International Journal of Systematic and Evolutionary Microbiology (2007), **57**, 2436–2446 **DOI** 10.1099/ijs.0.65430-0

Recommended minimal standards for describing new taxa of the family *Halomonadaceae*

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Following Recommendation 30b of the *Bacteriological Code* (1990 Revision), a proposal of minimal standards for describing new taxa within the family *Halomonadaceae* is presented. An effort has been made to evaluate as many different approaches as possible, not only the most conventional ones, to ensure that a rich polyphasic characterization is given. Comments are given on the advantages of each particular technique. The minimal standards are considered as guidelines for authors to prepare descriptions of novel taxa. The proposals presented here have been endorsed by the International Committee on Systematics of Prokaryotes Subcommittee on the Taxonomy of *Halomonadaceae*.

Development of minimal standards for describing new taxa is a task mentioned in Recommendation 30b of the *Bacteriological Code* (1990 Revision) (Lapage *et al.*, 1992). Accordingly, this paper provides a list of recommended minimal standards for descriptions of new genera and species of the family *Halomonadaceae*.

Taxonomic status of the *Halomonadaceae*

The family *Halomonadaceae* was proposed on the basis of results obtained with the 16S rRNA cataloguing technique of several closely related organisms (Franzmann *et al.*, 1988). This resulted in the inclusion of two genera that grouped moderately halophilic and marine gammaproteobacteria, *Halomonas* and *Deleya*, which respectively contained four and six species whose names had been validly published. Subsequently, several new genera related to these two were proposed, *Halovibrio* (Fendrich, 1988), *Chromohalobacter* (Ventosa *et al.*, 1989), *Volcaniella* (Quesada *et al.*, 1990) and *Zymobacter* (Okamoto *et al.*, 1993), each containing a single species. However, the degree of relatedness as derived from 16S rRNA gene sequence phylogenetic analysis motivated the reclassification of *Volcaniella eurihalina* into the genus *Halomonas* (Mellado *et al.*, 1995) and the transfer of the species of the genus *Deleya*, as well as *Halovibrio variabilis* and *Pseudomonas halodenitrificans*, to the genus *Halomonas* (Dobson & Franzmann, 1996), resulting in an increase in the number of *Halomonas* species to 15.

Though two *Halomonas* species have been reclassified into the genus *Chromohalobacter* (Arahal *et al.*, 2001a) and a third has been proposed as the type species of a new genus, *Cobetia* (Arahal *et al.*, 2002a), the number of *Halomonas* species has continued to grow as 34 new species proposals have been published between 1996 and May 2007. Compared with the large number of species currently classified in the genus *Halomonas* (42 and still growing), *Chromohalobacter* presently contains seven

species, including the recently reclassified *Pseudomonas beijerinckii* (Peçonek *et al.*, 2006), and the remaining genera of the family *Halomonadaceae* (*Carnimonas*, *Cobetia* and *Zymobacter*) each contain only one. A list of these species, including additional data, can be found in Table 1. Five novel *Halomonas* species and two novel *Chromohalobacter* species, as well as a new genus and species within the family *Halomonadaceae*, are described in papers listed as in press at the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM) website as of May 2007. In addition, it has to be mentioned that a request for an opinion regarding the identity of the strains *Pseudomonas halophila* DSM 3050 and *Halomonas variabilis* DSM 3051 has recently been formulated (Sorokin & Tindall, 2006), since these two catalogue numbers have been wrongly associated with names and descriptions. If the Judicial Commission adopts the proposals in that request, it would mean that (i) the type of the species name *Halovibrio variabilis* Fendrich 1989 would be DSM 3050 (not DSM 3051) and the name *Halomonas variabilis* would be rejected since it was created under a wrong assumption and (ii) DSM 3051 would be recognized as the type strain of *Pseudomonas halophila* and transferred to the genus *Halomonas* as *Halomonas utahensis* nom. nov. (Sorokin & Tindall, 2006).

