needs (Hieda 2019). Thirdly, the *protein import/export machinery* of the nucleus that allows the RNA molecules to be exported to the ribosomes, and enzymes and regulatory proteins to be imported into the nucleus (Görlich and Kutai 1999). In which way do these entities work in an orchestrated way so as to permit the functional integration between the nucleus and the rest of the eukaryotic cell? First of all, cytoplasmic signals and the protein import/export machinery must exhibit a form of

coordination in such a way that the flow of molecules for transcription and translation is regulated in relation to physiological needs (Hieda 2019); secondly, the displacement of the nucleus made by the cytoskeleton likely favours a better transmission of signals from the cytoplasm to the nucleoplasm (and back) in order to have a more efficient coordination of transcription and translation (Tapley and Starr 2012).

Membrane contact sites and vesicles play a fundamental role in the functional integration between the *endomembrane system* and the *cytoplasm*, because they allow the exchange of proteins and lipids between the ER, the Golgi complex, and the cytoplasm. The release of substances by membrane contact sites (Scorrano et al. 2019) and vesicles (Gomez-Navarro and Miller 2016) is controlled by a number of signals (e.g. GTPases) that permit the coordination between the metabolic and regulatory processes occurring in the organelles and the metabolic pathways in the cytoplasm. Furthermore, the regulation of the structure and the functions of the organelles of the endomembrane system depends on signalling pathways (e.g. MAP kinases) that are activated by extracellular signals (Farhan and Rabouille 2011).

To conclude, I may try to provide a more abstract (and general) account of the functional integration between microcompartments and the cell by employing a basic definition of cellular functional integration offered in chapter 2. There, I have proposed a basic definition of cellular physiological integration in terms of an organisational closure between *S* (signalling pathways), *R* (regulatory processes), and *C* (the constitutive procesesses of the cell). We may now ask whether this closure is kept in presence of the organelles and, if yes, how.

In the case of prokaryotic microcompartments, since they mostly isolate catabolic reactions, without synthetising constitutive components (e.g. proteins or lipids) of the cell, they only marginally contribute to C. Furthermore, they do not perform genetic regulation and their membranes do not seem to be involved in a complex signalling network with the receptors of the cellular membrane. As a result, the functional integration of prokaryotic microcompartments in the cell does not entail a profound transformation of the basic organisational closure of the cell between C, R, and S. In prokaryotic cells, most of the closure between C, R, and S occurs between the plasma membrane and the cytoplasm; only a minor part of the metabolism occurs in organelles.

In eukaryotes, instead, the closure between C, R, and S become highly complex for two main reasons. First, the patterns of interaction between *C, R, S* are no longer localised only (or mostly) in the cytoplasm, but rather they are *shared* between the cytoplasm, the nuclear envelope, the nucleoplasm, and the membranes and organelles of the endomembrane system. Indeed, eukaryotic

organelles are involved in the constitutive processes (e.g. biosynthesis of RNA molecules, proteins, and lipids), in their regulation (e.g. transcriptional, post-transcriptional, translational, and post-translational regulation), and in a variety of signalling pathways (e.g. the signalling pathways for nuclear import/export or for the secretory pathway) that are involved in the regulation of the biochemical processes occurring within the organelle lumen. Secondly, in order to ensure the physiological integration of C, R, and S among different cellular sub-regions, *inter-organelle communication* is required. This complex form of functional coordination is achieved through a variety of signalling pathways (e.g. the Ras signalling pathway, the cAMP-PKA pathway, the heterotrimeric G proteins), the receptors of which are located within the plasma membrane and within the membranes of the organelles. Inter-organelle communication is fundamental to the eukaryotic physiology: it coordinates the membrane fluxes along each of the trafficking segments (endoplasmic reticulum-Golgi complex, Golgi complex-plasma membrane, nuclear envelope-plasma membrane, nuclear envelope-mitochondria) so as to avoid gross imbalances in the system. Furthermore, trafficking fluxes respond to extracellular signals, thus adapting the interior of the cell to extracellular requests (Sallese et al. 2006).

4.7 CONCLUDING REMARKS

In this chapter, I have examined the structural and functional organisation of prokaryotic and eukaryotic membrane-bound compartments and I have analysed the main physiological mechanisms involved in the physiological integration of the prokaryotic microcompartments, the nucleus, and the endomembrane system within the cell. The case-studies have shown that the main functional difference between prokaryotic and eukaryotic organelles lies in the kind of biochemical reactions that take place within them: the former isolates and metabolically transform substances that would be toxic or volatile in the cytoplasm; the latter are involved in the synthesis and regulation of the constitutive components (e.g. RNA molecules, proteins, lipids) of the cell.

This functional diversity entails different mechanisms for the physiological integration of the organelles within the cell. In prokaryotic microcompartments, their constitutive components are synthetised and controlled by the *cytoplasmic genetic machinery*, and their metabolic functions depends on the control, made by intracellular membranes, over the flow of metabolic intermediates coming from and sent to the cytoplasm. In eukaryotic microcompartments, the integration between the organelles and the cytoplasm hinges on a variety of *signalling pathways*, the receptors of which

are located in both the plasma membrane and the membranes of the organelles. Furthermore, the *cytoskeleton* plays an important role in the functional integration among the organelles and the cytoplasm, inasmuch as it transmits to organelles mechanical stimuli coming from the cytoplasm and displaces organelles in response to physiological needs.

I have argued that the closure between constitutive processes, regulatory processes, and signalling pathways is kept both in prokaryotic and eukaryotic cells, although it is differently organised in prokaryotic and eukaryotic microcompartments. In prokaryotic cells, C, R, and S mostly occur in the cytoplasm, and microcompartments can be considered as an extension of C that does not substantially modify the organisation of the closure between C, R, and S. Eukaryotic cells, instead, *distribute* C, R, and S *among the organelles*, so that the constitutive dimension of the eukaryotic autonomy is acquired through the equal physiological contribution made by the organelles and the cytoplasm, and no longer by the only cytoplasm. This fundamental organisational change is grounded on *intra-cellular communication* that allows organelles to work in an orchestrated way among them and with the cytoplasm.

The difference in the organisation of C, R, and S in prokaryotic and eukaryotic membrane-bound compartments leads us to the third and last question of this chapter: why do prokaryotes and eukaryotes have such a distinct organisation of membrane-bound compartments and which evolutionary possibilities are opened up by each of them?

The membrane-bound compartments of prokaryotes are thought to have evolved multiple times independently, because the proteins that can influence organelles formation are unique to each of the organelle systems. These organelles stem from the inward folding (tubulation, invagination, or vesiculation) of the prokaryote's plasma membrane, and therefore they seem to have an autogenous origin (Murat et al. 2010). Two interesting considerations can be made: first, since membrane-bound compartments are *not* a *universal* feature of bacteria and archaea, but rather a peculiar characteristic of some of them, we may suppose that their evolutionary appearance is connected to very specific metabolic needs of some prokaryotic cells that likely constrained the synthesis of new proteins and determined the refunctionalisation of previous proteins for the formation of intracellular membranes. Secondly, the fact that prokaryotic cells mostly contain *one* or *a few* microcompartments performing *very limited* functions implies that there are some systemic constraints (e.g. cell size, bioenergetic constraints) that prevent prokaryotic cells from developing a high number of intracellular membranes and promoting their functional diversity.

Whereas the endosymbiotic origin of mitochondria and chloroplasts is universally accepted (Archibald 2014; Martin et al. 2015), the evolutionary roots of the nuclear envelope and the membranes of the secretory pathway is still a controversial issue. Two main theories have been proposed: the endosymbiotic origin and the autogenous one. According to the former, the nucleus and the other endomembranes derived from endosymbiotic events like those that originated mitochondria and chloroplasts (see Gupta and Golding 1996; Moreira and Lopez-García 1998); in the latter scenario, they stemmed from a series of autogenous modifications (i.e. invagination, tubulation, and vesiculation) of the prokaryotic ancestor's plasma membrane (Jékely 2007).

Again, I can make some observations and put forward some hypotheses. First, the fact that intracellular membranes are *ubiquitous* in eukaryotes is consistent with their *constitutive* functions (e.g. genetic and metabolic functions) that make them essential to any form of eukaryotic life. Secondly, the appearance of intracellular membranes is closely connected with the emergence of *signalling pathways* for intracellular communication. Thirdly, the distribution of functions, some of them previously performed in the cytoplasm, among different compartments likely favoured an overall *improvement* of biochemical processes (e.g. a more fine-tuned regulation of transcription and translation) that expanded the physiological capacities of the eukaryotic cell. Finally, the appearance of such a high number of organelles probably *co-evolved* with the increase in size of the eukaryotic cell. Indeed, the increase in size of the eukaryotic cell required an improvement in the genetic and metabolic functions in order to sustain very demanding bioenergetic requirements (Lane 2014). In turn, the physiological needs of a larger cell could have forced organelles to harbour a functional diversification, thus explaining most of the functional novelties found in the eukaryotic cell.

CHAPTER 5 MOTILITY CONTROL OF SYMBIONTS AND ORGANELLES BY THE EUKARYOTIC CELL: THE HANDLING OF THE MOTILE CAPACITY OF INDIVIDUAL PARTS FORGES A COLLECTIVE BIOLOGICAL IDENTITY[1]

5.1 INTRODUCTION

By *collective* (or *nested*) biological organisations, I mean biological entities consisting of different parts, each having their own genetic and phenotypic identity. Symbiotic associations and ecosystems are pre-eminent examples of nested organisations, as the biological members of these associations exhibit distinct genomes and specific phenotypic features. The eukaryotic cell is now a unique functionally integrated individual, but its evolutionary origin dates to two (so far proven) endosymbiotic events: the endosymbiosis between an α-proteobacterium and the proto-eukaryotic cell is at the origin of *mitochondria*, whereas the endosymbiosis between a cyanobacterium and the proto-eukaryotic cell gave rise to *plastids*. Accordingly, eukaryogenesis is currently explained as a progressive transformation of a *nested biological organisation* into a *functionally integrated individual* that still saves some traces of its symbiotic past (Martin et al. 2015).

The interaction among the members of a collective association is complex and includes a variety of processes ranging from metabolic fluxes to chemical signals involved in coordinated gene expression. An important, yet neglected, aspect of nested associations is the *motility of their parts*, because the motile capacities of components are severely constrained by the whole association. Since a living being can reach its nutrients in the environment and interact with its surroundings by means of motile capacities, the way in which motility is controlled and constrained affects the biological capacities not only of the parts but also of the collective association as a whole.

This chapter aims at exploring how the constraints imposed on the motility of the individual parts (i.e. symbionts and organelles) of a eukaryotic cell affect their autonomous interactive capacities and at evaluating how this affects the constitutive autonomy of the overall collective association. Accordingly, the key question of this chapter can be stated as follows: how can a *collective identity* emerge from the control and transformation of the motility of the individual parts?

[1] The ideas and most of the contents of this chapter have already been published in Militello (2019).

In order to address this issue, I will analyse how the motility of the symbionts of the eukaryotic cell is controlled by the host so as to[2] enable the self-maintenance of the whole symbiotic association. The control of motility occupies a decisive role not only in ongoing symbiotic associations but also in the transformation of endosymbiotic proto-mitochondria and proto-plastids into eukaryotic organelles: indeed, the eukaryotic cytoskeleton tightly controls the movement of eukaryotic organelles in such a way that physiological functions and homeostatic regulatory mechanisms can be performed. Accordingly, from an evolutionary point of view, the eukaryotic cytoskeleton has introduced biological novelties that permitted a proto-eukaryotic cell and its endosymbionts to achieve a functionally integrated individuality.

In the light of the above, the main issue of this chapter will be explored by addressing the following theoretical questions:

1. How is the motility of symbionts controlled by the host so as to enable the self-maintenance of the overall symbiotic association?
2. How is the motility of eukaryotic organelles controlled by cytoskeleton?
3. What is the role played by the eukaryotic cytoskeleton in controlling the interactive capacities of endosymbionts and organelles and how does it affect the biological identity of the eukaryotic cell?

The analysis of these three questions sheds light on the organisational role played by motility in symbiotic associations as well as in individuals (i.e. the eukaryotic cell) based on the integration of closely related units (i.e. eukaryotic organelles). Furthermore, the different interactive behaviors of symbionts and organelles will shed light on their different organisational roles within the eukaryotic cell and explain why they are differently controlled.

The chapter is divided as follows: in section 5.2, I present a critical review of the current debate on the individuality of symbiotic associations and some theoretical accounts of the relationship between 'interactive' and 'constitutive' autonomy. The following two sections will examine the physical constraints acting on the motility of eukaryotic symbionts (section 5.3) and eukaryotic

[2] In this chapter, I explore the relationship between motility and self-maintenance by employing some expressions ('so as to', 'in order to', etc.) that can suggest a teleological meaning. However, all these 'teleological' expressions should be understood within the organisational framework for biological functions, according to which biological functions (including motile capacities and sensorimotor abilities) are aimed at self-maintaining a biological organisation within a regime of organisational closure (see, for example, Moreno and Mossio, ch. 3; Mossio and Bich 2017).

organelles (section 5.4). Section 5.5 will explore the role played by the eukaryotic cytoskeleton in the control of motility and the evolutionary innovations that it has introduced. Finally, section 5.6 makes some concluding remarks concerning the relationship between motility and biological autonomy.

5.2 INTERACTIONS AS THE CORNERSTONE OF SYMBIOTIC ASSOCIATIONS AND AUTONOMOUS ORGANISMS

Over the past years, an increasing number of studies have stressed the importance of taking into consideration symbiotic interactions for defining a biological individual. The eukaryotic cell, notably in multicellular organisations, forms a nested ecosystem with their bacterial symbionts in such a way that they form a unique collective identity based on their mutual interactions (McFall-Ngai et al. 2013). Although the term 'holobiont' currently designates the relationship between a multicellular eukaryote with its bacterial symbionts, Margulis (1993) employed this term to refer to a general symbiotic association between a symbiont and a host. The variety of symbiotic associations is extremely wide, since they range from prokaryote-prokaryote interactions (e.g. the *Candidatus Tremblaya princeps-Candidatus Moranella endobia* consortium of *Planococcus citri* (McCutcheon and von Dohlen 2011) to bacterial communities of biofilms (Saxena et al. 2019)), protist-prokaryote relationships (e.g. the *Paulinella chromatophora-cyanobacteria* couple (Bodył et al. 2007)), protist-multicellular eukaryotes relationships (e.g. *Giardia lamblia* and the gut of many mammals (Adam 2001)), and prokaryotes-multicellular eukaryotes associations (e.g. the bacteria living within human gut (Thursby and Juge 2017)). On the basis of the location of the symbiont with respect to the host, I distinguish ectosymbionts (or epibionts) from endosymbionts (Moya et al. 2008): the former live on the surface of their host, whereas the latter within them.

All the aforementioned symbiotic associations are able to *self-maintain* by means of a number of *constitutive* interactions among symbiotic partners: metabolic, genetic, developmental and immunological interactions (Moya et al. 2008; Gilbert et al. 2012). Metabolic relationships occur when symbiotic partners interchange a number of metabolites, nutrients and enzymes in such a way that the host provides the symbiont with the nutrients and, in turn, the symbiont supplies the host with the necessary enzymes for assimilating these nutrients or for synthesising metabolic components (Moya et al. 2008). Genetic interactions consist in the interchange of genetic material among symbiotic partners; this phenomenon, also called as 'horizontal gene transfer' (HGT), favours

genetic variability and it is an important source of phenotypic complexity (Ochman and Moran 2001; Moran 2007). The development of many invertebrates and vertebrates is partly dependent on their symbionts, because symbionts may provide larvae or embryos of the host with nutrients in such a way that "development then becomes a matter of interspecies communication" (Gilbert et al. 2012, p. 328). Finally, the immune system of the host provides its symbionts with niches where they can grow and, in turn, symbionts enhance the pathogen immunity of their host (Chiu and Gilbert 2015; Gilbert and Tauber 2016).

