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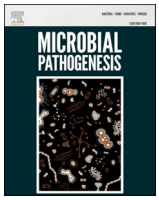

Genes involved in the adhesion and invasion of *Arcobacter butzleri*

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ABSTRACT

Arcobacter butzleri is a foodborne pathogen that mainly causes enteritis in humans, but the number of cases of bacteraemia has increased in recent years. However, there is still limited knowledge on the pathogenic mechanisms of this bacterium. To investigate how *A. butzleri* causes disease, single *knockout* mutants were constructed in the *cadF*, ABU_RS00335, *ciaB,* and *flaAB* genes, which might be involved in adhesion and invasion properties. These mutants and the isogenic wild-type (WT) were then tested for their ability to adhere and invade human Caco-2 and HT29-MTX cells. The adhesion and invasion of *A. butzleri* RM4018 strain was also visualized by a Leica CTR 6500 confocal microscope. The adhesion and invasion abilities of mutants lacking the invasion antigen CiaB or a functional flagellum were lower than those of the WTs. However, the extent of the decrease varied depending on the strain and/or cell line. Mutants lacking the fibronectin (FN)-binding protein CadF consistently exhibited reduced abilities, while the inactivation of the other studied FN-binding protein, ABU_RS00335, led to a reduction in only one of the two strains tested. Therefore, the *ciaB* and *flaAB* genes appear to be important for *A. butzleri* adhesion and invasion properties, while *cadF* appears to be indispensable.

1. Introduction

Arcobacter butzleri has been linked to severe cases of enteritis and bacteraemia in humans, including other single cases of peritonitis and endocarditis [1]. Unlike the closely related *Campylobacter jejuni* species, *A. butzleri* causes a more prolonged and watery form of diarrhoea, instead of bloody and acute diarrhoea [2]. Symptoms of *A. butzleri* infection can range from mild to severe and persistent [3], suggesting that the doses result in different progression of the disease. The general clinical prevalence of this pathogen is not clear, as it is not routinely detected in microbiology laboratories, with reported incidence rates ranging from 0.4 to 56.7 % [4,5]. In addition, *A. butzleri* is also involved as causative agent of diarrhoea, mastitis and abortion in animals [6]. The distribution of this microorganism is known to be widespread as it can be isolated from different environmental water sources, raw and ready-to-eat foods, gastrointestinal tract of animals, and foods of animal origin. In fact, the latter are the main transmission sources for humans [7].

The pathogenic potential of *A. butzleri* has been investigated, especially through *in vitro* studies. These studies have shown that this bacterium has the ability to adhere to and invade human and animal culture cells, produce toxins, and induce barrier dysfunction and host cell inflammation [8–10]. However, there have been limited *in vivo* studies aimed at elucidating the pathophysiology and pathogenicity of *A. butzleri* [11]. Furthermore, the genome of *A. butzleri* presents numerous putative-virulence genes that may be useful in understanding these mechanisms [12,13].

Despite all the efforts made to demonstrate the pathogenic potential of *A. butzleri*, the mechanisms of pathogenesis remain unclear. Some of its virulence genes, homologs to those of *C. jejuni,* are commonly used as virulence markers and are thought to be involved in cell adhesion and invasion of *A. butzleri* [6]. For example, *cadF* (ABU_RS02405), which encodes a FN-binding protein, has been shown in *C. jejuni* to be important to adhere to and invade tissue culture cells [14]. In a recent study [15], transcriptomic analysis showed that several genes, in particular *cadF,* are upregulated during the infection process. ABU_RS00335, a

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homologue of the *C. jejuni cj1349* gene that also encodes a FN-binding protein, has also been shown to be associated with cell adhesion and invasion [16]. The *hecA,* involved in attachment, aggregation, and epidermal cell killing, promotes adherence of diverse bacteria to host cells [17,18]. The *ciaB* gene (ABU_RS07735), which encodes the invasion antigen B, has been shown in some studies to be involved in the invasion process of *C. jejuni* into host cells [19]. In addition, *A. butzleri* possesses two flagellin genes, *flaA* (ABU_RS11245) and *flaB* (ABU_RS11250), which are located adjacent to each other. These flagelar genes are known to be important for motility in *A. butzleri* [20,21]. Their role in host cell adhesion and invasion has been extensively studied in *C. jejuni* [22–24].

In this study, we aimed to investigate the putative virulence genes *cadF*, ABU_RS00335, *hecA*, *ciaB*, and *flaAB* of *A. butzleri* in their role in enabling this bacterium to adhere to and/or invade human tissue culture cells. To our knowledge, this is the first time that these genes have been studied in *A. butzleri* in relation to these functions. Althought all gene inactivations had some effect on the adhesion and invasion properties of *A. butzleri*, the *cadF* gene appeared to be essential. This could be an important step towards understanding the mechanism by which this pathogen infects humans and for developing effective prevention and control strategies in the future.

2. Materials and methods

2.1. Bacterial strains, cell lines and growth conditions

A complete list of the bacterial strains and plasmids used in this study is provided in Table 1. *A. butzleri* strains were routinely grown aerobically at 30 °C for 12–16 h in Brain Heart Infusion (BHI) broth (Oxoid) or for 24–48 h on Columbia agar with 5 % sheep blood plates (BD™). *Escherichia coli* strains were routinely grown aerobically at 37 °C for 12–16 h in Luria-Bertani (LB) broth (Condalab) or for 24 h on LB agar plates (Condalab), supplemented with 100 μg/mL ampicillin (Amp; CAS: 69-52-3; Sigma-Aldrich) or 50 μg/mL kanamycin (Km; CAS: 25389-94-0; NZYTech) when necessary. Shaking (150 rpm) was applied when necessary.

