

Alteromonas genovensis sp. nov., isolated from a marine electroactive biofilm and emended description of *Alteromonas macleodii* Baumann *et al.* 1972 (Approved Lists 1980)

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Thirty-five isolates obtained from a marine electroactive biofilm grown on a stainless steel cathode (Genoa, Italy) were investigated by a polyphasic taxonomic approach. Whole-cell fatty acid methyl ester analysis indicated that the isolates belonged to the class *Gammaproteobacteria* and were closely related or belonging to the genus *Alteromonas*. Genomic fingerprinting using the BOX-PCR primer delineated five clusters of isolates with similar BOX-PCR fingerprints. This study demonstrated that isolates from four of the BOX-PCR clusters belonged to *Alteromonas macleodii* and that the 14 isolates representing BOX-PCR cluster 1 constituted a novel species, which shared 98.4% 16S rRNA gene sequence similarity with its nearest phylogenetic neighbour, *Alteromonas hispanica*. Both phenotypic and genotypic analyses enabled this novel species, for which the name *Alteromonas genovensis* sp. nov. is proposed, to be differentiated from established species of the genus *Alteromonas*. The DNA G+C content of *Alteromonas genovensis* sp. nov. is 44.5 mol% and the type strain is LMG 24078^T (=CCUG 55340^T).

The genus *Alteromonas* was created by Baumann *et al.* (1972, 1984) to accommodate Gram-negative, strictly aerobic, heterotrophic rods that are motile by a single polar flagellum. Gauthier *et al.* (1995) restricted the genus *Alteromonas* to the type species, *Alteromonas macleodii*, and transferred all the remaining species into the genus *Pseudoalteromonas*. Since then, several marine bacteria have been classified as novel *Alteromonas* species and eight further species have been described: *Alteromonas macleodii* (Baumann *et al.*, 1972), *A. marina* (Yoon *et al.*, 2003), *A. stellipolaris* (Van Trappen *et al.*, 2004), *A. litorea* (Yoon *et al.*, 2004), *A. addita* (Ivanova *et al.*, 2005), *A. hispanica*

(Martínez-Checa *et al.*, 2005), *A. tagae* (Chiu *et al.*, 2007) and *A. simiduii* (Chiu *et al.*, 2007).

The genus *Alteromonas* has gained increasing interest because of potential industrial applications (Hayase *et al.*, 2003; Kodama *et al.*, 1993; Martínez-Checa *et al.*, 2005; Ragueneas *et al.*, 2003, 1996; Shiozawa *et al.*, 1993).

Van Trappen *et al.* (2004) emended the description of the genus *Alteromonas* by reporting the formation of prosthecae and buds in several species when grown for 3 or more days at low temperatures. The presence of prosthecae and buds enhances the bacterial surface:volume ratio, which facilitates substrate uptake in oligotrophic marine environments (Van Gernerden & Kuenen, 1984). Marine prosthecae bacteria also play a role in the corrosion of metals as they biologically catalyse the ennoblement of stainless steel surfaces (Baker *et al.*, 2003).

The present study was part of an analysis of the microbial diversity of a marine electroactive biofilm grown on a

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 24078^T, LMG 24079, LMG 24080, LMG 24081, LMG 24082 and LMG 24083 are AM885866, AM887686, AM885868, AM885867, AM885870 and AM887685, respectively.

DNA–DNA hybridization results and additional electron micrographs are available with the online version of this paper.

stainless steel cathode (EA-BIOFILMS-508866, NEST) exposed to natural seawater at the ISMAR-CNR Marine Station, located in the port of Genoa, Italy. Stainless steel samples (25 × 10 mm), cut from a UNS S 31254 plate (1 mm thick), were drilled, screwed and then treated with emery papers up to P1200. They were immersed in a tank of about 100 l natural seawater at ambient temperature. The seawater was continuously renewed at a rate of about 1.5–2.0 l min⁻¹ with water pumped directly from the sea.