The capacity of self-maintenance of nested biological organisations needs to be studied in close connection with their ability to interact with the surroundings. Studies on prokaryotic endosymbionts of insects have suggested that these prokaryotes exhibit a highly reduced number of genes for cell motility (Moya et al. 2008; Degnan et al. 2010; Manzano-Marín et al. 2012). This suggests that endosymbiosis and maybe also ectosymbiosis impose some constraints on the motility of the individual parts in such a way that the motility of the symbiont(s) is modified and sometimes restricted. One of the reasons why symbiotic associations (particularly endosymbionts) exhibit different environmental conditions compared to the free-living lifestyle is that the micro-environment provided by the host generates a niche with different conditions of life compared to free-living organisms (Moya et al. 2008).

From a philosophical point of view, it has been emphasised that the autonomy of a biological organisation relies on two main dimensions: the *constitutive* aspect and the *interactive* dimension. The former includes all those aspects (e.g. metabolism, regulatory processes, immunology, development, etc.) that contribute to the self-maintenance of an individual. The latter entails the capacities (e.g. perception, motility, action) that allow an organism to interact with the environment and to change it according to its own internal norms (Moreno and Mossio 2015; Mossio and Bich 2017).

The constitutive and the interactive dimensions are *mutually dependent*, giving rise to an 'organisational closure' in such a way that the environment constrains the internal processes of an agent, and an agent exerts some constraints on its own boundary conditions (Moreno et al. 2008; Moreno and Mossio 2015, chap. 4; see also Arnellos and Moreno (2015) for how this relation can happen in multicellular system). Indeed, a living being could not undergo metabolic processes, if it had not access to the nutrients that are present in the environment. Therefore, minimal forms of agency are required to allow an organism to reach its nutrients, prey or escape from its predator. In this respect, we can state that the constitutive dimension requires the interactive one. Nonetheless,

the opposite holds true as well: the interactive capacities need not only the energy (e.g. in the form of ATP molecules) supplied by metabolic processes but also regulatory mechanisms that adapt agential capacities to the features of the environment. Accordingly, the interactive dimension entails the constitutive one and it could not exist without it.

The concept of *'agency'*, which plays a major role both in life and cognitive sciences, summarises the main aspects of the *autonomous interactive dimension*. Indeed, an individual is an agent if it exhibits a clear distinction between the interior (e.g. the cellular environment) and the exterior (e.g. the surroundings) (individuality criterion); if it is the source of activity (interactional asymmetry criterion); and if it acts according to its own norms or goals (normativity criterion) (Barandiaran et al. 2009). An agent must be able to *modulate* and *control* its behavior in accordance with environmental circumstances, which, in turn, is possible only if a system "is able to evaluate sequentially temporal situations and determine which possibility is functional at each moment in time. […] Thus, an agent has the ability not just to avoid negative tendencies, but to actively seek to improve its situation" (Moreno 2018, p. 293).

In this sense, agency is a kind of *adaptive behavior* that can be fulfilled by two different types of mechanisms: either by modifying the constitutive organisation of the system (i.e. metabolism or development) or by modifying the external conditions of the system (i.e. modification of the environmental conditions of the system). Moreno (2018) proposes a simple but valuable model for explaining an autonomous minimal agent: a system is a minimal agent if it has a regulatory subsystem that *modulates* all those inputs that produce *functional modifications* of the e*nvironmental* conditions. The regulatory subsystem consists of a self-production network (i.e. a metabolic system) and a dynamically decoupled regulatory subsystem exerting control actions (Moreno 2018, p. 295). Within this theoretical framework, agency is a cyclical process that requires that "the effector processes be modulated in accordance with the detected environmental conditions" (Moreno 2018, p. 296).

A very important aspect of agency is motility, which is "an agent's capacity to move under its own power, so that it is able to perform fast (relative to its size) directional movements aimed at changing its environment in search of more favorable conditions" (Moreno and Mossio 2015, p. 102). Motion favors a specific position of the agent with respect to its surroundings in such a way that "motility-based interaction (i.e., behavior) embeds the agent in an active sensorimotor coupling with the environment" (Arnellos and Moreno 2015, p. 334). It has been claimed that all agents (from the simplest prokaryotes to the most complex multicellular eukaryotes) exhibit a coupling between

sensory inputs (e.g. environmental cues, attractants or repellents) and motor capacities in such a way that perception and action are inextricably connected (Moreno and Exteberria 2005; Moreno and Mossio 2015; Di Paolo et al. 2017)[3]. Agential behavior is strongly influenced by environmental stimuli and also by size-time limitations[4] (Moreno and Exteberria 2005; Moreno and Mossio 2015).

To conclude, the concept of 'agency' has been studied in free-living organisms in close connection with their sensorimotor abilities. Nevertheless, symbiotic associations pose different constraints on the motility of their individual members in such a way that the *organisational conditions* for *agency* in nested biological associations are distinct from those of free-living organisms. This fundamental aspect of symbiotic interactions will be addressed in the following section.

5.3 THE CONTROL OF SYMBIOTIC MOTILITY

Some prokaryotic cells are endowed with very efficient motile systems that provide them not only with the essential means of locomotion but also with an important material constraint on metabolism. Indeed, the supply of nutrients is made possible by a specific system that links the picking up of environmental cues of nutrients with locomotion. The locomotion of prokaryotes is performed by three kinds of systems: flagella, type IV pili, and cytoskeletal- and cell surface-based movements (Jarrell and McBride 2008). Bacterial symbionts of unicellular and multicellular eukaryotes are broadly characterised by the modification of their motility systems and, more globally, interactive capacities. In this section I examine the role played by motility in the establishment of symbiotic relationships, notably I focus on three distinct symbiotic processes: biofilms[5], endosymbionts, and ectosymbionts.

As explained in chapter 3, biofilms are symbiotic communities of single- or multi-species bacteria that arise when they attach to an *abiotic* or *biotic* surface, by means of adhesins, leading to a

[3] A clear example of sensorimotor coupling is bacterial chemotaxis (e.g. in *E. coli*), since the detection of attractants or repellents in the environment triggers a signalling cascade that modifies the frequency and the direction of the motile system (i.e. flagella).

[4] As pointed out by Moreno and Exteberria (2005) and Barandiaran and Moreno (2008), motility and behavioral agency are strongly affected by the size of the organism, because the increase in size makes more difficult not only the correlation between sensor and effector surfaces "because of the slow velocity of diffusion processes" (Moreno and Mossio 2015, p. 103), but also the achievement of a bodily coordination for displacement.

[5] Although biofilms are a kind of symbiotic association that can live independently from a eukaryotic host (indeed, biofilms can attach to abiotic surfaces), they usually attach to biotic surfaces provided by a (multicellular) eukaryotic host. Accordingly, I think that biofilms can be considered as a specific kind of transient symbiont (i.e. a parasite) of eukaryotic cells and, therefore, it is useful to evaluate the constraints posed on the motility of the bacterial components by the extracellular polymeric matrix and how this affects the relationship with the eukaryotic host.

monolayer or multilayer biofilm (Karatan and Watnik 2009). The biofilm life cycle is characterised by important changes in the motility of its bacterial components. At the beginning, the attachment of bacteria to a surface is strongly favored by *flagella-mediated motility*, because flagella may facilitate the bacterial attachment to surfaces by overcoming *repulsive forces* at the surface-medium interface. Flagella may also promote the bacterial movement of growing cells along an abiotic surface in such a way that the spread of a biofilm is encouraged (Pratt and Kolter 1998). The attachment to a surface is also promoted by type IV pili, because they contain a specific adhesin (the mannose-specific adhesin, FimH) that allows a stable cell-to-surface attachment (O'Toole and Kolter 1998; Pratt and Kolter 1998).

When a bacterial population increases and overcomes a threshold, the motility of individual bacteria is *inhibited* in order to promote the constitution of the extracellular polymeric substance (EPS) matrix. The reduction of motility is achieved by means of post-translational modifications[6], transcriptional regulation[7], and quorum sensing (QS) system[8] (Guttenplan and Kearns 2013). During the existence of the EPS matrix, the motility of single bacteria is impeded. However, the EPS matrix is an ephemeral structure that disassembles in response to environmental substances concentration or bacterial lysis. The *re-activation of the genes* responsible for bacterial motility is a crucial aspect of the disassembly of the EPS matrix and, therefore, the destruction of a biofilm and the re-appearance of the planktonic state. Recent studies have shown that the dispersion of a biofilm can be promoted by the synthesis of bacterial flagella (as in *E. coli*) or by the production of mushroom-like pillars of bacteria (as in *P. aeruginosa*) (Karatan and Watnik 2009).

It is worth stressing that in biofilms the inhibition of bacterial motility is *not* performed by a (abiotic or biotic) surface, but it is rather the outcome of the signals triggered by the EPS matrix. Biofilm is an interesting case of how the *collective control of the motility of parts* allows the emergence of nested biological organisation. However, let us focus now on two kinds of symbiotic associations – endosymbiosis and ectosymbiosis- in which the motility of the symbiont is controlled by the *host*.

The *inhibition of motility* is common in bacterial endosymbionts and it is due either to the *loss of the genes for cell motility* or to the *recruitment of ancient motile genes to new functions*. The loss of

[6] One of the most relevant post-translational modifications is the bond between the second-messenger c-di-GMP and the PilZ domain in the *ycgR* gene (Ko and Park 2000, Hengge 2009).

[7] A number of transcriptional regulatory mechanisms may either activate (e.g. Rcs system and CsrA) or inhibit (e.g. FliZ and CsgD) the expression of flagellar genes in such a way that motility gene expression appears to be strongly controlled during the transition from motile to sessile state of bacteria.

[8] QS system plays an important role in the inhibition of chemotaxis and motion of bacteria. For example, the autoinducer 2 (AI-2) determines a cascade of events that dephosphorylate the response regulator CheY, leading to a counter-clockwise rotation of flagella and smooth swimming (Blat and Eisenbach 1994).

genes is a common aspect of intracellular bacteria and parasites (Moran and Wernegreen 2000; Gil et al. 2004), since the stable environment provided by the host and, sometimes, the existence of secondary endosymbionts make some genes redundant (Perez-Brocal et al. 2006). In endosymbionts, the loss of genes includes both those related to metabolic processes and those associated with the synthesis of the proteins of flagellar apparatus. As a result, their motility is completely lost. A representative example is provided by *Erwinia dacicola* (a prokaryotic symbiont of the Olive Fly *Bactrocera oleae*), which has a reduced number of genes for the amino acid and carbohydrate transport and metabolism and a nearly complete loss of genes for cell motility compared to its free-living state (Estes et al. 2018).

Some endosymbionts, like *Buchnera aphidicola* (an endosymbiotic bacterium of pea aphids) (fig. 5.1), keep their motile genes, but they cannot move, because the proteins expressed by their flagellar genes are supposed to be employed for protein transport functions, and not for motile functions (Maezawa et al. 2006). Flagellar genes are therefore used for a different purpose (likely protein transport), even though a potential pathogenic role cannot be excluded (Moya et al. 2008). As Toft and Fares (2008) pointed out, the endosymbiotic bacteria of insects usually lose their flagellar genes and they retain only the proteins of flagellum involved in protein export, whereas those involved in the synthesis of the hook and filament of flagella have generally been lost. Therefore, since the presence of flagella is unnecessary and energetically expensive, it has been suggested that the re-functionalisation of the flagellar genes of endosymbionts (like in *B. aphidicola*) is the outcome of the adaptation of the symbiont to the intracellular niche of the host (Toft and Fares 2008).

It has been shown that spirochaetes[9] live on the surface –as ectosymbionts- of many protists (within the hindgut of termites) *without performing locomotion* (Iida et al. 2000; König et al. 2005). In spite of having flagella, spirochaetes cannot use them to move. However, the *unique* (so far known) example of bacterial ectosymbionts performing locomotion is represented by the spirochaetes living on *Myxotricha paradoxa* (a protist of the order of *Trichomonadida*) (Wenzel et al. 2003; König et al. 2005) (Fig. 5.2). *M. paradoxa* contains both endosymbionts (rod-like bacteria) and ectosymbionts (spirochaetes). Although *M. paradoxa* possesses four flagella[10], its movement is performed by its spirochaetes. It has been proven that the loss of ectosymbionts or their inhibition by means of starvation or antibiotic treatment makes *M. paradoxa* unable to move (Radek and

[9] Spirochaetes are bacteria with spiral shapes.

[10] The flagella of *M. paradoxa* seem to be an ancient relic rather than a functional part of the protist.

Nitsch 2007). It is worth noting that many termite flagellates have been reported to have ectosymbionts with spirochaetes, but *only M. paradoxa* has spirochaetes that perform a coordinated movement in such a way that *M. paradoxa* can displace (Cleveland and Cleveland 1966). The association of *M. paradoxa* and its ectosymbionts seems to be obligate not only for the movement but also for the performance of other vital functions of the symbiotic inter-identity (Radek and Nitsch 2007). By contrast, the endosymbionts of *M. paradoxa*, as most of endosymbionts, cannot perform movement and are thought to perform a mitochondrion-like role.

The three symbiotic processes that I have so far examined reveal some important differences between them. In particular, biofilms use the motility of single bacteria for the primary attaching phase; then, when the EPS matrix begins to develop, the genes for motility are inhibited. During the breakdown of the EPS matrix, the genes for motility are re-activated and they allow single bacteria to get into the planktonic state. Endosymbiosis usually promotes the inhibition of symbiont motility especially through the loss or re-functionalisation of genes for motility. Finally, ectosymbionts exhibit flagella that cannot move, except for the ectosymbiotic spirochaetes of *M. paradoxa*.

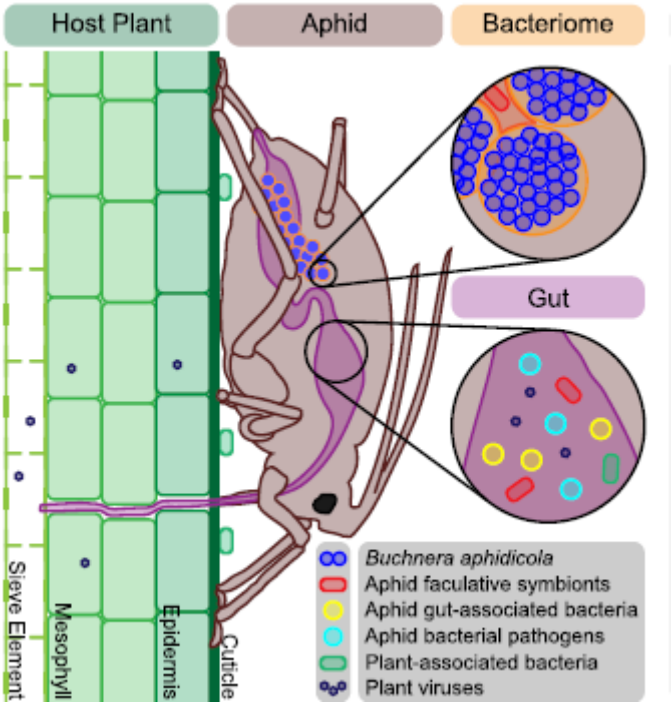

Figure 5.1 The endosymbiotic relationship between Buchnera aphidicola and aphids (Thompson et al. 2019).

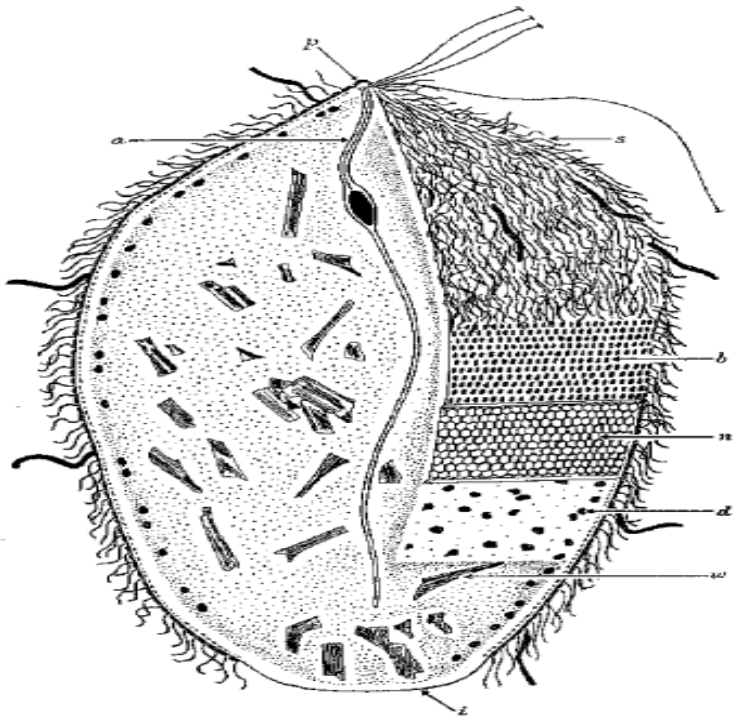

Figure 5.2 Mixotricha Paradoxa and its flagella (Maheshwari 2007, p. 903).

