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Universidad del País Vasco
Euskal Herriko Unibertsitatea

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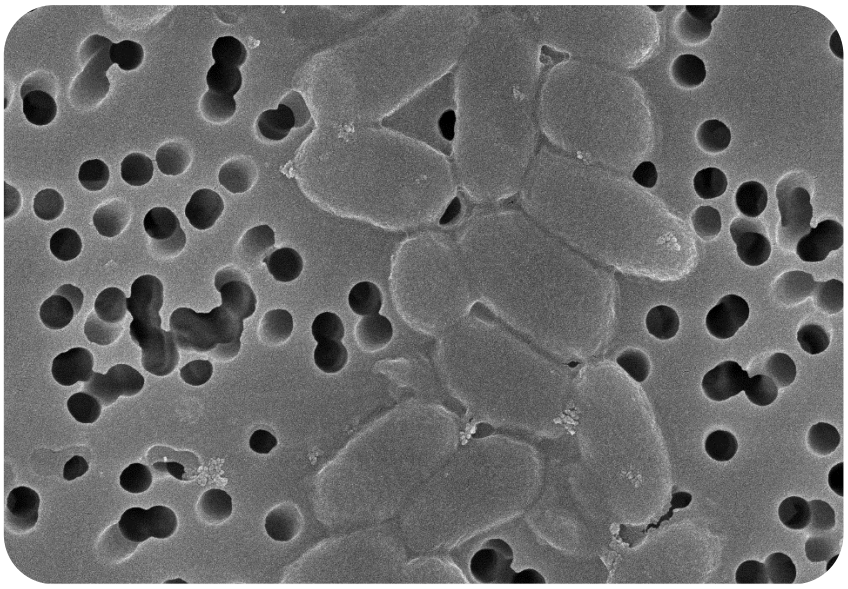
FACULTAD DE CIENCIA Y TECNOLOGÍA

Doctoral thesis

Analysis of the temperature-dependent adaptation of *Vibrio harveyi* in seawater microcosms



Itxaso Montánchez Alonso

Leioa, 2018

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Scientific contributions

Some of the results presented in this thesis have previously been published in two scientific articles and reported at national and international conferences.

Publications

- Montánchez, I., Arana, I., Parada, C., Garaizabal, I., Orruno, M., Barcina, I. and Kaberdin, V. R. (2014) **Reprogramming of *Vibrio harveyi* gene expression during adaptation in cold seawater.** *FEMS Microbiology Ecology*, *87*, 193-203.

- Kaberdin, V. R., Montánchez, I., Parada, C., Orruño, M., Arana, I. and Barcina, I. (2015) **Unveiling the metabolic pathways associated with the adaptive reduction of cell size during *Vibrio harveyi* persistence in seawater microcosms.** *Microbial Ecology*, *70*(3), 689-700.

Congress communications

- Kaberdin, V., Montánchez, I., Hernandez Plagaro, A., Parada, C., Orruño, M. and Arana, I. **Towards defining essential metabolic pathways and regulatory factors involved in *Vibrio harveyi* adaptation in marine environments.** 7th Congress of European microbiologists, FEMS. Valencia, Spain; July 9-13, 2017. Poster.

- Kaberdin, V, Arana, I, Orruño, M., Ruiz-Larrabeiti, O., Montánchez, I., Irastorza, M., Hernández Plágaro, A. and Arabiotorre, A. **Analysis of mechanisms involved in bacterial adaptation to stress and their regulation by antisense RNAs.** V Jornadas de Investigación de la Facultad de Ciencia y Tecnología. Leioa (España), 2016. Poster

- Kaberdin, V., Hernandez Plagaro, A., Ruiz Larrabeiti, O., Parada, C., Orruño, M., Montanchez, I. and Arana, I. **Metabolic pathways and regulatory factors involved in *Vibrio harveyi* adaptation in marine environments.** 4th Molecular Microbiology Meeting. Viena (Austria), September 16-18, 2015. Poster.

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Aita eta Amari

Table of contents

Abbreviations

AI	Autoinducer
AI-2	Autoinducer 2
ATCC	American Type Culture Collection
Ca.	*circa* (approximately)
CAI-1	Cholera autoinducer 1
cDNA	Complementary DNA
CFU	Colony Forming Unit
Chr1	Chromosome 1, the larger chromosome
Chr2	Chromosome 2, the smaller chromosome
CPR	Continuous Plankton Recorder
DNA	Deoxyribonucleic Acid
e. g.	*Exempli gratia* (For example)
ENSO	El Niño Southern Oscillation
EPS	Extracellular Polysaccharides
Et al.	*Et alii* (and collaborators)
etc.	*Et cetera*
GW	Global Warming
HAI-1	Harveyi autoinducer 1
i.e.	*Id est* (That is)
IPCC	Intergovernmental Panel on Climate Change
MA	Marine agar
MB	Marine broth
MEMB+	Intact cytoplasmic membrane
min	Minute(s)

MLSA	Multi-Locus Sequence Analysis
NAG	*N*-acetyl-D-glucosamine, monomeric component of chitin
NAO	North Atlantic Oscillation
NCBI	National Center for Biotechnology Information
NCMB	National Collection of Marine Bacteria
NOAA	National Oceanic and Atmospheric Administration of the USA
nt	Nucleotide
ORF	Open Reading Frame
P. monodon	*Penaeus monodon* (Black Tiger shrimp)
PCR	Polymerase Chain Reaction
QQ	Quorum Quenching
QS	Quorum Sensing
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RT-qPCR	Quantitative reverse transcription PCR
sRNA	Small RNA
SST	Sea Surface Temperature
TNC	Total number of bacteria
TTSS	Type III secretion system
TXT	Tetrodotoxin
UV	Ultraviolet
VBNC	Viable but Nonculturable
VHML	*Vibrio harveyi* mucovirus like phage

Summary

Summary

Vibrio species including *Vibrio harveyi* constitute one of the most diverse genera of Gram-negative bacteria ubiquitously present in natural aquatic systems.

In addition to striving in a free-living state, they can stay attached to biotic or abiotic surfaces (Stabili *et al.*, 2006). Moreover, some of them can be involved in symbiotic or host-pathogen interactions, occasionally eliciting infections in various fishes and marine invertebrates. Owing to their ubiquitous presence and ability to act as primary or opportunistic pathogens, Vibrio species greatly contribute to the diversity and evolution of marine ecosystems, especially in temperate and tropical climates. One of the major manifestations of the ongoing climate change is the gradual increase in sea surface temperature, which is believed to be the main reason for the recent spread of pathogenic Vibrio species and a concomitant increase in the number of disease outbreaks caused by these pathogens. Although some Vibrio species have recently been used to study ecology and evolution of bacterial population in natural aquatic ecosystems (Takemura *et al*, 2014; Vezzulli *et al.*, 2015), the effects of temperature and limitation of nutrients on the long-term survival and adaptation of many Vibrio species are still poorly characterized and merit further investigation.

In the present study, we used *V. harveyi* as a model organism to unveil the cellular strategies enabling this marine gammaproteobacterium to cope with limitation of nutrients during its persistence in seawater microcosm.

We found that *V. harveyi* incubation in seawater microcosm at moderate (20°C) and elevated (30^{o}C) temperatures led to a progressive reduction in the average cell size. Moreover, these morphological changes took place faster at 30^{o}C. Furthermore, in contrast to its persistence at 20 °C and preservation of cell culturability, incubation of *V. harveyi* at 30^{o}C apparently led to the gradual cell damage and death, thus pointing to its higher vulnerability at elevated temperatures. Analysis of the adaptation steps at the whole transcriptome level revealed that reduction of cell size at 20°C was accompanied by a profound decrease in gene expression affecting the central carbon metabolism, major biosynthetic pathways, and energy production. In contrast, *V. harveyi* increased expression of genes involved in lipid degradation via the β-oxidation pathway, thus promoting the use of endogenous fatty acids (discharged

with the onset of cell envelope shrinking) as a major energy and carbon source at 20°C. Likewise, *V. harveyi* upregulated a number of genes important for sustaining iron homeostasis, cell resistance to the toxic effect of reactive oxygen species, and recycling of amino acids. The above gene expression changes likely ensure the integrity of *V. harveyi* cells and their capacity to resume growth even after prolong incubation in seawater at 20°C. In contrast to their regulation at 20°C, some of these genes show counter adaptive expression at 30°C, for instance, downregulation of those that are essential for cell envelope remodeling and fitness, which apparently results in the cell damage and death observed at the elevated temperature. In contrast to its negative impact on expression of some gene essential for adaptation, incubation at 30°C increased expression of virulence genes apparently essential for the spread of Vibrio-associated diseases in the time of global warming.

Finally, analysis of Vibrio adaptation at 4°C enabled to disclose a number of additional cell stress responses primarily counteracting the negative effects of cold shock (e.g. protein misfolding etc.) on cell physiology. In summary, apart from revealing the distinct phenotypical and physiological changes linked to *Vibrio harveyi* adaptation at low, moderate and elevated temperatures, the present study disclosed a considerable number of stress-related genes and their products (e.g. genes encoding transporters, stress-related factors and essential metabolic enzymes) apparently important for adaptation of *V. harveyi* and other marine bacteria in natural aquatic systems.

Introduction

1. The taxonomy and general characteristics of the genus *Vibrio*

The first *Vibrio* species was described in 1854 by the Italian anatomist Filippo Pacini, who isolated *Vibrio cholerae* (Pacini, 1854) during a cholera outbreak in London. Following this discovery, many other *Vibrio* species (e.g. *Vibrio cincinnatiensis* (1896), *Vibrio splendidus* (1900), *Vibrio anguillarum* (1909), *Vibrio marinus* (1927), *Vibrio harveyi* (1936), *Vibrio costicola* (1938), *Vibrio parahaemolyticus* (1951), *Vibrio alginolyticus* (1961), *Vibrio natriegens* (1961), *Vibrio proteolyticus* (1964)) were gradually identified and characterized by other researchers (LPSN, 2017).

The family Vibrionaceae was formally coined by Véron in 1965 (Véron, 1965) to group several related species dispersed among other existing orders and families initially described in the seventh edition of Bergey's Manual of Determinative Bacteriology (Breed *et al.*, 1957). In the ninth edition of the same manual (Holt *et al.*, 1994), some additional criteria (such as exhibition of oxidase activity, possession of particular flagella type, Na^+ requirement for growth and sensitivity to the vibriostatic compound O/129 (2,4-diamino-6,7-diisopropyl-pteridine phosphate)) were introduced in addition to morphological characteristics, and this extended set of criteria made it possible to better differentiate most of the genera in the family Vibrionaceae from genera of other closely related families, in particular Enterobacteriaceae.

According to the one of the recent editions of *Bergey's Manual of Systematic Bacteriology* (Farmer and Janda, 2006), vibrios (i.e. *Vibrionaceae* strains) belong to the *Gammaproteobacteria* class, and represent a genetically, metabolically and ecologically diverse group of bacteria that contains many pathogenic species known for their negative effects on human and animal health.

Over the years, the taxonomy of the Vibrionaceae family has been undergoing continuous changes. In addition to the three genera (*Vibrio*, *Photobacterium* and *Salinivibrio*) initially described in 2006 (Farmer and Janda, 2006), seven additional genera (*Beneckea*, *Listonella*, *Lucibacterium*, *Allomonas*, *Catenococcus, Enterovibrio* and *Grimontia*) were included in the *Taxonomic Outline of the Bacteria and Archaea* published the following year (Garrity *et al.*, 2007). Although the same number of genera (10) was preserved in one of the recent classifications presented in the *Ribosomal Database Project* (2016) and included *Vibrio*, *Photobacterium*, *Salinivibrio,*

Listonella, *Lucibacterium*, *Allomonas*, *Catenococcus, Enterovibrio, Grimontia* and *Aliivibrio* (instead of *Beneckea*), the actual number and type of genera in the *Vibrionaceae* family remain controversial and vary from one source to another. These discrepancies indicate that the taxonomy of this family is still evolving and might likely include new genera (e.g. the *Thaumasiovibrio* genus proposed by Amin *et al.* (2017)) in the near future.

Among ten genera currently included in the Vibrionaceae family, vibrios represent one of the most diverse marine bacterial genera (Gomez-Gil *et al.*, 2014), and the genus is organized in clusters according to their phenotypic, physiological and genotypic similarities. In other words, the great diversity of the *Vibrio* genus makes it difficult to identify environmental *Vibrio* species based only on phenotype and biochemical tests and therefore genotyping (Gomez-Gil *et al.*, 2004) is normally needed to complete their identification. In addition, the task to distinguish strains within the Vibrio clade can be particularly challenging not only due to occasional gene transfer among closely related species (Chimetto Tonon *et al.*, 2015) but also owing to their high genotypic homology (Cano-Gomez *et al.*, 2009). For example, many species that have been assigned to the genus *Vibrio* based on the homology of 16S rRNA gene sequences share at least 85% sequence similarity in other genes (e.g. *recA*, *rpoA* and *pyrH*) frequently used in genotyping (Thompson *et al.*, 2005). To better discriminate species within the *Vibrio* genus, Sawabe *et al.* (2007) has recently proposed to define individual *Vibrio spp.* as groups of strains that share >95% gene sequence similarity and > 99.4% amino acid identity of proteins encoded by 8 protein-coding housekeeping genes (*ftsZ*, *gapA*, *gyrB*, *mreB*, *pyrH*, *recA*, *rpoA* and *topA*). The use of these genes for multi-locus sequence analysis (MLSA) provides a high discriminatory power, is reproducible, and straightforward since the reference sequences are available online in the NCBI database, and their availability facilitates strain identification and data exchange among laboratories worldwide. Moreover, the chosen housekeeping genes are not under selective pressure, and therefore their use allows a long-term classification of species (Thompson *et al.*, 2007). Despite its excellent discriminative capacity, MLSA based on the use of the above housekeeping genes is not the best option when the aim is to identify or distinguish pathogenic strains often differing in the number and nature of virulence genes present in closely related species (Cano-

Gomez *et al.*, 2009). In this case, the use of the virulent-gene specific primers can overcome the above limitation and can lead to development of efficient PCR-based approaches (Xu *et al.*, 2017). Very recently, Machado *et al.* (2017) have described a gene, the ferric-uptake regulator gene (*fur*), which can additionally be used as a genotyping marker that provides species discrimination and makes their identification easier and faster because the sequence homology of each *fur* gene of unknown species can easily be examined online (http://www.cbs.dtu.dk/services/ furIOS-1.0/).

Unlike many other Gram-negative bacteria, vibrios possess two circular chromosomes of different sizes. The larger chromosome (Chr1) mostly contains housekeeping genes (e.g. genes encoding ribosomal proteins, polar flagella, DNA replication machinery, *etc*.). The smaller chromosome (Chr2), on the contrary, hosts accessory genes such as those necessary for pathogenicity, antimicrobial resistance as well as genes involved in Vibrio adaptation to different environmental stresses (Grimes *et al.*, 2009). Due to the high plasticity of their genomes, vibrios are metabolically versatile and can be adapted to many situations to survive in highly diverse and rapidly changing environments. Their high adaptability is likely in charge, at least in part, for their dominance among the heterotrophic bacteria, in particular during their isolation from aquatic samples (Urakawa and Rivera, 2006). Members of the genus *Vibrio* are morphologically diverse and include straight, slightly curved, curved, or comma-shaped Gram-negative rods, ranging from 0.5 to 0.8 µm in width and from 1.4 to 2.6 µm in length (Farmer and Janda, 2006). When grown in liquid medium, they can exhibit a significant morphological variation. The curvature of vibrios is not generally seen during the exponential phase of growth but can become visible once the cells entered the stationary phase. Apart from the growth phase, other parameters such as salinity, temperature, nutrient availability and age of the culture are also among the key factors that influence cell morphology (Onarheim *et al.*, 1994).

Vibrios are motile and particularly fast swimmers. Some of them have two distinct flagellar systems that allow propelling at different viscosities. When grown planktonically in liquid media (i.e in their free-living state), all *Vibrio* species possess a polar flagellum that allows them to swim. Depending on the species, they have either one (monotrichous) or more (politrichous) polar flagella, which are covered with a sheath of the bacterial outer membrane (Follett and Gordon, 1963). Although the

average swimming speed of vibrios in liquid media is *ca*. 60 µm per second (McCarter, 2001), some of them can move as fast as 100 µm *per* second (Magariyama *et al.*, 1995). However, the polar flagellum is not efficient enough to propel the bacterium when the viscosity of the medium increases. Therefore, while being grown on solid or in viscous media, some species acquire numerous lateral (peritrichous) flagella that are shorter than the polar ones. The peritrichous flagella enable vibrios to swarm due to an increase in their capacity to move over semi-solid surfaces and through viscous environments (McCarter, 2004). In contrast to polar ones that propelled by the Na^+ motive force, peritrichous flagella are highly abundant (>100/cell), unsheathed and are driven by the H^+ motive force (Zhu *et al.*, 2013).

While being facultative anaerobes, vibrios are chemoorganotrophs able to carry out both fermentative and respiratory metabolism. Due to the extraordinary diversity of their metabolic enzymes, vibrios can use a wide spectrum of organic compounds as sole energy and carbon sources, and there is a considerable variation among the species regarding their metabolic preferences (Thompson and Polz, 2006). Most of the vibrios are able to grow in a mineral medium by using D-glucose, dextrin, glycogen, N-acetyl-D-glucosamine, D-fructose, maltose, D-trehalose, methyl pyruvate, L-asparagine aconitate, L-proline or inosine as the primary carbon source and NH_4^+ as the sole nitrogen source. Just a few of them are able to denitrify or fix nitrogen (Takemura *et al.*, 2014). Moreover, sodium is indispensable for their growth, and, depending on each particular *Vibrio* species, the optimal concentration of NaCl in growth media can vary from 1 to 6%. Under laboratory conditions, vibrios usually grow well on marine agar or on selective media such as thiosulfate-citrate-bile salt-sucrose agar (TCBS) (Thompson *et al.*, 2004).

Vibrios are widely present in natural aquatic ecosystems (Thompson *et al.*, 2004) represented by an enormous number of habitats: fresh water (Mishra *et al.*, 2010), estuaries, riverines, marine coastal waters and sediments, pelagic deep sea (Vezzulli *et al.*, 2009); and can easily be found in artificial aquaculture settings as well. They are mesophilic, and, with few exceptions, preferentially grow in warm (>18°C) waters at moderate salinity (<2.5% NaCl), thus being more abundant during the warmer months (Vezzulli *et al.*, 2015). With few exceptions (Takemura *et al.*, 2014), vibrios can often be found either in a free-living state or in association with other

marine organisms including corals, fish, molluscs, seagrass, sponges, shrimp and zooplankton (Thompson *et al.*, 2004; Chimetto *et al.*, 2015). Moreover, some of them can also be found attached to abiotic or biotic surfaces. The latter include chitinous zoo- and phytoplankton considered to be important reservoirs of these bacteria in nature (Turner *et al.*, 2009; Lutz *et al.*, 2013). Due to their ubiquitous presence and abundance in natural aquatic systems, many members of the Vibrionaceae family are known to greatly contribute to nutrient cycling by remineralizing organic matter in aquatic ecosystems. Namely, besides their major role in decomposition of zooplankton (Fukami *et al.*, 1985), vibrios also break down chitin, a homopolymer of *N*-acetyl-D-glucosamine (NAG), which is a major component of structural polymers in bacteria, plants and animals (Riemann and Azam, 2002). While being the structural material forming the exoskeleton of many marine invertebrate animals, fungi and algae, NAG is the largest source of amino sugars present in the ocean. Owing to their ability to consume a wide array of carbon substrates the biogeochemical significance of vibrios may vary with the nutrient status of each particular environment (Thompson and Polz, 2006). Moreover, their capacity to degrade various organic pollutants apparently contribute to the sustainability and preservation of marine ecosystems (Arnosti, 2010). For instance, some vibrios have been reported to be able to degrade toxic polycyclic aromatic hydrocarbons (West *et al.*, 1984; Berardesco *et al.*, 1998; Hedlund and Staley, 2001) and therefore can play important roles in natural bioremediation of toxic compounds of industrial origin.

Although most of the vibrios are commensal or mutualistic and can even be found in some symbiotic interactions with other organisms (Nyholm and McFall-Ngai, 2004; Stabili *et al.*, 2006; Takemura *et al.*, 2014), yet some of them are well known pathogens infecting animals. In addition to three main species causing diseases in humans (*V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*) (Chakraborty *et al.*, 1997; Grimes *et al.*, 2009), there are also a number of species that are pathogenic towards marine organisms, including molluscs, coral and fish (Chatterjee and Haldar, 2012). In summary, due to their significant contribution to carbon cycling and capacity to affect life-styles and persistence of numerous marine organisms, *Vibrio spp.* have recently emerged as key players of marine ecosystems that make important economical and environmental impacts (Vezzulli *et al.*, 2012).

2. *Vibrio harveyi*

2.1 Discovery, occurrence and general properties

V. harveyi was initially described as *Achromobacter harveyi* (Johnson and Shunk, 1936) in honour of Edmund Newton Harvey for his pioneering work on bioluminescence of this species. Similar to many other vibrios, *Vibrio harveyi* emits luminescence primarily in the blue and green light spectrum in response to some environmental signals (Baumann *et al.*, 1984). The species has first been placed into *Lucibacterium* (Hendrie *et al.*, 1970) and *Beneckea* (Reichelt and Baumann, 1973) genera, but was finally included in the *Vibrio* genus, where it belongs nowadays (Baumann *et al.*, 1981). Likewise, two other closely related strains, initially called *V. carchariae* (Grimes *et al.*, 1985) and *V. trachuri* (Iwamoto *et al.*, 1996), were also later renamed to *V. harveyi* based on genotyping data (Pedersen *et al.*, 1998; Gauger and Gomez-Chiarri, 2002; Thompson *et al.*, 2002). Although MLSA (see **section 1**) is normally sufficient for identification of *Vibrio* species, a number of biochemical tests (Table 1) are also currently being used for additional verification of *V. harveyi* (Farmer and Hickman-Brenner, 2006). According to the most recent classification, *V. harveyi* is placed within the Harveyi clade, known as the Vibrio core of the *Vibrio* genus (Urbanczyk *et al.*, 2013).

Table 1. Selected biochemical tests used to confirm the identity of *V. harveyi* (adopted from Farmer and Hickman-Brenner (2006)). The table summarizes some common properties of *V. harveyi* strains associated with detection of enzymatic activities (oxydase, lysine decarboxylase etc.), production of metabolites (e.g. indole or acetoin) or capacity to reduce nitrate. The tests are either positive for 90-100% (++) and 75-89.9% (+) species or yield negative results (-) for the most of them.

Test		
Oxidase activity		++
Nitrate reduction to nitrite		++
Indole production		++
Acetoin production (Voges-Proskauer test)		-
Lysine decarboxylase activity	(2-day)	++
"	(7-day)	++
Arginine dihydrolase activity	(2-day)	-
"	(7-day)	-
Ornithine decarboxylase activity	(2-day)	+
"	(7-day)	++

Depending on the strain, the GC content of *V. harveyi* DNA can vary within 44.5 - 45.6% (NCBI Genome Database, 2017). Up to now, 35 *Vibrio harveyi* strains have been fully sequenced. The length of their genomes is between 5.45 and 6.70 Mb. Moreover, three genomes (strains ATCC 33843, ATCC 43516 (also known as BAA-1116) and LG353) have already been annotated. Each of these annotated species has two chromosomes (i.e. Chr1 and Chr2) of approximately 3.6 Mb and 2.3 Mb, respectively, and two of them (strains LG353 (NCBI Genome Database, 2017) and ATCC 43516 (Grimes *et al.*, 2009)) are known to harbour plasmids.

V. harveyi is a ubiquitous marine bacterium found in temperate and tropical aquatic systems, both in a free-living state and in interaction with eukaryotes including coral mucus, plankton, surfaces of marine animals (Stabili *et al.*, 2006), light organs of certain marine fish and cephalopods and intestine of aquatic animals (O'Brien and Sizemore 1979; Ramesh and Venugopalan, 1989). Some symbiotic *V. harveyi* strains isolated from the pufferfish *Arothron hispidus* are known to be the source of tetrodotoxin (TTX) produced in the fish (Campbell *et al.*, 2009). TXT is a non-proteinaceus potent neurotoxin believed to be implicated in the protection of fish from predators, and considered to be a source of food poisoning affecting many people each year (Lago *et al.*, 2015). *V. harveyi* can also be found attached to abiotic surfaces including sediments or those of industrial origin. While being able to survive by colonizing these unusual habitats, vibrios also form biofilms on various biotic surfaces, possibly serving as a source of nutrition and shelter, which seems to provide these marine bacteria with a selective advantage to survive under adverse conditions (Yildiz and Visick, 2009).

Regarding their optimal growth conditions, *V. harveyi* strains grow well at NaCl concentration ranging from 0.5 to 6%. Moreover, *V. harveyi* is very versatile regarding carbon sources and is able to utilize 30 to 45 organic compounds (depending on the strain) as a primary carbon source (Reichelt and Baumann, 1973). With few exceptions, *Vibrio harveyi* strains can utilize a wide spectrum of sugars and related compounds (D-glucose, D-mannitol, maltose, trehalose, cellabiose, D-mannose, D-galactose), which apparently makes them highly adaptable in natural aquatic systems. The use of some sugars (e.g. melibiose or sucrose) can be dependent on the strain tested. Moreover, the sucrose-fermenting biotype seems to be attributed to (associated with) pathogenic

strains (Alavandi *et al.*, 2006). Apart from the genomic plasticity and metabolic versatility, *V. harveyi* life-styles are also influenced by quorum sensing (QS), an important regulatory system identified in many different *Vibrio* species (Bassler, 1999) being in charge of cell-to-cell communication. QS coordinates the expression of certain genes in response to the increasing concentrations of signal molecules called autoinducers (AIs). The latter are produced by bacteria and released at concentrations proportional to cell density. In other words, the QS response is population-dependent. Bacteria adjust gene expression in response to different levels of AIs, thus being able to coordinate gene expression of the entire populations, i.e. similar to multicellular organisms. The first reference to QS (initially called "autoinduction") appeared in Nealsen *et al.* (1970), when they described this phenomenon during their study of the related luminous marine bacterium *Vibrio fischeri*. However, it took many years to understand in detail how QS controls bioluminescence and other physiological functions in *Vibrio harveyi* (Fig. 1). Surprisingly, the QS regulation has been shown to be substantially different and more complicated than that of *V. fisheri* (Owens and Busico-Salcedo, 2006). Namely, *Vibrio harveyi* produces at least three distinct AIs: Harveyi autoinducer 1 (HAI-1), an acyl homoserine lactone; autoinducer 2 (AI-2), a furanosyl-borate-diester; and Cholera autoinducer 1 (CAI-1), a (Z)-3-aminoundec-2-en-4-one (Ea-C8-CAI-1). Thus, *V. harveyi* uses a three-channel QS system to control the expression of genes responsible for bioluminescence and some virulence factors of pathogenic vibrios (Henke and Bassler, 2004a).

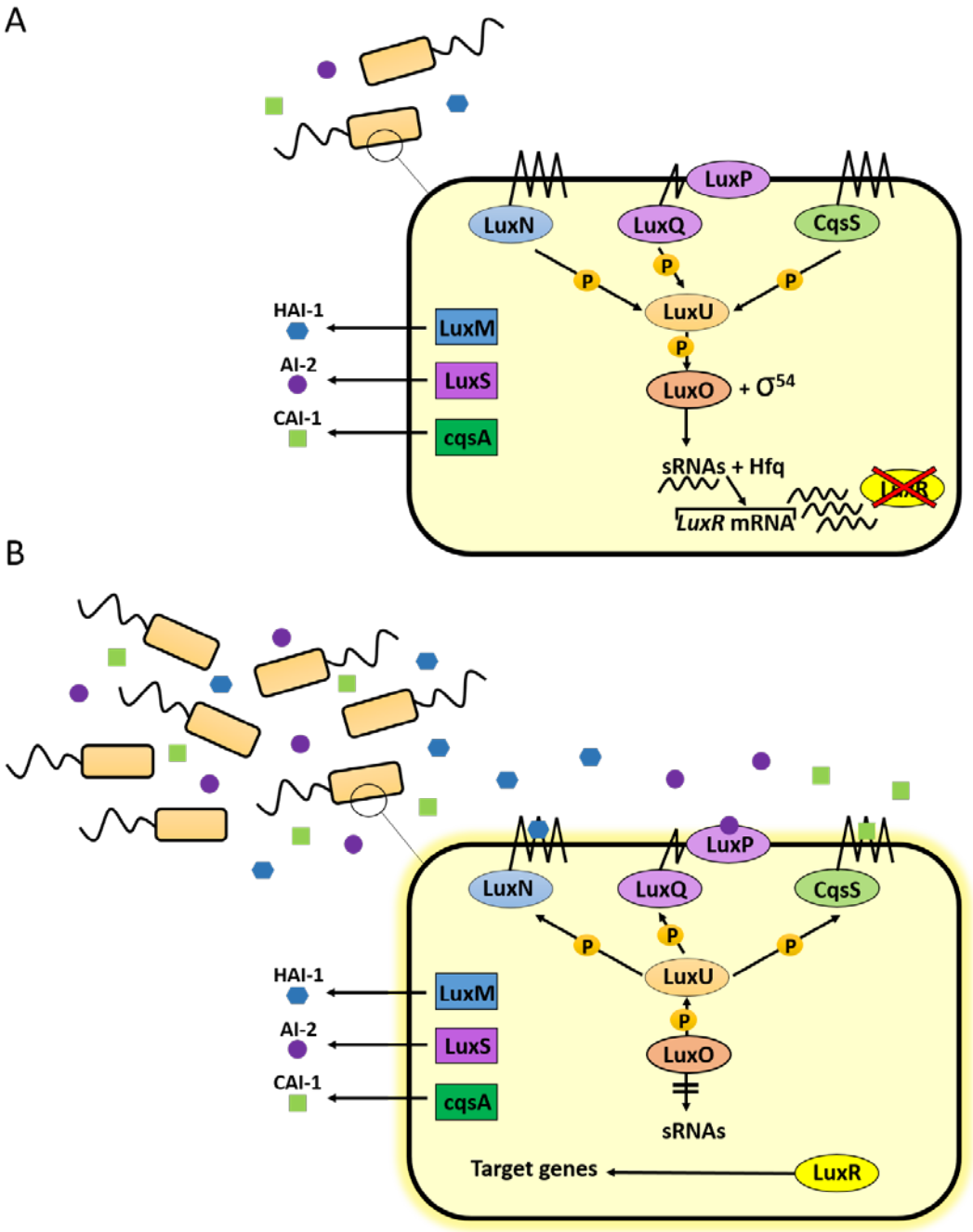

Fig. 1. A brief scheme depicting the major players and mechanism of QS in *Vibrio harveyi*. There are three *V. harveyi* enzymes, LuxM, LuxS and CqsA, being responsible for the synthesis of the autoinducers HAI-1, AI-2 and CAI-1, respectively. Once being secreted, these autoinducers can be recognized by the corresponding receptors (LuxN, LuxQ and CqsS, respectively) that are located at the cell surface **(Defoirdt *et al.*, 2008).** (A) When the autoinducers are absent or present at very low levels, the receptors become autophosphorylated and this reaction triggers the LuxU-mediated phosphorylation of LuxO. The latter activates the σ^{54}-dependent transcription of several small regulatory RNAs (sRNAs), Qrr1-5. These regulatory RNAs act in an Hfq-dependent manner to destabilize *luxR* mRNA, thereby preventing production of the transcriptional regulator LuxR. (B) Once the concentrations of autoinducers reach high levels, their cognate receptors (LuxN, LuxQ and CqsS) can bind them and subsequently change their own status from kinases to phosphatases. This leads to dephosphorylation of LuxO, thus converting it into an inactive form unable to promote Qrr1-5 transcription, and therefore derepress LuxR production.

2. 2. Pathogenic properties of *Vibrio harveyi*

2. 2. 1 Diseases caused by *V. harveyi*

Although many of the *V. harveyi* strains are non-pathogenic, some do cause diseases in a large number of marine organisms including fish and invertebrates (Table 2). In general, the naturally occurring *V. harveyi* infection of fish is more opportunistic than in invertebrates (Owens and Busico-Salcedo, 2006).

Recent studies have shown that *V. harveyi* can often cause infections in cultivated marine animals (Ruwandeepika *et al.*, 2010). The devastating statistics collected during the last 20 years revealed *V. harveyi* contribution to the mass mortality of artificially cultivated shrimps, causing a highly negative economic impact on the seafood industry, particularly in tropical and temperate climates. Some diseases caused by *V. harveyi* include eye-lesions (Ishimaru and Muroga, 1997), gastroenteritis Liu *et al.* (2004) and vasculitis in fish (Grimes *et al.*, 1984), as well as luminous vibriosis (Prayitno and Latchford, 1995) and *Bolitas negricans* (Robertson *et al.*, 1998) in shrimps. Luminous vibriosis is the most common disease of penaeids (crustaceans from Penaeidae family) caused by luminescent *V. harveyi*, being the leading cause of death among shrimps in aquaculture farms. Moreover, during the infection cycle *V. harveyi* can invade eggs and larvae, thus causing a major problem for shrimp farmers. While being often identifiable by glowing of the infected organisms in the dark, luminous vibriosis is known to occur in many other crustaceans (Diggles *et al.*, 2000). Interestingly, recent laboratory tests revealed that mortality of penaeid shrimps occurs only when they are exposed to a *V. harveyi* concentration of 10^2 CFU/ml or higher (Lightner, 1993).

Table 2. Examples of marine organisms affected by *V. harveyi*-associated diseases.

Host organism	Reference
Invertebrates	
Brine shrimp (*Artemia franciscana*)	Soto-Rodriguez *et al.* (2003)
Kuruma shrimp (*Penaeus japonicus*)	Liu *et al.* (1996)
Ridgeback rock shrimp (*Sicyonia ingentis*)	Martin *et al.* (2004)
Tiger shrimp (*Peneaus monodon*)	Lavilla-Pitogo *et al.* (1990), Karunasagar *et al.* (1994)
White shrimp (*Litopenaeus vannamei*)	Agiree-Guzmán *et al.* (2001)
Rock Lobster (*Jasus verreauxi*)	Diggles *et al.* (2000)
Abalone (*Haliotis tuberculata*)	Nicolas *et al*. (2002)
Japanese abalone (*Sulculus diversicolor supratexta*)	Nishimori *et al.* (1998)
Pearl oyster (*Pinctada maxima*)	Pass *et al*. (1987)
Sea cucumber (*Holothuria scabra*)	Becket *et al.* (2004)
Coral (*Pocillopora damicornis*)	Luna *et al*. (2009)
Vertebrates	
Cobia fish (*Rachycentron canadum*)	Liu *et al.* (2004)
Grouper (*Epinephelus coioides*)	Lee *et al.* (2002)
Red drum (*Scaienops ocellatus*)	Liu *et al.* (2003)
Atlantic salmon (*Salmo salar*)	Zhang and Austin (2000)
Rainbow trout (*Oncorhynchus Mykiss*)	Zhang and Austin (2000)
Seahorse (*Hippocampus* sp.)	Alcaide *et al.* (2001)
Summer flounder (*Paralichthys dentatus*)	Soffientino *et al.* (1999) and Lee *et al.* (2002)
Sandbark shark (*Carcharhinus plumbeus*)	Bertone *et al.* (1996)

2. 2. 2. Virulence factors involved in *V. harveyi* pathogenicity

Vibrios are able to invade a considerable number of marine organisms (e.g. see cucumber, sea horse etc.) and elicit infections. The infectious cycle of pathogenic bacteria begins with the initial interaction with their hosts followed by internalization of the pathogen, its establishment and multiplication, consequently causing temperate or severe damage of host tissues and cells (Vanmaele *et al.*, 2014). Each step of infection is controlled by specific virulence factors that allow pathogens to elicit infection, propagate and cause diseases (Defoirdt, 2013).

While enabling an interaction with host surface(s) (McCarter, 2004), flagellar motility is thought to be essential for attachment to host surfaces and successful colonization. Indeed, one of the recent studies demonstrated that the flagellar motility of *V. harveyi* plays an important role in the virulence of this bacterium (Yang and Defoirdt, 2015). Apart from the sheathed polar flagellum (24-30 nm in diameter) most of the strains synthesize unsheathed peritrichous flagella (14-16 nm in diameter) (Fig. 2), and both types of the flagellar seem to play a role in host's colonization (Fig. 3).

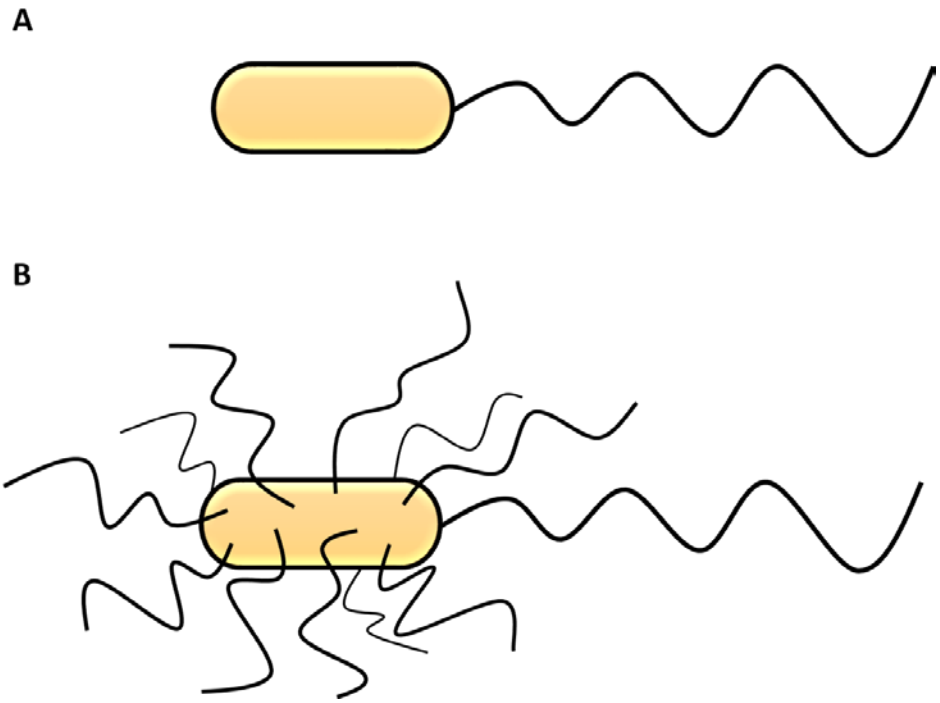

Fig 2. *V. harveyi* possesses polar (A) and peritrichous (B) flagella used to move in liquid media (A) or on semisolid surfaces (B), respectively.

Namely, while polar flagellum facilitate swimming during the initial approach to the host (Kirov, 2003), lateral flagella serves to confer swarming movement, thus helping pathogenic bacteria to move over the host surface and subsequently contributing to the colonization and biofilm formation (Fig. 3) (Merino *et al*., 2006).

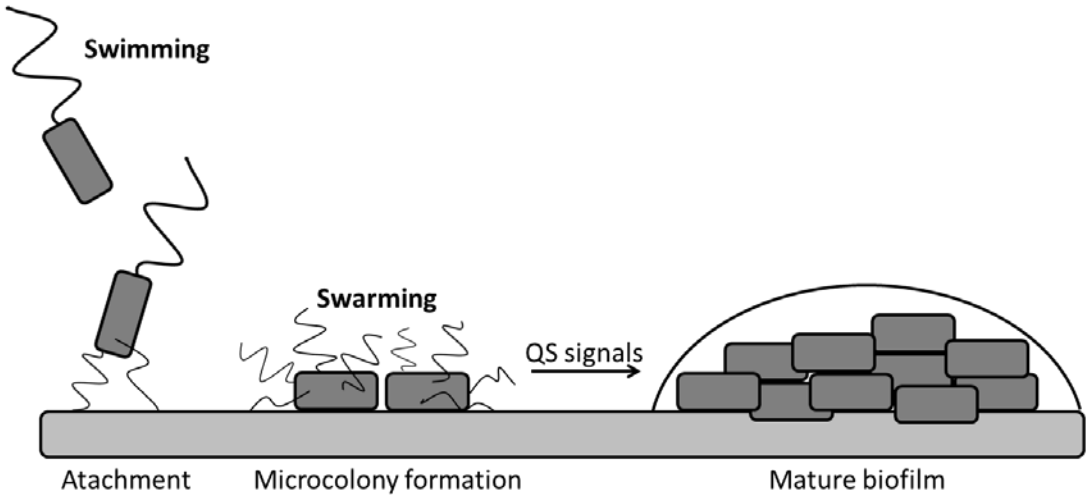

Fig. 3. Major steps leading to biofilm formation by *Vibrio spp*.

The ability of adhesion to external and mucosal surfaces of the host is another essential factor ensuring successful attachment and retention of vibrios on biotic surfaces. The major type of molecular structures involved in adhesion are pili, fibre-like assemblies composed of many subunits of a major structural protein (pilin) packed tightly into a helical array (Proft and Baker, 2008). It is thought that the molecules that are located on the tip of pili can vary depending on the surface they need to be attached (Yildiz and Visick, 2009). Moreover, chitinases and chitin-binding proteins may also play a role in adhesion, but more studies are needed to clarify whether expression of chitinase genes not only facilitates the use of chitin as a carbon source but is also needed for cell attachment.

After entering their hosts, pathogens secrete different types of lytic enzymes. These enzymes cause damage to the host tissues, thereby enabling the pathogen to obtain access to nutrients. While being able to lyse erythrocytes, haemolysins are among the major exotoxins identified in the core group of the lytic enzymes

contributing to the virulence of *V. harveyi* towards both shrimp and fish (Liu *et al.*, 1996; Sun *et al.*, 2007).

Moreover, some pathogenic vibrios such as *V. harveyi* VIB 645 strain (Zhang *et al.*, 2001) possess more than one closely related haemolysin genes associated with the virulence. While being able to digest a wide range of host proteins, proteases constitute the second important group of lytic enzymes. Ruwandeepica *et al.* (2011) observed a peak in the expression of the *srp* serine protease and *vhp* metalloprotease genes in pathogenic vibrios during infection of brine shrimp larvae, whereas the expression level of the same genes remained low in a non-virulent state. Liu and Lee (1999) also reported a cysteine protease as the major exotoxin lethal to the tiger prawn *Penaeus monodon*. Moreover, other proteases able to digest casein, collagen, fibropectin and gelatin have also been reported to contribute to infection and disease development (Teo *et al.*, 2003).

Another important class of lytic enzymes includes lipases. They break long chain triacylglycerols into fatty acids and glycerol and are believed to potentially cause damage to host tissues (Teo *et al.*, 2003), but still very little is known about their actual impact during infection and disease development. As mentioned above, *V. harveyi* can produce up to 10 chitinases. In addition to their potential role in adhesion, the presence of chitinases presumably helps pathogens to invade their hosts and survive in the host environment. While penetrating tissues that contain chitin, the pathogens can receive nutrients directly in the form of amino acids and/or indirectly by exposing other tissues to enzymatic degradation (Gooday, 1990). Analysis of the *V. harveyi* strain BAA-1116 has shown that it carries eight genes that are present on both chromosomes and encode different chitinases (Grimes *et al.*, 2009). Some of these chitinases are always present, while production and secretion of others is dependent on the presence of particular chitins in the environment (Svitil *et al.*, 1997).

As soon as the bacteria enter their hosts, the environment changes and the availability of nutrients become different from that of seawater, and therefore they must adjust their metabolisms and gene expression programs to be able to obtain various nutrients and ions necessary for their survival. The acquisition of some metal ions such as iron is not always easy during infection of host cells where it does not exist in a free form and is usually bound to a number of high-affinity iron-binding proteins

(Neilands, 1981). Due to its essential metabolic functions and involvement in numerous biological processes including intermediary metabolism, secondary metabolism and host cell interactions, iron is indispensable for bacteria. Therefore, to sustain its physiological function inside the host, *V. harveyi* has developed a highly efficient iron acquisition system enabling to scavenge iron from the host environment under iron-restricted conditions. Iron acquisition is linked to the ability of *V. harveyi* and other bacteria to synthesize ferric chelators (siderophores), small high-affinity compounds able to sequester iron from the cell environment and transport it by binding to specific outer membrane receptors. Furthermore, vibrios can also transport iron bound by exogenous siderophores secreted by other bacteria (Cornelis and Andrews, 2010). It has been demonstrated that the capacity to sequester iron is an important characteristic of pathogenic Vibrio species during their infection of vertebrates, but not invertebrates (Owens *et al.*, 1996; Skaar, 2010). This difference is possibly due to the lack of competition for iron between invertebrate hosts and bacteria, and therefore iron acquisition can be more efficient. Apart from facing limitation of nutrients and iron, vibrios also need to adapt to the acidic environment inside of the host's digestive system. Until recently, however, there have been no studies elucidating the molecular mechanism used by *V. harveyi* to deal with acidic environments.

To elicit infection, pathogenic bacteria use specific transport systems to secrete virulence factors outside of the cells or display them on the bacterial cell surface where they can exert their biological functions. Type III secretion system (TTSS) is the best-known one. Previous work has demonstrated that the genes encoding TTSS components are generally clustered in pathogenicity islands or on plasmids (Winstanley and Hart, 2001). The TTSS is well conserved in Gram-negative bacteria including *V. harveyi* (Henke and Bassler, 2004b) and is believed to be widely used by them to secrete and translocate virulence factors to the cytosol of eukaryotic cells (Lee, 1997).

2. 2. 3 Regulation of virulence gene expression and transfer

As production of virulence factors is metabolically costly and is needed only during infection and disease development, *V. harveyi* has different ways to tightly control their expression. One of them is mediated by ToxR, a transcriptional regulator essential in coordinating the expression of several virulence factors in *V. cholerae* (Miller *et al*., 1987) and *V. harveyi* (Pang *et al*., 2006).

In addition to the ToxR-mediated control, the *V. harveyi* QS is now known to also regulate the expression of different virulence factors (Natrah *et al*., 2011) including metalloproteases, siderophores, type III secretion system, chitinases, three phospholipases (Ruwandeepika *et al*. 2012) and flagellar proteins (Yang and Defoirdt, 2015). Despite considerable progress in understanding of both mechanisms, their actual regulation (activation) by host factors (such as noradrenaline (Nakano *et al*., 2007) or bile salts (Gotoh *et al*., 2010; Hsieh *et al*., 2003) able to influence *Vibrio spp.* growth and cytotoxicity or induce expression of virulence factors, respectively) is still poorly understood.

Horizontal gene transfer is a common mechanism used by many pathogenic species to exchange virulence genes with non-pathogenic species (Jackson *et al*., 2011). Besides conjugation and natural transformation (Arber, 2014), exchange of virulence genes can be mediated by bacteriophage transduction. During this process, phages are able to transfer virulence factors between bacteria by integrating their nucleic acid into either the host chromosome or in a plasmid (Howard-Varona *et al*., 2017), thus transforming non-pathogenic strains into virulent ones. Oakey and Owens (2000) have recently described a *Vibrio harveyi* mucovirus-like (VHML) bacteriophage isolated from a moribund prawn larva. Three years later, Munro and colleagues (Munro et al., 2003) demonstrated the ability of this phage to transform a non-pathogenic *V. harveyi* strain into virulent one by increasing its haemolysin activity, protein secretion and capacity to cause mortality in infected *Penaeus monodon* larvae. More recently, Khemayan *et al*. (2012) described another phage, the *Vibrio harveyi* Siphophage (VHS1), able to infect *V. harveyi* and enhance its virulence. This phage has been found to be associated with hemocyte agglutination and mortality of the giant tiger shrimp but not the white-leg shrimp. Interestingly, sequence comparison of both

phages revealed no significant sequence similarity at the nucleic acid or amino acid levels (Khemayan *et al.*, 2012).

2. 3. *V. harveyi* adaptation in natural aquatic systems

Living organisms normally possess a large arsenal of adaptation strategies to survive and successfully strive in changing environments. As mentioned above, due to the versatility of their metabolism and ability to resist many stress factors, *Vibrio* species are ubiquitously present in aquatic systems. To a large degree, such adaptability is possible due to genomic plasticity facilitated by horizontal gene transfer and elevated mutation rates (Johnson, 2013).

Nevertheless, a number of biotic and abiotic environmental factors also influence the distribution and dynamics of vibrio populations in their habitats, and temperature seems to be one of the most important ones. During the entire year, the presence of *Vibrio species* can be detected in various aquatic ecosystems, even though it is not always possible to culture them, especially when they originate from low-temperature environments (Cavallo and Stabili, 2004). One of the strategies to adapt to low temperature consists in acquisition of the viable but nonculturable phenotype (VBNC), a state of dormancy characterized by the prolong inability of the stressed cells to resume growth under standard laboratory conditions, while most of them still retain some basal physiological activities and cell integrity (Cavallo and Stabili, 2004). However, when the adverse conditions are eliminated, VBNC bacteria are able to recover their normal phenotype and pathogenic properties (Sun *et al.*, 2008). Many *Vibrio* species including *V. harveyi* seem to enter into the VBNC state in response to different natural stresses such as low temperature, limitation of nutrients, low salinity or ultra violet (UV) radiation (Ramaiah *et al.*, 2002). Prolong incubation of bacterial cultures at 4°C is widely used to stimulate the entry of *V. harveyi* into the VBNC state under laboratory conditions (Sun *et al.*, 2008). Consistent with the above observations, it has been demonstrated in many studies that the concentration of *V. harveyi* free-living cells in natural aquatic systems is strongly influenced by temperature and have a pattern of seasonality (O'Brien and Sizemore, 1979; Zhou *et al.*, 2012b). These studies revealed that the presence of *V. harveyi* in sediment was relatively constant during the

cold and warm seasons, but the number of free-living cells increases at higher temperatures. In fact, an increase in seawater temperature induces *V. harveyi* growth (Chimetto Tonon *et al.*, 2015), especially at temperatures exceeding 25^{o}C (Chimetto *et al.*, 2008) and the doubling time can be as short as 15 minutes. Apart from temperature, other parameters (e.g. salinity and dissolved oxygen) also affect the presence of this bacterium in nature (Cavallo and Stabili, 2004).

As it has been mentioned in previous section, vibrios demonstrate a notable pleomorphism in liquid media. There are several studies where changes in morphology (i.e. size reduction and shape alterations) due to starvation were reported (Holmquist and Kjelleberg, 1993; Stretton *et al.*, 1997; Chen *et al.*, 2009). Visible morphological changes are often observed in old cultures under adverse growth conditions including suboptimal salt (NaCl) concentrations or limitation of nutrients (i.e. starvation). These changes are manifested by a decrease in the diameter and volume of cells, often changing the morphological appearance of bacteria from rods to cocci (Farmer and Janda, 2006). The latter can often be found in natural aquatic systems in the form of ultra-small cells belonging to various species and are also referred to as "spherical ultramicrocells" or "round bodies" (Torrella and Morita, 1981). Likewise, there are some studies that refer to experiments performed with *Vibrio* species whose tiny cells could easily pass through 0.2 micron pores but were still able to recover normal size after culturing in rich media (Felter *et al.*, 1969; Novitsky and Morita, 1976; MacDonell and Hood, 1982). It is believed now that the reduction of size in response to the action of various stress factors represents one of the survival strategies, as it provides the cell with a bigger surface-to-volume ratio facilitating the nutrient uptake (McDougald and Kjelleberg, 2006), potentially minimizing energy needs (present study) and helping them to escape from predation (Pernthaler, 2005).

The large variability of organic compounds that *V. harveyi* can use as carbon and energy sources is believed to greatly contribute to the survival of vibrios when they compete with other microorganisms in their natural ecosystems (Ramesh *et al.*, 1989). At least in part, the survival of *V. harveyi* is reinforced by secretion of various hydrolytic enzymes (amylases, gelatinases, lipases and chitinases) involved in assimilation of biopolymers (e.g. proteins, polysaccharides, chitin etc.) naturally present in seawater. Previous work has shown that *V. harveyi* secretes at least ten

different chitinases, presumably utilized to hydrolyze various forms of chitin found in nature (Svitil *et al.*, 1997), thus contributing to the nutrient supply of the natural aquatic systems with a pool of metabolizable amino sugars. However, this process is partly inhibited by osmotic stress, reducing the yield and variety of organic carbon produced by vibrios and thus affecting the composition of ecosystems (Odic *et al.*, 2007).

As it has been mentioned above, *V. harveyi* is able to produce luminescence. Although bioluminescence could be *per se* a burden for bacteria due to an increase in energy consumption, it can also play some important symbiotic functions (Guerrero-Ferreira and Nishiguchi, 2007) or reduce the damaging effect of solar radiation. It has been recently reported that *V. harveyi* cells could reduce DNA damage by ionizing radiation (Czyz *et al.*, 2000) by channelling some of the reactive species generated upon exposure to UV light to bioluminescent pathways, thus repairing DNA by a photoreactivation process (Czyz *et al.*, 2000). Moreover, the GTP-binding protein CgtA appears to plays an important role in this process by stimulating *recA* gene expression, thus activating the RecA-dependent DNA repair pathways (Zielke *et al.*, 2003).

