Chromohalobacter salexigens sp. nov., a moderately halophilic species that includes Halomonas elongata DSM 3043 and ATCC 33174

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Two strains that were originally isolated and characterized as members of the moderately halophilic species Halomonas elongata, strains DSM 3043 (= 1H11) and ATCC 33174 (= 1H15), were studied in detail. Their complete 16S rRNA sequences were determined and, when compared to sequences available from the databases, they showed a close phylogenetic relationship to Chromohalobacter marismortui. In addition, DNA–DNA hybridization experiments showed that both strains are members of the same species, but their DNA relatedness to the type strains of Halomonas elongata, ATCC 33173T, and Chromohalobacter marismortui, ATCC 17056T, is very low. Phenotypically, the two strains showed very similar features, related to those of Chromohalobacter, but clear differences were found between these two strains and Chromohalobacter marismortui. On the basis of these data, it is proposed that Halomonas elongata DSM 3043 and ATCC 33174 should be included in a new species of the genus Chromohalobacter, Chromohalobacter salexigens sp. nov. The type strain is DSM 3043T (= ATCC BAA-138T = CECT 5384T = CCM 4921T = CIP 106854T = NCIMB 13768T).

Keywords: Chromohalobacter salexigens sp. nov., Halomonas, taxonomy, moderately halophilic bacteria, 16S rRNA sequence

The genus Halomonas includes straight or curved Gram-negative rods that are motile or non-motile, catalase-positive and aerobic, although some strains are able to grow anaerobically in the presence of nitrate (Vreeland et al., 1980; Dobson & Franzmann, 1996). They contain ubiquinone 9 as the major respiratory quinone (Franzmann & Tindall, 1990). This genus was proposed by Vreeland et al. (1980) with a single species, Halomonas elongata, which included some moderately halophilic bacteria isolated from a saltern located on the Netherlands Antilles. Later, Baumann et al. (1983) proposed the genus Deleya to accommodate some marine species of the genus Alcaligenes and Pseudomonas marina. The species of the two genera Halomonas and Deleya were included in the family Halomonadaceae (Franzmann et al., 1988). Other related genera that were proposed to include slightly or moderately halophilic Gram-negative rods are Halovibrio (Fendrich, 1988), Chromohalobacter (Ventosa et al., 1989) and Volcaniella (Quesada et al., 1990).

Two studies, based on 16S rRNA sequence comparisons, led to the conclusion that the species of the genera Volcaniella, Deleya and Halovibrio, as well as the species Paracoccus halodenitrificans, should be considered as members of an emended genus Halomonas (Mellado et al., 1995; Dobson & Franzmann, 1996).

The species of the genus Halomonas have been isolated from different terrestrial and aquatic environments, mainly with high salt concentrations and/or alkaline pH. The type species is Halomonas elongata, which has been used extensively during recent decades as a model organism for physiological and osmoregulatory studies (Cánovas et al., 1996, 1997, 1998a, b; Gölger et al., 1998; Hart & Vreeland, 1988; Martin et al., 1983; Vargas et al., 1995; Vreeland et al., 1983). In the
description of *Halomonas elongata*. Vreeland *et al.* (1980) studied nine isolates and proposed strain 1H9T (= ATCC 33173T = DSM 2581T) as the type strain. In addition, two other strains have been deposited in culture collections: strain 1H11 (= DSM 3043) and strain 1H15 (= ATCC 33174), the latter strain being considered to be a biovar of *Halomonas elongata* because its cells are lophotrichous and it is unable to grow at 37 °C in the presence of 32% solar salt (Vreeland *et al.*, 1980). In this study, we have compared strains DSM 3043 and ATCC 33174 with the type strains of the species *Halomonas elongata* (ATCC 33173T) and *Chromohalobacter marismortui* (ATCC 17056T). Phenotypic, genotypic and phylogenetic data show clearly that the former *Halomonas elongata* strains constitute a novel species of the genus *Chromohalobacter*, for which we propose the name *Chromohalobacter salexigens* sp. nov.

For our study, we used the strains listed in Table 1. They were cultured on a complex medium containing approximately 10% salts (Ventosa *et al.*, 1989). The phenotypic characteristics, including morphological, physiological, biochemical and nutritional features, were determined by using the methodology described previously (Quesada *et al.*, 1984; Ventosa *et al.*, 1982).