The stainless steel electrode was cathodically polarized at –200 mV Ag/AgCl (e.g. Faimali *et al.*, 2008). Current flowing from the 'n'th polarized sample was calculated from the ohmic drop, ΔV_n , measured across the resistor, R_n , whose value was chosen in such a way that a ΔV_n less than 10 mV was measured. During the tests, the cathodic current on all polarized samples was regularly measured and recorded.

The current increased and stabilized at 0.5 A m⁻² in less than 10 days and microscopic analysis (400 × magnification using an Olympus BX41 epifluorescence microscope coupled with a UV filter block for DAPI) showed biofilm formation on the cathode. The biofilm was removed from the stainless steel cathode by sonication (Branson 3200) (90 s) in a sterile plastic tube containing 30 ml 0.85 % NaCl solution. Diluted cell suspensions (10⁻¹ to 10⁻⁶) were inoculated on marine agar (MA; Difco 2216) and incubated aerobically at 20 °C for several days. Pure cultures were obtained and isolates were stored at –20 °C or –80 °C using MicroBank vials.

All isolates were tentatively identified using whole-cell fatty acid methyl ester analysis as described by Mergaert *et al.* (2001). For 35 isolates, comparison of the fatty acid methyl ester profiles with a commercial database (MIS) indicated that they belonged to the class *Gammaproteobacteria* and that they were closely related or belonged to the genus *Alteromonas*. These data correlated with *Alteromonas*-like bacteria constituting considerable fractions of attached marine microbial populations (Acinas *et al.*, 1999; Dang & Lovell, 2000). In addition, *Alteromonas*-like bacteria are also frequent producers of extracellular polymer substances, which is important in the context of biofilm formation (Branda *et al.*, 2005; Flemming & Wingender, 2001). The dominant fatty acids of the 35 isolates were C_{10:0} 3-OH (2.2 ± 0.8 %), C_{11:0} 3-OH (1.4 ± 0.5 %), C_{12:0} (3.3 ± 1.1 %), C_{12:0} 3-OH (2.2 ± 1.2 %), C_{14:0} (3.4 ± 0.9 %), C_{15:1}ω8c (1.2 ± 0.6 %), C_{16:0} (19.4 ± 2.3 %), C_{17:0} (2.6 ± 0.7 %), C_{17:1}ω8c (5.7 ± 1.9 %), C_{18:0} (1.1 ± 0.5 %), C_{18:1}ω7c (13.6 ± 3.4 %), summed feature 2 (which comprises any combination of C_{12:0} aldehyde, an unknown fatty acid of equivalent chain-length 10.928, iso-C_{16:1} I and C_{14:0} 3-OH; 4.5 ± 1.7 %) and summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 30.5 ± 3.0 %); the remaining fatty acids constituted minor fractions only (<1 %). These results correlated with previous fatty acid analyses of members of the genus *Alteromonas* (Ivanova *et al.*, 2000).

DNA was extracted according to Pitcher *et al.* (1989). The genetic diversity among the 35 isolates and type strains of

Alteromonas species was investigated by repetitive DNA-PCR (rep-PCR) fingerprinting using the BOX-A1R-primer (5'-CTACGGCAAGGCGACGCTGACG-3') (Versalovic *et al.*, 1994) as described by Rademaker & De Bruijn (1997) and Rademaker *et al.* (2000). Numerical analysis of the obtained DNA profiles using the BIONUMERICS 4.61 software (Applied Maths) revealed five clusters of isolates with similar BOX-PCR profiles (at least 55.2 % intra-cluster profile similarity) (Fig. 1): cluster 1 (14 isolates), cluster 2 (9 isolates), cluster 3 (5 isolates), cluster 4 (3 isolates) and cluster 5 (4 isolates). The BOX-PCR profiles of these isolates also differed from those of the *Alteromonas* type strains. Each of the clusters comprised some isolates with virtually indistinguishable profiles suggesting that they represent isolates of the same strain. However, each cluster also comprised isolates with marked differences in their PCR fingerprints, indicating the presence of multiple strains.

