NOTE

***Halomonas muralis* sp. nov., isolated from microbial biofilms colonizing the walls and murals of the Saint-Catherine chapel (Castle Herberstein, Austria)**

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A group of seven halophilic strains (optimal growth at 2.5–10.0% NaCl) was isolated from samples of a wall and a mural painting, both heavily contaminated by microbial growth, inside the Saint-Catherine chapel of Castle Herberstein (Austria). The strains were subjected to a polyphasic taxonomic study that included DNA–DNA relatedness studies, DNA base-ratio determinations, 16S rDNA sequence analysis, rep-PCR genomic fingerprinting, fatty acid analysis and phenotypic and biochemical characterization. The data obtained indicate that the strains belong to the genus *Halomonas* and represent a novel species, for which the name *Halomonas muralis* sp. nov. is proposed. The type strain is strain LMG 20969^T (= DSM 14789^T).

Keywords: *Halomonas muralis* sp. nov., polyphasic taxonomy

It is widely acknowledged that wall paintings can be attacked and destroyed by micro-organisms (Ciferri, 1999). An example of this can be found in the Saint-Catherine chapel of Castle Herberstein (Styria, Austria), the chancel walls of which are decorated with medieval paintings that have been badly damaged by microbial growth. The bacteria associated with the damage observed at this site and other mural painting sites were investigated in several studies using DNA-based techniques and these revealed that many of the bacteria found could not be attributed to any valid species (Altenburger *et al.*, 1996; Rölleke *et al.*, 1996; Wieser *et al.*, 1999; Gurtner *et al.*, 2000; Heyrman & Swings, 2001). In a previous study, five samples of different deterioration phenomena of the mural paintings and walls at Herberstein were investigated for the presence of heterotrophic bacteria (Heyrman *et al.*, 1999). Three of the samples showed heavy bacterial contamination (1.0×10^8 – 7.5×10^8 c.f.u. g⁻¹) on media with added salt (10% NaCl). One colony type was responsible for the bulk of these total counts and seven isolates of this colony type were purified. After

polyphasic characterization, this group of seven isolates can be accommodated within the genus *Halomonas* as a novel species, *Halomonas muralis* sp. nov.

Isolates were obtained from trypticase soy agar (TSA; BBL) supplemented with 10% (w/v) NaCl, as described previously (Heyrman *et al.*, 1999), and further maintained on marine agar (MA; Difco) at 28 °C. With the exception of strain LMG 20971, they were isolated from a sample taken from a brown-coloured coating below the east window of the chancel, an area without a paint layer. Strain LMG 20971 was isolated from a sample taken from paint-layer material covered with a black biofilm on the vault of the chancel. Four isolates were deposited in the BCCM/LMG Bacteria Collection (Laboratorium voor Microbiologie Gent, Gent, Belgium) as strains LMG 19418, LMG 20969^T, LMG 20970 and LMG 20971. The others, isolates R-5038, R-5056 and R-5059, are maintained in Microbank tubes (PRO-LAB diagnostics) at –80 °C as part of our laboratory research collection.

Total genomic DNA was purified for 16S rDNA sequencing and BOX-PCR using a slight modification of the method of Pitcher *et al.* (1989), as described previously by Heyndrickx *et al.* (1996). For determination of the G+C content and DNA–DNA hybridization, approximately 1 g biomass was harvested from agar plates and prepared further using a combination of the protocols of Marmur (1961) and

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Abbreviations: MA, marine agar; TSA, trypticase soy agar.

The EMBL accession number for the 16S rRNA gene sequence of *Halomonas muralis* sp. nov. LMG 20969^T is AJ320530.

Pitcher *et al.* (1989), as described previously by Logan *et al.* (2000).