In general, in each of these three cases, the control of the motile interaction is a way to *contribute* to the *self-maintenance* of the overall symbiotic association. Indeed, the inhibition of motility of the bacteria of a biofilm keeps them in a stable position so as to favor the formation and the maintenance of the EPS matrix which in turn allows bacteria to interchange nutrients, metabolites, and to increase their immune response to pathogens and antibiotics. Likewise, the control of motility of endosymbionts and ectosymbionts indirectly affects the self-maintenance of the overall symbiotic association, because the loss of motile genes allows symbionts to spare ATP molecules that can be employed for performing physiological (notably metabolic) processes that are crucial for the whole association. Furthermore, the re-functionalisation of motile genes allows symbionts to perform important mechanisms (e.g. protein transport) that improve the metabolic relationships between the symbiont and the host. Finally, the spirochaetes of *M. paradoxa* make a direct contribution to the motility of the overall symbiotic association and as such enable it to reach its nutrients and to autonomously interact with its surroundings.

A particular theoretical interest is aroused by endosymbionts, as this form of symbiosis is considered as the root of eukaryogenesis, notably of mitochondria and plastids (Margulis 1967). We may therefore suppose that the inhibition of motility, which plays a cardinal role in endosymbionts,

should be also an important feature for understanding the transition from the endosymbiotic to the organelle form of mitochondria and plastids.

5.4 MOBILITY OF EUKARYOTIC ORGANELLES

Both mitochondria and plastids exhibit extremely reduced genomes and can synthesise few proteins involved in the electron transport chain and F_0F_1ATPase (mitochondria) or in the photosynthetic apparatus and in the transcription/translation apparatus (plastids). Thus, they lack almost all the genes (of prokaryotic origin) for the most fundamental cellular physiological functions, including those for flagella. Although neither mitochondria nor plastids can *spontaneously move*, they *are instead moved* by the *eukaryotic cytoskeleton*. Since the motility of mitochondria and plastids is *hetero-driven* by cytoskeletal filaments and not self-driven by the organelle itself, they exhibit *mobility* and *not motility*. By the former, I mean the movement of an entity performed by another entity; whereas the latter is the motion performed by the entity itself.

Mitochondria and plastids *are moved* by two main cytoskeletal filaments: *microtubules* and *microfilaments*[11]. The former are composed of *polymers of tubulin* that are responsible not only for cell motility, but also for several cellular functions, such as the transport of chromosomes during cell division, the maintenance of cell shape, the transport of intracellular materials, and the movement of cell membrane components. The latter are *filaments of actin* that control cell motility and cell separation (cytokinesis). Microfilaments can generate movement in two ways: by a sliding movement of actin and myosin filaments against each other or assembling and disassembling the microfilament bundles. In the former case, when myosin heads bind ATP molecules, they have a high affinity for actin and this drives the bond between actin and myosin. The hydrolysis of ATP allows myosin heads to slightly rotate and to become disengaged from myosin[12]. In the latter case, actin filaments polymerise and depolymerise so as to produce motion.

Mitochondria use cytoskeletal proteins as tracks for their *directional* (anterograde or retrograde) *movement* by means of a coordinated action between microtubules and microfilaments (Anesti and Scorrano 2006). Both microtubules and microfilaments are important for mitochondrial movement

[11] A third system, which can be found in the eukaryotic cells of vertebrates and some invertebrates, is represented by the intermediate filaments which contribute to the maintenance of cell-shape.

[12] In muscle cells the sliding movement is mediated by tropomyosin and troponin, which bind to the actin filament (Cappuccinelli 1980).

and contribute to mitochondrial displacement in a different way. A protein (the mitochondria-microtubule binder protein, mmb1p) seems to be responsible for the bond between *mitochondria and microtubules* (Fu et al. 2011), giving rise to a functional interdependence between them. Indeed, on the one hand, mitochondria reduce microtubule shrinkage rate and contribute to the stabilisation of microtubules; on the other, they are controlled by microtubules, because microtubules are *scaffolds* to maintain the position of mitochondria (Pon 2011). Furthermore, the bond between *mitochondria and actin cables* (Fig. 5.3), mediated by the mitochore complex, drives mitochondrial movement both in an *anterograde and a retrograde direction*. The anterograde movement of mitochondria is driven by the Arp2/3 complex[13] that stimulates actin polymerisation for the generation of anterograde force (Boldogh and Pon 2006; Wu et al. 2013). Finally, intermediate filaments maintain cell shape by bearing tension, whereas microtubules resist compression (Wu et al. 2013). The movement of mitochondria along actin and tubulin is made possible by molecular motors[14] (myosin binds to actin, whereas dynein and kinesin bind to tubulin) which are proteins powered by ATP hydrolysis and consisting of three main parts: the *head domain* binding the cytoskeletal filament, the *neck domain* acting as a lever arm for transducing chemical energy into mechanical energy, and the *tail domain* binding the cargo (fig. 5.4). Molecular motors bind organelles at the tail domain and cytoskeletal filaments at the head domain in such a way as to act as a 'cart' for the movement of organelles.

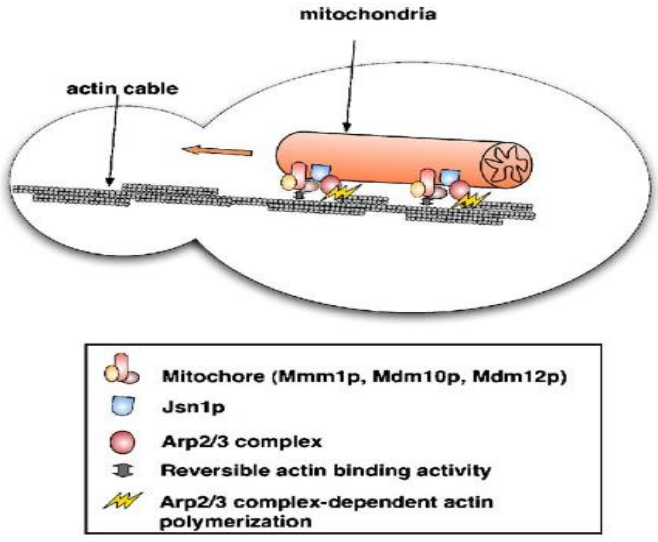

Figure 5.3 Mitochondrial movement on actin filaments (Boldogh and Pon 2006, p. 455).

[13] The Arp2/3 is a protein complex that regulates the polymerisation and depolymerisation of actin filaments.
[14] For a detailed discussion of molecular motors, see chapter 1, section 1.4.

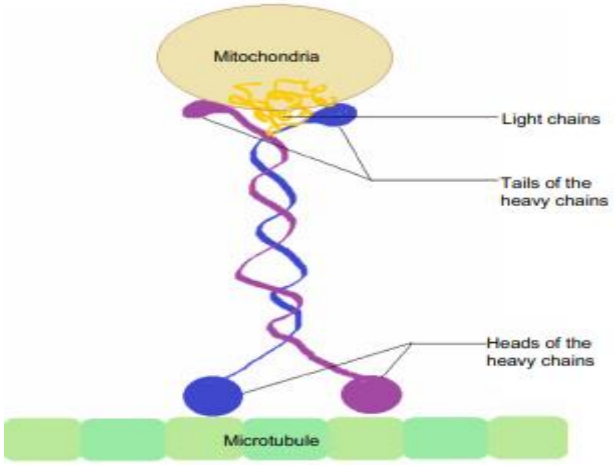

Figure 5.4 The movement of mitochondria along microtubules is mediated by molecular machines (Wu et al. 2013, p. 4038).

The movement of chloroplasts is mainly due to actin filaments which are localised at the interface between the chloroplast and the plasma membrane. In particular, *motor proteins* and the *polymerisation of actin filaments* are the main actors of chloroplast movement. The motor proteins responsible for plastid movement are different from those involved in mitochondrial movement (i.e. myosin, dynein, and kinesin) and are based on the *actomyosin system* (Shimmen and Yokota 2004). Actin polymerisation is induced by environmental stimuli (e.g. changes in light intensity or mechanical touch) and controlled by a number of mechanisms not yet clearly understood. It is believed that the protein CHUP1[15] may play a major role, because it binds to profilin which supports actin assembly (Wada and Kong 2018). The polymerisation of chloroplast-actin filaments is considered the most likely candidate mechanism to generate the force required for chloroplast movement (Wada and Kong 2018). Microtubules of plant cells are thought to contribute to chloroplast movement inasmuch as they support the functioning of actin filaments (Brandizzi and Wasteneys 2013).

Both *mitochondrial and plastid movement* make a substantial contribution to the physiology of the eukaryotic cell, insofar as mitochondria and plastids can be more spatially close to the other eukaryotic organelles and hence favor *intracellular communication*.

Cytoskeletal-driven movement is intimately connected with the so-called 'mitochondrial dynamics' consisting of cycles of fusion and division, as the disassembly of microtubules eliminates mitochondrial mobility and, as a result, makes possible fusion and fission events (Bartolak-Suki et

[15] CHUP1 stands for Chloroplast Unusual Positioning 1.

al. 2017). Fusion and fission events involve changes both in mitochondrial shape and in mitochondrial membranes, inasmuch as fusion entails the merger of mitochondrial membranes, whereas fission needs the formation of a septum within the membrane, leading to daughter mitochondria. Fusion and fission play a pivotal role in several eukaryotic cellular processes, insofar as they are involved in the maintenance of calcium homeostasis (through the connection with endoplasmic reticulum), cell development and cellular division. Furthermore, mitochondrial dynamics are involved in *cell survival processes*, including autophagy, apoptosis and necroptosis (Xie et al. 2018). The mobility of mitochondria involves not only their fusion and fission but also their capacity to interact with other eukaryotic organelles via *signaling pathways* in such a way as to regulate many cellular functions. More particularly, mitochondria interact with endoplasmic reticulum, peroxisomes, lysosomes and Golgi apparatus[16].

In plants, the movement of chloroplasts is important for plant growth and development. Depending on light intensity, plastids can distribute differently in the plant cells (randomly in bundle sheath cells, centripetally in the vascular tissue, and centrifugally around the periphery of the bundle sheath cells) so as to favor the exchange of metabolites. Both cytoplasmic ATP levels and CO_2 diffusion are important physiological factors affecting chloroplast movement and positioning (Takagi et al. 2009). Moreover, the spatial proximity of plastids to the plasma membrane permits the maximisation of the transport of CO_2 from the intercellular airspace to the site of CO_2 fixation (the chloroplast stroma) and, therefore, makes photosynthesis more efficient (Takagi et al. 2009).

In spite of playing a different role in the control of the movement of chloroplasts and mitochondria, both actin filaments and microtubules make a significant contribution to the positioning of the organelles within the eukaryotic cell. The molecular motors of the cytoskeleton (myosin, dynein, kinesin) are crucial to the *functional integration* of the cell, because they provide the force for the displacement of organelles. In turn, as already pointed in chapter 4, the intracellular displacement of organelles enables *intracellular communication* and other important *physiological cellular functions* (e.g. metabolic and reproductive[17]). The controlled motion of organelles occupies a crucial organisational role that, on the one hand, makes a dramatic difference with symbiotic

[16] Lysosomes play an important role in amino acid sensing, exocytosis, plasma membrane repair, transcriptional regulation and also acts as reservoir of amino acids, metabolites and ions. Endoplasmic reticulum is relevant for protein folding, $Ca2^+$ storage, and metabolism of carbohydrates and lipids. Peroxisomes perform the β-oxidation of fatty acids (Diogo et al. 2018).

[17] The relationship between mitosis and the displacement of organelles will be analysed in chapter 6.

association, and, on the other, suggests the critical importance of the *cytoskeleton* in the *transition* from prokaryotic to eukaryotic cell.

5.5 INTERACTIVE DYNAMICS AND THE ORGANISATIONAL ROLE OF THE EUKARYOTIC CYTOSKELETON

The previous two sections have examined the motility of symbionts and organelles, focusing on their different functional contributions to the eukaryotic cell. In both cases the control of the motility of the parts is aimed at satisfying physiological requirements of the eukaryotic cell. However, ongoing endosymbionts and organelles of endosymbiotic origin exhibit a different control of motile capacities which can be understood partly by exploring the evolutionary innovations introduced by the eukaryotic cytoskeleton (compared to the prokaryotic one), partly by analyzing the different roles played by endosymbionts and organelles within the eukaryotic cell.

Despite the discovery of bacterial homologs of actin (Bork et al. 1992), tubulin (de Boer et al. 1992; RayChaudhuri and Park 1992; Mukherjie et al. 1993) and intermediate filaments (Margolin 2004)[18], the eukaryotic cytoskeleton performs new functions, not present in the prokaryotic cell, which allow eukaryotes to move organelles or bacterial pathogens within themselves. Compared to the prokaryotic cytoskeleton, which is involved in the production of cell wall, the maintenance of cell shape and the support for cell division, the eukaryotic one permits a new spatial organisation of the intracellular space[19] and perform several different functions, including *intracellular transport of organelles* and *intracellular communication*. Intracellular transport of organelles is enabled by the *molecular machines* of actin filaments (myosin) and microtubules (dynein and kinesin): they bind to the organelle and to the cytoskeleton and displace the organelles along the cytoskeleton by exploiting ATP hydrolysis (Dawson and Paredez 2013; Jékely 2014). Since both endosymbionts (of protists and insects) and organelles are embedded in eukaryotic cells having a eukaryotic cytoskeleton, both should be moved and displaced by molecular motors along actin filaments and microtubules. Nevertheless, the fact that only organelles, and not also endosymbionts, have a

[18] Homologs proteins for actin are FtsA, MreB, MamK, ParM and Alf; for tubulin are FtsZ, TubZ, PhuZ, and BtubA/B; and for intermediate filaments the crescentin protein (Pilhofer and Jensen 2013).

[19] The eukaryotic cytoskeleton performs some mechanisms (*filament growth*, *filament shrinkage*, and *molecular motors walking on filaments*, see Jékely 2014) that provide it with the force to displace organelles and give rise to a new spatial order within the cell.

cytoskeleton-driven movement is closely connected with the different functional role that organelles and endosymbionts play within the eukaryotic cell.

The movement of organelles enabled by the cytoskeleton play an important role in *intracellular communication*: the interchange of molecules (e.g. ions, proteins, lipids, etc.) among mitochondria (and plastids), endoplasmic reticulum, Golgi apparatus, lysosomes, and nucleus would not occur if these organelles were not be *spatially close*[20] (Perico and Sparkes 2018). In turn, the delivery and the coordinated transfer of molecules enable organelles to perform important physiological tasks that collectively contribute to the self-maintenance of the eukaryotic cell. For example, the spatial proximity between endoplasmic reticulum and Golgi apparatus allows the movement of proteins between them as well as the closeness between mitochondria and other organelles favors the interchange of reducing equivalents and ATP molecules. Since organelle movement plays such a crucial role, the eukaryotic cell *modulates* the distribution of the organelles with *spatiotemporal accuracy* by means of changes in network and motor properties (e.g. polarisation, signaling, motor mobility, etc.) (van Bergeijk et al. 2015; Ando et al. 2015).

