Fodinicurvata halophila sp. nov., a moderately halophilic bacterium from a marine saltern

Carmen Infante-Dominguez,1 Paul A. Lawson,2 Crystal N. Johnson,2 Cristina Sánchez-Porro1 and Antonio Ventosa1

1Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, 41012 Sevilla, Spain
2Department of Microbiology and Plant Biology, University of Oklahoma, Norman, OK 73019, USA

The genus Fodinicurvata belongs to the family Rhodospirilaceae, within the order Rhodospirillales (Pfennig & Trüper, 1971) of the class Alphaproteobacteria. This genus was originally proposed by Wang et al. (2009) and, at the time of writing, comprises two species with validly published names: Fodinicurvata sediminis (Wang et al., 2009) and Fodinicurvata fenggangensis (Wang et al., 2009). Both species were isolated from a sediment sample collected from a salt mine in Yunnan, south-west China. Species of the genus Fodinicurvata stain Gram-negative, are facultatively anaerobic, non-motile, vibrioid or rod-shaped. Catalase and oxidase are produced and polyhydroxybutyrate (PHB) granules are observed. The predominant polar lipids consist of diphosphatidylglycerol, phosphatidylmethylethanolamine and phosphatidylcholine; phosphatidylinositol is variable between species. The DNA G+C content ranges from 61.5–62.3 mol% (Wang et al., 2009).

Recent metagenomic studies have provided new insights into the microbial diversity along the salinity gradient of salters located in Santa Pola and Isla Cristina in Spain (Ghai et al., 2011; Fernández et al., 2014a,b; León et al., 2014; Ventosa et al., 2014). During the course of a study to search for the most abundant micro-organisms in these salters, a novel, moderately halophilic, yellow- to cream-pigmented, Gram-stain-negative bacterium, strain BA45ALT, was isolated from a water sample. The pH of the water was 7.4 and the salinity was 12 % (w/v) salts. At the time of sampling, the temperature of the water was 28 °C. In this paper, we describe the taxonomic features of this novel bacterium and propose that it represents a novel species of the genus Fodinicurvata.

Aliquots of the saltern water sample were diluted with sterile salt solution, and 100 μl was plated on saline media and incubated aerobically at 37 °C. The isolation medium used was prepared with a 15 % (g l-1) salts mixture: NaCl, 117; MgCl2·6H2O, 19.5; MgSO4·7H2O, 30.5; CaCl2, 2H2O, 0.3;
Fodinicurvata halophila sp. nov.

KCl, 3; NaHCO₃, 0.06; NaBr, 0.23 (Subov, 1931), supplemented with 0.25 % (w/v) yeast extract (Difco) and 1.8 % (w/v) agar (Difco). The pH of the medium was adjusted to 7.5 with 1 M KOH. A pure culture of strain BA45ALᵀ was obtained after several transfers on the same medium. For routine growth, the strain was cultivated in the same medium but prepared with 10 % (w/v) total salts. The culture was maintained at −80 °C in the routine medium containing 50 % (v/v) glycerol. Fodinicurvata fenggangensis DSM 21160ᵀ and Fodinicurvata sediminis DSM 21159ᵀ obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures) were used as reference strains and were grown following the recommendations of the culture collection.

Cell morphology and motility were examined using an Olympus CX41 microscope equipped with phase-contrast optics. Cells were motile, both rod- and vibrioid-shaped (Fig. S1, available in the online Supplementary Material). Colony morphology was observed on routine medium under optimal growth conditions after incubation at 37 °C for 5 days. Colonies of strain BA45ALᵀ that formed on agar plates were circular, smooth, entire, opaque and yellow–cream-pigmented, with a size of 0.3–0.5 mm. The strain was able to accumulate PHB, as was observed by using an Olympus CX41 microscope equipped with phase-contrast optics.

Genomic DNA from strain BA45ALᵀ was obtained using the method described by Marmur (1961). The 16S rRNA gene of the isolate was amplified by PCR using forward primer 16F27 and reverse primer 16R1488 (Mellado et al., 1995). An almost complete 16S rRNA gene sequence (1392 nt) of strain BA45ALᵀ was obtained. The identification of the closest phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e tool (Kim et al., 2012). The 16S rRNA gene sequence similarities between strain BA45ALᵀ and the most closely related type strains, F. fenggangensis YIM D812ᵀ and F. sediminis YIM D82ᵀ were 98.2 and 97.4 %, respectively. The 16S rRNA gene sequence was aligned and checked against both primary and secondary structures of the 16S rRNA molecule using the alignment tool of the ARB software package. Phylogenetic trees were reconstructed using three different methods, namely the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) algorithms integrated in the ARB software. The maximum-likelihood phylogenetic tree is presented in Fig. 1. Similar tree topologies were obtained using the neighbour-joining and maximum-parsimony treeing methods (data not shown).