Rep-PCR genomic fingerprinting was performed with the BOX primer (Versalovic *et al.*, 1994) using the PCR conditions described previously by Rademaker & de Bruijn (1997). For each strain, 6 µl PCR product mixed with 2 µl loading buffer (Rademaker & de Bruijn, 1997) was electrophoresed in a 1.5% (w/v) agarose gel and TAE buffer [1.21 g Tris 2-amino-2(hydroxymethyl)-1,3-propanediol l⁻¹, 0.2 ml 0.5 M EDTA l⁻¹, pH 8] for 15 h at a constant 55 V and 4 °C. The first and every sixth lane were loaded with 6 µl of a size ladder [45.5% (v/v) 100 bp ladder, 36.5% (v/v) 500 bp ladder and 18% (v/v) loading buffer]. After staining with ethidium bromide (0.5 µg ml⁻¹) and visualization, the patterns were digitalized and a Pearson correlation of the resulting band patterns was performed using the BioNumerics 2.0 software (Applied Maths).

16S rDNA sequences were analysed as described previously by Heyrman & Swings (2001). Phylogenetic analysis was performed with the BioNumerics 2.0 software, including 16S rDNA sequences (between 1390 and 1540 bp) of all validly described members of the genera *Halomonas* and *Chromohalobacter* and *Pseudomonas beijerinckii*, using *Zymobacter palmae* and *Carnimonas nigrificans* as outgroups. Calculation of a pairwise similarity matrix (discarding unknown bases) was followed by global alignment and calculation of a multiple alignment similarity matrix. This matrix was corrected for multiple base changes at single positions by the formula of Jukes & Cantor (1969) and a tree was constructed by applying the neighbour-joining method of Saitou & Nei (1987). The stability of relationships was assessed by a bootstrap analysis of 1000 datasets.

The G + C content of genomic DNA was determined by HPLC (Mesbah *et al.*, 1989) using a Waters Symmetry Shield RP₈ column thermostatically controlled at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ with 1.5% (v/v) acetonitrile (pH 4.0). Non-methylated phage lambda DNA was used as the calibration reference. Percentage DNA–DNA binding was determined using a modification of the microplate method described by Ezaki *et al.* (1989), as described by Willems *et al.* (2001).

Analysis of the fatty acid contents of the cell wall was started from cells grown on MA for 24 h and on TSA + 10% (w/v) NaCl for 24 or 48 h, at 28 °C. These media were used because growth on TSA without added salt was very poor (a reliable fatty acid pattern on this medium was only obtained for LMG 20971 after 48 h at 28 °C). Quantitative analysis of cellular fatty acids was performed using the GLC procedure as previously described (Mergaert *et al.*, 1993) and the resulting profiles were analysed by the Microbial Identification System of MIDI Inc. Further computer analysis was performed as described previously (Heyrman *et al.*, 1999).

Cell morphology and flagella arrangement were ex-

Fig. 1. BOX-PCR patterns of the seven *Halomonas* isolates. Lanes: 2–6, isolates LMG 19418, R-5038, R-5056, LMG 20969^T and R-5059; 8–9, LMG 20970 and LMG 20971; 1, 7 and 11, molecular size markers; 10 and 12, positive (R-6384) and negative controls.

amined by phase-contrast microscopy, by using the staining method of Heimbrook *et al.* (1989). Gram stain, anaerobic growth (in an anaerobic chamber on MA), catalase and oxidase activity were recorded. Tolerance of different NaCl concentrations, pH and temperatures was measured spectrophotometrically as OD₅₅₀ in marine broth after 48 h incubation at 28 °C. API 20NE tests were performed according to the recommendations of the manufacturer (bioMérieux). Additional strips were prepared starting from a suspension in distilled water with 3% (w/v) NaCl added and following the recommendations given. Utilization of different carbon sources was analysed using marine broth as a base and replacing the peptone component with 1% (w/v) of each carbon source tested. Results were monitored after 72 h incubation at 28 °C and (as for all other tests) were scored positive if the change in OD₅₅₀ was greater than 0.300. Sensitivity reactions to antibiotics were tested by applying Sensi discs (6 mm, Oxoid) on inoculated MA plates and incubating for 24 h at 28 °C. Zone diameter interpretive standards of the NCCLS (1993) were used for interpretation.