The human gastrointestinal epithelial cell lines Caco-2 (ATCC-HTB-37) and HT29-MTX (a kind gift of Dr. Thécla Lessuffleur) [27] were routinely grown in 25 cm^2 flasks in 1X Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) containing 10 % fetal bovine serum (FBS; Thermo Fisher Scientific) at 37 °C in 10 % CO_2.

Table 1
Strains and plasmids used in this study.

Strains or plasmids	Characteristics/description[a]	Reference[b]
E. coli DH5α NCCB2955	Competent cells for cloning	NCCB
RM4018	Wild-type (WT) reference strain	[17]
RM4018Δ*cadF*::Km	*cadF* deletion containing mutant Km^r (FN-binding deficient)	This study
RM4018ΔABU_RS00335::Km	ABU_RS00335 deletion containing mutant Km^r (FN-binding deficient)	This study
RM4018Δ*ciaB*::Km	*ciaB* deletion containing mutant Km^r (invasion antigen B deficient)	This study
RM4018Δ*flaAB*::Km	*flaAB* deletion containing mutant Km^r (non-motile)	This study
Ab-PV16	WT strain isolated from turkey	[25]
Ab-PV16Δ*cadF*::Km	*cadF* deletion containing mutant Km^r	This study
Ab-PV16ΔABU_RS00335::Km	ABU_RS00335 deletion containing mutant Km^r	This study
Ab-PV16Δ*ciaB*::Km	*ciaB* deletion containing mutant Km^r	This study
Ab-PV16Δ*flaAB*::Km	*flaAB* deletion containing mutant Km^r	This study
pGEM-T Easy Δ*cadF*::Km	*cadF* deletion containing plasmid	This study
pGEM-T Easy ΔABU_RS00335:Km	ABU_RS00335 deletion containing plasmid	This study
pGEM-T Easy Δ*ciaB*::Km	*ciaB* deletion containing plasmid	This study
pGEM-T Easy Δ*flaAB*::Km	*flaAB* deletion containing plasmid	[21]
pMW2	Km^r cassette containing plasmid	[26]

[a] Km^r, kanamycin resistant.
[b] NCCB, Netherlands Culture Collection of Bacteria.

2.2. Isolation of nucleic acids

Genomic DNA from RM4018 was isolated using the PrepMan™ Ultra reagent (Applied Biosystems) according to the manufacturer's instructions. Plasmid DNA was prepared using the GeneJET™ Plasmid Miniprep Kit (Thermo Fisher Scientific) following the manufacturer's protocol.

2.3. Construction of the A. butzleri knockout mutant strains

Knockout strains for adhesion and invasion-associated genes were constructed using methods previously described [28]. Briefly, the genes and their flanking regions were amplified by PCR using the primers sets listed in Table 2 and the proofreading enzyme ACCUZYME™ DNA polymerase (Bioline). The resulting PCR fragments were 5′-A tailed using BIOTAQ™ DNA polymerase (Bioline) and cloned into the commercial cloning vector pGEM-T Easy (Promega). The resulting plasmids were linearized by an outward PCR using *Bam*HI restriction site containing primers, and subsequently ligated to a Km resistance cassette (*aph (3′)-III*) obtained from the plasmid pMW2 [26]. The orientation of the cassette and the mutated ORFs were the same in all constructed plasmids. These final plasmids were purified and electroporated into electrocompetent cells of the WT *A. butzleri* strains RM4018 and Ab-PV16. Colonies growing on Columbia agar with 5 % sheep blood plates (BD™) supplemented with 50 μg/mL Km were then selected as candidates for each *A. butzleri* mutant. The mutation of the genes was confirmed by PCR.

2.4. Growth analysis

The growth curve profiles of all the strains were obtained as previously described [21] using a SynergyTM HT plate reader (BioTek) to monitor the optical density (OD_{550}) at different point times during 24 h. The exponential growth rates were calculated from three separated growth experiments, and the results were expressed as exponential growth curves (h^{-1}) ± standard error (SE).

2.5. Motility assay

The motility of both WT strains, RM4018Δ*flaAB*::Km, and Ab-PV16Δ*flaAB*::Km was tested by stabbing them with a pipette tip into semisolid thioglycolate medium containing 0.4 % agar (Scharlau) and incubating at 30 °C for 24 h. The assay was performed in triplicate in three independent experiments. Results were expressed as the mean of the halos diameter (cm) ± SE.

2.6. Adhesion and invasion assays

For each adhesion and invasion assay, each cell line was split into two 24-well plates by adding 2×10^4 Caco-2 cells/well and 4×10^5 HT29-MTX cells/well, and subsequently incubating the plates for 48 h until approximately 90 % confluence was reached. Both Caco-2 and HT29-MTX cell line monolayers were then infected following previously described methods [29], with slight modifications. Briefly, overnight *A. butzleri* liquid cultures were diluted to an OD_{550} of 0.08 (approximately 10^9 CFU mL^{-1}), harvested by centrifugation, (900 × *g* for 5 min) and suspended in the same volume of tempered (37 °C) 1X DMEM (Thermo Fisher Scientific) with 10 % of FBS (Thermo Fisher Scientific). For the adhesion assays, cell monolayers were inoculated with 0.5 mL of the prepared bacterial suspension, which corresponds to an approximate multiplicity of infection (MOI) of 2500 on both cell lines, and incubated at 37 °C for 2 h. The cells were then washed twice with 1X Dulbecco's phosphate buffered saline containing calcium chloride and magnesium

Table 2
List of primers used to generate the mutants and verify the deletions.

