**Research Article**

**Arcobacter spp. possess two very short flagellins of which FlaA is essential for motility**

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**Abstract**

Like *Campylobacter* and *Helicobacter* spp., *Arcobacter* spp. possess two flagellin genes (*flaA* and *flaB*) located adjacent to each other. The aim of this study was to characterize the flagellin proteins of *Arcobacter* spp., because these proteins are known virulence factors in the *Epsilonproteobacteria*, to which these three species belong. With the exception of *Arcobacter nitrofigilis*, *Arcobacter* flagellins are almost half the size of those in other *Epsilonproteobacteria*. *Arcobacter* flagellins lack a large part of the variable central region. The low homology observed among flagellins of different *Arcobacter* species indicates genetic heterology between the members of this genus. Unlike in other *Epsilonproteobacteria*, the transcription of flagellin genes is not regulated by $\sigma^{28}$- or $\sigma^{34}$-dependent promoters, which suggests that transcription must be regulated in a different way in *Arcobacter* spp. Mutational studies revealed that only *flaA* is needed for the motility of *Arcobacter* spp. Quantitative PCR analysis showed that transcription of *flaB* is higher at 30 °C than at 37 °C. Mutation of *flaB* had no effect on motility or on *flaA* transcription while mutation of *flaA* abolished motility and increased the transcription of *flaB*. These results underline that the genus *Arcobacter* is an unusual taxon in the epsilon subdivision of the *Proteobacteria*.

**Introduction**

In 1991, *Arcobacter nitrofigilis* and *Arcobacter cryaerophilus* have been placed in a separated cluster of the rRNA superfamily VI i apart from *Campylobacter* spp., *Helicobacter* spp. and *Wolinella succinogenes* on the basis of rRNA gene analysis (Vandamme *et al.*, 1991). The genus *Arcobacter* was assigned, comprising two species: *A. nitrofigilis* and *A. cryaerophilus*, of which the former is the type species. *Arcobacter* spp. are Gram-negative, nonspore-forming rods, generally 0.2–0.5 μm wide and 0.5–3 μm long. They are motile and have one unsheathed single polar flagellum at one or both ends of the cells (Vandamme, 2000). Presently, the genus contains six species which inhabit extremely diverse environments. *Arcobacter nitrofigilis* is present in the roots of a marsh plant (McClung *et al.*, 1983) and *Arcobacter halophilus* was isolated from a hypersaline lagoon (Donachie *et al.*, 2005). Other *Arcobacter* species were found in association with humans and animals: *Arcobacter cibarius* was isolated from chicken carcasses (Houf *et al.*, 2005); *Arcobacter butzleri*, *Arcobacter skirrowii* and *A. cryaerophilus* were found in foods of animal origin (Kabeya *et al.*, 2004; Scullion *et al.*, 2006) and in the stool of healthy as well as sick animals and humans (Vandamme *et al.*, 1992; Anderson *et al.*, 1993; On *et al.*, 2002; Kabeya *et al.*, 2003; Vandenberg *et al.*, 2005). This mixture of species with very different habitats makes *Arcobacter* an ‘unusual’ and probably heterogeneous genus (Vandamme, 2000).

Several studies on adhesion, invasion, and cytotoxicity of *A. butzleri* and *A. cryaerophilus* have been performed, but the knowledge on their pathogenic potential still is limited (Ho *et al.*, 2006). In fact there are only a few reports on the molecular properties of the genus. The complete genome sequence of *A. butzleri* strain RM4018 contains 2 341 251 bp, and is the second largest genome among the characterized members of the *Epsilonproteobacteria* (Miller *et al.*, 2007). Interestingly, the genome sequence of this *A. butzleri* strain has the highest similarity to *W. succinogenes*.