The genomic diversity of the isolates was first tested by BOX-PCR, since it has been shown previously that rep-PCR fingerprinting is in close agreement with DNA–DNA relatedness and can be used as a genomic screening method to group novel isolates and to select representative strains for further analyses (Nick *et al.*, 1999; Rademaker *et al.*, 2000). The seven isolates showed identical BOX-PCR patterns (Fig. 1), indicating their high genomic similarity and supporting their classification into the same species.

Fig. 2. Phylogenetic position based on neighbour-joining of the 16S rDNA sequence of strain LMG 20969^T, representative of the mural painting isolates, among the species of the genera *Halomonas* and *Chromohalobacter*. *Zymobacter palmae* and *Carnimonas nigrificans* were used as outgroups. Bar, 0.01 substitutions per nucleotide position. Bootstrap values (expressed as percentages of 1000 replications) greater than 60 % are shown at the branch points. Groups 1 and 2 refer to the monophyletic groups within *Halomonas* described previously by Arahal *et al.* (2002).

Strains LMG 19418, LMG 20969^T, LMG 20970 and LMG 20971 were analysed further by sequencing of the 16S rDNA region between positions 27 and 1512 (*Escherichia coli* numbering). All the 16S rDNA sequences analysed were 100 % similar, confirming the mutual high overall genomic similarities as revealed by the BOX-PCR pattern. In the tree based on the neighbour-joining algorithm (Fig. 2), strain LMG 20969^T, representative of the group, clustered closest to *Halomonas pantelleriensis* and *Halomonas desiderata*, with respective pairwise alignment similarities of 97.1 and 96.7 %. The similarity to all other sequences was below 96 %. In the 16S rDNA clustering, the genus *Halomonas* is not monophyletic, and the novel isolates are related to the type species of *Halomonas* (*Halomonas elongata*) and *Chromohalobacter* (*Chromohalobacter marismortui*) with comparable sequence similarities, respectively 94.9 and 95.1 %. Based on 16S rDNA sequencing, *Chromohalobacter* was expanded by the addition of two species, *Chromohalobacter canadensis* and *Chromohalobacter israelensis* (Arahal *et al.*, 2001), previously described as members of *Halomonas*, and this resulted in a better agreement between the phylogenetic tree and the classification. This *Chromohalobacter* group is well delineated and has a bootstrap value of 100 % in the clustering presented

(Fig. 2). In addition, Arahal *et al.* (2002) recently discussed the phylogeny of the family *Halomonadaceae* on the basis of 23S and 16S rDNA sequence analyses and found two distinguishable phylogenetic groups in the genus *Halomonas*, marked as groups 1 and 2 in Fig. 2. Group 1 has a bootstrap value of 94 % and contains six species, including the type species, *H. elongata*, and the recently described *Halomonas maura* (Bouchotroch *et al.*, 2001). Group 2 has a bootstrap value of 99 % and contains seven *Halomonas* species. The other *Halomonas* species studied by Arahal *et al.* (2002) could not be attributed to one of these two groups, nor did they form a delineated additional group amongst themselves. However, the recently described species *Halomonas alimentaria* (Yoon *et al.*, 2002) clusters with *Halomonas halodenitrificans* in Fig. 2 (bootstrap value of 96 %). Both species lack the usual rod-shaped cell morphology (Yoon *et al.*, 2002). Further, the novel species described here groups with *H. pantelleriensis* and *H. desiderata*, with respective bootstrap values of 96 and 87 %. Finally, the recently described *Halomonas marisflavi* forms a distinct evolutionary lineage within the *Halomonadaceae* (Yoon *et al.*, 2001), as was already the case for *Halomonas marina*. Taking the current phylogenetic situation of *Halomonas* into account, it cannot be excluded that the genus will be

Table 1. Fatty acid composition of the group of novel isolates on different media

Data are expressed as percentages. The fatty acid compositions on MA and TSA + 10% (w/v) NaCl for all seven isolates are given as mean percentages with the standard deviation. The fatty acid composition on TSA could only be determined for strain LMG 20971. Fatty acids for which the mean amount was less than 1% are not included. The summed feature comprises $C_{16:1}\omega 7c$, iso- $C_{15:0}$ 20H or both.