Target gene	Primer name and sequence[a,b]	Amplicon size (bp)	Description	Reference
cadF	cadF-F 5′-AATAGTAGATGCTGGATACG-3′ cadF-R 5′-TGCTAGAGCTAGATTATATG-3′	1610	Amplification of *cadF* gene	This study
	ΔcadF-F 5′-GGATCCAAAGATAAACCAGCGTTAG-3′ ΔcadF-R 5′-GGATCCTCTGCTGTTAATGCTTTAG-3′	3924	*cadF* gene deletion	
ABU_RS00335	cj1349-F 5′-CGTTGCAGTAGGTGAAGAAG-3′ cj1349-R 5′-GTGCAGTAACAACAGACATC-3′	1934	Amplification of *ABU_RS00335* gene	
	Δcj1349-F 5′-GGATCCAATTATCGAGTTTGATTTAG-3′ Δcj1349R 5′-GGATCCATACCTCAATCAGTTATAATTC-3′	3955	*ABU_RS00335* gene deletion	
ciaB	ciaB-F 5′-GCACAATTACTATCTTTGAC-3′ ciaB-R 5′-AATAATGGCTTTGGATTGAC-3′	2549	Amplification of *ciaB* gene	
	ΔciaB-F 5′-GGATCCAAATACTTTATGCCAAATG-3′ ΔciaB-R 5′-GGATCCGCAAATCTTAAATCACTATTC-3′	3993	*ciaB* gene deletion	
hecA	hecA-F 5′-ATGGACTGCAACAAACTG-3′ hecA-R 5′-TCAAGTCGTTGTTCTC-3′		Amplification of *hecA* gene	
	ΔhecA-F 5′-GGATCCTCTGATAATCCAAGTTTAG-3′ ΔhecA-R 5′-GGATCCTCATCACCATCAATTATTC-3′		*hecA* gene deletion	
flaAB	flaAB-F 5′-CAGAAAATGGTGCTAAATTCTTAGG-3′ flaAB-R 5′-TACCTTCGTAGAATTTACAATGTGTC-3′	2569	Amplification of *flaAB* gene	[21]
	ΔflaAB-F 5′-AGGATCCGCTGGTGCAACTGCTGGTACAAC-3′ ΔflaAB-R 5′-AGGATCCTTTGCAATAGCCGTTCTACC-3′	5330	*flaAB* gene deletion	
aph(3′)-III	*Aph(3′)-III-F* 5′-GGGGATCAAGCCTGATTGGGAGA-3′	–	Used in combination of the reverse primer for each corresponding gene to confirm that *aph(3′)-III* is in the mutant DNA.	[26]

[a] F, forward; R, reverse.
[b] The *Bam*HI restriction site is underlined in the primer sequence.

chloride (DPBS; Sigma), and lysed with 1 % Triton X-100 (Sigma) by adding 0.5 mL per well and allowing to act for 10 min. The total number of bacteria associated with tissue cells was then calculated. For the invasion assays, the plates were inoculated and incubated as for the adhesion assays, followed by treatment with DMEM containing 125 μg/mL gentamicin (Thermo Fisher Scientific) during 1 h in order to kill extracellular bacteria. The cells were then washed and lysed as above-mentioned; and the number of intracellular bacteria was calculated. The total numbers of cell-associated bacteria and intracellular bacteria were determined by plating the respective lysates on BHI agar plates (Oxoid) supplemented with 5 % defibrinated sheep blood (Bio-Trading). The number of adhered bacteria was calculated as the difference between the total number of bacteria associated with tissue cells and the number of intracellular bacteria. Per experiment, each strain was studied in triplicate and the experiments were repeated on a minimum of three independent occasions. Results were expressed as the mean number of bacteria (CFU ml^{-1}) that adhered to or invaded cells ± SE.

2.7. Confocal microscopy

Immunofluorescence labelling was performed to visualize the adhesion and invasion abilities of the strain RM4018 WT in Caco-2 and HT29-MTX cells using confocal microscopy. The above-mentioned protocol for adhesion and invasion was followed, with some additional steps. Briefly, both cell lines were split into two 24-well plates containing cover slips and grown for 72 and 96 h, respectively. Once monolayers were formed, the nuclei of both culture cells were stained with 5 μg/mL Hoechst 33342 (Thermo Fisher Scientific) in 1 % bovine serum albumin (BSA)/DPBS (Sigma) for 10 min, and subsequently washed twice with 1X DPBS. Arcobacters were then stained according to the instructions of the Cell Tracer™ Far Red Cell Proliferation Kit (Invitrogen). The labelled bacteria were incubated for 2 h with the Hoechst stained Caco-2 and HT29-MTX cell lines. For the invasion assay, 125 μg/mL gentamicin (Thermo Fisher Scientific) was added for 1 h. After two washes with 1X DPBS the cells were fixed with 4 % of cold paraformaldehyde (Thermo Fisher Scientific) in DPBS for 20 min. The membrane of the culture cells was stained with (WGA)-Alexa 488 diluted 1:250 (W11262, Invitrogen) in 1 % BSA/DPBS for 20 min. The coverslips were then removed from the wells, washed three times with 1X DPBS (Sigma), twice with Milli-Q water, and dried on paper. Finally, the coverslips were placed on a slide containing a 5 μL drop of ProLong™ Diamond Antifade Mountant (Invitrogen). The Leica CTR 6500 confocal microscope was used to visualize adhered and internalized *A. butzleri* in Caco-2 and HT29-MTX cells using laser excitation spectral lines at 361, 495 and 630 nm.