Much research concerning *Campylobacter* and *Helicobacter* spp. has been done on the flagellum. Bacterial flagella are...
not only involved in cell motility and chemotaxis. Their role in colonization and invasion of host cells has also been extensively studied. Flagellin, the subunit of the flagellar filament, is one of the best characterized protein antigens of Campylobacter spp. (Guerry, 1997). Flagellins are important virulence factors and a primary target for the immune system (Ramos et al., 2004; Gewirtz, 2006). Apart from studies on their role in pathogenesis and their immunological properties, flagellins and the genes encoding them have been used to develop molecular methods for bacterial identification and diagnostics. Usually the flagellar filament is constituted of a single type of flagellin. However, some bacterial flagella contain several different flagellin subunits (Winstanley & Morgan, 1997). In Campylobacter spp., Helicobacter spp., and W. succinogenes, there are two homologous flagellin genes of which one is predominantly expressed. The regulation of these genes and the significance of their coexistence is still an important subject of research.

Herein, the genes encoding flagellins and their expression in several Arcobacter strains are described.

Materials and methods

Bacterial strains, plasmids, and growth conditions

All bacterial strains and plasmids used are listed in Table 1. Arcobacter strains were grown in brain heart infusion (BHI) broth (Oxoid) under shaking conditions or on BHI agar supplemented with 5% horse blood and incubated overnight or for 48 h, respectively, at 30 °C under microaerobic conditions (generated by the use of a BD CampyPak™ system, Becton, Dickinson and Company) unless special growth conditions were required (see below). Escherichia coli strains were grown in Luria–Bertani (LB) broth or on LB agar at 37 °C (Bertani, 1951). When antibiotic selection was required, ampicillin (100 µg mL⁻¹) or chloramphenicol (25 µg mL⁻¹) was added to the medium.

Isolation of flagellin proteins

To estimate the size and to isolate the Arcobacter flagellins, crude flagellin proteins were extracted by the acid pH disaggregation method (Ibrahim et al., 1985). Overnight broth cultures were harvested by centrifugation (5000 g, 30 min, 20 °C). The pellet was resuspended in 0.9% NaCl. The suspension was adjusted to pH 2.0 with 1 M HCl and centrifuged (8000 g, 15 min, 4 °C). After ultracentrifugation (100 000 g, 1 h, 4 °C), the supernatants were adjusted to pH 7.2 with 1 M NaOH and proteins were precipitated with ammonium sulfate. The samples were dialysed against running tap water for 2 h and next against distilled water containing activated charcoal for 18 h at 4 °C. Samples were stored at −70 °C for further analysis.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and N-terminal amino acid sequencing

N-terminal amino acid sequencing was performed on the putative flagellin extracted from A. butzleri strain UU-K32. The crude flagellin samples were electrophoresed on 12% polyacrylamide SDS gel. After overnight blotting the proteins from the gel onto nitrocellulose membrane (Schleicher & Schuell, BioScience) at 20 V, 4 °C, using a Tank Transfer System (Bio-Rad) bands were visualized by staining with Coomassie Brilliant Blue. The correct bands were excised, and sent to the Sequence Centre Utrecht (Faculty of Chemistry, Utrecht University, the Netherlands) for N-terminal amino acid sequence determination. This amino acid sequence was analyzed with BLAST and degenerated primers were designed in order to amplify a part of the gene encoding the flagellin.

Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacteria and plasmids</th>
<th>Origin/function</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. butzleri LMG 6620</td>
<td>Blood sample of a woman with cellulites</td>
<td>BCCM™</td>
</tr>
<tr>
<td>A. butzleri 13819</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. skirrowii LMG 6621</td>
<td>Feces of a lamb with persistent diarrhea</td>
<td>BCCM™</td>
</tr>
<tr>
<td>A. cryaerophilus LMG 7537</td>
<td>Aborted ovine fetus</td>
<td>BCCM™</td>
</tr>
<tr>
<td>A. cibarius LMG 21996</td>
<td>Chicken carcass</td>
<td>BCCM™</td>
</tr>
<tr>
<td>A nitrofigilis LMG 7604</td>
<td>Isolation from the root of a mash plant</td>
<td>BCCM™</td>
</tr>
<tr>
<td>E. coli DH5α, NCCB 2955</td>
<td>Competent cells for cloning</td>
<td>NCCB</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>Cloning vectors</td>
<td>Promega</td>
</tr>
<tr>
<td>pAV35</td>
<td>Plasmid with chloramphenicol resistance (Cm⁴) gene</td>
<td>van Vliet et al. (1998)</td>
</tr>
</tbody>
</table>

BCCM, The Belgian co-ordinated collections of microorganisms.
NCCB, The Netherlands culture collection of bacteria.
Detection of protein glycosylation

The glycosylation of *A. butzleri* flagellin proteins was examined using the digoxigenin Glycan Detection kit (Roche). To detect the presence of the N-glycosyl linkages the samples were treated with N-glycanase (Prozyme) and electrophoresed on 12% polyacrylamide SDS gel. The N-glycosylated surfactant protein D (SP-D) was used as a positive control. All assays were performed according to the manufacturers’ instructions.