Fatty acid	MA	TSA + 10% NaCl	TSA (LMG 20971)
$C_{10:0}$	2.43 ± 0.16	2.69 ± 1.11	3.13
$C_{12:0}$	3.82 ± 0.26	4.10 ± 0.42	4.27
$C_{12:0}$ 3OH	9.16 ± 0.89	8.78 ± 0.51	8.80
Summed feature	17.40 ± 0.44	18.68 ± 0.69	18.80
$C_{16:0}$	21.00 ± 1.32	17.30 ± 0.39	17.00
$C_{17:0}$ cyclo	1.42 ± 0.50	< 1.00	< 1.00
$C_{18:1}\omega 7c$	34.00 ± 2.09	43.60 ± 1.96	42.80
$C_{19:0}$ cyclo $\omega 8c$	7.56 ± 3.12	1.95 ± 0.50	2.31

rearranged taxonomically at the generic level; however, the group of novel isolates discussed here clearly falls within the genus as currently described.

As discussed above, the 16S rDNA sequence similarities of the novel isolates were around 97% to *H. pantelleriensis* and *H. desiderata*, but lower than 96% to all other sequences in the EMBL database. Generally recommended and accepted criteria for delineating bacterial species state that strains with a level of DNA relatedness below 70% or with a difference in 16S rDNA similarity greater than 3% are considered to belong to different species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). However, bacterial strains with a difference in 16S rDNA similarity of less than 3% cannot be allocated to the same species without performing DNA–DNA relatedness studies. Since the 16S rDNA similarity percentages obtained were just around this level for the two closest relatives, a representative of the group of novel isolates, LMG 20969^T, was used in a total DNA–DNA relatedness study with the type strains of *H. pantelleriensis* and *H. desiderata*. The values obtained were well below 70%, with LMG 20969^T showing relatedness values of 22 and 18% to *H. desiderata* and 22 and 23% to *H. pantelleriensis*, thus allocating the group of novel isolates to a novel species within the genus *Halomonas*.

It was determined previously (Heyrman *et al.*, 1999) that the seven isolates form a distinct group in a larger UPGMA clustering of the Canberra metric coefficients calculated between the fatty acid methyl ester profiles of 385 strains isolated from different samples of three mural painting sites. The group branched off in the clustering at approximately 75% Canberra metric similarity and the seven isolates show an internal similarity of more than 90%. The major fatty acids found for the isolates were $C_{18:1}\omega 7c$, $C_{16:0}$ and $C_{16:1}\omega 7c$ and/or iso- $C_{15:0}$ 20H (Table 1), which is in accordance with other *Halomonas* species (Franzmann & Tindall,

1990; Urakami *et al.*, 1990; Berendes *et al.*, 1996; Valderrama *et al.*, 1998; Bouchotroch *et al.*, 2001; Yoon *et al.*, 2001, 2002). Valderrama *et al.* (1998) reported that the fatty acid composition of *Halomonas salina* changes when cells are grown at different salt concentrations. Although a reliable fatty acid profile could be obtained for only one isolate (LMG 20971) from growth on TSA without added salt, the results show that there are no important differences in fatty acid composition with or without the addition of 10% (w/v) salt. In contrast, profile differences were observed between isolates grown on MA and TSA; the fatty acid profiles obtained from isolates grown on MA contain smaller relative amounts of the mono-unsaturated $C_{16:1}$ and $C_{18:1}$ fatty acids.

The seven isolates grow poorly on media without salt and have optimal growth with 2.5–10% (w/v) NaCl added, but do not grow in the presence of 20% (w/v) NaCl or more. No growth was observed at 5 or 40 °C, while the optimum temperature range for growth was 25–35 °C. All isolates showed a wide pH optimum, of 6.5–9.5, with pH limits for growth between 5.5 and 10.0 (strain LMG 20969^T also grew at pH 5.0). These isolates are therefore distinguished from the species related most closely in 16S rDNA sequence analysis, *H. pantelleriensis* and *H. desiderata*, which are both obligately alkaliphilic (Romano *et al.*, 1996; Berendes *et al.*, 1996). Additional characteristics that discriminate the isolates at the species level from their close relatives and the type species, *H. elongata*, are given in Table 2. Assimilation tests in API 20NE strips did not result in any positive reaction after 48 h incubation. Because it is very improbable that the isolates would score negative on all tests, these negative results are probably due to shortcomings or inhibitors of the medium used for inoculation and these results were therefore excluded from the final phenotypic description. Additional characteristics that apply for all isolates are given in the description below.