The DNA G+C contents of representatives of each BOX-PCR cluster were determined. DNA was degraded enzymically into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated using a Waters Breeze HPLC system and Xbridge Shield RP18 column thermostabilized at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5 % acetonitrile. Non-methylated lambda phage (Sigma) and *Escherichia coli* LMG 2093 DNA were used as calibration reference and control, respectively. The DNA G+C contents of representatives of BOX-PCR clusters 1, 2, 3, 4 and 5 were 44.6–45.4 mol%, which correlated with the DNA G+C data reported for members of the genus *Alteromonas* (Gauthier *et al.*, 1995) (see Supplementary Table S1 in IJSEM Online).

Almost-complete 16S rRNA gene sequences (1339–1501 bp) were subsequently obtained for two representatives per BOX-PCR cluster using the universal primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pH (5'-AAGGAGGTGATCCAGCCGCA-3'; Edwards *et al.*, 1989) as described previously (Mergaert *et al.*, 2001). The FASTA program was used to find the most similar sequences in public databases. Sequences were aligned using CLUSTAL_X (Thompson *et al.*, 1997) and edited using BIOEDIT (Hall, 1999) and FORCON (Raes & Van De Peer, 1999). A neighbour-joining dendrogram was constructed (Saitou & Nei, 1987) using the TREECON software (Van De Peer & De Wachter, 1994) (Fig. 2).

The similarity in 16S rRNA gene sequences among isolates of the same BOX-PCR cluster was always ≥99.1 % (data not shown). These results confirm that isolates with highly similar rep-PCR profiles are closely related (Rademaker & De Bruijn, 1997; Versalovic *et al.*, 1994).

Representatives of BOX-PCR clusters 2, 3, 4 and 5 exhibited relatively high 16S rRNA gene sequence similarities (97.6–99.2 %) and were 97.6–99.6 % similar to *A. macleodii* LMG 2843^T and *A. marina* LMG 22057^T, their nearest phylogenetic neighbours (Fig. 2). BOX-PCR cluster 1 isolates exhibited less than 97 % 16S rRNA gene sequence similarity to the