Determination of the nucleotide sequence of the genes encoding *Arcobacter* flagellin

The nucleotide sequence of *Arcobacter* flagellin genes was determined in three steps:

(i) Degenerate PCR: A degenerate forward primer based on the N-terminal amino acid sequence of the *A. butzleri* UU-K32 flagellin and degenerate reverse primers based on an alignment of the major flagellin genes in *Arcobacter*-related organisms (FlaA in *Campylobacter* and *Helicobacter* spp., FlaG in *W. succinogenes*) were designed and used in a PCR to generate an amplicon from the flagellin gene of *A. butzleri* UU-K32. The PCR product was sequenced. The deduced amino acid sequence was aligned with those in related bacteria to design a second set of degenerated primers for PCR to amplify the flagellin gene of other *Arcobacter* strains.

(ii) Southern blot hybridization: Southern hybridization was performed in order to determine the number of flagellin genes present in *Arcobacter* species. Total genomic DNA was restricted with the endonucleases EcoRI, HindIII or PstI (New England Biolabs). DNA fragments were transferred from agarose gels to Hybond-N+ membranes (Amersham Biosciences) by capillary blotting (Southern, 1975). The DNA was immobilized on the filters using a Stratalinker® UV Crosslinker (Stratagene). Hybridization was performed overnight at 60 °C with digoxigenin-labeled probes. Species-specific probes were synthesized by standard PCR reactions with the PCR digoxigenin Probe Synthesis kit (Roche), using primers that were designed based on the sequences generated with the degenerated PCR reactions in step (i) (Table 2). Color was developed using an anti-digoxigenin-alkaline phosphatase antibody and NPT/bromo-4-chloro-3-indolyl-phosphate substrates (Roche). All steps were performed following the manufacturers’ instructions.

(iii) Sequence extending: With the first fragment of each strain, obtained by the degenerated PCR in step (i), DNA walking was carried out in order to obtain the entire gene(s) sequence using the TOPO® Walk Kit (Invitrogen). Total genomic DNA was digested with the restriction enzyme PstI or NsiI (New England Biolabs) that leaves a 3′-overhang and nucleotide sequence extension was performed according to the manufacturer’s instructions.

Cloning for nucleotide sequencing

To determine the nucleotide sequence, PCR amplicons were ligated into the pGEM-T Easy vector (Promega) and the plasmids were transformed into *E. coli* DH5α (Table 1). The transformation was performed either by electroporation or by the heat-shock method depending on the length of the insert. Plasmids with inserts were purified (Miniprep, QIA-GEN) and sent to BaseClear (the Netherlands) for nucleotide sequence determination.

Preparation of *A. butzleri* LMG 6620 competent cells

*Arcobacter butzleri* LMG 6620 competent cells were prepared for transformation as described for *Campylobacter jejuni* (Wilson et al., 2003) with some modifications. An overnight culture was diluted (1:100) in prewarmed (30 °C) BHI broth and incubated for 4 h under shaking conditions. Bacterial cells were harvested by centrifugation (11 000 g, 5 min), washed in saline and resuspended in 10 ml of BHI broth that was adjusted to an absorbance of 1.0 at 600 nm. To this, 25 µl of competent cells (1 µl of competent cells/ml) were added and the mixture was incubated at 30 °C for 90 min. The competent cells were subsequently centrifuged (5 min, 5000 g, 4 °C). The resulting pellet was resuspended in 10 ml of BHI broth and incubated for 4 h under shaking conditions. The resulting culture was centrifuged (10 000 g, 10 min, 4 °C), washed twice in saline and resuspended in 10 ml of BHI broth.
3 min, 4 °C). Cell pellets were gently resuspended in ice-cold glycerol water (15% glycerol and 7% sucrose) and centrifuged (11 000 g, 3 min, 4 °C). This washing step was repeated twice. Finally, cells were resuspended in ice-cold glycerol water and left on ice for 1.5 h before electroporation. Forty microliters of competent cells (containing c. 3–5 × 10⁹ CFUs) was used for each transformation.