Although no precise correlation exists between the percentage of 16S rRNA gene sequence divergence and species delineation, it is generally recognized that divergence values of 1.3 % or more are significant (Stackebrandt & Goebel, 1994). However, due to the high sequence similarity values to its close relatives, DNA–DNA hybridization studies were performed. These studies were carried out using the competition procedure of the membrane method (Johnson, 1994) as described by Ventosa et al. (2004). DNA–DNA relatedness values for strain BA45ALᵀ with respect to F. fenggangensis DSM 21160ᵀ and F. sediminis DSM 21159ᵀ were 30 and 15 %, respectively. These data confirmed that strain BA45ALᵀ represents a novel species of the genus Fodinicurvata, having DNA–DNA hybridization values <70 % with respect to the type strains of the most closely related species of the genus Fodinicurvata (Wayne et al., 1987; Stackebrandt & Goebel, 1994).

Optimal conditions for growth and range were determined by growing the strain in routine medium prepared at salinity values of 0.5, 3, 5, 7, 7.5, 8, 10, 12, 15, 20 and 25 % (w/v) total salts, and at temperatures from 5 to 45 °C (in increments of 5 °C) and from 35 to 40 °C in increments of 1 °C. The pH range for growth of the isolate was determined in routine medium at optimal salinity with the pH adjusted to pH 4–10 (in increments of one pH unit). Growth was determined by monitoring the optical density at 600 nm using a spectrophotometer.

Strain BA45ALᵀ grew in media containing 5–20 % (w/v) salts and optimally in media containing 10 % (w/v) salts. No growth was observed in the absence of NaCl. The strain grew at temperatures in the range 14–45 °C (optimally at 37 °C) and at pH 5.0–9.0 (optimally at pH 7.5), and was able to grow anaerobically, determined after incubation in an anaerobic chamber (GasPak Anaerobic system, BBL).

All phenotypic features were determined when growing the strain in routine medium prepared at 10 % (w/v) total salts, pH 7.5 and at 37 °C. The production of acid from different carbohydrates was tested in a medium with 0.5 % (w/v) yeast extract and supplemented with 1 % (w/v) of the carbohydrate tested (sterilized separately) (Ventosa et al., 1982). To determine the utilization of different organic substrates as carbon and energy sources, a medium containing 0.05 % (w/v) yeast extract and supplemented with 1 % (w/v) of the tested substrate was used (Ventosa et al., 1982). The results for the utilization of different substrates are included in the species description.

Catalase activity was determined by adding 1 % (w/v) H₂O₂ solution to colonies on solid medium. The oxidase test was performed using a DrySlide assay (Difco). Strain BA45ALᵀ was catalase- and oxidase-positive. The following tests were conducted as described by Cowan & Steel (1977): hydrolysis of aesculin, casein, gelatin, Tween 80 and starch; urease, methyl red and indole production; Vogues-Proskauer test; nitrate and nitrite reduction; and Simmons citrate. The following tests were negative: methyl red, Voges–Proskauer, indole production from tryptophane and Simmons’s citrate, casein and starch hydrolysis. Strain BA45ALᵀ was positive for nitrate reduction but nitrite was not reduced. Both gelatin and aesculin were hydrolysed and the urease test was positive. Other phenotypic characteristics of strain BA45ALᵀ are included in the species description.
Antimicrobial compound sensitivity tests were performed by spreading the culture suspension on solid medium plates and applying discs impregnated with antimicrobial compounds following the technique of Bauer–Kirby (Bauer et al., 1966). Discs with the following concentrations of antimicrobial agents were used (μg per disc unless otherwise stated): ampicillin (10), chloramphenicol (30), erythromycin (15), gentamicin (10), nalidixic acid (30), neomycin (10), novobiocin (30), penicillin G (10 U) and rifampicin (30). Strain BA45ALT was resistant to ampicillin, chloramphenicol, novobiocin, penicillin G and rifampicin, but susceptible to erythromycin, gentamicin, nalidixic acid, neomycin, novobiocin, penicillin G and rifampicin, but susceptible to chloramphenicol.