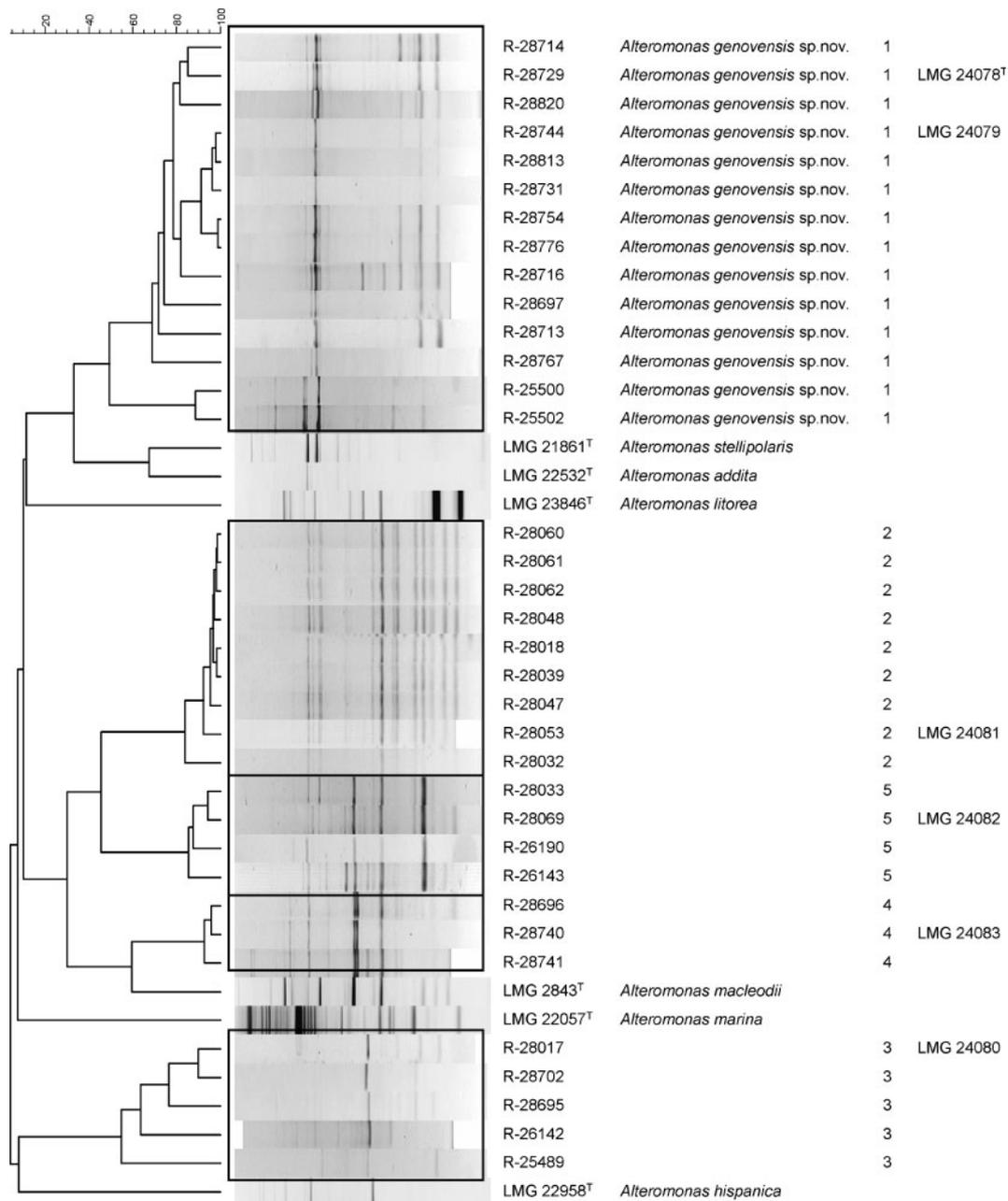


Fig. 1. Dendrogram representing the BOX-PCR fingerprints of *Alteromonas genovensis* sp. nov. (BOX-PCR cluster 1), BOX-PCR clusters 2, 3, 4 and 5, and *Alteromonas* reference strains. Fingerprints were clustered by UPGMA using Pearson's product correlation coefficient.

isolates of BOX-PCR clusters 2, 3, 4 and 5, suggesting that BOX-PCR cluster 1 isolates represent a distinct species (Stackebrandt & Goebel, 1994). The nearest phylogenetic neighbour of BOX-PCR cluster 1 isolates was *A. hispanica* with 98.4% 16S rRNA gene sequence similarity.

DNA–DNA hybridization experiments were performed to elucidate the taxonomic position of the isolates. Two representative isolates per BOX-PCR cluster were selected

on the basis of their BOX-PCR fingerprints to include isolates with the most diverse BOX-PCR profiles (Fig. 1). Their genomic relatedness towards *A. macleodii* LMG 2843^T, *A. hispanica* LMG 22958^T, *A. addita* LMG 22532^T, *A. stellipolaris* LMG 21861^T, *A. litorea* LMG 23846^T and *A. marina* LMG 22057^T was also determined. DNA–DNA hybridization experiments were carried out with photo-biotin-labelled probes in microplate wells, as described by Ezaki *et al.* (1989), using an HTS7000 Bio Assay Reader