Unlike organelles, endosymbionts require neither displacement nor a fine-tuned dynamic spatiotemporal control from the eukaryotic cell. Indeed, endosymbionts usually provide the host with the enzymes necessary for performing catabolic or anabolic pathways (e.g. the enzymes for amino acid anabolism of sap-feeding insects) which are absent or incomplete in the host. The enzymes synthesized by endosymbionts are targeted to the plasma membrane of the host through co-translation or post-translation pathway without the need for spatial proximity to the membrane contact sites of eukaryotic organelles. For these reasons, the host does not need to consume energy to displace endosymbionts and they can be kept in an extremely stable position during the symbiotic association. It is worthy of note that the eukaryotic cytoskeleton can be also employed by bacterial pathogens for performing invasion strategies (Haglund and Welch 2011; Gouin et al. 2015) by exploiting actin polymerisation. Therefore, the fact that (bacterial) endosymbionts are not moved by the cytoskeleton is likely not due to a cytoskeletal limitation, but rather to the uselessness of this displacement within the eukaryotic context[21].

The eukaryotic cytoskeleton is a fundamental step not only in the transition from prokaryotic to eukaryotic cell but also in the evolution of mitochondria and plastids from long-term stable

[20] As we have seen in chapter 4, spatial proximity is fundamental to all those exchanges that occur through membrane contact sites (zones of apposition between two organelles). Furthermore, an efficient vesicle transport requires the action of cytoskeleton (Kamal and Goldstein 2000).

[21] As shown in chapter 3, endosymbionts are usually segregated in very specific parts of the cell (e.g. bacteriocytes).

endosymbionts to organelles. The eukaryotic cytoskeleton has given rise to an extremely dynamic and interconnected network within the eukaryotic cell that has led to complex forms of intracellular *communication* and a fine-tuned *spatiotemporal localisation* of eukaryotic organelles in such a way that the degree of cohesion and mutual dependence among the parts considerably increased. This was a very important innovation during eukaryogenesis because it opened up a more sophisticated form of *intracellular communication* (vesicular transport instead of simple diffusion) and an *effective control over the positioning of organelles*. These important biological novelties have made an important contribution to the overall functional integration of the eukaryotic cell.

Special attention should be paid to the major contribution made by the eukaryotic cytoskeleton to the transition from endosymbiotic proto-mitochondria and proto-plastids to organelles. As discussed in chapter 3, both mitochondria and plastids have an endosymbiotic origin (α-proteobacteria were likely the ancestors of mitochondria, whereas cyanobacteria of plastids) and they transformed into organelles over millions of years (Martin et al. 2015). It has been stressed that the main events that allowed endosymbionts to become organelles were the massive transfer of genes to the eukaryotic nucleus (endosymbiotic gene transfer) and the appearance of protein import machineries in the membranes of proto-mitochondria and proto-plastids (Theissen and Martin 2006). I hypothesise that at some point in eukaryogenesis the eukaryotic cytoskeleton must have played a pivotal role in the transformation of proto-mitochondria and proto-plastids into organelles.

Indeed, given that mitochondria and plastids were endosymbionts, they lost most of their genes, including those for cell motility. It is therefore likely that in an initial phase of eukaryogenesis mitochondria and plastids were immobile or, at least, with a very reduced ability to move. Yet, since proto-mitochondria and proto-plastids were progressively performing regulatory and homeostatic mechanisms, it was necessary to provide some mechanisms for displacing and putting them close to other eukaryotic organelles in order to ensure intracellular communication. From this perspective, the eukaryotic cytoskeleton is no longer just a bunch of filaments for controlling cell shape, but an extremely dynamic structure that has allowed mitochondria, plastids, and the other eukaryotic organelles to achieve a high degree of functional integration.

5.6 CONCLUDING REMARKS: THE RELATIONSHIP BETWEEN MOTILITY AND BIOLOGICAL AUTONOMY

In the light of the theoretical results achieved in the previous sections, I shall explore in this concluding section how the control of the motility of the individual parts affects their *interactive autonomy* (i.e. agency) and the *constitutive autonomy* of the whole collective organisation.

The inhibition of motility is a biological phenomenon that both symbionts (except for the ectosymbionts of *M. paradoxa*) and organelles have in common. Nevertheless, I have shown that the eukaryotic cytoskeleton provides organelles with a mobility which is completely controlled by the eukaryotic cell. In the light of the distinction between mobility and motility (see section 5.4), it is therefore clear that the notion of 'motility' implies the concept of 'agency', inasmuch as the *autonomous movement* is a way to interact and functionally modify the surroundings. Since both symbionts and organelles have lost their motile capacities or, if they are present, they are driven by the eukaryotic cell, is it possible to consider (endo)symbionts and organelles genuine agents?

In order to address this question, let us consider what a minimal agent is and then evaluate whether or not symbionts and organelles satisfy the conditions for minimal agency. A definition of minimal agency has recently been provided by Moreno (2018), who has stressed that a minimal agent is a system detecting relevant features of the surroundings (e.g. nutrients) and triggering processes that can functionally modify the environmental conditions. The effector mechanisms must be controlled *from within* by means of a *self-production network* (i.e. metabolism) and *a regulatory system* that is dynamically decoupled from the self-production network (Moreno 2018, p. 295).

The bacteria forming a biofilm and attaching to the biotic surface of a multicellular eukaryote are able to detect environmental signals and nutrients which are present in the surface and to perform effector mechanisms that modify their host. For example, bacteria constituting the biofilm of dental plaque can detect environmental signals such as pH or the nutrients (amino acids, proteins, glycoproteins) provided by saliva and gingival fluid and they release enzymes that produce infectious diseases (like caries or periodontitis) or inflammatory states (like gingivitis) in the host. The release of enzymes of biofilms is tightly controlled by the QS system of biofilms. Likewise, endosymbionts detect the nutrients released by their host in the host cytoplasm and they synthesise and release enzymes for metabolic pathways (e.g. the enzymes for amino acid synthesis). The production of enzymes is controlled by the genes of the endosymbiont, not by the host.

Ectosymbionts (like the spirochaetes of *M. paradoxa.*) detect environmental signals that activate their flagella which in turn allow *M. paradoxa* to move. The regulation of the movement of spirochaetes is made by the symbiont and not by the host. In each of these three cases, even though motility can be inhibited or lost (in bacteria of biofilms or in endosymbionts), symbionts still preserve their ability to *autonomously* interact with their host and the interactive processes are controlled *from within* and not by the host. For this reason, they can be considered as genuine agents, even if in nested hierarchical organisations of symbionts "many functions of the individuated parts are transferred to the higher collective level. These facts often lead to an ultra-simplification of certain agents (e.g., endosymbionts)" (Moreno 2018, p. 306).

Organelles exhibit a pretty different organisation. They perform a wide variety of functions that go far beyond metabolic contributions (like in endosymbionts) and that include regulatory and homeostatic mechanisms of the eukaryotic cell. As such, their effector mechanisms functionally change their surroundings (i.e. the eukaryotic cell) by controlling the eukaryotic cell as a whole. A clear example is provided by mitochondrial dynamics (fusion and fission) which collectively control pivotal events of the eukaryotic cell, such as apoptosis, autophagy, cell development, etc. Furthermore, the mobility of organelles, fulfilled by the cytoskeleton, allows them to efficiently communicate with one another in such a way as to perform pivotal physiological processes. Apparently, the organelles of endosymbiotic origin seem genuine agents within a 'macro-agent' represented by the eukaryotic cell. However, since almost all of their genes have been transferred to the eukaryotic nucleus, the proteins controlling their functions are genetically expressed and controlled by the eukaryotic nucleus[22]. Accordingly, given that the regulation of their effector mechanisms is placed *outside the organelle*, and *not within*, they *cannot be considered genuine agents*. For example, the key proteins regulating mitochondrial fusion (Mtf1 and Mtf2, and OPA1) and fission (Drp1, Fis1, and DnmP1), in spite of being placed within the outer and inner mitochondrial membrane, are *expressed* and *genetically controlled* by the genes placed in the eukaryotic nucleus. The endosymbiotic gene transfer and the genetic control and expression made by the eukaryotic nucleus represent the dividing line between organelles of endosymbiotic origin and ongoing endosymbionts.

In line with the definition of 'minimal agency' provided by Moreno (2018), I think that what defines a minimal agent is the ability of functionally modifying its surroundings by virtue of some effector

[22] An exception is represented by those few genes already present in mitochondrial and chloroplast genomes which control oxidative metabolism (in mitochondria) and photosynthesis (in chloroplasts).

mechanisms that are controlled from within. If we accept this characterisation of minimal agents, symbionts can be considered agents, even though they do not exhibit the coupling between sensory inputs and motor outputs. Sensorimotor coupling is an important aspect of agency in prokaryotic and eukaryotic forms of life (Moreno and Exteberria 2005; Moreno and Mossio 2015; Di Paolo et al. 2017); however, it fails to explain why symbionts can be considered agents and why mitochondria and plastids cannot. Moreover, it is worth emphasising that the acknowledgement of symbionts as genuine agents allows a better characterisation of the biological status of symbiotic associations. Indeed, the identity of a symbiotic association relies on the kind of interactions (metabolic, immunological, developmental, etc.) among symbiotic partners. The *control of the motility* of the symbiont plays a very important role in the emergence of a *collective inter-identity*, insofar as it weakens the interactive capacities of the symbionts –without completely undermining them- to the benefit of the constitutive processes (metabolism, regulatory mechanisms, development, etc.) of the symbiotic association as a whole.

Considering symbionts as real agents is extremely important not only for explaining the emergence of collective inter-identities, but also for clarifying the difference between endosymbionts and organelles of endosymbiotic origin. The ultimate outcome of the transition from the former to the latter was the *loss* of *autonomy* and, therefore, *agential capacities*. This can be mostly attributed to the transference of genes to the host and the subsequent control of their functions by the eukaryotic cell. The reason why mitochondria and plastids are *not* agents is based on the fact that the genes responsible for their motility were likely lost and their movement is completely controlled by the cytoplasm (and cytoskeleton). Certainly, they perform functions that change the eukaryotic cell and exhibit motor capacities driven by cytoskeleton, but the absence of an *internal* regulation of these processes do not make them agents. The interactive capacities of mitochondria and plastids can be likened to the footballers of a table football: they 'kick' the little ball and they perform an action which modifies the position of the little ball; however, their movement is completely controlled by a human being who decides *when* and *how* a footballer moves so as to push the little ball towards the goal area of the opponent.

It is important to stress that, even though a biological system has lost its *autonomous* interactive capacities, this does not necessarily imply the complete loss of interactive capacities. The case of the organelles of endosymbiotic origin is extremely clear in this respect: although organelles have lost their autonomy and their agential abilities because of a massive endosymbiotic gene transfer

that has placed their genetic control in the eukaryotic nucleus, they *interact* with the other eukaryotic organelles by means of vesicle-mediated pathways and thanks to cytoskeletal proteins.

I have so far discussed the relationship between agency and interactive capacities in symbionts and organelles. I can now provide an answer to the key question of this chapter: how is the *motility of individual parts* related to the *constitutive dimension of a collective identity*? The answer lies in the fact that the control of the motility of the part is aimed at maintaining the collective identity as a whole by constraining a flux of energy and matter and, as such, it keeps the nested organisation far from thermodynamic equilibrium (Moreno and Mossio 2010; Mossio and Moreno 2015). Both the loss or inhibition of motility (in symbionts) and the cytoskeleton-driven mobility (in organelles) are ways to contribute to the self-maintenance of the nested organisation, inasmuch as they are a fundamental support for the maintenance of other pivotal interactions (e.g. the metabolic fluxes between the part and the whole, the intracellular communication among organelles, etc.) which collectively sustain a nested organisation as a whole.

CHAPTER 6 FUNCTIONAL INTEGRATION AND REPRODUCTION

6.1 INTRODUCTION

The previous chapters have examined the different dimensions of the physiological integration in both prokaryotic and eukaryotic cells, putting emphasis on how they affect the constitutive and interactive autonomy of the cell. A fundamental capacity of biological autonomous organisations is not only to self-maintain by interacting with the environment, but also to undergo *processes of reproduction* so as to transmit part of its identity (e.g. genetic and phenotypic features) to the offspring. As such, although single individuals die, the lineage, which they form, continues to exist across several generations of individuals. The persistence of a lineage relies upon two important conditions: first, both unicellular and multicellular organisms must be able to *reproduce as a whole* in such a way as to give rise to new biological organisations that are genotypically and phenotypically similar to the reproducer; secondly, the individuals of the lineage differentially reproduce and selectively transmit their genetic and phenotypic features to the offspring so as to undergo natural selection, leading to the evolution of the lineage across generations.

The nature of *reproduction* and its relationship with the *units of selection* (i.e. evolutionary individuality) have been at the core of a lived debate in theoretical biology and philosophy of biology, the main core of which is whether or not the existence of a collective reproductive system is a necessary condition for an organism to be a unit of selection. Two schools of thought can be identified: first, those who argue that an organism must reproduce as a whole to be an evolutionary individual (Hull 1980; Godfrey-Smith 2009). Secondly, those who claim that an evolutionary individual does not necessarily entail a collective reproductive system and the ability to reproduce as a whole, thus paving the way for a pluralistic view of the evolutionary individuality that could eventually be disentangled from the idea of a collective reproduction of the whole organism (Ereshefsky and Pedroso 2013, 2015).

A related, but not very explored, aspect is the relationship between *system-level coordinated reproduction* and *functional integration* in biological individuals. The third chapter has put forward the hypothesis that a certain degree of physiological integration is required in (collective) biological organisations to perform mechanisms for a unitary reproduction of the organisation as a whole. This chapter aims at confirming this working hypothesis by studying the type of physiological integration

required for a *cell* to reproduce as a whole, leading to a parent-offspring lineage. In order to achieve this purpose, I will analyse, as case-studies, the example of binary fission in bacteria and mitosis[1] in eukaryotes. Broadly speaking, the binary fission of bacteria (and archaea) occurs when the cell spits into *two* daughter cells that exhibit the same DNA and cellular organisation of the mother; mitosis is also a kind of binary fission, because the cell divides into two identical daughter cells. However, mitosis occurs only in eukaryotic cells, entails the formation of a *spindle apparatus* that is not present in prokaryotic binary fission, and involves a very complex interaction and reorganisation of the eukaryotic organelles and cytoskeleton. As such, I just focus on unicellular organisms, leaving aside the issue of reproduction in multicellular organisations. This study will allow us to understand not only the transformation of the proto-eukaryotic cell into a reproductive (and evolutionary) unit, but also to shed some new light on the relationship between reproduction and individuality by considering a fundamental, though usually forgotten, dimension of biological individuality, which is functional integration.

Thus, the research questions of this chapter can be framed as follows:

1 What kind of physiological integration is required for a cell to reproduce as a whole?

2 What is the relationship between physiological integration, system-level coordinated reproduction, and biological individuality?

3 Why did eukaryogenesis entail the transition from binary fission to mitosis and meiosis?

This chapter argues that a system-level coordinated reproduction implies functional integration with developmental[2] and metabolic processes, which is the result of three fundamentals levels of mechanisms that allow for an organisational closure between reproduction, growth, and metabolism. This has fundamental consequences for understanding the relationship between physiological and evolutionary individuality.

[1] Legitimately, a reader could ask why I do not examine the other fundamental mechanism of eukaryotic reproduction, that is *meiosis*. There are two basic reasons that justify this choice: first, mitosis and meiosis share many important mechanisms (e.g. regulatory proteins for the coordination of the different phases of cell division or cytoskeletal proteins for the spatial organisation of the cell during division), and thus the kind of physiological integration required for meiosis is not too different from that of mitosis. Secondly, mitosis is a *ubiquitous* feature of eukaryotes, whereas meiosis is a *facultative* mode of division that is not present in some unicellular eukaryotes (e.g. Giardia lamblia can exchange chromosomes without a true meiosis) (Carpenter et al. 2012). As such, the kind of functional integration required for mitosis can be applicable to all eukaryotes, whereas that underlying meiosis cannot be generalizable to all the eukaryotic species. Nevertheless, meiosis will be also addressed in some parts of the chapter, especially in relation to mitosis.

[2] In this chapter, when I employ the words “development” or “developmental processes”, I use them as synonyms of “growth” and vice versa.

The chapter is divided as follows. Section 6.2 critically reviews the current debate on the relationship between reproduction and biological individuality, understood as both physiologically and evolutionary individuality. Sections 6.3 and 6.4 examine the kind of physiological integration required for a prokaryotic and eukaryotic cell, respectively, to divide. Section 6.5 discusses the relationship between functional integration, reproduction, and biological individuality in the light of the case-studies. Finally, section 6.6 offers some concluding remarks.