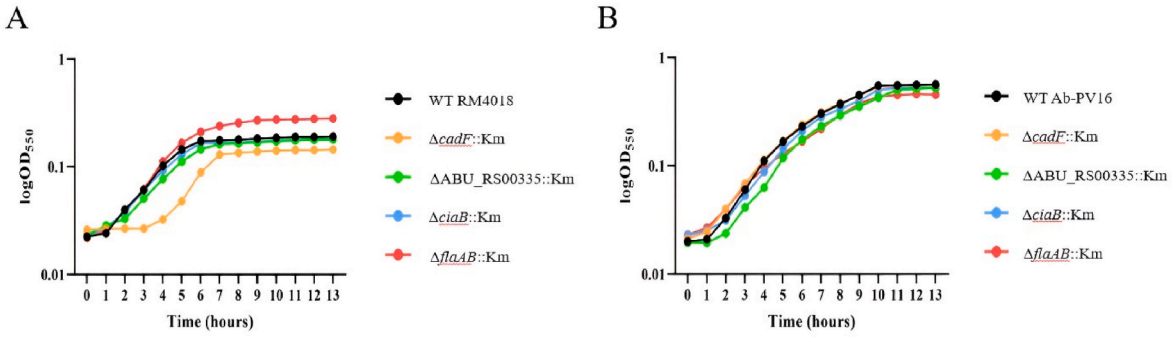
2.8. Statistical analysis

GraphPad Prism 9.0 software was used to analyse the data. All experiments were performed in triplicate and repeated three times. The statistical analysis was performed using one-way ANOVA. Significance was stablished at p level of <0.05.

3. Results

3.1. Mutagenesis, growth analysis, and motility

The *cadF*, *ABU_RS00335*, *ciaB*, and *flaAB* genes were successfully inactivated in *A. butzleri* RM4018 and Ab-PV16. The construction of the final *knockout* plasmid for the *hecA* gene presented certain challenges, which resulted in the inability to obtain *hecA* mutants. The function of the *cadF*, ABU_RS00335, *ciaB,* and *flaAB* genes was studied by deleting a fragment of each gene (703 bp, 996 bp, 1573 bp, and 1337 bp, respectively) and replacing by a Km resistance cassette. The resulting mutants were verified by PCR and differences in planktonic growth were investigated by means of growth curves. No differences in the growth rate in BHI broth were observed between the WT strains and their isogenic mutants (ANOVA-based $p > 0.05$) (Fig. 1A–C). In addition, no differences were observed in the colonies formed on blood agar plates (data not shown). Based on these results, none of the mutations led to a crucial growth defect. However, the RM4018 mutant in *cadF* showed a longer



C

Strain	Exponential growth rate (h^{-1}) ± SE
WT RM4018	0.180 ± 0.018
Δ*cadF*::Km	0.171 ± 0.022
ΔABU_RS00335::Km	0.177 ± 0.015
Δ*ciaB*::Km	0.179 ± 0.013
Δ*flaAB*::Km	0.183 ± 0.018
WT Ab-PV16	0.252 ± 0.011
Δ*cadF*::Km	0.253 ±0.010
ΔABU_RS00335::Km	0.246 ± 0.016
Δ*ciaB*::Km	0.250 ± 0.013
Δ*flaAB*::Km	0.248 ± 0.011

Fig. 1. Growth curves of (A) WT RM4018 strain and its isogenic mutants and (B) WT Ab-PV16 strain and its isogenic mutant strains. (C) Exponential growth rates (h^{-1}) with SE of both WT strains and their isogenic mutants. Experiments were repeated three times. Growth curves are representative.

lag phase compared to the other mutants. This suggests that the *cadF* mutation is hindering RM4018's ability to adapt to liquid medium after growing in solid medium, thus affecting the time it takes for RM4018 to start growing.

To verify the different motility of the WT strains and their isogenic *flaAB* mutants, their motility was investigated by a soft agar motility assays. These assays demonstrated that the motility between the WT strains and their *flaAB* mutants differs significantly [*** ANOVA based statistically significant differences ($p < 0.001$)] (Fig. 2A and B). The WT RM4018 strain showed a halo diameter of 1.96 ± 0.03 cm, while the RM4018Δ*flaAB*::Km mutant had a halo diameter of 0.27 ± 0.03. The WT Ab-PV16 strain had a halo diameter of 2.20 ± 0.15 cm and Ab-PV16Δ*flaAB*::Km showed a halo of 0.23 ± 0.03 cm. The results demonstrate that the deletion of the *flaAB* genes in both strains results in a lack of motility.

3.2. Adhesion to Caco-2 and to HT29-MTX cell lines

Adhesion assays were performed to determine whether the *cadF*, ABU_RS00335, *ciaB,* or *flaAB* genes play a role in the ability of *A. butzleri* to adhere to Caco-2 and HT29-MTX cells. All the strains adhered to both cell lines, but differences were observed among them and between cell lines (Fig. 3). In relation to strains, Ab-PV16 WT showed a significantly greater adhesion ability than RM4018 WT in both cell lines ($p < 0.001$). Regarding cell lines, both WT strains adhered significantly more ($p < 0.001$) to HT29-MTX than to Caco-2 cells.