**Construction of flagellin mutants**

Mutants in the flagellin genes of *A. butzleri* LMG 6620 were generated using the allelic exchange method (Wosten *et al.*, 2004). The target gene was amplified and the amplicon was ligated into the pGEM-T Easy vector. The plasmids containing the insert were linearized by a reverse amplification using primers with a BamHI site at the 5’ end in order to introduce a unique BamHI restriction site into the PCR products. The linear plasmid was digested with BamHI (New England Biolabs) and dephosphorylated with calf intestine alkaline phosphatase (Fermentas). Finally, the dephosphorylated plasmid was ligated to an 850-bp fragment containing the chloramphenicol resistance gene (Cmr) obtained by digestion of the pA235 plasmid with BamHI. All primers used to construct mutants are shown in Table 2.

The constructed plasmid was introduced into *A. butzleri* LMG 6620 competent cells by electroporation with settings of 2.5 kV, 300 Ω, and 25 μF. After stabilization of the bacterial cells in 100 μL SOC medium (Hanahan, 1983) for 10 min at room temperature, the cells were transferred to 2 mL prewarmed BHI broth and incubated for 3 h at 30 °C under microaerobic and shaking conditions and spread onto blood agar plates containing 25 μg mL⁻¹ chloramphenicol and incubated (2 days at 30 °C under microaerobic conditions). The resulting mutants with their target flagellin gene disrupted by double crossover events were verified by PCR.

**Motility assay**

To test for bacterial motility, individual colonies were spotted onto 0.4% thioglycolate plates (Fluka). The plates were incubated at 30 and 37 °C under microaerobic conditions, and the growth and expansion of colonies was examined.

**Electron microscopy**

Overnight cultures were inoculated into prewarmed (30 °C) BHI broth (1:100) and incubated for 4 h. Carbon-coated copper grids were floated for 5 min on top of 20 μL drops of broth culture and then immediately stained for 1 s with 2% potassium phosphotungstate pH 7.0. The grids were examined with a Philips electron microscope CM10.

**Quantitative PCR (qPCR) and study of flagellin gene expression**

The transcription of flagellin genes in *A. butzleri* LMG 6620 at different growth temperatures (30 vs. 37 °C) and at different oxygen concentrations (aerobic vs. microaerobic) was examined. Overnight cultures (grown at 30 °C under microaerobic conditions) of the wild-type and mutant strains of *A. butzleri* LMG 6620 were diluted in prewarmed BHI broth (1:100) and incubated at the experimental conditions to obtain OD₆₀₀nm = 0.6–0.8 (mid-log phase). Total bacterial RNA was isolated using TRIZol® reagent (Sigma). For each sample, 3 μg of total RNA was treated in 60 μL with DNase I (Invitrogen) before qPCR amplification and consequently 4 μL of treated RNA was used in 25 μL qPCR reaction mixture. The qPCR was performed using the one step reverse transcriptase (RT) qPCR MasterMix Plus for SYBR® Green I kit (Eurogentec) on an ABI Prism 7000 sequence detection system (Applied Biosystems). All procedures were performed as instructed by the manufacturers.

Relative gene expression to a housekeeping gene-gyrA (gyrase A from *Arcobacter* strains of which sequences were obtained from the NCBI database) was calculated in order to compensate for the variance in the amount of mRNA in the reactions. The results are presented in mRNA-fold difference between two growth conditions (30 vs. 37 °C; microaerobic vs. aerobic; wild type vs. mutant) according to the ΔΔCt method (Pfaffl, 2001). All qPCR reactions were performed in triplicate.

**Sequence and data analysis**

Known amino acid sequences used for comparison of similarity and designing degenerated primers were obtained from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Similarities between amino acid sequences obtained in this study and known sequences were determined using The Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast/). Multiple sequence alignments were performed by CLUSTALW (http://align.genome.jp/). Phylogenetic trees were constructed using MEGA 3.1 (Kumar *et al.*, 2004).