Finally, one of strategies used by many microbial species to increase the likelihood of survival under adverse environmental conditions, include their interactions with other organisms or biotic surfaces able to diminish (partly or completely) the negative effects of environmental stress factors and provide more nutrients. The ability of bacteria to adhere to biotic / abiotic surfaces and form biofilms (Fig. 3) increases their persistence and survival, resistance to disinfectants, predators and antibiotics (Kuranasagar *et al.*, 1994). Formation of mature biofilms requires extracellular matrix components that hold the cells together and keep the biofilm attached to the surface through extracellular polysaccharides (EPS) surrounding the cell. Analysis of their composition in the *V. harveyi* strain VB23 revealed that EPS are mainly composed of neutral sugars, proteins, uronic acids and methylated pentoses. The sugar moieties are primarily represented by hexoses (mostly by galactose and glucose), although residues of deoxyhexoses (rhamnose and fucose) as well as pentoses (ribose, arabinose, xylose and mannose) can also be found in some acidic heteropolysaccharides (Bramhachari and Dubey, 2006).

2.4. *Vibrio harveyi* resistance to antibiotics, antibacterial agents and new approaches to control diseases

To increase their chances of survival in the presence of antibacterial substances (antibiotics) secreted by other microbial species (e.g. fungi), environmental bacteria are able to acquire antibiotic resistance. Many antibiotic resistance genes are located on conjugative plasmids or other mobile genetic elements (transposons and bacteriophages) that can easily be transferred between species to quickly spread genetic information throughout the entire population. In 1993, Harris (1993) reported a conjugative plasmid (R-factor) found in a pathogenic *V. harveyi* strain causing diseases in *P. monodon* shrimp. The plasmid carries multiple genes that confer resistance to erythromycin, streptomycin, kanamycin, sulphafurazole and cotrimoxazole.

Apart from their sensitivity to classical antibiotics of fungal origin and their synthetic analogues, many bacteria including vibrios are sensitive to bacteriocins, a group of toxins produced by bacteria. Bacteriocins exhibit an antimicrobial mode of action against sensitive and usually closely related bacterial species. A bacteriocin-like toxin (BLIS), which is produced by some *Vibrio harveyi* strains, has also been reported (McCall and Sizemore, 1979). This toxin (also known as harveyicin) confers a competitive advantage to bacteriocin-producing strains over closely related, i.e. non-bacteriocinogenic strains present in the same environment (Hoyt and Sizemore, 1982). The production of harveyicin-like toxin was recently reported in *Beneckea harveyi* SY strain (*V. harveyi* SY) and was lethal to two other strains of *V. harveyi* (KN96 and BBP8). More recently, Prasad *et al.* (2005) described a novel BLIS produced by *V. harveyi* strain VIB 571 and demonstrated its ability to confer inter-strain (i.e against four isolates of the same species) and inter-species (i.e. against *Vibrio fischeri*, *Vibrio gazogenes* and *Vibrio parahaemolyticus*) antibacterial action.

In addition to expression of genes conferring antibiotic resistance, the capacity of *V. harveyi* to form biofilms provides them with an enhanced antibiotic resistance. A previous study of Kuranasagar *et al.* (1996) demonstrated that the formation of a biofilm on surfaces such as high density polyethylene plastics caused resistance to sanitizers.

The ability of pathogenic bacteria to acquire antibiotics resistance can create serious problems in seafood industry. It has been reported recently (Romero *et al.*, 2012) that the extended misuse of antibiotics to prevent spreading of diseases in many shrimp hatcheries has resulted in appearance of a large number of multidrug resistant bacteria. For instance, Kuranasagar *et al.* (1994) reported a highly virulent multidrug resistant strain of *V. harveyi* isolated from a larval tank hatchery that showed resistance to cotrimoxazole, chloramphenicol, streptomycin as well as the vibriostatic agent 0/129. The appearance and spread of multidrugresistant species is alarming and indicate that new strategies are needed to prevent and eradicate *V. harveyi* infections in aquaculture farms. For example, a physical removal of biofilm on surfaces and periodic drying of tanks would be a good way to reduce the chance of infection. On the other hand, as many pathogens rely on cell-to-cell communication mechanisms to control infection and survival in the host, probably the most efficient and promising strategy for disease control is quorum quenching (QQ) or quorum sensing disruption. Such approaches hamper bacterial infection by interfering with microbial cell-to-cell communication. As QQ mechanisms naturally occur in many prokaryotic and eukaryotic organisms, their exploration could help to develop a strategy for the generation of new antimicrobials (Dong *et al.*, 2007). Manefield *et al.* (2000) described a QQ mechanism able to inhibit luminescence and virulence in the *P. monodon* pathogen *V. harveyi*. They showed that a halogenated furanone produced by the red algae *Delisea pulchra* interferes with AI-1 regulated gene expression. In another work, Nakayama *et al.* (2007) studied the effect of copper concentration on the virulence of pathogenic *V. harveyi* and demonstrated its influence on the repression of luminescence and toxin production, which could probably be due to disruption of QS in the presence of copper-containing compounds.

3. *Vibrio* species in the time of ongoing climate change (effects of environmental and anthropogenic factors)

While having a great impact on nature (e.g. deforestation, greenhouse gases emissions, extensive farming, industrial pollution *etc.*), human activities are driving many environmental changes (IPCC, 2014) influencing ecosystem stability, productivity and diversity (Hautier *et al.*, 2015).

Earth warming and associated climate changes are unequivocally evidenced from observations of increases in the global average air and ocean temperatures. These changes promote worldwide melting of snow and ice, thereby reducing the areas of mountain glaciers and ice caps, and consequently increasing sea level. As the Intergovernmental Panel on Climate Change (IPCC) concluded in their last Climate Change Synthesis Report, many of these environmental changes are unprecedented (IPCC, 2014). During the last 45 years, global surface temperature has risen at an average rate of approximately 0.17°C per decade, i.e. more than twice compared to the rate (0.07°C per decade) observed during the entire period of recorded observations since the late nineteenth century to the year 2016, and global average temperatures have risen by nearly 0.95°C since 1880 (NOAA, 2017). In the Global Climate Report presented in May 2017 by the National Oceanic and Atmospheric Administration (NOAA) of the United States of America, it was reported that year 2016 was the second warmest year to date, and the forecast is warning, as this trend has continued into the first five months of year 2017.

It has been estimated that about 90% of the excess heat accumulated on the Earth since the 1960s has been stored in the oceans (Roemmich *et al.*, 2012). Indeed, one of the direct effects of the global warming is the rise of ocean temperature. Consistent with this trend, the regional seas in Europe have manifested an accelerated warming, with the Baltic, North, and Black Seas showing the greatest increase in temperature over the last 25 years (Reid *et al.*, 2011). At a global scale, the ocean warming is most profound near the surface, namely in the upper 75 m, where the average temperature has increased at a rate of over 0.1°C per decade between 1971 and 2010 (IPCC, 2014). Besides its natural inter-annual (seasonal) variability, the sea

surface temperature (SST) is also dependent on global climate patterns such as the North Atlantic Oscillation (NAO) and El Niño Southern Oscillation (ENSO) (Kirov and Georgieva, 2001).

One of the main risks of the global warming (GW) could be wider spread of disease outbreaks caused by some pathogenic or opportunistic infections (Harvell *et al.*, 2002), not only negatively affecting marine ecosystems, but also having impact on society and affecting human health in all continents across the world. Indeed, increases in SST were found to lead to expansion of microbial habitats and a concomitant spread of diseases caused by pathogenic species in marine organisms (Harvell *et al.*, 2002), in turn affecting human health through contaminated water and seafood (Newton *et al.*, 2012). As the cases of cholera in endemic countries are strongly dependent on the quality of water, more *V. cholerae* outbreaks are known to occur when water is warmer and rich in zooplankton (Lipp *et al.*, 2002). Furthermore, there is also a geographical expansion of *Vibrio*-related diseases to previously non-endemic areas, especially to temperate and cold regions of northern latitudes due to the ongoing reduction of salinity and warming (Vezzulli *et al.*, 2013). Baker-Austin *et al.* (2013) recently described the occurrence of waterborne *Vibrio* infections within Baltic Sea areas during three extremely warm summers, and they envisaged three factors likely contributing to the outbreaks of these diseases at elevated temperatures (>19°C): (i) an increase in the growth rate and number of pathogenic species, (ii) the temperature-induced upregulation of pathogenic competence of some *Vibrio* species, and (iii) an increase in water recreational activities.

In addition to humans, many ecologically and/or economically important marine organisms from temperate oceans, such as corals, seagrasses, oysters, sea urchins and fish (Harvell *et al.*, 1999) have been found to be influenced by large-scale epidemics as well. These changes potentially have negative impacts on many commercial activities such as production of seafood. The infection and mortality of many industrially important marine organisms caused by *V. harveyi* (e.g. various mollusc and crustaceous) are known to be increased at elevated temperatures (Fukui *et al.*, 2010; Travers *et al.*, 2009, Zhou *et al.*, 2012a). Moreover, recent studies revealed that abnormally warm environment also affects corals by making them more susceptible to *V. harveyi* infections (Luna *et al.*, 2010; Krediet *et al.*, 2013) and

therefore has a great impact on marine ecosystems. These findings were unexpected, as *V. harveyi* is not a common pathogen of corals and is normally involved in a symbiotic-like relationship with them possibly due to its ability to fix nitrogen (Chimetto *et al.*, 2008), thereby helping corals to maintain their normal growth rate at moderate temperatures. Thus, it seems likely that elevated temperatures can apparently stimulate V. *harveyi* conversion to an opportunistic pathogen and even help this marine bacterium to outcompete other species present in the coral mucus (Dinsale *et al.*, 2008).

The above findings point to a link between emerging diseases caused by *Vibrio* species and ongoing increase of SST or climatic events such as ENSO and NAO (Bruno *et al.*, 2007; Marcogliese, 2008). Another aspect of the GW is that the ice sheets of Greenland and Antarctic have been losing mass constantly over the last decades, likely at a larger rate between 2002 and 2011. These events along with the continuous shrinkage of glaciers worldwide (IPCC, 2014) and changes in the pattern of rainfalls and their intensity (likewise induced by GW) reduce salinity in estuaries and coastal wetlands (Hakkinen, 2002). Owing to its influence on *V. harveyi*'s growth, salinity has been shown to play an important role in the mortality of larval shrimps (Alavandi *et al.*, 2006). In their recent work, Subramanian and Rosamma (2013) studied *V. harveyi* virulence towards *Fenneropenaeus indicus* (Indian prawn) and concluded that *V. harveyi* showed higher growth rate and virulence with an increase in salinity from 0.5 to 3.5%. In contrast, a study presented by Prayitno and Latchford (1995) reported that low salinity during the rainy seasons (1.0 – 1.5%) can lead to an increase in *V. harveyi* virulence. These conflicting reports can likely be reconciled, given that salinity affects not only virulence but also the susceptibility of the host cells to infection that can be different *per se* for various organisms.

The pH of ocean is another parameter affected by human activities in the industrial era. The concentration of CO_2 readily absorbed by seawater has been increasing since the beginning of the industrial era, thus resulting in acidification of the ocean. The pH of ocean surface water has decreased by 0.1, corresponding to a 26% increase in acidity compared to preindustrial levels (Doney *et al.*, 2009). Moreover, oxygen concentration in the open ocean decreased by 3-5 µmol/kg per decade due to

increasing sea surface temperatures and expansion of anaerobic areas along the coastal zones (IPCC, 2014).

Given the gradual acidification of the world ocean, some studies examined the effect of pH on pathogenic properties of *V. harveyi.* While using a wide range of pH (i.e. 5.5 to 9.0) and testing its impact on virulence of this pathogen toward penaeid prawn larvae, Prayitno and Latchford (1995) have demonstrated that low pH, in particular pH 5.5, have a clear negative effect on the ability of this bacterium to elicit infection.

Even though pH does not seems to be one of the most crucial factors directly affecting *V. harveyi* presence in aquatic systems, ocean acidification can profoundly affect other members of the marine life, thereby indirectly influencing *Vibrio* spp. abundance and survival. Namely, previous work has shown that pH can differentially affect the phytoplankton community and does it to a greater degree than warming or nutrient availability (Dutkiewicz *et al*., 2015). It has been reported recently that a higher abundance of phytoplankton biomass normally leads to an increase in the number of vibrios, provided that other parameters such as temperature and salinity remain unchanged (Asplund *et al*., 2011). Moreover, acidification has negative effect on shell-building organisms such as oysters or mussels (Gazeau *et al*., 2013), apparently making them weaker and more susceptible to Vibrio-associated infections (Dorfmeier, 2012).

Solar radiation seems to be another factor that can potentially affect the microbial composition and variety in aquatic systems. Its intensity and damaging effects are gradually increasing due to ongoing deterioration of the ozone layer by toxic compounds of industrial or agricultural origin (Rowland, 2006). The solar radiation can arbitrarily be subdivided into three bands comprising different ranges of wavelength: infrared radiation (> 700 nm), visible radiation (400 - 700 nm) and ultraviolet (UV) radiation (< 400 nm), respectively. The harmful effect of UV radiation on microbial populations has extensively been documented, thus leading to the wide use of UV radiation for disinfection and sterilization (Meulemans, 1987). In the ocean, UV light can barely pass through the first meter of water and the depth of penetration is affected by water turbidity (i.e. the presence of some particulate and natural organic matter). Although previous studies have shown that members of the

Gammaproteobacteria are highly resistant to solar radiation (Alonso-Sáez *et al*., 2006) and pigmentation *per se* does not provide any significant advantage for survival (Agogué *et al*., 2005), this area of research is still in its infancy and more work has to be done to fully understand the actual effect of solar radiation on presence, virulence and diversity of *Vibrio* spp. in the time of GW.

Despite some evidence linking global warming and disease outbreaks, very few studies examined this trend over long timescales. One of the most comprehensive studies assessing a possible linkage between the occurrence of *Vibrio*-associated diseases and SST over a long-term scale has recently been carried out by Vezzulli *et al*. (2012). They employed molecular and pyrosequencing analysis of the microbial community present in formalin-fixed samples obtained from the historical archive of the Continuous Plankton Recorder (CPR) survey, one of the longest and most comprehensive collection covering the abundance and distribution of marine organisms in the world (http://www.sahfos.ac.uk) over long periods of time. The analysis was grounded on the fact that plankton represents a nutrient-rich reservoir for *Vibrio* species (Turner *et al*., 2009) and therefore vibrios should be found attached to it or present in the surrounding water, especially during the warmer months. Thus, it seemed likely that the CPR system should capture a substantial fraction of these bacteria and subsequently yield a long-term record for vibrio presence in aquatic systems. While examining a large number of samples, the authors succeeded to provide real evidence that vibrios increased their presence within the plankton-associated bacterial community of the North Sea over 44 years and the observed increase nicely correlated with the climate-dependent sea surface warming during the same period of time. Moreover, the outcome of this analysis was also supported by the results of previous work (Huq *et al*., 1984) showing that an increase in temperature not only have a positive effect on the growth rate of Vibrio species but also enhances their capacity to attach to and multiply on plankton. In spite of numerous reports, the biocomplexity of interactions between vibrios and their natural environment in the context of climate change is still poorly understood. In particular, little is known about other events triggered by the reorganization of global climate and able to change the availability of organic matter and iron, important factors controlling bacterial

variability and richness, that could dramatically change the composition and biological characteristics of marine ecosystems (Hayes *et al.*, 2001).

To clarify these factors more studies are needed to dissect this complexity in a systematic manner and define environmental and ecological factors affecting *Vibrio spp.* abundance and dynamics in a long-term and spatial scale. In other words, more work needs to be done to better understand the whole spectrum of conditions enabling vibrios to survive and proliferate in their natural ecosystems. These studies should help to reveal new major determinants of vibrio adaptation and survival and therefore will help to develop advanced measures to predict, prevent, and control Vibrio-related diseases. In this respect, the European Vibrio network (http://www.vibrionet.de/index.html) created recently represents an excellent platform to attract scientific and public attention to the recent spread of *Vibrio spp.* infections in marine and inland aquatic ecosystems and to foster discussion regarding their potential impact on society (Le Roux *et al.*, 2015).

Objectives

Objectives

Despite a number of studies reporting the impacts of environmental factors (e.g. temperature, salinity, pH and solar radiation) on microbial growth and persistence in natural aquatic systems (see **General Introduction**), the actual contribution of these factors to the long-term survival capacity and adaptation of many marine bacteria including numerous *Vibrio* species are still poorly characterized, in particular at the molecular level.

The **main objective** of this study was to use *Vibrio harveyi* as a model organism and learn more about responses and putative mechanisms this marine bacterium uses to survive in a free-living state and cope with limitation of nutrients in its natural habitats. Towards this goal, the **specific aims** of the experimental work were focused on:

- Study of survival, physiological responses and morphological changes of *V. harveyi* during its short- and long- term incubation in seawater microcosms.

- Analysis of *V. harveyi* adaptation at the whole transcriptome level and identification of the key metabolic pathways and cellular factors playing the major roles in the adaptation process.

- Comparison of adaptation mechanisms, physiological and phenotypic changes triggered in *Vibrio harveyi* during its incubation in seawater microcosms at optimal and suboptimal temperatures.

Chapter 1

Chapter 1

Reprogramming of *Vibrio harveyi* gene expression during adaptation in cold seawater

Introduction

Marine bacteria including those of the genus *Vibrio* can act as primary or opportunistic pathogens affecting a large number of sea organisms that serve as a source of seafood world-wide. Recent studies have revealed that Vibriosis (Egidius, 1987), a typical disease caused by *Vibrio* species in shrimps and in other industrially important sea organisms (lobster, oysters, *etc.*), is gradually becoming one of the major concerns during the ongoing climate changes (Vezzulli *et al.*, 2010). Some types of Vibriosis are caused by *Vibrio harveyi* (Martin *et al.*, 2004; Haldar *et al.*, 2010; Soto-Rodriguez *et al.*, 2010; Zhou *et al.*, 2012), a facultative anaerobic Gram-negative bacterium globally present in marine environments.

Many marine bacteria are highly adaptive and can survive and strive in natural aquatic systems under severe stress conditions including seasonal temperature downshifts and starvation. Moreover, regular trapping of marine microorganisms by ocean / sea currents (both cold and warm) as well as their subsequent release in new locations can also subject microorganisms to sudden environmental changes (including temperature shifts and metabolic stresses) specific for their new habitats. Adaptation of Gram-negative bacteria to low temperature and starvation has been studied previously by using different model microorganisms including *Vibrio* species (Carroll *et al.*, 2001; Yang *et al.*, 2009; Wood & Arias, 2011). Although these studies revealed a number of strategies developed by phylogenetically distant bacteria to resist cold temperatures and limitation of nutrients, they also demonstrated that the putative adaptation mechanisms as well as the genes controlling these mechanisms only partly coincide and can often vary from species to species.

The lack of systematic data related to survival and growth of *V. harveyi* at low temperatures under limitation of nutrients and the potential value of such data for understanding the ecology of *Vibrio* species led to the present study aimed at

investigating adaptation of *V. harveyi* to cold seawater. In the course of this study, we examined the dynamic of *V. harveyi* adaptation by monitoring the number of total and viable cells and their morphology using fluorescent and scanning electron microscopy as well as standard microbiological techniques. In addition, we compared *V. harveyi* cells at the transcriptome level before and after exposure to stress. Microarray analysis revealed a group of highly up- and downregulated genes controlling several major metabolic pathways and membrane functions. We discuss the putative roles of these genes in sustaining the *V. harveyi* capacity to survive and maintain cellular functions in natural aquatic systems.

Materials & Methods

Vibrio harveyi survival assays and viability tests

Vibrio harveyi strain ATCC 14126 was aerobically grown at 28ºC in marine broth (MB, Panreac). Stationary-phase *V. harveyi* populations were subjected to starvation and nonoptimal temperature by diluting (1:20) overnight *V. harveyi* cultures with cold (4ºC) natural seawater (from Port of Armintza in the North of Spain, 43º 26' 24" N and 2º 54' 24" W). The experiments were carried out in Erlenmeyer flasks cleaned with H_2SO_4 (97%, v/v) beforehand, rinsed with deionized water, and heated at 250°C for 24 h to avoid any presence of residual organic substances. Large Erlenmeyer flasks containing 2 L filtered and subsequently autoclaved seawater were inoculated and incubated at 4ºC with shaking (90 rpm). Periodically, samples were collected in triplicate to determine the total number of cells, the numbers of viable and culturable bacteria. The total number of bacteria (TNC) was determined by filtering bacterial cell populations through 0.22 µm pore-size polycarbonate membrane filters (Millipore), followed by staining of the attached cells with acridine orange and direct counting individual cells using epifluorescence microscopy (Hobbie *et al.*, 1977). Viable bacteria were estimated as bacteria with intact cytoplasmic membranes (MEMB+). These MEMB+ bacteria were counted with the aid of the Live/Deads BacLight™ kit (Invitrogen) as described by Joux *et al.* (1997). The bacteria with intact cytoplasmic membranes (green fluorescence, MEMB+) and the permeabilized bacteria (red fluorescence) were enumerated separately. Culturability, expressed as colony forming units (CFU), was evaluated by the spreading aliquots on marine agar (MA, Oxoid) followed by incubation for 24 h at 28°C.

The size measurements of unstressed and stressed cells were performed via image analysis of epifluorescence preparations (Massana *et al.,* 1997) by using an image analysis system, which included a video camera of high resolution (Hamamatsu 2400, Hamamatsu Photonics, Hamamatsu City, Japan). Digitized images of microscopic fields were analyzed by Scion Image 1.62ª software (for further details, see Table. S5).

Scanning electron microscopy

Samples unstressed (overnight culture) and stressed (after 12 h incubation in cold seawater) of *V. harveyi* cells were fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The suspensions of the fixed cells were filtered through 0.22 µm-pore size membrane filters (GTTP filters, Millipore). The filters with the attached *V. harveyi* cells were further dehydrated by applying increasing series of ethanol (30, 50, 70, 90 and 100%). The filters with the attached dehydrated *V. harveyi* cells were overlaid with 1 ml of hexamethyldisilazane, incubated for 5 min and air dried. Finally, the samples were coated with gold, and imaging was carried out by analyzing samples in a Hitachi S4800 scanning electron microscope.

RNA isolation and microarray analysis

V. harveyi ATCC 141126 cells were grown in triplicate in marine broth at 28^{o}C until the culture reached the stationary phase (12-16 h) and aliquots (20 ml) were diluted by sterile 4^{o}C seawater (1:20) and further incubated at 4°C with shaking (90 rpm). Aliquots of stressed cell cultures withdrawn after 5 min and 12 h of incubation in triplicate were mixed with stop solution (5% phenol in ethanol) at ratio 8:1, incubated on ice for 15-20 min and the cells were collected by centrifugation (15 min, 4°C, 4400 g). The pelleted *V. harveyi* cells were used to isolate total RNA by using Trizol reagent and PureRNA mini Kit (Invitrogen) according to the vendor's instructions. Further analysis of RNA samples was carried out at the General Genomic Service (SGiker) of the University of the Basque Country as previously described (Rutherford *et al.*, 2011). Briefly, after verifying RNA quality and integrity by Lab-chip technology on an Agilent 2100 Bioanalyzer with Agilent RNA 6000 Nano Chips, RNA has been retrotranscribed with Superscript III Reverse Transcriptase (Invitrogen) and labelled using the SuperScript Indirect cDNA labeling System (Invitrogen) to incorporate aminomodified nucleotides. After a purification step to remove unincorporated nucleotides, the aminomodified cDNA was coupled to fluorescent dyes (Cy5 or Cy3) and used for hybridization with custom microarray (Rutherford *et al.*, 2011) produced by Agilent Technologies according to the design kindly provided by the lab of Prof. Bonnie Bassler (Princeton University). Raw data from Feature Extraction Software (FE processed

signals) were subsequently processed on GeneSpring MultiOmic Analysis Software 12.0 (Agilent Technologies) and were subjected to further statistical analysis.

Validation of microarray data by quantitative real-time PCR

Total *V. harveyi* RNA was isolated from three independent cell cultures according to the procedure described in the previous section. Further analysis of RNA samples has been carried out at the General Genomic Service (SGiker) of the University of the Basque Country, Spain. Briefly, after verifying RNA quality and integrity by Lab-chip technology on an Agilent 2100 Bioanalyzer with Agilent RNA 6000 Nano Chips, RNA was used for cDNA synthesis using AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies). Aliquots (1 µg each) of each RNA sample were individually reverse-transcribed according to the vendor's instructions. The reaction mixtures containing cDNAs were diluted and used for quantitative PCR amplification in 7900HT Fast Real-Time PCR System (Applied Biosystems) to determine the relative changes in the level of 18 transcripts and the reference transcript (16S rRNA) used for normalization. The gene-specific primers used for RT-qPCR are listed in supplemental Table S3. The software to control the amplification process and obtain the quantitative PCR data was SDS 2.4 (Applied Biosystems). The Ct values obtained in this step were further used to calculate the relative expression (for details, see Table S4).

Results

The morphology and viability of *V. harveyi* is not affected during 12 h incubation in cold seawater

Incubation in cold seawater can have potentially adverse effects on morphology and survival of bacteria. We first compared the morphological appearance of unstressed and stressed *V. harveyi* cells by using fluorescent and scanning electron microscopy (Fig. 1). Aliquots of *V. harveyi* overnight cultures were diluted (1:20) by sterile cold sea water from the Bay of Biscay and the diluted cultures were further incubated for 12 h at 4^{o}C. Samples of *V. harveyi* cells taken before and after incubation in seawater were stained using acridine orange (Hobbie *et al.*, 1977) and were further examined by epifluorescent microscopy. The results of this examination (Fig. 1A) demonstrated that during their incubation in cold seawater up to 12 hours, *V. harveyi* cells did not show any significant changes in their morphology and size, when compared to the cells from the original overnight culture. Moreover, the results of three independent experiments obtained by analyzing the size of 150-210 cells (in each experiment) corroborate the initial observation that 12 h of incubation in cold sea water do not change the morphology and size of *V. harveyi* cells (see Table S5). The lack of phenotypical changes was also supported by images obtained using scanning electron microscopy. The shape and the integrity of unstressed (control overnight culture) and stressed (incubated for 12 h in cold seawater) cells were very similar (Fig. 1B).

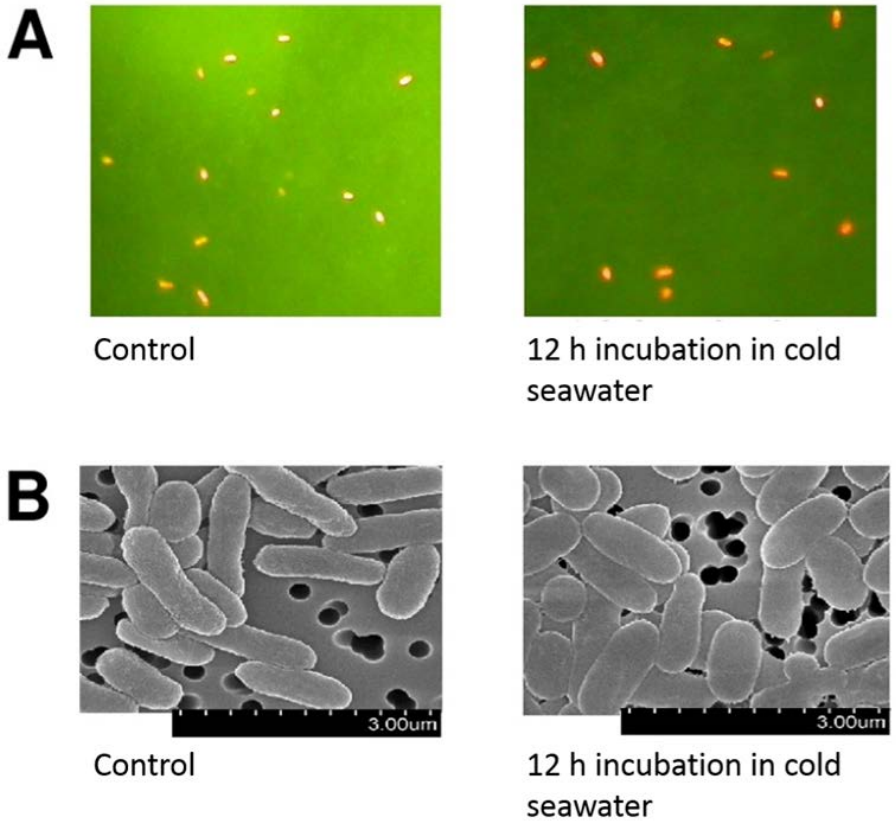

Fig. 1. Analysis of morphology and integrity of *Vibrio harveyi* cells exposed to cold seawater. Cells taken before (control) and after (12 hours) incubation in cold seawater were examined by epifluorescence microscopy (panel A) or fixed on 0.22 µm pore-size polycarbonate membrane and analyzed by scanning electron microscopy (panel B) as described in Materials & Methods.

In addition, enumeration of the cells present in aliquots taken after 0.5, 1, 2, 3, 4, 5, 6 and 12 hours of *V. harveyi* incubation in cold seawater by fluorescent microscopy revealed that the total number of *V. harveyi* cells remained essentially the same (Fig. 2). Likewise, the number of cells, which maintained the integrity of cytoplasmic membrane and retain capacity to form colonies on marine agar, remained unchanged.

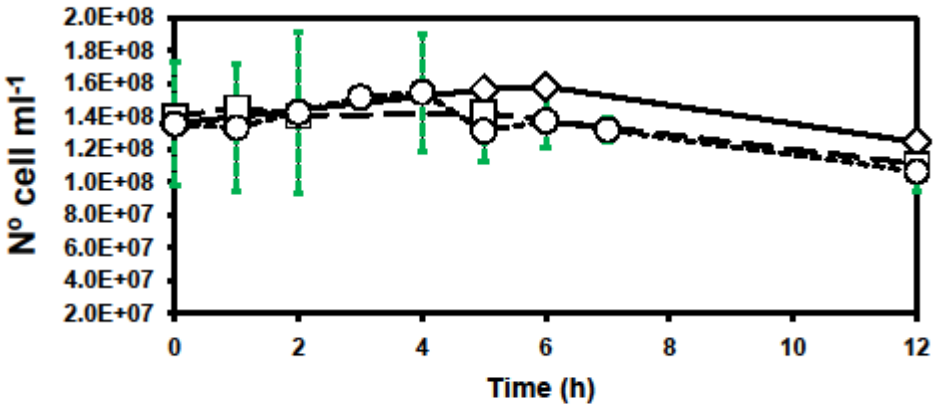

Fig. 2. ***Vibrio harveyi*** **counts obtained during adaptation to cold seawater.** The total number of cells **(◊)** and the number of viable cells **(□)** were counted via epifluorescence microscopy. The number of culturable bacteria **(○)** was determined by the spread plate method on Marine Agar. The experiment has been repeated three times and the average numbers are presented. The data are mean values from three independent experiments with errors bars (highlighted in green) representing the standard deviations calculated for culturable bacteria (○).

The standard deviations calculated for total number of cells (◊) and for number of viable cells (□) were smaller and are omitted for simplicity.

Gene expression analysis

To learn more about specific adaptation mechanisms triggered by *Vibrio harveyi* in order to survive during seasonal changes in temperature and nutrient availability, we compared *V. harveyi* transcriptome profiles after short and long exposure to cold sea water. *V. harveyi* cells were grown in Marine Broth at 28°C overnight, subsequently diluted with sterile cold seawater (1:20) and further incubated at 4°C. Total RNAs for microarray analysis was isolated after 5 min (control cells) and 12 hours (stressed cells) incubation in cold seawater. Comparison of these two gene expression points by hybridizing isolated total *V. harveyi* RNAs with a custom microarray revealed profound changes that occur at the transcriptome level. A large number of *V. harveyi* transcripts have showed 2 fold (or higher) changes in abundance (see Table S1 (downregulated genes) and Table S2 (upregulated genes)).

While analyzing these data, we selected a group of genes that are highly up- or downregulated (see Table 1) and therefore, likely play the major role in *V. harveyi*

responses to cold seawater. Apart from several stress-related genes (e.g. genes involved in stringent response) as well as numerous genes coding for the components of the protein-synthesizing (e.g. genes encoding ribosomal proteins and RNA-modifying enzymes), many other selected genes encodes transporters, various enzymes involved in biosynthesis and transport of amino acids, nucleotides, lipids and other essential biomolecules as well as enzymes that function in the central carbon metabolism (e.g. glycolysis, TCA cycle). Further analysis of their possible contribution to *V. harveyi* capacity to survive in and adapt to cold seawater (see Discussion), suggests that the observed changes in the *V. harveyi* transcriptome likely affect all major cellular functions including metabolism, transport, energy production and gene expression processes.

Table 1. *V. harveyi* genes highly up- or downregulated during adaptation to cold seawater. Annotation of the genes and their product is according to the classification used in the KEGG database (http://www.genome.jp/). Up- or downregulation of genes is indicated with upward (↑) or downward (↓) arrows, respectively.

General category	Specific biological pathway	Highly up- and downregulated genes		
		Gene product	Locus tag (systematic name)	Fold change
Amino acid metabolism	**Alanine, Aspartate & Glutamate metabolism**	L-aspartate oxidase	VIBHAR_03543	16.85 ↑
		Aspartate ammonia-lyase	VIBHAR_00154 (*aspA*)	14.06 ↓
		Glutamine synthetase	VIBHAR_00588 (*glnA*)	8.79 ↓
	Lysine biosynthesis	2,3,4,5-tetrahydropyridine-2,6-carboxylate N-succinyltransferase	VIBHAR_03309	15.74 ↓
		Dihydrodipicolinate synthase	VIBHAR_03187	9.01 ↓
	Cystein metabolism	Transcriptional regulator CysB	VIBHAR_01793 (cysB)	20.66 ↑
	Glycine, serine and threonine metabolism	Glycine dehydrogenase	VIBHAR_05973	11.15 ↓
	Valine, Leucine & Isoleucine biosynthesis	DNA-binding transcriptional regulator IlvY (LysR family transcriptional regulator, positive regulator for ilvC)	VIBHAR_00477	25.15 ↑
	Arginine & proline biosynthesis	Glutamine synthetase	VIBHAR_00588 (*glnA*)	8.79 ↓
	Histidine biosynthesis	ATP phosphoribosyltransferase	VIBHAR_01830 (*hisG*)	16.08 ↑
		Bifunctional phosphoribosyl-AMP cyclohydrolase / phosphoribosyl-ATP pyrophosphatase	VIBHAR_01837	10.13 ↑
		1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase	VIBHAR_01835	18.22 ↑
		Imidazole glycerol phosphate synthase subunit HisF	VIBHAR_01836 (*hisF*)	16.47 ↑
		Imidazole glycerol phosphate synthase subunit HisH	VIBHAR_01834 (*hisH*)	19.23 ↑
		Imidazole glycerol-phosphate dehydratase / histidinol phosphatase	VIBHAR_01833	20.24 ↑
		Histidinol-phosphate aminotransferase	VIBHAR_01832	17.12 ↑
		Histidinol dehydrogenase	VIBHAR_01831 (*hisD*)	22.61 ↑
	Purine metabolism	Adenosine deaminase	VIBHAR_06421	96.23 ↑
		Guanylate kinase	VIBHAR_00630 (*gmk*)	29.26 ↑
		Xanthine-guanine phosphoribosyltransferase	VIBHAR_01159	20.54 ↑
		Putative diguanylate ciclase, PleD	VIBHAR_05729	13.41 ↓

		Purine nucleoside phosphorylase	VIBHAR_03374	9.46 ↓
		Nucleoside triphosphate pyrophosphohydrolase	VIBHAR_03527 (*mazG*)	19.37 ↓
		Bifunctional UDP-sugar hydrolase/5'-nucleotidase periplasmic	VIBHAR_01256 (*ush*A)	17.41↓
		Anaerobic ribonucleoside triphosphate reductase	VIBHAR_05713	8.16↓
		Bifunctional 2',3'-cyclic nucleotide 2'-phosphodiesterase/3'-nucleotidase periplasmic protein	VIBHAR_00764 (*cpdB*)	8.90 ↓
		Ribonucleotide-diphosphate reductase subunit alpha	VIBHAR_02733 (*nrdA*)	7.89 ↓
		Ribonucleotide-diphosphate reductase subunit beta	VIBHAR_02734 (*nrdB*)	10.55 ↓
	Pyrimidine metabolism	Dihydroorotase	VIBHAR_06413	63.08 ↑
		Putative MFS transporter, AGZA family, xanthine/uracil permease	VIBHAR_01158	310,6 ↑
		Aspartate carbamoyltransferase regulatory subunit	VIBHAR_03648	16.72 ↓
		Deoxycytidylate deaminase	VIBHAR_05385	7.89 ↓
		Aspartate carbamoyltransferase catalytic subunit	VIBHAR_03647 (*pyrB*)	22.92 ↓
		Uridine phosphorylase	VIBHAR_01516	14.88 ↓
		Nucleoside triphosphate pyrophosphohydrolase	VIBHAR_03527 (*mazG*)	19.37 ↓
		Bifunctional UDP-sugar hydrolase/5'-nucleotidase periplasmic	VIBHAR_01256 (*ush*A)	17.41 ↓
		Anaerobic ribonucleoside triphosphate reductase	VIBHAR_05713	8.16 ↓
		Bifunctional 2',3'-cyclic nucleotide 2'-phosphodiesterase/3'-nucleotidase periplasmic protein	VIBHAR_00764 (*cpdB*)	8.90 ↓
		Ribonucleotide-diphosphate reductase subunit alpha	VIBHAR_02733 (*nrdA*)	7.89 ↓
		Ribonucleotide-diphosphate reductase subunit beta	VIBHAR_02734 (*nrdB*)	10.55 ↓
Central carbon metabolism	**Glycolysis/ Gluconeo-genesis**	6-phosphofructokinase	VIBHAR_00145	12.17 ↓
		Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	VIBHAR_03049	14.78 ↓
		Fructose-1-phosphate phosphatase YqaB	VIBHAR_05389	10.68 ↓
	Pentose phosphate pathway	Transaldolase B	VIBHAR_06256	9.34 ↓
	Galactose metabolism	Alpha/beta hydrolase	VIBHAR_01858	7.08 ↓
	Citrate Cycle	Type II citrate synthase	VIBHAR_01350	9.65 ↓
	Pyruvate metabolism	Pyruvate-formate lyase	VIBHAR_01546	7.25 ↓
	Starch & sucrose metabolism	Periplasmic alpha-amylase	VIBHAR_04858 (*malS*)	7.25 ↑
	Transport of Maltodextrins	Acetyltransferase, maltose O-acetyltransferase	VIBHAR_06688	11.63 ↓

	Glyoxylate & dicarboxylate metabolism	Formyltetrahydrofolate deformylase	VIBHAR_01373 (*purU*)	8.55 ↓
		Acetoacetyl-CoA reductase	VIBHAR_05365	11.73 ↓
		Phosphoglycolate phosphatase	VIBHAR_05409	16,59 ↓
		Glycine cleavage system protein H	VIBHAR_05972	9,18 ↓
Lipid biogenesis	**Lipoprotein biosynthesis**	Apolipoprotein N-acyltransferase	VIBHAR_01226 (*lnt*)	21.27 ↑
	Lipopolysaccharide biosynthesis	ADP-L-glycero-D-manno-heptose-6-epimerase, GmhD	VIBHAR_00682 (*rfaD*)	6.97 ↓
	Fatty acid biosynthesis	3-oxoacyl-ACP synthase, FabB	VIBHAR_03105	15.71 ↓
	Glycerophospholipid metabolism	Phosphatidate cytidylyltransferase	VIBHAR_03232	12.92 ↑
		Glycerophosphoryl diester phosphodiesterase	VIBHAR_06395	14.82 ↓
		Phospholipid phosphatase	VIBHAR_00821	24.06 ↓
Processing of genetic information	**tRNA biogenesis**	tRNA guanosine-2'-O-methyltransferase	VIBHAR_00626	23.21 ↑
		7-cyano-7-deazaguanine reductase (biosynthesis of queuosine)	VIBHAR_01193 (*queF*)	10.55 ↓
		RNA binding protein with homology to *Vibrio sp.* EJY3 tRNA uridine 5-carboxymethylamino-methyl modification enzyme	VIBHAR_00255	11.92 ↓
	Ribosome biogenesis	O-methyltransferase	VIBHAR_00267	83.58 ↑
		Ribosomal-protein-alanine acetyltransferase	VIBHAR_05581	25,77 ↑
		50S ribosomal protein L11 methyltransferase	VIBHAR_00173 (*prmA)*	12.84 ↓
		30S ribosomal protein S10	VIBHAR_00729 (*rpsJ*)	10.18 ↑
Transporters and ancillary factors	**ABC transporters**	Amino acid ABC transporter ATP-binding protein	VIBHAR_05950	16.86 ↑
		ABC-type vitamin B12-transporter protein BtuF	VIBHAR_00921 (*btuF*)	6.08 ↑
		Efflux ABC transporter ATP-binding/permease (high homology to *V. parahaemolyticus* BB22OP LolC)	VIBHAR_07028	37.62 ↑
		ABC amino acid transporter periplasmic component	VIBHAR_00441	7.26 ↓
		Sugar ABC transporter periplasmic protein	VIBHAR_06396	8.45 ↓
		Phosphate ABC transporter permease	VIBHAR_05138	10.72 ↓
	Energy-dependent transport TonB-ExbB-ExbD complex	Biopolymer transport protein ExbB	VIBHAR_00636 VIBHAR_00635	9.83 ↑
		Biopolymer transport protein ExbD	VIBHAR_00634	9.12 ↑
		Periplasmic protein TonB	VIBHAR_00633	8.86 ↑
	Outer membrane protein assembly	Outer membrane protein assembly factor YaeT / Omp85 /BamA	VIBHAR_03229	10.06↑
	Iron uptake, storage and utilization	Bacterioferritin-associated ferredoxin (see Mey *et al.*, 2005)	VIBHAR_00053	10.06 ↑
		Iron transport protein	VIBHAR_00638	12.86 ↑
		Co-chaperone HscB (Maturation of iron-sulfur cluster-containing proteins)	VIBHAR_01058	11.43 ↑
		Ferredoxin	VIBHAR_02274	14.31 ↓
		Cysteine desulfurase	VIBHAR_01055	10.98 ↑

Stress responses	**Stringent response**	Stringent starvation protein A	VIBHAR_00886	10.05↑
		Bifunctional (p)ppGpp synthetase II / guanosine-3',5'-bis pyrophos-phate 3'-pyrophosphohydrolase	VIBHAR_00627	11.52↑
	Oxidative stress	DNA-binding transcriptional regulator OxyR	VIBHAR_00035	10.61↑
	Detoxification of reactive electrophilic compounds	Glutathione S-transferase	VIBHAR_05193	10.01 ↓
	Cold-shock response	Ribosome-associated protein Y, putative sigma-54 modulation protein (Cold-shock protein inhibiting translation)	VIBHAR_03665	6.00 ↓
	DNA repair	LexA repressor	VIBHAR_00268	16.69 ↑
		Excinuclease ABC subunit C	VIBHAR_02747	15.63 ↑
		Exodeoxyribonuclease VII, small subunit	VIBHAR_01175	10.34 ↓
Protein turnover and folding	**Protein turnover**	Serine proteinase	VIBHAR_05576	90,01 ↑
		ClpXP protease specificity-enhancing factor	VIBHAR_00887	13.52 ↑
	Protein chaperones induced by heat-shock	Heat shock protein, molecular chaperone IbpA	VIBHAR_00446	11.99 ↓
		Chaperonin GroEL	VIBHAR_00142	10.32 ↓
		Co-chaperonin GroES	VIBHAR_00143 (*groES*)	12.26 ↓
		Molecular chaperone DnaK	VIBHAR_01134	16.21 ↓
		ATP-dependent protease peptidase subunit, ATP-dependent HslUV protease, peptidase subunit HslV	VIBHAR_00724	11.15 ↓
Energy production	**Respiration**	Cytochrome c oxidase subunit II	VIBHAR_06272	8.17 ↓
	Oxidative phosphorrylation	Inorganic pyrophosphatase	VIBHAR_00784	8.39 ↓
Miscellaneous functions	**Porphyrin and Chlorophyll Metabolism**	Corrin/porphyrin methyltransferase	VIBHAR_00892	21.82 ↑
		Coproporphyrinogen III oxidase	VIBHAR_00581	5.90 ↓
		Uroporphyrinogen decarboxylase	VIBHAR_06129 (*hemE*)	11.62 ↓
	Nitrogen metabolism	Carbonic anhydrase	VIBHAR_06412	75.48 ↑
	Biotin metabolism	BirA family transcriptional regulator, biotin operon repressor / biotin-[acetyl-CoA-carboxylase] ligase	VIBHAR_00240	14.57 ↑
	Quorum sensing	Transcriptional regulator LuxR	VIBHAR_00157	16.85 ↓
	Multidrug resistance	Bicyclomycin/multidrug efflux system protein	VIBHAR_02654	25.75 ↑
	Antibiotic resistance	Multiple antibiotic transporter	VIBHAR_00158	46.32 ↑
	Control of Na+/H+ balance	Na+/H+ antiporter	VIBHAR_01828	8.10 ↑
	Antisense control by sRNAs	CsrB/RsmB RNA family	VIBHAR_00349	2.39↑
		Spot 42 RNA	VIBHAR_00573	44.47 ↑

Validation of microarray data by quantitative real-time PCR

To validate the microarray data, the alterations in the level of transcripts (18 in total), selected from the group of highly up- and downregulated genes that control the key biological pathways (Table 1), have also been assessed by quantitative real-time PCR (Table S4). In pilot experiments, we have determined that the level of 16S rRNA after 5 min (control cells) and 12 hours (stressed cells) incubation in cold seawater remained unaltered (data not shown), and therefore we used this rRNA as a reference to normalize qRT-PCR data. The analysis has been performed with RNA corresponding to three biological replicates using gene-specific primers (Table S3). As seen in Table 2, the result of the qRT-PCR analysis confirmed up- and downregulation of the above genes, thus corroborating microarray data.

Table 2. Validation of microarray data by quantitative real-time PCR (qRT-PCR). Total RNA was isolated in triplicate from *V. harveyi* cells after 5 min and 12 h incubation in cold seawater and the differences in the level of individual transcripts determined by qRT-PCR and microarray analysis are presented for highly up- and downregulated transcripts.

Locus tag (systematic name)	**Gene product**	**Regulation** (12 h *versus* 5 min)	
		qRT-PCR	Microarray
VIBHAR_01158	Putative MFS transporter, AGZA family, xanthine / uracil permease	up	up
VIBHAR_07028	Efflux ABC transporter ATP-binding / permease	up	up
VIBHAR_00268	LexA repressor	up	up
VIBHAR_03105	3-oxoacyl-ACP synthase, FabB	down	down
VIBHAR_01134	Molecular chaperone DnaK	down	down
VIBHAR_01833 (*hisB*)	Imidazole glycerol-phosphate dehydratase / histidinol phosphatase	up	up
VIBHAR_00729 (*rpsJ*)	30S ribosomal protein S10	up	up
VIBHAR_06412	Carbonic anhydrase	up	up
VIBHAR_00573	Spot42 (sRNA)	up	up
VIBHAR_00588 (*glnA*)	Glutamine synthetase	down	down
VIBHAR_00630	Guanylate kinase	up	up
VIBHAR_00627	Bifunctional (p)ppGpp synthetase II / guanosine-3',5'-bis pyrophosphate 3'-pyrophosphohydrolase	up	up
VIBHAR_01159	Xanthine-guanine phosphoribosyltransferase	up	up
VIBHAR_03309	2,3,4,5-tetrahydropyridine-2,6-carboxylate N-succinyltransferase	down	down
VIBHAR_04858 (*malS*)	Periplasmic alpha-amylase	up	up
VIBHAR_03229	Outer membrane protein assembly factor YaeT / Omp85 /BamA	up	up
VIBHAR_00633	Periplasmic protein TonB	up	up
VIBHAR_00638	Iron transport protein	up	up

Discussion

Although sea microorganisms possess a tremendous potential to adapt and adequately respond to environmental changes, which they face in natural aquatic systems, the specific mechanisms involved in their adaptation are still poorly understood. This is particularly true for some *Vibrio* pathogens including *V. harveyi*, an emerging pathogen infecting a large number of sea organisms (e.g. shrimps) important for seafood industry. The survival of *Vibrio* species in natural aquatic systems can be challenged by numerous environmental factors (e.g. temperature, composition of seawater as well as presence of symbiotic or predator organisms) potentially limiting their growth and survival (Johnson, 2013).

Here we investigated adaptation of *V. harveyi* in cold seawater by monitoring phenotypical and gene expression changes. The experiments were performed on stationary-phase bacteria in order to study responses of non-dividing bacteria to temperature and nutritional stress. We found that the overall shape, size and integrity of *V. harveyi* cells have not been affected upon 12 h of incubation in sterile seawater. Moreover, the number of viable and culturable cells remained the same during the time course, which implies that *V. harveyi* likely elicits specific adaptation mechanisms maintaining its culturability under these stress conditions.

To learn more about the putative nature of these mechanisms we employed microarray analysis and assessed gene-specific variations in the level of individual transcripts. We found that incubation in cold seawater induces global changes in the *V. harveyi* gene expression program affecting a large number of transcripts (supplementary Table S1 and S2). The observed changes were further analyzed with a major focus on highly up- and downregulated genes (Table 1) and in the context of the action of two major stress factors (i.e. (i) limitation of nutrients / microelements and (ii) low temperature) affecting *V. harveyi* survival.

In our experimental system, the first factor is partly associated with adaptation to stress caused by a decrease in the concentration of amino acids (partly eliminated when *V. harveyi* cells were transferred from marine broth to cold seawater) and by limited availability of alternative carbon sources. We found that limitation of nutrients

leads to significant downregulation of genes controlling the central carbon metabolism, biosynthesis of lipids, amino acids and nucleotides (Table 1).

Several downregulated genes that control the central carbon metabolism encode the key enzymes of glycolysis, tricarboxylic acid cycle and glyoxylate / dicarboxylate metabolism. Moreover, the fine tuning of these metabolic pathways appears to be attained through the action of small regulatory RNAs known for their essential roles in post-transcriptional control of gene expression (Kaberdin and Bläsi, 2006). Among several sRNAs that have been upregulated in *V. harveyi* (namely, CsrB/RsmB; M1 RNA, an RNA component of RNase P, 6S / SsrS RNA, tmRNA and Spot 42 RNA, see Table S2), two sRNAs (CsrB / RsmB and Spot 42) are specifically known for their role in sugar metabolism. Previous studies of sugar metabolism in bacteria revealed that the first one (CsrB / RsmB) is involved in antagonizing the action of the global regulator CsrA able to positively (or negatively) control various biological pathways including quorum sensing, glycolysis, acetate metabolism, motility, gluconeogenesis, biofilm formation and others (Babitzke and Romeo, 2007; Timmermans and Van Melderen, 2010). Moreover, the role of CsrB in regulation of quorum sensing in *Vibrio cholerae* has been demonstrated recently (Lenz *et al.*, 2005). Thus, an increase in the abundance of this small RNA in *V. harveyi* is likely linked to a regulatory mechanism(s) serving to adjust one (or several) biological pathways affected by the stress conditions used. Even higher degree of upregulation (more than 40 fold) has been observed for Spot 42. This sRNA is highly conserved in bacteria including the *Vibrionaceae* family (Hansen *et al.*, 2012). Although the precise function of this sRNA in adaptation of *V. harveyi* to cold seawater remains to be determined, previous characterization of its mode of action in *E. coli* (Beisel and Storz, 2011) and the nature of the recently predicted targets of this sRNA in other bacteria such as *Aliivibrio salmonicida* (Hansen *et al.*, 2012) suggest that *V. harveyi* Spot 42 likely plays a critical role in regulating the central carbon metabolism.

Besides transcriptome changes affecting the central carbon metabolism, there were also significant alterations in the expression level of several genes known for their role in lipid biogenesis (Table 1). A considerable decrease in the level of the *fab* mRNA encoding the second enzyme of fatty acid biosynthesis (i.e. 3-oxoacyl-ACP synthase) suggests a reduction in *de novo* synthesis of lipids.

With few exceptions, significant downregulation was also detected for many genes involved in biosynthesis of amino acids. However, in contrast to genes involved in biosynthesis of other amino acids, expression of genes encoding several enzymes of histidine biosynthesis including those involved in purine metabolism was increased dramatically (Table 1). The anticipated reduction of protein synthesis caused by amino acid starvation and temporal arrest of *V. harveyi* growth is likely linked to co-regulation of other genes known to control protein functionality under normal conditions. Indeed, our microarray data (Table 1) point to a significant decrease in the level of transcripts encoding the main players of chaperone-mediated protein folding (IbpA, GroEL, GroES, and DnaK). This response is reminiscent of a cold-induced reduction of chaperone expression previously observed in *V. parahaemoliticus* (Yang *et al.*, 2009).