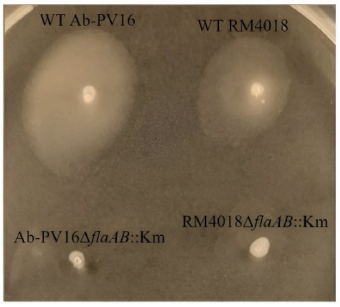
In terms of adhesion ability of the mutants compared to their isogenic WT, only the RM4018 mutant in *cadF* showed a significant reduction ($p < 0.001$) on Caco-2, while all the Ab-PV16 mutants adhered significantly less than the WT ($p < 0.001$) on the same cell line (Fig. 3A and B). On HT29-MTX cells, all RM4018 and Ab-PV16 mutants were significantly less able to adhere compared to their isogenic WT strain ($p < 0.001$) except the ABU_RS00335 mutant of RM4018, which adhered similarly to the WT strain (Fig. 3C and D). These results indicate that the *cadF* gene is essential for adhesion of *A. butzleri* to epithelial cells, while the *ciaB* and *flaAB* genes are somehow involved in the process.

3.3. Invasion into Caco-2 and to HT29-MTX cell lines

Invasion assays were performed to determine whether the *cadF*, ABU_RS00335, *ciaB*, and *flaAB* genes are involved in the invasion process of *A. butzleri* in Caco-2 and HT29-MTX. All the strains were able to invade both cell lines. Although the cell invasion ability was similar for both WT strains on Caco-2 cells, the WT strain RM4018 exhibited a significantly higher invasiveness than that of PV16 on HT29-MTX ($p < 0.001$). Indeed, RM4018 WT exhibited significantly higher invasiveness in HT29-MTX compared to Caco-2 ($p < 0.001$), while Ab-PV16 did not show a significant difference between the two cell lines.

Regarding the invasive ability of the mutants and their isogenic WT strains, all RM4018 and Ab-PV16 mutants were found to significantly

A



B

Strain	Mean halo diameter (cm) ± SE
WT RM4018	1.967 ± 0.033
RM4018Δ*flaAB*::Km	0.266 ± 0.033***
WT Ab-PV16	2.200 ± 0.151
Ab-PV16Δ*flaAB*::Km	0.233 ±0.033***

Fig. 2. (A) Representative image of the motility assays. (B) Results of the motility assays for *A. butzleri* WT and their isogenic mutant strains.

differ from their WT strains on both cell lines ($p < 0.001$), with the exception of the RM4018 ABU_RS00335 mutant (Fig. 4A–D). In particular, the invasion ability of the *flaAB* and *cadF* mutants was greatly reduced compared to their isogenic WT strains (approximately 4 to 8-fold for the former, and over 50-fold for the latter). These results demonstrate that *cadF*, *ciaB,* and *flaAB* genes are important in the process of *A. butzleri* epithelial cell invasion, with *cadF* playing the most prominent role.

3.4. Visualization of A. butzleri RM4018 WT strain by confocal microscopy

Confocal microscopy was used to support the ability of *A. butzleri* to adhere to and invade the cell lines studied here, Caco-2 and HT29-MTX. For this purpose, bacterial cells of the reference strain RM4018 WT and the nuclei of both cell lines were stained with Far Red and Hoechst, respectively, prior to the adhesion and invasion assay. After the incubation, the membranes of eukaryotic cells were stained with wheat germ agglutinin (WGA)-Alexa 488 (Fig. 5A). Several RM4018 bacteria were

