Arcobacter mytili sp. nov., an indoxyl acetate-hydrolysis-negative bacterium isolated from mussels

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Three Arcobacter isolates, recovered from mussels (genus Mytilus), and one isolate from brackish water in Catalonia (north-east Spain) showed a novel pattern using a recently described identification method for members of the genus Arcobacter, 16S rRNA gene RFLP. Enterobacterial repetitive intergenic consensus PCR fingerprinting demonstrated that the three isolates from mussels belonged to two genotypes and that the fourth isolate from water belonged to a third genotype. Analysis of the 16S rRNA and rpoB gene sequences showed that the new isolates formed a separate lineage within the genus Arcobacter. This was also confirmed by the low DNA–DNA relatedness values (16–30 %) of the isolates with the type strains of recognized Arcobacter species. Hydrolysis of indoxyl acetate, a characteristic trait for all species of the genus Arcobacter, was negative for the novel isolates. The susceptibility of the novel isolates to cefoperazone, together with the lack of urease production and nitrate reduction, further enabled them to be differentiated from recognized Arcobacter species based on physiological characteristics. Genotypic and phenotypic characteristics indicated that the new isolates represent a novel species of the genus Arcobacter, for which the name Arcobacter mytili sp. nov. is proposed, with the type strain F2075T (=CECT 7386T =LMG 24559T). The DNA G+C content of strain F2075T was 26.9 mol%.

In 1991, Vandamme and colleagues reclassified the ‘aerotolerant campylobacters’ in the genus Arcobacter, including Arcobacter nitrofigilis and Arcobacter cryaerophilus (Vandamme et al., 1991). The genus was emended a year later with the addition of Arcobacter butzleri and Arcobacter skirrowii (Vandamme et al., 1992). Two more novel species have since been described, Arcobacter cibarius, isolated from broiler carcasses in Belgium (Houf et al., 2005) and Arcobacter halophilus, isolated from a hypersaline lagoon in Hawaii (Donachie et al., 2005) and the genus currently comprises six species. Moreover, an autotrophic, obligate microaerophilic sulfide-oxidizing bacteria named ‘Candidatus Arcobacter sulfidicus’ of marine origin was described in 2002 as a possible additional novel taxon (Wirsen et al., 2002).

The type species of the genus, A. nitrofigilis, was first recovered from the roots of Spartina alterniflora, a salt marsh plant (McClung et al., 1983), and since then there have been few reports on this species (Figueras et al., 2008). A. butzleri is the most common species in environmental water, food and clinical samples (Ho et al., 2006). In fact, this species was ranked as the fourth most common campylobacterium isolated from human faeces in two independent studies performed in Belgium and France (Vandenberg et al., 2004; Prouzet-Mauleon et al., 2006). Recently, this species was considered to be a serious hazard to human health by the International Commission on Microbiological Specification for Foods (ICMSF, 2002). A. cryaerophilus, the second most commonly isolated species of the genus, has been recovered from cases of diarrhoea and bacteraemia in humans, as well as from the meat of several animals (Ho et al., 2006). This species was also recovered from faeces of 1.4 % of healthy people (Houf & Stephan, 2007). A. skirrowii is usually isolated from...
preputial fluids of bulls and faeces of animals, including sheep and cattle (Vandamme et al., 2005). It has also been associated with chronic diarrhoea in an old man (Wybo et al., 2004) and has been recently detected in humans with and without diarrhoea in South Africa (Samie et al., 2007). A. butzleri, A. cryaerophilus and A. skirrowii are frequently isolated from animal faeces (Van Driessche et al., 2003) and have recently been associated with faecally polluted environmental waters (Collado et al., 2008). Although the epidemiology of Arcobacter species is not clear, it has been suggested that water and foods are the transmission route of arcobacters to humans (Ho et al., 2006).

As part of a study on the prevalence of arcobacters in meat and shellfish products in Catalonia (north-east Spain), three isolates (F2026, F2075T and F2076) were recovered from mussels (genus Mytilus) in 2006 and an additional strain (T234) was isolated from brackish water in 2008. Using a recently proposed Arcobacter species identification method, 16S rRNA gene RFLP, these isolates showed a specific pattern that was different from those defined for the six recognized species of the genus (Figueras et al., 2008). In the present study, a polyphasic approach was used to establish the taxonomic position of these novel isolates. For this purpose, phylogenetic analyses of the 16S rRNA and rpoB gene sequences, DNA–DNA hybridization experiments, DNA G+C content determination, enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) genotyping and phenotypic analysis were performed. Based on the results obtained, we propose the allocation of these isolates to a novel species of the genus Arcobacter.