Several phenotypic differences were observed between strain BA45ALT and the two closely related species of the genus *Fodinicurvata*, including those concerning motility, physiological and cultural features, nitrate reduction, hydrolysis of gelatin and aesculin, H2S production, and the late-exponential stage of growth according to the four-quadrants streak method (Sasser, 1990). Fatty acid methyl esters were prepared and analysed according to the standard protocol of the Microbial Identification System (MIDI, version 6.1; identification library TSBA40 4.1; Microbial ID). Extracts were analysed using a Hewlett Packard model HP6890A gas chromatograph equipped with a flame-ionization detector as described by Kempfer & Kroppenstedt (1996). The fatty acid profile of strain BA45ALT included C₁₈:₁ω₇c (35.6 %), iso-C₁₆:₀ (16.3 %) and iso-C₁₅:₀ (10.1 %) as the major fatty acids, followed by C₁₇:₀ (5.5 %), C₁₆:₁ (3.3 %), C₁₈:₀ 2-OH (3.0 %). The fatty acid profile of strain BA45ALT was determined from the mid-point value (Tₘ) of the thermal denaturation profile (Marmur & Doty, 1962), obtained with a Perkin-Elmer UV-Vis Lambda 20 spectrophotometer at 260 nm. The DNA G+C content of strain BA45ALT was 58.0 mol%, a value close to those reported for species of the genus *Fodinicurvata* (Wang et al., 2009).

Cell biomass for fatty acid determination, polar lipid analyses and quinone composition of strain BA45ALT and the reference strains was determined on cultures reaching the late-exponential stage of growth according to the four-quadrants streak method (Sasser, 1990). Fatty acid methyl esters were prepared and analysed according to the standard protocol of the Microbial Identification System (MIDI, version 6.1; identification library TSBA40 4.1; Microbial ID). Extracts were analysed using a Hewlett Packard model HP6890A gas chromatograph equipped with a flame-ionization detector as described by Kempfer & Kroppenstedt (1996). The fatty acid profile of strain BA45ALT included C₁₈:₁ω₇c (35.6 %), iso-C₁₆:₀ (16.3 %) and iso-C₁₅:₀ (10.1 %) as the major fatty acids, followed by C₁₇:₀ (5.5 %), C₁₆:₁ (3.3 %), C₁₈:₀ 2-OH (3.0 %). The fatty acid profile of strain BA45ALT was determined from the mid-point value (Tₘ) of the thermal denaturation profile (Marmur & Doty, 1962), obtained with a Perkin-Elmer UV-Vis Lambda 20 spectrophotometer at 260 nm. The DNA G+C content of strain BA45ALT was 58.0 mol%, a value close to those reported for species of the genus *Fodinicurvata* (Wang et al., 2009).

Cell biomass for fatty acid determination, polar lipid analyses and quinone composition of strain BA45ALT and the reference strains was determined on cultures reaching the late-exponential stage of growth according to the four-quadrants streak method (Sasser, 1990). Fatty acid methyl esters were prepared and analysed according to the standard protocol of the Microbial Identification System (MIDI, version 6.1; identification library TSBA40 4.1; Microbial ID). Extracts were analysed using a Hewlett Packard model HP6890A gas chromatograph equipped with a flame-ionization detector as described by Kempfer & Kroppenstedt (1996). The fatty acid profile of strain BA45ALT included C₁₈:₁ω₇c (35.6 %), iso-C₁₆:₀ (16.3 %) and iso-C₁₅:₀ (10.1 %) as the major fatty acids, followed by C₁₇:₀ (5.5 %), C₁₆:₁ (3.3 %), C₁₈:₀ 2-OH (3.0 %). The fatty acid profile of strain BA45ALT was determined from the mid-point value (Tₘ) of the thermal denaturation profile (Marmur & Doty, 1962), obtained with a Perkin-Elmer UV-Vis Lambda 20 spectrophotometer at 260 nm. The DNA G+C content of strain BA45ALT was 58.0 mol%, a value close to those reported for species of the genus *Fodinicurvata* (Wang et al., 2009).