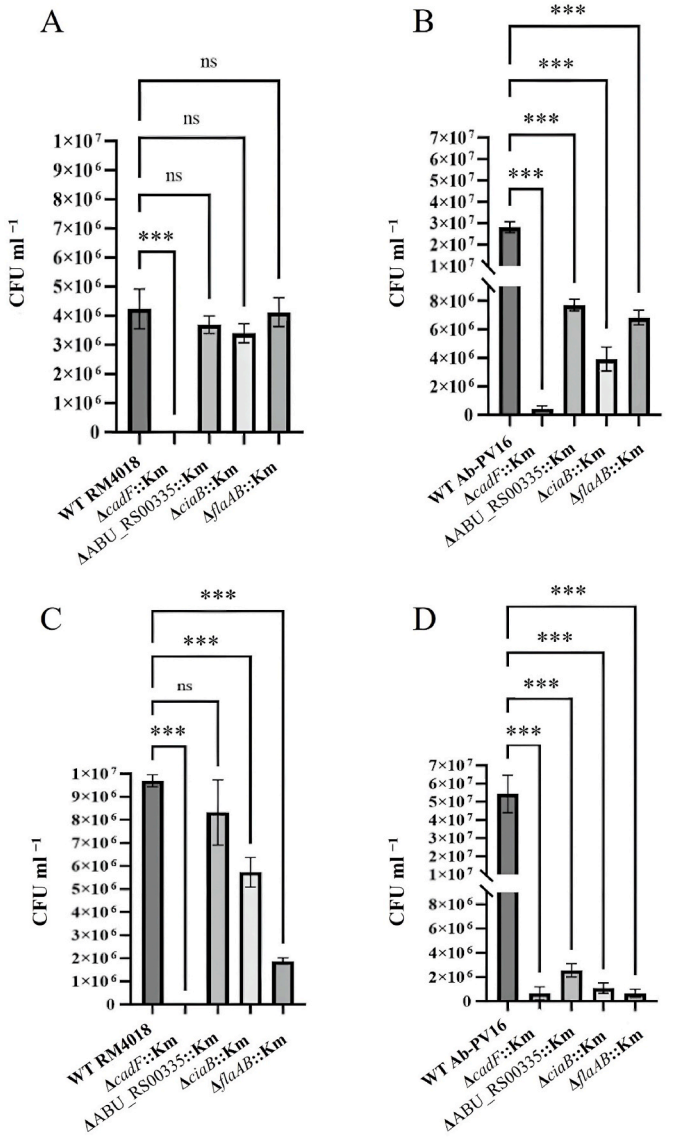


Fig. 3. Adhesion ability of (A and C) WT RM4018 strain and (B and D) WT Ab-PV16 strain and their isogenic mutants on (A and B) Caco-2 and (C and D) HT29-MTX cell lines.*** ANOVA based statistically significant differences ($p < 0.001$). ns, not significant. The experiments were repeated three times.

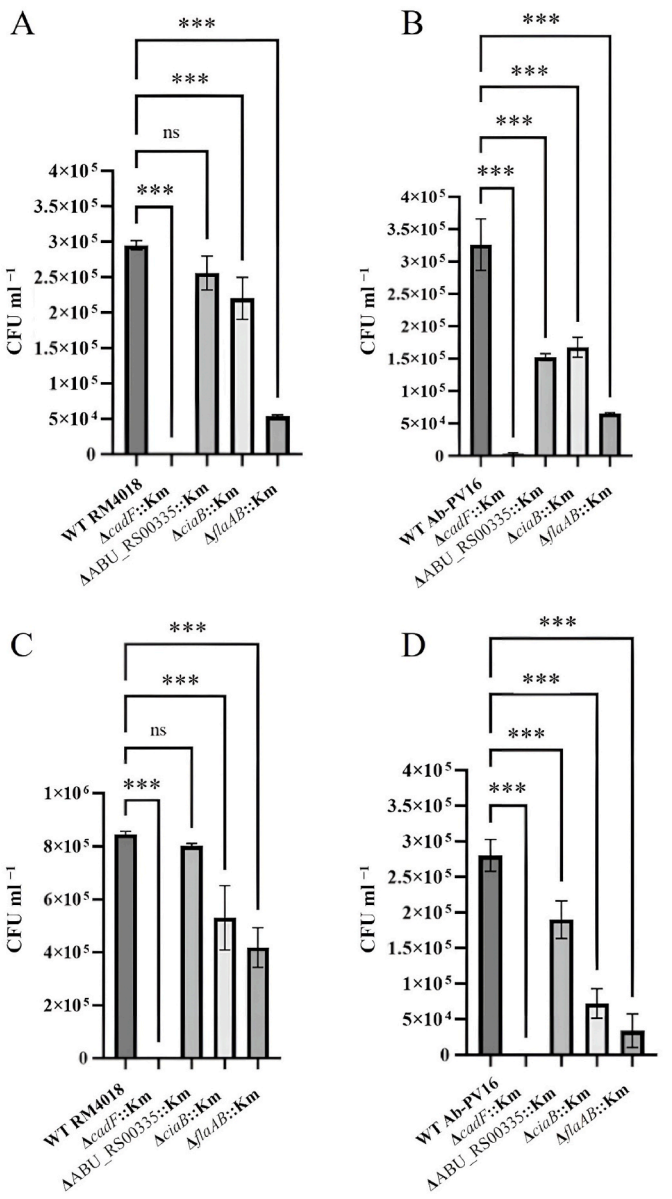


Fig. 4. Invasion ability of (A and C) WT RM4018 strain and (B and D) WT Ab-PV16 strain and their isogenic mutants on (A and B) Caco-2 and (C and D) HT29-MTX cell lines. *** ANOVA based statistically significant differences ($p < 0.001$). ns, not significant. The experiments were repeated three times.

observed adhering to or invading both cell types (Fig. 5B and C). This supports the ability of *A. butzleri* to adhere to and invade Caco-2 and HT29-MTX cell lines.

4. Discussion

The majority of diarrheal diseases caused by *Arcobacter* in humans are associated with *A. butzleri* infection [6]. Despite the recent developments in *A. butzleri* research regarding the pathogenic potential and the large number of virulence factors present in the genome, the characterization of these genes remains to be determined [9,12,15]. The aim of this study was to determine whether the genes *cadF*, ABU_RS00335, *ciaB,* and *flaAB*, which are frequently detected in *A. butzleri*, are associated with the adhesion and/or invasion of human culture cells. We found that both processes involve the *ciaB* and *flaAB* genes, while *cadF* is essential.