Cells of *Halomonas elongata* DSM 3043 and ATCC 33174 were Gram-negative, straight or slightly curved, motile rods, 0.7–1.0 µm in width and 2.0–3.0 µm in length, occurring singly or in pairs. On solid complex medium with 10% (w/v) salts, colonies of the two strains appeared cream, opaque and circular and were 0.5–1.0 mm in diameter at 48 h growth. They were able to grow in media containing 0.9–25% (w/v) salts, showing optimal growth at 7.5–10% (w/v) salts. They are obligately aerobic. Table 2 shows the differential biochemical and nutritional features of the two strains in comparison with other related species.

For determination of the DNA base composition, the DNA was extracted and purified by the method of Marmur (1961) and its G + C content was determined from the mid-point value (Tm) of the thermal denaturation profile (Marmur & Doty, 1962) by using the equation of Owen & Hill (1979). The G + C contents of the DNA of strains DSM 3043 and ATCC 33174 were 64.2 and 66.0 mol%, respectively (Table 1).

DNA–DNA hybridization studies were performed by the competition procedure of the membrane method of Johnson (1994), described in detail elsewhere (Arahal *et al.*, 2001). The hybridization temperature for *Halomonas elongata* DSM 3043 was 58 °C, which is within the limits of validity for the filter method (De Ley & Tijtgat, 1970). The DNA–DNA hybridization results are shown in Table 1. The level of DNA relatedness between strain DSM 3043 and the type strain of *Halomonas elongata*, ATCC 33173T, was too low (25%) to consider them as members of the same species. However, strain DSM 3043 had 100% DNA relatedness to strain ATCC 33174.

Phylogenetic analysis of *Halomonas elongata* DSM 3043 and ATCC 33174 was performed as described elsewhere (Arahal *et al.*, 2001) and by using the ARB software (Ludwig & Strunk, 1996). The complete 16S rDNA sequences of the two strains were determined and deposited in the EMBL database. The sequence similarity between the two strains was 99.9%. However, the nearest similarities were to *Chromohalobacter israelensis* (99.9%), *Chromohalobacter canadensis* (97.0%) and *C. marismortui* (96.7%) and not to *Halomonas elongata* ATCC 33173T (94.1%). Fig. 1 shows a consensus phylogenetic tree based on three methods (distance-matrix, maximum-parsimony and maximum-likelihood methods). Strains DSM 3043 and ATCC 33174 belong phylogenetically to a branch that also contains *C. israelensis*, *C. canadensis* and *C. marismortui*. *C. israelensis* and *C. canadensis* are two former *Halomonas* species that are transferred to the genus *Chromohalobacter* by Arahal *et al.* (2001).

*Halomonas elongata* was described on the basis of the morphological, biochemical and physiological characteristics of nine bacterial isolates (Vreeland *et al.*, 1980). This species has been used in recent years as a model for the study of osmoregulatory mechanisms in

### Table 1. DNA G + C content and levels of DNA–DNA relatedness between *Halomonas elongata* DSM 3043, ATCC 33174 and ATCC 33173T and species of the genus *Chromohalobacter*

<table>
<thead>
<tr>
<th>Source of unlabelled DNA</th>
<th>G + C content (mol%)</th>
<th>Relatedness to 3H-labelled DNA from <em>H. elongata</em> DSM 3043 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. elongata</em> DSM 3043</td>
<td>64.2</td>
<td>100</td>
</tr>
<tr>
<td><em>H. elongata</em> ATCC 33174</td>
<td>66.0</td>
<td>100</td>
</tr>
<tr>
<td><em>H. elongata</em> ATCC 33173T</td>
<td>60.5</td>
<td>25</td>
</tr>
<tr>
<td><em>C. canadensis</em> ATCC 43984T</td>
<td>57</td>
<td>6</td>
</tr>
<tr>
<td><em>C. israelensis</em> ATCC 43985T</td>
<td>64</td>
<td>22</td>
</tr>
<tr>
<td><em>C. marismortui</em> ATCC 17056v</td>
<td>62.3</td>
<td>36</td>
</tr>
<tr>
<td><em>C. marismortui</em> A-492</td>
<td>63.9</td>
<td>58</td>
</tr>
</tbody>
</table>

* Data were taken from this study and from Vreeland *et al.* (1980) (*a*), Huval *et al.* (1995) (*b*) and Ventosa *et al.* (1989) (*c*).
Chromohalobacter salexigens sp. nov.