In addition, similar to regulation in other Gram-negative bacteria (Jin*, et al.*, 2012), amino acid starvation appeared to trigger the so-called stringent response. We anticipate the occurrence of this response due to the observed increase in the level of mRNA encoding the putative ppGpp synthase (see Table 1). The regulator molecule ppGpp and other factors such as CgtA and DksA have been previously shown to play important roles in *V. cholerae* adaptation to amino acid starvation and to other stress conditions (Pal *et al.*, 2011) and therefore might also have similar functions in *V. harveyi.* Moreover, we also observed upregulation of the *sspA* gene encoding the stringent starvation protein A (SspA). Previous studies in *Escherichia coli* revealed that this protein is important in *E. coli* stress responses during stationary phase and when cells face limitation of nutrients (Williams *et al.*, 1994). The *E. coli* SspA was found to function as transcription factor and at least in part it can functionally be substituted by SspA orthologs of other bacteria including the *V. cholerae* counterpart (Hansen *et al.*, 2005).

The anticipated reduction in metabolic activities and efficiency of gene expression implies that *V. harveyi* starvation in seawater should inevitably lead to a reduced consumption of energy. Consistently, we observed a considerable decrease in the abundance of transcripts encoding cytochrome *c* oxidase subunit II and inorganic pyrophosphatase, i.e. enzymes involved in energy production (see Table 1 and 4). In agreement with the downregulation of these genes, our microarray data also revealed a concomitant decrease in the expression level of transcripts encoding the enzymes

(namely, coproporphyrinogen III oxidase and uroporphyrinogen decarboxylase) involved in the biosynthesis of heme, an important co-factor of energy-generating enzymes during aerobic growth.

Reactions of the central carbon metabolism, amino acid and fatty acid biosynthesis as well as biosynthetic pathways of modified tetrapyrroles (e.g. heme and cobalamin) involve carbon dioxide (CO_2) and bicarbonate ion (HCO_3^-) endogenously produced in bacteria and required for cell growth under normal conditions. Downregulation of several genes (e.g. *hemE*) encoding CO_2-generating enzymes such as uroporphyrinogen, oxaloacetate and orotidine 5'-phosphate decarboxylases (see Table 1 and supplementary Table S1 and S2) likely leads to a decrease in carbon dioxide production. The anticipated reduction in CO_2 production is, in turn, consistent with upregulation of *birA* expression known to negatively control the biosynthesis of biotin, a co-factor of decarboxylases. Moreover, it is also correlated with a strong upregulation of the gene encoding carbon anhydrase, an enzyme facilitating rapid interconversion of carbon dioxide and water to bicarbonate, thus suggesting an important role for this enzyme in maintaining the proper CO_2 / HCO_3^- equilibrium in *V. harveyi* under stress (Merlin *et al.*, 2003).

Apart from numerous genes encoding metabolic enzymes and regulatory proteins whose expression was altered in response to starvation, significant up- and downregulation of many other genes is apparently linked to the function of *V. harveyi* cell envelope representing the front line of defence against environmental threats in bacteria. Some regulatory mechanisms apparently serve to adjust the composition and physical properties of the cellular envelope to preserve the maximal functionality of the outer and inner membranes in response to the drastic temperature downshift (cold shock). One of the major challenges that bacteria face under cold-shock conditions is a partial loss of the membrane fluidity, in turn impairing important membrane functions such as energy production, cell division and transport. In this regard, our finding that temperature downshift leads to the upregulation of genes involved in lipoprotein biogenesis (*lolC, int* (Table 1) and to a lesser degree of *lolA* and *lolB* (Table S1)) suggests that efficient production and incorporation of lipoproteins into the outer membrane may play a critical role in *V. harveyi* adaptation to low temperatures.

A somewhat different role can be envisaged for another ancillary protein, BamA (YaeT), encoded by the *V. harveyi yeaT* gene. An increase in expression of this gene (see Table 1) is likely critical to provide a high level of YeaT production. This protein is required for efficient insertion of several beta-barrel proteins (into the outer membrane of bacteria (Dautin and Bernstein, 2007) and therefore could be critical for *V. harveyi* cell envelope biogenesis under stress.

One of the important functions of the bacterial envelope is linked to transport of solutes and biomolecules. Control of nutrient / metal ions transporter expression plays a central role in regulation of bacterial growth and adaptation to changing environmental conditions. We found that several *V. harveyi* transporter-encoding genes were up- and downregulated by exposure to cold seawater. Microarray data suggest that, at least in part, *V. harveyi* attempts to compensate the reduced expression of biosynthetic genes by upregulation of transporter genes controlling the uptake of amino acids (e.g. amino acid ABC transporter ATP-binding protein) and nucleotides (e.g. putative xanthine / uracil permease).

In addition to the *lolC* gene (discussed above), some highly upregulated genes are involved in the control of polar amino acids and iron uptake. The latter encode the energy-dependent transport TonB-ExbB-ExbD complex, bacterioferritin-associated ferredoxin, iron transport protein and co-chaperone HscB. The upregulation of iron acquisition and downregulation of the iron storage genes known to be involved in iron homeostasis in other *Vibrio* species (e.g. *Vibrio cholerae*; Mey *et al.* (2005)) suggests that *V. harveyi* adjusts its metabolism to strive in the seawater under iron-limiting conditions.

Finally, a somewhat unexpected group of transcripts with a significant change in expression was found to be related to DNA stability and genetic information processing. Exposure of *V. harveyi* to cold sea water appears to affect the integrity of DNA and subsequently change the expression level of several DNA repair genes (e.g. *lexA*) listed in Table 1. We also found that, similar to genes regulated in the human pathogen *V. vulnificus* by cold shock (Wood & Arias, 2011), a large group of genes controlling protein synthesis, in particular several genes involved in ribosome and tRNA biogenesis (e.g. *rpsJ*), were likewise upregulated in *V. harveyi* (Table 1). Their upregulation appears to be redundant and, unlike downregulation of other genes

controlling ribosome and tRNA biogenesis (Table S1), contrasts with the reduced capacity of *V. harveyi* to carry out protein synthesis under starvation conditions. A possible explanation for this paradox is likely rooted in the partial loss of autoregulation usually exerted by the products of these genes (i.e. ribosomal proteins) by means of their binding to the 5' UTR regions of their cognate polycistronic RNAs in order to inhibit ribosome binding and subsequent translation of these transcripts (Babitzke *et al.*, 2009). Thus, it seems likely that low temperature (4^{o}C) prevents the correct folding of the corresponding 5' UTR, thereby preventing their autoregulation and subsequently leading to the excessive production (upregulation) of these transcripts under cold shock conditions. Based on these observations, we propose that, in addition to the increased propensity of RNA to form structures under cold shock conditions that potentially prevent the correct translation / transcription of bacterial transcripts (Phadtare *et al.*, 2000), bacterial adaptation to low temperature can also be impaired by misfolding of RNA structures (e.g. translational operators or riboswitches) that have essential regulatory functions *in vivo*.

In summary, although microarray analysis revealed several group of genes that were affected by starvation and cold shock in the same manner as they are regulated in some other *Vibrio* species (Carroll *et al.*, 2001; Yang *et al.*, 2009; Wood & Arias, 2011), it also disclosed a considerable number of new stress-related genes (e.g. genes encoding guanylate kinase and putative xanthine / uracil permease that are involved in nucleotide salvage pathways as well as periplasmic alpha-amylase, an enzyme involved in utilization of polysacharides) most likely important for adaptation *of V. harveyi* and other marine bacteria in natural aquatic systems.

Chapter 2

Chapter 2

Unveiling the metabolic pathways associated with the adaptive reduction of cell size during *Vibrio harveyi* persistence in seawater microcosms

Introduction

During their life cycle, marine bacteria including those of the genus *Vibrio* face diverse and continuously changing environments. Besides acting as primary or opportunistic pathogens and causing diseases (Egidius, 1987) that affect a large group of marine invertebrates (lobster, oysters *etc.*) essential for seafood industry, vibrios can also dwell in a free-living state. Their adaptation and survival in marine ecosystems was examined by using *V. cholerae* (reviewed in (Lutz *et al.*, 2013; Oliver, 2010; Colwell, 2000)) and other *Vibrio* species (Oliver, 2010; Kjelleberg *et al.*, 1993; Chen *et al.*, 2009; Chen *et al.*, 2014). These studies revealed that exposure to prolong starvation in seawater microcosms (i.e. under conditions largely mimicking their natural habitats) not only affects cell culturability but also leads to transformation of *Vibrio* cells from rods to cocci (Felter *et al.*, 1969; Novitsky and Morita, 1976; Baker *et al.*, 1983; Nilsson *et al.*, 1991; Stretton *et al.*, 1997; Vattakaven *et al.*, 2006; Zhong *et al.*, 2009) or even to minicells (Baker *et al.*, 1983; Chaiyanan *et al.*, 2007) largely resembling ultramicrobacterial forms prevailing in natural marine microbiota (Atlas and Bartha, 1997). Moreover, analysis of *Vibrio* responses to starvation suggested a link between the appearance of coccoid-like cells and their entry into the viable but not culturable (VBNC) state (reviewed in Oliver (2010)) occasionally activated in free or biofilm-associated cells. Although the cells that have acquired the VBNC state lose the ability to grow on standard laboratory media used for cultivation of marine bacteria (Zhong *et al.*, 2009; Oliver *et al.*, 1991; Roszak *et al.*, 1984; Xu *et al.*, 1982; Sun *et al.*, 2008), they are still alive and can recover by elimination of abiotic factors inducing nonculturability (Oliver, 2010) or upon the action of resuscitation factors (e.g. the quorum sensing autoinducer AI-2 (Ayrapetyan *et al.*, 2014; Bari *et al.*, 2013).

In addition to its effects on cell morphology, nutrient deprivation is known to alter the biochemical composition of the starved cells by decreasing the level of lipids, carbohydrates and proteins (Hood *et al.*, 1986). Moreover, some of these changes can potentially be in charge of the altered cell surface properties likewise observed under nutrient-limiting conditions and likely involved in biofilm formation under stress (Mizunoe *et al.*, 1999; Wai *et al.*, 1998).

Apart from disclosing morphological transformation of vibrios under starvation and defining alterations in their biochemical content, previous studies also identified a number of fitness factors with putative roles in adaptation of *Vibrio* species (reviewed in (Johnson, 2013)). Some starvation-related genes and protein factors were additionally revealed by analyzing adaptation changes at the transcriptome (Carroll *et al.*, 2001; Montánchez *et al.*, 2014; Wood and Arias, 2011; Yang *et al.*, 2009) and proteome (Nystrom *et al.*, 1988; Nystrom *et al.*, 1990; Nystrom *et al*, 1992; Kim *et al.*, 2012; Ostling *et al.*, 1997) levels. Despite significant progress, the specific roles of many starvation-dependent genes and their products (for instance, numerous membrane and periplasmic proteins (Nystrom *et al.*, 1988) with respect to their actual contribution to the regulation of *Vibrio* metabolism during starvation still remain largely uncertain and merit further analysis.

With the aim to learn more about specific metabolic pathways playing critical roles in survival of *Vibrio* species in natural aquatic systems, we studied adaptation of *V. harveyi,* a well-known pathogenic bacterium able to infect many industrially important sea organisms (Haldar *et al.*, 2010; Martin *et al.*, 2004; Soto-Rodriguez *et al.*, 2010; Zhou *et al.*, 2012). In the following, we present a time course analysis of *V. harveyi* adaptation in seawater microcosm at 20^{o}C and discuss phenotypical and gene expression changes that occur during the initial phase of the adaptation process. This analysis enabled to discern a number of strategies and adaptation mechanisms apparently employed by *V. harveyi* to sustain its key physiological functions and likely implicated in maintaining its capacity to strive in a free-living state in marine environments.

Materials and Methods

Vibrio harveyi survival assays and bacterial cell count

The survival tests were carried out with *Vibrio harveyi* strain ATCC 14126 incubated in natural sterile seawater collected from the Port of Armintza in the North of Spain, 43° 26' 24" N and 2° 54' 24" W). The experiments were performed in triplicate in large Erlenmeyer flasks beforehand cleaned with H_2SO_4 (97%, v/v), rinsed with deionized water, and heated at 250°C for 24 h to avoid any presence of residual organic substances. For each experiment, *V. harveyi* was aerobically grown at 26°C (optimal for *V. harveyi* growth) in marine broth (MB, Panreac), a standard laboratory medium resembling natural seawater and additionally containing yeast extract and bacteriological peptone to provide nitrogen, minerals, vitamins and amino acids essential for the balanced growth of marine bacteria under laboratory conditions. *V. harveyi* cells in the stationary phase of growth were further used to inoculate two liters of filtered and subsequently autoclaved natural seawater to get a final density of 10^8 cells ml^{-1}. The Erlenmeyer flasks were further incubated in the dark with shaking (90 rpm) at 20°C. Periodically, samples were collected in triplicate to determine the number of total, viable and culturable bacteria.

The total bacterial count (TBC) was determined by filtering aliquots (100-200 μl) of bacterial cell populations (diluted 1:100) through 0.22 μm pore-size polycarbonate membrane filters (Millipore), followed by staining of the filter-bound cells with acridine orange and direct counting individual cells using epifluorescence microscopy (Hobbie *et al.*, 1977). Viable bacteria, estimated as bacteria with intact cytoplasmic membranes (MEMB+), were counted with the aid of the Live/Dead BacLight™ kit (Invitrogen) as described by Joux *et al.* (1997). The bacteria with intact cytoplasmic membranes (green fluorescence) and the permeabilized bacteria (red fluorescence) were enumerated separately. Culturability, expressed as colony-forming units, was evaluated by spreading aliquots on Marine Agar (MA, Oxoid) followed by incubation for 24 h at 26°C. To estimate the size of the cells present in the control and test samples we employed epifluorescence microscopy. The measurements of bacterial size were performed *via* image analysis of epifluorescence preparations as described by Massana *et al.* (1997) using samples stained with acridine orange and collected on

0.22 μm pore-size black filters (Millipore) as described above. The image analysis system included a high-resolution video camera (Hamamatsu C2400; Hamamatsu Photonics). Images of very flat fields with enough bacteria and lacking very bright particles were selected to be digitized and analyzed by Scion Image 1.62ª software. For each sample, 150-200 bacteria were measured and, according to their length, the cells fell into four groups: ≤ 0.75 μm, >0.75-1.5 μm, >1.5-2.5 μm, > 2.5 μm.

Scanning electron microscopy

V. harveyi cells present in the control and test samples were fixed with 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The suspensions of the fixed cells were filtered through 0.22 μm pore-size membrane filters (GTTP filters, Millipore). The cells on the filters were further examined by scanning electron microscopy at the Advance Research Core Facility Unit (SGIker) of the University of the Basque Country. Briefly, the filters with the attached *V. harveyi* cells were further dehydrated by applying increasing series of ethanol (30, 50, 70, 90 and 100%). The filters with the attached dehydrated *V. harveyi* cells were overlaid with 1 ml of hexamethyldisilazane, incubated for 5 min and air dried. Finally, the samples were coated with gold, and imaging was carried out by analyzing samples in a Hitachi S4800 scanning electron microscope.

RNA isolation and microarray analysis

V. harveyi ATCC 14126 cells were grown in triplicate in marine broth at 26^{o}C until the culture reached the stationary phase (12-16 h) and aliquots (20 ml) were diluted by sterile room temperature seawater (1:20) and further incubated with shaking (90 rpm) at 20°C roughly representing the average summer temperature of the coastal water in the Bay of Biscay. Aliquots of cell cultures withdrawn in triplicate after 5 min, 12 hours and 6 day of incubation were mixed with stop solution (5% phenol in ethanol) at ratio 8:1, incubated on ice for 15-20 min and the cells were collected by centrifugation (15 min, 4^{o}C, 4400 g). The pelleted *V. harveyi* cells were used to isolate total RNA by using

Trizol reagent and PureRNA mini Kit (Invitrogen) according to the vendor's instructions. Further analysis of RNA samples was carried out at the General Genomic Service (SGIker) of the University of the Basque Country as previously described (Montánchez *et al.*, 2014). Briefly, after verifying RNA quality and integrity by Lab-chip technology on an Agilent 2100 Bioanalyzer with Agilent RNA 6000 Nano Chips, RNA has been retrotranscribed with Superscript III Reverse Transcriptase (Invitrogen) and labelled using the SuperScript Indirect cDNA labeling System (Invitrogen) to incorporate aminomodified nucleotides. After a purification step to remove unincorporated nucleotides, the aminomodified cDNA was coupled to fluorescent dyes (Cy5 or Cy3) and used for hybridization with custom microarray previously described in (Rutherford *et al.*, 2011) and produced by Agilent Technologies according to the custom design kindly provided by the lab of Prof. Bonnie Bassler (Princeton University). Raw data from Feature Extraction Software (FE processed signals) were subsequently processed on GeneSpring MultiOmic Analysis Software 12.0 (Agilent Technologies) and were further subjected to LIMMA statistical analysis and k-means clustering by using MultiExperiment Viewer application (version 4.7.1) available at http://www.tm4.org/mev.html (Saeed *et al.*, 2003; Saeed *et al.*, 2006). During this procedure, the p-value threshold was set to 0.05 to select significant genes for further clustering according to K-means algorithm (Hartigan and Wong, 1979).

Validation of microarray data by quantitative real-time PCR

Total RNA was isolated from *V. harveyi* cells in triplicate according to the protocol we used to prepare RNA for microarray analysis (see above). Further analysis of RNA samples has been carried out at the General Genomic Service (SGIker) of the University of the Basque Country, Spain. Briefly, after verifying RNA quality and integrity by Lab-chip technology on an Agilent 2100 Bioanalyzer with Agilent RNA 6000 Nano Chips, RNA was used for cDNA synthesis using AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies). Aliquots (1μg each) of each RNA sample were individually transcribed according to the vendor's instructions. The reaction mixtures containing cDNAs were diluted and used for quantitative PCR amplification in 7900HT

Fast Real-Time PCR System (Applied Biosystems) to determine the relative changes in the level of 16 transcripts including two reference transcripts used for normalization. The gene-specific primers used for RT-qPCR are listed in supplemental Table S2. The software to control the amplification process and obtain the quantitative PCR data was SDS 2.4 (Applied Biosystems). The comparative Ct method (Livak and Schmittgen, 2001) was used to calculate fold change in expression level of individual transcripts. Further statistical analysis was performed by using the unpaired T-test.

Results

Effects of incubation of *V. harveyi* cells in seawater on their viability and morphology

Survival pattern of a *V. harveyi* population subjected to starvation in natural seawater at 20°C is presented in Fig. 1. During the experimental period (14 days), the total and viable bacteria counts remained essentially unchanged as well as the number of culturable bacteria determined on Marine Agar.

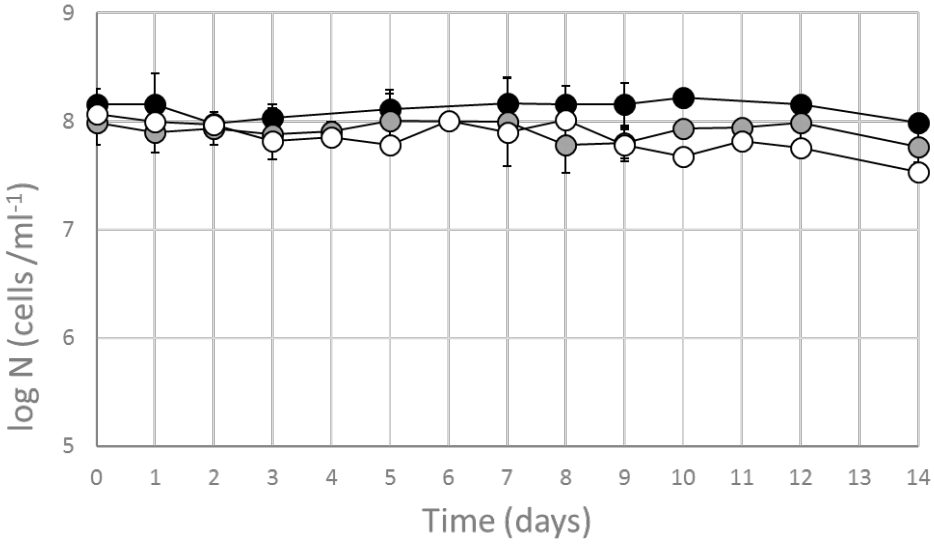

Fig. 1. *Vibrio harveyi* counts obtained during survival under starvation in seawater at 20°C. The number of total (●), viable (⊙) and culturable (○) bacteria were estimated as described in Materials & Methods. The data are mean values from three independent experiments with errors bars representing the standard deviations calculated.

During their incubation in seawater at 20°C, *V. harveyi* cells endured progressive changes affecting their morphology and size. When compared to the cells present in the control culture, the cells withdrawn after 6 and 14 days of incubation in seawater were apparently shorter. Indeed, further quantitative analysis confirmed this observation (Fig. 2C). We found that after two weeks of incubation in seawater about 10% of *V. harveyi* cells had the length shorter than 0.75 µm and possessed a nearly spherical shape, i.e. acquired the so-called coccoid-like morphology. Moreover, the fraction of cells with the size ranging from > 0.75 to 1.5 µm increased by 30%, whereas the percentages of longer cells decreased progressively. These morphological changes in greater detail were also seen on images obtained by scanning electron microscopy (Fig. 2B), thus pointing to the overall reduction of cell size during starvation.

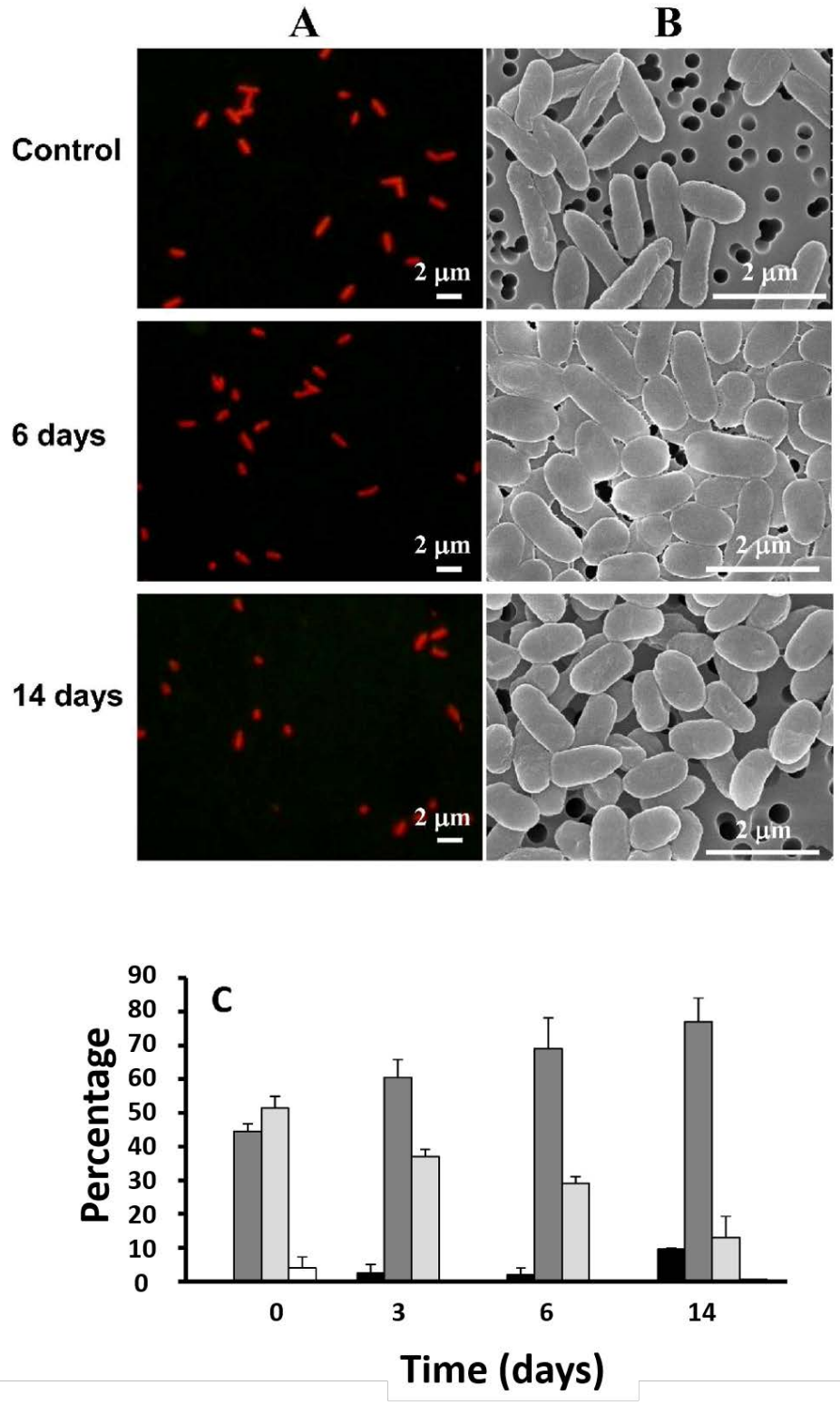

Fig. 2. Morphology, integrity and size distribution of *Vibrio harveyi* populations under starvation conditions in seawater at 20ºC. A, epifluorescence microscopy images of *V. harveyi* cells stained with acridine orange and analyzed as described in Materials & Methods; B, scanning electron microscopy images and C, size distribution of population examined by epifluorescence microscopy (percentage ± standard deviation). ■, ≤0.75 µm; ■, >0.75-1.5 µm; ■, >1.5-2.5 µm and □, >2.5 µm.

Gene expression analysis

To identify the genes that play the major role in maintaining *V. harveyi* viability in natural aquatic systems, we analyzed the effect(s) of short- and long-term incubation of *V. harveyi* cells in sterile seawater on their transcriptome profiles. Total RNA was isolated from the initial inoculate (overnight culture) and from *V. harveyi* cells incubated for 5 min, 12 hours and 6 days in seawater and then was labelled with Cy3 or Cy5 followed by hybridization to a custom microarray. Analysis of the gene expression patterns corresponding to each time point disclosed a considerable number of transcripts differentially affected by incubation in seawater.

Statistical analysis of gene expression data enabled to group up- and downregulated genes into 13 genes clusters (Fig. 3). We further used bioinformatics tools available in the STRING database (Franceschini *et al.*, 2013) to discover the key interaction networks containing nodes of highly up- and downregulated genes in each cluster and subsequently assigned them to the specific biological pathways as summarized in Table 1. The complete set of the affected genes grouped according to their biological role is presented in supplementary Table S1.

According to their putative contribution to different stages of *V. harveyi* adaptation, the genes listed in Table S1 arbitrary fall into three major categories. The first one includes genes whose expression is mainly affected during the early adaptation phase (cluster 5) and therefore these genes are likely involved in the first wave of adaptation changes that occurred immediately after *V. harveyi* cells were transferred to seawater. The genes in cluster 5 primarily control fatty acid degradation, envelope biogenesis, iron homeostasis and assimilation of sulphur.

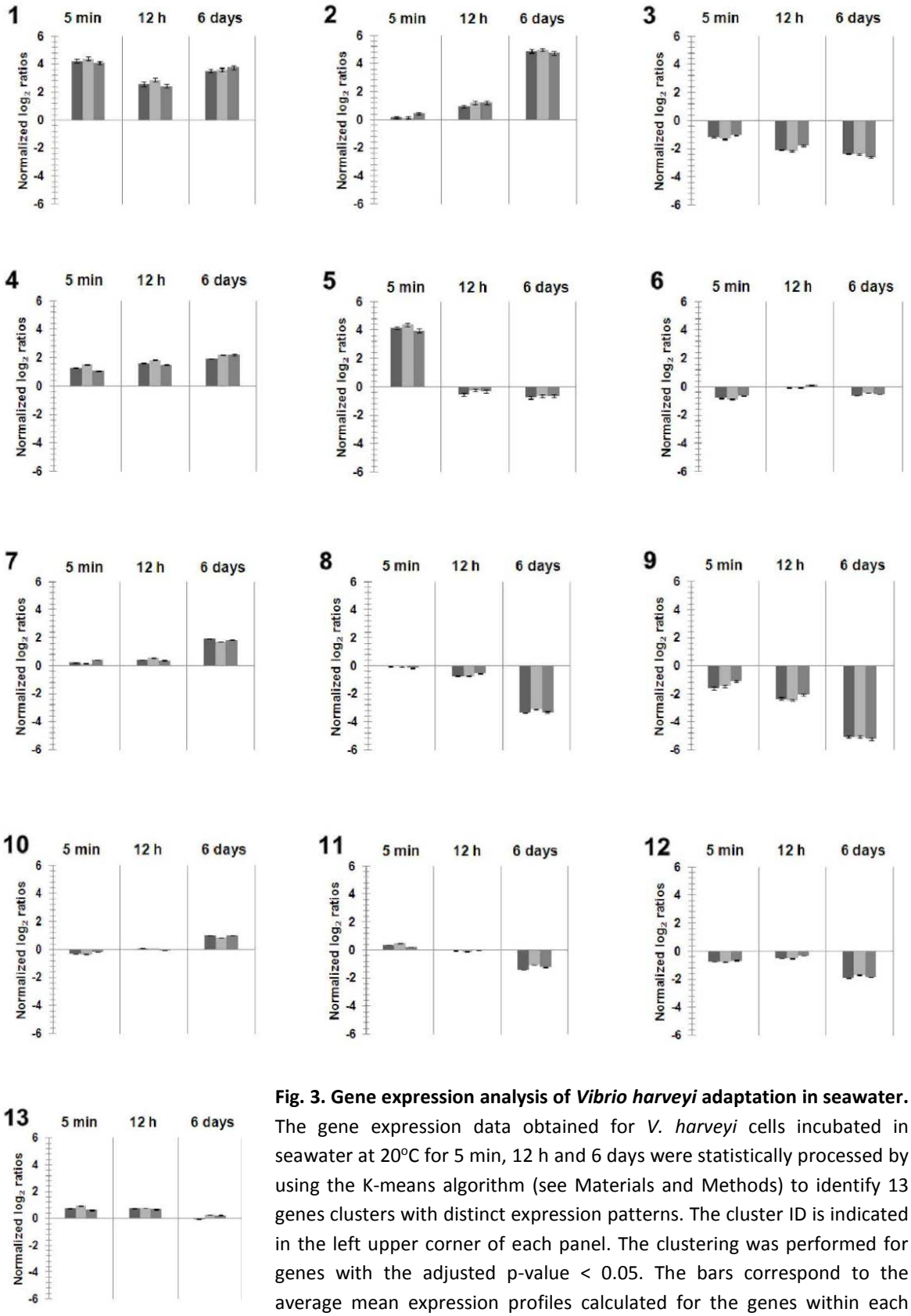

Fig. 3. Gene expression analysis of *Vibrio harveyi* adaptation in seawater. The gene expression data obtained for *V. harveyi* cells incubated in seawater at 20°C for 5 min, 12 h and 6 days were statistically processed by using the K-means algorithm (see Materials and Methods) to identify 13 genes clusters with distinct expression patterns. The cluster ID is indicated in the left upper corner of each panel. The clustering was performed for genes with the adjusted p-value < 0.05. The bars correspond to the average mean expression profiles calculated for the genes within each cluster and expressed as normalized log_2 ratios (i.e. fold change shown on a logarithmic scale with reference to the control time at 0 h). Each time point is represented by three grouped bars corresponding to three biological replicates. The error bars show the standard deviations of the mean values.

Table 1. Major biological pathways associated with gene expression clusters revealed by microarray analysis. Some examples of the genes (p-value < 0.001) whose expression was considerably affected during adaptation in seawater are given in the last column of the table. The complete sets of genes belonging to each biological pathway and their brief annotation are presented in Table S1.

Biological pathways		Gene cluster	Examples of highly up- (⇑) and downregulated (↓) genes	
General category	Specific pathway		Locus tag	Fold change
Lipid metabolism	Fatty acid degradation	1, 5, 7, 10	VIBHAR_00459 (*fadA*)	23.43 (5 min) ↑
	Fatty acid biosynthesis	3, 8	VIBHAR_02907 (*acpP*)	19.83 (6 days) ↓
	Glycerophospholipid metabolism	5, 13	VIBHAR_06179 (*betA*)	20.23 (5 min) ↑
	Metabolism of glycerophosphodiesters	9	VIBHAR_06396 (*ugpB*)	219.14 (6 days)↓
	Lipid A biosynthesis	11	VIBHAR_03227 (*lpxD*)	4.18 (6 days) ↓
	Lipoprotein biosynthesis	11	VIBHAR_00970 (*lgt*)	5.57 (6 days) ↓
Central carbon metabolism	Glyoxylate & 2-methylcitrate cycle	5, 7	VIBHAR_02397 (*prpB*)	20.77 (5 min) ↑
	Citrate cycle	9	VIBHAR_01350 (*gltA*)	43.51 (6 days)↓
	Glycolysis and gluconeogenesis	8, 9, 12	VIBHAR_03525 (*eno*)	14.86 (6 days)↓
Stress responses	Cell envelope stress (phage-shock-protein response)	1	VIBHAR_00008 (*pspG*)	32.51 (5 min) ↑
	Antioxidant stress responses	2, 7, 8	VIBHAR_04761	37.57 (6 days)↑
	Stringent response	7, 10, 11	VIBHAR_00886 (*sspA*)	5.96 (6 days)↑
	DNA damage, repair and synthesis	4, 5, 7, 10, 12	VIBHAR_00268 (*lexA*)	7.38 (6 days)↑
Iron uptake, storage & utilization	Iron cluster biogenesis	1	VIBHAR_01058 (*hscB*)	13.82 (5 min)↑
	Tricarboxilic transport	2	VIBHAR_02414	449.58 (6 days)↑
Transport	Energy-dependent transport (TonB-ExbB-ExbD complex)	4, 13	VIBHAR_00634 (*exbD*) VIBHAR_00637	6.31 (12 h)↑ 7.96 (5 min)↑
	Type III secretion apparatus	3, 9	VIBHAR_01742 (*vopD*)	18.46 (6 days)↓
	Maltose transport	7, 8	VIBHAR_04822 (*malM*)	29.68 (6 days)↓
	Arginine transport	3, 9	VIBHAR_05528 (*artP*)	25.16 (12 h)↓
	ABC transporters	8, 12	VIBHAR_06841	11.11 (6 days)↓
	Phosphate transport	9	VIBHAR_05137 (*pstS*)	180.39 (6 days)↓
Protein biogenesis	Protein turnover	2	VIBHAR_01024	16.82 (6 days)↑
	Protein folding	8, 11, 12	VIBHAR_00143	26.79 (6 days)↓
	Sec-dependent translocation	11	VIBHAR_00909 (*secA*)	4.18 (6 days)↓
Amino acid metabolism	Breakdown of tyrosine, phenylalanine and tryptophan	3	VIBHAR_02177 VIBHAR_06709 (*tnaA*)	24.21 (12 h) ↓ 16.05 (12 h) ↓
	Glutamate metabolism	3, 9	VIBHAR_00927 (*gltD*)	19.52 (6 days)↓
	Valine, leucine and isoleucine biosynthesis / degradation	3, 12	VIBHAR_00826 (*ilvH*)	6.60 (6 days) ↓
	Arginine and proline metabolism	3, 8, 9	VIBHAR_00042 (*argB*)	27.10 (6 days)↓
	Serine biosynthesis / degradation	5	VIBHAR_02609 (*dsdA*)	18.90 (5 min)↑
	Amino acid recycling	5, 10, 13	VIBHAR_01332 (*asnB*)	4.11 (5 min) ↑
	Biosynthesis of L-cysteine	5	VIBHAR_00004 (*cysI*)	14.41 (5 min)↑
Nucleic acid metabolism	Purine metabolism	4, 13	VIBHAR_03200 (*purM*)	5.12 (5 min) ↑
	Pyrimidine metabolism	8, 9	VIBHAR_03648 (*pyrI*)	37.05 (6 days)↓
Energy production	Aerobic respiration	3, 9	VIBHAR_01351 (*sdhC*)	22.08 (6 days)↓
	Anaerobic respiration	8, 9	VIBHAR_00423 (*atpA*)	16.68 (6 days)↓

The second category unites a large number of genes (cluster 1, 3, 4, 9 and 12) that were up- or downregulated at the beginning of the adaptation process and this regulation trend was either maintained (cluster 1, 3 and 4) or further reinforced (cluster 9 and 12) at later time points, thus suggesting that the pathways these genes regulate are involved in both short- and long-term adaptation. Besides numerous genes downregulated to reduce the efficiency of the central carbon metabolism (glycolysis, TCA cycle *etc.*) and associated biosynthetic pathways (e.g. amino acid biosynthesis), upregulation of other genes from this category is in charge of a concomitant increase in the level of transcripts encoding enzymes involved in recycling of biomolecules, in particular lipids.

Finally, a somewhat different type of genes highly up- or downregulated after prolong incubation (> 12 h) of *V. harveyi* cells in seawater (cluster 2, 7, 8, 10 and 11) seem to contribute to the long-term adaptation responses. Apart from making further adjustments in metabolic pathways, their regulation may enable to coordinate antioxidant stress responses (e.g. up- and downregulation of stress-related genes in cluster 2 and 7). The latter could play an essential role in *V. harveyi* adaptation by minimizing the damaging effect of reactive oxygen species accumulating during degradation / recycling of lipids as discussed below.

Validation of microarray data by quantitative real-time PCR

To validate the microarray data, the changes in the level of transcripts (14 in total) selected among the group of up- and downregulated genes that control the key biological pathways, have alternatively been assessed by quantitative real-time PCR (Table 2). The housekeeping gene VIBHAR_00479 was used as a reference to normalize qRT-PCR data. The analysis has been performed with RNA corresponding to three biological replicates using gene-specific primers (Table S2). As seen in Table 2, the result of the qRT-PCR analysis confirmed up- and downregulation of the above genes, thus corroborating microarray data.

Table 2. Validation of microarray data by quantitative real-time PCR (qPCR). Total RNA was isolated in triplicate from overnight culture (control) and *V. harveyi* cells incubated in seawater for 6 days. The differences in the level of individual transcripts determined by qPCR and microarray analysis are presented for several up- (↑) and downregulated (↓) transcripts. The fold changes for the transcript marked by an asterisk (VIBHAR_06715) were determined for the time points, at which the differences in the transcript's level were maximal (i.e. 6 days *vs* 5 min).

Locus tag (Systematic name)	**Gene product**	**qRT-PCR**		**Microarray**	
		Fold change (6 days *vs* control)	**p-value**	**Fold change** (6 days *vs* control)	**p-value**
VIBHAR_00157	Transcriptional regulator LuxR	7.10↓	0.0064	6.05↓	0.004
VIBHAR_01058	Co-chaperone HscB	2.63↑	0.1086	13.82↑	<0.001
VIBHAR_01182	GcvB, regulatory RNA	147.33↓	0.0019	231.01↓	<0.001
VIBHAR_01352	Succinate dehydrogenase hydrophobic membrane anchor protein, SdhD	37.96↓	0.0088	24.1↓	<0.001
VIBHAR_02416	Putative tricarboxylic transport protein TctA	6.97↑	0.1251	148.03↑	<0.001
VIBHAR_03105	3-oxoacyl-[acyl-carrier-protein] synthase I	3.09↓	0.0294	12.65↓	<0.001
VIBHAR_03245	CsrB / RsmB, regulatory RNA	2.25↑	0.1736	9.96↑	0.048
VIBHAR_03284	RNA polymerase, sigma-24 subunit	3.80↑	0.0209	16.40↑	<0.001
VIBHAR_03286	Putative protease Lon	54.20↑	0.0008	22.17↑	0.018
VIBHAR_03525	Phosphopyruvate hydratase	4.36↓	0.0059	14.86↓	<0.001
VIBHAR_04761	Alkyl hydroperoxide reductase, subunit F	2.64↑	0.0427	37.57↑	<0.001
VIBHAR_05192	Catalase	6.14↑	0.0247	17.52↑	<0.001
VIBHAR_05322	Qrr4, regulatory RNA	2.35↑	0.0166	4.45↑	<0.001
VIBHAR_06715	Hydroxyglutarate oxidase	7.26↓	0.0291	15.56↓	<0.001

Discussion

Recent studies documented the enormous potential of marine bacteria including *Vibrio* spp. to sustain growth and survive when they face temperature up- or downshifts, limitation of nutrients, exposure to pollutants of industrial origin or interact with symbiotic and predator organisms. Although analysis of *Vibrio* responses to these environmental factors revealed some common features (Johnson, 2013), still little is known about the regulation and specific contribution of the major metabolic pathways during the adaptation process.

To gain insights into the molecular mechanisms underlying the capacity of *Vibrio* species to survive in a free-living state, we decided to further investigate their adaptation in seawater by employing *V. harveyi* as a model organism. Analysis of *V. harveyi* adaptation in seawater microcosm studied for 14 days at 20^{o}C revealed that the majority of *V. harveyi* cells preserved their viability and culturability, and therefore did not enter the VBNC state during that time. This observation is congruent with the results of previous studies suggesting that the entry of a *Vibrio* species into the VBNC state at temperatures that are close to 20^{o}C (Vattakaven *et al*., 2006; Pruzzo *et al.*, 2003; Wolf and Oliver, 1992) or higher (Duncan *et al.*, 1994) might require much longer periods of time. In contrast, persistence of vibrios at low temperatures seems to proceed differently and can frequently lead to appearance of VBNC cells within few days (Zhong *et al.*, 2009; Du *et al.*, 2007; Jiang and Chai, 1996), thus suggesting that a combined action of different stress factors (e.g. cold and nutrient deprivation) can likely reduce the time required for activation of the VBNC state.

Further inspection of *V. harveyi* morphology (Fig. 2) disclosed a gradual increase in the number of cells with a reduced size and coccoid-like appearance. Similar phenotypical changes were previously reported for several *Vibrio* species subjected to nutrient deprivation (Felter *et al.*, 1969; Novitsky and Morita, 1976; Baker *et al.*, 1983; Nilsson *et al.*, 1991; Stretton *et al.*, 1997; Vattakaven *et al.*, 2006; Zhong *et al.*, 2009; Du *et al.*, 2007). Moreover, the results presented in Fig. 2 as well as those reported in previous work (Novitsky and Morita, 1976; Baker *et al.*, 1983) suggest that appearance of cells with a decreased size and coccoid-like morphology does not necessarily involve the loss of culturability and entry into the VBNC state.

The observed morphological changes and preservation of culturability after 14 days incubation in seawater microcosm under nutrient-limiting conditions implies that *V. harveyi* likely triggers specific regulatory mechanisms adjusting its shape, size and the key metabolic pathways. To learn more about these putative mechanisms, we employed microarray analysis and assessed the time-dependent gene-specific variations that occur at the transcriptome level. Statistical analysis of the microarray data revealed 13 genes clusters (Fig. 3). The members of each cluster show similar transcription profiles likely corresponding to individual transcriptional programs implemented by *V. harveyi* at different phases of adaptation.

The gradual reduction of the cell size and acquisition of the coccoid-like morphology suggests that some regulatory pathways control the biogenesis and functions of the *Vibrio harveyi* cell envelope. Indeed, nearly all clusters contained genes (Table 1) known for their contribution to the control of the membrane composition and membrane-associated biological pathways.

One of the responses globally affecting the cellular envelope functions is known to be controlled by the so-called phage-shock protein (Psp) response (Joly *et al.*, 2010). A drastic increase in the level of transcripts transcribed from the putative *V. harveyi pspABC* operon (cluster 1) encoding the major players of the Psp response suggests that it might play an important regulatory role by coordinating multiple gene regulation mechanisms activated during the adaptation process.

Remarkably, we found that a considerable number of the affected genes are involved in lipid biogenesis. Analysis of their expression profiles revealed that, in contrast to downregulation of genes encoding enzymes involved in lipid biosynthesis (clusters 3 and 8), nearly all genes controlling lipid degradation *via* β-oxidation pathway (reviewed in (Fujita *et al.*, 2007)) were upregulated (clusters 1, 5 and 7). This expression pattern suggests that limitation of nutrients (in particular, carbon sources) in seawater microcosm prompts *V. harveyi* to use endogenous carbon sources, thereby triggering degradation of fatty acids. This scenario is consistent with the results of previous studies revealing that prolong starvation of *Vibrio* species (Hood *et al.*, 1986) and other bacteria (e.g. *Salmonella typhimurium* (Galdiereo *et al.*, 1994)) leads to a decrease in their lipid content. Therefore, it is conceivable that degradation of lipids and concomitant decrease of their presence in the membrane not only permits to

produce energy to maintain physiological activities of *V. harveyi* but also affects the size and shape of its cellular envelope, thereby contributing to the observed morphological changes. The efficient turnover of lipids during *V. harveyi* adaptation and survival is also supported by upregulation of genes involved in utilization of acetyl-CoA, a key cellular metabolite generated *via* beta-oxidation pathway. In particular, we observed a considerable increase in the level of transcripts encoding isocitrate lyase (VIBHAR_01042), malate synthase (VIBHAR_01041) and other enzymes involved in the glyoxylate cycle (see Fig. 4), an anaplerotic pathway bypassing two decarboxylation steps in the tricarboxylic acid (TCA) cycle, thereby allowing to use acetyl-CoA as a primary carbon source for other biosynthetic pathways (reviewed in (Nunn, 1986)).

Lipid turnover usually leads to the formation of additional product, propionyl-CoA, generated along with acetyl-CoA by β-oxidation of odd-chain-length fatty acids. Consistent with the anticipated increase in the level of this metabolite and its subsequent utilization *in vivo*, we observed upregulation of genes controlling the methylcitrate cycle, a common pathway for conversion of propionyl-CoA to pyruvate *in vivo* (Fig. 4). Apart from this pathway, bacteria also employ a 2-hydroxyglutarate synthase-dependent mechanism enabling to convert propionyl-CoA together with glyoxylate (one of the intermediates of the glyoxylate cycle) to alpha-hydroxyglutarate (Reeves *et al.*, 1967). The latter is converted to alpha-ketoglutarate (Fig. 4) yielding hydrogen peroxide (H_2O_2) as a side product (Kalliri *et al.*, 2008).

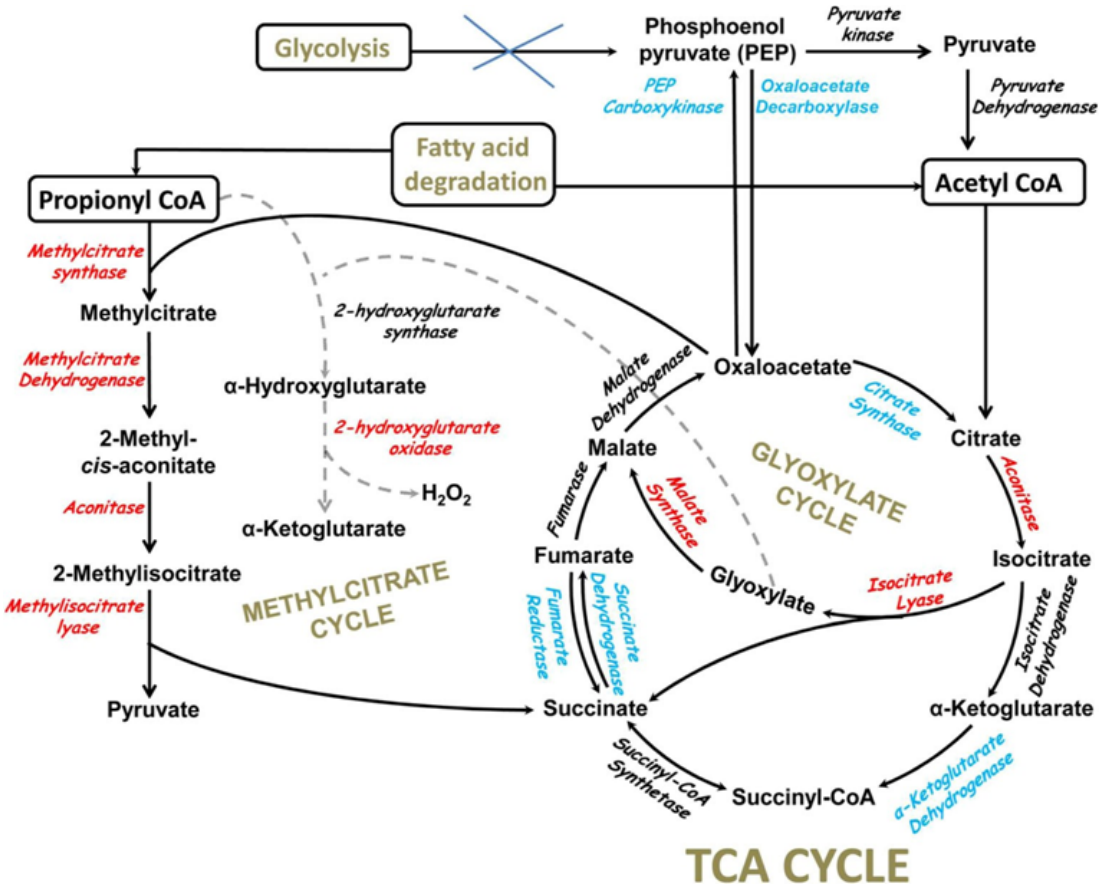

Fig. 4. Selected biochemical pathways involved in the central carbon metabolism in bacteria. Metabolic enzymes encoded by genes that are activated, repressed or show no significant changes in their expression level after *V. harveyi* has been exposed to deprivation of nutrients are indicated by red, light blue or black colour, respectively. In contrast to a drastic decrease in gene expression affecting the level of glycolytic enzymes (cluster 8, 9 and 12) and some enzymes (e.g. fumarate reductase and α-ketoglutarate dehydrogenase) of the tricarboxylic acid (TCA) cycle (cluster 9), a number of genes involved in lipid degradation *via* the β-oxidation pathway (cluster 1, 5 and 7) as well as those controlling further processing of the final products of lipid turnover in glyoxylate and methylcitrate cycles (cluster 5 and 7) were strongly upregulated.

As endogenously produced hydrogen peroxide (see above) can yield free hydroxyl radicals (•OH) able to severely damage important macromolecules including lipids, pro- and eukaryotic cells possess specific defence mechanisms that trigger production of enzymes (catalyses, superoxidases *etc.*) and specialized ancillary proteins (e.g. glutaredoxins) to eliminate hydrogen peroxide and repair the damaged molecules (for review, see (Imlay, 2013)). In agreement with the necessity to exert protection from the toxic effects of H_2O_2 and presumably other reactive oxygen species, *V. harveyi* upregulates a number of genes (cluster 2 and 7) whose products (e.g. catalase, alkylhydroxyperoxide reductase and glutaredoxin 1) can confer cell resistance to these

toxic species and repair DNA damage (e.g. with the help of deoxyribodipyrimidine photolyase). Likewise, upregulation of genes (i.e. VIBHAR_03283 and VIBHAR_03284) previously shown to be involved in *Rhodobacter sphaeroides* stress response to the presence of singlet oxygen and phylogenetically conserved in other bacteria including *Vibrio* species (Dufour *et al*., 2008) strongly suggests that *V. harveyi* apparently needs to mobilize several antioxidative systems to "neutralize" the action of reactive oxygen species generated during its short- and long-term adaptation in seawater microcosm.

The availability of some microelements, in particular iron, can have a great impact on cell growth. The apparently limited concentration of iron in seawater as well as its possible loss due to the damaging effect of reactive oxygen species can considerably hinder the ability of *V. harveyi* to survive in natural aquatic systems. We found that already at the very early time-point (i.e. after 5 min) *V. harveyi* highly upregulated numerous genes involved in iron homeostasis (cluster 1 and 4). These genes (for details, see Table S1) encode iron storage proteins (ferredoxin and bacterioferritin-associated ferredoxin; cluster 1 and 4), components of iron transport systems including the energy-dependent TonB-ExbB-ExbD transport complex (cluster 4), as well as factors (e.g. co-chaperones HscA and HscB) implicated in assembly and maturation of Fe-S clusters, essential structural elements present in a large variety of metabolic enzymes (Roche *et al*., 2013). The upregulation of these genes is reminiscent of that observed in *V. harveyi* during its incubation in seawater microcosm at 4^{o}C (Montánchez *et al*., 2014). These observations imply that, regardless of environmental temperature, an efficient and proper control of iron homeostasis enables *V. harveyi* to maintain its physiological activities at the level sufficient for sustaining its long-term survival under iron-limiting conditions.

The microarray data indicate that deprivation of nutrients (i.e. their scarcity in seawater microcosm) inevitably leads to significant downregulation of genes controlling the central carbon metabolism, biosynthesis of lipids, amino acids and nucleotides with the exception of several upregulated genes that are involved in purine biosynthesis (Table S1). The upregulation of these genes is likely coupled to the control of the nucleotide salvage pathway also activated in this case and usually serves to increase the efficiency of nucleotide recycling in cells facing starvation conditions (Zalkin and Nygaard, 1996).