Two cell lines with different characteristics were used: Caco-2, a non

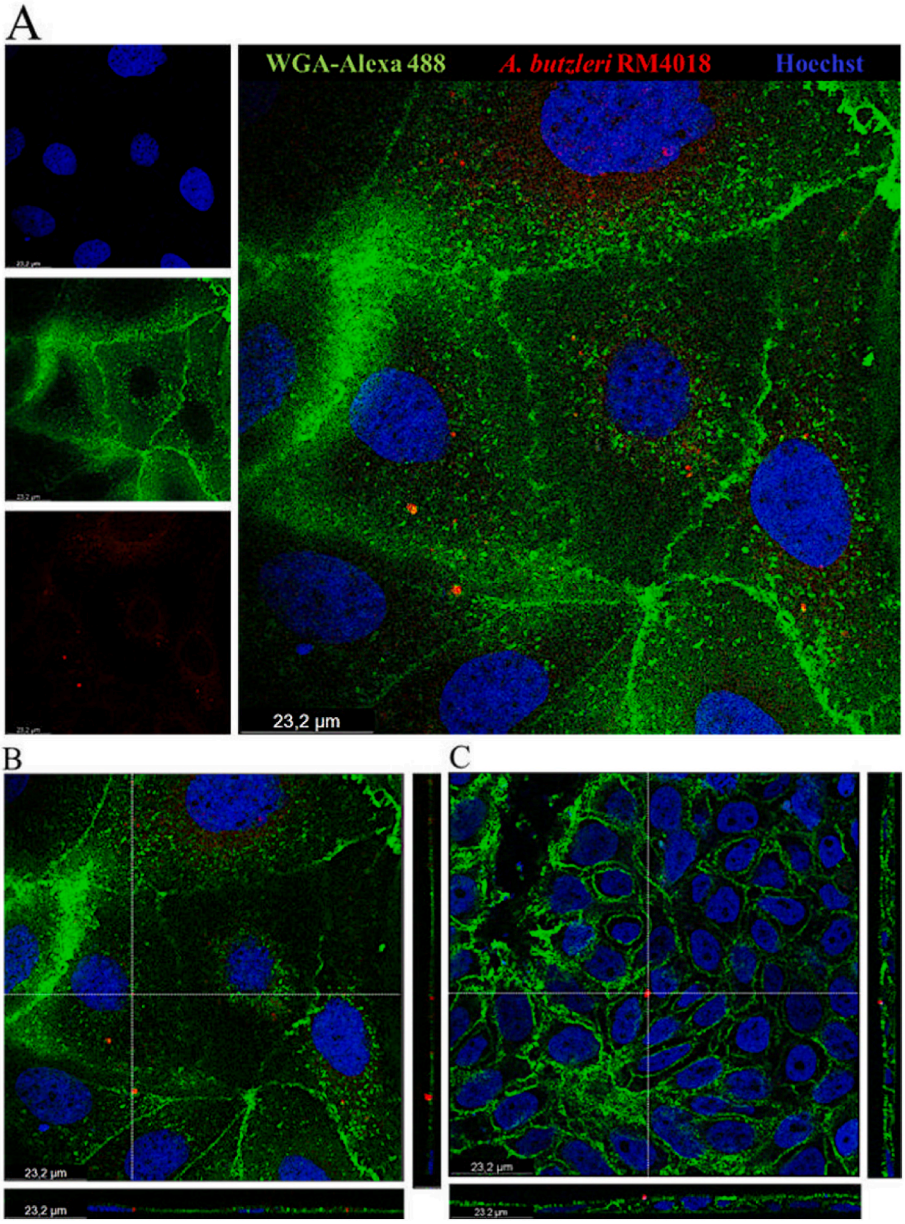


Fig. 5. Adherence and invasion of RM4018 WT strain in Caco-2 and HT29-MTX cell lines. (A) Fluorescence confocal microscopy image of confluent Caco-2 cells stained for membranes with WGA-Alexa 488 (green), RM4018 bacteria with Far Red (red) and nucleic acids DNA with Hoechst (blue); (B) RM4018 attached to and inside Caco-2 cells; (C) RM4018 attached to HT29-MTX cells. Magnification, × 63. Scale bar, 23.2 µm.

mucus-producing cell line, and HT29-MTX, a mucus-producing cell-line [27,30]. Infection assays were conducted using two WT *A. butzleri* strains and their isogenic mutants. Ab-PV16 WT, a food-borne isolate [25], was selected due to its high adhesion ability on Caco-2 [31]. RM4018 WT, a human clinical isolate, was included as a reference strain [17]. Regardless of the higher adhesion shown by Ab-PV16 in both cell lines, our results suggest that the mucus produced by the cells promotes the adherence of *A. butzleri*, as the adhesion of both WT strains significantly increased on HT29-MTX. This finding is supported by a previous study conducted by Buzzanca et al. [9], which also reported an increased adhesion ability of *A. butzleri* in a mixed culture of Caco-2/HT29-MTX cells over a Caco-2 culture. Increased virulence properties in the presence of mucus secreted by HT29-MTX have been reported elsewhere for *C. jejuni*, *Helicobacter pylori*, and *Salmonella* [32,33]. Adhesion of microorganisms to host tissue is an important initial step in the infection process [34]; which can be followed or not by cell invasion. The invasiveness of *Arcobacter* is well-documented [9,10,29,35,36]. However, our results suggest that in *A. butzleri* it does not necessarily correlate proportionally with the ability to adhere. Although Ab-PV16 demonstrated superior adhesion to RM4018, the latter exhibited superior invasiveness when HT29-MTX cells were used. This let us hypothesize that RM4018, which was isolated from a human with diarrhoea, may be better adapted to infecting humans than the food-borne Ab-PV16. For instance, the presence of MUC2, the major intestinal mucin, leads to an increase in the expression of several virulence genes associated with the infection process in *C. jejuni*, including *flaA* and *ciaB* [37]. It is possible that a similar process is occurring in this *A. butzleri* RM4018 clinical strain.