Table 2. Phenotypic features that differentiate Halomonas elongata DSM 3043 and ATCC 33174 (C. salexigens) from species of the genus Chromohalobacter

Data were taken from Huval et al. (1995), Ventosa et al. (1989), Vreeland et al. (1980) and this study. Characteristics are scored as: +, positive; −, negative.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>H. elongata DSM 3043 and ATCC 33174</th>
<th>C. israelensis ATCC 43985T</th>
<th>C. canadensis ATCC 43984T</th>
<th>C. marismortui ATCC 17056T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indole</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Oxidase</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>H₂S production</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Citrate (Simmons)</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Anaerobic growth with nitrate</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Lysine decarboxylase</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Ornithine decarboxylase</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Acid production from:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arabinose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>D-Trehalose</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of casein</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Growth on:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-Cellobiose</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
</tbody>
</table>

halophilic bacteria, since it is able to grow over a wide range of salinities, probably one of the most eurihaline of the bacteria (Ventosa et al., 1998). However, parallel studies carried out on two Halomonas elongata strains (ATCC 33173T and DSM 3043) have shown that important differences were evident between them, suggesting that they could represent different species.

Studies carried out on Halomonas elongata DSM 3043 have been related to its accumulation of compatible solutes as osmoprotectant compounds, enabling growth at different salt concentrations. Osmoadaptation is achieved by accumulation of glycine betaine, which is taken up from the medium or synthesized from choline (Cánovas et al., 1996, 1998b), and by synthesis of ectoine and hydroxyectoine (Cánovas et al., 1997). In Halomonas elongata DSM 3043, the pattern of ectoine accumulation in the
presence of betaine seems to be different from that of *Halomonas elongata* ATCC 33173² (Cánovas et al., 1997; Wohlfarth et al., 1990). In agreement with this, the homology between the ectoine synthesis genes of the two strains is significantly lower than one would expect from strains belonging to the same species (Cánovas et al., 1998a; Göller et al., 1998). These data, together with important differences in the salinity requirements of the two strains (Cánovas et al., 1996; Vreeland & Martin, 1980), suggested strongly that these two strains of *Halomonas elongata* could be phylogenetically distant. This has been approached in the present study.

Comparison of the 16S rRNA sequences of strains DSM 3043 and ATCC 33174 with other related 16S rRNA sequences showed that they are not related as closely to *Halomonas elongata* ATCC 33173 as would be expected. In fact, they are phylogenetically more related to other moderately halophilic microorganisms, *C. israelensis*, *C. canadensis* and *C. marismortui*, than to *H. elongata* (Fig.1). Indeed, *Halomonas elongata* DSM 3043 and ATCC 33174 constitute a monophyletic branch (Fig.1) with *C. israelensis*, *C. canadensis* and *C. marismortui*.

DNA–DNA hybridization experiments permitted us to determine that strains DSM 3043 and ATCC 33174 are very closely related to each other, but not to any of the phylogenetically related (16S rRNA sequence similarity higher than 97%) species of the genus *Chromohalobacter* or to *Halomonas elongata*. Our results show that the DNA relatedness to the type strain of *Halomonas elongata* is very low (25%), lower than the 70% value that is currently accepted for strains that belong to the same species (Wayne et al., 1987). In addition, the low values to *C. marismortui*, *C. israelensis* or *C. canadensis* (Table 1) support their placement in a separate species.

Vreeland et al. (1980) reported that the G+C content of strain 1H11 (= DSM 3043) was 60.5 mol% (determined by the Tm and bouyant-density methods). The same value was obtained for the type strain, ATCC 33173. In our study, the G+C content of strain DSM 3043 is 64.2 mol%, a value slightly higher than that reported previously, but within the range of G+C content defined for the genera *Halomonas* (Dobson & Franzmann, 1996) and *Chromohalobacter* (Ventosa et al., 1989).

Concerning the phenotypic features, we have found some differences with respect to the biochemical response (methyl red, lysine decarboxylase, ornithine decarboxylase, H₂S production) of strains DSM 3043 and ATCC 33174 between our study and the work of Vreeland et al. (1980), which could be due to differences in the methodology used.