Mussel samples were taken from the Ebro river delta (north-east Spain) on 29 July (one sample) and 21 September 2006 (two samples) and were processed by homogenizing 10 g of mussel flesh with 90 ml Arcobacter enrichment broth (Oxoid) supplemented with CAT comprising (mg l\(^{-1}\)) cefoperazone (8), amphotericin B (10) and teicoplanin (4). After incubation for 48 h at 30 °C under aerobic conditions, 200 µl broth was inoculated on a blood agar (BA) plate following the procedure described by Collado et al. (2008). Small, colourless or beige-to-off-white, translucent colonies, the characteristic form of colonial growth for members of the genus Arcobacter, were selected from each sample for genetic identification. This was performed using a multiplex PCR (m-PCR) for simultaneous detection of A. butzleri, A. cryaerophilus and A. skirrowii (Houf et al., 2000) and a 16S rRNA gene RFLP method, which consists of an amplification of 1026 bp of the 16S rRNA gene and posterior digestion with MseI endonuclease to obtain restriction patterns that enable characterization of the six recognized species (Figueras et al., 2008). Three mussel isolates, one recovered from the July sample (F2026) and one from each of the two September samples (F2075T and F2076), were identified as A. skirrowii with the m-PCR method due to the presence of a 653 bp band on the agarose gel that could not be differentiated from the 641 bp band that is typical of A. skirrowii. However, the isolates showed a novel RFLP pattern (650/143/138 bp) that did not correspond to any of the recognized Arcobacter species (Figueras et al., 2008). One additional isolate (T234) was recovered (June 2008) from brackish water (salinity 14.4%) of the Ebro river delta using the isolation procedure described by Collado et al. (2008). This isolate was also identified as A. skirrowii with m-PCR and showed the same RFLP pattern as the mussel isolates.

To investigate the genetic relatedness of the four isolates, ERIC-PCR (Houf et al., 2002) was performed. Isolates F2075T and F2076, despite having been isolated from different samples, were considered to have the same genotype on the basis of sharing the same ERIC-PCR pattern. This pattern was clearly different from those of isolates F2026 and T234 (see Supplementary Fig. S1, available in IJSEM Online).

The 16S rRNA genes of the four isolates (around 1460 bp) were amplified according to Martinez-Murcia et al. (1992) and sequenced in both directions using a BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), according to the manufacturer’s instructions. Sequence assembly was performed with AUTO ASSEMBLER (Applied Biosystems). In addition, the rpoB genes of the four novel isolates and 11 Arcobacter strains [two strains for each recognized species of the genus Arcobacter, with the exception of A. halophilus (see Supplementary Table S1)], were amplified using the PCR primers CamrpoB-L and RpoB-R and the conditions that have been established for use with the genus Campylobacter (Korcza et al., 2006). The expected PCR product size (524 bp) was obtained for all strains, with additional unexpected bands in some cases. The bands of the expected size were purified from the agarose gel with the GFX PCR DNA and Gel Band Purification kit (GE Healthcare) according to the manufacturer’s instructions. The PCR products were sequenced in both directions and in duplicate. The rpoB gene sequences were used to calculate the percentage nucleotide substitutions for a continuous stretch of 487 bp (positions 1552–2039 according to Escherichia coli numbering).

Both sets of sequence data, for the 16S rRNA and rpoB genes, from strains F2075T and F2076 were identical and confirmed the ERIC-PCR results. Therefore, strain F2076 was not subjected to further phylogenetic or phenotypic analysis.

Using sequences obtained from this study and from GenBank, separate alignments of 16S rRNA gene sequences (1409 bp) and rpoB sequences (487 bp) were performed with CLUSTAL W (Thompson et al., 1994). Genetic distances were obtained using Kimura’s two-parameter model (Kimura, 1980) and evolutionary trees were constructed by the neighbour-joining method with the MEGA4 program (Tamura et al., 2007). The stability of each relationship was assessed by bootstrap analysis (1000 replicates).