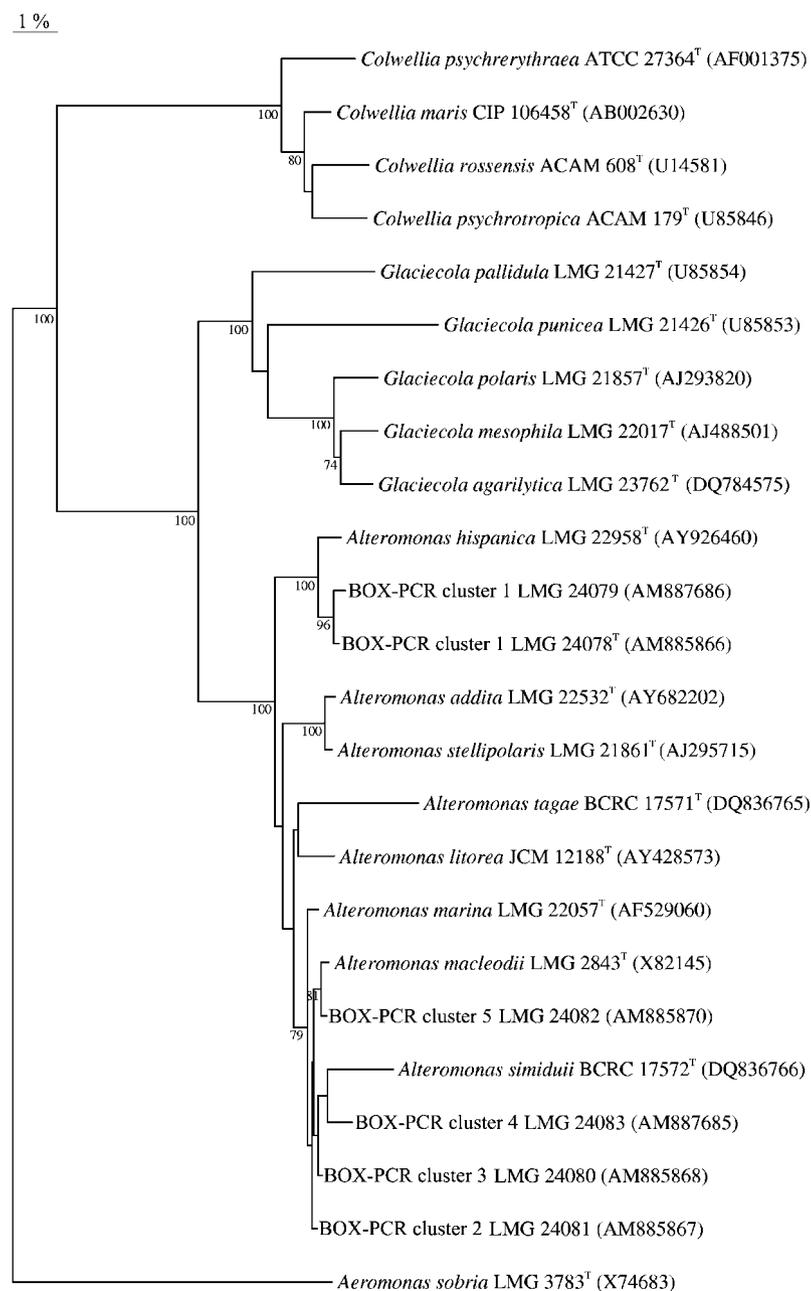


Fig. 2. Dendrogram depicting the 16S rRNA gene sequences of *Alteromonas genovensis* sp. nov., representatives of the BOX-PCR clusters and reference strains of the genera *Alteromonas*, *Colwellia* and *Glaciecola*. Bar, 1% 16S rRNA gene sequence diversity. Bootstrap values (1000 replicates) above 50% are shown.

(Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 39 °C and reciprocal reactions were performed for every pair of strains. DNA–DNA hybridization values are available in Supplementary Table S1 in IJSEM Online.

The DNA–DNA hybridization values among isolates of the same BOX-PCR cluster were very high, ranging from 80 ± 1% (BOX-PCR cluster 5) to 98 ± 2% (for BOX-PCR cluster 4). This again confirms that the isolates within one BOX-PCR cluster are closely related and belong to the same species (Wayne *et al.*, 1987).