In addition, deprivation of nutrients is known to induce the so-called stringent response (Jin *et al.*, 2012) discovered in many Gram-negative bacteria including *Vibrio* species (Flardh *et al.*, 1994; Flardh *et al.*, 1997). We found that some genes with the putative roles in the stringent response (i.e. *gppA* (VIBHAR_00339) encoding guanosine pentaphosphate phosphohydrolase and *sspA* (VIBHAR_00886) encoding the stringent starvation protein A) were upregulated, thus suggesting a role for this mechanism in adaptation of *V. harveyi.*

It is conceivable that the overall decrease in metabolic activities (due to poor availability of nutrients) should render a subset of free ('unemployed') metabolic enzymes as well as other non-functional polypeptides that could further be recycled to yield amino acids subsequently used for other cellular needs. Consistent with this idea, we observed upregulation of genes encoding the ATP-dependent protease La (Lon), Peptidase U32, a ClpP-like membrane-bound serine protease and tmRNA known to be involved in protein turnover. Moreover, expression of several genes encoding enzymes that catalyze mutual interconversion of free amino acids (e.g. asparagine synthase B and aminotransferase), their degradation (e.g. methylmalonatesemialdehyde dehydrogenase) or conversion to other essential metabolites (e.g. arginine deaminase) was increased considerably, thus pointing to important ancillary functions of these enzymes during protein turnover and amino acid recycling. Interestingly, some of the pathways involved in amino acid biogenesis are likely controlled by the small RNA GcvB phylogenetically conserved in diverse Gammaproteobacteria (Sharma *et al.*, 2007). Similarly to other sRNAs (Kaberdin and Bläsi, 2006), GcvB is known to act as an antisense RNA by baseparing with the complementary regions of its target mRNAs to repress their translation (Sharma *et al.*, 2007; Urbanowski *et al.*, 2000). As we found a dramatic decrease in the level of this antisense RNA during *V. harveyi* adaptation in seawater microcosm (Table 2 and S1), it seems likely that the GcvB-dependent control likely plays an essential regulatory role in the adaptation process.

Furthermore, due to the predicted decline in the number of functional polypeptides, the key players of chaperone-mediated protein folding (GroEL, GroES, DnaK, DnaJ, Hsp15 *etc.*) should become partly redundant, which is consistent with the decreased expression of their cognate genes (cluster 8, 11 and 12). Some of these genes are likewise downregulated in *V. harveyi* (Montánchez *et al.*, 2014) and other

Vibrio species (Carroll *et al.*, 2001; Wood and Arias, 2011; Yang *et al.*, 2009) exposed to nutrient deprivation under cold-shock conditions and therefore might belong to a common set of *Vibrio* genes activated in response to prolong starvation.

Finally, the anticipated slowdown of cellular metabolism is in agreement with downregulation of genes (cluster 8 and 9) encoding many components of the cell's respiration system (i.e. components of the FOF1 ATP synthase and fumarase reductase complexes), arguably the major systems responsible for generation of ATP *in vivo*. Their gradual downregulation implies the involvement of these genes in the short- and long-term adaptation responses.

In summary, our data demonstrate that *V. harveyi* adaptation in seawater reduces cell size and profoundly affects gene expression. As physiological needs and energy expenses decrease with the reduction of cell size, the gradual shrinkage of *V. harveyi* cells and acquisition of the coccoid-like morphology might play a crucial role in *V. harveyi* adaptation under nutritional stress. In addition, we found that along with the global decrease of gene expression due to the anticipated reduction of metabolic activities and inhibition of the main biosynthetic pathways (e.g. amino acid and lipid biosynthesis), a number of central carbon metabolism genes that control the utilization of the final products of lipid turnover (namely, through their processing in glyoxylate and methylcitrate cycles) were readily upregulated. The upregulation of these genes and a concomitant increase in the level of transcripts encoding enzymes involved in degradation of lipids *via* the β-oxidation pathway strongly suggest that *V. harveyi* uses lipids as a major carbon and energy source.

Other mechanisms potentially implicated in cell fitness and survival of *V. harveyi* in seawater microcosm are linked to iron homeostasis, the stringent response, molecular salvage pathways as well as stress envelope and antioxidative responses. Future studies will enable to address to which extent the above adaptation mechanisms and stress-related genes are conserved in Vibrionaceae and define their possible contribution to biofilm formation and/or acquisition of the VBNC state, frequently used by *Vibrio* species during their adaptation in marine ecosystems (Johnson, 2013).

Chapter 3

Analysis of *Vibrio harveyi* persistence in seawater microcosms revealed a number of genes potentially related to its adaptation in the time of global warming

Introduction

Vibrio harveyi is a heterotrophic Gram-negative luminous bacterium inhabiting marine environments and showing a preference for temperate and tropical waters. In its natural habitats, *Vibrio* spp**.** can be found attached to either biotic or abiotic surfaces (Yildiz and Visick, 2009), in a free living state as well as in symbiotic (Campbell *et al.*, 2009) or host-pathogen (Owens and Busico-Salcedo, 2006) interactions with other organisms, occasionally causing diseases or even death of the infected organisms (Ruwandeepika *et al.*, 2010).

The most recent studies have revealed a wider spread of pathogenic vibrios and this phenomenon was documented along with a gradual increase in the temperature of the sea surface water taking place in the time of global warming (GW) (Le Roux *et al.*, 2015). Consistent with the above trends, there have been a concomitant increase in the cholera or/and gastroenteritis outbreaks (Jones and Oliver, 2009; Morris Jr., 2003) apparently caused by the consumption of water contaminated with pathogenic vibrios (Martínez-Urtaza *et al.*, 2010; McMichael *et al.*, 2006; Paz *et al.*, 2007; Vezzulli *et al.*, 2013). As the average temperature of water increases towards the equator, more cases of Vibrio-related diseases were reported in countries such as India or those in the Caribbean (Letchumanan *et al.*, 2014; GHO, 2016). Moreover, a number of recent reports clearly point to the emergence of Vibrio-associated diseases in Europe including some countries where the presence of virulent variants of *V. vulnificus* and other pathogenic strains could hardly been detected previously (Vezzulli *et al.*, 2016). This alarming statistics includes discovery of pathogenic *Vibrio* species in several unexpected locations such as finding of *V. cholerae* in estuaries of Portugal (Albuquerque *et al.*, 2013), *V. parahaemolyticus* on the French Atlantic coast (Deter *et al.*, 2010) and the highly pathogenic strain *V. parahaemolyticus serovar* O3:K6 in Spain (Baker-Austin *et al.*, 2013; Martinez-Urtaza *et al.*, 2008, 2013 and 2016).

In accordance with the overall increase in the occurrence of Vibrio-associated diseases (Harvell *et al.*, 2002), several highly virulent *V. harveyi* strains able to provoke mass mortality in both marine invertebrates (Soto-Rodriguez *et al.*, 2003; Martin *et al.*, 2004) and various fishes (Lee *et al.*, 2002; Liu *et al.*, 2004) have been reported as well. *V. harveyi* is notoriously known for being an important pathogen of cultured penaeid shrimp (Austin and Zhang, 2006) and its wider occurrence in aquaculture farms causes serious problems for the seafood industry, especially in tropical countries where temperatures are generally higher. As the forecast is not promising, it is conceivable that in coming years, ocean is going to experience even more devastating consequences of GW, which could likely lead to further spread of *Vibrio*-associated infections.

The ubiquitous presence and further spread of *Vibrio* spp. indicate that these marine bacteria are able to easily adapt to changing environmental conditions including those caused by climate change. Several studies have previously examined their adaptation by analyzing phenotypical and gene expression changes taking place in vibrio species facing different stress conditions including deprivation of nutrients at normal or cold temperatures (Lutz *et al.*, 2013; Townsley *et al.*, 2016). These studies revealed that deprivation of nutrients (starvation) can trigger profound morphological changes leading to reduction of cell size and conversion of rod-shaped bacteria into their coccoid-like variants (Farmer and Janda, 2006; present study). In addition, *V. harveyi* can occasionally acquire the viable but nonculturable (VBNC) phenotype when environmental conditions are not favorable (e.g. limitation of nutrients, low temperature, low salinity or ultra violet (UV) radiation) for growing (Ramaiah *et al.*, 2002) and preserves it until the adverse conditions are eliminated, thus being able to recovery from this state of dormancy and resume growth.

An important characteristics that enables vibrios to successfully strive in marine ecosystems is their metabolic versatility, in particular, their capacity to use a wide variety of organic substances (e.g. D-glucose, dextrin, glycogen, N-acetyl-D-glucosamine, D-fructose, maltose, D-trehalose, methyl pyruvate, L-asparagine aconitate, L-proline or inosine) as a primary carbon source. Moreover, the use of different carbon sources is, in turn, facilitated by the ability of *Vibrio* spp. to secrete various hydrolytic enzymes (amylases, gelatinases, lipases and chitinases) converting

the naturally occuring biopolymers (e.g. proteins, polysaccharides, chitin etc.) into smaller, easily metabolizable compounds (Overbeck and Chróst, 1990).

Although changing environments are known to have a great impact on marine bacteria, still little is known how the physiology, metabolic preferences and adaptation mechanisms of *Vibrio* spp. are affected by the main environmental factors (elevated temperature, ocean acidification, suboptimal salinity concentration, etc.) caused by the ongoing climate changes. In order to learn more about *Vibrio* spp. responses to global warming and their possible contribution to survival and pathogenicity of *Vibrio* spp., we studied phenotypical and gene expression changes that occur during the time-dependent adaptation of *Vibrio harveyi* (ATCC® 14126™) in seawater microcosm at 30°C. This temperature is nearly optimal for *V. cholerae* (Huq *et al.*, 1984) and *V. harveyi* (Fukui *et al.*, 2010) growth under laboratory conditions and is also close to the upper limit of sea surface temperature (SST) recently observed in certain regions of the globe (Kizhakudan *et al.*, 2014; Salas-Perez and González-Gándara, 2016). Analysis of experimental data and their comparison with those obtained while studying *Vibrio harveyi* adaptation at 20°C and 4°C (Montánchez *et al.*, 2014; Kaberdin *et al.*, 2015; Parada *et al.*, 2016), disclosed the major cluster of genes controlling the main biochemical pathways and stress-resistance mechanisms at elevated temperatures. Moreover, our study revealed upregulation of many virulence genes potentially being in charge of the increased pathogenic properties and survival of *Vibrio* species in the time of global warming.

Materials and Methods

Vibrio harveyi survival assays, bacterial cell count and size measurements

The survival assays were performed in triplicate with *Vibrio harveyi* (ATCC® 14126™) incubated in natural seawater, which was sampled in the Port of Armintza in the Bay of Biscay, 43° 26′ 24″ N and 2° 54′ 24″ W and then filter sterilized and autoclaved. The experiments were carried out in glass flasks beforehand cleaned with H_2SO_4 (97 %, v/v), rinsed with deionized water, and heated at 250°C for 24 h to avoid any presence of residual organic matter. For each experiment, *V. harveyi* was semi-aerobically grown overnight at 26°C (optimal temperature for *V. harveyi* growth) in marine broth (MB) (Panreac) and aliquots of the stationary phase cultures were used to inoculate sterile natural seawater to obtain a final density of 10^8 cells mL^{-1}. The resulting inoculates of *V. harveyi* were incubated in dark with shaking (90 rpm) at 30°C.

Periodically, samples were collected to determine the total number of cells and culturable bacteria. The total number of cells was determined by filtering aliquots (100–200 µl) of diluted (1:100) *V. harveyi* suspensions through 0.22 µm pore-size polycarbonate membrane filters (Millipore), followed by staining of the attached cells with acridine orange and direct counting individual cells using epifluorescence microscopy (Hobbie *et al.*, 1977). The microscope (Eclipse E400, Nikon) was equipped with a video camera Hamamatsu 2400 (Hamamatsu Photonics, Japan) enabling to obtain high resolution images for their subsequent analysis by Scion Image 1.62[a] software to determine the size of individual cells as described by Massana *et al.* (1997). Approximately 150-200 cells were measured in each sample, and, according to their length, the cells fell into three groups: ≤ 1.2 µm, > 1.2-2 µm and > 2 µm.

Cultivability expressed as colony-forming units was evaluated by spreading aliquots obtained by consecutive dilutions of *V. harveyi* suspensions on Marine Agar (MA, Oxoid) followed by incubation for 24 h at 26 °C.

Scanning electron microscopy

V. harveyi cells present in the control (overnight culture) and test samples (i.e. after 3, 6 and 21 days of incubation in seawater at 30°C) were fixed by adding 50 µl of 3 %

formalin to each sample (1 ml) and were further stored at 4°C. The suspensions of the fixed cells were filtered through 0.22 µm pore-size membrane filters (GTTP filters, Millipore) and the cells attached to the filters were further examined by scanning electron microscopy (SEM) at the Advance Research Core Facility Unit (SGIker) of the University of the Basque Country. Briefly, the filters with the attached *V. harveyi* cells were dehydrated by sequentially immersing them (for 5 min each time) in water/ethanol solutions containing increasing concentrations of ethanol (30, 50, 70, 90, and 100%, respectively) followed by overlaying with hexamethyldisilazane, incubation for 5 min and drying on air. Finally, the samples were coated with a layer of 15 nm gold by using an Emitech K550X Sputter Coater, and imaging was further carried out by examining samples in a Hitachi S4800 scanning electron microscope.

RNA isolation, sequencing and *in silico* analysis of RNAseq data

V. harveyi ATCC 14126 cultures were grown in triplicate overnight (12–16 h) at 26°C in MB, and 20 mL of each culture were individually diluted (1:20) with sterile seawater and further incubated with shaking (90 rpm) at 30°C. Aliquots of *V. harveyi* suspensions were withdrawn after 12 hours, 3 and 6 days of incubation, mixed (8:1) with stop solution (5% phenol in ethanol) and then incubated on ice for 15–20 min. The cells were collected by centrifugation (15 min, 4°C, 4400 g) and the resulting cell pellets were further used to isolate total RNA by employing TRIzol reagent and PureRNA mini Kit (Invitrogen) following the vendor's instructions.

After verifying the quality of the purified RNA by using Bioanalyzer (Agilent, United States), it was further processed with the assistance of the General Genomic Service (SGIker) of the University of the Basque Country. The Ribo-Zero (Bacteria) kit was used to deplete 275-400 ng of total RNA with ribosomal RNA following the low input protocol. The samples depleted with rRNA were then concentrated with RNA Clean & Concentrator-5 kit (Zymo Research) for recovery and preservation of large RNA fragments > 200 nt, and afterwards were used to prepare stranded RNA-Seq libraries. The libraries were prepared from total RNA by using ScriptSeq Complete kit (Illumina) optimized for bacterial RNA. The kit included the Ribo-Zero™ (Bacteria) rRNA removal kit and the ScriptSeq™ v2 Kit with ScriptSeq Index PCR Primers Set 1,

containing Index 1 to Index 12. The preparation of libraries was carried out following the manufacturer's instructions with some minor modifications.

RNA sequencing and bioinformatic analysis were carried out at the National Center for Genomic Analysis (CNAG) in Barcelona integrated with the Center for Genomic Regulation (CRG). The libraries were sequenced on HiSeq 4000 (Illumina, Inc) in a paired-end mode with a read length of 2x76 bp using HiSeq 4000 SBS kit in a fraction of a HiSeq 4000 PE Cluster kit sequencing flow cell lane following the standard protocol.

Image analysis, base calling and quality scoring of the run were processed using the manufacturer's software Real Time Analysis (RTA 2.7.6) and followed by generation of FASTQ sequence files by CASAVA. The processed RNA-seq reads were mapped against *Vibrio harveyi* reference genome (*Vibrio harveyi* ATCC 43516) (available at ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank/; accession number GCA_001558435.1) with STAR (Dobin *et al.*, 2012) and were further quantified with RSEM (Li and Dewey, 2011). Information about some sRNAs initially absent in the annotation of *V. harveyi* ATCC 43516 genome were added to the annotation file by performing a highly restrictive BLAST search by using the sequences of *V. campbellii* ATCC BAA-116 sRNAs retrieved from RFAM and BSRD databases as queries. Functional annotation of other genes was performed by blasting each assembled gene sequence to find homology to other V*ibrio harveyi* genes already annotated in the NCBI database and the functional annotations of the best hits was used for the final annotations of transcripts revealed by RNAseq. Differential expression analysis of genes with P adjusted value < 0.05 and significant fold change (> 2 fold change) as well as gene ontology enrichment of the differentially expressed genes were performed with the assistance of DESeq2 R (Love *et al.*, 2014) and GOstats (Falcon and Gentleman, 2007) packages, respectively. Time series cluster analysis was performed with Mfuzz bioconductor package (Futschik and Carlisle, 2005) with k=6. Heat-maps were generated with the heat-map R package (https://cran.r-project.org/web/packages/pheatmap/index.html) and Principal Component Analysis (PCA) with the 'prcomp' R function and ggplot2.

Results

Effects of prolonged incubation of *V. harveyi* cells in seawater at 30°C on their viability and morphology

To assess the effects of elevated temperatures on physiology and appearance of *Vibrio harveyi*, its overnight culture diluted (1:20) with sterile seawater and continuously incubated at 30°C was examined within 21 days. While determining culturable populations in aliquots withdrawn at different time points and analyzed by spreading on Marine Agar plates, a 10-fold decrease in the number of culturable cells was observed after three weeks of incubation (Fig. 1).

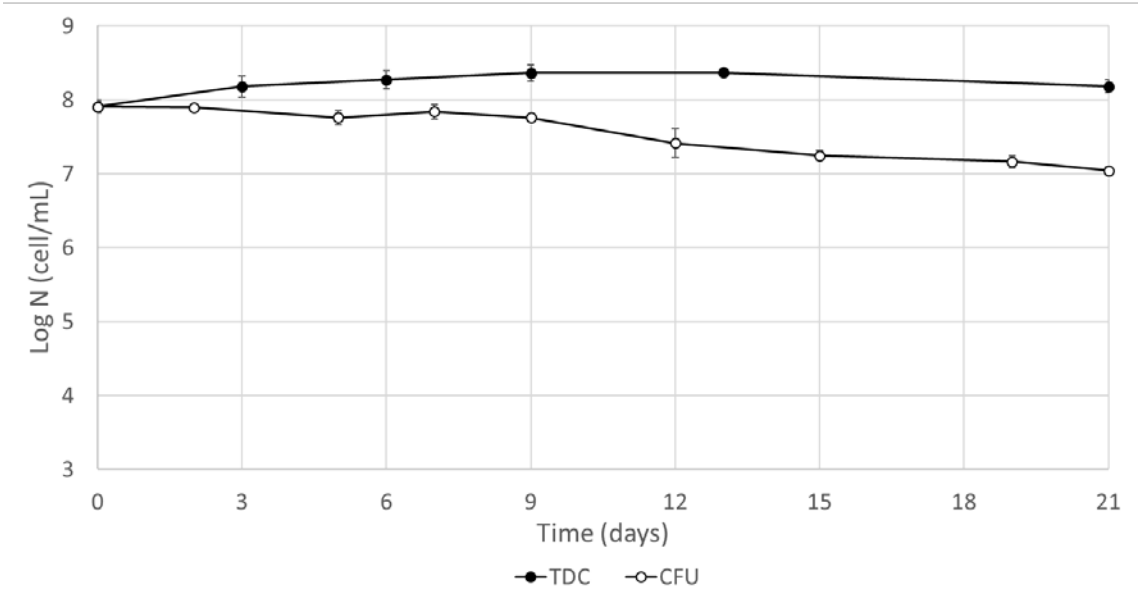

Fig. 1. *Vibrio harveyi* counts obtained during its persistence in seawater at 30°C. The number of total (●) and culturable (○) bacteria were estimated as described in Materials and Methods. The data are mean values from three independent experiments with errors bars representing the standard deviations calculated.

Moreover, the loss of culturability was accompanied by a marked reduction of cell size that became readily distinguishable already after 3 days of incubation and the gradual increase of the fractions of smaller cells was observed until the last day of the measurements (Fig. 2). In other words, while 75.5% of the cells in the control sample represented by the overnight culture (day 0) had size between 1.2 to 2 µm, this number has decreased to 27.4% after 3 days of incubation and the percentage of longer cells continued decreasing progressively until day 6, when nearly all cells (i.e. 95%) were shorter than 1.2 µm.

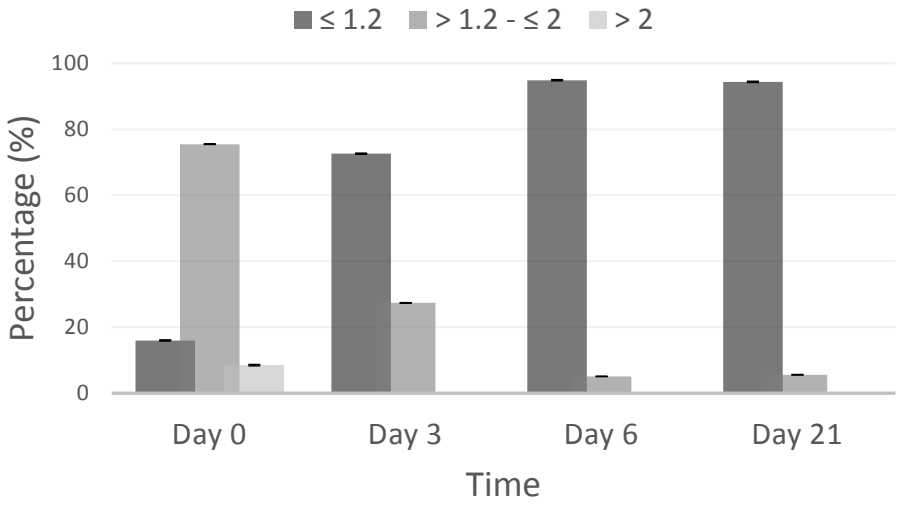

Fig. 2 Size distribution of *Vibrio harveyi* cells during their persistence in seawater at 30°C. The values were obtained by analyzing cell images captured by epifluorescence microscopy and processed as described in Materials and Methods. The bars show the percentage of cell within each size range (■, ≤ 1.2 µm; ■, > 1.2-2 µm and ■, > 2 µm, respectively).

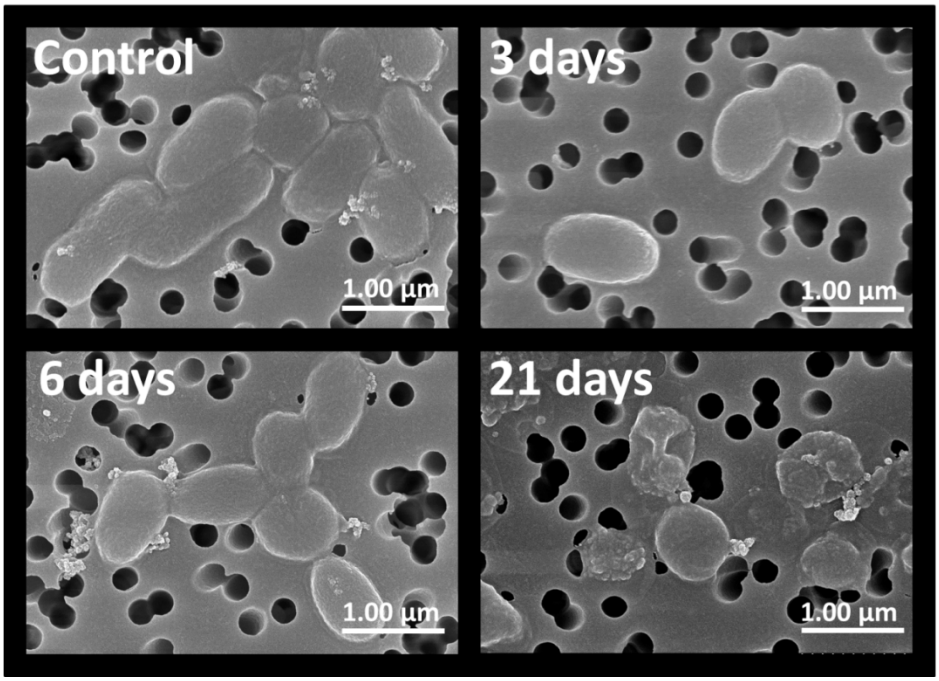

Fig. 3. Morphology and integrity of *Vibrio harveyi* populations incubated in seawater at 30°C. Scanning electron microscopy images were obtained for the cells present in the initial inoculate (control) and well as those incubated at 30°C for 3, 6 and 21 days, respectively.

The observed size reduction was further corroborated by the results of scanning electron microscopy (Fig. 3). They confirmed the appearance of cells with a coccoid-like morphology (i.e. with a nearly spherical shape) after 3 to 6 days of incubation. Moreover, the electron microscopy data also revealed a considerable number of damaged cells, thus indicating that prolong incubation (> 6 days) at 30^{o}C can lead to the loss of cell integrity and death.

Gene expression analysis

Besides monitoring cell viability and morphological changes, *V. harveyi* adaptation was also analyzed at the whole transcriptome level. To carry out this analysis, total RNA was isolated from the initial inoculate (control) and from *V. harveyi* cells after their incubation in sterile seawater for 12 hours, 3, 6, 14 and 21 days. Total RNA samples were prepared in triplicate, i.e. they were originated from three independent assays carried out in parallel. Since the profiles of RNA samples isolated from cells incubated for 14 and 21 days revealed that they contained a considerable fraction of degraded RNA (apparently accumulating due to cell damage beginning after prolong incubation of *V. harveyi* cells in sea water, Fig. 3), only RNAs corresponding to three initial points (12 h, 3 and 6 days) along with the control (i.e. RNA isolated from the initial inoculate) were used for RNA sequencing.

After completing RNA sequencing according to the procedure described in Materials and Methods and mapping the obtained reads to the reference genome (*V. harveyi* ATCC 43516), the read counts corresponding to each individual transcript were processed further to identify transcripts that show significant (fold change > 2) and statistically reliable (p value 0.05) variations between the control and test samples. The complete list of the differentially expressed genes is presented in the supplementary Table S1 (see Supplementary Data).

The differentially expressed genes were further analyzed *in silico* to identify six clusters, each comprising the genes with similar expression profiles (Fig. 4). Two of them (clusters 1 and 5) were gradually upregulated during the course of the experiment and included a number of genes (see Table S1) primarily related to transport**,** virulence (clusters 1 and 5) and stress responses (cluster 5). The other four

clusters (i.e clusters 2, 3, 4 and 6) contained many genes controlling various cellular functions. The expression of these genes was initially decreased and then remained low (clusters 2, 3 and 6) or was transitionally upregulated (cluster 4) at later time points. While clusters 2 and 3 were primarily represented by metabolic genes (e.g. genes controlling carboxylic acid and nucleotide metabolism, respectively), the other two (clusters 4 and 6) comprised the genes playing important roles in the processing of genetic information (namely, transcription and translation, respectively).

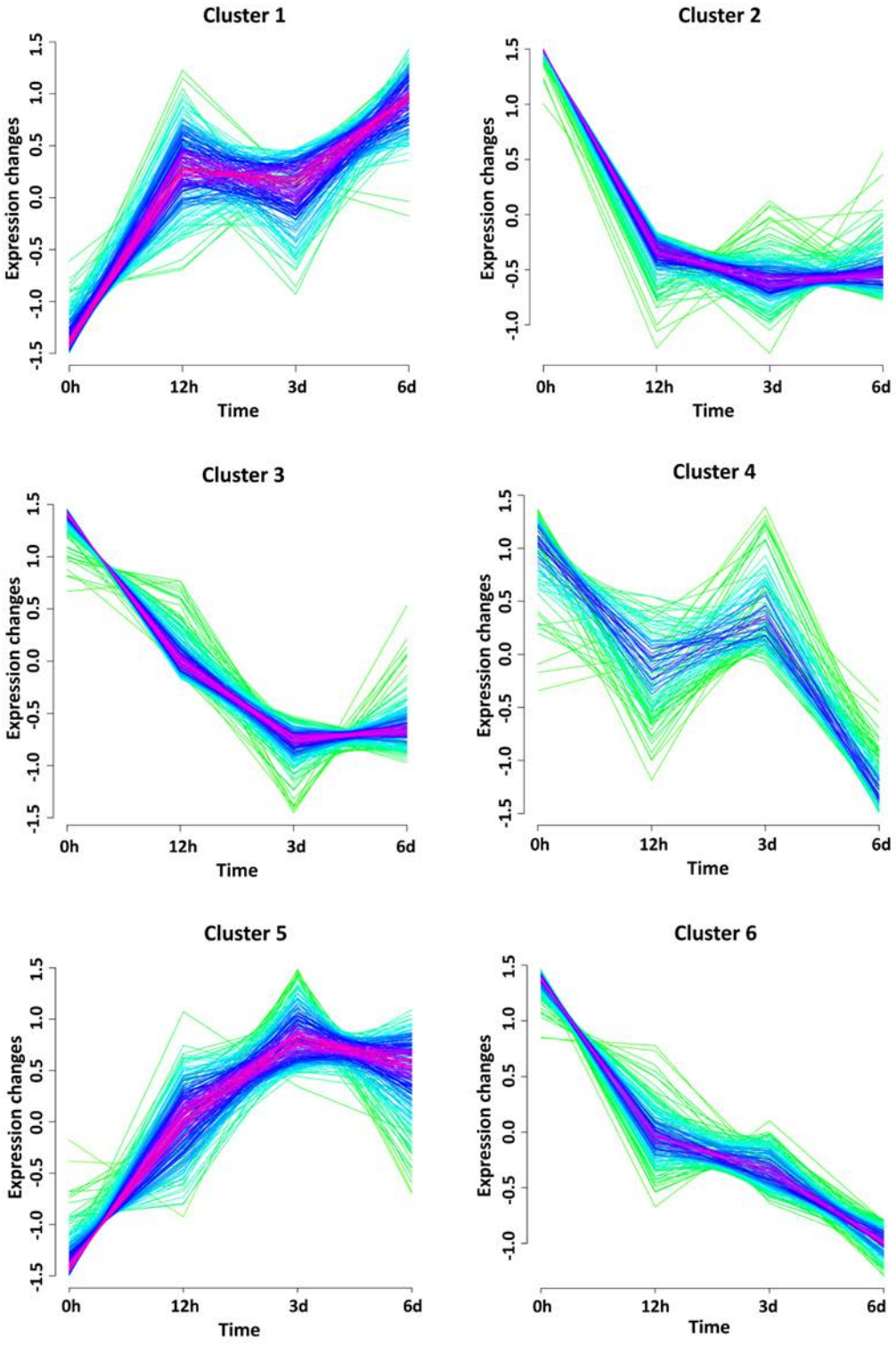

Fig. 4. Gene expression analysis of *Vibrio harveyi* adaptation in seawater at 30°C. Temporal expression patterns were obtained for the time-points corresponding to *Vibrio harveyi* incubation in seawater at 30°C for 12 hours, 3 days and 6 days. Gene membership values are color-encoded with red shades denoting high membership values and green shades denoting low membership values of genes. Differentially expressed genes (False Discovery Rate < 5% and fold change > 2) with respect to time 0 were used to construct temporal clusters with soft clustering (Mfuzz). Principal components of the clusters showed that k=6 was the optimum number of clusters showing minimal overlap. Scaled expression values (z-score) are used for cluster visualization.

Discussion

Marine bacteria including many members of the *Vibrionaceae* family have an enormous potential to survive and sustain their growth by adequately responding to many adverse and continuously changing conditions they face in their natural habitats. Despite a considerable number of studies that revealed a plethora of mechanisms related to adaptation of *Vibrio* spp., comparatively little is known about the key adaptation strategies employed by these marine bacteria in the time of global warming profoundly affecting the physicochemical parameters (e.g. sea surface temperature, salinity, acidity and nutrient availability) and biological variety of natural aquatic systems.

To investigate the impact of elevated temperatures on adaptation of *Vibrio* species, we used *Vibrio harveyi* as a model organism and studied its persistence in seawater microcosms at 30^{o}C, which is known to be within the upper limit of sea surface temperature observed in some areas of the global oceans (Pierrehumbert, 2000)**.** The experiments were done in triplicate by diluting aliquots of the stationary phase cultures (control) with sterile seawater and analyzing the appearance and culturability of vibrio population after 12 hours, 3, 6, 14 and 21 days of incubation.

Analysis of cell morphology by fluorescence and electron microscopy revealed a discernible reduction of cell size already after 3 days of incubation. *In silico* processing of cell images corroborated the overall reduction of cell size until the sixth day of incubation with no significant changes afterwards (Fig. 2 and 3). Similar changes in morphology (i.e. size reduction and shape alterations) have been reported to happen in old cell cultures of *V. parahaemoliticus* and *V. angustum* (among others) subjected to carbon starvation in marine mineral liquid medium under laboratory conditions (Holmquist and Kjelleberg, 1993; Stretton *et al.*, 1997; Chen *et al.*, 2009). In natural aquatic systems, carbon concentration varies and, brief periods of carbon abundance are normally interspersed with long periods of its scarcity. Therefore, it is quite common that free-living bacteria have reduced size in their natural habitats due to shortage of carbon sources (McDougald and Kjelleberg, 2006). In other words, the acquisition of coccoid-like phenotype by *V. harveyi* seems to be a natural response of this marine bacterium to limitation of nutrients. Comparison of three independent

experiments performed with the same *V. harveyi* strain at 4°C (Parada *et al.*, 2016), 20°C (Kaberdin *et al.*, 2015) and 30°C (present work) demonstrates that, although reduction of cell size and occasional acquisition of a coccoid-like phenotype occurs in a wide range of temperatures (i.e. from 4°C to 30°C), these morphological changes take place faster and to a greater degree as the temperature increases. Besides acceleration of morphological changes, elevated temperature (i.e. 30°C) increases the rate of metabolic reactions and energy consumption. However, the increased metabolic and energy demands seem to limit the cell's capacity to cope with stress at 30°C and ultimately lead to the appearance of dead (heavily damaged) cells clearly seen on images of 21 day old cultures obtained by scanning electron microscopy (Fig. 3). The presence of cells with damaged membranes is congruent with a higher percentage of degraded RNA in samples isolated from *V. harveyi* cells after 14 and 21 days of incubation (data not shown) and excluded from further analysis due to their poor quality. Moreover, an increase in the number of damaged cells is also consistent with a 90% loss of cultivability within the entire population (Fig. 1) observed during the same period of time (i.e. after 14 days). Thus, in contrast to their good persistence and preservation of culturability at 20°C (Kaberdin *et al.*, 2015), *V. harveyi* cells *per se* become more vulnerable at 30°C, which apparently leads to their faster damage and death.

To learn about the major adaptation mechanisms that *V. harveyi* and likely other *Vibrio* species trigger to cope with elevated temperatures in their natural habitats, we used RNA sequencing to obtain transcriptome profiles of *V. harveyi* following its incubation in seawater microcosm at 30°C for 12 hours, 3 and 6 days. Comparison of these transcriptome data with those obtained for the control samples (initial inoculate) revealed a large number of genes whose expression was up- or downregulated during the incubation time. Further statistical analysis revealed six distinct gene clusters (Fig. 4), each comprising a set of genes with similar time-dependent expression profiles. The differentially expressed genes were further grouped according to their biological functions and presented in Table S1.

The fast morphological changes and accumulation of cells with damaged membranes (see above) indicate that *V. harveyi* might struggle to properly control expression of genes involved in cell envelope biogenesis and associated metabolic

pathways. This scenario apparently finds its support in expression of phage shock proteins PspA, PspB and PspC (cluster 4) (Table 1), known for their role in relieving membrane stress (Joly *et al.*, 2010). In contrast to their gradual upregulation during persistence of *V. harveyi* in sea water microcosms at 20°C (Kaberdin *et al.*, 2015), expression of these genes at 30°C decreases after 6 day of incubation, thus pointing to the failure of *V. harveyi* to engage the product of the phage shock operon in the adaptation process.

Another example of "unexpected" response concerns lipid biogenesis and transport. Although the shrinkage of *V. harveyi* cell envelope associated with the reduction of cell size should inevitably lead to a discharge of extra lipids and other membrane components for further recycling, we surprisingly found that expression of genes controlling lipid turnover decreased. In other words, a number of genes that are involved in lipid degradation via β-oxidation pathway (i.e. gene encoding phosphoglycerate kinase, glycerophosphoryl diester phosphodiesterase, acyl-CoA dehydrogenase, enoyl-CoA hydratase, beta-ketoacyl-ACP reductase, malonyl CoA-acyl carrier protein transacylase) (clusters 2 and 3) were strongly downregulated. The downregulation of genes controlling β-oxidation pathway as well as those involved in further conversion of acetyl-CoA, the key cellular metabolite generated via β-oxidation pathway, by the enzymes of the glyoxylate cycle (clusters 2 and 3) was unexpected and, in fact, was opposite to their upregulation at 20°C observed in our previous work (Kaberdin *et al.*, 2015). Thus, it seems likely, that, despite the considerably fast shrinkage of cell envelope and concomitant release of free lipids at 30°C, *V. harveyi* fails to upregulate the key genes whose products involved in lipid recycling. Nevertheless, the upregulation of some genes encoding for long-chain fatty acid transporters (AL538_RS25015) could indicate that *V. harveyi* might try to deal with an excess of lipids accumulated due to reduction of cell size by changing their subcellular localization or exporting them outside of cells. In addition to some anticipated problems with lipid recycling, the shrinkage of cell envelope could additionally be hindered by discoordinate expression of major enzymes (for example, murein L,D-transpeptidase (cluster 4) and D-alanyl-D-alanine carboxypeptidase (cluster 3) involved in peptidoglycan remodeling. Besides alterations in expression of genes controlling cell envelope biogenesis, another large group of the affected genes included those related

Table 1. Some examples of *Vibrio harveyi* genes and its products highly up- or down-regulated during persistence in seawater microcosm at 30°C.

Systematic name	Gene product (specific biological pathway)	Fold change (time after exposure to seawater)			Gene expression cluster
		12h	3d	6d	
Amino acid metabolism					
AL538_RS23670	Methylmalonate-semialdehyde dehydrogenase (CoA acylating)	-2.325	-3.377	-2.733	2
AL538_RS15645	Alanine dehydrogenase	-2.877	-2.910	-3.289	2
AL538_RS01790	Ornithine cyclodeaminase	-2.714	-4.302	-3.209	2
AL538_RS07820	Argininosuccinate synthase	-2.225	-2.890	-2.258	2
AL538_RS07290	Aspartate carbamoyltransferase	-1.401	-1.345	-1.897	6
AL538_RS07295	Aspartate carbamoyltransferase regulatory subunit	-1.251	-1.976	-1.289	2
AL538_RS25000	Glutamate synthase	-0.858	-1.233	-1.597	6
AL538_RS11745	Glutamate synthase subunit beta	-1.595	-2.359	-1.828	2
AL538_RS01370	Alkaline serine protease	-1.378	-3.486	-2.372	4
AL538_RS14635	Serine protein kinase PrkA	-1.385	-1.333	-2.215	6
AL538_RS01665	4-hydroxyphenylpyruvate dioxygenase	-2.624	-2.794	-3.415	2
AL538_RS03245	Ribonucleotide-diphosphate reductase subunit beta	-1.553	-2.794	-2.503	3
AL538_RS14510	Uridine phosphorylase	-1.682	-2.946	-3.119	3
Carbon metabolism					
Citrate, glyoxylate & 2-methylcitrate cycles					
AL538_RS17025	Citrate synthase/methylcitrate synthase	-2.020	-1.656	-2.315	6
AL538_RS06380	Aconitate hydratase B	-1.588	-2.634	-2.372	3
AL538_RS10235	Phosphoenolpyruvate carboxykinase (ATP)	-1.458	-3.015	-2.504	3
Glycolysis and gluconeogenesis					
AL538_RS06930	Phosphoglycerate kinase	-1.341	-2.545	-2.503	3
AL538_RS10235	Phosphoenolpyruvate carboxykinase (ATP)	-1.458	-3.015	-2.504	3
AL538_RS04660	Type I glyceraldehyde-3-phosphate dehydrogenase	-1.325	-2.871	-2.660	3
Lipid biogenesis					
AL538_RS16785	Lipid A biosynthesis lauroyl acyltransferase	0.664	0.993	1.203	1
AL538_RS25885	Choline dehydrogenase	1.176	1.370	0.993	5
AL538_RS03425	Acyl-CoA thioesterase	0.878	1.704	1.073	5
AL538_RS06930	Phosphoglycerate kinase	-1.341	-2.545	-2.503	3
AL538_RS26865	Glycerophosphoryl diester phosphodiesterase	-2.629	-3.534	-2.947	2
AL538_RS23675	Acyl-CoA dehydrogenase	-2.053	-2.768	-2.308	2
AL538_RS23680	Enoyl-CoA hydratase	-2.034	-2.947	-2.470	2
AL538_RS27060	Beta-ketoacyl-ACP reductase	-1.347	-2.564	-2.983	3
AL538_RS03900	Malonyl CoA-acyl carrier protein transacylase	-1.815	-2.683	-2.368	3
Acetyl-CoA-dependent metabolism					
AL538_RS14965	Peptidoglycan-associated lipoprotein	-1.663	-2.943	-2.801	3
AL538_RS04550	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase	-1.470	-3.161	-2.871	3
AL538_RS08465	Acetyl-CoA synthetase	-1.601	-2.312	-2.177	3
Processing of genetic information					
AL538_RS06035	Elongation factor G	-1.316	-2.547	-2.559	3
AL538_RS12075	Energy-dependent translational throttle protein EttA	-1.550	-2.247	-2.244	3
AL538_RS02125	Translation initiation factor IF-3	-1.436	-2.093	-2.476	6

Ribosome biogenesis					
AL538_RS07900	30S ribosomal protein S12	-1.454	-2.108	-2.716	6
AL538_RS10850	50S ribosomal protein L22	-0.954	-2.066	-2.065	6
AL538_RS03245	Ribonucleotide-diphosphate reductase subunit beta	-1.553	-2.794	-2.503	3
Transporters and ancillary factors					
Energy-dependent transport TonB-ExbB-ExbD complex					
AL538_RS26550	Biopolymer transporter ExbB	1.623	2.108	1.740	5
AL538_RS19445	TonB-system energizer ExbB	0.201	1.186	1.025	5
ABC transporters					
AL538_RS26870	ABC transporter substrate-binding protein	-2.683	-4.749	-3.279	2
AL538_RS19380	Amino acid ABC transporter	-1.236	-1.698	-1.907	3
AL538_RS00950	Amino acid ABC transporter substrate-binding protein	-1.188	-2.888	-2.524	3
AL538_RS01680	Peptide ABC transporter substrate-binding protein	-2.146	-4.524	-3.021	3
AL538_RS01780	Polyamine ABC transporter ATP-binding protein	-2.676	-3.963	-3.018	2
AL538_RS01795	Spermidine/putrescine ABC transporter substrate-binding protein	-2.751	-4.780	-3.382	2
Other transporters					
AL538_RS01055	EamA family transporter	0.950	1.603	1.870	5
AL538_RS25015	long-chain fatty acid transporter	0.640	0.909	1.212	1
AL538_RS21850	MFS transporter	1.249	1.445	1.537	1
AL538_RS17670	PTS mannitol transporter subunit IIA	1.036	1.597	1.373	5
Protein turnover and folding					
Protein degradation					
AL538_RS01370	Alkaline serine protease	-1.378	-3.486	-2.372	4
AL538_RS12810	Aminoacyl-histidine dipeptidase	-1.960	-2.991	-2.215	2
AL538_RS10020	Oligopeptidase A	-1.105	-1.895	-2.198	6
AL538_RS01150	Peptidase A24	0.249	1.061	1.581	1
AL538_RS01675	Peptidase M20	0.859	0.886	1.368	1
AL538_RS24140	Peptidase M50	1.118	0.888	1.354	1
AL538_RS25255	Peptidase S8	0.698	0.813	1.005	1
AL538_RS18135	D-alanyl-D-alanine carboxypeptidase	-1.318	-2.530	-2.336	3
AL538_RS01705	Xaa-Pro aminopeptidase	-2.220	-4.042	-3.019	3
Protein folding					
AL538_RS25315	Molecular chaperone	0.594	1.004	1.011	5
AL538_RS05430	Molecular chaperone	-1.777	-1.784	-2.056	2
AL538_RS00890	RNA chaperone ProQ	-1.198	-1.487	-2.016	6
Energy production					
AL538_RS03195	Cytochrome c nitrite reductase subunit NrfD	0.565	1.438	0.764	5
AL538_RS00765	Cytochrome-c oxidase, cbb3-type subunit III	-1.764	-2.531	-2.308	2
ATP synthesis coupled proton transport					
AL538_RS09605	F0F1 ATP synthase subunit delta	-1.768	-3.170	-2.672	3
AL538_RS09595	F0F1 ATP synthase subunit gamma	-1.825	-3.548	-2.778	3
Fumarate reductase (complex II)					
AL538_RS08265	Fumarate reductase flavoprotein subunit	-1.862	-2.041	-1.584	2
AL538_RS08280	Fumarate reductase subunit D	-1.508	-2.045	-1.847	2
Stress responses					
Cell envelope stress: Phage-shock-protein response					
AL538_RS15985	Phage shock protein PspA	-0.908	0.819	-1.526	4
AL538_RS15995	Envelope stress response membrane protein PspC	-1.042	-0.263	-1.819	4

Stringent response					
AL538_RS11565	Stringent starvation protein B	-1.121	-0.789	-1.168	2
Antioxidative defence					
AL538_RS17640	Catalase	1.260	2.075	1.668	5
AL538_RS21845	Glutaredoxin, GrxB family	0.713	1.418	0.700	5
Nitric oxide detoxification					
AL538_RS24635	Nitrite reductase large subunit	1.472	1.227	1.433	1
DNA damage, repair and synthesis					
AL538_RS18185	Deoxyribodipyrimidine photolyase	1.446	1.734	1.280	5
AL538_RS23750	DNA mismatch repair protein MutT	1.004	0.776	1.593	1
Iron uptake, storage & utilization					
AL538_RS26680	Ferric reductase	0.938	1.185	1.140	5
Iron uptake/transport					
AL538_RS21155	Iron permease	0.959	1.295	1.249	1
AL538_RS23515	Iron ABC transporter	1.203	1.009	0.937	1
Iron storage					
AL538_RS07875	Bacterioferritin	-2.859	-2.868	-3.496	2
AL538_RS12375	Ferredoxin, 2Fe-2S type, ISC system	-0.365	-0.256	-1.152	4
Iron cluster biogenesis					
AL538_RS16405	Cysteine desulfurase	-2.740	-3.362	-3.312	2
Virulence factors involved in *V. harveyi* pathogenicity					
AL538_RS26540	Putative heme utilization radical SAM enzyme HutW	1.028	1.301	0.458	5
AL538_RS20930	VirK protein	0.708	1.912	1.755	5
Quorum Sensing					
AL538_RS06465	LuxR family transcriptional regulator	-1.543	-2.549	-2.843	3
AL538_RS19310	Sensor histidine kinase	1.253	1.080	1.148	1
AL538_RS25660	TonB-dependent siderophore receptor	1.338	1.520	1.380	5
Chemotaxis, Motility & Biofilm					
AL538_RS25425	Chemotaxis protein	0.892	1.340	1.235	5
AL538_RS21340	Flagellar biosynthesis protein FlhB	0.891	1.557	1.256	5
AL538_RS25995	Pilus assembly protein CpaF	1.152	1.522	1.409	5
Secretion systems					
AL538_RS15160	EscJ/YscJ/HrcJ family type III secretion inner membrane ring protein	1.077	1.212	1.477	1
AL538_RS23415	Type VI secretion protein	1.769	1.838	1.741	5
Drug efflux & Antibiotic resistance					
AL538_RS20560	Bcr/CflA family drug resistance efflux transporter	0.695	1.286	1.148	5
AL538_RS25145	Polyketide cyclase	0.622	1.104	1.046	5

to the *V. harveyi* central carbon metabolism. Analysis of their regulation revealed a fast downregulation of many genes involved in carboxylic acid metabolism (e.g. aconitate hydratase B (*acnB*), phosphoenolpyruvate carboxykinase; see Table S1 in Supplementary Data) as well as those controlling carbon storage such as the one encoding CsrB known for its roles in post-transcriptional control of gene expression. The downregulation of these genes is likely caused by limitation of nutrients in seawater and it was observed already after 12 hours of incubation. Moreover, the initial rate of downregulation was considerably faster in present study (i.e. at 30^{o}C) than that previously observed at 20^{o}C (Kaberdin *et al.*, 2015), thus pointing to the overall increase in the rate of the cellular response at elevated temperatures. In addition, we found that, while decreasing the intensity of the central carbon metabolism, *V. harveyi* apparently attempts to switch to utilization of alternative carbon sources by upregulating a number of genes (e.g. alpha-amylase (cluster 1) and several alpha- and beta-mannosidase genes (belonging to clusters 1 and 5)) encoding various hydrolases able to convert some polysaccharides naturally present in sea water into smaller metabolizable sugars.

Another option to scavenge carbon sources from a nutrient-limiting environments consists in secretion of proteinases able to degrade polypeptides potentially present in the environment (or within the host cells) into oligopeptides or amino acids for their further uptake by the starved cells. This strategy seems to be less costly when compared to *de novo* synthesis and is likely preferred by *V. harveyi* struggling to maintain the required level of amino acids during its persistence in sea water, i.e. under conditions when the key genes necessary for biosynthesis of amino acids are downregulated (i.e. arginine-succinate synthase, hydroxyproline-2-epimerase or ornithine cyclodeaminase from cluster 2; carbamoyl-phosphate synthase small subunit, cluster 3; glutamate synthase, cluster 6). Consistent with this scenario, *V. harveyi* upregulates a number of genes (e.g. those in cluster 1) coding for various proteinases such as the subtilisin-serine peptidase S8 and protease M20. Most members of the peptidase S8 family are secreted and they are probably implicated in the pathogenesis by damaging host tissues. The periplasmic peptidases belonging to M20 family are carboxy-metallo-proteases (Rawlins and Barrett, 1995) appear to assist the uptake of peptides by converting them into free amino acids in the periplasm. In

contrast to the above examples, several protease genes were found to be downregulated. Their repression could be caused by different factors including the high cost of their operation (such as ATP-dependence of oligopeptidase A), reduced cellular needs in some specialized enzymes (e.g. the proteases HtpX and RseP involved in protein quality control) or simply due to their redundancy (see Table S1).

While striving under nutrient-limiting conditions and looking for alternative way to supply its needs, *V. harveyi* also up- and downregulates a large number of transporter-encoding genes primarily found in clusters 1 and 5 (Fig. 4). Some upregulated genes control (i) the energy-dependent transport mediated by the TonB-ExbB-ExbD complex, (ii) multidrug efflux transporters that belong to the multidrug and toxic compound extrusion (MATE) family and (iii) membrane transport proteins of the major facilitator superfamily (MFS) that facilitate movement of small solutes across cell membranes. In contrast to the above examples, many genes coding for ABC- and PTS-type transporters (clusters 2, 3 and 5) were found to be downregulated, thus indicating that the transport of the corresponding molecules (particular sugars and amino acids) is likely taken over by alternative transport systems or is deactivated due to its redundancy.

The scarcity of iron is a particular challenge normally faced by marine bacteria during their persistence in their natural habitats (Johnson *et al.*, 1997). Consistent with the necessity to deal with low concentrations of iron in seawater microcosms, we observed that a number of genes involved in iron uptake/transport and storage were up- and downregulated, respectively. In addition to regulation of carbon metabolism and acquisition of iron, incubation in seawater seems to activate some adaptive mechanisms aimed at protecting *V. harveyi* cells from the possible damage potentially caused by hydrogen peroxide, other reactive oxygen species and/or nitric oxide. Namely, we found that *V. harveyi* upregulated several stress-responsive genes (cluster 5) encoding catalase, alkyl hydroperoxide reductase subunit F, glutaredoxin as well as some nitrate reductases and cytochrome *c* nitrite reductase subunit NrfD able to minimize (or reduce) the damaging effects of the aforementioned toxic species.

As the spread of Vibrio–associated diseases is believed to be dependent on the unprecedented increases of seawater surface temperature observed during the ongoing climate changes, we also checked whether elevated temperature affects

regulation of *V. harveyi* genes controlling its virulence and ancillary mechanisms (i.e. production and secretion of virulence factors, biofilm formation and motility). We found that, in addition to upregulation of lytic enzymes including various glycosidases and proteinases (see above), *V. harveyi* also upregulated a number of genes encoding the Type III secretion system (TTSS) well conserved in *V. harveyi* (Henke and Bassler, 2004b) and best known for its involvement in transport of virulence factors. Moreover, some genes related to other secretion systems (i.e. Type I secretion system (Delepelaire, 2004), Type IV secretion system (Juhas *et al.*, 2008) and Type VI secretion system (Ho *et al.*, 2014)) that are likewise involved in pathogenicity were upregulated as well.

Another important finding was concerned upregulation of genes encoding structural proteins of flagella and pilus (flagellar biosynthesis proteins FlhB and FliR from cluster 5; pilus assembly proteins CpaB, CpaF, PapD, PilN, PilZ, TadB and TadE FliR from cluster 1 and 5) as well as ancillary enzymes such as peptidase A24 (gene AL538_RS01150). This membrane inserted endopeptidase, also known as type IV prepilin peptidase, processes type 4 pilin precursor proteins (prepilins) that are required for the type IV pilus formation, secretion of toxin and enzymes, gene transfer and biofilm formation (LaPointe and Taylor, 2000). The upregulation of the above genes can play an important role in cell motility and attachment during the initials steps of infection. Moreover, the overall increase in motility and capacity to attach to biotic surfaces could also increase *V. harveyi* chances to find new sources of food (e.g. *via* attachment to sea shells whose components (e.g. amino polysaccharides) can further be processed by secreted hydrolyzes to yield amino sugars in nutrient-limiting environments.