It is noteworthy that both strains show very similar phenotypic features (Table 2). Although they are described as extremely salt-tolerant micro-organisms, they should be considered as moderately halophilic (Ventosa et al., 1988), showing optimal growth at 7.5–10% (w/v) salts, rather than halotolerant. The phenotypic features of strains DSM 3043 and ATCC 33174 are clearly related to those of the genus *Chromohalobacter* (Arahal et al., 2001; Ventosa et al., 1989) and phylogenetically they are within the monophyletic branch of this genus; however, they are not genetically related to the type species, *C. marismortui*, or to the other two species of the genus *Chromohalobacter* and, thus, we propose to include them in a new species, *Chromohalobacter salexiaxigen* sp. nov. The phenotypic features that differentiate it from the other species of the genus *Chromohalobacter* are shown in Table 2.

**Description of *Chromohalobacter salexiaxigen* sp. nov.**

*Chromohalobacter salexiaxigen* (sal.ex.i.gens. L. n. sal salt; L. v. exigo to demand; M.L. part. adj. salexiaxigen, salt-demanding).

Gram-negative, non-spore-forming rods, 0.7–1.0 x 2.0–3.0 μm. Cells occur singly or in pairs. Motile. On solid, complex medium containing 10% (w/v) salts, colonies are cream, opaque and circular and less than 2 mm in diameter; spreading may occur after extended incubation. In liquid medium containing 10% (w/v) salts, they produce homogeneous turbidity. Growth occurs in media containing 0–9–25% (w/v) salt and optimal growth occurs at 7.5–10% (w/v) salts. Growth occurs from pH 5 to 10 on liquid media containing 10% total salts (optimal growth at pH 7.5) and between 15 and 45 °C. The optimum temperature is 37 °C. Strictly aerobic. Catalase-positive and oxidase-negative. Acid is produced from L-arabinose, D-fructose, D-galactose, glyceral, D-glucose, lactose, maltose, D-mannose, sucrose and D-xylene but not from trehalose. Nitrate is reduced to nitrite but nitrite is not reduced. Citrate-positive. Gelatin, starch, aesculin, DNA and Tween 80 are not hydrolysed. Casein is hydrolysed. H₂S is produced from cysteine. Indole and acetoin are not produced. Methyl red-positive. Phenylalanine deaminase, lysine decarboxylase and ornithine decarboxylase are not produced. The following compounds are utilized as sole carbon and energy sources: L-arabinose, erythritol, D-fructose, D-galactose, D-glucose, D-mannitol, D-mannose, D-sorbitol, sucrose, D-trehalose, D-ribose, D-xylene, ethanol, glycerol, meso-inositol, dulcitol, acetate, α-aminovalerate, α-ketoglutarate, citrate, fumarate, DL-glyceralde, glutamate, malate, malonate, propionate, D-saccharate, succinate and D-tartarate. The following compounds are not utilized as sole carbon and energy sources: adonitol, cellobiose, L-fucose, α-lactose, D-melibiose, D-raffinose, L-rhamnose, galactosamine, gluconolactone, inulin, DL-α-aminoxybutyrate, butyrate, caprylate, lactate and oxalate. The following compounds are utilized as sole sources of carbon, nitrogen and energy: L-arginine, L-asparagine, betaine, glycine, L-glutamine, L-lysine, L-...
ornithine, l-proline and l-serine. The following com-
ounds are not utilized as sole sources of carbon,
nitrogen and energy: l-alanine, creatine, l-methion-
ine, putrescine, sarcosine, l-threonine and l-valine.
The G+C content of the DNA is 64.2–66.0 mol % (Tm
method). The EMBL accession numbers of the 16S
rRNA gene sequences of strains DSM 3043T and
ATCC 33174 determined in this study are AJ295146
and AJ295147, respectively.

Isolated from salterns. The type strain is DSM 3043T
(= ATCC BAA-1387T = CECT 5384T = CCM 4921T
= CIP 106854T = NCIMB 13768T).

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References

Arahal, D. R., García, M. T., Ludwig, W., Schleifer, K. H. &
Ventosa, A. (2001). Transfer of Halomonas canadensis and
Halomonas israelensis to the genus Chromohalobacter as
Chromohalobacter canadensis comb. nov. and Chromo-
halobacter israelensis comb. nov. Int J Syst Evol Microbiol 51,
1443–1448.

Baumann, L., Bowditch, R. D. & Baumann, P. (1983). Description of
Deleya gen. nov. created to accommodate the marine species
Alcaligenes acutus, A. pacificus, A. cupidis, A. venustus, and

Cánovas, D., Vargas, C., Csonka, L. N., Ventosa, A. & Nieto, J. J.
(1996). Osmoprotectants in Halomonas elongata: high-affinity
betaine transport system and choline-betaine pathway. J Bacteriol
178, 7221–7226.