**Nucleotide sequence accession numbers**

Flagellin gene sequences of *Arcobacter* spp. in this study have been assigned Genbank accession numbers from EF667144 to EF667148 and EU056204.

**Results**

**Identification of *Arcobacter* flagellins**

To isolate the *Arcobacter* flagellins an acid pH disaggregation method was used to obtain crude flagellin protein extracts.
Only extracts from *A. butzleri* strains contained a major band upon polyacrylamide-SDS gel electrophoresis, with a molecular weight between 36 and 43 kDa.

Amino acid sequence determination of the purified flagellin protein from strain *A. butzleri* UU-K32 resulted in an N-terminal sequence of twenty nine amino acids: M R I N T N V S S L T A Q E A A V N T N K N I S S S L E K which resembled the N-terminal sequences of flagellin subunits from *Helicobacter* spp. and *W. succinogenes*.

**Nucleotide sequence of Arcobacter flagellin genes**

To obtain the nucleotide sequence of the flagellin genes PCR reactions with degenerated primers were performed on *A. butzleri* UU-K32 using a forward primer based on the sequence A Q E A A V N T of the N-terminal sequence above and a reversed primer based on the sequence A Q A(S) G S Y A(S) of the conserved C-terminal sequence of the flagellins in *Campylobacter* spp., *Helicobacter* spp., and *W. succinogenes*. The reaction produced an amplicon of 849 bp. TBLASTX analysis (search translated nucleotide database using a translated nucleotide query) of the nucleotide sequence of this amplicon demonstrated a resemblance with sequences coding for flagellins in the organisms mentioned above.

The deduced amino acid sequence of *A. butzleri* UU-K32 flagellin was aligned with flagellin sequences of closely related bacteria and a conserved sequence – A A D D A S G was chosen to design another degenerated forward primer which was used together with the previous degenerated reverse primers to amplify the nucleotide sequences encoding flagellins of other *Arcobacter* strains. This PCR produced fragments of 678–786 nucleotides for all strains except *A. nitrofigilis* for which an amplicon of 1332 bp long was obtained, approximately twice as long as that of the others. Again, TBLASTX analysis of the sequences obtained from these amplicons showed resemblance with sequences coding for flagellin subunits in *Campylobacter* spp., *Helicobacter* spp., and *W. succinogenes*. Based on the obtained sequences, primers were designed to synthesize species-specific probes as well as for gene walking.

To determine the number of flagellin genes present in *Arcobacter* species Southern blots were performed (data not shown). In the hybridization one band was visible in all DNA samples cleaved with EcoRI. Hybridization of DNA cleaved with HindIII showed two bands for *A. cryaerophilus* and one band for the other strains. Hybridization of DNA digested with PstI showed two bands for *A. butzleri* and *A. skirrowii*. Only one band was detected for *A. cibarius* DNA restricted with either HindIII or PstI. These results indicated the existence of at least two flagellin genes in *A. butzleri*, *A. skirrowii*, and *A. cryaerophilus* as seen for other members of the epsilon subdivision.

In order to obtain the entire gene sequence, the TOPO® gene walker kit was used for each strain. The walking process was repeated until the complete desired sequence was obtained. The primer set for the next walking was designed based on the sequence generated in the previous step. In this way the two adjacent *fla* genes for *A. butzleri*, *A. skirrowii*, *A. cryaerophilus*, and *A. nitrofigilis* were completely sequenced. In the case of *A. cibarius*, the walking process failed to yield the second gene due to difficulties with nonspecific amplification.

**Analysis of Arcobacter flagellin genes**

Like in *Campylobacter* species, the two flagellin genes of *Arcobacter* species are orientated, head-to-tail separated by a noncoding region of 158–168 nucleotides, which is 217 nucleotides in *A. nitrofigilis*. The deduced amino acid sequences of *Arcobacter* flagellins, with the exception of *A. nitrofigilis*, are half the size of those in *Campylobacter* spp. (Table 3). Promoter elements resembling conserved promoter sequences in other bacteria could not be identified from the sequences upstream of the start codon of the various genes, but a well-positioned ribosome-binding site was present.