6.2 THE PHILOSOPHICAL AND BIOLOGICAL DEBATE ON REPRODUCTION AND INDIVIDUALITY

Over the last five decades, the biological and philosophical debate has linked the question of reproduction with two fundamental dimensions of biological individuality: the evolutionary and the physiological one. An evolutionary individual must have reproductive capacities that allow it to generate offspring that may undergo variation. At the same time, reproduction is part of the life cycle and developmental processes of a physiological individual. Thus, reproduction seems to have a very peculiar, perhaps *ambiguous*, status that lies in between the ontogenetic and phylogenetic dimension of biological organisations. This section aims at critically reviewing the main views on reproduction, putting emphasis on its relationship with the evolutionary and physiological dimensions of biological individuality.

The problem of the *reproducibility* of a biological system was firstly posed by Dawkins (1976, 1982) and Hull (1980), who designated as "replicator" any entity capable of transmitting its biological features to a descent. Neither in Dawkins' nor in Hull's account reproduction is conceptualised as such, but rather it is implicitly evoked as a matter of *replication* (understood as *copying*) of a biological organisation, which obeys to the principles of *fecundity*, *fidelity, and longevity* (Dawkins 1976, 1982; Hull 1980). According to Dawkins, replicators give rise to a high number of copies (fecundity) and transmit the (genetic) information with a high fidelity in the replication, thus allowing for the preservation of (genetic) information over time (Dawkins 1982). Genes are the best candidates to be qualified as replicators, because they can transmit genetic and phenotypic aspects of the overall organisation to new systems. Both Dawkins (1976, 1982) and Hull (1980) emphasise that the act of replication cannot occur by itself and need to be assisted by vehicles (Dawkins) or

interactors (Hull)[3], which are produced by replicators and help them to increase in numbers by interacting effectively with their environments (Dawkins 1982).

In recent years, a shift in the attention from replication to reproduction has occurred, because the replication of the basic units of life (i.e. cells) takes the form of reproduction. In spite of being related, *replication* and *reproduction* are two distinct concepts: the former refers to a mere resemblance between the generator and the generated and, in the case of genetic replication, to the transmission of genetic information from the replicator to the replicated. The latter, instead, refers to biological processes (e.g. fission and fusion) characterised by a *material continuity* between parents and offspring (Griesemer 2000, 2016). Thus, Griesemer (2000) has proposed to employ the term "reproducer", instead of "replicator", to designate all those entities that have the capacity to multiply and transmit their material structure to the offspring.

Reproduction is at the core of the Darwinian view of natural evolution by means of natural selection, because *heritability* and *fitness* require that organisms transmit their traits to their offspring (Godfrey-Smith 2009). For this reason, Godfrey-Smith (2009, 2013) has argued that the individuals with evolutionary capacities (i.e. *evolutionary* or *Darwinian* individuals) need to include the ability to reproduce, giving rise to parent-offspring lineages. Taking Griesemer's concept of *reproducer*, Godfrey-Smith has suggested that it is possible to distinguish three categories of reproducers with *evolutionary* capacities: simple, collective, and scaffolded reproducers. Simple reproducers (e.g. unicellular organisms) produce new entities (other cells), the components of which (i.e. cell components) cannot reproduce. Collective reproducers (e.g. multicellular organisms or symbiotic relationships) generate new organisms, the components of which (i.e. germ cells) are able to reproduce. Scaffolded reproducers (e.g. viruses) reproduce as part of the reproduction of some larger units (Godfrey-Smith 2009, 2013).

Unlike the concept of replicator, "reproducer" is intimately connected with the *life cycles* and *developmental processes* of a biological organisation, because *development* is a process of *growth* and *differentiation* of the parts together with the maturation necessary to reproduce. Indeed, the life cycle (or biological cycle) of an organism includes "a series of developmental transformations and reproductive phases that lead from a given developmental stage of a given organisational form, to the same developmental stage of the same organisational form in a following generation, through all the organisational forms of the organism" (Fusco and Minelli 2019, p. 23). According to Griesemer

[3] There is a slight difference between Dawkins' vehicles and Hull's interactors: the former are considered in a passive way, whereas the latter in a more active sense.

(2016), *development* is not a mere life cycle phase preceding *reproduction*, but rather they are two mutually embedding, and *entwined*, aspects of life. The connection between reproduction and development is made by "*scaffolds"*, which are temporary structures that help developing entities to undergo developmental (e.g. assembly and construction) and reproductive processes (Griesemer 2016, p. 806). Furthermore, reproduction is characterised by material overlapping parts (notably genetic and phenotypic traits) that convey or confer developmental capacities on offspring via transfer of material parts (Griesemer 2000).

The characterisation of a life cycle is based on the idea that it is possible to sharply distinguish between reproduction, which entails the transition to a new generation, and development, which is instead a transformation of the same individual (Fusco and Minelli 2019, p. 23). Nevertheless, the development-reproduction relationship is somewhat problematic in nature, because it is often difficult to distinguish "the transformations of an individual that do not alter its identity, and those that, at some point, will result instead in a new distinct individual, somehow emerging from it" (Fusco and Minelli 2019, p. 25). Moreover, in many biological organisations, life cycles are complex[4], because they "involve relatively discontinuous and substantial changes of form, behavior, or environment" (Griesemer 2016, pp. 803-804), thus complicating the relationships between reproduction and development.

A further problem posed by reproduction lies in the *persistence* of *biological identities* across the generations of a same lineage. While it is true that reproduction entails a material overlap between parents and offspring (Griesemer 2000, 2016), it is also true that reproduction is characterised by an *organisational discontinuity*[5]: "a functional change (an alteration, disappearance, or appearance of one or more functional constraints) occurring in the temporal unfolding of constraint dependencies" (Mossio and Pontarotti 2019). The organisational discontinuity is the outcome of three different types of reproductive processes: fission, fusion, and sexual reproduction. Reproduction may entail the fact that parents cease to exist when reproduction occurs. This is what happens in *fission* events such as cell division or also in *fusion* events. Fission entails the division of a system (e.g. a cell) into two or more parts, so that the previous system ceases to exist and two

[4] Fusco and Minelli (2019) distinguish between two broad categories of life cycles: first, monogenerational life cycles, in which there is one generation, one kind of organisation, one developmental process, and one reproductive phase; secondly, multigenerational life cycles, in which multiple generations occur, characterized by multiple organisational forms, multiple developmental processes, and multiple reproductive phases.

[5] As pointed out by DiFrisco and Mossio (2020), the *organisational continuity* (often referred as "diachronic identity") is characteristic of the developmental stages of the *same* individual, presupposing a spatiotemporal continuity between the material and functional states of an organism.

new individuals appear. Fusion events lead to a spatiotemporal separation (asymmetrical dependence relation) between the fusing systems once they have come together in space. However, there are also forms of reproduction in which parents persist after reproduction: this is the case of *sexual reproduction* that entails both fusion and fission events of gametes from parents, and then the integration of the zygote with the mother. The zygote exhibits a spatiotemporal separation from the mother both from an evolutionary and developmental point of view (DiFrisco and Mossio 2020).

Although reproduction makes an organisational change in the transition from parents to offspring, the conservation of genetic and phenotypic traits between the individuals of the same lineage means that a part of the biological organisation of the parent(s) is preserved in the offspring. This phenomenon is often referred as *biological heredity* and it has usually been understood as a synonym of 'genetic heredity' during the twentieth century, though it can be used in a more extensive way by designating the *cross-generation conservation* of functional elements which, in turn, are defined as constraints subject to cross-generation closure[6] (Mossio and Pontarotti 2019). Heredity designates the specific way in which the functional constituents of a biological system remain *stable* over generations ("cross-generation stability"), in contrast to the stability of various elements in the environment. If there were no cross-generation similarities, the discontinuity between the successive generations would be interpreted as the production of new (completely different) organised systems, rather than as reproduction (Mossio and Pontarotti 2019). Thus, reproduction contributes to *cross-generation stability* insofar as it permits a biological organisation to transmit its functions to the offspring in such a way that the hereditary object is subject to a cross-generation closure, thus allowing for an *organisational stability* across generations[7] (Saborido et al. 2011).

In the light of the above, it is apparent that reproduction is a fundamental link between physiological (life cycles and growth) and evolutionary individuality (transmission of traits to the offspring and variation of them). Normally, evolutionary individuals entail system-level coordinated capacities, ending with parent-offspring lineages. Thus, a basic question, though neglected in the

[6] By "cross-generation closure", the authors mean the fact that the biological functions of an organism depend on those of the parent(s). The connection is ensured by reproductive functions (cross-generation functions) that reproduce the same biological functions of the parents in the biological organisation of the offspring.

[7] The emergence of functional variation over time shows that biological systems involved in hereditary processes do not reproduce faithfully. Biological heredity requires the cross-generation time interval at which functional conservation is observed to be specified. Most constraints, which are subject to intra-generation closure, are also subject to cross-generation closure. This means that their existence in each generation depends not only on the organisation of the intra-generation system, but also on the constraints exerted by previous instances of the organisation endowed with the same functional constraints (Mossio and Pontarotti 2019).

current literature on biological reproduction, is what type and degree of *functional integration* is required for the developmental processes of the life cycles of a biological organisation to give rise to a *unitary form of reproduction*, ending with *parent-offspring lineage*. In order to study this issue, the next two sections examine the degrees and kinds of functional integration that are required for having a unitary reproduction both in prokaryotic and eukaryotic cells.

6.3 BINARY FISSION: HOW BACTERIAL REPRODUCTION IS FUNCTIONALLY INTEGRATED WITH METABOLIC AND DEVELOPMENTAL PROCESSES

Prokaryotic forms of life (i.e. Bacteria and Archaea) reproduce by *binary fission* that is an *asexual* type of reproduction -characterised by DNA replication, segregation of chromosomes, and formation of a cell wall (septum)- that enable the split of the prokaryote into two identical daughter cells. In this section, I focus on the mechanisms that allow binary fission to be integrated with development and metabolism and that allow for a system-level coordinated reproduction. Here, I limit myself to analysing bacterial fission in bacteria, and not also in archaea[8].

Before studying the relationship between reproduction, development, and metabolism, I would like to spend a few words on the organisation of bacterial life cycle, because it is the main object of study of this section and it clearly shows the remarkable *continuity* between developmental processes and the reproductive ones. The cell cycle of bacteria is divided into three stages: the *birth* phase (B), the *chromosome* phase (C), and the *division* phase (D). The period between *cell birth* and chromosome replication is the B phase and is characterised by bacterial growth in response to nutrient availability. Then, the C stage is a connecting point between the developmental phase and the reproductive one, because it is characterised by *chromosome segregation* and *DNA replication*. Finally, during the D phase, bacteria split into two daughter cells each containing a full copy of the genome of the parent (Dewachter et al. 2018) (Fig. 6.1).

The growth (and the reproduction) of a bacterium (phase B) critically depends on the *availability* of *nutrients* that are present in the environment. Multiple *signaling pathways* transmit nutritional and growth rate information to the cell cycle machinery so as to permit cells to adapt to nutrient fluctuations and fine-tune cell cycle processes (Wang and Levin 2009). If bacteria detect the presence of the nutrients they need, they increase their *size* and *mass*; if not, they cannot grow and

[8] The main mechanisms underlying binary fission in bacteria exhibit many similarities with those of archaea. Accordingly, for reasons of simplicity, I just focus on bacteria, taking them as a paradigmatic case of prokaryotic reproduction.

their size and mass decrease (Wang and Levin 2009). For example, carbon availability and the metabolic processes for its transformation are the primary determinants of cell size in bacteria: under carbon-rich conditions, a regulatory network triggers the increase in cell size (Wang and Levin 2009). The dependence of developmental processes on nutrient availability is extremely important, because it ensures that the average cell size is maintained under specific *growth conditions*.

Not only the B phase, but also the C and the D phases depend on nutrient availability and metabolic control. If there is nutrient availability, both DNA replication and chromosome segregation (phase C) are activated. The presence of metabolic substrates triggers the initiation and elongation phases of replication, which in turn activate chromosome segregation. By contrast, if substrates lack, both replication initiation and chromosome segregation are inhibited. For example, in E. coli (a gram-negative bacterium), amino acid starvation induces the production of guanosine tetraphosphate and pentaphosphate[9] that are responsible for the *nutrient-dependent control* of DnaA[10] expression and replication initiation (Wang and Levin 2009). Sugar metabolic signals seem to control DNA replication and chromosome segregation also in gram-positive bacteria, such as B. subtilis (Wang and Levin 2009). The D phase is also controlled by nutrients and metabolites. For example, in E. coli and B. subtilis, the accumulation of the substrate UDP-glucose (a precursor of glycogen) inhibits cell division, because it hampers the activity of the cytoskeletal-like protein FtsZ that plays a pivotal role in binary fission. Instead, when UDP-glucose levels are low, cell division can occur (Wang and Levin 2009).

A key aspect of the integration between bacterial reproduction and development is represented by their temporal coordination that is enabled by a set of *regulatory proteins* that *synchronise* bacterial growth with the events of chromosome segregation, DNA replication, and bacterial fission. The temporal coordination between reproduction and development entails two main aspects. First, *DNA replication* and *binary fission* occur only when bacteria have reached a critical *cell size* (under given growth conditions), which usually coincides with the double of their original mass (Wang and Levin 2009; Willis and Huang 2017). The proteins responsible for the coordination between bacterial growth, DNA replication, and bacterial division are still unknown, but it is thought that the key protein regulators of bacterial growth (the MreB protein), DNA replication (the DnaA protein), and cell division (the FtsZ protein) could interact one each other so as to coordinate cell cycle events and

[9] Guanosine tetraphosphate and pentaphosphate are molecules that inhibit RNA synthesis when there is a low concentration of amino acids.

[10] DnaA is a transcription factor that regulates its own expression and that of several other genes.

cell growth (Willins and Huang 2017). Secondly, *DNA replication* and *chromosome segregation* (phase C) are coordinated with *bacterial reproduction* (phase D) so as to ensure one round of chromosome replication per cell division (Westfall and Levin 2017). For example, the temporal coordination between DNA replication and binary fission is regulated by the accumulation and degradation of a protein complex (the active DNA-ATP complex) throughout the cell cycle. Likewise, the coordination between chromosome segregation and binary fission is mostly based on two mechanisms (the nucleoid occlusion[11] and the Ter linkage[12]) that allows the Z ring[13] to form after chromosome segregation and in a specific part of the cytoplasm (Dewachter et al. 2018).

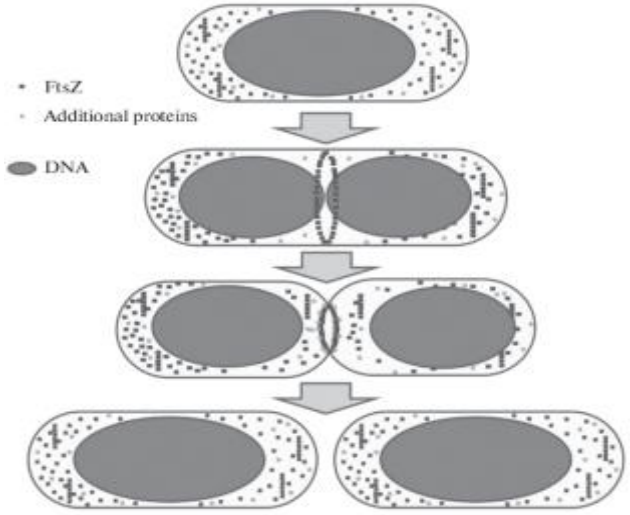

Figure 6.1 Binary fission in bacteria (Vedyakin et al. 2019, p. 247).