In both of the phylogenetic trees derived from the 16S rRNA and rpoB gene sequences (Fig. 1 and Supplementary
and cibarius butzleri A. halophilus species: similarity values with the type strains of the six (Ade´kambi proposed as the cut-off value for species delineation with a rpoB DNA–DNA relatedness value of more than 70 % correlated similarity and DNA–DNA hybridization for 230 bacteria, a rpoB that analysed the relationship between variability (see Supplementary Fig. S2). In a recent study the novel isolates being at the lower range of intra-species similarities between strain F2075 T and strains F2026 and 5.3 %, with the sequences from 10.7 %, while the intra-species variation ranged from 0.2 to 88.0 %, 86.7 %, 85.8 %, 83.0 %, 82.1 % and 81.9 % for A. halophilus species were below 87.9 %. The 16S rRNA gene sequence of strain F2075 T showed 99.9 and 99.8 % sequence similarities with those of strains F2026 and T234, respectively. The rpoB gene sequence similarities between strain F2075 T and each Arcobacter type strain were 88.0 %, 86.7 %, 85.8 %, 83.0 %, 82.1 % and 81.9 % for A. halophilus, A. butzleri, A. nitrofigilis, A. cryaerophilus, A. cibarius and A. skirrowii, respectively. The inter-species rate of nucleotide substitutions for the rpoB gene was over 10.7 %, while the intra-species variation ranged from 0.2 to 5.3 %, with the sequences from A. skirrowii, A. butzleri and the novel isolates being at the lower range of intra-species variability (see Supplementary Fig. S2). In a recent study that analysed the relationship between rpoB gene sequence similarity and DNA–DNA hybridization for 230 bacteria, a DNA–DNA relatedness value of more than 70 % correlated with a rpoB gene sequence similarity of 97.7 %, and this was proposed as the cut-off value for species delineation (Adékambi et al., 2008). The rpoB gene sequence similarities between strain F2075 T and strains F2026 and T234 were 99.8 and 99.6 %, respectively, which are clearly above this cut-off value.

For DNA–DNA hybridization experiments and for the determination of DNA G + C content, genomic DNA was prepared according to the procedure of Wilson (1987) with the modification by Cleenwerck et al. (2002). DNA–DNA hybridizations were performed at 32 °C according to a modification (Goris et al., 1998; Cleenwerck et al., 2002) of the method described by Ezaki et al. (1989). Reciprocal reactions were performed for every comparison pair and the variation found was within the limits of this method (Goris et al., 1998). The DNA–DNA relatedness values reported are the means of a minimum of four hybridizations. The DNA–DNA relatedness value obtained for strain F2075 T with A. halophilus LA31B T, the most closely related species on the basis of the 16S rRNA and rpoB gene sequences, was 30 %, while values with all other recognized Arcobacter species were 23 % or below (Table 1). Although these DNA–DNA hybridization values may seem low, they are not low in comparison with those recently published in the description of A. halophilus, which ranged between 4 and 12 % with the other recognized Arcobacter species (Donachie et al., 2005). The G + C content of each DNA sample was determined by three independent analyses using the HPLC technique (Mesbah et al., 1989). The DNA G+C content of strain F2075 T was 26.9 mol%, which is within the previously defined range of 26.8–35 mol% for the genus (Donachie et al., 2005; Houf et al., 2005; Vandamme et al., 2005).

Phenotypic characterization of strains F2026, F2075 T and T234 was performed using the biochemical identification scheme of Vandamme et al. (2005) (Table 2). NaCl tolerance and susceptibility to cefoperazone were tested on nutrient broth no. 2 (Oxoid) supplemented with 5 % whole sheep blood and 1.5 % agar. The indoxyl acetate hydrolysis test was performed according to Mills & Gherna (1987) and confirmed using indoxyl acetate diagnostic tablets (IAC)-DIETABS (Rasco Diagnostica). All tests were conducted at least twice. The novel isolates were biochemically different from the recognized species of the genus

Fig. 1. Neighbour-joining phylogenetic tree showing the relationship of Arcobacter mytili sp. nov. with other Arcobacter species on the basis of 1409 nucleotides from the 16S rRNA gene. Numbers at nodes represent percentage bootstrap values (1000 replications). Bar, 2 substitutions per 100 nt.

Table 1. DNA–DNA relatedness between strain F2075 T and the type strains of other Arcobacter species

<table>
<thead>
<tr>
<th>Strain</th>
<th>DNA–DNA relatedness with strain F2075 T (%)</th>
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<tbody>
<tr>
<td>A. halophilus LA31B T</td>
<td>30 ± 12</td>
</tr>
<tr>
<td>A. butzleri LMG 10828 T</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>A. skirrowii LMG 6621 T</td>
<td>19 ± 8</td>
</tr>
<tr>
<td>A. nitrofigilis CECT 7204 T</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>A. cibarius CECT 7203 T</td>
<td>16 ± 6</td>
</tr>
<tr>
<td>A. cryaerophilus LMG 9904 T</td>
<td>16 ± 7</td>
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Arcobacter in that they did not hydrolyse indoxyl acetate (Table 2). Since the studies of On et al. (1996) demonstrated that A. nitrofigilis was able to hydrolyse indoxyl acetate, which contradicted previous data (Mills & Gherna, 1987), all species of the genus Arcobacter have been considered to be indoxyl-acetate-hydrolysis-positive. In the present study, using four strains of A. nitrofigilis (CECT 7204T, LMG 7547, F2173 and F2176), the results of On et al. (1996) were corroborated as, despite the reactions being slower and weaker than that observed for the other species, they were clearly positive. The susceptibility to cefoperazone (64 mg l\(^{-1}\)) differentiated the novel isolates from A. butzleri, A. cibarius, A. cryaerophilus and A. skirrowii. The lack of urease activity enabled the novel strains to be differentiated from A. nitrofigilis. The growth in MacConkey agar and the inability to reduce nitrate to nitrite differentiated the novel strains from A. halophilus. In addition, strains F2026, F2075 T and T234 differed from the other Arcobacter species by their fast growth in BA (growth was observable after 24 h incubation in aerobic conditions).