General outlines

Many novel species have been described within the family *Halomonadaceae* during the past few years. As a consequence, there has been an increase in both the number of species and the phenotypic heterogeneity, especially within the genus *Halomonas*. The main purpose of this article is to present a framework for describing new taxa within the *Halomonadaceae*. The recommendations given here are aimed at preventing the establishment of insufficiently characterized 'new' taxa, which are later difficult to handle by other microbiologists and quite often represent a source of confusion in taxonomic classification. To be truly useful and complete, the list of features recommended in this paper had to be necessarily large; however, this should not discourage scientists from publishing the descriptions of novel species or genera. On the contrary, adhering to these standards will benefit all, in both the short and long run.

The recent re-evaluation of the species definition in bacteriology (Stackebrandt *et al.*, 2002) reinforces the necessity for and value of minimal characteristics, stating that they 'should be provided and follow the guidelines set forth by various subcommittees of the ICSP'. The methodology employed should be sufficiently explained in the text or through an appropriate reference. A large compilation of morphological, physiological, biochemical, nutritional and antimicrobial susceptibility tests conducted on *Halomonas* species can be found in Mata *et al.* (2002).

One of the consensus criteria in modern bacterial systematics is the adoption of polyphasic approaches that integrate phenotypic (including chemotaxonomic markers) with genotypic methods. Especially for the latter, a wealth of new techniques and methodologies of great promise has become available, and many more are being developed (Stackebrandt *et al.*, 2002).

Salt/temperature/pH as preliminary tests

Standardization is a key issue. Studies should be performed on actively growing cultures. Members of the *Halomonadaceae* have diverse physiological requirements, so it is not possible to recommend one single culture medium and growth conditions that fit all. This is especially true when considering the salt, temperature and pH requirements. It is important that at least these three variables are studied in order to determine the ranges that enable growth for each parameter and the optimal conditions within those ranges. Moreover, these parameters are interdependent, so it is recommended that a reasonable number of salt content/temperature/pH combinations are tested. We emphasize the importance not only of assessing the ability to grow under different conditions but also of determining the optimal set of conditions to be used in further tests.

All members of the *Halomonadaceae* described so far are able to grow at salinities between 5 and 10 % (w/v) (except *Zymomonas palmae*, which has been described as osmotolerant but whose degree of halophilism and halotolerance has not been reported), at temperatures from 20 to 35 °C and pH 7.0–8.0, though *Halomonas campisalis* (Mata *et al.*, 2002) and *Halomonas gudaonensis* (Wang *et al.*, 2007) require pH 8.0 or above to grow. For more than 90 % of the species, their physiological boundaries embrace at least 3–15 % salinity, temperatures from 15 to 37 °C and pH 6–9. Table 2 lists all current members of the family *Halomonadaceae* and their response towards salt concentration, temperature and pH. Determination of the minimal amount of salts required for growth is not as obvious as it might seem. When using commercial media, it is important to take the salt content of the media into account. For instance, for a strain growing in TSB (tryptone soy broth), it cannot be concluded that it grows in the absence of salts since this medium contains 0.5 % (w/v) NaCl. To test growth in the absence of salts, minimal media are recommended, as well as cell inocula free of the medium in which they were grown. Occasionally, in addition to the values suggested in Table 2, further tests might be required to assess better the physiological boundaries of the organism being described [for instance, if growth occurs at 25 % (w/v) salts then growth at 30 % should be tested as well]. Moreover, following the first round of tests, intermediate values should be tested as well to define better the boundaries of growth. It has to be noted as well that many strains respond better to salt mixtures (particularly those resembling the original habitat) than to NaCl alone. Some species have even been reported to require Mg^{2+} (Valderrama *et al.*, 1991). The presence of compatible solutes also has to be taken into account, since they may enhance the salt tolerance. Therefore, all these circumstances have to be indicated in the description of the methodology.

Table 1. Validly published species names within the family *Halomonadaceae*, updated to May 2007

More information (synonyms, historical record of the names, type strains, etc.) can be obtained from the List of Prokaryotic names with Standing in Nomenclature available at http://www.bacterio.cict.fr (Euzéby, 1997). For each species, an accession number for the 16S rRNA gene sequence from the type strain is given with an indication of the total length and the number of ambiguities (if any, in parentheses) (for some strains, additional sequences are available, but they are shorter and/or contain more ambiguities).