Cell biomass for fatty acid determination, polar lipid analyses and quinone composition of strain BA45ALT and the reference strains was determined on cultures reaching the late-exponential stage of growth according to the four-quadrants streak method (Sasser, 1990). Fatty acid methyl esters were prepared and analysed according to the standard protocol of the Microbial Identification System (MIDI, version 6.1; identification library TSBA40 4.1; Microbial ID). Extracts were analysed using a Hewlett Packard model HP6890A gas chromatograph equipped with a flame-ionization detector as described by Kempfer & Kroppenstedt (1996). The fatty acid profile of strain BA45ALT included C₁₈:₁ω₇c (35.6 %), iso-C₁₆:₀ (16.3 %) and iso-C₁₅:₀ (10.1 %) as the major fatty acids, followed by C₁₇:₀ (5.5 %), C₁₆:₁ (3.3 %), C₁₈:₀ 2-OH (3.0 %). The fatty acid profile of strain BA45ALT was determined from the mid-point value (Tₘ) of the thermal denaturation profile (Marmur & Doty, 1962), obtained with a Perkin-Elmer UV-Vis Lambda 20 spectrophotometer at 260 nm. The DNA G+C content of strain BA45ALT was 58.0 mol%, a value close to those reported for species of the genus *Fodinicurvata* (Wang et al., 2009).
clearly different from those of the related taxa. Although all three species produce C\textsubscript{18}:1\textit{v} \textit{c} as a major product, the novel organism also produces major amounts of iso-C\textsubscript{16}:0 and iso-C\textsubscript{15}:0 which are lacking in \textit{F. sediminis} DSM 21159\textsuperscript{T} and \textit{F. fenggangensis} DSM 21160\textsuperscript{T} (Table S1).

The polar lipids of strain BA45\textsuperscript{ALT} and the reference strains were analysed as described by Groth \textit{et al.} (1996). The polar lipids detected in strain BA45\textsuperscript{ALT} were diphosphatidylglycerol, phosphatidylmethylethanolamine and a number of unknown phospholipids and lipids (Fig. S2). It is important to note that in our hands TLC plates from the novel strain, BA45\textsuperscript{ALT}, or neighbours, \textit{F. sediminis} DSM 21159\textsuperscript{T} and \textit{F. fenggangensis} DSM 21160\textsuperscript{T}, did not possess choline-containing lipids as previously described (Wang \textit{et al.}, 2009). The total polar lipid pattern of strain BA45\textsuperscript{ALT}, along with other physiological properties, more closely resembles \textit{F. sediminis} DSM 21159\textsuperscript{T} but clear differences are also observed.

Quinone analyses were carried out by the Identification Service of the DSMZ, Braunschweig, Germany (Tindall \textit{et al.}, 2007). The novel strain contained a ubiquinone with ten isoprene units (Q-10) that is commonly found in species belonging to the genus \textit{Fodinicurvata} (Wang \textit{et al.}, 2009).

Based on phenotypic, chemotaxonomic and phylogenetic data presented in this study, strain BA45\textsuperscript{ALT} represents a novel species of the genus \textit{Fodinicurvata}, for which the name \textit{Fodinicurvata halophila} sp. nov. is proposed.

**Description of \textit{Fodinicurvata halophila} sp. nov.**

\textit{Fodinicurvata halophila} (ha.lo'phi.la. Gr. masc. n. \textit{hals}, \textit{halos} salt; Gr. adj. \textit{philos} loving; N.L. fem. n. \textit{halophila} salt-loving).

Cells are motile, rod- to vibrioid-shaped, 0.05–0.1 × 0.2–1.5 μm in size and stain Gram-negative. Endospores are not produced. Colonies are circular, smooth, entire, opaque and yellow–cream-pigmented, with a size of 0.3–0.5 mm diameter, after 5 days at 37 °C on plates containing 10% (w/v) total salts. In liquid medium, growth results in a strong tendency to form aggregates at the bottom. Grows over a wide range of salts concentrations (5–20%, w/v), with optimal growth at (10%, w/v) salts. No growth is observed in the absence of NaCl. Grows at 14–45 °C (optimally at 37 °C) and at pH 5.0–9.0 (optimally at pH 7.5). PHB is accumulated. Facultatively anaerobic. Catalase- and oxidase-positive. Nitrate but not nitrite is reduced. Aesculin and gelatin are hydrolysed, but DNA, starch, Tween 80 and casein are not. H\textsubscript{2}S is