Cánovas, D., Vargas, C., Iglesias-Guerra, F., Csonka, L. N., Rhodes,
D., Ventosa, A. & Nieto, J. J. (1997). Isolation and charac-
terization of salt-sensitive mutants of the moderate halophile
Halomonas elongata and cloning of the ectoine synthesis genes.

Cánovas, D., Vargas, C., Calderón, M. I., Ventosa, A. & Nieto, J. J.
(1998a). Characterization of the genes for the biosynthesis of the
compatible solute ectoine in the moderately halophilic bac-
terium Halomonas elongata DSM 3043. Syst Appl Microbiol 21,
487–497.

Cánovas, D., Vargas, C., Csonka, L. N., Ventosa, A. & Nieto, J. J.
(1998b). Synthesis of glycine betaine from exogenous choline in
the moderately halophilic bacterium Halomonas elongata. Appl
Environ Microbiol 64, 4095–4097.

methods for DNA–DNA hybridization. Antonie Leeuwenhoek
36, 461–474.

genera Deleya (Baumann et al. 1983), Halomonas (Vreeland et
al. 1980), and Halovibrio (Fendrich 1988) and the species
Paracoccus halodenitrificans (Robinson and Gibbons 1952) into a
single genus, Halomonas, and placement of the genus
Zymobacter in the family Halomonadaceae. Int J Syst Bacteriol
46, 550–558.

Fendrich, C. (1988). Halovibrio variabilis gen. nov. sp. nov.,
Pseudomonas halophila sp. nov. and a new halophilic aerobic

coccoid eubacterium from Great Salt Lake, Utah, USA. Syst
Appl Microbiol 11, 36–43.

Franzmann, P. D. & Tindall, B. J. (1990). A chemotaxonomic
study of members of the family Halomonadaceae. Syst Appl
Microbiol 13, 142–147.

Halomonadaceae fam. nov., a new family of the class Proteo-
bacteria to accommodate the genera Halomonas and Deleya.
Syst Appl Microbiol 11, 16–19.

terization of an NaCl-sensitive mutant of Halomonas
elongata impaired in ectoine biosynthesis. FEMS Microbiol
Lett 161, 293–300.

hydrophilic cell surface character of Halomonas elongata in

Huval, J. H., Latta, R., Wallace, R., Kushner, D. J. & Vreeland, R. H.
israelensis sp. nov. and Halomonas canadensis sp. nov. Can J Microbiol 41,
1124–1131.

for General and Molecular Bacteriology, pp. 655–682. Edited by
Washington, DC: American Society for Microbiology.

Ludwig, W. & Strunk, O. (1996). ARB—a software environ-
ment for sequence data. http://www.mikro.biologie.tu-
uemchen.de/pub/ARB/documentation/arb.ps

Marmur, J. (1961). A procedure for the isolation of deoxy-

composition of deoxyribonucleic acid from its thermal

Martin, E. L., Duryea-Rice, T., Vreeland, R. H., Hilsabeck, L. &
Davis, C. (1983). Effects of NaCl on the uptake of x-
[14C]Chaminoisobutyric acid by the halotolerant bacterium

Phylogenetic inferences and taxonomic consequences of 16S
ribosomal DNA sequence comparison of Chromohalobacter
marismortui, Volcaniella eurihalina, and Deleya salina and
reclassification of V. eurihalina as Halomonas eurihalina comb.

Owen, R. J. & Hill, L. R. (1979). The estimation of base
compositions, base pairing and genome sizes of bacterial
deoxyribonucleic acids. In Identification Methods for Micro-

Quesada, E., Ventosa, A., Ruiz-Berraquero, F. & Ramos-
Cormenzana, A. (1984). Deleya halophila, a new species of

Quesada, E., Valderrama, M. J., Bejar, V., Ventosa, A., Gutiérrez,
Volcaniella eurihalina gen. nov., sp. nov., a moderately
halophilic nonmotile gram-negative rod. Int J Syst Bacteriol
40, 261–267.

Vargas, C., Fernández-Castillo, R., Cánovas, D., Ventosa, A. &
Nieto, J. J. (1995). Isolation of cryptic plasmids from moder-
ately halophilic eubacteria of the genus Halomonas. Characteri-
sation of a small plasmid from H. elongata and its use for

Ventosa, A., Quesada, E., Rodríguez-Valera, F., Ruiz-Berraquero,

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