Species	Reference*	Strains (*n*)	Isolation source	16S rRNA gene accession no.	Length (bp)
Carnimonas					
Car. nigrificans	Garriga *et al.* (1998)	9	Raw cured-meat products (Spain)	Y13299	1519 (2)
Chromohalobacter					
Chr. beijerinckii	Peçonek *et al.* (2006)	2	Salted beans and herrings	AB021386	1495 (1)
Chr. canadensis	Arahal *et al.* (2001a)	1	Contaminant on medium containing 25 % NaCl	AJ295143	1532
Chr. israelensis	Arahal *et al.* (2001a)	1	Dead Sea	AJ295144	1532
Chr. marismortui	Ventosa *et al.* (1989)	8	Dead Sea and solar salterns	X87219	1423 (1)
Chr. nigrandesensis	Prado *et al.* (2006)	52	Hypersaline sediment of Lake Tebenquiche (Chile)	AJ277205	1505
Chr. salexigens	Arahal *et al.* (2001b)	2	Solar salterns	AJ295146	1531 (3)
Chr. sarecensis	Quillaguamán *et al.* (2004b)	1	Saline soil around a hypersaline lake (Bolivia)	AY373448	1448
Cobetia					
Cob. marina	Arahal *et al.* (2002a)	7	Marine	AJ306890	1536
Halomonas					
H. alimentaria	Yoon *et al.* (2002)	1	Jeotgal (traditional Korean fermented seafood)	AF211860	1493
H. almeriensis	Martínez-Checa *et al.* (2005)	1	Solar saltern (Spain)	AY858696	1459 (2)
H. anticariensis	Martínez-Cánovas *et al.* (2004b)	3	Saline soil (Spain)	AY489405	1464 (5)
H. aquamarina	Dobson & Franzmann (1996)	1	Marine	AJ306888	1528
H. avicenniae	Soto-Ramírez *et al.* (2007)	1	Salty leaves of *Avicennia germinans* (Puerto Rico)	DQ888315	1433
H. axialensis	Kaye *et al.* (2004)	1	Deep-sea hydrothermal-vent environments	AF212206	1438
H. boliviensis	Quillaguamán *et al.* (2004a)	2	Soil around a hypersaline lake (Bolivia)	AY245449	1441
H. campaniensis	Romano *et al.* (2005)	1	Mineral pool (Italy)	AJ515365	1480 (3)
H. campisalis	Mormile *et al.* (1999)	1	Soil below a crystalline salt surface	AF054286	1400 (2)
H. cupida	Dobson & Franzmann (1996)	5	Marine	L42615†	1471 (19)
H. denitrificans	Kim *et al.* (2007)	3	Saline water (Korea)	AM229317	1489
H. desiderata	Berendes *et al.* (1996)	6	Municipal sewage	X92417	1495
H. elongata	Vreeland *et al.* (1980)	9	Solar salterns	X67023	1470 (5)
H. eurihalina	Mellado *et al.* (1995)	16	Hypersaline habitats (soils, salt ponds) and seawater	X87218	1473 (6)
H. gomseomensis	Kim *et al.* (2007)	2	Saline water (Korea)	AM229314	1484
H. gudaonensis	Wang *et al.* (2007)	2	Saline soil contaminated by crude oil (China)	DQ421808	1462
H. halmophila	Dobson *et al.* (1990)	1	Dead Sea	AJ306889	1530
H. halocynthiae	Romanenko *et al.* (2002)	1	Gill tissues of the ascidian *Halocynthia aurantium*	AJ417388	1514
H. halodenitrificans	Dobson & Franzmann (1996)	1	Meat-curing brines	L04942	1531 (5)
H. halodurans	Hebert & Vreeland (1987)	29	Estuarine water	L42619†	1474 (27)
H. halophila	Dobson & Franzmann (1996)	38	Saline soils	M93353†	1478 (76)
H. hydrothermalis	Kaye *et al.* (2004)	1	Deep-sea hydrothermal-vent environments	AF212218	1409 (1)
H. indalinina	Cabrera *et al.* (2007)	1	Solar saltern (Spain)	AJ427627†	1527 (14)
H. janggokensis	Kim *et al.* (2007)	2	Saline water (Korea)	AM229315	1478
H. koreensis	Lim *et al.* (2004)	1	Solar saltern (Korea)	AY382579†	1399
H. magadiensis	Duckworth *et al.* (2000)	3	Littoral sediments of haloalkaline East African lakes	X92150	1473
H. marisflavi	Yoon *et al.* (2001)	1	Marine	AF251143	1495
H. maura	Bouchotroch *et al.* (2001)	4	Solar saltern (Morocco)	AJ271864†	1396 (8)
H. meridiana	James *et al.* (1990)	7	Antarctic saline lakes	AJ306891	1528
H. muralis	Heyrman *et al.* (2002)	7	Biofilm covering a wall and a mural (Austria)	AJ320530	1493 (1)
H. neptunia	Kaye *et al.* (2004)	1	Deep-sea hydrothermal-vent environments	AF212202	1454 (2)
H. organivorans	García *et al.* (2004)	4	Saline soils (Spain)	AJ616910	1472