Table 2. Characteristics that differentiate between *H. muralis* sp. nov., its closest relatives *H. pantelleriensis* and *H. desiderata* and the type species, *H. elongata*

Data for reference taxa were taken from Romana *et al.* (1996) (*H. pantelleriensis*), Berendes *et al.* (1996) (*H. desiderata*) and Vreeland *et al.* (1980) (*H. elongata*). Characteristics are scored as: +, > 85% positive; w+, weakly positive; –, 0–15% positive; ND, not determined.

Characteristic	<i>H. muralis</i> sp. nov. (7 isolates)	<i>H. pantelleriensis</i> DSM 9661 ^T	<i>H. desiderata</i> DSM 9502 ^T	<i>H. elongata</i> ATCC 33174 ^T
pH range	5.0–10.0	7.5–11.0	7.0–11.0	5.0–9.0
NaCl range (% w/v)	0–15	1.25–15	0–18	0–32
Oxidase	+	+	+	–
Urease	–	+	–	+
Gelatin hydrolysis	–	–	–	+
Anaerobic growth	–	–	+	+
Growth on:				
D-Arabinose	–(6/7)	–	ND	+*
Cellobiose	–(6/7)	–	w+	+
Lactose	+	–	ND	+
Raffinose	+	–	ND	ND
D-Xylose	–	w+	+	–*
Susceptibility to:				
Chloramphenicol	–	+	ND	ND
Gentamicin	+	–	ND	ND
G+C content of the type strain (mol %)	62.4	65.0	66.0	60.5

* Additional data determined in this study for *H. elongata* LMG 9076^T.

Description of *Halomonas muralis* sp. nov.

Halomonas muralis (mu.ra'lis. L. adj. *muralis* pertaining or belonging to walls).

Cells are rods, 0.4–0.6 µm wide and 1.5–3.0 µm long, that occur singly or in pairs, are motile by peritrichous flagella, do not form endospores and stain Gram-negative. Colonies are small (< 1 mm) and colourless on MA, smooth, glistening, circular in outline and low convex in height. Strictly aerobic. Oxidase- and catalase-positive. The temperature range for growth is 10–35 °C with optimal growth at 25–35 °C. Growth occurs at pH 5.5–10.0 and is optimal in a range pH 6.5–8.5. The NaCl concentration range for growth is 0–15% (w/v) and the optimal range is 2.5–10% (w/v). Aesculin is hydrolysed but gelatin is not hydrolysed. Nitrate is reduced to nitrite. Indole is not produced. The following carbon sources can be used for growth: D-glucose, DL-lactose, D-melezitose, raffinose, sucrose and D-trehalose. No growth on D-fructose or D-xylose. Usually, no growth is observed on D-arabinose (except R-5056), cellobiose (except R-5056) or D-galactose (except LMG 20970 and R-5056). Growth on D-mannose is variable. Cells are susceptible to erythromycin, gentamicin, kanamycin and rifampicin. They are resistant to ampicillin, bacitracin, chloramphenicol, clindamycin, oxytetracycline, streptomycin, tetracycline and vancomycin. The major fatty acids are C_{18:1}ω7c, C_{16:0} and C_{16:1}ω7c and/or iso-C_{15:0} 2OH. Isolated from samples of biofilm covering a wall

and a mural of the Saint-Catherine chapel of Castle Herberstein (Austria). The G + C content is 62.4 mol % for the type strain, LMG 20969^T (= DSM 14789^T). In the variable characters listed above, the type strain scores negative for growth on D-mannose. Additionally, the type strain also grows at pH 5.0.

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