Cytoskeletal-like proteins (e.g. the FtsZ protein and the MinCDE) play an important role in the system-level coordinated reproduction of bacteria, because they are involved in the formation of the *Z-ring* and *septum formation*, thus permitting a bacterium to divide its cytoplasm and generate two daughter cells (Wallden et al. 2016; Dewachter et al. 2018). A prominent role is played by the FtsZ protein that is homologous to eukaryotic tubulin and assemblies the ring structure (the Z-ring) at midcell. The Z-ring is a dynamic ring-like polymer structure acting as a scaffold to recruit the components of the divisome, constrain liposomes, and establish the location of septum synthesis (Dewachter et al. 2018). Furthermore, the FtsZ has the ability to bind GTP and to transform it into

[11] Nucleoid occlusion is a negative regulatory system that allows the Z-ring to assemble after the chromosome segregation so as to prevent chromosome fragmentation (Dewachter et al. 2018).
[12] Ter linkage is a protein complex (consisting of the proteins MatP, ZapA, and ZapB) that provides a spatiotemporal coordination between chromosome segregation and Z-ring assembly by coupling the chromosomal terminus region to the divisome (Dewachter 2018).
[13] The Z-ring is a polymer structure involved in binary fission.

GDP, thus acting as an energetic source. According some authors, this energetic source could provide the Z-ring with the force to contract and to divide the prokaryotic cell (Margolin et al. 2005; Lan et al. 2009).

From the above, I may draw some conclusions about the enabling conditions for a system-level reproduction in bacteria. First, bacterial growth and reproduction depend on *nutritional cues*, and the overall *metabolic* status. Secondly, developmental processes and reproduction are *coordinated* with one another to ensure that reproduction is linked to a specific cell size and that there is one round of chromosome replication per cell division. Thirdly, *cytoskeletal-like proteins* (notably the FtsZ) allow for the formation of the septum and the Z-ring, thus creating the machinery required for bacterial division.

6.4 FUNCTIONAL INTEGRATION BETWEEN MITOSIS, GROWTH, AND METABOLISM IN EUKARYOTIC CELLS

Following the same method of the previous section, I explore now the kind of physiological integration required for a eukaryotic cell to have a system-level reproduction. I focus on mitosis - the most representative example of eukaryotic reproduction- and on three organisational dimensions related to its integration: first, the dependence of mitosis on the *growth* processes of the cell; secondly, the functional connection between mitosis and the *spatial reorganisation* of the eukaryotic organelles and cellular membrane; thirdly, functional integration between *mitosis*, *growth*, and *metabolism*. Let me study these three issues in order.

The eukaryotic life cycle can be divided into two main parts: the interphase, during which the cell grows and replicates its DNA; and the mitotic phase, in which the cell divides into two daughter cells with the same genetic and phenotypic traits. The interphase is usually divided into three phases: the G_1 phase during which the cell grows in size and synthesises mRNAs and proteins; the S phase in which the DNA replication occurs; and the G_2 phase during which the cell grows and synthesises proteins. The mitotic and cytokinesis (the division of the cytoplasm into two daughter cells) phases occur after the G_2 phase and, between them and the G_1 phase, there is a quiescence phase (G_0 phase). Mitosis consists of five phases: *prophase*, *prometaphase*, *metaphase*, *anaphase*, and *telophase* (Fig. 6.2). Prophase is characterised by *chromosome condensation*, and the movement of centrosomes and microtubules at the opposite poles of the cell. The *mitotic spindle* forms during the *prometaphase* and it attaches to the chromosomes. In metaphase, the mitotic spindle organises

into a *two-fold symmetric structure*. Finally, anaphase is characterised by the successful *segregation of chromosomes*, thus leading to two fully functional nuclei in distant parts of the cell (a process called "telophase") (McIntosh 2016).

We can now ask how is mitosis integrated with the developmental processes occurring in G_1 and G_2 phases of the life cycle. Basically, mitosis is spatiotemporally coordinated with the G_2 phase (preceding mitosis) and with the G1 phase (following mitosis) through *regulatory proteins*, notably the cyclin-dependent kinases (CdKs), which coordinate the events of the cell cycle, so as to produce a sequential order. For example, the progression from G_2 to the M phase is made possible by the Cdk1/cyclinB1 complexes: the CDKs complexes together with the Polo-like kinase 1 (Plk1) inhibit the kinase Wee1 and activate the Cdc25 phosphatase, thus triggering mitosis. Once in mitosis, chromosomes attach to the mitotic spindle and align with the metaphase plate so as to activate the anaphase promoting complex/cyclosome (APC/C). This protein complex, in turn, drives the exit from mitosis and the entrance into G1 phase, and guarantees the proper chromosome segregation (Ovejero et al. 2020).

Another important aspect of mitosis is the *controlled duplication* and *segregation of intracellular organelles*, and their transmission from parent to offspring. This entails a functional and spatiotemporal coordination of the main component parts of the eukaryotic cell: the action of *cytoskeletal filaments*, the modification of the *nuclear membrane*, the reshaping of *plasma membrane*, and the *reshaping/disassembly of the organelles*. These events are not only coordinated among each other but also with the other stages of the interphase, thus providing a functionally integrated organisation for mitosis.

The formation of the *mitotic spindle*[14] is enabled by *microtubules* and *tubulins*. Microtubules play a fundamental role in mitosis regulation, because they control the shape of the *mitotic spindle* and promote the alignment of *chromosomes* at the spindle zone. The connection between microtubules and chromosomes is mediated by *kinetochores* that are protein complexes located on each chromatid and include several fibrous proteins that bind to microtubule walls. Kinetochores require the action of *molecular machines*[15]: *motor proteins*[16] (e.g. dynein and kinesin) bind microtubules and *generate forces* that can change both chromosome position and microtubule dynamics. All

[14] The mitotic spindle is a way of organising the DNA: one copy of each chromosome attaches to each end of the spindle; then, the movement of the spindle separates the duplicated chromosomes into two distinct sets and push them toward the opposite ends of the cell.

[15] See chapter 1 for a broader discussion of molecular machines.

[16] The motor proteins of the cytoskeleton have also been discussed in chapters 4 and 5.

kinetochores bind a minus end-directed motors and are actively motile on the microtubules to which they bind (Wieser and Pines 2015). During telophase, microtubules and their associated proteins are involved in the construction of the cytokinetic machinery that allows the cytoplasm to be divided into two daughter cells. Subsequently, eukaryotic cells use microfilaments and actin in order to divide the cell into two parts (Wieser and Pines 2015; D'Avino et al. 2015).

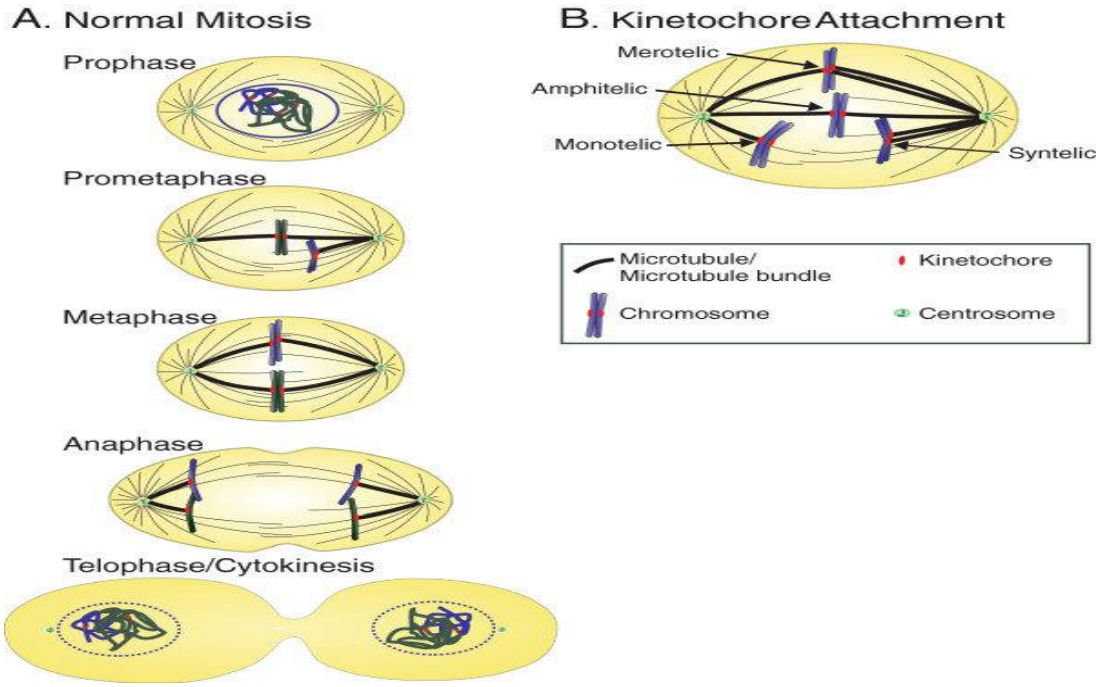

Figure 6.2 Schematic representation of mitosis (Silkworth and Cimini 2012).

All the stages of mitosis entail a fundamental reshaping of the *nuclear envelope* so as to enable *microtubules* to reach chromosomes and allow for their faithful segregation[17]. The change in the shape of the nuclear envelope *may* or *may not* include the *breakdown* of the *nuclear envelope*: the former case is often referred to as *open mitosis*, the latter as *closed mitosis*. Open mitosis occurs in multicellular eukaryotes and is characterised by the disruption of the nuclear envelope and the fusion of the nucleoplasm and the cytoplasm. Chromosomes condense and attach to spindle microtubules in a nuclear envelope-like structure. Then, the nuclear envelope disassembles and is incorporated into the endoplasmic reticulum (Boettcher and Barral 2017). Closed mitosis takes place in unicellular eukaryotes (e.g. yeasts and protozoa): the nuclear envelope does *not* break down and the mitotic spindle forms in the cytoplasm and interacts with the chromosomes through

[17] As already shown in section 4.4, cytoskeletal filaments interact with the nuclear envelope so as to control its position in the cell, promote the spatial connection and communication between the nuclear and the plasma membrane, and enable mitosis.

the nuclear envelope. Each kinetochore attaches to the inner surface of the nuclear envelope and forms a microtubule attachment site on the cytoplasmic face of the envelope (McIntosh 2016). The nucleus undergoes a process of fission through which nuclear components (e.g. transcription factors and non-chromosomal DNA) compartmentalise and segregate so as to be equally distributed in the nuclei of the daughter cells (Boettcher and Barral 2017).

During mitosis, cell membrane undergoes an important reshaping because of the change in the surface-to-volume ratio. At the beginning of mitosis (prophase), the surface area of the cell membrane decreases at the beginning of mitosis because of the interruption of membrane recycling from endosomal compartments. At the end of mitosis (anaphase), the surface area increases through the massive fusion of endosomal membranes. The synchronous and coordinated fusion of the plasma membrane with the endomembranes, which is enabled by Ca^{2+} signaling pathways, allow endomembranes to be stored within the new cells (Boucrot and Kirchhausen 2007).

The *secretory pathway* undergoes some important changes during mitosis: there is a general cessation of membrane traffic, including endocytosis and endosome fusion, at the beginning of mitosis. Endosomes and lysosomes remain intact and separate during mitosis. The segregation into daughter cells requires coordinated movements, and during cytokinesis, these organelles accumulate near the microtubules (Bergeland et al. 2001). The mechanisms underlying organelle fragmentation involve a transient inhibition of fusion machinery and the continuous synthesis of transport vesicles. For example, both the endoplasmic reticulum and the Golgi apparatus break down from a single copy organelle into several vesicle clusters. Endosomes and lysosomes are maintained during cell division and do not fragment or fuse (Bergeland et al. 2001); they are partitioned as separate, intact vesicles[18]. Mitochondria and plastids divide and segregate into the two daughter cells in a process driven by cytoskeletal proteins, thus transmitting the mitochondrial and plastid DNA to the daughter cells (Imoto et al. 2011).

The spatiotemporal coordination between the cytoskeletal activities and the reshaping of nuclear, plasma- and endo- membranes is mainly controlled by *cyclin-dependent kinases*[19], thus ensuring a *coordination* among the distinct phases of mitosis (mitosis onset, sister chromatid separation, and

[18] According to Bergeland et al. (2001), there is not a specific mechanism for the exact distribution of endosomes to daughter cells. This process is stochastic in nature, though the endosomal compartments are clustered by directional movements, suggesting that microtubules are involved in the process (Bergeland et al. 2001).

[19] A part from CDKs, other kinases like Aurora Kinase, Polo-like kinase, and their partner phosphatases regulate the coordination between the cytoskeletal activities and the reshaping of nuclear, plasma- and endo- membranes (Huang and Zhang 2011).

mitotic exit). For example, the bond between cyclin B and Cdk1 protein [20]*activate* key mitotic events (e.g. the *nuclear envelope breakdown*, the *chromosome condensation*, the *mitotic cell rounding*[21], the *APC/C activation*[22], the *SAC signaling*[23], the *kinetochore assembly*) and inhibit others (e.g. *transcription/translation*, *intracellular trafficking*, *Golgi integrity*, *cytokinesis*, perhaps also *ER reorganisation* and *endocytosis*) (Wieser and Pines 2015). The inactivation of the complex cyclin B1-Cdk1 determines the last events of the mitotic phase (i.e. mitotic exit and cytokinesis), thus generating two genetically identical daughter cells.

Mitosis is globally coordinated not only with the cellular growth but also with *metabolism* in such a way that the *cell cycle regulates metabolism* to meet the specific requirements of mitosis. In yeasts, for example, the cyclin-dependent kinase (Cdk1) regulates not only the cell cycle but also the carbon metabolism: at the G1/S transition, the Cdk1 phosphorylates and activates the trehalase Ntk1[24]; afterward, during the S/G2/M phases, the active Ntk1 releases trehalose so as to fuel glycolyis (Ewald et al. 2016). Thus, the mechanisms underlying cell cycle progression are coordinated with nutrient sensing kinases in order to coordinate metabolism with cell cycle progression. At the same time, it has been shown that *metabolism regulates cell cycle* progression. In mammals, for example, some enzymes involved in glucose metabolism (e.g. 6-phosphofructo-2-kinase/fructose-2,6-bisphophatase 3) control the passage from G_1 to S and also from S to G2-M: the presence of substrates for glycolysis allows for the transition from one phase to another one of the cell cycle; by contrast, the reduction of glucose availability impairs the passage from the different steps of the cell cycle (Kalucka et al. 2015).

To conclude, we may distinguish three levels of functional integration required for mitosis. First, the *spatiotemporal coordination* between the *stages of the interphase* and the *mitotic phase*, which establishes a level of functional interdependence between the cellular growth and division. Secondly, the *spatiotemporal coordination* between the cytoskeleton and the modifications of the intracellular organelles. In both cases, the coordination is ensured by regulatory proteins (notably cyclin-dependent kinases) that act as cell cycle activators and inhibitors. More specifically, they a)

[20] In animals, the cyclin B-Cdk1 shuttles between the nucleus and the cytoplasm in interphase and then it accumulates in the nucleus at prophase, thus promoting the breakdown of nuclear envelope and the disassembly of the nuclear lamina (Gavet and Pines 2010).
[21] Mitotic cell rounding is a shape change that occurs during mitosis in most animal cells.
[22] APC/C plays a pivotal role in the regulation of eukaryotic cell reproduction. Ubiquitin-mediated proteolysis regulates sister chromatid separation by altering the local balance of protein kinases and phosphatases, and by activating separase, a protease that cleaves the cohesion rings holding sister chromatids (Wieser and Pines 2015).
[23] SAC stands for spindle assembly checkpoint, which is a set of mechanisms that control mitosis and meiosis, preventing the separation of the duplicated chromosomes (anaphase) before their proper attachment to the mitotic spindle.
[24] The activation of the Ntk1 also requires the nutrient signaling kinase PKA1 (Ewald et al. 2016).

activate some processes of the cell cycle (e.g. DNA replication) by inhibiting others (e.g. cell division); and b) trigger some morphological transformations and functions of the cellular structures (e.g. chromosome condensation) by repressing others (e.g. the inhibition of transcription and translation) during mitosis. Finally, the coordination of metabolism, growth, and division, which entails both the regulation of cell cycle through metabolites acting as signals and the regulation of metabolism by the regulators of cell cycle (e.g. CDKs).

6.5 FUNCTIONAL INTEGRATION, REPRODUCTION, AND BIOLOGICAL INDIVIDUALITY

In the previous sections, I have examined the mechanisms that permit cell metabolism, growth, and division to be physiologically integrated. This section has a twofold purpose: first, it summarises and compares the *levels* of *mechanistic integration* in prokaryotes and eukaryotes so as to appreciate the similarities and the differences in how reproduction, growth, and metabolism are integrated among each other; secondly, it evaluates the philosophical consequences of the interdependence between metabolism, growth, and reproduction for thinking the issues of *biological autonomy* and *biological individuality*.