Table 1. cont.

Species	Reference*	Strains (*n*)	Isolation source	16S rRNA gene accession no.	Length (bp)
H. pacifica	Dobson & Franzmann (1996)	6	Marine	L42616	1480 (4)
H. pantelleriensis	Romano *et al.* (1996)	1	Hard sand from Pantelleria island (Italy)	X93493	1477
H. salaria	Kim *et al.* (2007)	3	Saline water (Korea)	AM229316	1492
H. salina	Dobson & Franzmann (1996)	26	Hypersaline soils, salt ponds, salt lakes, seawater	AJ295145	1532
H. subglaciescola	Franzmann *et al.* (1987)	29	Antarctic saline lake (Organic Lake)	AJ306892	1531
H. sulfidaeris	Kaye *et al.* (2004)	1	Deep-sea hydrothermal-vent environments	AF212204	1430 (1)
H. taeanensis	Lee *et al.* (2005)	1	Solar saltern (Korea)	AY671975	1408
H. variabilis	Dobson & Franzmann (1996)	1	North arm of the Great Salt Lake (USA)	AJ306893	1528
H. ventosae	Martínez-Cánovas *et al.* (2004a)	3	Saline soils (Spain)	AY268080†	1395
H. venusta	Dobson & Franzmann (1996)	14	Marine	AJ306894	1530
Zymomonas					
Z. palmae	Okamoto *et al.* (1993)	4	Palm sap in Okinawa Prefecture (Japan)	AF211871	1509

*For names derived from new combinations, only the most recent reference is given.
†Sequences that do not reach the quality standards defined in the text.

Testing of multiple strains and deposition in culture collections

Following the recommendations of the ad hoc committee for the re-evaluation of the species definition in bacteriology (Stackebrandt *et al.*, 2002), which addressed the question raised by Christensen *et al.* (2001) relating to the need for multiple strains for the delineation of species, we recommend that species descriptions be based on more than a single strain. Of the current 52 species descriptions within the family *Halomonadaceae*, 22 are based on a single strain, 23 on two to nine strains and only seven on more than ten strains (Table 1). It has been noted that some species said to be based on more than one strain are in fact described with no information about the intraspecies variability. For future descriptions of novel species, an effort in collecting and characterizing a larger number of isolates and, where possible, from a variety of sources is recommended. If multiple strains are available, numerical analysis on a sufficient number of phenotypic traits is advised in order to obtain clusters of strains (Sneath & Sokal, 1973). The evidence of species co-identity of multiple strains can also be obtained from rapid DNA typing methods.