The amino acid sequence homology between the two flagellin subunits varies between 80% and 90% in *A. butzleri*, *A. skirrowii*, and *A. cryaerophilus* strains and is 62% in *A. nitrofigilis*. The similarity of each subunit ranged from 73% to 79% among human/animal-associated *Arcobacter* species and 61–72% between this group and *A. nitrofigilis*. Furthermore a relatively low homology (75%) was observed between the flagellins of two different *A. butzleri* strains.

In *Campylobacter* species *flaA* and *flaB* are flanked by genes coding for motility accessory factors (*maf*). In *A. cryaerophilus* a gene encoding a plasmid stabilization system protein (RelE/ParE family) is located upstream of the *flaA* gene, while in *A. skirrowii* and *A. nitrofigilis*, the gene coding for RNA methyltransferase (*TrmA* family) was found. No

**Table 3.** Similarity of the amino acid sequences between FlaA and FlaB subunits

<table>
<thead>
<tr>
<th>Strains</th>
<th>Length (amino acid)</th>
<th>Homology between FlaA and FlaB (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FlaA</td>
<td>FlaB</td>
</tr>
<tr>
<td><em>A. butzleri</em> LMG 6620</td>
<td>283</td>
<td>282</td>
</tr>
<tr>
<td><em>A. butzleri</em> 13819</td>
<td>307</td>
<td>307</td>
</tr>
<tr>
<td><em>A. skirrowii</em> LMG 6621</td>
<td>300</td>
<td>302</td>
</tr>
<tr>
<td><em>A. cryaerophilus</em> LMG 7537</td>
<td>316</td>
<td>316</td>
</tr>
<tr>
<td><em>A. cibarius</em> LMG 21996</td>
<td>280</td>
<td>–</td>
</tr>
<tr>
<td><em>A. nitrofigilis</em> LMG 7604</td>
<td>498</td>
<td>495</td>
</tr>
<tr>
<td><em>C. jejuni</em> NCTC 11168</td>
<td>572</td>
<td>572</td>
</tr>
<tr>
<td><em>H. pylori</em> NCTC 11637</td>
<td>510</td>
<td>514</td>
</tr>
<tr>
<td><em>W. succinogenes</em> DSM 1740</td>
<td>518</td>
<td>513</td>
</tr>
</tbody>
</table>
homology was found for the 571 nucleotides upstream of the flagellin in A. cibarius.

**Glycosylation of A. butzleri flagellins**

The digoxigenin Glycan Detection assay showed that the flagellin protein of A. butzleri strains were glycosylated. The assay with N-glycanase indicated that A. butzleri flagellin did not contain glycan N-linkages (data not shown).

**Phenotypic characterization of the flaA and flaB mutants of A. butzleri LMG 6620**

Mutants in flaA or flaB of A. butzleri LMG 6620 were obtained by insertion of a chloramphenicol resistance gene cassette in the opposite orientation to the fla genes.

It was noted that natural chloramphenicol resistance for A. butzleri LMG 6620 was present at a rate of one in $1 \times 10^6$. The natural resistance was still present when the concentration of the antibiotic in the medium was increased to 40 μg mL$^{-1}$. The same was observed for the other A. butzleri and A. cryaerophilus strains. The use of a kanamycin resistance gene for selection of mutants was contemplated but was disregarded because the same problems with natural kanamycin resistance as with chloramphenicol resistance were encountered. This phenomenon caused difficulties in the selection of the correct mutant colonies. Therefore, 100 colonies from each transformation (for either flaA or flaB disruption) were screened by PCR and subsequently three transformants for each were obtained, in which the target fla gene was interrupted by the Cmr gene.

The motility of these mutants was examined on thioglycolate agar plates at different temperatures and oxygen tension. flaA mutants were nonmotile under all growth conditions (30 and 37 °C and aerobic and microaerobic).

In contrast, flaB mutants exhibited comparable motility to the wild-type strain under all growth conditions as seen on the agar plates (Fig. 1). Electron microscopy showed that the flaA mutant did not possess a flagellum while the flaB mutant still produced a flagellum with a comparable length to that of the wild-type strain (Fig. 2).