Finally, we found that the gene coding for LuxR, a transcription factor mediating the quorum sensing (QS) response in a large variety of *Vibrio* spp. (Bassler, 1999) was markedly downregulated, thereby suggesting that QS might not play a significant role the adaptation process. Meanwhile, many genes encoding for proteins of two-component (sensor histidine kinase/response regulator) systems (CreC, UhpB and others, cluster 1 and 5) playing essential roles in sensing environmental signals and accordingly adjusting gene expression (Capra and Laub, 2012) were upregulated. Several two-component systems previously described in vibrios and shown to be

upregulated in the present study are involved in pathogenicity (Geszvain and Visick, 2008; Martinez-Wilson *et al.*, 2008; Shikuma *et al.*, 2009). Therefore, their upregulation might enhance the pathogenic potential of *V. harveyi* facing environmental challenges.

In summary, our data demonstrate that *V. harveyi* faces a significant size reduction and acquisition of coccoid-like morphology during its long-term incubation under deprivation of nutrients at 30^{o}C, which mimics the upper limit of sea surface temperature recently recorded in some areas of global oceans. Moreover, our data show that morphological changes, loss of viability and cell death apparently occur much faster at elevated temperatures (i.e. 30^{o}C) than at 20^{o}C, thus suggesting that elevated temperatures and starvation (substantial lack of nutrition in natural sea water) *per se* have a negative effect on *V. harveyi* viability. Despite its negative effect on *V. harveyi* survival due to counter adaptive downregulation of some genes (e.g. those controlling β-oxidation pathway), incubation at 30^{o}C readily enhances expression of many virulence factors likely essential for the spread of Vibrio-associated diseases in the time of global warming. Thus, the putative increase in their pathogenicity and capacity to enter host cells could likely provide a major strategy for Vibrio spp. to escape from adverse conditions at elevated temperatures.

General Discussion

General Discussion

Vibrio species represent one of the most diverse genera of marine bacteria known for their ubiquitous presence in natural aquatic systems. Some members of this genus including *Vibrio harveyi* are receiving increasing attention lately because they are becoming a source of health problems, especially for some marine organisms grown in aquaculture farms (Ruwandeepika *et al.*, 2010). The situation is getting particularly severe and alarming in the countries whose economy is strongly dependent on seafood industry based on cultivation of crustaceans (e.g. shrimps) and other marine organisms susceptible to Vibrio-associated infections. A number of studies suggest a link between the fast spread of *Vibrio*-associated diseases including cholerae outbreaks and the gradual increase in sea surface temperature (SST) caused by the ongoing climate change.

The omnipresence of vibrios in natural aquatic systems, their ability to tolerate a wide range of temperatures and recent spread of *Vibrio*-associated diseases indicate that *Vibrio* spp. possess an enormous potential to adapt and respond to environmental challenges (e.g. limitation of nutrients, suboptimal temperatures, pH and salinity, diverse life styles etc.) they face in their natural habitats. The above features of *Vibrio* spp. make them nice model organisms to study ecology and evolution of bacterial populations in aquatic ecosystems (Vezzulli *et al.*, 2015). Indeed, several *Vibrio* species have been used recently to reveal physiological responses and certain adaptation mechanisms employed by marine bacteria to persist in their natural environments, exert their pathogenic properties and adequately respond to ongoing climate changes. For instance, some of these studies proposed that survival and persistence of *Vibrio spp.* in their natural habitats could be associated with acquisition of the VBNC state suggested to be induced by low temperatures, starvation or/and high salinity (Wong *et al.*, 2004; Gauthier, 2000; Parada *et al.*, 2016).

Nevertheless, by the time we initiated this study, still little was known about the global regulatory network and key metabolic pathways contributing to specific adaptation steps that enable many different *Vibrio* species (such as *V. harveyi*) in their free-living state to cope with a large variety of adverse conditions and successfully strive in natural aquatic systems.

Therefore, in order to learn about adaptation changes triggered by this marine bacterium during its long-term persistence in seawater in a temperature-dependent manner, we selected *Vibrio harveyi* ATCC® 14126™ as a model organism and studied its adaptation in seawater microcosms at 4°C, 20°C and 30°C. While 20°C represented the average temperature of the coastal water of the Biscay Bay during the summer season (Ibañez-Artica, 1990), 4°C and 30°C closely mimicked the lower and upper limits of sea surface temperatures recorded around the globe (Fig. 1). Thus, it was anticipated that a combination of the above temperatures along with incubation in seawater microcosms (i.e. under limitation of nutrients known to negatively affect the cell's ability to survive and maintain physiological activities) could represent a wide range of stress conditions facing by *V. harveyi* in its natural environments.

Analysis of *V. harveyi* populations incubated at 20°C in seawater microcosm revealed that the majority of cells remained intact and preserved their culturability. In contrast, after 14 days of incubation at 30°C, nearly 90% of cells were not culturable and partly damaged, thus suggesting the negative effect of elevated temperatures on *V. harveyi* survival.

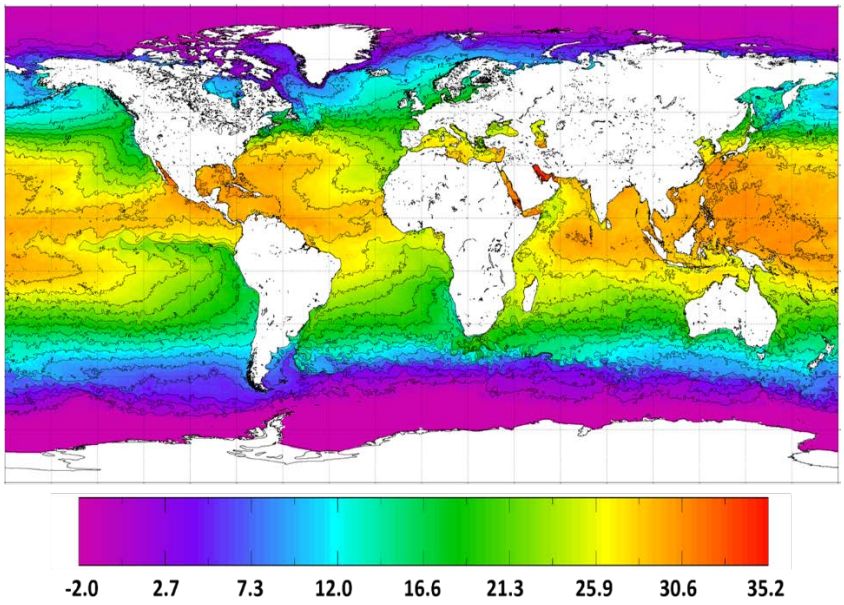

Fig. 1. Sea surface temperature contour chart. The weather map obtained based on measurement of sea surface temperatures recorded across the globe on 29th of August, 2017. Areas with different gradients of temperature defined according to the gradient bar beneath the map are separated by isotherm lines. The temperature map was adopted from the National Environmental Satellite, Data and Information service of National Oceanic and Atmospheric Administration (NOAA), United States (http://www.noaa.gov/).

Likewise, although the relatively short incubation of *V. harveyi* at 4°C (Chapter 1) revealed only a minor decrease in its ability to resume growth, other work from our laboratory demonstrated a dramatic decrease in the number of culturable cells after 14 days of permanence at 4°C, which ultimately led to a nearly complete loss of culturability found after three weeks of incubation (Parada *et al.*, 2016).

The apparently higher resistance of *V. harveyi* to starvation and its capacity to remain fully cultivable after relatively long incubation at 20°C (but not at 4°C and 30°C) is consistent with the preference of *V. harveyi* to inhabit temperate waters with temperatures usually ranging from 10 to 20°C.

Moreover, its high capacity to cope with limitation of nutrients at 20°C for long time is also corroborated by previous findings demonstrating that the actual loss of *V. harveyi* cultivability at temperatures close to 20°C followed by acquisition of the viable but non-culturable (VBNC) phenotype required much longer periods of time than those we tested in the present study (Vattakaven et el., 2006; Pruzzo *et al.*, 2003; Wolf and Oliver, 1992).

The actual mechanisms that contribute to reduction of *V. harveyi* cultivability and damage at suboptimal (i.e. low or elevated) temperatures are not fully understood. For instance, low temperatures are known for their negative impacts on protein folding and function and therefore induce cold-shock response. Although this response could temporally counteract the negative consequences of low temperature, it might not always fully 'neutralize' them and prolonged incubations of bacterial cultures at 4°C still lead to *V. harveyi* entry into the VBNC state (Sun *et al.*, 2008; Parada *et al.*, 2016).

The fast loss of *V. harveyi* culturability and cell damage observed at 30°C were somewhat surprising, given the optimal growth temperature for *V. harveyi* under laboratory conditions (i.e. ca. 26°C) as well as its ability to spread and cause disease outbreaks in the time of global warming. On the other hand, since the optimal survival temperature for many bacteria exposed to starvation is often below the optimum growth temperature (Randa *et al.*, 2004; Arana *et al.*, 2010; Hernroth *et al.*, 2010), one can envisaged that the lower survival capacity of *V. harveyi* in a free living state could be attributed to poor efficiency of some adaptation mechanisms used by this marine bacterium at elevated temperatures.

In addition to study cell culturability, we also monitored cell morphology (i.e. size reduction and shape alterations). Analysis of changes in *V. harveyi* morphology by fluorescence and electron microscopy revealed a gradual increase in the number of cells with a reduced size and coccoid-like appearance after incubation at 20^{o}C during 14 days accompanied by preservation of cell viability and capacity to resume growth under nutrient-rich conditions. These results indicate that a decrease in cell size and coccoid-like morphology does not necessarily involve the loss of culturability (Novitsky and Morita, 1976; Baker *et al.*, 1983). Although similar alterations in morphology could also be observed at 4^{o}C and 30^{o}C (Parada *et al.*, 2016; Chapter 3), the dynamics of these changes was different. The appearance of considerably smaller cells was faster at 30^{o}C, i.e. the reduction of cell size became noticeable already after 3 days of incubation and continued for another 3 days with no significant change in cell size afterwards. However, in contrast to their high persistence at 20^{o}C, longer incubations (>14 days) of *V. harveyi* cells at 30^{o}C led to the appearance of cells with damaged membranes and loss of culturability, thus suggesting that *V. harveyi* cells become more vulnerable at 30^{o}C, which likely leads to their faster degeneration and death. Finally, the above morphological changes seem to occur at low temperatures as well. Even though our experiment carried out for 12 h at 4^{o}C (chapter 1) did not reveal changes in length, shape and integrity of *V. harveyi* cells, a similar experiment performed in our laboratory at 4^{o}C for 21 days (Parada *et al.*, 2016) demonstrated a clear reduction of cell size. Comparison of our data from chapters 2 and 3 with the latter study indicates that although cell size reduction and acquisition of coccoid-like phenotype occur in a wide range of temperatures (i.e. from 4^{o}C to 30^{o}C), these morphological changes take place faster and to a greater degree at elevated temperatures. The fact that similar phenotypical changes occur at different temperatures and were also observed in *Vibrio* spp. populations subjected to carbon starvation (Holmquist and Kjelleberg, 1993; Stretton *et al.*, 1997; Chen *et al.*, 2009) indicates that the above morphological changes are controlled by a general mechanism likely activated by *V. harveyi* to adjust its shape, size and physiological needs in response to starvation. In other words, *V. harveyi* adaptation to starvation likely involves a thoroughly orchestrated regulatory network aimed at minimizing the major energy and metabolic needs of bacteria through reduction of their size.

To learn more about specific adaptation mechanisms potentially representing this regulatory network, *V. harveyi* adaptation was also analyzed at the whole transcriptome level. The result of this analysis revealed downregulation of genes controlling glycolysis, TCA cycle as well as major biosynthetic pathways (e.g. amino and nucleic acid biosynthesis etc.), thus suggesting the overall reduction of central carbon metabolism under all three temperature conditions. In contrast, a number of genes involved in nucleotide salvage pathways and recycling of major macromolecules (i.e. polypeptides and lipids) were generally upregulated (with some exceptions discussed below), thus enabling to minimize energy expenses. In this context, the observed reduction of cell size was critical for discharging free lipids in the process of shrinkage of cell envelope. It means that bacteria could benefit from size reduction not only (i) by decreasing the resources and energy necessary for maintaining some minimal physiological activities in smaller cells but also (ii) by receiving free lipids that can be recycled *via* beta-oxidation pathway to provide extra energy and carbon sources for cellular needs. This scenario apparently takes place at 4^{o}C and 20^{o}C. At these temperatures, the corresponding expression profiles revealed that genes encoding enzymes involved in lipid biosynthesis were downregulated, whereas nearly all genes controlling lipid degradation via β-oxidation pathway were upregulated. Lipid turnover is also supported by the upregulation of genes encoding enzymes involved in the glyoxylate cycle that utilizes acetyl-CoA generated via β-oxidation pathway. Likewise, we observed upregulation of genes controlling the methylcitrate cycle, a common pathway for conversion of propionyl-CoA (another product also generated in the β-oxidation pathway) to pyruvate. Therefore, it seems likely that starvation triggers degradation of cell wall fatty acids, thus making it possible for *V. harveyi* to mobilize internal carbon sources to combat nutrient limitation.

In contrast to what was observed at 4^{o}C and 20^{o}C, incubation at elevated temperature does not seem to promote lipid turnover by increasing expression of the corresponding genes, even though the size reduction was greater and faster at 30^{o}C.

Besides facing limitation of nutrients, *V. harveyi* should also cope with the scarcity of some trace elements (e.g. iron) in their natural environments (Johnson *et al*., 1997). Although bacteria normally need them in small quantities, they are absolutely essential for their survival. This is particularly true for the cellular role of

iron, an important metal ion present in many metabolic enzymes. Consistent with the importance of this microelement and necessity to maintain proper iron homeostasis (Andrews *et al.*, 2003), we found that *V. harveyi* adjusts expression of genes involved in iron acquisition and storage within the entire temperature range.

Some additional challenges apparently faced by *V. harveyi* in attempts to protect itself from the possible damage that could cause reactive oxygen species (e.g. hydrogen peroxide, superoxide ion etc.) apparently accumulating in stressed cells at different temperatures. We found that, in addition to upregulation of genes whose products (e.g. catalase, alkyl hydroperoxide reductase and glutaredoxin) are involved in inactivation of these toxic oxygen species, *V. harveyi* also upregulated some genes encoding DNA repair proteins such as MutT (Lu *et al.*, 2001) (at 30^{o}C) or LexA (at 4^{o}C and 20^{o}C) known to be activated in the so-called SOS response (Michel, 2005), i.e. when bacteria are exposed to DNA-damaging agents.

Most of the above regulations and adaptations seem to properly work at 20^{o}C. However, extreme temperatures lead to additional challenges. At low temperature bacteria must adapt its cellular physiology and face a number of changes such as a decrease in membrane fluidity or inefficient folding of some proteins (Phadtare, 2004). Moreover, the so-called cold-shock response, which happens after a rapid decrease in temperature, leads to the cessation of most proteins synthesis except production of specialized 'cold shock' proteins able to lessen the harmful effects of temperature downshift, thus facilitating cell growth at low temperatures (Wolffe, 1995). While being efficient in sustaining cell growth at some low temperatures, the cold-shock response apparently loses its efficiency at 4^{o}C ultimately leading to reduction in efficiency of metabolic pathways (Montánchez *et al.*, 2014) and cell entry into the VBNC state (Parada *et al.*, 2016). Likewise, despite their attempts to activate adaptation mechanisms to maintain cellular functions at elevated temperature, *V. harveyi* cells fail to adequately respond to this environmental challenge. Namely, the cells cannot cope with elevated temperatures for long periods of time (i.e. over 14 days) and ultimately become damaged quickly losing their potential to resume growth under nutrient-rich conditions.

In summary, our data demonstrate that *V. harveyi* incubation in seawater and its responses to limitation of nutrients and essential microelements such as iron lead

to cell size reduction and strongly influence gene expression, differentially affecting the physiology of *Vibrio harveyi* cells in a temperature-dependent manner. Despite the overall reduction in metabolic activities due to starvation, the stress response is more profound and happens faster at 30^{o}C. However, further gene expression analysis strongly suggests that the major *V. harveyi* adaptive mechanisms do not seem to be well coordinated and do not ensure cell survival at this temperature when compared to their action at 20^{o}C. On the other hand, elevated temperature promotes expression of many virulence genes potentially enhancing the pathogenic potential of this bacterium. These findings suggests that more frequent occurrence of *Vibrio* spp. outbreaks in the time of global warming might not be a result of its higher fitness in aquatic systems but, instead, could represent a possible strategy enabling *V. harveyi* (and likely other *Vibrio* species) to escape from adverse environment by getting involved in symbiotic or host-pathogen interactions.

Conclusions

Conclusions

1. *Vibrio harveyi* adaptation to limitation of nutrients and iron in seawater is differentially affected by temperature.

2. The long-term response of *V. harveyi* to starvation at moderate and elevated temperatures is accompanied by a gradual reduction of cell size and acquisition of a coccoid-like phenotype.

3. Although cell size reduction and acquisition of a coccoid-like phenotype can occur over a wide range of temperatures, these morphological changes take place faster and to a greater degree as the temperature increases.

4. The adaptive morphological changes are not necessarily coupled to loss of culturability and/or entry of *V. harveyi* into the VBNC state.

5. *V. harveyi* adaptation in seawater at 20^{o}C appears to be well orchestrated at the transcriptional level.

6. Analysis of gene expression suggests that *V. harveyi* incubation in seawater leads to the overall reduction of central carbon metabolism via downregulation of genes controlling glycolysis, TCA cycle as well as major biosynthetic pathways (e.g. amino and nucleic acid biosynthesis etc.) regardless of the temperatures tested.

7. Several genes involved in nucleotide salvage pathways and recycling of major macromolecules (i.e. polypeptides and lipids) were generally upregulated at 4^{o}C and 20^{o}C, but not at 30^{o}C.

8. *V. harveyi* promotes lipid turnover by increasing expression of the corresponding genes upon incubation at 4^{o}C and 20^{o}C. In contrast, this regulatory mechanism does not seem to work at 30^{o}C.

9. *V. harveyi* promptly responds to limitation of iron in seawater microcosms at optimal and suboptimal temperatures by adjusting expression of genes involved in iron acquisition and storage.

10. In the process of adaptation in seawater, *V. harveyi* upregulates a number of genes whose products are involved in oxidative stress response and, therefore might protect this bacterium from the possible damage caused by reactive oxygen species.

11. The efficiency of the cold-shock response induced by *V. harveyi* upon incubation in cold seawater is apparently maximal during the initial period of adaptation.

12. *V. harveyi* fails to adequately respond to starvation at elevated temperatures and prolong (> 14 days) incubation at 30^{o}C leads to cell damage and a sharp decrease in its capacity to resume growth under nutrient-rich conditions.

13. *V. harveyi* adaptation at 30^{o}C is accompanied by upregulation of virulence genes.

14. The more frequent occurrence of disease outbreaks caused by *V*. *harveyi* in the time of global warming might not be a result of its increased fitness in aquatic systems but, instead, could represent a possible strategy enabling *V. harveyi* (and likely other *Vibrio* species) to escape from adverse environments by getting involved in symbiotic or host-pathogen interactions.

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Supplementary data

Supplementary data for Chapter 1

Table S1. *V. harveyi* genes downregulated during adaptation to cold seawater (12 h *versus* 5 min). Annotation of the genes and their products is according to the classification used in the KEGG database (http://www.genome.jp/).

Biological Pathway	Locus tag (systematic name)	Fold change	Gene Name	Gene product
Amino acid metabolism				
Alanine, aspartate and glutamate metabolism	VIBHAR_04875	-3.76		4-aminobutyrate aminotransferase
	VIBHAR_01795	-3.58		Alanine dehydrogenase
	VIBHAR_00154	-14.06	*aspA*	Aspartate ammonia-lyase
	VIBHAR_00928	-3.23		Glutamate synthase, large subunit
	VIBHAR_03608	-4.23		Succinate-semialdehyde dehydrogenase
Arginine and proline biosynthesis	VIBHAR_00078	-4.60		Arginine N-succinyltransferase
	VIBHAR_07098	-5.29		Proline dehydrogenase / delta 1-pyrroline-5-carboxylate dehydrogenase
	VIBHAR_00077	-2.92	*astD*	Succinylglutamic semialdehyde dehydrogenase
Arginine and proline biosynthesis & pyruvate metabolism	VIBHAR_03491	-3.02		Oxaloacetate decarboxylase
	VIBHAR_03490	-2.33		Oxaloacetate decarboxylase subunit beta
Arginine and proline biosynthesis & alanine, aspartate and glutamate metabolism	VIBHAR_00588	-9.32	*glnA*	Glutamine synthetase
Arginine and proline metabolism	VIBHAR_02131	-5.15		Succinylglutamate desuccinylase
	VIBHAR_03644	-2.35		Arginine deiminase
Cysteine and methionine metabolism	VIBHAR_01309	-6.71		Cysteine synthase A
	VIBHAR_03568	-4.29		S-adenosylmethionine synthetase
	VIBHAR_03484	-4.26		S-ribosylhomocysteinase
	VIBHAR_00125	-3.79		Serine acetyltransferase
Glycine decarboxylation system's regulation	VIBHAR_03188	-4.38	*gcvR*	Glycine cleavage system transcriptional repressor, GcvR
Glycine, serine and threonine metabolism	VIBHAR_05973	-11.15		Glycine dehydrogenase
	VIBHAR_05668	-2.71		L-allo-threonine aldolase
	VIBHAR_05001	-3.36	*tdh*	L-threonine 3-dehydrogenase
	VIBHAR_05971	-7.53		Serine hydroxymethyltransferase
	VIBHAR_05000	-3.34		2-amino-3-ketobutyrate coenzyme A ligase
	VIBHAR_04899	-4.29		L-serine dehydratase
	VIBHAR_01908	-4.26		
	VIBHAR_03510	-4.83		Aspartate kinase
	VIBHAR_02623	-4.38		Phosphoserine aminotransferase
	VIBHAR_03710	-2.82		Aminotransferase
Histidine biosynthesis	VIBHAR_02072	-4.31		Histidine ammonia-lyase
	VIBHAR_04882	-2.01		Histidine utilization repressor
	VIBHAR_02073	-2.49		Urocanate hydratase
Lysine biosynthesis	VIBHAR_03187	-9.01		Dihydrodipicolinate synthase
	VIBHAR_03309	-15.74		2,3,4,5-tetrahydropyridine-2,6-carboxylate N-succinyltransferase
Lysine & arginine and proline biosynthesis	VIBHAR_00079	-4.61	*argD*	Bifunctional N succinyldiaminopimelate-aminotransferase/acetylornithine transaminase protein

Lysine biosynthesis & cysteine and methionine metabolism	VIBHAR_03103	-6.72		Aspartate-semialdehyde dehydrogenase
Phenylalanine, tyrosine and tryptophan biosynthesis	VIBHAR_02761	-2.28		Bifunctional indole-3-glycerol Phosphate synthase / phosphoribosylanthranilate isomerase
	VIBHAR_01001	-2.35		Chorismate mutase/prephenate dehydrogenase
	VIBHAR_00417	-3.42		Prephrenate dehydratase
Phenylalanine, tyrosine and tryptophan biosynthesis & glycine, serine and threonine metabolism	VIBHAR_02763	-2.32	*trpA*	Tryptophan synthase subunit alpha
	VIBHAR_02762	-2.74		Tryptophan synthase subunit beta
Beta-alanine metabolism & pantothenate and CoA biosynthesis	VIBHAR_03451	-3.40	*panC*	Pantoate--beta-alanine ligase
Thiamine metabolism	VIBHAR_04996	-4.46		Cysteine desulfurase
	VIBHAR_01178	-4.35		Thiamine biosynthesis protein ThiI
Tryptophan biosynthesis	VIBHAR_00999	-3.48		Trp operon repressor
Valine, leucine and isoleucine biosynthesis	VIBHAR_00825	-2.39		Acetolactate synthase 3 catalytic subunit
	VIBHAR_00826	-2.33	*ilvH*	Acetolactate synthase 3 regulatory subunit
	VIBHAR_05477	-2.90		Hydroxymethylglutaryl-CoA lyase
	VIBHAR_00478	-3.02		Ketol-acid reductoisomerase
Valine, leucine and isoleucine degradation	VIBHAR_05480	-3.47		Acyl-CoA dehydrogenase
	VIBHAR_05478	-4.94		Enoyl-CoA hydratase/isomerase
	VIBHAR_05487	-2.91		3-hydroxyisobutyrate dehydrogenase
	VIBHAR_05479	-4.88		Propionyl-CoA carboxylase subunit beta
Production of cyclic-di-GMP, a second messenger	VIBHAR_06147	-1.95		Diguanylate cyclase/phosphodiesterase
Tyrosine metabolism	VIBHAR_06282	-3.98	*hpaG*	2-hydroxyhepta-2,4-diene-1,7-dioate isomerase
	VIBHAR_02179	-3.18		2-oxopent-4-enoate hydratase
Purine / Pyrimidine metabolism				
Purine metabolism	VIBHAR_00582	-5.03		Adenosine deaminase
	VIBHAR_01029	-5.82		Exopolyphosphatase
	VIBHAR_06224	-4.81		Guanosine 5'-monophosphate oxidoreductase
	VIBHAR_01581	-2.40		LacI family transcriptional regulator
	VIBHAR_00767	-3.86	*cysN*	Sulfate adenylyltransferase subunit 1
	VIBHAR_00766	-3.30		Sulfate adenylyltransferase subunit 2
Purine and pyrimidine metabolism	VIBHAR_05714	-5.19		Anaerobic ribonucleoside-triphosphate reductase activating protein
	VIBHAR_05713	-8.12		Anaerobic ribonucleoside triphosphate reductase
	VIBHAR_00764	-8.90	*cpdB*	Bifunctional 2',3'-cyclic nucleotide 2'-phosphodiesterase/3'-nucleotidase periplasmic protein
	VIBHAR_01064	-2.00	*ndk*	Nucleoside diphosphate kinase
	VIBHAR_03527	-19.37	*mazG*	Nucleoside triphosphate pyrophosphohydrolase
	VIBHAR_03374	-9.46	*deoD*	Purine nucleoside phosphorylase
	VIBHAR_02733	-7.89		Ribonucleotide-diphosphate reductase subunit alpha
	VIBHAR_02734	-10.56	*nrdB*	Ribonucleotide-diphosphate reductase subunit beta
	VIBHAR_03520	-2.83	*surE*	Stationary phase survival protein SurE

Purine and pyrimidine metabolism & Nicotinate and nicotinamide metabolism	VIBHAR_01256	-17.41	*ushA*	Bifunctional UDP-sugar hydrolase/5'-nucleotidase periplasmic
Purine metabolism & sulfur metabolism	VIBHAR_00769	-2.74		Adenylylsulfate kinase
Pyrimidine metabolism	VIBHAR_03648	-16.72		Aspartate carbamoyltransferase regulatory subunit
	VIBHAR_03647	-12.05	*pyrB*	Aspartate carbamoyltransferase catalytic subunit
	VIBHAR_02116	-3.82		Cytidine deaminase
	VIBHAR_05385	-7.88		Deoxycytidylate deaminase
	VIBHAR_00649	-3.84	*pyrE*	Orotate phosphoribosyltransferase
	VIBHAR_02856	-2.98		Orotidine 5'-phosphate decarboxylase
	VIBHAR_02606	-4.57		Thioredoxin reductase
	VIBHAR_00006	-4.76		Thymidylate kinase
	VIBHAR_03376	-5.25	*deoA*	Thymidine phosphorylase
	VIBHAR_03235	-3.15	*pyrH*	Uridylate kinase
	VIBHAR_01516	-14.88		Uridine phosphorylase
Nicotinate and nicotinamide metabolism	VIBHAR_05230	-2.93	*nadE*	NAD synthetase
	VIBHAR_05733	-5.80	*pntA*	NAD(P) transhydrogenase subunit alpha
	VIBHAR_00222	-4.15	*nudC*	NADH pyrophosphatase
	VIBHAR_06577	-5.15		Pyrazinamidase/nicotinamidase
	VIBHAR_05734	-4.32	*pntB*	Pyridine nucleotide transhydrogenase
	VIBHAR_00265	-2.62		Soluble pyridine nucleotide transhydrogenase
Pyrimidine metabolism & alanine, aspartate and glutamate metabolism	VIBHAR_00912	-4.81		Carbamoyl phosphate synthase small subunit
Central carbon metabolism				
Amino sugar and nucleotide sugar metabolism & starch and sucrose metabolism	VIBHAR_01575	-5.05	*glgC*	Glucose-1-phosphate adenylyltransferase
Amino sugar and nucleotide sugar metabolism	VIBHAR_03430	-4.17		Beta-N-hexosaminidase
	VIBHAR_00419	-3.02	*gluM*	Bifunctional N-acetylglucosamine-1-phosphate uridyltransferase / glucosamine-1-phosphate acetyltransferase
	VIBHAR_05944	-2.36	*glgC*	Glucose-1-phosphate adenylyltransferase
	VIBHAR_01335	-3.30	*nagA*	N-acetylglucosamine-6-phosphate deacetylase
	VIBHAR_02257	-3.36		N-acetyl-D-glucosamine kinase
	VIBHAR_03432	-3.59		Phosphoglucomutase / phosphomannomutase
	VIBHAR_05793	-2.39		UDP-glucose 4-epimerase
Carbon storage	VIBHAR_03509	-4.41		Carbon storage regulator, CsrA
Citrate cycle	VIBHAR_03440	-2.92		Bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase
	VIBHAR_00131	-6.32		Fumarate reductase flavoprotein subunit
	VIBHAR_01915	-4.22		Fumarate hydratase, class I
	VIBHAR_00133	-3.70		Fumarate reductase subunit C
	VIBHAR_00134	-2.92		Fumarate reductase subunit D
	VIBHAR_06715	-3.15		Hydroxyglutarate oxidase
	VIBHAR_00795	-6.63		Malate dehydrogenase
	VIBHAR_01351	-4.82	*sdhC*	Succinate dehydrogenase cytochrome b556 large membrane subunit
	VIBHAR_01353	-3.53	*sdhA*	Succinate dehydrogenase flavoprotein

				subunit
	VIBHAR_01354	-3.71	*sdhB*	Succinate dehydrogenase iron-sulfur subunit
	VIBHAR_01350	-9.81	*gltA*	Type II citrate synthase
Glycolysis / Gluconeogenesis	VIBHAR_00458	-4.75		Alcohol dehydrogenase
	VIBHAR_05412	-7.63		
	VIBHAR_05634	-3.39		Aldehyde dehydrogenase coniferyl-aldehyde dehydrogenase
	VIBHAR_03463	-5.42	*aceF*	Dihydrolipoamide acetyltransferase
	VIBHAR_00012	-4.86	*pgi*	Glucose-6-phosphate isomerase
	VIBHAR_03049	-15.83		Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
	VIBHAR_00121	-1.96		Phosphoglyceromutase
	VIBHAR_03525	-6.42	*eno*	Phosphopyruvate hydratase
	VIBHAR_06724	-3.68		Phospho-beta-glucosidase B
	VIBHAR_00145	-12.18		6-phosphofructokinase
	VIBHAR_00709	-2.97	*tpiA*	Triosephosphate isomerase
	VIBHAR_02420	-2.92		NAD-dependent aldehyde dehydrogenase
	VIBHAR_03018	-3.32		Bifunctional acetaldehyde-CoA / alcohol dehydrogenase
	VIBHAR_00785	-2.55		Fructose-1,6-bisphosphatase
	VIBHAR_01345	-2.60		Phosphoglucomutase
	VIBHAR_00169	-6.56		Acetyl-CoA synthetase
	VIBHAR_01635	-8.46		
	VIBHAR_03565	-3.014.00	*pgk*	Phosphoglycerate kinase
	VIBHAR_05553	-6.02		Branched-chain alpha-keto acid dehydrogenase subunit E2
	VIBHAR_03462	-5.66		Dihydrolipoamide dehydrogenase
Glyoxylate and decarboxylate	VIBHAR_06362	-2.55		Oxidoreductase
	VIBHAR_05972	-9.36		Glycine cleavage system protein H
	VIBHAR_05365	-11.73		Acetoacetyl-CoA reductase
	VIBHAR_05409	-16.60		Phosphoglycolate phosphatase
	VIBHAR_01373	-8.55	*purU*	Formyltetrahydrofolate deformylase
Nucleotide sugar metabolism	VIBHAR_06725	-3.82		dTDP-D-glucose 4,6-dehydratase
Pentose phosphate pathway	VIBHAR_03377	-6.86		Deoxyribose-phosphate aldolase
	VIBHAR_03375	-5.18		Phosphopentomutase
	VIBHAR_06344	-4.00		6-phosphogluconolactonase
	VIBHAR_06142	-2.96		Ribokinase
	VIBHAR_03555	-3.02		Ribose-5-phosphate isomerase A
	VIBHAR_06256	-9.34		Transaldolase B
	VIBHAR_06257	-4.85		Transketolase
	VIBHAR_03567	-4.25		
Pentose phosphate pathway & arginine and proline biosynthesis	VIBHAR_00515	-3.02		Keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate aldolase
Pyruvate metabolism	VIBHAR_02942	-2.45		Acetate kinase
	VIBHAR_02367	-8.21		acylphosphatase
	VIBHAR_05675	-4.44		D-lactate dehydrogenase
	VIBHAR_02177	-2.70		4-hydroxyphenylpyruvate dioxygenase
	VIBHAR_06584	-9.28		Lactoylglutathione lyase
	VIBHAR_02034	-2.91		Malate dehydrogenase
	VIBHAR_06121	-2.53	*mgsA*	Methylglyoxal synthase
	VIBHAR_02943	-4.55		Phosphate acetyltransferase
	VIBHAR_06517	-4.36		Phosphoenolpyruvate synthase
	VIBHAR_00597	-2.82		Phosphoenolpyruvate carboxykinase
	VIBHAR_03464	-6.99	*aceE*	Pyruvate dehydrogenase subunit E1
	VIBHAR_05555	-2.82		Pyruvate dehydrogenase E1 component

				subunit alpha
	VIBHAR_05554	-3.22		Pyruvate dehydrogenase E1 component subunit beta
	VIBHAR_01546	-10.09		Pyruvate-formate lyase
	VIBHAR_00829	-4.01		Pyruvate kinase
	VIBHAR_03465	-8.75	*pdhR*	Transcriptional regulator PdhR
	VIBHAR_05366	-3.39		Acetyl-CoA acetyltransferase
Pyruvate metabolism & fatty acid biosynthesis	VIBHAR_00172	-4.60		Acetyl-CoA carboxylase biotin carboxylase subunit
Starch and sucrose metabolism	VIBHAR_05253	-2.22		Alpha-amylase
	VIBHAR_05679	-5.97		
	VIBHAR_06938	-3.60		Beta-glucosidase
	VIBHAR_04849	-2.05		Maltodextrin phosphorylase
	VIBHAR_05411	-3.01		Glucose dehydrogenase
Sugar metabolism	VIBHAR_06618	-5.45		Epimerase
	VIBHAR_05199	-2.66		Glycosyltransferase
	VIBHAR_00832	-1.96	*mtlR*	Mannitol repressor protein, mannitol operon repressor
	VIBHAR_05389	-10.69		Phosphatase YqaB
	VIBHAR_03445	-5.49		Sugar fermentation stimulation protein A, predicted DNA-binding transcriptional regulator
Acetylates maltose & other sugar metabolism	VIBHAR_06688	-11.63		Acetyltransferase, maltose O-acetyltransferase
Lipid biogenesis				
Biosynthesis of unsaturated fatty acids	VIBHAR_02637	-6.96		Trans-2-enoyl-CoA reductase
Biosynthesis of unsaturated fatty acids & fatty acid biosynthesis	VIBHAR_02908	-4.72		3-ketoacyl-ACP reductase
	VIBHAR_06461	-6.26		3-oxoacyl-ACP reductase
Fatty acid biosynthesis	VIBHAR_02907	-2.92	*acpP*	Acyl carrier protein
	VIBHAR_02909	-5.31	*fabD*	Malonyl CoA-acyl carrier protein transacylase
	VIBHAR_05488	-2.31	*fabG*	3-ketoacyl-ACP reductase
	VIBHAR_03105	-15.71		3-oxoacyl-ACP synthase, FabB
	VIBHAR_02910	-3.87		3-oxoacyl-ACP synthase, FabH
	VIBHAR_02906	-5.35		3-oxoacyl-(acyl carrier protein)synthase II, FabF
Fatty acid biosynthesis & pyruvate metabolism	VIBHAR_03221	-5.08		Acetyl-CoA carboxylase carboxyltransferase subunit alpha
	VIBHAR_03100	-4.80		Acetyl-CoA carboxylase subunit beta
	VIBHAR_00171	-5.38		Acetyl-CoA carboxylase biotin carboxyl carrier protein subunit
Fatty acid catabolism	VIBHAR_06892	-2.53		Methylmalonyl CoA epimerase
Fatty acid metabolism	VIBHAR_01379	-2.76		Long-chain-fatty-acid--CoA ligase
	VIBHAR_03121	-3.50	*fadI*	3-ketoacyl-CoA thiolase
Fatty acid metabolism, valine, leucine and isoleucine degradation & lysine degradation	VIBHAR_03120	-5.59	*fadJ*	Multifunctional fatty acid oxidation complex subunit alpha
Glycerolipid metabolism	VIBHAR_03651	-2.01		1-acyl-sn-glycerol-3-phosphate acyltransferase
Glycerolipid metabolism, glycerophospholipid metabolism	VIBHAR_00269	-2.52		Glycerol-3-phosphate acyltransferase
Glycerophospholipid	VIBHAR_03310	-6.84	*glpQ*	Glycerophosphodiester phosphodiesterase

metabolism	VIBHAR_06395	-14.82		Glycerophosphoryl diester phosphodiesterase
	VIBHAR_00124	-4.30	*gpsA*	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase
	VIBHAR_01172	-2.56		Phosphatidylglycerophosphatase A
Lipid metabolism	VIBHAR_03016	-8.96		Short chain dehydrogenase
Lipoic acid metabolism	VIBHAR_01212	-3.70	*lipB*	Lipoate-protein ligase B
	VIBHAR_01211	-3.86	*lipA*	Lipoyl synthase
Lipopolysacharide biosynthesis	VIBHAR_00682	-6.97	*rfaD*	ADP-L-glycero-D-manno-heptose-6-epimerase, GmhD
	VIBHAR_03203	-5.03	*gmhA*	Phosphoheptose isomerase
	VIBHAR_00907	-3.00	*lpxC*	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase
	VIBHAR_01254	-3.10		2-dehydro-3-deoxyphosphooctonate aldolase
Lipoprotein biogenesis	VIBHAR_00981	-3.72	*lspA*	Lipoprotein signal peptidase
Lipoprotein biosynthesis	VIBHAR_00868	-4.18		Bifunctional heptose 7-phosphate kinase/heptose 1-phosphate adenyltransferase, GmhC
Phospholipid metabolism	VIBHAR_06649	-4.80		Thioesterase, Orthology: *ybgC*, acyl-CoA thioester hydrolase
Processing of genetic information				
Conjugative DNA transfer	VIBHAR_02112	-2.18	*ihfA*	Integration host factor subunit alpha
DNA replication	VIBHAR_00430	-3.15		Chromosome partitioning protein, ParB family
	VIBHAR_00213	-2.49		DNA-binding protein, DNA-binding protein HU-alpha
	VIBHAR_03705	-3.19		Single-stranded DNA-binding protein, SSB
DNA synthesis	VIBHAR_02730	-3.38		DNA gyrase subunit A
	VIBHAR_03222	-6.94	*dnaE*	DNA polymerase III subunit alpha
	VIBHAR_00168	-8.48		DNA polymerase III subunit epsilon
	VIBHAR_03636	-3.17		DNA polymerase III subunit chi
	VIBHAR_03383	-3.66		DNA polymerase III subunit psi
	VIBHAR_00849	-4.50	*dnaG*	DNA primase
	VIBHAR_01574	-2.80		DNA topoisomerase I
	VIBHAR_03115	-2.13		N5-glutamine S-adenosyl-L-methionine-dependent methyltransferase (putative adenine-specific DNA-methyl-transferase)
Inhibition of translation	VIBHAR_01002	-4.28		Ribosome-associated protein Y
Inhibitor of DNA replication	VIBHAR_00651	-3.36	*slmA*	Nucleoid occlusion protein, TetR/AcrR family transcriptional regulator
Plasmid replication	VIBHAR_02859	-6.73	*ihfB*	integration host factor subunit beta
Ribosome biogenesis	VIBHAR_00762	-2.54		ATP-dependent RNA helicase DbpA DEAD-box helicase
	VIBHAR_00952	-2.12		ATP-dependent RNA helicase SrmB
	VIBHAR_00808	-2.94	*ksgA*	Dimethyladenosine transferase, 16S rRNA (adenine1518-N6/adenine1519-N6)-dimethyltransferase
	VIBHAR_00175	-2.63	*fis*	DNA-binding protein Fis, Transcription factor
	VIBHAR_00432	-2.41	*gidB*	16S rRNA methyltransferase GidB
	VIBHAR_03529	-5.39	*rumA*	23S rRNA 5-methyluridine methyltransferase
	VIBHAR_00056	-2.47		30S ribosomal protein S7
	VIBHAR_00057	-2.47	*rpsL*	30S ribosomal protein S12
	VIBHAR_00851	-3.39	*rpsU*	30S ribosomal protein S21
	VIBHAR_00226	-2.35	*rplL*	50S ribosomal protein L7/L12
	VIBHAR_00173	-12.84	*prmA*	50S ribosomal protein L11

				methyltransferase
	VIBHAR_00728	-3.51	*rpmE*	50S ribosomal protein L31
	VIBHAR_00438	-4.13	*rpmH*	50S ribosomal protein L34
	VIBHAR_03674	-7.80		Ribosomal large subunit pseudouridine synthase A
	VIBHAR_00529	-2.45		SAM-dependent methyltransferase (orthology: 16S rRNA (guanine1516-N2)-methyltransferase
RNA metabolism	VIBHAR_02501	-2.62		ATP-dependent RNA helicase HrpA
	VIBHAR_02501	-2.95		ATP-dependent RNA helicase HrpA DEAH-box helicase
	VIBHAR_00224	-5.02		DNA-directed RNA polymerase subunit beta'
	VIBHAR_01198	-4.22		2,3-cyclic-nucleotide 2' phosphodiesterase
RNA processing and decay	VIBHAR_00112	-4.25		Oligoribonuclease
	VIBHAR_03448	-6.69		Nucleotidyltransferase/poly(A) polymerase
	VIBHAR_01030	-3.09	*ppk*	Polyphosphate kinase
	VIBHAR_04827	-2.80		Ribonuclease
	VIBHAR_05500	-2.04		Ribonuclease UK114
	VIBHAR_00721	-7.44		Ribonuclease activity regulator protein RraA
	VIBHAR_00593	-3.74	*rbn*	Ribonuclease BN
RNA-mediated regulation	VIBHAR_03444	-2.51		ATP-dependent RNA helicase HrpB
Transcription	VIBHAR_00223	-2.64		Anti-RNA polymerase sigma 70 factor
	VIBHAR_03676	-5.20		ATP-dependent helicase HepA
	VIBHAR_00225	-5.27	*rpoB*	DNA-directed RNA polymerase subunit beta
	VIBHAR_00527	-2.22		DNA-binding transcriptional regulator AsnC, Lrp/AsnC family transcriptional regulator, regulator for *asnA*, *asnC* and *gidA*
	VIBHAR_03542	-6.77		RNA polymerase sigma factor RpoE
	VIBHAR_05265	-4.55		RNA polymerase sigma factor SigZ
	VIBHAR_03664	-4.61		RNA polymerase factor sigma-54
	VIBHAR_01170	-3.54	*nusB*	Transcription antitermination protein NusB
	VIBHAR_01107	-7.83		Transcriptional regulator
	VIBHAR_06732	-2.32		
Translation	VIBHAR_01897	-2.89	*asnC*	Asparaginyl-tRNA synthetase
	VIBHAR_03369	-2.06		Elongation factor G
	VIBHAR_00055	-2.31		
	VIBHAR_00137	-3.39		Elongation factor P
	VIBHAR_03236	-8.39	*tsf*	Elongation factor Ts
	VIBHAR_00054	-2.32		Elongation factor Tu
	VIBHAR_00233	-2.18		
	VIBHAR_03381	-2.69	*prfC*	Peptide chain release factor 3
	VIBHAR_02333	-2.20		Ribosome modulation factor
tRNA biogenesis	VIBHAR_02293	-6.83		C32 tRNA thiolase
	VIBHAR_01068	-3.28	*hisS*	Histidyl-tRNA synthetase
	VIBHAR_01594	-2.02		Methyltransferase (orthology: tRNA (mo5U34)-methyltransferase)
	VIBHAR_03106	-2.13	*mnmC*	5-methylaminomethyl-2-thiouridine methyltransferase
	VIBHAR_01245	-3.07		Peptidyl-tRNA hydrolase
	VIBHAR_01046	-2.74	*tgt*	Queuine tRNA-ribosyltransferase
	VIBHAR_01053	-5.06		rRNA methylase
	VIBHAR_02114	-4.87		Thiopurine S-methyltransferase

	VIBHAR_00174	-2.76		tRNA-dihydrouridine synthase B
	VIBHAR_00095	-3.67	*miaA*	tRNA delta(2)-isopentenylpyrophosphate transferase
	VIBHAR_03590	-5.98	*trmB*	tRNA (guanine-N(7)-)-methyltransferase
	VIBHAR_00435	-3.95	*trmE*	tRNA modification GTPase TrmE
	VIBHAR_03521	-2.84	*truD*	tRNA pseudouridine synthase D
	VIBHAR_01045	-2.30	*queA*	S-adenosylmethionine--tRNA ribosyltransferase-isomerase
	VIBHAR_01193	-10.55	*queF*	7-cyano-7-deazaguanine reductase
tRNA charging	VIBHAR_01368	-2.85	*argS*	Arginyl-tRNA synthetase
	VIBHAR_01597	-2.67	*aspS*	Aspartyl-tRNA synthetase
	VIBHAR_03447	-2.43		Glutamyl-Q tRNA(Asp) synthetase
	VIBHAR_01271	-6.66	*gltX*	Glutamyl-tRNA synthetase
	VIBHAR_02079	-2.33		Threonyl-tRNA synthetase
	VIBHAR_03637	-2.96	*valS*	Valyl-tRNA synthetase
DNA repair				
Base excision repair	VIBHAR_02522	-3.29		3-methyladenine DNA glycosylase
DNA repair	VIBHAR_00483	-2.63		ATP-dependent DNA helicase
DNA synthesis and repair	VIBHAR_02456	-2.26		DNA polymerase III subunit epsilon
Recombination	VIBHAR_01025	-2.85	*rdgC*	Recombination associated protein
Homologous recombination	VIBHAR_03301	-3.75	*recB*	ATP-dependent exonuclease V subunit beta
	VIBHAR_03302	-3.31	*recC*	Exonuclease V subunit gamma
	VIBHAR_01601	-3.05	*ruvB*	Holliday junction DNA helicase RuvB
	VIBHAR_01598	-2.73	*ruvC*	Holliday junction resolvase
Mismatch repair	VIBHAR_01175	-10.35		Exodeoxyribonuclease VII small subunit
Nucleotide excision repair	VIBHAR_02961	-2.27		Excinuclease ABC subunit B
	VIBHAR_01528	-4.46		Transcription-repair coupling factor
Metal ion homeostasis				
Iron homeostasis	VIBHAR_03284	-5.33	*rpoE, fecI*	RNA polymerase sigma factor
	VIBHAR_03122	-2.08		
	VIBHAR_00052	-6.21		Bacterioferritin
	VIBHAR_00943	-2.58		Autonomous glycyl radical cofactor GrcA
	VIBHAR_00312	-8.47	*cyaY*	Frataxin-like protein
Molybdenum uptake and assimilation	VIBHAR_02953	-3.83		Pterin-4-alpha-carbinolamine dehydratase, molybdenum cofactor biosynthesis protein B
	VIBHAR_02260	-3.41		Putative bifunctional molybdopterin-guanine dinucleotide biosynthesis protein MobB/MoeA
	VIBHAR_02261	-4.43	*mobA*	Molybdopterin-guanine dinucleotide biosynthesis protein MobA
	VIBHAR_02951	-5.36	*moaD*	Molybdopterin synthase small subunit
	VIBHAR_02954	-3.82	*moaA*	Molybdenum cofactor biosynthesis protein A
	VIBHAR_02952	-4.93	*moaC*	Molybdenum cofactor biosynthesis protein MoaC
Transporters and ancillary factors				
ABC transporter	VIBHAR_00441	-5.83		ABC amino acid transporter periplasmic component
	VIBHAR_06396	-8.45		Sugar ABC transporter periplasmic protein
	VIBHAR_00439	-3.27		ABC polar amino acid transporter ATPase component
	VIBHAR_02364	-4.40		ABC-type polar amino acid transport system, ATPase
	VIBHAR_00493	-4.77		ABC-type dipeptide transporter periplasmic component

	VIBHAR_05403	-2.04		ABC transporter ATPase
	VIBHAR_00279	-2.17		ABC transporter ATPase
	VIBHAR_02335	-4.30		ABC transporter ATPase
	VIBHAR_02362	-4.64		ABC transporter permease
	VIBHAR_00495	-2.31		ABC transporter permease
	VIBHAR_00494	-2.82		ABC transporter permease
	VIBHAR_02262	-8.24		Cobalt ABC transporter ATPase
	VIBHAR_01200	-6.86	*metQ*	DL-methionine transporter substrate-binding subunit
	VIBHAR_06143	-4.18	*rbsB*	D-ribose transporter subunit RbsB (monosaccharide transporter)
	VIBHAR_04877	-4.82		Fe3+ ABC transporter periplasmic protein
	VIBHAR_05215	-3.65	*malF*	Maltose transporter membrane protein
	VIBHAR_05216	-4.52	*malG*	Maltose transporter permease
	VIBHAR_05214	-3.31	*malE*	Maltose ABC transporter periplasmic protein
	VIBHAR_05213	-4.59		Maltose/maltodextrin transporter ATP-binding protein
	VIBHAR_04881	-3.24		Molybdenum ABC transporter ModB
	VIBHAR_05139	-6.74		Molybdate ABC transporter permease
	VIBHAR_05449	-3.82	*modF*	Molybdenum transporter ATP-binding subunit ModF, molybdate transport system
	VIBHAR_01033	-2.27	*pstB*	Phosphate transporter ATP-binding protein
	VIBHAR_05140	-1.99		Phosphate ABC transporter ATP-binding protein
	VIBHAR_05138	-10.72		Phosphate ABC transporter permease
	VIBHAR_05137	-4.97		Phosphate ABC transporter periplasmic protein
	VIBHAR_00997	-2.89		Putative ABC transporter ATP-binding protein
	VIBHAR_02678	-2.67		Putative ABC transporter solute-binding protein
	VIBHAR_06144	-2.78	*rbsC*	Ribose ABC transporter permease
	VIBHAR_06937	-4.64		Sugar ABC transporter ATP-binding protein
	VIBHAR_06941	-4.25		Sugar ABC transporter periplasmic protein
	VIBHAR_06398	-2.58		Sugar ABC transporter permease
	VIBHAR_04880	-3.33		Spermidine/putrescine ABC transporter ATPase
Anaerobic C4-dicarboxylate transporter DcuA	VIBHAR_00155	-6.04		Anaerobic C4-dicarboxylate transporter
Peptide transport	VIBHAR_04898	-6.99		Dipeptide/tripeptide permease
Sugar transport	VIBHAR_05091	-6.58		L-lactate permease, lactate transporter, LctP family
	VIBHAR_00514	-2.25		Permease, gluconate:H+ symporter, GntP family
	VIBHAR_04848	-4.32		Transcriptional regulator MalT, DNA-binding transcriptional activator for the mal regulon and maltotriose-ATP-binding protein
Other transport related proteins	VIBHAR_03084	-2.08		Aquaporin Z
	VIBHAR_02455	-2.69		Choline/carnitine/betaine transporter
	VIBHAR_00120	-2.06		Permease EamA-like transporter family
Transport of hydrophobic molecules	VIBHAR_05664	-4.40		Lipocalin (outer membrane lipoprotein Blc)
Export of sodium ions	VIBHAR_03275	-2.40	*nqrA*	Na(+)-translocating NADH-quinone

				reductase subunit A Catalyzes transport of Na+ ions from the inside of a bacterial cell to the periplasmic space
Potassium:proton antiporter	VIBHAR_00068	-2.30		Glutathione-regulated potassium-efflux system protein KefB
PTS systems	VIBHAR_01308	-3.88		Phosphocarrier protein PtsH
	VIBHAR_01307	-4.36		Phosphoenolpyruvate-protein phosphotransferase, PtsI
	VIBHAR_01306	-5.65		PTS system glucose-specific transporter, Crr
	VIBHAR_02900	-5.13		PTS system glucose-specific transporter subunit IIBC, PtsG
	VIBHAR_01336	-2.98		PTS system, N-acetylglucosamine-specific IIABC component
	VIBHAR_05186	-2.82		PTS system nitrogen regulatory IIA component, PtsN
Bacterial secretion system	VIBHAR_00604	-2.19	*gspG*	General secretion pathway protein G
	VIBHAR_00869	-3.10	*tolC*	Outer membrane channel protein
	VIBHAR_01047	-5.77	*yajC*	Preprotein translocase subunit YajC
	VIBHAR_01709	-2.17		Type III secretion system protein
	VIBHAR_00909	-3.39	*secA*	Preprotein translocase subunit SecA
	VIBHAR_01049	-3.48	*secF*	Preprotein translocase subunit SecF
	VIBHAR_00567	-2.77	*tatC*	Sec-independent protein translocase protein, TatC
Nitrogen metabolism				
Nitrogen metabolism	VIBHAR_01341	-4.15		Flavodoxin FldA
	VIBHAR_05377	-3.78		Nitrate reductase catalytic subunit
	VIBHAR_07090	-2.80		Nitrogen regulation protein NtrX
	VIBHAR_05376	-2.52		Cytochrome c-type protein NapB
	VIBHAR_05375	-2.50		Cytochrome c-type protein NapC
	VIBHAR_02686	-3.83		Anaerobic nitric oxide reductase transcriptional regulator
	VIBHAR_05378	-4.87		NapD protein subunit of nitrate reductase
Nitrogen metabolism, oxidative phosphorylation	VIBHAR_06269	-4.06		cytochrome c oxidase subunit III
Nitrotoluene degradation	VIBHAR_06498	-2.23		NADH-flavin oxidoreductase
Metabolism of nitrosubstituted compounds	VIBHAR_00135	-7.95		Nitroreductase
Protein turnover and folding				
Protein degradation	VIBHAR_02344	-2.92	*pepN*	Aminopeptidase N
	VIBHAR_01419	-2.05	*lon*	ATP-dependent protease, Lon
	VIBHAR_00723	-5.32	*hslU*	ATP-dependent protease, ATP-binding subunit HslU
	VIBHAR_01417	-2.67	*clpP*	ATP-dependent Clp protease proteolytic subunit
	VIBHAR_00724	-11.15		ATP-dependent protease peptidase subunit, ATP-dependent HslUV protease, peptidase subunit HslV
	VIBHAR_02477	-2.67		Carboxypeptidase Taq
	VIBHAR_04872	-2.89		Cold-active alkaline serine protease
	VIBHAR_05872	-2.36		ClpA/B type protease
	VIBHAR_04979	-5.70		Elastase
	VIBHAR_06926	-2.68		Esterase
	VIBHAR_06870	-2.68		Oligopeptidase 1
	VIBHAR_06836	-3.59		Peptidase
	VIBHAR_06306	-8.25		
	VIBHAR_06837	-2.18		