A first level of mechanistic integration is represented by *nutrient-dependent signals* (e.g. metabolites) and *regulatory proteins* that trigger cell growth (e.g. increase in cell size or DNA replication) and also division in response to *nutrient availability*. In bacteria, the intermediates of glucose metabolism and regulatory proteins (e.g. guanosine tetra- and pentaphosphate) can trigger or inhibit cell growth and division, so that the two phases of the life cycle occur in a coordinated way. Eukaryotic growth and division also depend on nutrient availability and metabolic processes; however, there is an important difference with prokaryotes: metabolic signals trigger growth and division, but the reverse is also true, insofar as the life cycle can regulate metabolism. Indeed, the proteins controlling cell growth and mitosis (CDKs) also control metabolism, and at the same time some enzymes (e.g. involved in carbohydrate metabolism) control not only metabolism but also the transition between the different phases of the eukaryotic life cycle (Kalucka et al. 2015).

The second level of mechanistic integration is provided by a number *regulatory proteins* that allow both prokaryotic and eukaryotic cells to *grow* and *divide* in an interdependent way, as shown by their life cycles. These regulatory proteins allow for a *spatiotemporal coordination* between development phases (e.g. increase in cell size, DNA replication and transcription, chromosome segregation) and cell division (i.e. bacterial binary fission, eukaryotic mitosis and meiosis). By

"spatiotemporal coordination", I mean that the growth and development phases of the life cycle occur not only in a *sequential* order, sometimes strictly determining the other phases of the life cycle (as in eukaryotic cells), but also in very *specific sites* of the intracellular space (e.g. the plasma membrane, the cytoplasm, the nucleoplasm). In bacteria, *binary fission* is spatiotemporally coordinated with both *DNA replication* through regulatory proteins such as GidA and MioC, and also with *chromosome segregation* by means of nucleoid occlusion, the Ter linkage and other protein complexes (e.g. the MukBEF and the MinCDE) (Dewachter et al. 2018). In eukaryotes, *mitosis* is spatiotemporally coordinated with the *DNA replication* (S phase) as well as the *growth in cell size* and with the *protein synthesis* (G_1 and G_2 phases) mostly through cyclin-dependent kinases and other regulatory proteins (e.g. the anaphase promoting complex/cyclosome or the spindle assembly checkpoint)[25].

A third level of mechanistic integration is provided by *cytoskeletal proteins* that act as a *scaffold* allowing for not only the chromosome segregation and septum formation but also, and very importantly, the *spatial coordination* among the *different parts* of the *cell* during cell division, thus enabling the cell to reproduce as a whole. In the case of bacteria, the protein FtsZ plays a fundamental role inasmuch as it is responsible for the assembly of the Z-ring, the recruitment of the components of the divisome, the constraining of liposomes, and the establishment of the septum (Dewachter et al. 2018). Likewise, in eukaryotic cells, microtubules and their motor proteins (dynein and kinesins) not only control the shape of the mitotic spindle and the attachment of chromosomes to it, but also promotes the reshaping of the nuclear envelope, the division and segregation of mitochondria, plastids, and of the endomembranes of the secretory pathway, so that the compartments composing the eukaryotic cell can divide and segregate in a coordinated fashion to create a daughter cell that is morphologically similar to the parent (Bergeland et al. 2001; Wieser and Pines 2015).

These three levels of mechanistic integration enable a cell to reproduce as a whole, thus recreating the same organisation in the daughter cells. Moreover, since these three levels of mechanistic integration allow cellular division to be coordinated with the the metabolic and developmental processes, reproduction depends on *systemic* conditions (e.g. nutrient availability, metabolic processes and energy production, cell size, DNA replication), so that the biological organisation

[25] It is interesting to observe that in prokaryotes chromosome segregation is not considered as a part of the division cycle. Instead, in eukaryotes, chromosome segregation is a part of the mitotic process occurring during anaphase.

exhibits a *system-level coordinated reproduction*. The fact that a cell must exhibit a very specific kind of physiological (mechanistic) integration to ensure the interdependence between metabolism, growth, and reproduction has far-reaching consequences for thinking *biological autonomy* and *biological individuality*.

As already pointed out in the previous chapters, an organisational account of biological autonomy relies on the concepts of *constitutive* and *interactive autonomy*: the former entails all those processes (e.g. metabolism, gene expression, and development) involved in the self-maintenance of a biological organisation; the latter the capacity (e.g. sensorimotor capacities) to actively interact with the environment by transforming and being transformed by it (Moreno and Mossio 2015). *Metabolism* is definitely the most representative example of constitutive process, because it provides the cell with the amount of energy necessary for its self-maintenance. At the same time, *growth* and *developmental processes* are a significant aspect of constitutive autonomy, because the self-maintenance of a biological organisation always entails a *life cycle* during which an organism is born, grows, (usually) reproduces, and dies. From an organisational point of view, metabolism and growth are significantly interdependent among each other, giving rise to an organisational closure: metabolism provides the cell with the energy for sustaining the quiescent and growth states; likewise, growth sustains metabolism, as some of the proteins regulating development also control metabolism in such a way that growth dynamics control metabolic ones. In a sense, we may think metabolism and development as complementary (and interdependent) processes from a thermodynamic point of view: metabolism keeps a cell *far* from thermodynamic equilibrium, thus temporarily violating the second law of thermodynamics; however, the processes of growth ends with the death of the organism, which is the achievement of *thermodynamic equilibrium*, therefore being the fulfilment of the second law of thermodynamics.

The ontological status of reproduction is rather complex, because it seemingly does not contribute to the self-maintenance (metabolism and growth) of the current biological organisation (Saborido et al. 2011). Accordingly, reproductive capacities have been defined as “cross-generation function”, basically because they “would contribute to the autonomous organisation of the lineage, the species or the biological community in question” (Saborido et al. 2011, p. 596) and they differ from intra-generation functions that “would contribute to the autonomous organisation of individual organisms” (Saborido et al. 2011, p. 596).

Despite this difference, the authors consider intra- and cross-generation functions as having the same ontological status, insofar as both contribute to the *self-maintenance* of the current

organisation: *intra-generation* functions (including metabolic and development functions) contribute to the self-maintenance on an *ontogenetic* scale related to the life cycle of a single organism; *cross-generation* functions, instead, contribute to the self-maintenance of organisms as a species covering several generations on a *philogenetic* scale (Saborido et al. 2011). A question arises: are intra- and cross-generation functions physiologically connected (and integrated)?

According to what has been presented in this chapter, I suggest that there is a circular relationship between metabolism, development, and reproduction. Reproduction depends on metabolism in order to get the amount of energy required for processes of division. Cellular reproduction also hinges on development, because division processes can occur only when the organism has reached a certain developmental phase. At the same time, metabolism and development depend on reproduction, insofar as the metabolic and developmental processes occur in an organism, which has been generated by another by means of reproduction. In this sense, reproduction allows for the generation of a new biological organisation that exhibits a material connection with the reproducer, so that the intra-generation functions of the reproduced depend on the reproductive capacitites (cross-generation functions) of the reproducer.

The functional integration between intra- and cross-generation functions has important consequences for defining a *biological individual*, and particularly for conceptualising the link between physiological and evolutionary individuality (Pradeu 2016). As already pointed out in chapter 3, the *physiological* individuality refers to the fact that a biological individual exhibits a certain degree of physiological *integration* such that it appears as a cohesive physiological unit capable of functionally coordinated behaviours. The *evolutionary* individuality designates a biological organisation that satisfies Lewontin's (1970) three conditions for *natural selection*: first, genetic and phenotypic variation; secondly, a differential fitness produced by variation; thirdly, the heritability of variation. With regard to physiological and evolutionary individuality, I make two important remarks: first, the closure between metabolism, growth, and system-level coordinated reproduction is the core of *physiological individuality*; secondly, system-level coordinated reproduction is a necessary condition for being an *evolutionary individual* (i.e. a unit of selection) and it relies upon physiological individuality, and thus on the physiological integration between metabolism, growth and reproduction.

As regards the first point, most of contemporary accounts of physiological individuality have underlined the metabolic (Dupré and O'Malley 2009), the immunological (Pradeu 2010, 2016), and the developmental (Griesemer 2016) dimensions of a biological organisation. According to my view,

physiological individuality cannot be understood if we do not recognise the role of the *physiological integration* between *metabolism*, *development*, and *reproduction*, exactly because they are *constitutive processes* of a cell that contribute to its self-maintenance, and hence to its physiology[26]. This in turn relies upon the three levels of mechanistic integration above described: first, the *nutrient-dependent signals* that connect the metabolic status to growth and, indirectly, reproduction; secondly, the *regulatory proteins* for the integration of growth and division; finally, the *cytoskeletal proteins* and *motor proteins* for the spatial coordination among the different parts of a cell for performing a system-level reproduction.

Concerning the second point, my account goes beyond the current views of evolutionary individuality. Indeed, some of them have stressed the importance of *reproductive bottlenecks* -a single cell proliferate so as to lead to a multicellular organism- (Maynard-Smith and Szathmary 1995; Godfrey-Smith 2009) and of germ-soma distinction (Godfrey-Smith 2009). Nevertheless, these criteria suffer from two main weaknesses: first, they fit well multicellular organisms (notably the process of embryogenesis), but cannot be applied to unicellular (prokaryotes as well as eukaryotes) organisms; secondly, they do not explain the biological link between the evolutionary dimension of an individual and the physiological processes responsible for its self-maintenance. With this regard, I suggest that, for a *unicellular* organism to be an evolutionary individual, it requires a system-level coordinated reproduction being organisationally closed with metabolic and developmental processes and exhibiting the above mentioned three levels of mechanistic integration. Therefore, my line of reasoning is that the notion of evolutionary individual cannot be disentangled from the physiological one[27].

6.6 CONCLUDING REMARKS

[26] The notion of physiology covers all those phenomena that allow a unicellular and multicellular organism to self-maintain in a steady state, keeping a number of parameters (e.g. pH, temperature, intracellular calcium concentration, etc.) within certain "physiological" ranges. By contrast, pathophysiology occurs when a number of parameters are not in physiological ranges, thus progressively preventing the organism from self-maintaining.

[27] If it is true that evolutionary individuality entails the physiological one, the reverse is *not always* true. As already pointed out by Pradeu (2016), there are physiological individuals that do not necessarily have a system-level collective reproduction. For example, in collective systems (e.g. colonies of bacteria or of insects), there is no system-level coordinated reproduction, but rather independent forms of reproduction of the parts of the whole (see also sections 3.3 and 3.5). Another case of physiological individuals that are not evolutionary units is represented by sexually reproducing organisms (through meiosis) that are sterile: in spite of having reproductive functions, they cannot perform them.

In this chapter I have examined the relationship between biological reproduction, functional integration, and implicitly heredity, addressing the question of whether, and how, physiological integration is a necessary condition for a system-level coordinated reproduction that enables a biological organisation to transmit its genetic and phenotypic traits to the offspring, thus leading to a parent-offspring lineage. In order to address this issue, I have examined two representative examples of cellular reproduction: the binary fission of prokaryotes (bacteria and archaea) and the mitosis of eukaryotic cells. The comparative analysis of these two forms of reproduction has shown a common theoretical core: first, system-level coordinated reproduction is mutually dependent on developmental processes so as to generate a life cycle that is sustained by and also sustains metabolic processes; secondly, this functional interdependence between system-level reproduction, growth, and metabolism relies on three levels of mechanisms that are functionally integrated: the nutrient-dependent signals coordinating the life cycle with metabolism, the regulatory proteins controlling life cycle, and the cytoskeletal proteins and motor proteins controlling the spatial coordination during cellular fission.

I have also observed that this organisational closure between reproduction, growth, and development sheds new light on the issues of biological autonomy and individuality. I have underlined that the integration between reproduction, growth, and development represents the *physiological basis* for unifying intra- and cross-generation functions and for understanding the continuity between constitutive processes and reproductive functions. I have also stressed that the above-mentioned organisational closure is the linchpin of physiological and evolutionary individuality, thus providing us with a unified view of what a biological individual is.

I can now address the third and last question of this chapter: why did the proto-eukaryotic cell need to evolve the prokaryotic mechanism of binary fission into mitosis and meiosis? As usual, it is not easy to answer to this question because of the lack of fossils or still living organisms that are intermediate between prokaryotes and eukaryotes. Nevertheless, I can suggest some theoretical clues.

First, the transition from prokaryotic to eukaryotic cell entailed the appearance of many intracellular organelles that produced a global change of the cytoplasmic space. For a proto-eukaryotic cell to divide and reproduce faithfully, it needed to develop some *reproductive mechanisms* that allowed for a precise *division* and *segregation* of the *organelles* so as to faithfully transmit them to the daughter cells. Since the prokaryotic protein FtsZ does not form microtubules

and cannot segregate organelles[28] (Margolin 2005), it seems very likely that the appearance of microtubules and eukaryotic motor proteins[29] was an *enabling condition* for the appearance of mitosis: without them, neither the construction of the *spindle*, nor the *division* of the *nucleus* and of the *organelles*, nor *cytokinesis* would have been possible (Wickstead and Gull 2011; Koumandou et al. 2013). Since the faithful transmission of the intracellular structures was a necessary condition for the preservation and the evolution of the eukaryotic cell across time, we may reasonably hypothesise that the appearance of the eukaryotic cytoskeletal and motor proteins was an event that *preceded*, or at least *co-evolved* with, the emergence of intracellular membranes and the intracellular division between cytoplasm and nucleoplasm (see also Cavalier-Smith 2010). In other words, what I suggest is that mitosis was a quite *early* event in eukaryogenesis that responded to a new physiological need: the faithful reproduction of the internal division of the cytoplasmic space.

Secondly, the emergence of a new set of regulatory proteins, notably the cyclin-dependent kinases, was required for temporally coordinating mitosis with interphase. In spite of being poorly understood, the CDKs seem to be a *de novo* class of proteins that do not evolve from the bacterial regulators of cell cycle (two-component signal transduction proteins) (Liu and Kipreos 2000). Furthermore, the appearance of the CDKs has been extremely important for the coordination of energy and carbon metabolism with proliferation, since they can directly and indirectly control metabolic fluxes through, for example, the phosphorylation of metabolic enzymes (Solaki and Ewald 2018).

Thirdly, the transition from closed to open mitosis is intimately connected with the increase in genome size of eukaryotic cells: since nuclear volume scales linearly with genome size, the length of the mitotic spindle would have increased and would not be able to fit into the nucleus, thus requiring the breakdown of the nuclear envelope (i.e. open mitosis) (Sazer et al. 2014, p. R1101). The passage from closed to open mitosis is not very clear, but it is possible that transposable elements[30] are responsible for a transition from closed to open mitosis in plants and animals (Sazer et al. 2014). Transposable elements would eventually allow for the opening of the mitotic spindle, thus facilitating quick access of the mitotic spindle to the pool of cytoplasmic microtubule protein (Pickett-Heaps 1974).

[28] The FtsZ is reported to allow organelles of endosymbiotic origin (i.e. chloroplasts and mitochondria) to undergo fission (Margolin 2005).
[29] They are examples of molecular machines.
[30] Transposable elements are fragments of DNA that can change their position within a genome. They can lead to genetic mutations that may alter the cell's genetic identity and genome size.

Finally, the transition from mitosis to meiosis is also a difficult issue, but an interesting hypothesis has been proposed by Wilkins and Holliday (2009), who consider meiosis as an event that evolved from mitosis. According to the authors, there is a close correspondence between meiotic and mitotic stages, thus suggesting that the former may have evolved from the latter through a change in the mechanisms underlying mitotic cycle[31] (Wilkins and Holliday 2009).

[31] According to Wilkins and Holliday (2009), meiosis would have arisen from mitosis through *only one step*, namely *homolog synapsis* (i.e. the pairing of two homologous chromosomes during meiosis) that was inserted into the mitotic cycle.