**Transcription of the flagellin genes in A. butzleri strain LMG 6620**

To study the transcription of the two fla genes, the mRNA levels of the flaA and flaB genes were analyzed by qPCR. It has been reported that the regulation of flaB in Campylobacter spp. is modulated by the growth environment (Alm et al., 1993). Therefore, the wild type and mutants of A. butzleri LMG 6620 were grown under different temperatures (30 and 37 °C) and oxygen tensions (microaerobic and

![Fig. 1. Motility phenotypes of the wild type, flaA, and flaB mutants of Acrobacter butzleri LMG 6620 on thioglycolate medium.](image1)

![Fig. 2. Electron micrographs of Acrobacter butzleri wild type (a), flaA mutant (b), and flaB mutant (c). Scale bar = 500 nm.](image2)
Arcobacter flagellins

Fig. 3. Comparing relative expression of flagellin genes by qPCR. (a) Difference in mRNA levels at 30 vs. 37 °C in the wild type (mRNA levels of flaB were approximately three- to sixfold higher at 30 °C than at 37 °C), (b) difference in mRNA levels of flaA or flaB between the wild type and flaB mutant or flaA mutant, respectively (mutation of flaA caused an increase in the expression flaB). The experiments have been performed in triplicate. The results are expressed as the means of these three experiments (error bar = SD).

Discussion

The genus Arcobacter has been considered a ‘peculiar’ group because both plant- and human/animal-associated organisms are present in this taxa (Vandamme, 2000). The present study showed that the plant-associated A. nitrofigilis possesses remarkably different flagellins, both in size and amino acid sequence, compared to the human- and animal-associated Arcobacter species. Only low homology between each flagellin subunit was found among the members of the genus and even between two strains of one species. Furthermore, the protein encoding sequences found upstream of flaA in different Arcobacter strains resembled genes encoding various proteins in Campylobacter and Helicobacter spp., which may indicate a difference in gene order among these strains. This underlines the substantial genetic variation among members of the genus, which has been observed before in studies on the prevalence of Arcobacter spp. in animals (Van Driessche et al., 2004, 2005). Nevertheless, the flagellins of all Arcobacter species form a separate group in a phylogenetic tree derived from flagellin amino acid sequences apart from other related genera including Campylobacter spp. (Fig. 4).

Except for A. nitrofigilis, the flagellins in Arcobacter species associated with humans and animals are almost half the size of those in Campylobacter spp., Helicobacter spp., and W. succinogenes. Small flagellin proteins (<300 amino acids) have been found in several Gram-positive and Gram-negative bacteria (Winstanley & Morgan, 1997). A functional flagellin should have a minimum size of c. 250 amino acids with conserved N- and C-terminal sequences of about 140 and 90 residues, respectively (Beaton et al., 2006). These conserved regions, which are embedded in the inner core of the flagellar filament play important roles in the filament structure and flagellar motility (Yonekura et al., 2003). Among bacterial flagellins the central domain of the protein is highly variable, both in amino acid sequence and length (Winstanley & Morgan, 1997). Experiments with insertions and deletions in the central domain of the
flagellins in Salmonella and E. coli demonstrate that modifications in amino acid sequence and size of this region do not affect the motility of flagella (Kuwajima, 1988; Newton et al., 1989; Lu et al., 1995). The 142-N- and 87-C- terminal amino acid residues of the Arcobacter flagellin proteins share high sequence similarities with those in related bacteria. In
Fig. 6. Phylogenetic tree (neighbor-joining) of flagellin promoter regions (120 nucleotides upstream the start codon ATG) of Arcobacter spp. and their related organisms (A: flaA; B: flaB; G: flaG in Wolinella succinogenes; Helicobacter hepaticus ATCC 51 449 possesses 2 identical flaA genes, flaA-1 and flaA-2).

Conclusion the short length of Arcobacter flagellins is fully accommodated in the center part of the proteins.

Apart from the N- and C-termini, flagellins in other genera of the Epsilonproteobacteria share two other conserved sequences within the central region, from residues 210 to 229 and 280 to 311 (residue numbers are according to the W. succinogenes FlaG sequence) (Schuster et al., 1994). These two regions were also found in the flagellin sequences of A. nitrofigilis (albeit with a lower level of similarity than among Campylobacter spp., Helicobacter spp., and W. succinogenes). However they are hardly present in the protein sequences of the other Arcobacter species, where substantial deletions occurred (Fig. 5).