	VIBHAR_03671	-2.08		Peptidase PmbA
	VIBHAR_06184	-2.02		Prolidase (proline dipeptidase)
	VIBHAR_06914	-2.90		Prolyl oligopeptidase
	VIBHAR_03193	-2.55		Protease
	VIBHAR_00091	-2.00		Serine protease
	VIBHAR_05222	-1.98		Thioesterase
	VIBHAR_00526	-2.48		Zn-dependent oligopeptidase
	VIBHAR_04891	-3.23		Zinc-carboxypeptidase
Protein export	VIBHAR_00566	-5.92		Sec-independent translocase
Protein translocation	VIBHAR_06666	-3.15		Phosphatase
	VIBHAR_00123	-5.93		Preprotein translocase subunit SecB
	VIBHAR_01048	-4.26	*secD*	Preprotein translocase subunit SecD
	VIBHAR_01049	-3.47	*secF*	Preprotein translocase subunit SecF
	VIBHAR_00565	-3.79	*tatA*	Twin arginine translocase protein A
	VIBHAR_01610	-2.30	*tolB*	Translocation protein TolB
Protein folding	VIBHAR_01135	-3.07		Chaperone protein DnaJ
	VIBHAR_00142	-10.32	*groEL*	Chaperonin GroEL
	VIBHAR_00143	-12.26	*groES*	Co-chaperonin GroES
	VIBHAR_00404	-2.46		Disulfide bond formation protein, thiol:disulfide interchange protein DsbA
	VIBHAR_00446	-11.00		Heat shock protein, molecular chaperone IbpA
	VIBHAR_01134	-16.21	*dnaK*	Molecular chaperone DnaK
	VIBHAR_01416	-3.62	*tig*	Trigger factor
Protein maturation	VIBHAR_00391	-2.94	*def*	Peptide deformylase
	VIBHAR_01421	-3.09		Peptidyl-prolyl cis-trans isomerase
	VIBHAR_07023	-2.93		peptidyl-prolyl cis-trans isomerase B, (cyclophilin B)
	VIBHAR_00066	-7.02		Peptidyl-prolyl cis-trans isomerase
	VIBHAR_00957	-3.10		Protein-disulfide isomerase
Protein repair	VIBHAR_03048	-3.75	*msrB*	Methionine sulfoxide reductase B
Repair of protein damage	VIBHAR_03519	-2.61	*pcm*	Protein-L-isoaspartate O-methyltransferase
Periplasmic protein biogenesis	VIBHAR_00156	-3.13	*dipZ*	Thiol:disulfide interchange protein
Energy production				
Oxidative phosphorylation	VIBHAR_03274	-2.13		Na(+)-translocating NADH-quinone reductase subunit B
	VIBHAR_00428	-2.35		F0F1 ATP synthase subunit A
	VIBHAR_00426	-4.64		F0F1 ATP synthase subunit B
	VIBHAR_06106	-6.05		F0F1 ATP synthase subunit alpha
	VIBHAR_00421	-5.41		F0F1 ATP synthase subunit beta
	VIBHAR_00424	-5.44		F0F1 ATP synthase subunit delta
	VIBHAR_00420	-3.72	*atpC*	F0F1 ATP synthase subunit epsilon
	VIBHAR_00591	-3.95		ATPase
	VIBHAR_00479	-3.91		ATP synthase subunit ETA
	VIBHAR_00429	-2.12		F0F1 ATP synthase subunit I
	VIBHAR_00423	-6.35		F0F1 ATP synthase subunit alpha
	VIBHAR_06104	-5.19		F0F1 ATP synthase subunit beta
	VIBHAR_00427	-4.30		F0F1 ATP synthase subunit C
	VIBHAR_00422	-6.12		F0F1 ATP synthase subunit gamma
	VIBHAR_06270	-3.26		Cytochrome C oxidase assembly protein
	VIBHAR_02301	-3.27		Cytochrome c oxidase subunit CcoQ
	VIBHAR_06271	-4.11	*coxA*	Cytochrome c oxidase subunit I
	VIBHAR_06272	-8.17	*coxB*	Cytochrome c oxidase subunit II
	VIBHAR_00879	-2.12		Cytochrome d ubiquinol oxidase subunit III
	VIBHAR_05546	-4.68		Electron transfer flavoprotein-ubiquinone

				oxidoreductase
	VIBHAR_02274	-13.48		Ferredoxin
	VIBHAR_00434	-7.64		Flavodoxin, FMN-binding protein MioC
	VIBHAR_05806	-7.19		Formate dehydrogenase, anaerobic metabolism
	VIBHAR_00132	-5.07	*frdB*	Fumarate reductase iron-sulfur subunit, FrdB
	VIBHAR_00784	-8.39		Inorganic pyrophosphatase
	VIBHAR_06175	-2.22		NAD(P)H-dependent flavin reductase, FAD-dependent and NAD(P)H-dependent enzymes able to metabolize nitrosubstituted compounds
	VIBHAR_00402	-3.82		NADH:ubiquinone oxidoreductase subunit I
	VIBHAR_03273	-2.64		Na(+)-translocating NADH-quinone reductase subunit C
	VIBHAR_03270	-3.11		Na(+)-translocating NADH-quinone reductase subunit F
	VIBHAR_01860	-2.97		Putative manganese-dependent inorganic pyrophosphatase
	VIBHAR_00885	-3.71		Ubiquinol-cytochrome c reductase
	VIBHAR_00884	-4.89		Ubiquinol-cytochrome c reductase, cytochrome b
Oxidative phosphorylation & porphyrin and chlorophyll metabolism	VIBHAR_06264	-3.40		Protoheme IX farnesyltransferase
Electron-transport	VIBHAR_04984	-4.41		Cytochrome b562
Stress adaptation and responses				
Stress response	VIBHAR_00532	-8.95		Universal stress protein
Oxidative stress defense	VIBHAR_05229	-2.38		Ferredoxin/oxidoreductase predicted 2Fe-2S cluster-containing protein; 6-N-hydroxylaminopurine resistance protein
	VIBHAR_05192	-2.45		Catalase
	VIBHAR_01773	-3.62		Catylase/peroxidase
	VIBHAR_01773	-2.54		
	VIBHAR_01510	-4.25		Ferredoxin
	VIBHAR_00036	-2.39		Glutaredoxin
	VIBHAR_02635	-2.58	*grxA*	Glutaredoxin, redox coenzyme for ribonucleotide reductase (RNR1a)
	VIBHAR_05596	-2.23		Glutathione S-transferase
	VIBHAR_05193	-6.40		
	VIBHAR_06617	-3.72		Lactoylglutathione lyase
	VIBHAR_01039	-5.91		Peroxiredoxin (alkyl hydroperoxide reductase subunit C)
	VIBHAR_06431	-3.39		Putative glutathione S-transferase YghU
	VIBHAR_03014	-4.15		Superoxide dismutase
	VIBHAR_05698	-2.56		Thioredoxin 2
	VIBHAR_03189	-2.59	*bcp*	Thioredoxin-dependent thiol peroxidase
	VIBHAR_00348	-3.25		Gamma-glutamyltransferase
	VIBHAR_00523	-6.76		Glutathione reductase
	VIBHAR_03635	-5.07	*pepA*	Leucyl aminopeptidase
Cold-shock protein inhibiting translation	VIBHAR_03665	-6.00		Ribosome-associated protein Y, putative sigma-54 modulation protein
DNA protection from stress	VIBHAR_00213	-2.14		Histone-like DNA-binding protein
Multidrug resistance	VIBHAR_06903	-5.32		Histidine kinase
Membrane and peptidoglycan biogénesis				
Beta-barrel outer	VIBHAR_01070	-3.13		Outer membrane protein assembly

membrane protein biogenesis				complex subunit YfgL
Terpenoid backbone biosynthesis	VIBHAR_01174	-6.33		Geranyltranstransferase
	VIBHAR_04924	-3.32		Isopentenyl pyrophosphate isomerase
	VIBHAR_00798	-5.81		Octaprenyl-diphosphate synthase
	VIBHAR_01173	-4.46		1-deoxy-D-xylulose-5-phosphate synthase
	VIBHAR_03522	-2.30	*ispF*	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase
	VIBHAR_03523	-3.11	*ispD*	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase
	VIBHAR_00983	-4.77	*ispH*	4-hydroxy-3-methylbut-2-enyl diphosphate reductase
Peptidoglycan biogenesis	VIBHAR_06653	-4.79		D-alanyl-D-alanine carboxypeptidase
	VIBHAR_03466	-2.48	*amiD*	N-acetyl-anhydromuranmyl-L-alanine amidase
	VIBHAR_04852	-5.96	*mtgA*	Monofunctional biosynthetic peptidoglycan transglycosylase
	VIBHAR_05410	-3.93	*ldcA*	Muramoyltetrapeptide carboxypeptidase
	VIBHAR_07057	-2.47	*mpaA*	Murein peptide amidase A, MpaA
Isoprenoid biosynthesis	VIBHAR_05413	-5.68		Isoprenoid biosynthesis protein with amidotransferase-like domain
Membrane biogenesis	VIBHAR_03186	-4.17		Lipoprotein
Membrane potential	VIBHAR_06233	-3.13		Chloride channel protein
Putative membrane receptor	VIBHAR_06904	-2.95		Outer membrane protein W
Control of membrane proteins	VIBHAR_01813	-7.69		Heat shock protein HtpX (Membrane-localized protease)
Miscellaneous functions				
Adenylation of glutamate	VIBHAR_00867	-4.05		Bifunctional glutamine-synthetase adenylyltransferase / deadenyltransferase
Anti-sigma factor	VIBHAR_03541	-2.70		Negative regulator of sigma E activity, RseA
Arsenite oxidase	VIBHAR_05169	-7.02		Azurin
Cell attachment	VIBHAR_06691	-2.33	*csgA*	C factor cell-cell signaling protein
Cell division	VIBHAR_00905	-3.89	*ftsA*	Cell division protein FtsA
	VIBHAR_00906	-5.80		Cell division protein FtsZ
	VIBHAR_01311	-5.35		Cell division protein ZipA
	VIBHAR_01588	-2.29		Condesin subunit E Orthology: chromosome partition protein MukE
Chemotaxis	VIBHAR_03140	-2.24		Chemotaxis-specific methylesterase
	VIBHAR_04951	-2.00	*fliA*	DNA-directed RNA polymerase sigma subunit/flagellum specific
	VIBHAR_01288	-3.52	*flgD*	Flagellar basal body rod modification protein
	VIBHAR_01281	-2.26	*flgA*	Flagellar basal body P-ring biosynthesis protein FlgA
	VIBHAR_01286	-2.49	*flgB*	Flagellar basal body rod protein FlgB
	VIBHAR_01287	-2.20	*flgC*	Flagellar basal body rod protein FlgC
	VIBHAR_03163	-4.02	*fliE*	Flagellar hook-basal body protein FliE
	VIBHAR_01289	-3.35	*flgE*	Flagellar hook protein FlgE
	VIBHAR_04950	-2.78		Flagellar motor protein MotA
	VIBHAR_01176	-4.28		Flagellar motor protein PomA
	VIBHAR_01177	-3.95	*motB*	Flagellar motor protein MotB
	VIBHAR_03153	-2.52	*fliN*	Flagellar motor switch protein
	VIBHAR_03154	-2.09	*fliM*	Flagellar motor switch protein FliM

	VIBHAR_03170	-3.95		Flagellar protein FlaG
	VIBHAR_03167	-2.04	*fliS*	Flagellar protein FliS
	VIBHAR_03171	-3.07		Flagellin
	VIBHAR_01300	-3.81		
Folate metabolism	VIBHAR_01391	-3.49		Bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase/ 5,10-methylene-tetrahydrofolate cyclohydrolase
	VIBHAR_00047	-2.47	*metF*	5,10-methylenetetrahydrofolate reductase
Galactose metabolism	VIBHAR_01858	-7.08		Alpha/beta hydrolase
Oxidoreductase	VIBHAR_00355	-3.20		NADH dehydrogenase
	VIBHAR_05194	-5.60		Oxidoreductase
	VIBHAR_05303	-12.09		
Pantothenate and CoA biosynthesis	VIBHAR_03450	-4.36	*panB*	3-methyl-2-oxobutanoate hydroxymethyltransferase
Phosphonate and phosphinate metabolism	VIBHAR_04876	-5.97		2-aminoethylphosphonate--pyruvate transaminase
	VIBHAR_04874	-7.87		Phosphonoacetaldehyde hydrolase
Porphyrin and Chlorophyll metabolism	VIBHAR_00581	-5.90		Coproporphyrinogen III oxidase
	VIBHAR_00570	-6.08	*hemB*	Delta-aminolevulinic acid dehydratase,
	VIBHAR_03417	-3.41		Glutamate-1-semialdehyde, HemL aminotransferase
	VIBHAR_00314	-2.44	*hemC*	Porphobilinogen deaminase, HemC
	VIBHAR_06129	-11.62	*hemE*	Uroporphyrinogen decarboxylase
	VIBHAR_00316	-2.82		Uroporphyrin-III C methyltransferase
	VIBHAR_00765	-2.19		
	VIBHAR_00315	-4.00	*hemD*	Uroporphyrinogen-III synthase
Quorum sensing	VIBHAR_06242	-2.20	*luxA*	Alkanal monooxygenase subunit alpha
	VIBHAR_00299	-6.58	*rhtB*	Homoserine / homoserine lactone efflux protein
	VIBHAR_02960	-2.88		miscRNA quorum regulatory RNA Qrr
Recycling of muropeptides during cell elongation and / or cell división	VIBHAR_03593	-7.34		Lytic murein transglycosylase
Redox protein	VIBHAR_02782	-3.44		Coenzyme A disulfide reductase
	VIBHAR_03272	-2.80		Na(+)-translocating NADH-quinone reductase subunit D
	VIBHAR_03271	-3.04		Na(+)-translocating NADH-quinone reductase subunit E
Reductive cleavage of azo bond in aromatic azo compounds	VIBHAR_02502	-3.20	*azoR*	Azoreductase, FMN-dependent NADH-azoreductase
Riboflavin metabolism	VIBHAR_01169	-4.72	*ribH*	6,7-dimethyl-8-ribityllumazine synthase
	VIBHAR_01168	-5.56		3,4-dihydroxy-2-butanone 4-phosphate synthase
	VIBHAR_01166	-2.30		Pyrimidine deaminase
	VIBHAR_01167	-2.77		Riboflavin synthase subunit alpha
Sulfur metabolism	VIBHAR_00005	-2.22		Sulfite reductase (NADPH) flavoprotein subunit alpha
	VIBHAR_00004	-2.84		Sulfite reductase subunit beta
	VIBHAR_00003	-2.98		Phosphoadenosine phosphosulfate reductase
Signal transduction (Two-component system)	VIBHAR_06241	-2.44	*luxB*	Alkanal monooxygenase subunit beta, luciferase operon
	VIBHAR_01582	-2.48	*torD*	Chaperone protein TorD
	VIBHAR_01583	-4.35		DNA-binding transcriptional regulator TorR
	VIBHAR_05292	-3.21		Histidine kinase

	VIBHAR_02702	-4.40		Histidine kinase (two-component system, NarL family, sensor kinase)
	VIBHAR_03530	-3.28		Hybrid sensory histidine kinase BarA
	VIBHAR_06240	-2.01	*LuxE*	Long-chain-fatty-acid ligase
	VIBHAR_00585	-3.34	*glnL*	Nitrogen regulation protein NR(II)
	VIBHAR_00621	-4.53	*ompR*	Osmolarity response regulator
	VIBHAR_05729	-13.41		Putative diguanylate ciclase, PleD
	VIBHAR_05381	-3.77		Transcriptional dual regulator NarL NarQ-NarP (nitrate respiration)
	VIBHAR_05158	-2.16	*cpxR*	Transcriptional regulatory protein CpxR
	VIBHAR_00157	-16.85		Transcriptional regulator LuxR
Ubiquinone and other terpenoid –quinine biosynthesis	VIBHAR_00564	-6.05	*ubiB*	Putative ubiquinone biosynthesis protein UbiB
Ubiquinone and other terpenoid - quinone biosynthesis, tyrosine metabolism, phenylalanine metabolism	VIBHAR_02177	-6.93		4-hydroxyphenylpyruvate dioxygenase
Ubiquinone and other terpenoid –quinone biosynthesis	VIBHAR_01432	-2.94		O-succinylbenzoate synthase, MenC
	VIBHAR_01430	-5.80		Acyl-CoA thioester hydrolase YfbB, MenH
	VIBHAR_00722	-2.46		1,4-dihydroxy-2-naphthoate octaprenyltransferase, MenA
	VIBHAR_01431	-4.51		Naphthoate synthase, MenB
	VIBHAR_01232	-2.24		2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase, UbiF
	VIBHAR_03549	-2.07		2-octaprenyl-6-methoxyphenol hydroxylase, UbiH
	VIBHAR_05419	-2.39	*ubiE*	Ubiquinone / menaquinone biosynthesis methyltransferase
Vitamin B6 metabolism	VIBHAR_07092	-7.79		Pyridoxamine 5'-phosphate oxidase
Not known				
Not known	VIBHAR_02663	-4.89		Acetyltransferase
	VIBHAR_00595	-3.53		
	VIBHAR_05632	-2.40		
	VIBHAR_05101	-2.98		
	VIBHAR_04762	-2.79		Alkyl hydroperoxide reductase c22 protein
	VIBHAR_00807	-4.16	*apaG*	ApaG protein
	VIBHAR_00431	-3.03		Chromosome partitioning ATPase
	VIBHAR_02633	-5.07		DNA-binding transcriptional regulator HexR
	VIBHAR_00590	-7.51		GTPase
	VIBHAR_01244	-2.82		GTP-dependent nucleic acid- binding protein EngD
	VIBHAR_00317	-5.29		Heme biosynthesis protein
	VIBHAR_05646	-2.69		Histidine kinase
	VIBHAR_04770	-2.61		Hydrolase
	VIBHAR_05147	-3.93		MoxR-like ATPase
	VIBHAR_06230	-3.06		Oxidoreductase
	VIBHAR_01197	-5.30		Phosphatase/nucleotidase
	VIBHAR_00821	-24.06		Phospholipid phosphatase
	VIBHAR_00852	-3.36		Putative DNA-binding/iron metalloprotein/AP endonuclease
	VIBHAR_04983	-8.28	*ycdX*	Putative hydrolase
	VIBHAR_01228	-3.37		Putative metalloprotease
	VIBHAR_02347	-4.07		Putative solute / DNA competence effector

	VIBHAR_00255	-11.92		RNA binding protein
	VIBHAR_06253	-3.36		Sensory transduction protein kinase
	VIBHAR_01913	-2.73		Signal transduction histidine kinase
	VIBHAR_00559	-2.78		Signal transduction protein
	VIBHAR_00122	-5.38		Sulfurtransferase
	VIBHAR_00618	-3.15		Transcriptional accessory protein
	VIBHAR_05195	-3.28		Transcriptional regulator
	VIBHAR_02423	-9.26		
	VIBHAR_06999	-2.38		
	VIBHAR_05858	-5.17		
Transport	VIBHAR_00531	-2.36		Permease
	VIBHAR_00118	-2.59		Sodium-dependent transporter

Table S2. *V. harveyi* genes upregulated during adaptation to cold seawater (12 h *versus* 5 min). Annotation of the genes and their products is according to the classification used in the KEGG database (http://www.genome.jp/).

Biological pathway	Systematic name	Fold change	Gene Name	Gene product
Amino acid metabolism				
Alanine, aspartate and glutamate metabolism	VIBHAR_03095	3.68		Amidophosphoribosyltransferase
	VIBHAR_01332	3.30	*asnB*	Asparagine synthetase B
	VIBHAR_03543	16.85		L-aspartate oxidase
Alanine, aspartate and glutamate metabolism & arginine and proline biosynthesis	VIBHAR_00040	3.64		Bifunctional argininosuccinate lyase/N-acetylglutamate synthase
Amino acid metabolism	VIBHAR_01796	3.78		Leucine-responsive transcriptional regulator (Lrp/AsnC family transcriptional regulator, leucine-responsive regulatory protein)
Amino acid transport	VIBHAR_05335	76.86		Inner membrane protein YjeH putative amino acid efflux transporter
Arginine and proline biosynthesis	VIBHAR_00042	2.69		Acetylglutamate kinase
	VIBHAR_00044	2.36		Acetylornithine deacetylase
	VIBHAR_06738	2.70		Agmatinase
	VIBHAR_06737	3.01		Arginine decarboxylase
	VIBHAR_00794	6.63		Arginine repressor
	VIBHAR_05528	3.82	*artP*	Arginine transporter ATP-binding subunit
	VIBHAR_05531	4.32	*artM*	Arginine transporter permease subunit ArtM
	VIBHAR_00043	3.88	*argC*	N-acetyl-gamma-glutamyl-phosphate reductase
	VIBHAR_04833	2.27		Ornithine decarboxylase
	VIBHAR_02383	8.16		Response regulator
	VIBHAR_05719	2.81		Aromatic amino acid aminotransferase (Alanine, aspartate and glutamate metabolism)
	VIBHAR_02289	1.95	*potA*	Putrescine/spermidine ABC transporter ATPase
	VIBHAR_02288	3.33	*potB*	Spermidine / putrescine ABC transporter membrane protein
Cystein metabolism	VIBHAR_01793	20.67	*cysB*	Transcriptional regulator CysB, a LysR family transcriptional regulator
D-alanine metabolism	VIBHAR_00017	5.07	*alr*	Alanine racemase
Glycine, serine and threonine metabolism	VIBHAR_03711	7.38		Aspartate kinase III
	VIBHAR_01183	2.52		DNA-binding transcriptional activator, GcvA LysR family transcriptional regulator, glycine cleavage system transcriptional activator
	VIBHAR_06354	4.63		D-serine deaminase is equivalent to D-serine dehydratase
	VIBHAR_06355	5.19		
	VIBHAR_02609	2.76		D-serine dehydratase
	VIBHAR_00940	2.18		Homoserine kinase
	VIBHAR_02611	2.04		Transcriptional regulator LysR family transcriptional regulator, D-serine deaminase activator
	VIBHAR_05968	3.11		Glycine cleavage system protein T2
Histidine biosynthesis	VIBHAR_01835	18.23		1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase
	VIBHAR_01830	16.81	*hisG*	ATP phosphoribosyltransferase
	VIBHAR_01837	10.14		Bifunctional phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP

				pyrophosphatase
	VIBHAR_01831	22.61	*hisD*	Histidinol dehydrogenase
	VIBHAR_01832	17.12		Histidinol-phosphate aminotransferase
	VIBHAR_01836	16.48		Imidazole glycerol phosphate synthase subunit HisF
	VIBHAR_01834	19.23	*hisH*	Imidazole glycerol phosphate synthase subunit HisH
	VIBHAR_01833	20.24		Imidazole glycerol-phosphate dehydratase/histidinol phosphatase
	VIBHAR_06625	4.88		Sensory histidine kinase CreC
Lysine degradation	VIBHAR_00205	2.35		Lysine decarboxylase
Phenylalanine, tyrosine and tryptophan biosynthesis	VIBHAR_00028	2.09	*aroK*	Shikimate kinase I
Polar amino acid transport	VIBHAR_05950	16.86		Amino acid ABC transporter ATP-binding protein
Thiamine metabolism	VIBHAR_00364	2.93		Adenylyltransferase, ThiS
	VIBHAR_01055	10.90		Cysteine desulfurase
	VIBHAR_06838	2.54		Phosphomethylpyrimidine kinase
	VIBHAR_00366	2.19		Thiamine biosynthesis protein ThiC
	VIBHAR_00365	2.50	*thiE*	Thiamine-phosphate pyrophosphorylase
Valine, leucine and isoleucine biosynthesis	VIBHAR_00816	2.42		3-isopropylmalate dehydrogenase
	VIBHAR_00410	6.17		Acetolactate synthase 2 catalytic subunit
	VIBHAR_00411	3.99	*ilvM*	Acetolactate synthase 2 regulatory subunit
	VIBHAR_00412	3.11		branched-chain amino acid aminotransferase
	VIBHAR_00477	25.15		DNA-binding transcriptional regulator IlvY (LysR family transcriptional regulator, positive regulator for ilvC)
Purine / Pyrimidine metabolism				
Purine metabolism	VIBHAR_06421	96.23		Adenosine deaminase
	VIBHAR_00313	4.00	*cyaA*	Adenylate cyclase
	VIBHAR_00089	3.14		Adenylosuccinate synthetase
	VIBHAR_00613	3.86	*nudE*	ADP-ribose diphosphatase NudE
	VIBHAR_00627	11.53		Bifunctional (p)ppGpp synthetase II/ guanosine-3',5'-bis pyrophosphate 3'-pyrophosphohydrolase
	VIBHAR_00210	6.11	*purH*	Bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
	VIBHAR_01077	2.37	*guaA*	GMP synthase
	VIBHAR_00339	2.47		Guanosine pentaphosphate phosphohydrolase
	VIBHAR_00630	26.96	*gmk*	Guanylate kinase
	VIBHAR_00211	2.58		Phosphoribosylamine-glycine ligase
	VIBHAR_01159	20.54		Xanthine-guanine phosphoribosyltransferase
Pyrimidine metabolism	VIBHAR_05319	1.99		Cytosine deaminase
	VIBHAR_05320	2.77	*codB*	Cytosine permease
	VIBHAR_06413	63.08		Dihydroorotase
	VIBHAR_00971	2.08	*thyA*	Thymidylate synthase
	VIBHAR_02924	2.19		Uridine kinase
Xanthine / Uracil transport	VIBHAR_01158	310.62		Permease
Central carbon metabolism				
Citrate cycle	VIBHAR_02395	4.40		aconitate hydratase
	VIBHAR_00536	16.38		Oxidoreductase Fumarate reductase flavoprotein subunit
	VIBHAR_05539	15.90		
	VIBHAR_06495	2.80		
Glucose metabolism	VIBHAR_06346	2.10		2-keto-3-deoxy-6-phosphogluconate aldolase

	VIBHAR_06348	2.19		
Glycolysis	VIBHAR_05341	2.69		Glyceraldehyde-3-phosphate dehydrogenase
Glyoxylate and dicarboxylate metabolism, one carbon pull by folate	VIBHAR_05174	2.58		Phosphoglycolate phosphatase
Pentose phosphate pathway	VIBHAR_00719	2.64	*glpX*	Fructose 1,6-bisphosphatase II
	VIBHAR_02429	2.24		Glucose-6-phosphate 1-dehydrogenase
Pyruvate metabolism	VIBHAR_05640	3.36		Lactoylglutathione lyase
	VIBHAR_02963	5.54		Pyruvate-formate lyase
Starch and sucrose metabolism	VIBHAR_04858	7.25	*malS*	Periplasmic alpha-amylase
Sugar metabolism	VIBHAR_03702	2.27		Regulatory protein CsrD
Amino sugar and nucleotide sugar metabolism	VIBHAR_05945	2.62		Chitinase
	VIBHAR_07002	3.24	*nagB*	Glucosamine-6-phosphate deaminase
	VIBHAR_05593	8.24		UDP-N-acetylglucosamine pyrophosphorylase
Lipid biogenesis				
Biosynthesis of unsaturated fatty acids	VIBHAR_06094	3.99		Acyl-CoA desaturase
Fatty acid biosynthesis	VIBHAR_03226	2.82	*fabZ*	(3R)-hydroxymyristoyl-ACP dehydratase
	VIBHAR_05699	2.91		3-oxoacyl-ACP synthase
	VIBHAR_05256	2.45	*fabG*	short-chain dehydrogenase / reductase SDR
Fatty acid metabolism	VIBHAR_06607	7.85		Enoyl-CoA hydratase
	VIBHAR_02929	8.41		Fatty acid metabolism regulator
	VIBHAR_01631	2.01		Acetyl-CoA acetyltransferase
Glycerophospholipid metabolism	VIBHAR_03232	12.93		Phosphatidate cytidylyltransferase
Lipopolysacharide biosynthesis	VIBHAR_03227	5.80	*lpxD*	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase
	VIBHAR_03225	2.95		UDP-N-acetylglucosamine acyltransferase
	VIBHAR_00664	2.16		3-deoxy-D-manno-octulosonic-acid kinase
	VIBHAR_01538	3.54		3-deoxy-manno-octulosonate cytidylyltransferase
	VIBHAR_01536	5.41	*lpxK*	Tetraacyldisaccharide 4'-kinase
Lipoprotein biosynthesis	VIBHAR_01226	21.27	*lnt*	Apolipoprotein N-acyltransferase
Processing of genetic information				
DNA relication	VIBHAR_00442	5.54	*dnaA*	Chromosomal replication initiation protein
	VIBHAR_03558	4.06		Chromosome replication initiation inhibitor protein
	VIBHAR_00727	2.66		Primosome assembly protein PriA
	VIBHAR_00018	4.21		Replicative DNA helicase
	VIBHAR_03211	2.01	*rnhA*	Ribonuclease H
	VIBHAR_03223	2.05	*rnhB*	Ribonuclease HII
DNA uptake	VIBHAR_00614	2.56		Putative DNA uptake protein
Ribosome biogenesis	VIBHAR_03277	2.16		16S RNA G1207 methylase RsmC
	VIBHAR_03479	2.08	*rimM*	16S rRNA-processing protein RimM
	VIBHAR_00084	1.92		23S rRNA (guanosine-2'-O-)-methyltransferase
	VIBHAR_03529	1.92	*rumA*	23S rRNA 5-methyluridine methyltransferase
	VIBHAR_01005	4.93	*rluD*	23S rRNA pseudouridine synthase D
	VIBHAR_00736	3.51		30S ribosomal protein S3
	VIBHAR_00747	3.90	*rpsE*	30S ribosomal protein S5
	VIBHAR_00744	6.06	*rpsH*	30S ribosomal protein S8
	VIBHAR_00729	10.19	*rpsJ*	30S ribosomal protein S10
	VIBHAR_00743	5.58	*rpsN*	30S ribosomal protein S14

	VIBHAR_03480	1.98	*rpsP*	30S ribosomal protein S16
	VIBHAR_00739	2.45	*rpsQ*	30S ribosomal protein S17
	VIBHAR_00734	4.49		30S ribosomal protein S19
	VIBHAR_00733	6.29	*rplB*	50S ribosomal protein L2
	VIBHAR_00730	9.36	*rplC*	50S ribosomal protein L3
	VIBHAR_00731	8.30	*rplD*	50S ribosomal protein L4
	VIBHAR_00742	4.67	*rplE*	50S ribosomal protein L5
	VIBHAR_00745	6.00	*rplF*	50S ribosomal protein L6
	VIBHAR_00740	3.69	*rplN*	50S ribosomal protein L14
	VIBHAR_00749	2.97	*rplO*	50S ribosomal protein L15
	VIBHAR_00737	2.71	*rplP*	50S ribosomal protein L16
	VIBHAR_00746	4.76	*rplR*	50S ribosomal protein L18
	VIBHAR_03477	2.20	*rplS*	50S ribosomal protein L19
	VIBHAR_00735	4.57	*rplV*	50S ribosomal protein L22
	VIBHAR_00732	7.22	*rplW*	50S ribosomal protein L23
	VIBHAR_00741	2.92	*rplX*	50S ribosomal protein L24
	VIBHAR_02662	5.21		50S ribosomal protein L25
	VIBHAR_00738	2.43		50S ribosomal protein L29
	VIBHAR_00748	3.37	*rpmD*	50S ribosomal protein L30
	VIBHAR_01071	7.32	*engA*	GTP-binding protein EngA
	VIBHAR_00267	83.58		O-methyltransferase
	VIBHAR_02918	6.07		Ribonuclease E
	VIBHAR_03681	1.99		Ribonuclease G
	VIBHAR_03536	2.31	*rnc*	Ribonuclease III
	VIBHAR_06087	2.35	*rbgA*	Ribosomal biogenesis GTPase
	VIBHAR_02655	1.93		Ribosomal small subunit pseudouridine synthase A
	VIBHAR_05581	25.77		Ribosomal-protein-alanine acetyltransferase
	VIBHAR_00574	6.50	*engB*	Ribosome biogenesis GTP-binding protein YsxC
	VIBHAR_01218	2.11		rRNA large subunit methyltransferase
RNA folding	VIBHAR_00522	2.25		Cold shock protein (beta-ribbon, CspA family)
RNA polymerase-associated protein	VIBHAR_00886	10.05	*sspA*	Stringent starvation protein A (amino acid starvation)
RNA turnover	VIBHAR_05125	5.58		ATP-dependent RNA helicase DEAD-box helicase
Transcription	VIBHAR_00848	3.14		RNA polymerase sigma factor RpoD
Translation	VIBHAR_03538	6.61		GTP-binding protein LepA (Elongation factor)
	VIBHAR_00093	4.11		Putative GTPase HflX
	VIBHAR_00384	2.93		Translation factor
Trans-translation	VIBHAR_01123	1.72		tmRNA
tRNA biogenesis	VIBHAR_01193	4.59	*queF*	7-cyano-7-deazaguanine reductase
	VIBHAR_02293	1.96		C32 tRNA thiolase
	VIBHAR_03478	2.45	*trmD*	tRNA (guanine-N(1)-)-methyltransferase
	VIBHAR_00626	11.61		tRNA guanosine-2'-O-methyltransferase
	VIBHAR_03241	2.36		tRNA pseudouridine synthase C
	VIBHAR_01791	3.75		tRNA-hydroxylase
	VIBHAR_00450	8.45	*glyQ*	glycyl-tRNA synthetase subunit alpha
	VIBHAR_01842	4.83	*cysS*	cysteinyl-tRNA synthetase
	VIBHAR_00449	6.37	*glyS*	glycyl-tRNA synthetase subunit beta
Small RNAs	VIBHAR_00893	2.22		M1 RNA, RNA component of RNase P
	VIBHAR_03553	2.50		6S / SsrS RNA (sRNA)
	VIBHAR_00349	1.93	*miscRNA*	CsrB/RsmB RNA family (sRNA)
	VIBHAR_00573	44.47		Spot 42 RNA (sRNA)
Anaerobic transcription	VIBHAR_02295	3.58	*fnr*	Fumarate/nitrate reduction transcriptional regulator

DNA repair				
Translesion, DNA synthesis	VIBHAR_00268	16.69		LexA repressor Other SOS response factors
Non-homologous recombination	VIBHAR_03262	3.11		ATP-dependent DNA helicase RecQ (RecFor pathway proteins)
	VIBHAR_00445	3.23	*gyrB*	DNA gyrase subunit B (Facilitator)
Nucleotide excision repair	VIBHAR_03087	2.81		DNA polymerase III subunits gamma and tau
	VIBHAR_00351	7.90	*uvrD*	DNA-dependent helicase II
	VIBHAR_00629	2.90		DNA-directed RNA polymerase subunit kappa/omega
	VIBHAR_03708	6.08	*uvrA*	Excinuclease ABC subunit A
Recombination	VIBHAR_01799	7.85		Recombination factor protein RarA
Homologous recombination	VIBHAR_00625	3.96		ATP-dependent DNA helicase RecG
	VIBHAR_03513	7.14	*recA*	Recombinase A (RecFOR pathway proteins)
	VIBHAR_00444	4.71	*recF*	Recombination protein F
	VIBHAR_03512	7.92		Recombination regulator RecX
DNA repair	VIBHAR_05122	2.24		Deoxyribodipyrimidine photolyase
	VIBHAR_05654	3.07		DNA repair exonuclease
	VIBHAR_02747	15.64		Excinuclease ABC subunit C
	VIBHAR_01130	7.06		Recombination and repair protein
	VIBHAR_00956	1.99	*xerD*	Site-specific tyrosine recombinase XerD
	VIBHAR_03571	4.91		Endonuclease I
Base excision repair	VIBHAR_00217	2.34		Uracil-DNA glycosylase
Mismatch excision repair	VIBHAR_00443	5.99		DNA polymerase III subunit beta
Metal iron homeostasis				
Iron uptake	VIBHAR_00053	10.06		Bacterioferritin-associated ferredoxin
Iron uptake, storage and utilization	VIBHAR_01059	8.69		Chaperone protein HscA
	VIBHAR_01058	11.43		Co-chaperone HscB
Zinc uptake	VIBHAR_00010	3.32		Zinc uptake regulation protein
Co2+ and Mg2+ efflux	VIBHAR_00807	2.19	*apaG*	ApaG protein
Transporters and ancillary factors				
ABC transporter	VIBHAR_06570	2.63		ABC amino acid transporter periplasmic ligand binding protein
	VIBHAR_06023	2.31		ABC efflux transporter permease / ATP-binding protein
	VIBHAR_05951	11.26		ABC transporter ATP-binding protein/permease (Polar amino acid transport)
	VIBHAR_01552	2.24		ABC transporter permease, AotM
	VIBHAR_05241	2.77		ABC-type hemin transport system periplasmic protein (iron complex)
	VIBHAR_07029	47.74		ABC-type transporter ATPase component (K02004+K02003 system)
	VIBHAR_00921	6.08		ABC-type vitamin B12-transporter protein BtuF (BtuF)
	VIBHAR_01549	2.75		Amino acid ABC transporter, ATP-binding protein, AotP
	VIBHAR_05530	2.77		Arginine transporter permease subunit ArtQ
	VIBHAR_07028	37.62		Efflux ABC transporter ATP-binding/permease
	VIBHAR_06134	5.45		Enterochelin ABC transporter permease, FhuB
	VIBHAR_06564	3.72		Glycine betaine ABC transporter ATP-binding protein (ProV)
	VIBHAR_06563	4.42		Glycine betaine/L-proline ABC transporter permease (ProW)
	VIBHAR_03132	3.47		Heme exporter protein D, CcmD
	VIBHAR_06840	3.88		Nitrate / sulfonate/bicarbonate ABC transporter permease, ThiX

	VIBHAR_06839	3.86		Nitrate/sulfonate/bicarbonate ABC transporter ATPase, ThiZ
	VIBHAR_05588	2.35		Nitrate/sulfonate/bicarbonate ABC transporter periplasmic protein
	VIBHAR_06841	2.27		Nitrate/sulfonate/bicarbonate transport systems, periplasmic proteins,ThiY
	VIBHAR_02946	3.17	*oppD*	Oligopeptide ABC transporter ATP-binding protein
	VIBHAR_02948	4.27	*oppB*	Oligopeptide transporter permease
	VIBHAR_00788	2.39	*tbpA*	Thiamine transporter substrate binding subunit
	VIBHAR_02132	2.53		Vitamin B12-transporter permease (BtuC)
Control of membrane fluidity	VIBHAR_06161	3.25		C-5 sterol desaturase
Transport of C4-dicarboxylates during anaerobic growth	VIBHAR_06095	2.39	*dcuC*	C4-dicarboxylate transporter DcuC
Translocation of lipoproteins from the inner membrane to the outer membrane	VIBHAR_01248	4.43	*lolB*	Outer membrane lipoprotein LolB
	VIBHAR_01532	2.55		Outer membrane-specific lipoprotein transporter subunit LolE
	VIBHAR_01798	5.48	*lolA*	Outer-membrane lipoprotein carrier protein
Iron transport	VIBHAR_00638	12.86		Iron transport protein
	VIBHAR_00345	12.06		Permease
	VIBHAR_00395	2.07		K+ transport system protein
Energy-dependent transport TonB-ExbB-ExbD complex	VIBHAR_00636	9.66		Biopolymer transport protein ExbB
	VIBHAR_00635	10.06		
	VIBHAR_00634	9.12		Biopolymer transport protein ExbD
	VIBHAR_00633	8.86		Periplasmic protein TonB
Control of Na+/H+ balance	VIBHAR_01078	3.00		Na+/H+ antiporter
	VIBHAR_01828	8.01		
Electron transfer	VIBHAR_06497	1.96		Glutaredoxin
Bacterial secretion system, protein export	VIBHAR_05362	2.50	*secF*	Preprotein translocase subunit SecF
Threonine efflux	VIBHAR_06605	3.55		Putative threonine efflux protein
	VIBHAR_00347	4.47		Threonine efflux system
Nitrogen metabolism				
Nitrogen fixation protein NifU and related proteins	VIBHAR_01056	8.77		Scaffold protein
Nitrogen metabolism	VIBHAR_06412	75.48		Carbonic anhydrase
	VIBHAR_00087	4.63		Nitric oxide dioxygenase
Nitric oxide-sensitive transcriptional repressor	VIBHAR_00086	7.12		Transcriptional repressor NsrR
Protein turnover and folding				
Protein biogenesis	VIBHAR_05306	8.46		Thiol:Disulfide interchange protein DsbA
	VIBHAR_02331	3.57		ATP-dependent protease
	VIBHAR_05814	2.06		Esterase
	VIBHAR_03388	2.89		Protease
	VIBHAR_00624	3.17		Prolyl oligopeptidase
Protein folding	VIBHAR_05559	2.84		Putative chaperone
Protein translocation	VIBHAR_03537	2.05		Signal peptidase
Protein turnover	VIBHAR_03238	2.04		Methionine aminopeptidase
	VIBHAR_05576	90.01		Serine proteinase
Energy production				
Oxidative	VIBHAR_03135	4.24		Cytochrome c biogenesis protein CcmA

phosphorylation	VIBHAR_03131	2.24		Cytochrome c-type biogenesis protein CcmE
	VIBHAR_02965	4.48		Electron transport complex protein RnfB
	VIBHAR_02967	2.92	*rnfD*	Electron transport complex protein RnfD
	VIBHAR_06108	2.06		ATP synthase subunit B
	VIBHAR_02966	3.83		Electron transport complex protein RnfC
	VIBHAR_06109	3.16		F0F1 ATP synthase subunit C
Stress responses				
Envelope stress	VIBHAR_03539	3.39		Positive regulator of sigma E activity
	VIBHAR_04764	5.84		Redox protein, regulator of disulfide bond formation
Oxidative stress response	VIBHAR_00035	10.61		DNA-binding transcriptional regulator OxyR
	VIBHAR_00741	2.96		Peroxiredoxin
Trans-translation (Stress protein)	VIBHAR_01124	3.40	*smpB*	SsrA-binding protein
Glutathione metabolism	VIBHAR_05534	4.23		Glutathione S-transferase
	VIBHAR_01618	2.70		Glutathionylspermidine synthase
Trehalose operon repressor	VIBHAR_01204	2.82	*treR*	Trehalose repressor
Membrane and peptidoglycan biogenesis				
Terpenoid backbone biosynthesis	VIBHAR_03231	4.73		1-deoxy-D-xylulose 5-phosphate reductoisomerase
	VIBHAR_01247	4.57	*ipk*	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
	VIBHAR_03233	5.37		Undecaprenyl diphosphate synthase
The outer membrane protein assembly machine	VIBHAR_03229	10.06		Outer membrane protein assembly factor YaeT
Multidrug resistance	VIBHAR_00266	9.04		Multidrug efflux pump
Peptidoglycan biosynthesis	VIBHAR_01217	4.53		Cell division protein FtsI/penicillin-binding protein 2, MrdA
	VIBHAR_06597	3.10		Alpha-1,2-mannosidase
Miscellaneous functions				
Antibiotic resistance	VIBHAR_00158	23.16		Multiple antibiotic transporter
Benzoate degradation	VIBHAR_00333	1.97		Phenol hydroxylase
	VIBHAR_05532	5.36		Protocatechuate 3,4-dioxygenase subunit beta protein
Biotin metabolism	VIBHAR_01806	6.08		Adenosylmethionine-8-amino-7-oxononanoate aminotransferase
	VIBHAR_00240	14.57		biotin--protein ligase BirA family transcriptional regulator, biotin operon repressor / biotin-[acetyl-CoA-carboxylase] ligase
	VIBHAR_01809	2.27		Methyltransferase (malonyl-CoA O-methyltransferase)
Butanoate metabolism	VIBHAR_05420	2.11		Acetoacetyl-CoA synthetase
Cell division	VIBHAR_01797	5.95		Cell division protein FtsK
	VIBHAR_02914	2.10		Maf-like protein is likely involved in inhibition of septum formation
Chemotaxis	VIBHAR_01283	3.12		Chemotaxis methyltransferase CheR
	VIBHAR_00011	2.35		chemotaxis protein CheX
	VIBHAR_04911	3.25	*flgD*	Flagellar basal body rod modification protein
	VIBHAR_00274	4.30		Flagellar basal body-associated protein FliL-like protein
	VIBHAR_01300	2.45		Flagellin
	VIBHAR_01301	2.99		
	VIBHAR_01302	2.29		
	VIBHAR_03173	2.64		

Folate biosynthesis	VIBHAR_03403	2.86		Dihydropteroate synthase
	VIBHAR_03098	2.58		Folylpolyglutamate synthase
Geraniol degradation	VIBHAR_02638	8.40		Acyl-CoA dehydrogenase
Morphology	VIBHAR_03685	2.54		Rod shape-determining protein MreB
	VIBHAR_03684	7.46		Rod shape-determining protein MreC
	VIBHAR_03683	4.90		Rod shape-determining protein MreD
Oxidation of phenolic compounds	VIBHAR_00368	2.32		Laccase
Porphyrin and chlorophyll metabolism	VIBHAR_05724	2.12		Cobyric acid synthase
	VIBHAR_00383	2.10		Coproporphyrinogen III oxidase
	VIBHAR_00892	21.82		Corrin/porphyrin methyltransferase
	VIBHAR_00332	2.10	*fre*	FMN reductase
	VIBHAR_05583	2.08		Siroheme synthase
Signal transduction	VIBHAR_01913	2.07		Signal transduction histidine kinase
	VIBHAR_07025	4.73		Histidine kinase
Sodium / Proton balance	VIBHAR_02930	5.16	*nhaB*	Sodium/proton antiporter
Signal transduction (Two-component system)	VIBHAR_05690	2.11		Anaerobic C4-dicarboxylate transporter DcuB (fumarate)
	VIBHAR_00887	13.52		ClpXP protease specificity-enhancing factor
	VIBHAR_06112	2.14		Transcriptional regulatory protein CpxR
	VIBHAR_00933	2.18		Two-component response regulator, ArcA
Ubiquinone and other terpenoid –quinone biosynthesis	VIBHAR_00334	4.63	*ubiD*	3-polyprenyl-4-hydrozybenzoate decarboxylase
	VIBHAR_00272	2.35	*ubiA*	4-hydroxybenzoate octaprenyltransferase
	VIBHAR_00273	2.53		Chorismate lyase
Pathogenic factor in other Vibrio species	VIBHAR_00046	2.67		Transcriptional regulator, PadR family transcriptional regulator, regulatory protein AphA
Not known				
Not known	VIBHAR_00127	2.72		Acetyltransferase
	VIBHAR_05103	7.15		
	VIBHAR_02654	25.75		Bicyclomycin/multidrug efflux system protein
	VIBHAR_04978	2.05		Diguanylate cyclase/phosphodiesterase
	VIBHAR_00603	3.02		General secretion pathway protein F
	VIBHAR_00306	4.00		Hydrolase
	VIBHAR_06337	10.57		Modulator of drug activity B
	VIBHAR_06353	4.04		N-acyl-D-glutamate deacylase protein
	VIBHAR_00389	37.91		Nucleotide-binding protein
	VIBHAR_06386	4.69		Peptidase
	VIBHAR_00646	7.44		Permease
	VIBHAR_00074	5.09		Phosphoribulokinase
	VIBHAR_06895	4.13		Putative glycosyl hydrolase
	VIBHAR_00455	6.19		Transcriptional regulators
	VIBHAR_00830	6.60		
	VIBHAR_01389	2.82		
	VIBHAR_05092	2.75		
	VIBHAR_05259	2.14		
	VIBHAR_05356	4.28		
	VIBHAR_05418	2.07		
	VIBHAR_05594	5.52		
	VIBHAR_05843	3.78		
	VIBHAR_06369	1.91		
	VIBHAR_06494	2.17		
	VIBHAR_06568	1.99		
	VIBHAR_06683	4.60		
	VIBHAR_06958	3.23		
	VIBHAR_07024	4.73		

	VIBHAR_07031	7.69		
	VIBHAR_06604	4.95		
	VIBHAR_00297	1.91		Transcriptional regulator ArsR family transcriptional regulator
	VIBHAR_08189	6.57		Transposase
	VIBHAR_06736	6.04		Zinc metallohydrolase

Table S3. Primers used for quantitative real-time PCR.