DNA–DNA hybridization values among representatives of BOX-PCR clusters 2, 3, 4 and 5 were moderate to high (a

mean value of 66 ± 1% was obtained). Isolates of BOX-PCR clusters 2 and 5 revealed high (>90%) DNA–DNA hybridization values towards *A. macleodii* LMG 2843^T; BOX-PCR clusters 3 and 4 isolates revealed DNA–DNA hybridization values of 65 ± 2% towards *A. macleodii* LMG 2843^T. DNA–DNA hybridization values towards *A. marina* and all other *Alteromonas* species examined were low (ranging from 34 ± 2% to 43 ± 1%). In addition, DNA–DNA hybridization values of BOX-PCR cluster 1 isolates towards isolates from BOX-PCR clusters 2, 3, 4 and 5 were low (mean of 19 ± 2%), as were the DNA–DNA hybridization values between BOX-PCR cluster 1 isolates and *A. hispanica* LMG 22958^T (37 ± 2%), their nearest phylogenetic neighbour, and all other *Alteromonas* species examined (23 ± 2% or less).

The following morphological, physiological and biochemical tests were performed on one representative isolate per BOX-PCR cluster. Colony morphology was described after 4 days incubation at 20 °C on MA. Cells were tested for their Gram reaction and catalase and oxidase activities.

Growth on nutrient agar (NA), trypticase soy agar (TSA), R₂A and peptone/yeast extract/glucose agar (PYG) (Tan & Rüger, 1999) was recorded. The optimal NaCl concentration and the optimal growth temperature were determined using R₂A supplemented with 1–20 % NaCl and incubated for 2 weeks at 20 °C and MA incubated at 4–45 °C for 2 weeks, respectively. The effect of pH on growth was analysed using marine broth growth medium (Difco 2216) at pH 5.0–10.0 (at intervals of 0.5 pH units) incubated at 20 °C for 7 days.

Degradation of casein and chitin (Reichenbach & Dworkin, 1981), DNA [using DNA agar (Difco) containing 0.01 % toluidine blue (Merck)], starch and L-tyrosine (Barrow & Feltham, 1993) was tested and the reactions were read after 5 days incubation at 20 °C. The isolates were inoculated on Sierra's medium to determine their lipolytic activity and incubated for 10 days at 20 °C (Sierra, 1957).

The susceptibility to the following antibiotics (Oxoid) was tested on MA plates using the disc diffusion method: ampicillin (25 µg), cefoxitin (30 µg), gentamicin (10 µg), erythromycin (15 µg) and kanamycin (30 µg). Results were read after 3 days incubation at 20 °C.

Biochemical characteristics from commercial microtest galleries (API ZYM, API 20E and API 20NE) were assessed according to the manufacturer's instructions. API ZYM was read after 4 h incubation at 20 °C, whereas API 20E and API 20NE were read after 24 h incubation at 20 °C.

The cell morphology of BOX-PCR cluster 1 isolate LMG 24078^T was determined by transmission electron microscopy. Cells were negatively stained with 2 % uranyl acetate. Ultrathin sections were prepared and analysed as described by Mast *et al.* (2005) (Fig. 3). Additional electron micrographs are available as Supplementary Fig. S1 in IJSEM Online.

Results of the phenotypic tests are listed in Table 1. Isolates representing BOX-PCR clusters 2, 3, 4 and 5 had the same phenotypic profile as *A. macleodii* LMG 2843^T. In addition, phenotypic characteristics clearly discriminated the isolates

of BOX-PCR cluster 1 from *A. hispanica*, their nearest phylogenetic neighbour, and from the other *Alteromonas* species.

In conclusion, although DNA–DNA hybridization experiments revealed some diversity among isolates of clusters 2, 3, 4 and 5, this polyphasic taxonomic study demonstrated that they are all most appropriately classified as *A. macleodii*. Isolates of BOX-PCR cluster 1 constituted a novel species within the genus *Alteromonas*, for which the name *Alteromonas genovensis* sp. nov. is proposed.

