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***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 33173^T, and *Chromohalobacter marismortui*, ATCC 17056^T, 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 3043^T (= ATCC BAA-138^T = CECT 5384^T = CCM 4921^T = CIP 106854^T = NCIMB 13768^T).

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; Göller *et al.*, 1998; Hart & Vreeland, 1988; Martin *et al.*, 1983; Vargas *et al.*, 1995; Vreeland *et al.*, 1983). In the

The EMBL accession numbers for the 16S rRNA gene sequences of *Halomonas elongata* DSM 3043 and ATCC 33174 are AJ295146 and AJ295147, respectively.

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

Source of unlabelled DNA	G + C content (mol %)*	Relatedness to ³ H-labelled DNA from <i>H. elongata</i> DSM 3043 (%)
<i>H. elongata</i> DSM 3043	64.2	100
<i>H. elongata</i> ATCC 33174	66.0	100
<i>H. elongata</i> ATCC 33173 ^T	60.5 ^a	25
<i>C. canadensis</i> ATCC 43984 ^T	57 ^b	6
<i>C. israelensis</i> ATCC 43985 ^T	64 ^b	22
<i>C. marismortui</i> ATCC 17056 ^T	62.3 ^c	36
<i>C. marismortui</i> A-492	63.9 ^c	58

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

description of *Halomonas elongata*, Vreeland *et al.* (1980) studied nine isolates and proposed strain 1H9^T (= ATCC 33173^T = DSM 2581^T) 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 33173^T) and *Chromohalobacter marismortui* (ATCC 17056^T). 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 less than 2 mm in diameter after 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 (T_m) 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 33173^T, 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 33173^T (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 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.

Characteristic	<i>H. elongata</i> DSM 3043 and ATCC 33174	<i>C. israelensis</i> ATCC 43985 ^T	<i>C. canadensis</i> ATCC 43984 ^T	<i>C. marismortui</i> ATCC 17056 ^T
Indole	–	+	+	–
Oxidase	–	–	–	+
H ₂ S production	+	–	–	–
Urease	+	–	–	–
Citrate (Simmons)	+	–	–	+
Nitrate reduction	+	+	+	–
Anaerobic growth with nitrate	+	–	–	–
Lysine decarboxylase	–	+	+	–
Ornithine decarboxylase	–	+	+	–
Acid production from:				
Arabinose	+	+	+	–
Maltose	+	+	–	+
Sucrose	+	+	–	+
D-Trehalose	–	–	–	+
Hydrolysis of casein	+	–	–	–
Growth on:				
D-Cellobiose	–	+	+	–
Sucrose	+	+	–	+

Fig. 1. Phylogenetic tree derived from analysis of the 16S rRNA sequences of strains DSM 3043 and ATCC 33174 and related species of *Chromohalobacter* and *Halomonas*. The collapsed branches correspond to those whose topology was affected by the methods applied. Bar, 5% sequence difference. Outgroup sequences are not shown.

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 33173^T 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. Osmo-adaptation 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 33173^T (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^T as would be expected. In fact, they are phylogenetically more related to other moderately halophilic micro-organisms, *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 T_m and bouyant-density methods). The same value was obtained for the type strain, ATCC 33173^T. 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, H_2S 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.*, 1998), 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 salexigens* sp. nov. The phenotypic features that differentiate it from the other species of the genus *Chromohalobacter* are shown in Table 2.

Description of *Chromohalobacter salexigens* sp. nov.

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

Gram-negative, non-spore-forming rods, 0.7–1.0 × 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, glycerol, D-glucose, lactose, maltose, D-mannose, sucrose and D-xylose 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. H_2S 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, α-D-glucose, maltose, D-mannitol, D-mannose, D-sorbitol, sucrose, D-trehalose, D-ribose, D-xylose, ethanol, glycerol, *meso*-inositol, dulcitol, acetate, α-aminovalerate, α-ketoglutarate, citrate, fumarate, DL-glycerate, glutamate, malate, malonate, propionate, D-saccharate, succinate and D-tartrate. 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-aminobutyrate, 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 compounds are not utilized as sole sources of carbon, nitrogen and energy: L-alanine, creatine, L-methionine, putrescine, sarcosine, L-threonine and L-valine. The G + C content of the DNA is 64.2–66.0 mol % (T_m method). The EMBL accession numbers of the 16S rRNA gene sequences of strains DSM 3043^T and ATCC 33174 determined in this study are AJ295146 and AJ295147, respectively.

Isolated from salterns. The type strain is DSM 3043^T (= ATCC BAA-138^T = CECT 5384^T = CCM 4921^T = CIP 106854^T = NCIMB 13768^T).

Acknowledgements

D.R.A. and D.C. were recipients of fellowships from the Ministerio de Educación y Cultura, Spain. This investigation was supported by grants from the Ministerio de Educación y Cultura, Spain (grants PB97-0722, 1FD97-1162 and PB98-1150) and from the Junta de Andalucía.

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