Systematic name	Gene product	Forward primer (5' to 3')	Reverse primer (5' to 3')	R^2	Efficiency*
VIBHAR_01158	Putative MFS transporter, AGZA family, xanthine / uracil permease	CTTTCTCTACGCACTGGTATCT	CGTTGCTGGGTTGTCTACTA	0.976	0.85
VIBHAR_07028	Efflux ABC transporter ATP-binding / permease	GTAATGGCGTTAGTGGTCTTTG	GCTACCAAGTGCTGATAATGTG	0.976	1.02
VIBHAR_00268	LexA repressor	GTGAAGCGTTTAGAGCGTAAAG	CGATGTTAGGTCAACTTGGATTG	0.997	0.96
VIBHAR_03105	3-oxoacyl-ACP synthase, FabB	CGCAGTTCGTTGTATGAAGATG	GCTCTTTCACGTCACCTACT	0.990	1.03
VIBHAR_01134	Molecular chaperone DnaK	CTTGGTGGTGAAGACTTTGATAC	CGCTAGTGGGTCGTTCTTTA	0.993	1.07
VIBHAR_01833 (*hisB*)	Imidazole glycerol-phosphate dehydratase / histidinol phosphatase	GACCGTGATGGCACCTTAAT	CGACAACAGGCTTGGGATAA	0.999	0.99
VIBHAR_00729 (*rpsJ*)	30S ribosomal protein S10	CTCTCCACACGTTAACAAGAAAG	GACGCATTAGAGCATCAACAG	0.996	0.99
VIBHAR_06412	Carbonic anhydrase	CCGTGGTGTTTAATGCTGTG	CATGGAAATTCTCTGGCAAGTC	0.999	1.01
VIBHAR_00573	Spot42 (sRNA)	TCTCGTAGGGTACAGAGGTAAG	CTGAATCAGCCTAATCCAATAACG	0.998	0.99
VIBHAR_00588 (*glnA*)	Glutamine synthetase	GGCTGGTCTTGACGGTATTAAG	TGGGATTTCTGCTGCTTCTTC	0.996	0.97
VIBHAR_00630	Guanylate kinase	CGTCAAATCCGTGAGCAAATG	TGTCTTGACCACGAGTGTTTAG	0.995	0.96
VIBHAR_00627	Bifunctional (p)ppGpp synthetase II	GCTCTGCGTCAATCTTATGTG	CGACTGGGTGGATGATGTAT	0.996	1.01
VIBHAR_01159	Xanthine-guanine phosphoribosyl-transferase	CTACGTGAAATGTACCCGAAAG	GTGTCTTGAGCGATGTCTACTA	0.998	1.03
VIBHAR_03309	2,3,4,5-tetrahydropyridine-2,6-carboxylate N-succinyltransferase	GCATGGTTGAAGGTCGTATCTC	CCAGATAGCGTACCCATGATAGA	0.994	0.95
VIBHAR_04858 (*malS*)	Periplasmic alpha-amylase	CTACGCTTACCACGGTTACT	CCTCTTCCACCAGCAATTTAAG	0.993	1.01
VIBHAR_03229	Outer membrane protein assembly factor YaeT / Omp85 /BamA	CATGGACACGTAACAACCTAAAC	GCCAGGTACGGTCATCTTATAG	0.997	1.02
VIBHAR_00633	Periplasmic protein TonB	TGGTTCAGTACTTGGTCACTAC	GAGGCATTTGAGGTTGGAAAG	0.999	0.97
VIBHAR_00638	Iron transport protein	CAGGTGGTAGTGCAGAATGTA	CTGGTGGTTAAGGCTAGTTAGG	0.994	0.93
VIBHAR_00254	16S ribosomal RNA	CGGTGGAGCATGTGGTTTAAT	GCACCAATCCATCTCTGGAAAG	0.999	1.04
	***The qPCR amplification efficiency was calculated for each primer pair using the slope of the standard curve obtained with serial dilutions of a concentrated cDNA sample using the following equation: Efficiency (E)=[10(-1/slope)]-1**				

Table S4. Total RNA was isolated in triplicate from *V. harveyi* cells after 5 min and 12 h incubation in cold seawater, and the differences in the level of individual transcripts were determined by qRT-PCR using the gene-specific primers listed in Table S3. The comparative Ct method (Livak, K.J. & Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) method. Methods 25:402-408) was used to calculate fold change in expression level of individual transcripts. Further statistical analysis was performed by using the unpaired T-test.

Systematic name	**Fold Change** (absolute)	**Log2 Fold Change**	**Regulation** (12 h *versus* 5 min)	**P-Value**
VIBHAR_01158	29.37961	4.87674	**up**	2.02E-05
VIBHAR_07028	101.47777	6.66502	**up**	0.0001183
VIBHAR_00268	8.29353	3.05199	**up**	0.0002046
VIBHAR_03105	-33.92788	-5.0844	**down**	0.0004162
VIBHAR_01134	-51.00178	-5.67248	**down**	0.0009135
VIBHAR_01833	10.14587	3.34282	**up**	0.0010808
VIBHAR_00729	4.63747	2.21334	**up**	0.0012411
VIBHAR_06412	87.28644	6.44769	**up**	0.0013318
VIBHAR_00573	59.55411	5.89613	**up**	0.0016205
VIBHAR_00588	-12.01373	-3.58661	**down**	0.0026475
VIBHAR_00630	32.43951	5.01968	**up**	0.0033051
VIBHAR_00627	6.01847	2.5894	**up**	0.0063703
VIBHAR_01159	4.61004	2.20478	**up**	0.0069932
VIBHAR_03309	-18.16101	-4.18277	**down**	0.0127378
VIBHAR_04858	5.09099	2.34795	**up**	0.0262371
VIBHAR_03229	2.67609	1.42013	**up**	0.0463068
VIBHAR_00633	7.22226	2.85245	**up**	0.0753135
VIBHAR_00638	7.45341	2.8979	**up**	0.0774724

Table S5. Average bacterial dimensions of *V. harveyi* cells examined before (control) and after (12 hours) incubation in cold seawater by epifluorescence microscopy.

The measurement of bacterial size was performed as described by Massana *et al.* (1997). Briefly. the cells were stained with acridine orange and placed on 0.2 µm black filters (Milipore) to capture cell images by a Nikon epifluorescence microscope equipped with a B-2A filter block (EX450-490 excitation filter. DM510 dichroic mirror. and BA520 barrier filter) and a video camera (Hamamatsu 2400. Hamamatsu Photonics. Hamamatsu City. Japan). The microscopic fields were directly visualized on the computer screen. Three independent areas of very flat fields with the representative number of bacteria (150-200) and absence of very bright particles were captured and processed by using the Scion Image 1.62ª software. The average length. width as well as number of randomly selected cells analyzed in each independent experiment are indicated.

Experiment	Control	12 h
1	Length = 2.030 ± 0.834 µM Width = 0.539 ± 0.141 µM Cell number = 150	Length = 1.988 ± 0.486 µM Width = 0.532 ± 0.118 µM Cell number = 150
2	Length = 2.266 ± 0.980 µM Width = 0.512 ± 0.132 µM Cell number = 210	Length = 2.052 ± 0.1176 µM Width = 0.492 ± 0.1243 µM Cell number = 200
3	Length = 2.196 ± 0.773 µM Width = 0.534 ± 0.199 µM Cell number = 200	Length = 2.108 ± 0.527 µM Width = 0.529 ± 0.183 µM Cell number = 200

Reference

Massana. R., Gasol. J. M., Bjørnsen. P. K., Blackburn. N., Hagstrom. Å., Hietanen. S., Hygum. B. H., Kuparinen. J. and Pedrós-Alió. C. (1997) Measurement of bacterial size via image analysis of epifluorescence preparations: Description of an inexpensive system and solutions to some of the most common problems. *Scientia Marina*. *61*. 397–407.

Supplementary data for Chapter 2

Table S1. Highly up- (↑) and downregulated (↓) genes grouped according to their putative functions *in vivo*. Annotation of the genes and their products is according to the classification used in the KEGG database (http://www.genome.jp/). Assignment to the specific gene cluster is indicated in accordance to the results of gene clustering presented in Fig. 3 of the main text.

Systematic name	Gene name	Gene product (specific biological pathway)	Fold change (time after exposure to seawater)	Gene expression cluster
Cell envelope stress				
Phage-shock-protein response				
VIBHAR_00008	*(pspG)*	Phage shock protein G	32.51 ↑ (5 min)	1
VIBHAR_01872	*(pspA)*	Phage shock protein A	18.81 ↑ (5 min)	1
VIBHAR_01873	*(pspB)*	Phage shock protein B	18.26 ↑ (5 min)	1
VIBHAR_01874	*(pspC)*	Phage shock protein C	14.62↑ (5 min)	1
Cell wall metabolism				
VIBHAR_00989	*(murQ)*	N-acetylmuramic acid-6-phosphate etherase (cell wall recycling)	4.87 ↑ (6 days)	7
VIBHAR_00998		Soluble lytic murein transglycosylase (growth and recycling of peptidoglycan)	4.56 ↑ (6 days)	7
VIBHAR_03362		Lytic murein transglycosylase (growth and recycling of peptidoglycan)	4.18 ↑ (6 days)	7
Lipid biogenesis				
Glycerophospholipid metabolism				
VIBHAR_06662		Hypothetical patatin-like phospholipase	6.44 ↑ (6 days)	7
VIBHAR_06957		Hypothetical esterase/lipase	14.87 ↑ (5 min)	5
VIBHAR_02696		Triacylglycerol lipase	5.47 ↑ (5 min)	5
VIBHAR_06845		Phosphatidylglycerophosphatase B	5.91 ↑ (5 min)	13
VIBHAR_02464		Phosphoglycerol transferase (biosynthesis of osmoregulated periplasmic glucans)	5.66↑ (5 min)	13
VIBHAR_00269		Glycerol-3-phosphate acyltransferase (phospholipid biosynthesis)	4.28 ↑ (5 min)	13
VIBHAR_00216		Phosphoglycerate dehydrogenase	5.17 ↓ (6 days)	12
VIBHAR_05290		Diacylglycerol kinase	8.09 ↑ (5 min)	5
VIBHAR_06179	*(betA)*	Choline dehydrogenase	20.23 ↑	5

	(betA)		(5 min)	
VIBHAR_06159			6.90 ↑ (5 min)	4
VIBHAR_06181		Transcriptional regulator BetI	43.13↑ (5 min)	1
Metabolism of glycerophosphodiesters				
VIBHAR_03310	*(glpQ)*	Glycerophosphodiester phosphodiesterase	16.78 ↓ (6 days)	9
VIBHAR_06394	*(glpR)*	Repressor of the glycerol-3-phosphate regulon	42.38 ↓ (6 days)	9
VIBHAR_06395		Glycerophosphoryl diester phosphodiesterase UgpQ	56.99 ↓ (6 days)	9
Fatty Acid Degradation				
VIBHAR_00824	*(fadD)*	Putative long-chain acyl-CoA synthetase	3.07 ↑ (6 days)	7
VIBHAR_00459	*(fadA)*	3-ketoacyl-CoA thiolase	23.43 ↑ (5 min)	5
VIBHAR_03121	*(fadI)*		13.012 ↑ (5 min)	
VIBHAR_05482		Acyl-CoA thiolase	13.07 ↑ (12 h)	10
VIBHAR_02638		Acyl-CoA dehydrogenase	91.60 ↑ (5 min)	1
VIBHAR_05549		Acyl-CoA dehydrogenase. short-chain specific	3.32 ↑ (12 h)	10
VIBHAR_01815		Short chain dehydrogenase	3.38 ↑ (6 days)	7
VIBHAR_00460	*(fadB)*	Multifunctional fatty acid oxidation complex subunit alpha	18.79 ↑ (5 min)	5
VIBHAR_03120	*(fadJ)*	Multifunctional fatty acid oxidation complex subunit alpha	29.10 ↑ (5 min)	5
			2.09 ↑ (6 days)	7
VIBHAR_02639		Transcriptional regulator PsrA	81.11 ↑ (5 min)	1
VIBHAR_03204	*(fadE)*	Acyl-CoA dehydrogenase	24.88 ↑ (5 min)	1
			16.79 ↑ (6 days)	
VIBHAR_03043	*(fadH)*	2.4-dienoyl-CoA reductase	22.85 ↑ (5 min)	1
			25.73 ↑	5
VIBHAR_05817	*(fadL)*	Long-chain fatty acid outer membrane transporter FadL	9.35 ↑ (5 min)	5
VIBHAR_02398	*(fadR)*	FadR homologue	60.42 ↑ (5 min)	5
VIBHAR_06094		Acyl-CoA desaturase	4.59 ↑ (5 min)	13
VIBHAR_06715		Hydroxyglutarate oxidase	10.58 ↑ (5 min)	5
			15.56↓	

			(6 days vs 5 min)	
Fatty acid biosynthesis				
VIBHAR_02907	*(acpP)*	Acyl carrier protein	19.83 ↓ (6 days)	8
VIBHAR_02907	*(acpP)*	Acyl carrier protein	31.51 ↓ (6 days)	9
VIBHAR_00171		Acetyl-CoA carboxylase biotin carboxyl carrier protein subunit	13.46 ↓ (6 days)	8
VIBHAR_00172		Acetyl-CoA carboxylase biotin carboxylase subunit	9.31 ↓ (6 days)	8
VIBHAR_02906	*(fabF)*	3-oxoacyl-(acyl carrier protein) synthase II	16.84 ↓ (6 days)	8
VIBHAR_06461	*(fabG)*	3-oxoacyl-(acyl carrier protein) reductase FabG	14.79 ↓ (6 days) 4.78 ↓ (5 min)	9 3
VIBHAR_03105	*fabB*	3-oxoacyl-[acyl-carrier-protein] synthase I	12.65 ↓ (6 days) 7.06 ↓ (5 min)	9 3
VIBHAR_02909	*(fabD)*	Malonyl CoA-acyl carrier protein transacylase	16.25 ↓ (6 days)	9
VIBHAR_02332	*(fabA)*	3-hydroxydecanoyl-(acyl carrier protein) dehydratase	3.80 ↓ (5 min) 4.35 ↓ (12 h) 6.56 ↓ (6 days)	6 & 12
VIBHAR_02910	*(fabH)*	3-oxoacyl-(acyl carrier protein) synthase III	8.09 ↓ (6 days)	12
Lipid A biosynthesis				
VIBHAR_03227	*(lpxD)*	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase	4.18 ↓ (6 days)	11
Lipoprotein biosynthesis				
VIBHAR_01215	*(rlpA)*	Hypothetical rare lipoprotein A	4.61 ↓ (6 days)	11
VIBHAR_00970	*(lgt)*	Prolipoprotein diacylglyceryl transferase	5.57 ↓ (6 days)	
Acetyl-CoA-dependent metabolism				
Butanoate metabolism				
VIBHAR_05365	*(phhB)*	Acetoacetyl-CoA reductase	15.21 ↓ (6 days)	9
VIBHAR_05366	*(phaA)*	Acetyl-CoA acetyltransferase	19.78 ↓ (6 days)	9
VIBHAR_05367	*(phaP)*	PHA granule-associated protein	296.47 ↓ (6 days)	9
Ethanol biosynthesis / biodegradation				
VIBHAR_03018	*(adhE)*	Bifunctional acetaldehyde-CoA / alcohol dehydrogenase	18.51 ↓ (6 days)	8
VIBHAR_03018	*(adhE)*	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase	22.70 ↓ (6 days)	9

VIBHAR_05412	*(adhB)*	Alcohol dehydrogenase	4.64↓ (5 min)	9
			7.14 ↓ (12 h)	3
			21.24 ↓ (6 days)	
Acetate biosynthesis (ATP production)				
VIBHAR_00169		Acetyl-CoA synthetase (generation of ATP from AMP and pyrophos-phate)	8.33 ↑ (5 min)	5
VIBHAR_01635			5.17 ↑ (5 min)	
Energy production				
VIBHAR_05548		Electron transfer flavoprotein subunit beta	4.99 ↑ (6 days)	7
VIBHAR_05546		Electron transfer flavoprotein-ubiquinone oxidoreductase	3.07 ↑ (6 days)	7
VIBHAR_00355		NADH dehydrogenase	6.93↓ (6 days)	12
VIBHAR_06269		Cytochrome c oxidase. subunit III	5.14 ↓ (5 min)	6
VIBHAR_06270		Cytochrome C oxidase assembly protein	4.38 ↓ (5 min)	6
VIBHAR_06271		Cytochrome c oxidase. subunit I	4.75 ↓ (5 min)	6
VIBHAR_06272		Cytochrome c oxidase. subunit II	4.60 ↓ (6 days)	12
VIBHAR_01602		Cytochrome d ubiquinol oxidase. subunit I	6.35 ↓ (6 days)	3
VIBHAR_01603		Cytochrome d ubiquinol oxidase. subunit II	8.03 ↓ (6 days)	3
VIBHAR_05492		Cytochrome o ubiquinol oxidase. subunit I	3.89 ↓ (5 min)	6
VIBHAR_05466		Cbb3-type cytochrome oxidase subunit I	4.15 ↓ (5 min)	6
ATP synthesis coupled proton transport				
VIBHAR_00423	*(atpA)*	F0F1 ATP synthase subunit alpha	16.68 ↓ (6 days)	8
VIBHAR_00428	*(atpB1)*	F0F1 ATP synthase subunit A	16.36 ↓ (6 days)	9
VIBHAR_00420	*(atpC)*	F0F1 ATP synthase subunit epsilon	10.08 ↓ (6 days)	8
VIBHAR_00421	*(atpD1)*	F0F1 ATP synthase subunit beta	18.56 ↓ (6 days)	9
VIBHAR_06104	*(atpD2)*	F0F1 ATP synthase subunit beta	13.58 ↓ (6 days)	8
VIBHAR_00427	*(atpE)*	F0F1 ATP synthase subunit C	21.79 ↓ (6 days)	8
VIBHAR_00426	*(atpF1)*	F0F1 ATP synthase subunit B	22.39 ↓ (6 days)	9
VIBHAR_00424	*(atpH1)*	F0F1 ATP synthase subunit delta	19.28 ↓ (6 days)	8

Fumarate reductase (complex II)				
VIBHAR_01351	*(sdhC)*	Succinate dehydrogenase cytochrome b556 large membrane subunit	22.08 ↓ (6 days)	9
VIBHAR_01352	*(sdhD)*	Putative succinate dehydrogenase hydrophobic membrane anchor protein	24.19 ↓ (6 days)	9
VIBHAR_01354	*(sdhB)*	Succinate dehydrogenase iron-sulfur subunit	28.83 ↓ (6 days)	9
			8.69 ↓ (6 days)	3
VIBHAR_00131	*(frdA)*	Fumarate reductase flavoprotein subunit	25.82 ↓ (6 days)	9
VIBHAR_00132	*(frdB)*	Fumarate reductase iron-sulfur subunit	20.59 ↓ (6 days)	9
VIBHAR_00133	*(frdC)*	Fumarate reductase subunit C	25.58 ↓ (6 days)	9
VIBHAR_00134	*(frdD)*	Fumarate reductase subunit D	15.17 ↓ (6 days)	9
VIBHAR_06362		Formate dehydrogenase. major subunit	12.51 ↓ (12 h)	3
VIBHAR_01860		Putative manganese-dependent inorganic pyrophosphatase	4.27 ↑ (5 min)	13
Amino acid biosynthesis				
VIBHAR_01182		GcvB RNA	231.01 ↓ (6 days)	9
Amino acid recycling				
VIBHAR_01332	*(asnB)*	Asparagine synthetase B (interconversion of asparagines to aspartate)	4.11 ↑ (5 min)	13
VIBHAR_03710		Alanine-glyoxylate transaminase / serine-glyoxylate transaminase / serine-pyruvate transaminase	8.74 ↑ (5 min)	5
VIBHAR_05483		Methylmalonate-semialdehyde dehydrogenase (degradation of amino acids)	9.68 ↑ (12 h)	10
Alanine degradation				
VIBHAR_01795		Alanine dehydrogenase	29.60 ↓ (6 days)	3
Arginine and proline metabolism				
VIBHAR_03644	*(arcA)*	Arginine deiminase (urea cycle)	21.44 ↑ (6 days)	2
VIBHAR_03645	*(argF)*	Ornithine transcarbamoylase (urea cycle)	14.74 ↓ (6 days)	8
VIBHAR_00912	*(carA)*	Carbamoyl phosphate synthase small subunit (urea cycle)	22.26 ↓ (6 days)	8
VIBHAR_00912	*(carA)*	Carbamoyl phosphate synthase small subunit CarA (urea cycle)	24.21 ↓ (6 days)	9
VIBHAR_00913	*(carB)*	Carbamoyl phosphate synthase large subunit (urea cycle)	16.55 ↓ (6 days)	8
VIBHAR_00041	*(argG)*	Argininosuccinate synthase (urea cycle)	23.81 ↓ (6 days)	9
VIBHAR_00040		Bifunctional argininosuccinate lyase/N-acetylglutamate synthase (urea cycle)	9.64 ↓ (12 h)	3

			5.27 ↓ (12 h)	3
VIBHAR_06738		Agmatinase (urea cycle)	5.83 ↓ (5 min)	3
			9.05 ↓ (6 days)	
VIBHAR_00042	*(argB)*	Acetylglutamate kinase (urea cycle)	27.10 ↓ (6 days)	9
VIBHAR_00043	*(argC)*	N-acetyl-gamma-glutamyl-phosphate reductase ArgC (urea cycle)	14.68 ↓ (6 days)	9
			16.44 ↓ (12 h)	3
Glutamate metabolism				
VIBHAR_02342		Glutamate dehydrogenase	5.54 ↓ (6 days)	12
VIBHAR_00926	*(gltB)*	Glutamate synthase subunit alpha	5.96 ↓ (6 days)	3
VIBHAR_00928		Glutamate synthase. large subunit	27.32 ↓ (6 days)	9
			11.05 ↓ (6 days)	3
VIBHAR_00927	*(gltD)*	Glutamate synthase subunit beta	19.52 ↓ (6 days)	9
			13.51 ↓ (6 days)	3
VIBHAR_07098		Delta 1-pyrroline-5-carboxylate dehydrogenase (proline biosynthesis)	5.11 ↓ (6 days)	3
Serine biosynthesis/degradation				
VIBHAR_02609	*(dsdA)*	D-serine dehydratase	18.90 ↑ (5 min)	5
VIBHAR_01908		L-serine dehydratase 1	30.25 ↓ (6 days)	9
VIBHAR_01909		Serine transporter	38.23↓ (6 days)	9
VIBHAR_00216		Phosphoglycerate dehydrogenase (serine biosynthesis)	5.18 ↓ (6 days)	12
Valine, leucine and isoleucine biosynthesis / degradation				
VIBHAR_00823	*(leuO)*	Leucine transcriptional activator	5.12 ↓ (5 min)	3
VIBHAR_00478		Ketol-acid reductoisomerase	3.70 ↓ (6 days)	12
VIBHAR_00826	*(ilvH)*	Acetolactate synthase 3 regulatory subunit	6.60 ↓ (6 days)	12
VIBHAR_05487		3-hydroxyisobutyrate dehydrogenase	9.68 ↓ (6 days)	3
VIBHAR_00477	*(ilvY)*	DNA-binding transcriptional regulator IlvY	5.66 ↑ (6 days)	4
Breakdown of tyrosine. phenylalanine and tryptophan				
VIBHAR_02177		4-hydroxyphenylpyruvate dioxygenase	24.21 ↓ (12 h)	3
VIBHAR_02178		Homogentisate 1.2-dioxygenase	19.42 ↓	3

			(12 h)	
VIBHAR_02179		2-oxopent-4-enoate hydratase	21.97 ↓ (12 h)	3
VIBHAR_02180		Maleylacetoacetate isomerase	22.42 ↓ (12 h)	3
VIBHAR_06709	*(tnaA)*	Tryptophanase	13.14 ↓ (5 min) 16.05 ↓ (12 h)	3
Stringent response				
VIBHAR_00886	*(sspA)*	Stringent starvation protein A	5.96↑ (6 days)	7
VIBHAR_00339	*(gppA)*	Guanosine pentaphosphate phosphohydrolase	1.92↑ (6 days)	10
VIBHAR_00627	*(spoT)*	Bifunctional (p)ppGpp synthetase II / guanosine-3'.5'-bis pyrophosphate 3'-pyrophosphohydrolase	2.31↓ (6 days)	11
Purine /pyrimidine biosynthesis				
VIBHAR_06142		Ribokinase (purine biosynthesis)	4.04 ↑ (6 days)	7
VIBHAR_03095	*(purF)*	Amidophosphoribosyl-transferase *purF* (purine biosynthesis)	3.71 ↑ (5 min)	13
VIBHAR_00385	*(purE. K)*	Phosphoribosylaminoimidazole carboxylase catalytic subunit (purine biosynthesis)	5.09 ↑ (5 min)	4
VIBHAR_03200	*(purM)*	Phosphoribosylaminoimidazole synthetase (purine biosynthesis)	5.12 ↑ (5 min)	13
VIBHAR_00210	*(purH)*	Bifunctional phosphoribosylaminoimidazole carboxamide formyltransferase / IMP cyclohydrolase (purine biosynthesis)	4.12↑ (5 min)	13
VIBHAR_00210		Bifunctional phosphoribosyl aminoimidazol ecarboxamide formyltransferase/IMP cyclohydrolase (purine nucleotides *de novo* biosynthesis)	5.09 ↑ (5 min)	4
VIBHAR_03374	*(deoD)*	Purine nucleoside phosphorylase	29.50 ↓ (6 days)	9
VIBHAR_03375	*(deoB)*	Phosphopentomutase	16.39 ↓ (6 days)	9
VIBHAR_02733	*(nrdA)*	Ribonucleotide-diphosphate reductase subunit alpha	15.33 ↓ (6 days)	8
VIBHAR_02734	*(nrdB)*	Ribonucleotide-diphosphate reductase subunit beta	19.06 ↓ (6 days)	8
VIBHAR_00764	*(cpdB)*	Bifunctional 2'.3'-cyclic nucleotide 2'-phosphodiesterase / 3'-nucleotidase (ribonucleic acid degradation in periplasm)	22.88 ↓ (6 days)	8
VIBHAR_01516		Uridine phosphorylase	11.51 ↓ (6 days)	8
VIBHAR_03648	*(pyrI)*	Aspartate carbamoyltransferase regulatory subunit	37.05 ↓ (6 days)	9

VIBHAR_05227	*(pyrC)*	Dihydroorotase	3.73 ↓ (6 days)	11
Nucleotide salvage pathways (nucleotide recycling)				
VIBHAR_00630	*(gmk)*	Guanylate kinase	4.83 ↑ (12 h)	
VIBHAR_00006		Thymidylate kinase	6.43 ↑ (6 days)	7
VIBHAR_01328	*(adk)*	Adenylate kinase	12.37 ↑ (5 min)	5
Carbon metabolism				
Citrate. glyoxylate & 2-methylcitrate cycles				
VIBHAR_01350	*(gltA)*	Type II citrate synthase (citric acid cycle)	43.51 ↓ (6 days)	9
VIBHAR_01355	*(sucA)*	2-oxoglutarate dehydrogenase E1 component (citric acid cycle)	22.78 ↓ (6 days)	9
VIBHAR_01356	*(sucB)*	Dihydrolipoamide succinyltransferase *sucB* (citric acid cycle)	16.06 ↓ (6 days)	9
VIBHAR_01041		Malate synthase (glyoxylate cycle)	58.91 ↑ (5 min)	5
VIBHAR_06695			10.13 ↑ (5 min)	
VIBHAR_01042		Isocitrate lyase (citric acid cycle. glyoxylate cycle)	15.40 ↑ (5 min)	5
VIBHAR_02396	*(prpC)*	Methylcitrate synthase (2-methyl citrate cycle)	8.92 ↑ (5 min)	5
VIBHAR_02396	*(prpC)*	Methylcitrate synthase (2-methyl citrate cycle)	6.57 ↑ (6 days)	7
VIBHAR_02397	*(prpB)*	2-methylisocitrate lyase (2-methyl citrate cycle)	20.77 ↑ (5 min)	5
VIBHAR_02395		Aconitate hidratase (2-methyl citrate cycle)	8.55 ↑ (6 days)	7
VIBHAR_02394		2-methylcitrate dehydratase accessory protein PrpF (2-methyl citrate cycle)	6.03 ↑ (6 days)	7
VIBHAR_00045	*(ppc)*	Phosphoenolpyruvate (PEP) carboxylase	6.49 ↓ (6 days)	12
VIBHAR_03491		Oxaloacetate decarboxylase	6.96 ↓ (6 days)	3
VIBHAR_03492		Oxaloacetate decarboxylase subunit gamma	8.84 ↓ (6 days)	3
Glycolysis and gluconeogenesis				
VIBHAR_00597	*(pckA)*	Phosphoenolpyruvate carboxykinase (gluconeogenesis)	17.33 ↓ (6 days)	9
VIBHAR_03525	*(eno)*	Phosphopyruvate hydratase	14.86 ↓ (6 days)	8
VIBHAR_00296		Glyceraldehyde-3-phosphate dehydrogenase (GAPDH. glycolysis)	259.32 ↑ (5 min)	5
VIBHAR_03565	*(pgk)*	Phosphoglycerate kinase	4.39 ↓ (6 days)	12
VIBHAR_03049	*(gapA)*	Glyceraldehyde 3-phosphate dehydrogenase	5.49 ↓ (6 days)	12
VIBHAR_00012	*(pgi)*	Glucose-6-phosphate isomerase	6.05 ↓	12

			(6 days)	
VIBHAR_06724		Phospho-beta-glucosidase B	4.75 ↓ (6 days)	12
VIBHAR_00296		Glyceraldehyde-3-phosphate dehydrogenase (GAPDH. glycolysis)	259.32 ↑ (5 min)	5
Pyruvate-dependent pathways				
VIBHAR_04773		Isochorismatase (shikimate and Chorismate Biosynthesis)	10.28 ↓ (6 days)	8
VIBHAR_00028		Shikimate kinase I (shikimate and Chorismate Biosynthesis)	4.02 ↑ (5 min)	4
Protein turnover and folding				
Protein degradation				
VIBHAR_01024		Peptidase U32	16.82 ↑ (6 days)	2
VIBHAR_06173		Membrane-bound serine protease ClpP	4.17↑ (6 days)	10
VIBHAR_03286		Lon-like ATP-dependent protease La (amino acids recycling. trans-translation)	22.17 ↑ (6 days)	2
VIBHAR_01123		tmRNA (trans-translation)	9.04 ↑ (6 days)	2
VIBHAR_01419		ATP-dependent Lon protease	4.85 ↓ (6 days)	12
VIBHAR_01157	*(pepD)*	Aminoacyl-histidine dipeptidase	5.90 ↓ (12 h)	3
VIBHAR_04872		Cold-active alkaline serine protease	24.03 ↓ (6 days)	8
VIBHAR_06914		Prolyl oligopeptidase	15.70 ↓ (6 days)	8
Protein folding				
VIBHAR_00142		Chaperonin GroEL	14.14 ↓ (6 days)	8
VIBHAR_00143		Co-chaperonin GroES	26.79 ↓ (6 days)	8
VIBHAR_00599		Ribosome-associated heat shock protein Hsp15	2.40↓ (6 days)	11
VIBHAR_01135	*(dnaJ)*	Heat shock protein J (DnaJ)	2.75 ↓ (6 days)	11
VIBHAR_01327	*(htpG)*	Heat shock protein 90	5.65 ↓ (6 days)	12
VIBHAR_01134	*(dnaK)*	Molecular chaperone DnaK	5.23 ↓ (6 days)	12
VIBHAR_03446		DnaK suppressor protein	4.43 ↓ (6 days)	12
VIBHAR_00143	*(groS)*	Co-chaperonin GroES	5.02 ↓ (6 days)	12
Antioxidative defence				
VIBHAR_04761		Alkyl hydroperoxide reductase. subunit F	37.57 ↑ (6 days)	2
VIBHAR_04762		Alkyl hydroperoxide reductase. c22	67.51 ↑	2

		protein	(6 days)	
VIBHAR_05192		Catalase	17.52 ↑ (6 days)	2
VIBHAR_06580		Catalase	9.04 ↑ (6 days)	2 & 7
VIBHAR_02635	*(grxA)*	Glutaredoxin 1	3.92 ↑ (6 days)	7
VIBHAR_02635	*(grxA)*	Glutaredoxin 1	3.34 ↑ (6 days)	10
Nitric oxide detoxification				
VIBHAR_00086	*(nsrR)*	Transcriptional repressor NsrR	3.15 ↑ (6 days)	10
VIBHAR_00294		Nitric oxide reductase cytochrome b subunit	61.58 ↑ (5 min)	5
Sigma E – ChrR system				
VIBHAR_03283		Transcriptional activator ChrR (photooxidative stress response)	12.74 ↑ (6 days)	2 & 7
VIBHAR_03284		RNA polymerase sigma E. factor. ECF subfamily	16.40 ↑ (6 days)	2
DNA damage, repair and synthesis				
VIBHAR_00442	*(dnaA)*	Chromosomal replication initiation protein	4.71 ↑ (5 min)	13
VIBHAR_00268		LexA repressor (SOS response)	7.38 ↑ (6 days)	7
VIBHAR_05122		Deoxyribodipyrimidine photolyase (DNA repair)	6.817 ↑ (6 days)	7
VIBHAR_01557		ATP-dependent DNA helicase DinG	3.85 ↑ (6 days)	10
VIBHAR_00022		Primosomal replication protein N	5.84 ↑ (12 h)	4
VIBHAR_00168		DNA polymerase III subunit epsilon	19.65 ↑ (5 min)	5
VIBHAR_p08270		DNA polymerase V subunit (plasmid-encoded)	4.057 ↑ (6 days)	10
VIBHAR_p08174		Relaxase NikB (plasmid-encoded)	4.73↑ (6 days)	10
VIBHAR_00213		DNA-binding protein HU-alpha	22.41 ↓ (6 days)	9
VIBHAR_01420		DNA-binding protein HU-beta	22.88 ↓ (6 days)	8
VIBHAR_03222	*(dnaE)*	DNA polymerase III subunit alpha	12.44 ↓ (6 days)	8
VIBHAR_00958		ssDNA exonuclease RecJ	2.87 ↓ (6 days)	11
VIBHAR_02730		DNA gyrase subunit A	3.32↓ (12 h)	11
VIBHAR_03302	*(recC)*	Exonuclease V subunit gamma	5.13 ↓ (6 days)	12
VIBHAR_01130		DNA repair protein RecN	3.08 ↓ (6 days)	12

VIBHAR_01312	*(ligA)*	NAD-dependent DNA ligase LigA	6.84 ↓ (6 days)	12
Transport				
Energy-dependent transport TonB-ExbB-ExbD complex				
VIBHAR_00634	*(exbD)*	Biopolymer transport protein ExbD	6.31 ↑ (12 h)	4
VIBHAR_00636	*(exbB)*	Biopolymer transport protein ExbB	4.46 ↑ (12 h)	4
VIBHAR_00637		TonB system biopolymer transport component	7.96 ↑ (5 min)	13
ABC transporters				
VIBHAR_06841		Nitrate / sulfonate / bicarbonate transport systems. periplasmic components	11.11 ↓ (6 days)	8
			4.64 ↓ (6 days)	12
VIBHAR_06839		ABC-type nitrate / sulfonate / bicarbonate transport system ATPase component	4.54 ↓ (6 days)	12
VIBHAR_06840		ABC-type nitrate / sulfonate / bicarbonate transport system permease component	4.16 ↓ (6 days)	12
VIBHAR_04880		ABC-type spermidine/putrescine transport system ATPase component	16.81 ↓ (6 days)	8
Tripartite ATP-independent periplasmic transport				
VIBHAR_02703		TRAP dicarboxylate transporter. DctM subunit	10.21 ↑ (5 min)	5
VIBHAR_06716		TRAP transporter. DctM-like subunit	21.98 ↑ (5 min)	5
VIBHAR_06717		Putative TRAP transporter solute receptor	23.23 ↑ (5 min)	5
Sec-dependent translocation				
VIBHAR_00909	*(secA)*	Preprotein translocase subunit SecA	4.18 ↓ (6 days)	11
VIBHAR_00123	*(secB)*	Preprotein translocase subunit SecB	4.55 ↓ (6 days)	11
VIBHAR_01048	*(secD)*	Preprotein translocase subunit SecD	3.85 ↓ (6 days)	11
TolAB-dependent translocation				
VIBHAR_01610	*(tolB)*	Translocation protein TolB	7.80 ↓ (6 days)	3
Maltose / maltodextrines transport				
VIBHAR_04858	*(malS)*	Periplasmic alpha-amylase precursor	4.45 ↑ (5 min)	5
VIBHAR_05679		Cytoplasmic alpha-amylase	4.12 ↑ (6 days)	7
			13.81 ↓ (6 days)	8
VIBHAR_05253		Alpha-amylase	20.29 ↓ (6 days)	8
VIBHAR_04822	*(malM)*	Maltose operon periplasmic protein MalM	29.68 ↓ (6 days)	8

VIBHAR_04849	*(malP)*	Maltodextrin phosphorylase	53.33 ↓ (6 days)	8
VIBHAR_05213	*(malK)*	Maltose/maltodextrin transporter ATP-binding protein	20.99 ↓ (6 days)	8
VIBHAR_05214	*(malE)*	Maltose ABC transporter periplasmic protein	36.68 ↓ (6 days)	8
VIBHAR_06226		Sugar kinase	4.06 ↑ (6 days)	7
Phosphate transport (*pstSCAB operon*)				
VIBHAR_03057	*(phoA)*	Alkaline phosphatase	4.76 ↑ (5 min)	13
VIBHAR_01026	*(phoB)*	Response regulator PhoB	17.45 ↓ (6 days)	9
VIBHAR_05137	*(pstS)*	ABC-type phosphate transport system periplasmic component *pstS*	180.39 ↓ (6 days)	9
VIBHAR_05138	*(pstC)*	ABC-type phosphate transport system permease component	33.71 ↓ (6 days)	9
VIBHAR_05139	*(pstA)*	Phosphate transporter inner membrane protein PstA	53.70 ↓ (6 days)	9
VIBHAR_05140	*(pstB)*	Phosphate ABC transporter ATP-binding protein	38.62 ↓ (6 days)	9
sn-Glycerol 3-phosphate transport system				
VIBHAR_06394	*(glpR)*	Hypothetical repressor of the glycerol-3-phosphate regulon	42.39 ↓ (6 days)	9
VIBHAR_06395	*(ugpQ)*	Glycerophosphoryl diester phosphodiesterase UgpQ	56.99 ↓ (6 days)	9
VIBHAR_06396	*(ugpB)*	sn-Glycerol 3-phosphate transport system substrate-binding protein	219.14 ↓ (6 days)	9
VIBHAR_06397	*(ugpA)*	sn-glycerol 3-phosphate transport system permease protein	123.98 ↓ (6 days)	9
VIBHAR_06398	*(ugpE)*	sn-glycerol 3-phosphate transport system permease protein	58.82↓ (6 days)	9
Arginine transport				
VIBHAR_05528	*(artP)*	Arginine transporter ATP-binding subunit	28.53 ↓ (6 days)	9
			25.16 ↓ (12 h)	3
VIBHAR_05529	*(artI)*	ABC amino acid transporter periplasmic ligand binding protein ArtI	36.19↓ (6 days)	9
VIBHAR_05530	*(artQ)*	Arginine transporter permease subunit ArtQ	23.46 ↓ (6 days)	9
VIBHAR_05531	*(artM)*	Arginine transporter permease subunit ArtM	22.70 ↓ (6 days)	9
Other transporters				
VIBHAR_00347		Putative threonine efflux protein	22.80↑ (6 days)	2
VIBHAR_04744		Putative threonine efflux protein	12.03 ↑ (6 days)	2
VIBHAR_04898		Dipeptide / tripeptide permease	26.79 ↓ (6 days)	8
			6.71 ↓ (12 h)	3
VIBHAR_00155		Anaerobic C4dicarboxylate transporter	35.88 ↓	9

			(6 days)	
VIBHAR_00155		Anaerobic C4-dicarboxylate transporter	35.88 ↓ (6 days)	9
VIBHAR_06592		PTS system fructose-specific IIBC component	6.03 ↑ (6 days)	4
VIBHAR_07028		Putative LolE	53.84 ↑ (6 days)	2
Translation				
VIBHAR_00594		D-tyrosyl-tRNA(Tyr) deacylase	6.00 ↑ (5 min)	5
VIBHAR_01245		Peptidyl-tRNA hydrolase	4.18 ↑ (5 min)	4
VIBHAR_01250	*(prfA)*	Peptide chain release factor 1	4.84 ↓ (6 days)	11
VIBHAR_00054		Elongation factor Tu	3.67 ↓ (6 days)	12
tRNA and Ribosome biogenesis				
VIBHAR_02293		C32 tRNA thiolase	4.18 ↑ (6 days)	7
VIBHAR_02753		16S rRNA uridine-516 pseudouridylate synthase	5.57 ↑ (6 days)	4
VIBHAR_00174		tRNA-dihydrouridine synthase B	7.93 ↑ (6 days)	4
Ribosomal proteins				
VIBHAR_00728		50S ribosomal protein L31	8.89 ↑ (6 days)	4
VIBHAR_00742		50S ribosomal protein L5	7.99 ↑ (6 days)	4
VIBHAR_00744		30S ribosomal protein S8	7.51 ↑ (6 days)	4
VIBHAR_00977		30S ribosomal protein S20	11.38 ↑ (6 days)	4
VIBHAR_04827		Periplasmic endonuclease I	21.44 ↓ (6 days)	8
VIBHAR_00082		Tryptophanyl-tRNA synthetase	3.95 ↓ (6 days)	11
VIBHAR_03681		Ribonuclease G	4.41 ↓ (6 days)	12
Iron uptake. storage & utilization				
Iron uptake				
VIBHAR_00638		Iron transport protein (TonB-dependent)	18.54 ↑ (5 min)	1
VIBHAR_03435	*(fbpA)*	Iron(III) transport system substrate-binding protein	11.87↑ (5 min)	1
VIBHAR_02414		Tricarboxylic transport protein TctC	449.58 ↑ (6 days)	2
VIBHAR_02415		Tricarboxylic transport protein TctB	184.40 ↑ (6 days)	2
VIBHAR_02416		Tricarboxylic transport protein TctA	148.03 ↑	2

			(6 days)	
Iron storage				
VIBHAR_00053		Bacterioferritin-associated ferredoxin	8.83 ↑ (5 min)	1
VIBHAR_02274		Ferredoxin	9.68 ↑ (5 min)	4
Iron cluster biogenesis				
VIBHAR_01054	*(iscR)*	Putative IscR regulator (control of operons and genes involved in the biogenesis of Fe-S clusters and Fe-S-containing proteins)	31.09 ↑ (5 min)	1
VIBHAR_01055	*(iscS)*	Cysteine desulfurase	28.77 ↑ (5 min)	1
VIBHAR_01056		Scaffold protein	21.37 ↑ (5 min)	1
VIBHAR_01057	*(putative iscA)*	Iron-binding protein IscA (iron-sulfur cluster assembly)	13.82 ↑ (5 min)	1
VIBHAR_01059	*(hscA)*	Chaperone protein HscA (maturation of iron-sulfur cluster-containing proteins)	5.75 ↑ (5 min)	4
VIBHAR_01058	*(hscB)*	Co-chaperone HscB (maturation of iron-sulfur cluster-containing proteins)	13.82 ↑ (5 min)	1
Biosynthesis of L-cysteine				
VIBHAR_00003	*(cysH)*	Phosphoadenosine phosphosulfate reductase	9.25 ↑ (5 min)	5
VIBHAR_00004	*(cysI)*	Sulfite reductase. subunit beta	14.41 ↑ (5 min)	5
VIBHAR_00005	*(cysJ)*	Sulfite reductase (NADPH) flavoprotein. subunit alpha	17.43 ↑ (5 min)	5
VIBHAR_00766	*(cysD)*	Sulfate adenylyltransferase subunit 2	23.96 ↑ (5 min)	5
VIBHAR_00767	*(cysN)*	Sulfate adenylyltransferase subunit 1	19.94 ↑ (5 min)	5
VIBHAR_00768		Putative sodium/sulfate symporter	7.05 ↑ (5 min)	5
VIBHAR_00769		Adenylylsulfate kinase	10.59↓ (6 days)	8
Other pathways				
Porphyrin & chlorophyll metabolism				
VIBHAR_01249	*(hemA)*	Glutamyl-tRNA reductase;	6.38 ↑ (6 days)	4
VIBHAR_06129	*(hemE)*	Uroporphyrinogen decarboxylase (Heme biosynthesis)	4.70 ↓ (5 min)	6
VIBHAR_05246	*(hemN)*	Oxygen-independent coproporphyrinogen III oxidase (Heme biosynthesis)	8.05 ↑ (6 days)	2
VIBHAR_00765	*(cysG)*	Uroporphyrin-III C-methyltransferase (biosynthesis of siroheme)	45.72 ↑ (5 min)	5
Nicotinate and nicotinamide metabolism				
VIBHAR_06575	*(pncB)*	Nicotinate phosphoribosyltransferase	4.41 ↓ (5 min)	6
VIBHAR_06577		Pyrazinamidase/nicotinamidase	4.50 ↓	3

			(5 min) 9.23 ↓ (12 h)	
VIBHAR_03543		L-aspartate oxidase	4.04 ↑ (5 min)	13
Thiamin metabolism				
VIBHAR_00363		Sulfur carrier protein ThiS	10.29 ↓ (6 days)	8
VIBHAR_00365		Thiamine-phosphate pyrophosphorylase	17.72 ↓ (6 days)	8
VIBHAR_00366		Thiamine biosynthesis protein ThiC	4.25 ↓ (6 days)	11
VIBHAR_00363		Sulfur carrier protein ThiS	5.74 ↓ (6 days)	12
VIBHAR_00364		Adenylyltransferase	5.17 ↓ (6 days)	12
Glutathione metabolism				
VIBHAR_06584		Lactoylglutathione lyase	6.35 ↓ (6 days)	11
VIBHAR_06603			3.70 ↓ (6 days)	
VIBHAR_06431		Putative glutathione S-transferase YghU	16.02 ↓ (6 days)	8
VIBHAR_01157	*(pepD)*	Aminoacyl-histidine dipeptidase (γ-glutamyl cycle)	5.90 ↓ (12 h)	3
VIBHAR_00348		Gamma-glutamyltransferase (γ-glutamyl cycle)	13.13 ↓ (6 days)	8
Quorum sensing				
VIBHAR_00157		Transcriptional regulator LuxR	6.05 ↓ (6 days)	12
VIBHAR_00046		PadR family transcriptional regulator. regulatory protein AphA	22.05 ↑ (6 days)	2
VIBHAR_04846		Small RNA Qrr2	2.23 ↑ (6 days)	7
VIBHAR_05322		Small RNA Qrr4	4.45 ↑ (6 days)	7
VIBHAR_06697		Small RNA Qrr3	2.19 ↑ (6 days)	7
Type III secretion apparatus				
VIBHAR_01738	*(vcrG)*	Homologue of low calcium response protein G from *Yersinia pestis*	23.82 ↓ (6 days)	9
VIBHAR_01740	*(vcrH)*	Low calcium response chaperone VcrH1	15.40 ↓ (6 days)	9
VIBHAR_01741	*(vopB)*	Putative translocator protein VopB1	58.67 ↓ (6 days)	9
VIBHAR_01742	*(vopD)*	Putative translocator outer membrane protein PopD precursor	18.46 ↓ (6 days)	9
VIBHAR_01731	*(vopN)*	Outer membrane regulator VopN1	4.77 ↓ (12 h)	9
			15.86 ↓ (6 days)	3

VIBHAR_01732	*(vcr1)*	Hypothetical protein homologous to Yop secretion and targeting control protein	9.69 ↓ (6 days) 11.37 ↓ (12 h)	3
VIBHAR_01734	*(vscX)*	Type III secretion protein SctX	7.66 ↓ (6 days)	3
VIBHAR_01736	*(vcrD)*	Type III secretory pathway. component EscV	6.55 ↓ (6 days)	3
Chemotaxis				
VIBHAR_04912	*(flgE)*	Flagellar hook protein FlgE	13.345 ↑ (5 min)	5
VIBHAR_04910	*(flgC)*	Flagellar basal body rod protein FlgC	4.28 ↑ (6 days)	4
VIBHAR_03155	*(fliL)*	Flagellar basal body-associated protein FliL	4.05 ↑ (6 days)	10
VIBHAR_03140		Response regulator CheB	5.52↓ (6 days)	11
Miscellaneous				
VIBHAR_00295		ADP-ribosyl-(dinitrogen reductase) hydrolase	64.54 ↑ (5 min)	5
VIBHAR_06482		Putative outer membrane usher protein	9.89 ↑ (6 days)	2
VIBHAR_00175	*(fis)*	DNA-binding protein Fis (upstream activation of rRNA promoters)	8.98 ↑ (6 days)	4
VIBHAR_00830		Putative glucitol operon repressor	12.50 ↑ (6 days)	2 & 7
VIBHAR_01432		O-succinylbenzoate synthase (anaerobic synthesis of enterobactin)	5.32 ↓ (6 days)	12
VIBHAR_00313	*(cyaA)*	Adenylate cyclase	5.65 ↑ (6 days)	7
VIBHAR_05311	*(ribB)*	3.4-dihydroxy-2-butanone 4-phosphate synthase (riboflavin biosynthesis)	8.50 ↑ (6 days)	4
VIBHAR_00921	*(btuF)*	ABC-type Fe^{3+}-siderophore transporter (vitamin B12 import)	3.05 ↓ (6 days)	11
VIBHAR_01807	*(bioB)*	Biotin synthase	8.01 ↓ (6 days)	11
VIBHAR_00689		UDP-N-acetylglucosamine 2-epimerase (sialic acid biosynthesis)	2.25 ↑ (6 days)	7
VIBHAR_00283		Multiple antibiotic resistance protein	2.50 ↑ (6 days)	7
VIBHAR_02236		Multidrug efflux protein	2.19 ↑ (6 days)	7
VIBHAR_03390	*(vmrA)*	Multidrug efflux pump VmrA	2.38 ↑ (6 days)	7
VIBHAR_07054		Chloramphenicol O-acetyltransferase (antibiotic resistance)	2.63↑ (6 days)	7
VIBHAR_01065	*(rlmN)*	Ribosomal RNA large subunit methyltransferase N (antibiotic resistance)	8.39 ↑ (6 days)	4
VIBHAR_03053	*(rlmA)*	23S rRNA (guanine745-N1)-methyltransferase (potentially involved in antibiotic resistance)	9.51 ↑ (12 h)	4

VIBHAR_00397		Hypothetical protein with homology to hemolysin III	6.76 ↑ (6 days)	7
VIBHAR_06598		Alpha-1.2-mannosidase (degradation of eukaryotic glycoproteins)	4.55 ↑ (6 days)	4
VIBHAR_03337		Beta-D-galactosidase. alpha subunit (hydrolysis of β-galactosides into monosaccharides)	6.25 ↑ (5 min)	5
			5.46 ↑ (5 min)	13
VIBHAR_01128		Outer membrane protein assembly factor BamE	4.70↑ (6 days)	4
VIBHAR_03245		CsrB/RsmB RNA family (carbon storage regulation)	9.96↑ (6 days)	7
VIBHAR_06731			2.93 ↑ (12 h)	1
Uncategorized				
VIBHAR_06409		Hypothetical protein	15.94 ↑ (6 days)	2
VIBHAR_06410		Hypothetical protein	22.21 ↑ (6 days)	2
VIBHAR_05708		Hypothetical protein	18.82 ↑ (6 days)	2
VIBHAR_02138		Hypothetical protein	6.83 ↑ (6 days)	7
VIBHAR_05709		Hypothetical protein	5.25 ↑ (6 days)	7
VIBHAR_05708		Hypothetical protein	5.14 ↑ (6 days)	7
VIBHAR_07026		Hypothetical protein	36.345 ↑ (6 days)	2
VIBHAR_07027		Hypothetical protein	70.60 ↑ (6 days)	2
VIBHAR_07030		Hypothetical protein	168.85 ↑ (6 days)	2
VIBHAR_02275		Hypothetical protein	4.93 ↑ (5 min)	4

Table S2. Primers used for quantitative real-time PCR.