Motility was observed in young cultures by examining wet mounts in nutrient broth no. 2 by phase-contrast microscopy.

Cell size, morphology and presence of flagella (Fig. 2) were determined with transmission electron microscopy (JEOL 1011) after negative staining with 2 % (w/v) phosphotungstic acid solution (pH 6.9) for 1 min and with scanning electron microscopy after fixing pieces of agar containing cells of growing strain F2075\(^{T}\) in 2.5 % glutaraldehyde in phosphate buffer for 24 h. Subsequently, the samples were post-fixed in 1 % osmium tetroxide for 2 h. After dehydration and critical-point drying, specimens were mounted and coated with a thin layer of gold before examination with a JEOL JSM 6400 scanning electron microscope.

The data presented here support the suggestion that the four novel strains belong to a previously unrecognized species of the genus Arcobacter, for which the name Arcobacter mytili sp. nov. is proposed.

The recently described 16S rRNA gene RFLP Arcobacter identification protocol (Figuera et al., 2008) is currently the only fast method that enables the differentiation of these four novel strains from the rest of the species of the genus on the basis of specific restriction patterns. In contrast, the m-PCR method (Houf et al., 2000) misidentifies the four novel strains as A. skirrowii.

### Table 2. Differential characteristics of Arcobacter mytili sp. nov. and other species of the genus Arcobacter

<table>
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<tr>
<th>Characteristic</th>
<th>1</th>
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<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<tbody>
<tr>
<td>Growth condition</td>
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<tr>
<td>Air at 25°C</td>
<td>++</td>
<td>+</td>
<td>v</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>MacConkey agar</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>v</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Minimal media</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−*</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>NaCl 4 % (w/v)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<td>−</td>
<td>+</td>
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<tr>
<td>Cefoperazone (64 mg l(^{-1}))</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Enzyme activity</td>
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<tr>
<td>Oxidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>v</td>
<td>+</td>
<td>v</td>
<td>+</td>
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<tr>
<td>Urease</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Nitrate reduction</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Indoxyl acetate hydrolysis</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
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</table>

*Two of the four strains tested in this study (LMG 7537 and LMG 10241) were positive.
†Two of the four strains tested in this study (LMG 9904\(^{T}\) and LMG 9065) were negative.

![Fig. 2](image-url) Images of cells of strain F2075\(^{T}\) as observed with scanning electron microscopy (a) and transmission electron microscopy, negatively stained (b). Bars, 1 μm.
Description of *Arcobacter mytili* sp. nov.

*Arcobacter mytili* (my’t.li. L. gen. n. *mytili* of a mussel, from the genus name *Mytilus*, from which the species was first isolated).

Cells are Gram-negative, non-encapsulated, non-spore-forming, slightly curved rods, some S-shaped, 0.4–0.6 μm wide and 1–3 μm long. Motile by means of a single polar flagellum. Colonies on BA inoculated in aerobic conditions at 30 °C for 48 h are 2–4 mm in diameter, beige to off-white, circular with entire margins, convex and non-swarming. Pigments are not produced. All strains grow on BA at room temperature (18–22 °C) and at 30 and 37 °C under aerobic or microaerobic culture conditions with no significant differences. Under aerobic conditions, all strains grow at 30 °C on MacConkey agar and on media containing 2.0–4.0% (w/v) NaCl. No growth is obtained on casein, minimal medium or media containing 64 mg cepheperazone L⁻¹. Weak growth is obtained in anaerobic conditions at 30 °C and in aerobic conditions at 42 °C, and no growth is observed at 4 °C. Oxidase-positive and weakly catalase-positive. Strains are not haemolytic and do not hydrolyse indoxyl acetate. Urease is not produced and nitrate is not reduced. Hydrogen sulfide is not produced in triple-sugar iron agar medium.

The type strain, F2075 T ( = CECT 7386 T = LMG 24559 T), was isolated from mussels from Catalonia, Spain. The DNA G+C content of the type strain is 26.9 mol%.

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References