A type strain, which should be a representative strain of the new taxon, must be designated when a novel species is proposed. Prior to the valid publication of a novel species name, it is required that the type strain is deposited in two or more recognized culture collections located in different countries (Labeda, 2000). Information about the collections where the type strains of the current species of the family *Halomonadaceae* have been deposited can be obtained at http://www.bacterio.cict.fr (Euzéby, 1997). In addition to the culture collections that received the type strains by direct deposit from the authors of the species descriptions, other collections may have acquired them later (usually on an exchange basis between culture collections). The StrainInfo.net bioportal (http://www.straininfo.ugent.be/) provides a straightforward search tool for locating type strains (or additional strains) that are catalogued in the main culture collections around the world.

Studies aimed at the proposal of novel species should include the type strains of all related species for comparison, whether or not they belong to the same genus, as well as the type strain of the type species of the genus. Based on 16S rRNA gene sequences, selecting the most related species to a new taxon is a straightforward matter. Both sequence similarity and tree topology should be taken into account. The final number of related species to be included for comparative purposes should be decided by the authors and depends largely on the situation. Additional strains may be necessary as positive or negative controls for certain tests.

Sequence-based phylogenetic analyses

Regarding molecular phylogeny, the 16S rRNA approach remains the backbone of prokaryotic systematics (Ludwig & Schleifer, 1999; Ludwig & Klenk, 2001). In the case of the family *Halomonadaceae*, the first additional gene employed was the 23S rRNA gene (Arahal *et al.*, 2002b), a molecule that contains more phylogenetic information than the 16S rRNA gene (Ludwig & Schleifer, 1994, 1999). Also, being part of the same operon, good agreement can be expected between the two phylogenies, as shown in the study of Arahal *et al.* (2002b). Thus, the 23S rRNA gene sequence can provide supplementary phylogenetic information to the 16S rRNA gene (Ludwig & Schleifer, 1994; Ludwig &

Table 2. Response of the members of the family *Halomonadaceae* to salt, temperature and pH

Data taken from original and emended descriptions and Mata *et al.* (2002). +, Growth detected; −, no growth obtained; ND, no data available.

Species	Salts (%, w/v)											Temperature (°C)							pH							
	0	0.5	1	2	3	3.5	5	10	15	20	25	4–5	10	15	20	35	37	45	5	6	7	8	9	10	11	12
Car. nigrificans	+	+	+	+	+	+	+	+	−	−	−	−	ND	ND	ND	ND*	−	−	ND	ND	ND	ND	ND	ND	ND	ND
Chr. beijerinckii	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	−	−
Chr. canadensis	−	−	−	−	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−
Chr. israelensis	−	−	−	−	−	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−
Chr. marismortui	−	−	−	+	+	+	+	+	+	+	+†	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Chr. nigrandesensis	−	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−
Chr. salexigens	−	−	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	−	−	−
Chr. sarecensis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Cob. marina	−	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	−	+	+	+	+	+	+	−	−
H. alimentaria	−	ND	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	ND	ND	ND	ND	ND
H. almeriensis	−	−	−	−	−	−	+	+	+	+	+	−	−	+	+	+	+	−	−	+	+	+	+	+	−	−
H. anticariensis	−	+	+	+	+	+	+	+	+	−	−	−	−	−	+	+	+	+	−	+	+	+	+	−	−	−
H. aquamarina	−	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	−	+	+	+	+	+	+	−	−
H. avicenniae	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	−	+	+	+	+	+	−	−	−
H. axialensis	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+
H. boliviensis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	ND
H. campaniensis	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	−	−	−	+	+	+	+	−	−
H. campisalis	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+‡	−	+	+	+	+	+	+	+
H. cupida	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+	+	−	+	+	+	+	+	+	−	−
H. denitrificans	−	−	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+‡	−	−	+	+	+	+	−	−
H. desiderata	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−	+	+	+	+	+	−
H. elongata	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
H. eurihalina	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
H. gomseomensis	−	−	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−
H. gudaonensis	−	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	−	−	−	−	+	+	−	−	−
H. halmophila	−	−	−	−	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	−	−
H. halocynthiae	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	−	−	+	+	+	+	+	+	+	−
H. halodenitrificans	−	−	−	−	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−
H. halodurans	−	−	−	−	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−
H. halophila	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
H. hydrothermalis	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
H. indalinina	−	−	ND	ND	+	+	+	+	+	+	+	−	−	+	+	+	+	−	+	+	+	+	+	−	−	−
H. janggokensis	−	−	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−
H. koreensis	−	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	−	+	+	+	+	+	−	−
H. magadiensis	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	−
H. marisflavi	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−
H. maura	−	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−
H. meridiana	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
H. muralis	+	+	+	+	+	+	+	+	+	−	−	−	+	+	+	+	−	−	−	+	+	+	+	+	−	−
H. neptunia	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+
H. organivorans	−	−	−	+	+	+	+	+	+	+	+†	−	−	+	+	+	+	+	−	+	+	+	+	+	−	−
H. pacifica	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
H. pantelleriensis	−	−	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	−	+	+	+	+	+	+	−
H. salaria	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−
H. salina	−	−	−	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
H. subglaciescola	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
H. sulfidaeris	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	−	+	+	+	+	+	+	−	−
H. taeanensis	−	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	−	+	+	+	+	−	−
H. variabilis	−	−	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	−	−	+	+	+	+	−	−	−
H. ventosae	−	−	−	−	+	+	+	+	+	ND	ND	−	−	+	+	+	+	+‡	−	+	+	+	+	+	−	−
H. venusta	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Z. palmae	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	−	−	−	+§	+	+	−	+‖	+	+	+	+	+	−	−