Locus tag (Systematic name)	Gene product	Forward primer (5' to 3')	Reverse primer (5' to 3')	R^2	Efficiency*
VIBHAR_00479	ATP synthase. subunit ETA	GACCCAAAGAAATTAGAGCAAATC	CCTTGGATTACTTGGCGAAC	0.992	0.8
VIBHAR_01519	Hypothetical protein	CGCCAAGCCATATCCTTATC	CCCATCATTGCCTCATCTTC	ND	ND
VIBHAR_04761	Alkyl hydroperoxide reductase. subunit F	TCTGCCTGATTTGCTCTCTC	GGATCGCTTGGTCTAACATAGT	0.998	0.82
VIBHAR_05192	Catalase	CTTCCTACGTGACCCACTAAA	GTCCAGAAATCCCAGTTGTTG	0.985	0.82
VIBHAR_03284	RNA polymerase. sigma-24 subunit	GAGTTGTTCGACATACCTTTGG	TGTTCATAATTGCTCCGTGTG	0.995	0.95
VIBHAR_03286	Putative protease Lon	AGTGATGGGCTTCTTGGTATTA	TGTCTAACCACTCGACTTCAG	ND	ND
VIBHAR_06715	Hydroxyglutarate oxidase	ACCATATCCTGCGGGTATTC	GCAAACGTGTAGGCTTCTTG	0.982	0.97
VIBHAR_03105	3-oxoacyl-[acyl-carrier-protein] synthase I	CGCAGTTCGTTGTATGAAGATG	GCTCTTTCACGTCACCTACT	0.996	0.93
VIBHAR_01352	Succinate dehydrogenase hydrophobic membrane anchor protein. SdhD	GGCTATGTCTTCTCTGGTCTATT	CGATTACTACGGCATCAAACTC	0.992	0.91
VIBHAR_01182	GcvB. regulatory RNA	GCTTCGTTGTTGCGATGT	ACAGGGAGTGTGGACAAA	0.996	0.82
VIBHAR_03525	Phosphopyruvate hydratase	CAGGTCAAATCAAGACTGGTTC	CACCTAGAGCTTCCTCGATAC	0.997	0.93
VIBHAR_02416	Putative tricarboxylic transport protein TctA	GGCTTCTTTACTGGTGTTCTTC	TATCCTTTGGAGCTAGGTTACG	0.993	0.96
VIBHAR_03245	CsrB / RsmB. regulatory RNA	GGACTAAGCTGACGGAATCA	ATAGTCTTACTCGCAGCAGTC	0.998	0.86
VIBHAR_01058	Co-chaperone HscB	ACGAGTCCAGAAGGCTTTAC	CTGAGAAGAAAGAAGGCTACCA	0.990	0.94
VIBHAR_00157	Transcriptional regulator LuxR	GCCCTCAACCAAGTTCTTAAT	TTTCTCTGCCAAGTCATTACAC	ND	ND
VIBHAR_05322	Qrr4. regulatory RNA	TTTAGTGTGACCCTTCTTAAGCC	TTTGTGAACAAATTCGAGTCCAC	ND	ND

***The qPCR amplification efficiency was calculated for each primer pair using the slope of the standard curve obtained with serial dilutions of a concentrated cDNA sample using the following equation: Efficiency (E)=[10(-1/slope)]-1; the efficiency of qPCR could not be calculated for four genes (ND) due to low expression (or low regression coefficient R^2<0.98) and consequently all data analysis steps for these five genes have been done without efficiency correction).**

Supplementary data for Chapter 3

Table S1. Highly up- and downregulated genes grouped according to their putative functions *in vivo*. The systematic names are given according to the annotation of the reference *Vibrio harveyi* ATCC 43516 (available at ftp://ftp.ncbi.nlm.nih.gov/genomes/genbank/; accession number GCA_001558435.1)

Systematic name	Gene product (specific biological pathway)	Fold change (time after exposure to seawater)			Gene expression cluster
		12h	3d	6d	
Cell envelope stress					
AL538_RS20835	Heat-shock protein	-0.616	-1.048	-1.215	6
AL538_RS16135	Heat-shock protein HslJ	-1.093	-0.796	-1.732	4
Phage-shock-protein response					
AL538_RS15985	Phage shock protein PspA	-0.908	0.819	-1.526	4
AL538_RS15990	Envelope stress response membrane protein PspB	-0.760	0.304	-1.244	4
AL538_RS15995	Envelope stress response membrane protein PspC	-1.042	-0.263	-1.819	4
Cell wall metabolism					
AL538_RS12080	Lytic murein transglycosylase	-0.639	-0.791	-1.009	6
AL538_RS07100		0.759	0.747	1.007	1
AL538_RS11095	UDP-N-acetylmuramate:L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase	-1.112	-0.950	-1.084	2
Lipid biogenesis					
AL538_RS16785	Lipid A biosynthesis lauroyl acyltransferase	0.664	0.993	1.203	1
AL538_RS25885	Choline dehydrogenase	1.176	1.370	0.993	5
AL538_RS03425	Acyl-CoA thioesterase	0.878	1.704	1.073	5
AL538_RS13865	Long-chain-fatty-acid--CoA ligase	-0.837	-1.115	-1.038	2
AL538_RS26865	Glycerophosphoryl diester phosphodiesterase	-2.629	-3.534	-2.947	2
AL538_RS23675	Acyl-CoA dehydrogenase	-2.053	-2.768	-2.308	2
AL538_RS23680	Enoyl-CoA hydratase	-2.034	-2.947	-2.470	2
AL538_RS23685		-2.182	-2.589	-2.503	2
AL538_RS27060	Beta-ketoacyl-ACP reductase	-1.347	-2.564	-2.983	3
AL538_RS03900	Malonyl CoA-acyl carrier protein transacylase	-1.815	-2.683	-2.368	3
AL538_RS15600	Phospholipase C	-1.253	-1.882	-1.093	2
AL538_RS15740	Cyclopropane-fatty-acyl-phospholipid synthase	-0.985	-1.285	-1.411	3
AL538_RS14940	Tol-pal system-associated acyl-CoA thioesterase	-1.008	-1.088	-1.538	6
AL538_RS14125	FAD-dependent oxidoreductase	0.347	0.746	1.139	1
AL538_RS20940		0.378	1.121	0.854	5
AL538_RS04910	Acetyl-CoA C-acyltransferase FadI	-0.092	-1.133	-0.269	3
Acetyl-CoA-dependent metabolism					
AL538_RS08475	Acetyl-CoA carboxylase biotin carboxyl carrier protein subunit	-1.682	-2.790	-2.670	6
AL538_RS08480	Acetyl-CoA carboxylase biotin carboxylase subunit	-1.694	-2.530	-2.391	3
AL538_RS05395	Acetyl-CoA carboxylase carboxyl	-1.273	-2.388	-2.011	3

	transferase subunit alpha				
AL538_RS04825	Acetyl-CoA carboxylase carboxyl transferase subunit beta	-1.208	-2.079	-2.351	3
AL538_RS14965	Peptidoglycan-associated lipoprotein	-1.663	-2.943	-2.801	3
AL538_RS14110	3-oxoacyl-ACP synthase	0.933	1.219	1.249	5
Ethanol biosynthesis / biodegradation					
AL538_RS04550	Bifunctional acetaldehyde-CoA/alcohol dehydrogenase	-1.470	-3.161	-2.871	3
AL538_RS17730	Alcohol dehydrogenase	0.799	0.465	1.083	1
AL538_RS09760	Alcohol dehydrogenase	-1.727	-3.140	-2.750	3
AL538_RS23985	NADH-dependent alcohol dehydrogenase	-1.359	-1.956	-2.513	6
AL538_RS18755	S-(hydroxymethyl)glutathione dehydrogenase/class III alcohol dehydrogenase	-1.552	-2.100	-1.968	2
AL538_RS08550	Zinc-dependent alcohol dehydrogenase	1.133	0.660	1.148	1
Acetate biosynthesis (ATP production)					
AL538_RS08465	Acetyl-CoA synthetase	-1.601	-2.312	-2.177	3
Energy production					
AL538_RS25345	Electron transfer flavoprotein subunit alpha	-1.977	-2.101	-2.265	2
AL538_RS27345	NADH dehydrogenase	1.037	0.665	0.830	1
AL538_RS22660	Cytochrome c	-0.669	-1.578	-1.741	3
AL538_RS10145	Cytochrome c	-0.548	-0.823	-1.085	6
AL538_RS18830	Cytochrome c biogenesis protein	0.750	1.228	0.600	5
AL538_RS03195	Cytochrome c nitrite reductase subunit NrfD	0.565	1.438	0.764	5
AL538_RS26195	Cytochrome c oxidase assembly protein	-0.492	-0.705	-1.359	4
AL538_RS26200	Cytochrome c oxidase subunit I	-0.842	-1.153	-1.471	6
AL538_RS00780	Cytochrome c oxidase. cbb3-type subunit I	-1.295	-1.551	-2.156	6
AL538_RS24750	Cytochrome C554	-1.499	-1.123	-1.848	4
AL538_RS14920	Cytochrome d terminal oxidase subunit 1	-1.139	-1.270	-1.210	2
AL538_RS17915	Cytochrome d ubiquinol oxidase subunit II	0.911	1.224	1.292	5
AL538_RS14925	Cytochrome d ubiquinol oxidase subunit II	-0.934	-0.369	-1.066	4
AL538_RS07615	Cytochrome-c oxidase. cbb3-type subunit III	-1.112	-1.702	-2.363	6
AL538_RS07640	Cytochrome-c oxidase. cbb3-type subunit III	-1.112	-1.702	-2.363	6
AL538_RS00765	Cytochrome-c oxidase. cbb3-type subunit III	-1.764	-2.531	-2.308	2
AL538_RS07650	Cytochrome-c oxidase. cbb3-type subunit III	-1.142	-1.068	-1.850	6
AL538_RS26785	Cytochrome-c peroxidase	1.040	1.270	1.267	5
ATP synthesis coupled proton transport					
AL538_RS09620	F0F1 ATP synthase subunit A	-1.654	-1.656	-2.178	6
AL538_RS09605	F0F1 ATP synthase subunit delta	-1.768	-3.170	-2.672	3
AL538_RS09595	F0F1 ATP synthase subunit gamma	-1.825	-3.548	-2.778	3
AL538_RS09625	F0F1 ATP synthase subunit I	-1.568	-2.783	-2.312	3
Fumarate reductase (complex II)					
AL538_RS08270	Succinate dehydrogenase/fumarate reductase iron-sulfur subunit	-0.974	-1.628	-0.798	2
AL538_RS08265	Fumarate reductase flavoprotein subunit	-1.862	-2.041	-1.584	2

AL538_RS08275	Fumarate reductase subunit C	-1.183	-1.221	-2.146	6
AL538_RS08280	Fumarate reductase subunit D	-1.508	-2.045	-1.847	2
AL538_RS00660	Formate dehydrogenase	-0.490	-0.967	-1.359	6
AL538_RS00665	Formate dehydrogenase	-0.359	-1.587	-1.551	6
Amino acid metabolism					
Amino acid recycling					
AL538_RS23670	Methylmalonate-semialdehyde dehydrogenase (CoA acylating)	-2.325	-3.377	-2.733	2
Alanine degradation					
AL538_RS15645	Alanine dehydrogenase	-2.877	-2.910	-3.289	2
AL538_RS21930	Alanine--glyoxylate aminotransferase	-1.603	-2.442	-1.489	2
AL538_RS06680	Alanine--tRNA ligase	-0.928	-1.365	-1.566	6
Arginine and proline metabolism					
AL538_RS20570	Arginine decarboxylase	0.980	1.229	1.257	5
AL538_RS08005	Arginine N-succinyltransferase	-1.016	-1.592	-1.194	2
AL538_RS13825	Arginine--tRNA ligase	-0.526	-0.715	-1.032	6
AL538_RS10110	Twin-arginine translocase subunit TatB	-0.944	-1.268	-1.630	6
AL538_RS27070	Twin-arginine translocation pathway signal protein	-0.986	-1.267	-1.232	2
AL538_RS05580	Proline--tRNA ligase	-0.750	-1.298	-0.870	3
AL538_RS01805	Hydroxyproline-2-epimerase	-2.242	-2.887	-2.763	2
AL538_RS19730	Ornithine carbamoyltransferase	1.065	0.873	1.132	1
AL538_RS01790	Ornithine cyclodeaminase	-2.714	-4.302	-3.209	2
AL538_RS18480	Ornithine decarboxylase SpeF	0.812	1.325	0.968	5
AL538_RS11685	Carbamoyl-phosphate synthase small subunit	-1.555	-2.798	-2.228	3
AL538_RS07820	Argininosuccinate synthase	-2.225	-2.890	-2.258	2
AL538_RS20565	Agmatinase	0.640	1.324	1.106	5
AL538_RS07825	Acetylglutamate kinase	-1.058	-1.119	-1.333	2
Aspartate metabolism					
AL538_RS07290	Aspartate carbamoyltransferase	-1.401	-1.345	-1.897	6
AL538_RS07295	Aspartate carbamoyltransferase regulatory subunit	-1.251	-1.976	-1.289	2
AL538_RS19820	Aspartate aminotransferase	0.593	1.274	1.104	5
AL538_RS12535	Aspartate aminotransferase family protein	-0.366	-1.016	-0.330	3
AL538_RS08010	Aspartate aminotransferase family protein	-1.598	-1.871	-1.686	2
AL538_RS04840	Aspartate-semialdehyde dehydrogenase	-0.658	-1.609	-1.441	3
AL538_RS04565	Aspartate-semialdehyde dehydrogenase	-1.499	-1.302	-1.248	2
AL538_RS14895	Aspartate--tRNA ligase	-1.120	-0.774	-1.138	2
Glutamate metabolism					
AL538_RS25000	Glutamate synthase	-0.858	-1.233	-1.597	6
AL538_RS11745	Glutamate synthase subunit beta	-1.595	-2.359	-1.828	2
AL538_RS13365	Glutamate--tRNA ligase	-0.758	-0.936	-1.068	2
AL538_RS19575	1-pyrroline-5-carboxylate dehydrogenase	-0.487	-0.696	-1.220	6
Serine biosynthesis/degradation					
AL538_RS16375	D-serine transporter DsdX	0.814	1.221	0.964	5
AL538_RS16340	3-phosphoserine/phosphohydroxythreonine aminotransferase	-1.012	-1.479	-0.766	2
AL538_RS11815	Homoserine kinase	-0.937	-1.165	-1.003	2

AL538_RS20985	L-serine ammonia-lyase	-1.086	-1.335	-1.044	2
AL538_RS03000	L-serine ammonia-lyase	-1.173	-1.149	-0.798	2
AL538_RS06050	Phosphoserine phosphatase	-0.716	-1.015	-0.727	2
AL538_RS14635	Serine protein kinase PrkA	-1.385	-1.333	-2.215	6
AL538_RS09945	Serine/threonine protein kinase	0.869	1.140	1.459	1
AL538_RS15670	Serine--tRNA ligase	-1.094	-1.263	-1.430	2
AL538_RS06895	D-3-phosphoglycerate dehydrogenase	-1.182	-1.327	-1.111	2
Valine. leucine and isoleucine biosynthesis / degradation					
AL538_RS09830	Ketol-acid reductoisomerase	-0.120	-1.001	-0.870	3
Breakdown of tyrosine. phenylalanine and tryptophan					
AL538_RS01665	4-hydroxyphenylpyruvate dioxygenase	-2.624	-2.794	-3.415	2
AL538_RS01660	Homogentisate 1.2-dioxygenase	-2.467	-2.629	-3.241	2
AL538_RS01650	Maleylacetoacetate isomerase	-2.354	-1.922	-2.926	6
Stringent response					
AL538_RS11565	Stringent starvation protein B	-1.121	-0.789	-1.168	2
Purine /pyrimidine biosynthesis					
AL538_RS25720	Ribokinase	0.859	0.909	1.231	1
AL538_RS15875	Nucleoside-diphosphate sugar epimerase	-1.163	-1.580	-1.245	2
AL538_RS06065	Phosphopentomutase	-1.607	-2.371	-2.171	3
AL538_RS03240	Ribonucleoside-diphosphate reductase subunit alpha	-1.121	-1.960	-2.065	3
AL538_RS03245	Ribonucleotide-diphosphate reductase subunit beta	-1.553	-2.794	-2.503	3
AL538_RS14510	Uridine phosphorylase	-1.682	-2.946	-3.119	3
AL538_RS07295	Aspartate carbamoyltransferase regulatory subunit	-1.251	-1.976	-1.289	2
AL538_RS22215	Dihydroorotase	-0.689	-0.495	-1.008	4
Nucleotide salvage pathways (nucleotide recycling)					
AL538_RS13630	Adenylate kinase	-1.563	-2.207	-2.102	3
Carbon metabolism					
Citrate. glyoxylate & 2-methylcitrate cycles					
AL538_RS17025	Citrate synthase/methylcitrate synthase	-2.020	-1.656	-2.315	6
AL538_RS13760	2-oxoglutarate dehydrogenase subunit E1	-1.543	-1.706	-1.613	2
AL538_RS13765	Dihydrolipoamide succinyltransferase	-1.233	-1.028	-1.384	2
AL538_RS12280	Isocitrate lyase	-1.318	-1.596	-1.452	2
AL538_RS06380	Aconitate hydratase B	-1.588	-2.634	-2.372	3
AL538_RS17035	2-methylaconitate cis-trans isomerase PrpF	-1.876	-1.908	-1.961	2
AL538_RS10235	Phosphoenolpyruvate carboxykinase (ATP)	-1.458	-3.015	-2.504	3
AL538_RS11240	2-isopropylmalate synthase [Vibrio]	-1.441	-1.289	-1.370	
AL538_RS07840	Phosphoenolpyruvate carboxylase	-0.996	-1.292	-1.183	2
Glycolysis and gluconeogenesis					
AL538_RS06930	Phosphoglycerate kinase	-1.341	-2.545	-2.503	3
AL538_RS10235	Phosphoenolpyruvate carboxykinase (ATP)	-1.458	-3.015	-2.504	3
AL538_RS04660	Type I glyceraldehyde-3-phosphate dehydrogenase	-1.325	-2.871	-2.660	3
AL538_RS01195	Glucose-6-phosphate dehydrogenase	-0.672	-0.609	-1.126	6
AL538_RS20635	6-phospho-beta-glucosidase	-0.557	-0.777	-1.112	6
Pyruvate-dependent pathways					
AL538_RS14160	Isochorismatase	0.839	1.241	1.159	5

AL538_RS14780	3-phosphoshikimate 1-carboxyvinyltransferase	-1.126	-1.715	-1.390	3
Protein turnover and folding					
Protein degradation					
AL538_RS12295	Serine protease	0.744	1.079	1.240	1
AL538_RS01370	Alkaline serine protease	-1.378	-3.486	-2.372	4
AL538_RS11515	Serine endoprotease DegQ	-0.449	-1.373	-1.586	3
AL538_RS07400	Metalloprotease PmbA	-0.849	-0.801	-1.178	6
AL538_RS06130	Protease	1.034	1.137	1.308	1
AL538_RS15715	Protease HtpX	-0.821	-0.978	-1.552	6
AL538_RS05440	RIP metalloprotease RseP	-0.197	-1.289	-1.086	3
AL538_RS20315	Zn-dependent protease	-1.482	-1.687	-2.175	6
AL538_RS12810	Aminoacyl-histidine dipeptidase	-1.960	-2.991	-2.215	2
AL538_RS10020	Oligopeptidase A	-1.105	-1.895	-2.198	6
AL538_RS01615	Peptidase	-0.896	-1.139	-1.123	2
AL538_RS20505	Peptidase	1.067	1.355	1.071	5
AL538_RS18730	Peptidase	-0.014	-1.023	-0.910	3
AL538_RS08240	Peptidase	-0.446	-1.010	-0.269	3
AL538_RS01150	Peptidase A24	0.249	1.061	1.581	1
AL538_RS05230	Peptidase M15	-0.419	-0.572	-1.261	4
AL538_RS25460	Peptidase M19	0.957	0.900	1.036	1
AL538_RS01675	Peptidase M20	0.859	0.886	1.368	1
AL538_RS24140	Peptidase M50	1.118	0.888	1.354	1
AL538_RS00775	Peptidase S41	-1.639	-2.045	-1.936	2
AL538_RS25255	Peptidase S8	0.698	0.813	1.005	1
AL538_RS11905	Peptide chain release factor 1	-0.366	-1.288	-0.954	3
AL538_RS12385	Aminopeptidase PepB	-1.212	-1.429	-0.934	2
AL538_RS07245	Cytosol aminopeptidase	-0.912	-1.460	-1.109	3
AL538_RS18135	D-alanyl-D-alanine carboxypeptidase	-1.318	-2.530	-2.336	3
AL538_RS14260	Endopeptidase La	-0.946	-1.745	-1.151	3
AL538_RS03145	Murein L.D-transpeptidase	-0.373	0.473	-1.237	4
AL538_RS01935	Oligoendopeptidase F	-1.118	-1.260	-1.409	2
AL538_RS17705	Xaa-His dipeptidase	1.352	1.540	1.276	5
AL538_RS01705	Xaa-Pro aminopeptidase	-2.220	-4.042	-3.019	3
AL538_RS09315	Xaa-Pro aminopeptidase	-1.018	-1.271	-1.375	2
AL538_RS24480	Xaa-Pro dipeptidase	-1.700	-2.530	-2.663	3
Protein folding					
AL538_RS25315	Molecular chaperone	0.594	1.004	1.011	5
AL538_RS05430	Molecular chaperone	-1.777	-1.784	-2.056	2
AL538_RS12735	Molecular chaperone DnaJ	-0.636	-1.307	-0.720	3
AL538_RS10240	Molecular chaperone Hsp33	-0.578	-1.187	-1.147	3
AL538_RS00770	CcoQ/FixQ family Cbb3-type cytochrome c oxidase assembly chaperone	-1.696	-1.968	-2.171	6
AL538_RS16020	Lipase chaperone	1.174	0.964	1.124	1
AL538_RS00890	RNA chaperone ProQ	-1.198	-1.487	-2.016	6
AL538_RS12125	ATP-dependent chaperone ClpB	-1.046	-1.499	-1.898	6
Antioxidative defence					
AL538_RS19980	Alkyl hydroperoxide reductase subunit F	0.443	0.980	1.170	5
AL538_RS17640	Catalase	1.260	2.075	1.668	5
AL538_RS15575	Catalase-peroxidase	-1.254	-3.259	-2.811	3
AL538_RS05275	Arsenate reductase (glutaredoxin)	-1.339	-0.582	-1.802	4

AL538_RS21845	Glutaredoxin. GrxB family	0.713	1.418	0.700	5
AL538_RS04530	Monothiol glutaredoxin. Grx4 family	-0.871	-0.911	-1.563	6
Nitric oxide detoxification					
AL538_RS03195	Cytochrome c nitrite reductase subunit NrfD	0.565	1.438	0.764	5
AL538_RS22675	Nitrate reductase	-0.473	-0.429	-1.369	4
AL538_RS25185		0.673	1.166	0.939	5
AL538_RS25225		0.607	0.929	1.040	5
AL538_RS24635	Nitrite reductase large subunit	1.472	1.227	1.433	1
AL538_RS13585	Nitrogen regulatory protein P-II	-1.303	-1.864	-1.960	6
AL538_RS05380	Nitrogen regulatory protein P-II	-1.132	-1.471	-1.631	6
Sigma E – ChrR system					
AL538_RS06820	Anti-sigma E factor	-0.557	-0.095	-1.517	4
AL538_RS06810	Positive regulator of sigma E activity	-0.326	-0.313	-1.179	4
DNA damage. repair and synthesis					
AL538_RS13710	Replication initiation regulator SeqA	-1.042	-1.042	-1.632	6
AL538_RS18185	Deoxyribodipyrimidine photolyase	1.446	1.734	1.280	5
AL538_RS10985	ATP-dependent RNA helicase	-0.826	-1.149	-0.665	2
AL538_RS19845	DEAD/DEAH box helicase	0.542	1.216	1.155	5
AL538_RS14190	RNA helicase	0.815	-0.026	1.028	1
AL538_RS06915	DNA-binding protein	-2.431	-2.659	-2.544	2
AL538_RS15785		-1.204	-1.867	-1.031	2
AL538_RS26130		1.140	1.660	1.295	1
AL538_RS07560	Single-stranded DNA-binding protein	-1.535	-1.806	-1.838	2
AL538_RS23750	DNA mismatch repair protein MutT	1.004	0.776	1.593	1
AL538_RS09695	DNA polymerase III subunit beta	-1.011	-1.309	-1.308	2
AL538_RS14655	Exonuclease	0.843	1.358	1.059	5
AL538_RS03225	DNA gyrase subunit A	-0.368	-0.454	-1.216	4
Transport					
Energy-dependent transport TonB-ExbB-ExbD complex					
AL538_RS26550	Biopolymer transporter ExbB	1.623	2.108	1.740	5
AL538_RS19445	TonB-system energizer ExbB	0.201	1.186	1.025	5
ABC transporters					
AL538_RS01240	ABC transporter	0.153	1.407	0.028	5
AL538_RS25410		-2.188	-1.852	-1.608	2
AL538_RS27075		0.982	0.893	1.015	1
AL538_RS17835		0.662	0.415	1.057	1
AL538_RS06445		0.775	0.867	1.108	1
AL538_RS01700	ABC transporter ATP-binding protein	-2.419	-3.833	-2.944	3
AL538_RS00900		-1.257	-1.754	-1.936	3
AL538_RS18590		0.725	1.253	1.153	5
AL538_RS09900		-1.267	-2.130	-2.044	3
AL538_RS22125		-1.420	-1.759	-1.554	2
AL538_RS00590		-0.943	-0.944	-1.249	6
AL538_RS20535		1.253	1.520	0.914	5
AL538_RS06320		1.171	0.921	1.160	1
AL538_RS01775	ABC transporter permease	-2.482	-3.027	-2.565	2
AL538_RS09915		-0.508	-1.138	-0.746	3
AL538_RS25680		0.789	1.230	0.841	5
AL538_RS19325		1.212	1.748	1.369	5
AL538_RS20070		1.311	1.792	1.402	5
AL538_RS20295		1.509	2.157	1.920	5

AL538_RS09910		-1.454	-1.581	-1.890	2
AL538_RS26870	ABC transporter substrate-binding protein	-2.683	-4.749	-3.279	2
AL538_RS17590		-0.742	-1.337	-1.215	3
AL538_RS10445	ABC transporter substrate-binding protein	-0.466	-1.105	-0.901	2
AL538_RS22820		0.646	0.898	1.237	1
AL538_RS14680		-1.806	-2.848	-2.324	3
AL538_RS17540	ABC transporter substrate-binding protein	-0.222	-0.550	-1.112	6
AL538_RS19380	Amino acid ABC transporter	-1.236	-1.698	-1.907	3
AL538_RS23960	Amino acid ABC transporter permease	-0.887	0.650	-1.101	4
AL538_RS00950	Amino acid ABC transporter substrate-binding protein	-1.188	-2.888	-2.524	3
AL538_RS09685		-1.417	-1.807	-2.100	6
AL538_RS23965		-1.390	-2.612	-2.090	3
AL538_RS08400	C4-dicarboxylate ABC transporter	-1.633	-1.850	-1.721	2
AL538_RS15465		0.267	1.105	0.605	5
AL538_RS05790		0.957	1.072	0.681	5
AL538_RS20665	C4-dicarboxylate ABC transporter permease	0.868	1.211	1.186	5
AL538_RS14220		0.884	1.272	0.951	5
AL538_RS11130	C4-dicarboxylate ABC transporter substrate-binding protein	-1.327	-2.314	-2.223	3
AL538_RS25870	Choline ABC transporter ATP-binding protein	0.930	0.579	1.050	1
AL538_RS17400	Cobalt ABC transporter permease	0.847	0.781	1.114	1
AL538_RS17205	Heme acquisition ABC transporter HasD	0.602	0.754	1.029	1
AL538_RS26560	Hemin ABC transporter substrate-binding protein	1.333	0.179	0.996	1
AL538_RS14675	Histidine/lysine/arginine/ornithine ABC transporter ATP-binding protein HisP	-1.671	-1.556	-1.824	2
AL538_RS14685	Histidine/lysine/arginine/ornithine ABC transporter permease HisQ	-1.310	-1.695	-1.841	2
AL538_RS14605	Lipid ABC transporter permease/ATP-binding protein	-0.666	-0.898	-1.634	6
AL538_RS03595	Macrolide ABC transporter ATP-binding protein	1.481	0.502	1.199	1
AL538_RS25190	Nitrate ABC transporter ATP-binding protein	1.037	0.927	1.429	1
AL538_RS01690	Peptide ABC transporter permease	-1.649	-2.492	-2.032	3
AL538_RS04075		-1.067	-0.772	-0.902	2
AL538_RS01680	Peptide ABC transporter substrate-binding protein	-2.146	-4.524	-3.021	3
AL538_RS01780	Polyamine ABC transporter ATP-binding protein	-2.676	-3.963	-3.018	2
AL538_RS01770	Polyamine ABC transporter permease	-2.572	-2.637	-2.720	2
AL538_RS20900	Putative 2-aminoethylphosphonate ABC transporter substrate-binding protein	-2.145	-3.945	-2.776	3
AL538_RS01795	Spermidine/putrescine ABC transporter substrate-binding protein	-2.751	-4.780	-3.382	2
AL538_RS04140	Excinuclease ABC subunit B	0.672	0.777	1.042	1
Sec-dependent translocation					
AL538_RS08685	Preprotein translocase subunit SecE	-0.640	-1.550	-1.241	3
AL538_RS14365	Preprotein translocase subunit Tim44	-0.922	-1.678	-1.686	3
Maltose / maltodextrines transport					
AL538_RS09720	Alpha-amylase	0.858	0.653	1.055	1
AL538_RS20165	Maltose operon protein	-1.820	-2.931	-2.592	3

Phosphate transport (*pstSCAB operon*)					
AL538_RS17175	Phosphate ABC transporter permease subunit PstC	1.465	1.515	1.782	1
AL538_RS17280		-1.142	-1.560	-1.800	6
AL538_RS25505	Phosphate ABC transporter substrate-binding protein	-0.948	-1.124	-1.076	2
AL538_RS17285	Phosphate ABC transporter substrate-binding protein	-2.034	-4.274	-3.377	3
AL538_RS17275	Phosphate ABC transporter. permease protein PstA	-1.414	-2.649	-2.232	3
AL538_RS11445	Phosphate transport regulator	-0.741	-1.150	-1.351	6
AL538_RS04700	Alkaline phosphatase	-1.825	-2.799	-2.727	3
sn-Glycerol 3-phosphate transport system					
AL538_RS26875	Sn-glycerol-3-phosphate transport system.-binding protein inner membrane component	-1.535	-2.186	-1.711	2
Arginine transport					
AL538_RS00965	Arginine ABC transporter ATP-binding protein	-0.989	-1.246	-0.880	2
AL538_RS25420	Arginine ABC transporter ATP-binding protein ArtP	-1.795	-3.056	-1.844	2
AL538_RS25415	Arginine ABC transporter substrate-binding protein	-2.022	-3.010	-2.415	2
Other transporters					
AL538_RS19885	Threonine transporter RhtB	1.013	1.686	0.990	5
AL538_RS19740	Amino acid permease	0.523	1.022	1.190	5
AL538_RS06910	Amino acid transporter	-2.510	-3.611	-2.367	2
AL538_RS13610	Anion permease	-0.068	1.425	-0.175	5
AL538_RS17480	L-lactate permease	-1.622	-1.498	-1.687	2
AL538_RS12815	NCS2 family permease	0.674	1.177	0.826	5
AL538_RS24605	Anaerobic C4-dicarboxylate transporter	0.868	1.553	1.429	5
AL538_RS18075	AI-2E family transporter	-1.038	-1.552	-1.368	3
AL538_RS25455	BCCT transporter	0.519	1.145	0.450	5
AL538_RS26905	Cation transporter	0.774	2.088	0.807	5
AL538_RS13110	Cobalt transporter	-1.115	-1.292	-1.490	2
AL538_RS26660	Copper transporter	1.044	1.275	1.432	1
AL538_RS17580	EamA family transporter	-1.354	-0.854	-1.822	4
AL538_RS17655		0.699	1.191	0.524	5
AL538_RS03560		0.335	0.381	1.063	1
AL538_RS10080		0.859	0.884	1.222	1
AL538_RS25595		0.723	1.235	1.678	1
AL538_RS01055		0.950	1.603	1.870	5
AL538_RS23630		0.589	0.843	1.046	1
AL538_RS16860	Efflux transporter periplasmic adaptor subunit	0.290	1.217	1.291	5
AL538_RS26665		1.095	1.036	1.424	1
AL538_RS23890		1.262	0.254	1.730	1
AL538_RS25350	Electron transporter RnfB	-2.009	-2.692	-2.495	2
AL538_RS15895	Formate transporter FocA	-1.227	-2.626	-2.400	3
AL538_RS24075		1.210	1.168	1.754	1
AL538_RS26880	Glycerol-3-phosphate transporter	-1.884	-1.757	-1.192	2
AL538_RS26795	Long-chain fatty acid transporter	0.754	1.261	0.299	5
AL538_RS25015		0.640	0.909	1.212	1
AL538_RS17795	Lysine transporter LysE	0.978	0.909	1.002	1
AL538_RS20975	Manganese transporter	1.600	1.992	1.690	5

AL538_RS01420	MATE family efflux transporter	0.906	0.972	1.080	1
AL538_RS08350		1.061	0.762	1.096	1
AL538_RS24425		0.825	1.474	1.177	5
AL538_RS18805		1.097	1.564	1.286	5
AL538_RS19005		0.350	1.011	0.598	5
AL538_RS05180	Melibiose:sodium transporter MelB	0.816	0.661	1.085	1
AL538_RS23230	MFS transporter	1.150	1.416	1.364	1
AL538_RS01870		-1.241	-0.311	-1.380	4
AL538_RS25050		0.720	1.015	1.083	1
AL538_RS01445		0.887	1.458	1.150	5
AL538_RS22405		0.689	1.119	1.177	5
AL538_RS18600		0.597	1.427	1.206	5
AL538_RS03545		1.197	1.520	2.155	1
AL538_RS26190		-1.089	-1.177	-2.073	6
AL538_RS24565		0.497	1.197	0.699	5
AL538_RS17405		1.207	1.630	1.251	5
AL538_RS18425		0.914	1.609	1.494	5
AL538_RS21850		1.249	1.445	1.537	1
AL538_RS20680	NADH:ubiquinone reductase (Na(+)-transporting) subunit B	0.921	1.504	1.047	5
AL538_RS05660	NADH:ubiquinone reductase (Na(+)-transporting) subunit C	-1.130	-1.552	-2.129	6
AL538_RS21195		0.334	1.104	0.678	5
AL538_RS05655	NADH:ubiquinone reductase (Na(+)-transporting) subunit D	-0.517	-0.993	-1.483	6
AL538_RS05650	NADH:ubiquinone reductase (Na(+)-transporting) subunit E	-1.335	-1.664	-2.340	6
AL538_RS05645	NADH:ubiquinone reductase (Na(+)-transporting) subunit F	-1.208	-1.823	-1.941	3
AL538_RS25705	Nicotinamide mononucleotide transporter	0.583	1.267	0.486	5
AL538_RS06080	NupC/NupG family nucleoside CNT transporter	-1.506	-2.402	-2.384	3
AL538_RS19095	PTS ascorbate transporter subunit IIBC	1.090	1.290	1.338	5
AL538_RS22020	PTS fructose transporter subunit IIB	-0.993	-0.833	-1.364	6
AL538_RS22005	PTS fructose transporter subunit IIC	0.486	1.119	0.653	5
AL538_RS14995	PTS fructose-like transporter subunit EIIC	0.510	1.145	1.203	5
AL538_RS13505	PTS glucose transporter subunit IIA	-1.322	-2.533	-2.634	3
AL538_RS14335	PTS glucose transporter subunit IIB	0.735	0.892	1.420	1
AL538_RS03855	PTS glucose transporter subunit IIBC	-1.263	-1.644	-2.161	6
AL538_RS17670	PTS mannitol transporter subunit IIA	1.036	1.597	1.373	5
AL538_RS26355	PTS mannitol transporter subunit IIBC	1.188	1.438	1.043	5
AL538_RS17700	PTS mannose transporter subunit IIC	0.757	1.002	1.159	1
AL538_RS13670	PTS N-acetylmuramic acid transporter subunit IIBC	-1.265	-1.290	-1.867	6
AL538_RS15325	PTS sucrose transporter subunit IIBC	1.044	1.311	1.225	5
AL538_RS02080	RND transporter	0.833	1.032	1.159	1
AL538_RS26620	Sodium:proline symporter	0.931	1.445	1.042	5
AL538_RS19580		-1.148	-1.084	-1.286	2
AL538_RS16040	Sodium-dependent transporter	-0.590	-0.627	-1.064	6
AL538_RS16130	Sodium-independent anion transporter	0.648	2.154	-0.150	5
AL538_RS17905	Transporter	0.444	1.747	1.361	5
AL538_RS20000		-0.927	-1.686	-1.117	3
AL538_RS24445		0.726	0.507	1.104	1

AL538_RS15560		0.707	1.240	1.270	5
AL538_RS21740		1.129	1.214	1.357	1
AL538_RS09840		-0.707	-0.943	-1.187	6
AL538_RS15480		0.688	0.856	1.230	1
AL538_RS15315	Porin	1.228	1.243	1.429	1
Translation					
AL538_RS10220	D-tyrosyl-tRNA(Tyr) deacylase	-1.153	-1.650	-0.910	2
AL538_RS06035	Elongation factor G	-1.316	-2.547	-2.559	3
AL538_RS08295	Elongation factor P	-0.416	-0.686	-1.560	4
AL538_RS03335	Elongation factor P-like protein YeiP	-0.811	-1.366	-2.202	6
AL538_RS12075	Energy-dependent translational throttle protein EttA	-1.550	-2.247	-2.244	3
AL538_RS06175	Translation initiation factor IF-2	-0.946	-1.702	-1.504	3
AL538_RS02125	Translation initiation factor IF-3	-1.436	-2.093	-2.476	6
AL538_RS10200	Translational GTPase TypA	-0.795	-1.083	-1.677	6
tRNA and Ribosome biogenesis					
AL538_RS14735	23S rRNA pseudouridylate synthase	0.435	0.471	1.027	1
AL538_RS16205	16S rRNA pseudouridine(516) synthase	0.829	1.243	0.717	5
AL538_RS16200	16S rRNA pseudouridine(516) synthase	1.080	0.921	1.034	1
Ribosomal proteins					
AL538_RS10940	30S ribosomal protein S11	-0.742	-1.618	-1.978	6
AL538_RS07900	30S ribosomal protein S12 [Vibrionaceae]	-1.454	-2.108	-2.716	6
AL538_RS10935	30S ribosomal protein S13	-1.239	-1.082	-1.742	6
AL538_RS10845	30S ribosomal protein S19	-1.068	-0.698	-1.896	4
AL538_RS11980	30S ribosomal protein S20	-1.096	-0.304	-1.460	4
AL538_RS10855	30S ribosomal protein S3	-0.631	-0.915	-1.570	6
AL538_RS10945	30S ribosomal protein S4	-0.871	-1.974	-2.224	3
AL538_RS20585	30S ribosomal protein S6--L-glutamate ligase	-0.896	-1.369	-1.560	6
AL538_RS07895	30S ribosomal protein S7	-0.665	-1.414	-1.753	6
AL538_RS10895	30S ribosomal protein S8	-0.477	-0.576	-1.438	4
AL538_RS11540	30S ribosomal protein S9	-1.169	-0.686	-1.635	4
AL538_RS11535	50S ribosomal protein L13	-0.915	-0.815	-1.966	4
AL538_RS10875	50S ribosomal protein L14 [Vibrionaceae]	-0.529	-0.802	-1.056	6
AL538_RS10860	50S ribosomal protein L16	-0.716	-1.463	-1.771	6
AL538_RS10840	50S ribosomal protein L2	-0.827	-0.764	-1.574	4
AL538_RS10850	50S ribosomal protein L22	-0.954	-2.066	-2.065	6
AL538_RS10865	50S ribosomal protein L29	-1.083	-0.671	-2.028	4
AL538_RS10825	50S ribosomal protein L3	-1.234	-1.966	-2.312	6
AL538_RS10900	50S ribosomal protein L6	0.001	-0.629	-1.019	6
AL538_RS03580	Endonuclease	0.868	0.364	1.120	1
AL538_RS20180	Endonuclease I	-0.445	-0.916	-1.033	3
AL538_RS04175	Endonuclease III	-0.766	-1.634	-1.222	3
AL538_RS11800	Ribonuclease	-1.242	-0.500	-1.299	4
AL538_RS03935	Ribonuclease E	-0.757	-1.385	-0.973	3
AL538_RS10780	Ribonuclease E activity regulator RraA	-1.298	-2.066	-2.651	6
AL538_RS08050	Ribonuclease R	-0.902	-1.215	-1.291	3
AL538_RS25010	Ribonuclease Z	0.814	1.160	1.314	1
AL538_RS04915	RNA polymerase sigma factor	-0.994	-1.647	-1.399	3
AL538_RS05025	RNA polymerase sigma factor FliA	-0.755	-0.910	-1.455	6
AL538_RS06825	RNA polymerase sigma factor RpoE	-0.591	-0.781	-1.899	4
AL538_RS08980	RNA polymerase sigma factor RpoH	-0.788	-1.491	-1.994	6

AL538_RS06705	RNA polymerase sigma factor RpoS	-1.885	-3.163	-3.052	2
AL538_RS07370	RNA polymerase sigma-54 factor	-0.759	-1.047	-1.281	6
AL538_RS22840	RNA polymerase subunit sigma-70	-0.345	-0.173	-1.762	4
AL538_RS26860	RNA polymerase subunit sigma-70	-1.093	-0.744	-0.901	2
AL538_RS07425	RNA polymerase-binding ATPase	-0.474	-0.649	-1.135	6
AL538_RS06965	Deoxyribonuclease I	0.845	1.282	1.406	5
Iron uptake. storage & utilization					
AL538_RS26680	Ferric reductase	0.938	1.185	1.140	5
Iron uptake/transport					
AL538_RS12515	Fe-S cluster assembly protein SufD	0.737	1.237	1.065	5
AL538_RS21155	Iron permease	0.959	1.295	1.249	1
AL538_RS06355	Iron ABC transporter permease	0.363	1.014	0.651	1
AL538_RS25685	Iron ABC transporter	-0.667	-0.229	-1.637	4
AL538_RS23515		1.203	1.009	0.937	1
AL538_RS20075		1.074	0.899	1.422	1
AL538_RS09135	Iron donor protein CyaY	-0.208	-1.088	-1.515	6
Iron storage					
AL538_RS07875	Bacterioferritin	-2.859	-2.868	-3.496	2
AL538_RS12375	Ferredoxin. 2Fe-2S type. ISC system	-0.365	-0.256	-1.152	4
Iron cluster biogenesis					
AL538_RS12520	Bifunctional cysteine desulfurase/selenocysteine lyase	0.660	0.922	1.077	5
AL538_RS16405	Cysteine desulfurase	-2.740	-3.362	-3.312	2
AL538_RS12365	Co-chaperone protein HscB	-0.393	-1.289	-0.823	3
Biosynthesis of L-cysteine					
AL538_RS07655	Sulfite reductase subunit beta	-1.257	-1.630	-1.620	2
AL538_RS20130	Sulfite reductase subunit C	0.657	0.874	1.315	1
Other pathways					
Nicotinate and nicotinamide metabolism					
AL538_RS17615	Nicotinate phosphoribosyltransferase	0.127	1.043	0.996	5
AL538_RS18270	Glutamate-aspartate symport protein	1.114	1.428	1.169	5
Thiamin metabolism					
AL538_RS25360	Thiamine biosynthesis protein ApbE	1.047	1.221	1.312	1
AL538_RS11010	Sulfate adenylyltransferase	-0.732	-1.077	-1.065	3
AL538_RS11005	Sulfate adenylyltransferase subunit 2	-0.987	-1.234	-1.033	2
Glutathione metabolism					
AL538_RS04180	Lactoylglutathione lyase	-1.666	-3.005	-2.321	3
AL538_RS25390	Glutathione S-transferase	-1.089	-1.186	-1.405	6
AL538_RS22050		-1.390	0.111	-0.768	4
AL538_RS06975	Glutathione synthase	-1.310	-1.297	-1.993	6
Quorum sensing					
AL538_RS00010	LuxR family transcriptional regulator	-1.042	-1.200	-1.147	2
AL538_RS06465		-1.543	-2.549	-2.843	3
AL538_RS00610	Two-component sensor histidine kinase	0.675	0.721	1.106	1
AL538_RS20460		0.881	1.319	1.131	5
AL538_RS17990	Two-component system sensor histidine kinase CreC	0.864	0.419	1.090	1
AL538_RS24500	Two-component system sensor histidine kinase UhpB	0.913	0.719	1.342	1
AL538_RS22485	Sensor histidine kinase	0.822	1.252	0.725	5
AL538_RS19550		0.720	1.090	1.059	5
AL538_RS19310		1.253	1.080	1.148	1

AL538_RS18275		0.490	1.532	1.194	5
AL538_RS02980	Hybrid sensor histidine kinase/response regulator	-0.563	-0.792	-1.030	6
AL538_RS00795		0.812	0.670	1.073	1
AL538_RS06770		-0.218	-0.424	-2.037	4
Miscellaneous					
Stress response					
AL538_RS26850	Universal stress global response regulator UspA	-1.278	-0.854	-1.887	4
AL538_RS10050	Universal stress global response regulator UspA	-0.992	-0.978	-1.660	6
Others					
AL538_RS05435	Outer membrane protein assembly factor BamA	-0.779	-1.599	-1.684	3
AL538_RS12420	Outer membrane protein assembly factor BamB	-0.812	-1.599	-1.444	3
AL538_RS05240	Outer membrane protein assembly factor BamC	-1.080	-1.393	-1.286	2
AL538_RS05720	Outer membrane protein OmpK	-1.980	-2.639	-2.290	2
AL538_RS14325	O-succinylbenzoate synthase	-1.153	-1.844	-1.224	2
AL538_RS22395	3.4-dihydroxy-2-butanone 4-phosphate synthase	0.737	1.074	0.926	5
AL538_RS15695	Biotin synthase BioB	-1.330	-0.713	-1.181	2
AL538_RS07310	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	-0.667	-0.920	-1.088	6
AL538_RS17760	Alpha-mannosidase	0.477	1.090	1.019	5
AL538_RS17770		0.790	1.085	1.166	5
AL538_RS17780		0.932	1.368	1.351	5
AL538_RS17765		1.126	0.913	1.411	1
AL538_RS17775		1.381	1.424	1.632	1
AL538_RS21700	Beta-mannosidase	1.160	1.126	1.271	1
gene.csv.7	CsrB	-1.626	-0.840	-1.902	4
gene.csv.11		-1.027	-0.188	-1.776	4
AL538_RS08095	RNA-binding protein Hfq	-1.770	-2.431	-2.078	2
gene.csv.32	Cold shock protein (CspA)	-0.318	-0.419	-1.767	4
gene.csv.35		1.675	1.647	1.371	5
Virulence factors involved in V. harveyi pathogenicity					
AL538_RS11345	Hemolysin	-1.757	-2.962	-2.300	3
AL538_RS26170	Heme A synthase	-0.724	-1.094	-1.258	3
AL538_RS26535	Heme degradation protein HemS	0.323	1.350	0.204	5
AL538_RS04975	Heme exporter protein CcmB	0.544	1.360	0.304	5
AL538_RS15910	Pentaheme c-type cytochrome TorC. partial	-0.908	-1.358	-1.371	3
AL538_RS26165	Protoheme IX farnesyltransferase	-1.184	-1.763	-2.012	3
AL538_RS23720	Protoheme IX farnesyltransferase	0.985	1.324	1.312	5
AL538_RS26540	Putative heme utilization radical SAM enzyme HutW	1.028	1.301	0.458	5
AL538_RS01310	Sulfoxide reductase heme-binding subunit YedZ	0.569	1.017	1.110	1
AL538_RS20930	VirK protein	0.708	1.912	1.755	5
AL538_RS23520	TonB-dependent receptor	1.039	1.028	1.106	1
AL538_RS16585	TonB-dependent siderophore receptor	1.011	0.645	0.918	1
AL538_RS23490	TonB-dependent siderophore receptor	0.791	1.106	0.958	5

AL538_RS01205	TonB-dependent siderophore receptor	0.832	0.827	1.152	1
AL538_RS25660	TonB-dependent siderophore receptor	1.338	1.520	1.380	5
AL538_RS24600	Siderophore ferric iron reductase	0.894	1.072	0.767	5
Chemotaxis. Motility & Biofilm					
AL538_RS19295	Chemotaxis protein	0.963	1.041	1.099	5
AL538_RS23565		1.116	0.788	1.205	1
AL538_RS11460		0.774	1.121	0.939	5
AL538_RS25425		0.892	1.340	1.235	5
AL538_RS21280	Chemotaxis protein LafU	0.997	1.305	0.946	5
AL538_RS10030	Diguanylate cyclase	0.503	1.289	0.778	5
AL538_RS26965		1.059	0.697	0.922	1
AL538_RS21410	Diguanylate phosphodiesterase	0.746	1.152	1.034	5
AL538_RS23540		0.951	0.980	1.254	1
AL538_RS25840		0.598	0.468	1.126	1
AL538_RS27365		0.825	1.345	1.024	5
AL538_RS13415	Flagellar biosynthesis anti-sigma factor FlgM	-1.020	0.077	-1.683	4
AL538_RS21335	Flagellar biosynthesis pathway. component FlhA	0.720	1.116	0.759	5
AL538_RS21340	Flagellar biosynthesis protein FlhB	0.891	1.557	1.256	5
AL538_RS21345	Flagellar biosynthetic protein FliR	0.808	1.413	1.439	5
AL538_RS21315	Flagellar capping protein	1.304	0.850	1.534	1
AL538_RS05055	Flagellar export apparatus protein FliQ	1.030	1.865	0.874	5
AL538_RS21310	Flagellar export chaperone FliS	1.346	1.556	1.531	5
AL538_RS21075	Flagellar hook-associated protein FlgK	1.127	1.588	0.855	5
AL538_RS05085	Flagellar hook-length control protein FliK	0.388	1.025	0.232	5
AL538_RS21300	Flagellar hook-length control protein FliK	0.463	1.081	0.755	5
AL538_RS24925	Flagellar motor protein	0.760	0.999	1.049	5
AL538_RS21365	Flagellar motor switch protein FliM	1.127	1.619	1.423	5
AL538_RS21385	Flagellar M-ring protein FliF	1.264	1.483	1.241	5
AL538_RS21070	Flagellar protein	0.834	1.240	1.240	5
AL538_RS21295	Flagellar protein	1.335	1.228	1.508	1
AL538_RS05165	Flagellin	0.446	1.218	1.041	5
AL538_RS04190	Sodium-type flagellar protein MotY	1.146	1.543	1.596	5
AL538_RS16835	Pilus assembly protein	-0.034	-1.399	-0.470	3
AL538_RS01140	Pilus assembly protein CpaB	1.085	0.936	1.697	1
AL538_RS05990	Pilus assembly protein CpaC	-0.569	-1.303	-1.285	3
AL538_RS05975	Pilus assembly protein CpaF	-0.596	-0.775	-1.455	4
AL538_RS01120		0.939	1.560	1.340	5
AL538_RS25995		1.152	1.522	1.409	5
AL538_RS22750	Pilus assembly protein PapD	1.277	0.933	1.301	1
AL538_RS07780	Pilus assembly protein PilN	0.421	0.652	1.299	1
AL538_RS22580	Pilus assembly protein PilZ	0.500	1.024	0.860	5
AL538_RS01115	Pilus assembly protein TadB	0.998	0.673	1.619	1
AL538_RS01100	Pilus assembly protein TadE	0.701	1.752	1.274	5
Secretion					
AL538_RS01135	General secretion pathway protein GspD	0.912	0.806	1.300	1
AL538_RS10300	General secretion pathway protein GspM	-0.116	-0.269	-1.203	2
AL538_RS01740	Hydrophobe/amphiphile efflux-1 family RND transporter	0.687	0.878	1.078	1
AL538_RS22720		0.934	1.207	0.960	5
Type I secretion apparatus					
AL538_RS22870	HlyD family type I secretion periplasmic	0.673	1.107	0.793	5

	adaptor subunit				
AL538_RS00995	Type I secretion C-terminal target domain-containing protein	-0.831	-1.608	-2.062	6
Type II secretion apparatus					
AL538_RS05980	Type II secretion protein	-0.377	-1.184	-0.698	3
Type III secretion apparatus					
AL538_RS15295	Translocator protein PopB	-1.081	-1.179	-1.296	2
AL538_RS15300	Translocator protein PopD	-1.118	-1.594	-1.132	2
AL538_RS15275	Type III secretion system regulator LcrR	0.367	1.750	0.793	5
AL538_RS15125	EscC/YscC/HrcC family type III secretion system outer membrane ring protein	1.239	0.974	1.136	1
AL538_RS15130	EscD/YscD/HrpQ family type III secretion system inner membrane ring protein	0.795	1.100	1.732	1
AL538_RS15160	EscJ/YscJ/HrcJ family type III secretion inner membrane ring protein	1.077	1.212	1.477	1
AL538_RS15270	EscV/YscV/HrcV family type III secretion system export apparatus protein	1.152	1.169	1.261	1
Type IV secretion apparatus					
AL538_RS24855	Type IV secretion protein Rhs	0.961	0.747	1.322	1
Type VI secretion apparatus					
AL538_RS16980	Type VI secretion protein	-0.970	-3.070	-2.631	3
AL538_RS16990	Type VI secretion protein	-0.612	-1.910	-1.804	3
AL538_RS24870	Type VI secretion protein	1.023	0.684	1.063	1
AL538_RS24905	Type VI secretion protein	0.537	0.496	1.178	1
AL538_RS23415	Type VI secretion protein	1.769	1.838	1.741	5
AL538_RS16935	Type VI secretion protein	-0.665	-1.550	-1.467	3
AL538_RS16975	Type VI secretion protein EvpB	-1.048	-3.869	-2.889	3
AL538_RS23390	Type VI secretion protein IcmF	0.435	1.398	0.462	5
AL538_RS24920	Type VI secretion protein IcmF	1.105	0.733	1.124	1
AL538_RS16930	Type VI secretion system-associated protein	-0.795	-1.004	-0.975	2
Drug efflux & antibiotic resistance					
AL538_RS20560	Bcr/CflA family drug resistance efflux transporter	0.695	1.286	1.148	5
AL538_RS15440	Multidrug transporter	-0.153	-1.599	-0.219	3
AL538_RS19345		1.040	0.341	1.281	1
AL538_RS22455		1.175	1.032	1.447	1
AL538_RS20920		1.644	1.584	1.755	1
AL538_RS23900	Multidrug transporter AcrB	1.071	1.484	1.265	5
AL538_RS18015	Multidrug transporter subunit MdtL	0.553	1.173	1.141	5
AL538_RS22715	MexE family multidrug efflux RND transporter periplasmic adaptor subunit	0.948	0.803	1.183	1
AL538_RS22070	Antibiotic resistance protein VanZ	-0.297	-0.232	-1.158	4
AL538_RS14540	Penicillin-binding protein activator LpoB	-1.185	-2.204	-2.303	3
AL538_RS12170	Penicillin-insensitive murein endopeptidase	-0.514	-1.273	-1.278	3
AL538_RS12395	Bifunctional tRNA (adenosine(37)-C2)-methyltransferase TrmG/ribosomal RNA large subunit methyltransferase RlmN	0.290	1.152	0.301	5
AL538_RS25145	Polyketide cyclase	0.622	1.104	1.046	5