CONCLUSIONS

In this last part of the work, I shall connect the results of the six chapters to draw some conclusions about the *nature* and the *role* of functional integration in one of the earliest evolutionary transitions: eukaryogenesis. I firstly introduce a rather general concept of functional integration and I critically assess its explanatory value for understanding eukaryogenesis. Then, I present a more precise (and stronger) characterisation of functional integration and consider its theoretical implications in the current philosophical debate on organismality, individuality, collective organisations, autonomy, and major evolutionary transitions, and different forms of collective synchronic organisations.

On the basis of what has come out so far, a first, loose definition of functional integration can be framed as follows: *the degree of functional interdependence among the component parts of a system that enables the whole to exhibit systemic behaviours*. This formulation, which is in line with current formulations of functional integration (see the Introduction), indicates that functional integration is a *matter of degree*: it occurs *gradually* and can be more or less *strong* in different biological organisations. As such, functional integration makes *qualitative* differences in how biological systems are functionally organised and also *quantitative* differences in how they are integrated.

Such a definition of functional integration allows us to distribute biological organisations on a *spectrum of functional integration*, evaluating their biological properties and behaviours on the basis of it. Different biological systems[1], such as a *molecular machine*[2], a *single cell*, and a *colony of bacteria* exhibit distinct *kinds* and *degrees* of functional integration. In a machine, functional integration depends on the degree of cohesion of the parts that permit them to function in a coordinated and organised way in order to achieve a new and more complex function. In a single

[1] Here, I employ the expression "biological system" in a very broad sense to designate a set of biological parts (or components) that perform one (or more) function(s). For this reason, biological system can refer both to the parts of cells (like molecular machines), cells themselves, or a set of cells collaborating among them (like colonies).

[2] A clarification is necessary here. As pointed out in chapter 1, I do *not* claim that molecular machines are organisms, nor that they exhibit the same functional integration of a cell. I just state that, if we accept a very general (and loose) definition of functional integration as a functional interdependence of parts performing a collective behaviour or function, we can legitimately claim that a molecular machine is a functionally integrated system. The reason is that, as repeated several times in the thesis, a molecular machine performs a function because of the way how its parts are arranged and integrated between them. Furthermore, as stressed in chapter 1, a molecular machine works and performs a function because of its functional interdependence with other macromolecules in the cellular context. For sure, if I provide a stricter and rigourous definition of functional integration (as I do a bit later in this section of the thesis), it is glaringly apparent that a molecular machine is *not* functionally integrated *as* a cell or as a colony of cells.

cell, functional integration is the degree of interdependence between the cellular components that allow cell to self-maintain and interact with the environment. In a collective organisation, functional integration entails the degree of cooperation and interdependence among the individual components that favours the appearance of systemic capacities and behaviours.

However, the above-mentioned definition of functional integration can grasp only coarse-grained features of biological organisations and does not help us to understand the origin of a strongly different and new form of integrated organisation such as the eukaryotic cell. As pointed out in chapter 3, one of the key events of eukaryogenesis was the *engulfment* of one (or more) prokaryote(s) by another and the progressive transformation of symbiont(s) into an organelle, leading to a full-fledged individual. Therefore, the main problem is to formulate a concept of functional integration that is useful for distinguishing the qualitative differences between current *symbiotic associations* of prokaryotic cells and the *organism-like organisation* of a eukaryotic cell, resulting of a very special and long process of prokaryotic association. If we assume functional integration as a mere functional interdependence of parts that comes in stages, we *cannot* appreciate the structural and functional differences between a symbiotic association (e.g. a biofilm) and an organism-like organisation (e.g. the endosymbiosis between *Tremblaya* and *Moranella*): in both cases, the component parts (i.e. bacteria) are functional interdependent on each other, because they share metabolic pathways, they have common developmental processes, and they can respond to external stimuli in a very coordinated way.

We therefore need a *stronger* and *more specific* concept of functional integration that allows us to distinguish the qualitative differences between collective individuals and new forms of cohesive individuals This means a *refinement* of the concept of functional integration in the light of four specific *organisational* aspects that have resulted from the case-studies:

1) functional integration is the result of specific *spatial constraints*;

2) functional integration is the outcome of *system-level regulation*;

3) functional integration is the product of *spatio-temporal coordination* among the component parts;

4) functional integration is the result of a *system-level reproduction*.

As regards the first point, chapters 1 and 3 have underlined that the *architecture* of a system imposes important constraints on how the parts work. In the case of a machine, it is the specific *design* and *cohesiveness* of the component parts that enable them to do work and perform specific functions. In biological organisations, *boundaries* (e.g. membranes) act as spatial constraints on the

space that they surround (this point was already stressed by Maturana and Varela 1973, 1980). We have seen in chapter 3 that *extracellular matrix* and *engulfment* impose different spatial constraints on the behaviour of the component parts, thus leading to a different functional integration of the symbionts. The extracellular matrix provides biofilm bacteria with *global cohesion* that enhances metabolic exchanges, intercellular communication, common development, and immune response, making biofilm bacteria stronger than in their free-living lifestyle. Nevertheless, biofilm bacteria still keep a certain degree of autonomy and independence that prevents most of biofilms from exhibiting some important system-level capacities such as reproduction. Furthermore, the extracellular matrix is highly sensitive to environmental changes, thus leading to a rather ephemeral, though effective, organisation.

Instead, engulfment provides two bacteria with a common boundary that constrains their behaviour in a selective manner and allows for the achievement of a very strong integration between two organisms. The engulfment promotes a *massive transference of genes* from the endosymbiont to the host, so that the host progressively establishes a complete genetic control over the symbiotic association as a whole. Moreover, the transference of genes is accompanied by an efficient *transport* and *targeting* of functional components between the partners through the endosymbionts' membranes. As a result, two endosymbionts not only share metabolic pathways, but they also exhibit system-level reproductive capacities that enable the collective association to persist over generations and potentially to evolve. For this reason, I have suggested in the third chapter that the engulfment between two prokaryotes is a fundamental *requirement* for the appearance of a strong, and non-facultative, functional integration between different symbiotic partners.

Spatial constraints contribute to determine *system-level regulation* that establishes a functional control of the whole over the parts by responding to cues and signals (see chap. 2). As pointed out in chapter 3, the extracellular matrix of a biofilm and the engulfment are extremely different from the point of view of system-level regulation. In spite of exhibiting many coordinated physiological processes (e.g. common metabolic pathways, collective developmental processes, or common immune responses), biofilms do not have a collective genetic control system for their regulation. Thus, biofilm bacteria keep a certain degree of *autonomy* in performing their functions and exhibit *distributed* forms of control, without establishing a fine-tuned system-level control of the development and reproduction. Neither exhibits the consortium *Tremblaya-Moranella* a collective genetic control, most likely because it is an intermediate form between a symbiotic relationship and

a new organism. Nonetheless, the endosymbiotic relationship has led to a strong genomic reduction of both organisms, which in turn has entailed that *Tremblaya* controls some of the functions performed by *Moranella* and viceversa. I have labelled this peculiar situation as "interlocked regulation" (see chapter 3) and it could eventually be considered as a very primitive form of system-level regulation.

An important aspect of system-level regulation in symbiotic associations, as stressed in chapter 5, is the *control* of the *sensorimotor capacities* of individual components. In biofilms, the motility capacities of the bacteria are transiently inhibited to favour the formation and maintenance of the whole biofilm. By contrast, the endosymbiotic events that led to the eukaryotic cell entailed the progressive loss of the sensorimotor abilities of endosymbionts and the control of their movement by the eukaryotic cytoskeleton. The *loss* of the autonomous movement (or *interactive autonomy*) of *organelles* in favour of the autonomy of the overall organisation was a major step in eukaryogenesis and represents an important difference with many symbiotic associations in which component parts still keep the genes for producing the proteins for sensorimotor capacities and interactive autonomy.

Another fundamental aspect of functional integration, which is also the outcome of spatial constraints, is the establishment of an effective *communication (*through signals exchange) among constituent parts so as to *spatially* and *temporally* coordinate their functions. Again, we can appreciate a fundamental difference between symbiotic associations and organism-like organisations. As shown in chapter 3, the extracellular matrix keeps bacteria close together so as to enhance the exchange of signals among them. However, the signal exchange is *not specific* (i.e. does not rely on single interactions for a certain effect) and can lead to a high variety of behaviours within the collective system. Instead, engulfment favours the implementation of *fine-tuned* mechanisms for transporting proteins across the membrane of the endosymbiont and it also promotes the synthesis of complex control macromolecules acting on endosymbiont's membrane, so as to send the right components in the right place at a given time.

In other words, engulfment paved the way for a *strong* spatio-temporal coordination between the component parts of a new individual. As stressed in chapter 4, the appearance of the eukaryotic cell entailed the division of the cytoplasmic space through intracellular membranes (or endomembranes) that promoted the spatial separation between the functions performed by the cell. For this organisation to be viable, it was necessary not only a systemic genetic regulation, but also a system of *intracellular signals* that allow the *organelles* to *communicate* among each other so

as to *spatiotemporally coordinate* their functions. I have shown that a major role in the spatiotemporal coordination of eukaryotic organelles is performed by the cytoskeleton that acts as a scaffold favouring the exchange of signals between the organelles and the cytoplasm, thus enabling intracellular communication.

A fourth dimension of functional integration that I want to stress in relation to the case-studies is the achievement of *system-level reproduction*. The ability to reproduce as a whole is a fundamental feature of full-fledged individuality (organisms) and often lacks in collective organisations, thus being an eventual dividing line between them. As argued in chapter 6, system-level reproduction can be achieved through a set of mechanisms that enables the overall system to divide and originate a new organisation with the same genetic and phenotypic features. In order to be viable, such a machinery must exhibit a reciprocal causal loop with developmental and metabolic processes. The interdependence between reproduction, development, and metabolism is enabled by both (cytoskeletal) molecular machines, system-level regulation and spatio-temporal coordination of the parts.

My proposal for functional integration can shed some new light on important issues of the current philosophical and biological debate. First of all, a clarification of functional integration can help to better understand the issue of organismality. Current philosophical accounts have rightly emphasised that functional integration is a fundamental aspect of *organisms*, being identified with structural and functional cohesion of the parts (Wolvekamp 1966; Sober 1991; Ruiz-Mirazo et al. 2000; Collier 2004), a clear boundary between the organism and the environment (Sterelny and Griffiths 1999; Godfrey-Smith 2011), high cooperation and low conflict among the parts (Queller and Strassmann 2009), and a system-level reproduction (Okasha 2011; Godfrey-Smith 2011). My proposal is in line with these accounts, but it lays emphasis on the *organisational aspects* underlying the relationship between the organism as a whole and its components parts.

More precisely, structural and functional cohesion, as well as the distinction between the interior and the exterior, are *not* the outcome of a mere spatial *contiguity* among the parts, but they rather depend on a very specific assembly of the parts (i.e. their *architecture* or *design*) and on a *common boundary* that acts as a spatial constraint over the behaviour of the parts. Queller and Strassmann's (2009) criterion of high cooperation and low conflict among the parts is somehow general and does not explain how it can be achieved: in my view, the low conflict of the parts is produced by the capacity of a system to *systemically regulate* its component parts in such a way as to avoid or settle their potential conflict. A high cooperation among the parts is determined by an effective

communication among the parts and by their spatio-temporal coordination. It has also been pointed out that system-level reproduction should be considered an important aspect for defining the functional integration of an organism (Okasha 2011; Godfrey-Smith 2011). I agree, but I emphasise that system-level reproduction has to be considered in conjunction with developmental processes (see Griesemer 2000, 2016) and with the metabolic processes that sustain it. I stress, in other words, that the reciprocal loop between metabolism, development, and reproduction is a fundamental aspect of functional integration, thus placing reproduction in a more systemic (physiological) context.

For these reasons, I consider that this propososal has far-reaching consequences also for thinking about *biological individuality*. The prevailing trend in the current philosophical debate is to consider individuality in a *pluralistic* way, leading to a "promiscuous individualism" (Dupré 2012). The explanatory strategy of such a pluralistic stance is to decompose the term "individual" into five main biological dimensions: structural unit, genetic unit, physiological unit, reproductive unit, evolutionary unit (see the Introduction). Pluralists argue that we can understand the variety of biological living beings by providing a very general (and loose) concept of individuality that encompasses a huge variety of biological organisations. Quite differently, I frame a concept of functional integration lying at the intersection between the five dimensions of individuality, which provides a *criterion* to distinguish cohesive individuals (organism-like individuals) from the high variety of collective associations (colonies, societies, etc.). It is not my aim to say if a collective association is an individual or not, but rather to provide a concept of functional integration that clearly explains *why* and *how* an *organism-like individual* is *more* integrated than a *collective association* and, on the basis of it, being able to appreciate their qualitative differences. I believe that *spatial constraints*, *system-level regulation*, *spatio-temporal coordinatio*n of the parts, and *system-level reproduction* are valuable parameters for the definition (and the evaluation) of biological individuality.

A number of authors (Dupré and O'Malley 2009; Ereshefsky and Pedroso 2013, 2015; Pradeu 2016) have argued that symbiotic associations and colonies can be considered as *physiological individuals*, inasmuch as they share functional capacities (e.g. metabolism), thus generating cohesive physiological units. In most cases, symbiotic associations, colonies, and societies do not exhibit system-level reproduction and they cannot give rise to parent-offspring lineage, thus posing the problem of whether or not they are levels of selection (or evolutionary individuals) (Clarke 2016; Skillings 2016). My proposal for functional integration turns out to be useful for explaining this

aspect of synchronic organisations: they differ from organism-like individuals because of a lower internal functional integration that usually takes the form of weak spatial constraints, a distributed (not hierarchical) regulation, the absence of fine-tuned spatio-temporal coordination of the parts, and finally the lack of a system-level regulation. In other words, functional integration provides a norm to distinguish between different biological organisations that are usually labelled as "individuals".

A redefinition of functional integration has also some effects on contemporary accounts of biological autonomy (Bickhard 2000; Collier 2000; Kauffman 2000; Christensen 2007; Rosslenbroich 2014; Moreno and Mossio 2015). In my view, biological autonomy is a property of full-fledged individuals and the outcome of a *strong functional integration* that determines a reciprocal causal loop between the processes involved in the self-maintenance of the organisation (i.e. constitutive processes) and those deployed by the organism with the aim of functionally modifying its environment (i.e. interactive processes, see also Arnellos et al. 2014). Indeed, the autonomy of full-fledged individuals (e.g. unicellular or multicellular organisms) entails the existence of a common (selective) border that constrains the parts so as to produce a systemic regulation and spatio-temporal coordination not only over the constitutive (including reproduction) processes, but also over the interactive ones. The close relationship between functional integration and biological autonomy is also useful for understanding collective organisations: they exhibit a high functional integration of the individual living entities ("parts") at the expense of the whole, so that the individual parts keep their autonomy and the overall synchronic organisation is prevented from being autonomous as a whole. In other words, the individual living entities, which are parts of such a collective organisation, keep their organismic condition.

A final theoretical issue that could benefit from my proposal for functional integration is the evolutionary (and philosophical) debate on major evolutionary transitions. For some evolutionary biologists (Buss 1987; Maynard-Smith and Szathmáry 1995; Michod 1999), major evolutionary transitions are characterised by the appearance of new forms of (cohesive) individuals (e.g. unicellular eukaryotic organisms, multicellular organisms), which are the outcome of a strong interdependence among the members of a group, so that they "become so integrated that they evolve into new higher-level individuals" (Michod 2007, p. 8613). This means that the previously mentioned aspects of functional integration contribute to explain how a group of individuals can develop such a strong internal division of labour, high cooperation of the parts and reduction of the

conflict among them, that it could no longer be a group of individuals, but rather a new full-fledged, functionally integrated, organism.

All in all, this work represents a first theoretical effort to clarify and define the concept of functional integration. The red thread of this thesis is a reflection on the nature of the relationship between *functional integration* and *biological individuality*, which is at the core of any definition of organism both in biology and philosophy of biology. In spite of its theoretical relevance, few biologists and philosophers have developed in-depth analyses and formulated a theoretical framework of the concept of functional integration. This work has tried to fill this conceptual gap and can be especially helpful to shed new light on critical conceptual issues that are at the intersection between theoretical biology and philosophy.

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