The differences in Arcobacter flagellins compared with related bacteria can be explained by gene shortening and genomic shaping during evolution of the bacterial genomes (Mira et al., 2001; Batut et al., 2004; Schneider & Ebert, 2004). It has been observed that members of the Alphaproteobacteria associated with mammals – and arthropods possess considerably smaller genomes than plant-associated or aquatic relatives. Genomic analysis suggests that mutation by deletion rather than insertion is the primary force that drives the streamlining of bacterial genomes (Mira et al., 2001). This would be beneficial for bacteria in terms of metabolic efficiency, growth rate and avoidance of accumulation of deleterious mutations. It may also explain why despite high frequencies of horizontal gene transfer and gene duplication, bacterial genomes still remain small in size (Mira et al., 2001).

A difference with respect to the regulation of Arcobacter fla gene expression compared with that in Campylobacter and Helicobacter spp. was also found. In these organisms flaA is transcribed from a typical $\sigma^{28}$-type promoter while flaB transcription is the under control of a $\sigma^{54}$ promoter (Guerry et al., 1990; Nuijten et al., 1990; Kostrzynska et al., 1991; Josenhans et al., 1999). However, the consensus promoter elements for those sigma factors were not detected upstream of the fla genes in any of the Arcobacter species. A phylogenetic tree derived from the 120 nucleotides upstream of the start codon (ATG) of fla genes in Arcobacter spp. and related organisms (the putative promoter regions) yielded three well-separated clusters (Fig. 6). One includes all sequences of the fla genes regulated by $\sigma^{28}$ and another consists of those from flaB, regulated by $\sigma^{54}$ in Campylobacter spp., Helicobacter spp., and W. succinogenes. All sequences upstream Arcobacter fla genes cluster in the third group.

Although different systems may regulate their gene expression, the study on motility phenotype and transcription of fla genes in wild type and flaA or flaB mutants of A. butzleri LMG 6620 indicates a similar pattern as found in Campylobacter spp. (Guerry et al., 1991; Wassenaar et al., 1991). In the wild type flaB was expressed at low levels. The transcription of flaB was influenced by the growth temperature, but not that of flaA. Mutation of flaA had no influence on the transcription of flaA, the mutant still produced a flagellum with a comparable length and was equally motile as the wild type strain. In contrast, mutation of flaA clearly increased the transcription of flaB indicating a compensation effect which is often seen for duplicate genes (Gu et al., 2003). However, some amino acid substitutions in the
N- and C-terminal-coding parts of flaB may prevent the incorporation of the FlaB protein into functional flagella. Consequently, the flaA mutant was aflagellated and non-motile.

The simultaneous shortening of both flagellin subunits and the clustering of both FlaA and FlaB shown in the phylogenetic tree (Fig. 4) suggest a concerted evolution of the two Arcobacter fla genes. This was also observed in C. jejuni (Meinersmann & Hiett, 2000). So far, the significance of concerted evolution in multigene families still needs to be clarified (Sugino & Innan, 2006). It has been suggested that unequal crossover or/and gene conversion are involved in the process of evolution of duplicated genes (Nei & Rooney, 2005; Eickbush & Eickbush, 2007), which assures the preservation of functional parts of the gene while still allowing genetic diversity (Santoyo & Romero, 2005). The preservation of functional parts of the gene while still allowing genetic diversity (Santoyo & Romero, 2005). The coexistence of two paralogous genes in tandem and the indications for their concerted evolution demonstrate that although flaB may not play an essential role in motility, the gene itself and its protein might serve for antigenic variation which is important in host-parasite interaction or/and might be involved in bacterial pathogenicity (such as adhesion and invasion).

In conclusion, in the present study some specific features of the genes encoding flagellins in Arcobacter spp. are described and compared to those in related organisms. Human- and animal-associated Arcobacter species possess much smaller flagellins than A. nitrofigilus. A similar transcription pattern for the two fla genes in A. butzleri as in related bacteria was found. However, in the expression of the genes in Arcobacter spp. different sigma factors than those employed in other related bacteria seem to play a role.

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References


Arcobacter flagellins