---

**Table 1. Characteristics that differentiate strain BA45\textsuperscript{ALT} from related species of the genus \textit{Fodinicurvata}**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony pigmentation</td>
<td>Yellow–cream</td>
<td>Cream–white</td>
<td>Cream–white</td>
</tr>
<tr>
<td>Cell size (width × length; µm)</td>
<td>0.05–0.1 × 0.2–1.5</td>
<td>0.2–04 × 0.5–1.3</td>
<td>0.3–0.5 × 0.7–1.5</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Temperature range for growth (°C)</td>
<td>14–45</td>
<td>15–42</td>
<td>15–42</td>
</tr>
<tr>
<td>NaCl range for growth (% w/v)</td>
<td>5–20</td>
<td>1.5–20</td>
<td>1.5–20</td>
</tr>
<tr>
<td>pH range for growth</td>
<td>5–9</td>
<td>6.5–8.5</td>
<td>6.5–8.5</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Aesculin</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Utilization of carbon sources:</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Fumarate</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Hippurate</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>L-Ornithine</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Simmons citrate</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>H\textsubscript{2}S production</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Major fatty acids</td>
<td>C\textsubscript{18}:1\textit{v} \textit{c}, iso-C\textsubscript{16}:0, iso-C\textsubscript{15}:0</td>
<td>C\textsubscript{18}:1\textit{v} \textit{c}, 2-OH-C\textsubscript{18}:1, C\textsubscript{16}:0</td>
<td>C\textsubscript{18}:1\textit{v} \textit{c}, 2-OH-C\textsubscript{18}:1, C\textsubscript{16}:0</td>
</tr>
<tr>
<td>Polar lipids*</td>
<td>DPG, PME 7 PL, 3 UL</td>
<td>DPG, PME, 4 PL, 2 UL</td>
<td>DPG, PME, 4 PL, 2 UL</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>58.0</td>
<td>62.3</td>
<td>61.5</td>
</tr>
<tr>
<td>Source</td>
<td>Salt water</td>
<td>Deposit of salt mine†</td>
<td>Deposit of salt mine†</td>
</tr>
</tbody>
</table>

*DPG, diphosphatidylglycerol; PME, phosphatidylmethylethanolamine; PL, unknown phospholipids; UL, unknown lipids.
†Data from Wang \textit{et al.} (2009).
produced. Indole, Simmons citrate, methyl red and Voges–Proskauer tests are negative. Urease is positive. Acid is not produced from various carbohydrates including: glycerol, D-arabinose, D-glucose, D-fructose, D-mannitol, galactose, lactose, sucrose, maltose, D-mannose, melezitose, melibiose, raffinose and sorbitol. The following compounds are utilized as sole sources of carbon and energy: D-glucose, D-galactose, D-fructose, D-arabinose, maltose, lactose, xylose, L-valine, L-ornithine, L-glutamine, L-serine, L-threonine, formate, fumarate, propionate, hippurate, butanol, dulcitol, ethanol and glycerol. The following compounds are not utilized as sole source of carbon and energy: cellobiose, D-ribose, D-mannose, D-melibiose, trehalose, sucrose, raffinose, melezitose, salicin, L-isoleucine, L-methionine, L-cysteine, L-phenylalanine, L-lysine, benzoate, citrate, DL-malate, DL-tartrate, succinate, D-mannitol, myo-inositol, propanol, D-sorbitol, xylitol and methanol. The predominant fatty acids are C18:1ω7c, iso-C16:0 and iso-C15:0. Polar lipids include diphosphatidylglycerol, phosphatidylethanolamine, and several unknown phospholipids and lipids. The respiratory quinone is Q-10.

The type strain is strain BA454AL\(^T\) (=CCM 8504\(^T\)=CEPT 8472\(^T\)=UCM 19075\(^T\)=LMG 27945\(^T\)), isolated from water of a marine saltern located in Santa Pola, Alicante, Spain. The DNA G+C content of the type strain is 58.0 mol%.

Acknowledgements

This research was supported by grants from the Spanish Ministry of Science and Innovation (CGL2013-46941-P and BIO2011-12879-E), and the Junta de Andalucía (P10-CVI-6226). FEDER funds and the Plan Andaluz de Investigación also supported this project. C.I.-D. was recipient of a post-graduate fellowship from the Spanish Ministry of Science and Innovation.

References