Description of *Alteromonas genovensis* sp. nov.

Alteromonas genovensis (ge.no.ven'sis. N.L. fem. adj. *genovensis* pertaining to Genoa, Italy, where the seawater electroactive biofilms originated).

Cells are Gram-negative rods (0.9 × 1.8 µm), motile by a single polar flagellum. Buds and prosthecae are formed when isolates are grown at lower temperatures (15 °C) for more than 3 days. Prostheca formation is peritrichous and prosthecae are short and straight. The prosthecae are most often found on aggregated cells derived from the bacterial colony. These cell aggregates and, to a lesser extent, isolated bacteria are covered by a layer of fibrillar material. Buds are formed on mother and daughter cells (Fig. 3). Colonies are beige, round and 2 mm in diameter. The surface is smooth and convex. Growth occurs after 2 days incubation on MA at 20 °C; no growth occurs on TSA, R₂A or PYG medium. Moderately halophilic and psychrotolerant. Temperature range for growth is 4–37 °C; no growth occurs at 40 °C or more. NaCl range for growth is 2–15 %; no growth occurs at 1 or 20 % NaCl. The pH range for growth is 6.0–8.5; the optimal pH for growth is 7.0–8.0. Catalase and oxidase activities are observed. Degrades aesculin and casein, but not tyrosine or DNA. Weakly hydrolyses starch and gelatin; weak lipolysis of Tween 80 is observed. Positive for α-chymotrypsin, alkaline phosphatase, leucine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, esterase (C4) and esterase lipase (C8) activities. Weak lipase (C14) and trypsin activities. No activity is detected for β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase or urease. Nitrate is not reduced to nitrite or nitrogen. No indole production, fermentation of glucose or assimilation of glucose, arabinose, mannose, mannitol, N-acetylglucosa-

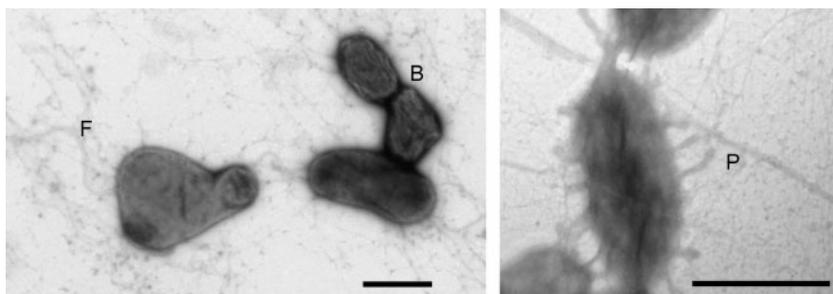


Fig. 3. Electron micrographs of negatively stained cells of strain LMG 24078^T showing flagella (F), buds (B) and prosthecae (P). Bars, 1 µm.

Table 1. Summary of the results of phenotypic tests for *Alteromonas genovensis* sp. nov., BOX-PCR clusters 2, 3, 4 and 5, and *Alteromonas* reference strains

Strains: 1, *A. genovensis* sp. nov. LMG 24078^T; 2, *A. hispanica* LMG 22958^T (data from Martínez-Checa *et al.*, 2005); 3, *A. litorea* LMG 23846^T (Yoon *et al.*, 2004); 4, *A. marina* LMG 22057^T (Yoon *et al.*, 2003); 5, *A. macleodii* LMG 2843^T, LMG 24081 (BOX-PCR cluster 2), LMG 24080 (BOX-PCR cluster 3), LMG 24083 (BOX-PCR cluster 4) and LMG 24082 (BOX-PCR cluster 5) (Baumann *et al.*, 1972, 1984; Gauthier *et al.*, 1995; Van Trappen *et al.*, 2004; Yi *et al.*, 2004 and this study). +, Positive; -, negative; w, weakly positive; ND, no data; I, intermediate antibiotic susceptibility.