Adhesins are crucial for bacterial adhesion to host cells [34]. The genes *cadF* and ABU_RS00335 encode two different FN-binding proteins [17] and are widely distributed in *A. butzleri* [25,38,39]. However, their role in the *Arcobacter* infection process has not yet been characterised and that is why they were selected for the present study. Adhesion assays showed that the *cadF* gene is essential for this first step in *A. butzleri* as,

regardless of the cell line used, both *cadF*-deficient mutants have significantly reduced adhesion ability, virtually loosing it. (Fig. 2). These results are consistent with those obtained for *C. jejuni cadF* mutants [14, 40–43]. In contrast to the CadF FN-binding protein, ABU_RS00335 does not seem to be as important for *A. butzleri* adhesion. Only in the Ab-PV16 strain was this gene able to contribute to it. This suggests that this gene may be less important for adhesion in *A. butzleri* compared to the homologous gene *cj1349* in *C. jejuni* [16]. Similar results were obtained in the invasion assay, where mutation of ABU_RS00335 only reduced the invasion in the Ab-PV16 strain. In contrast to ABU_RS00335 mutation, *cadF* inactivation strongly inhibited the invasion ability of both *A. butzleri* strains (>50 fold), which is much stronger than the 50 % reduction in invasion that has been observed in *C. jejuni cadF*-deficient mutants [41]. The majority of the studies investigating the function of the *cadF* gene in *C. jejuni* have focused on determining its association with adhesion, but not invasion [14,40,42,43]. The strong reduction in adhesion and invasion of the *cadF* mutants observed here suggests that the adhesion plays a more important role in facilitating invasion in *A. butzleri* than in *C. jejuni*. In *C. jejuni* CadF has been shown to activate the small Rho GTPases Rac1, which results in bacterial entry into host target cells [44]. A similar but perhaps stronger activation may occur with the CadF protein of *A. butzleri*.

The *ciaB* gene encodes the invasion antigen B and emerges as a key player in the internalization of some bacterial pathogens such as *C. jejuni*, *C. coli, C. upsaliensis*, *C. lari*, *Wolinella succinogenes*, and *H. hepaticus* [19,45]. This gene is frequently detected in *A. butzleri* and has been shown to be highly conserved [29,46], suggesting its potential role in the infection process of this bacterium. However, its function is still unknown in *A. butzleri*. In *C. jejuni*, mutation of the *ciaB* gene only results in a reduction in the invasion but not in adhesion according to some studies [47–49], while other authors have also observed no significant differences in invasion [50]. Interestingly, our results show a decrease in adhesion and invasion properties of *A. butzleri ciaB* mutants in both cell lines, except for the *ciaB*-deficient RM4018 mutant. This mutant did not exhibit reduced adherence in Caco-2 cells, but did show reduced invasiveness (Figs. 2 and 3). These observations suggest that *ciaB* may have a role in adhesion as well as invasion. However, the role of this gene in adhesion may only be detectable in specific cell lines, such as HT29-MTX, that produce mucus. Previous studies on the function of the *ciaB* gene in *C. jejuni* may have failed to identify it due to the absence of mucus-secreting cell lines [47–50]. The function of the CiaB protein in *A. butzleri* needs to be further investigated before any conclusions can be drawn, but on the basis of our results it seems that the CiaB protein is involved in both steps of the infection process: adhesion and invasion.

Flagella are often involved in the infection process [51]. The flagellum of *A. butzleri* is composed of two flagellin proteins encoded by the *flaA* and *flaB* genes [17]. Both are widely distributed in *A. butzleri*, allowing this bacterium to be motile [13,20] and promoting its adhesion, at least to abiotic surfaces [21]. To date, no studies have been conducted on relationship between these genes and *Arcobacter* infection processes. However, there have been descriptions of aflagellated strains able of invading human epithelial cells (Baztarrika et al., 2023). In the present study, mutation of the *flaAB* genes in both *A. butzleri* strains resulted in reduced adhesion and invasion in both cell lines, except for the *flaAB*-deficient RM4018 mutant that did not show any difference in adhesion compared to its WT when using Caco-2 cells (Figs. 2 and 3). Unmotile bacteria lacking FlaA from *C. jejuni* have also been shown to invade tissue culture cells less; with reduced adhesion in some reports and not in others [22–24]. This discrepancy in adherence may be due to the use of different adhesion protocols, strains and/or cell lines. In fact, our results show that the inactivation of *flaAB* genes mainly reduces the adhesiveness of *A. butzleri* in HT29-MTX cell, and not in Caco-2. This difference is likely due to the loss of motility derived from the inactivation of the *fla* genes [20,21]. A non-motile strain would face greater difficulty in crossing the mucosal barrier produced by HT29-MTX. Regardless of adhesion, our results show that the flagellum plays a significant role in the *A. butzleri* internalization, as differences between the *flaAB* mutants and WT strains were significant even in the Caco-2.

In conclusion, our results show that *A. butzleri* is able to adhere to and invade different cell lines and elucidate that the genes *cadF*, *flaAB,* and *ciaB* play an important role in the *A. butzleri* infection process, with *cadF* playing a remarkable role. Adhesion of *A. butzleri* to cells may be a more important intermediate stage in the invasion process than in *C. jejuni*. Further research is needed to understand how *A. butzleri* causes infection.

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CRediT authorship contribution statement

Itsaso Baztarrika: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Marc M.S.M. Wösten:** Writing – review & editing, Visualization, Validation, Supervision, Methodology. **Rodrigo Alonso:** Writing – review & editing, Supervision, Conceptualization. **Ilargi Martínez-Ballesteros:** Writing – review & editing, Project administration, Funding acquisition. **Irati Martinez-Malaxetxebarria:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Writefull for Word in order to improve scientific writing style. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Itsaso Baztarrika Uria reports financial support was provided by Basque Government. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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