| Characteristic | 1 | 2 | 3 | 4 | 5 |
|--------------------------|--|--|--|---------------------------|--|
| Origin | Marine electroactive biofilm, Genoa, Italy | Hypersaline water, Fuente de Piedra, Malaga, Spain | Intertidal sediment, Yellow Sea, Korea | Seawater, East Sea, Korea | Seawater, Hawaii + Marine electroactive biofilm (Genoa, Italy) |
| Growth in NaCl at: | | | | | |
| 1 % | - | - | + | ND | + |
| 10 % | + | + | + | + | + |
| 15 % | + | - | - | ND | w |
| Growth at: | | | | | |
| 4 °C | + | + | + | + | - |
| 40 °C | - | - | + | + | + |
| 45 °C | - | - | + | - | + |
| Growth on TSA | - | - | - | w | + |
| Hydrolysis of: | | | | | |
| DNA | - | + | - | ND | + |
| Starch | w | + | + | + | + |
| Gelatin | w | + | + | + | + |
| Tyrosine | - | + | + | + | + |
| Casein | + | + | + | + | - |
| Tween 80 | w | + | + | + | + |
| Enzymic activity: | | | | | |
| α-Chymotrypsin | + | - | - | - | - |
| Lipase (C14) | w | w | w | + | + |
| Valine arylamidase | - | w | w | - | + |
| Trypsin | w | w | w | - | - |
| α-Glucosidase | - | - | - | - | + |
| α-Galactosidase | - | - | - | - | + |
| β-Glucosidase | - | - | - | - | + |
| Susceptibility to: | | | | | |
| Ampicillin (10 µg) | - | + | I | - | + |
| Erythromycin (15 µg) | + | + | + | I | + |
| Gentamicin (10 µg) | + | ND | + | + | + |
| Kanamycin (30 µg) | I | + | I | I | + |
| Cefoxitin (30 µg) | - | - | - | - | + |
| DNA G + C content (mol%) | 44.5 | 46.3 | 46.0 | 44.5 | 45.2 |

mine, maltose, potassium gluconate, capric acid, adipic acid, malate, citrate or phenylacetic acid. Resistant to ampicillin (25 µg) and cefoxitin (30 µg), but not to gentamicin (10 µg) or erythromycin (15 µg). Intermediately susceptible to kanamycin (30 µg). The dominant fatty acids are C_{10:0} 3-OH, C_{11:0} 3-OH, C_{12:0}, C_{12:0} 3-OH, C_{14:0}, C_{16:0}, C_{17:0}, C_{17:1}ω8c, C_{18:0}, C_{18:1}ω7c, summed feature 2 and summed feature 3.

The type strain, LMG 24078^T (=CCUG 55340^T), was isolated from a marine electroactive biofilm grown on a stainless steel cathode (Genoa, Italy). A second isolate with a distinct BOX-PCR fingerprint is LMG 24079 (Fig. 1). The DNA G + C content of the type strain is 44.5 mol%.

Emended description of *Alteromonas macleodii* Baumann *et al.* 1972 (Approved Lists 1980)

The description is as given by Baumann *et al.* (1972, 1984), Gauthier *et al.* (1995), Van Trappen *et al.* (2004) and Yi *et al.* (2004) with the following additions. Hydrolyses DNA and L-tyrosine. Grows on TSA, but not on NA, R₂A or PYG. The NaCl range for growth is 1–12 %; no growth occurs in the presence of 15 % NaCl. Grows at 45 °C. No H₂S production. Susceptible to the following antibiotics: ampicillin (10 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg) and cefoxitin (30 µg). Lipase (C14), α-glucosidase, α-galactosidase, β-glucosidase, cystine arylamidase and β-galactosidase activities have been recorded, although these

data conflict with results obtained by Yi *et al.* (2004). The reason for this discrepancy is unclear.

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