*Information available is very limited: described as not able to grow at 5 or 37 °C and having optimal growth at 28–30 °C.
†Also grows with 30 % (w/v) salts.
‡Also grows at 50 °C.
§Growth reported at 21 °C.
||Lower limit pH 3.0.

Klenk, 2001). More recently, Okamoto *et al.* (2004) conducted a comparative phylogenetic analysis of *Halomonas variabilis* and related organisms of various origins based on 16S rRNA, *gyrB* and *ectBC* gene sequences. The recommendation of introducing the sequencing of housekeeping or other protein-coding genes (Stackebrandt *et al.*, 2002) to increase the level of phylogenetic information has so far had little impact on the taxonomy of the family. As of May 2007, more than 1300 nucleotide sequences of members of the *Halomonadaceae* can be retrieved from public databases, of which the vast majority correspond to the 16S rRNA gene (around 1200, but half of them are shorter than 1400 nucleotides), whereas the number of sequences from alternative phylogenetic markers is scarcely representative. The complete genome sequence of *Chromohalobacter salexigens* DSM 3043^T (GenBank accession no. CP000285) can be of help for new phylogenetic studies within the family, especially for the task of designing amplification primers for the desired genes.

Given the role of 16S rRNA gene sequence comparison in modern microbial taxonomy, guidance on some key points seems necessary. The first is the quality and length of the sequence itself. Stackebrandt *et al.* (2002) indicated that 'all species descriptions should include an almost complete 16S rRNA gene sequence (>1300 nt, <0.5 % ambiguity)'. Garrity *et al.* (2004) further defined high-quality 16S rRNA gene sequences as >1400 nt, <4 % ambiguity and fewer than 10 missing positions. Recently, Stackebrandt & Ebers (2006) criticized the careless handling of 16S rRNA gene sequences and its negative consequences for bacterial taxonomy.

Considering the existing background of 16S rRNA gene sequences among the species belonging to the family *Halomonadaceae* (Table 1) where, for all described species, there is at least one sequence available of the type strain, and the overall quality is very high, it is appropriate to preserve that level of quality. Thus, we recommend following the most demanding criteria (>1400 nt, <0.5 % ambiguity), since they are met by almost all species within the family *Halomonadaceae*. The only exceptions are *Halomonas cupida*, *H. halodurans*, *H. halophila*, *H. indalinina*, *H. koreensis*, *H. maura* and *H. ventosae*, whose sequences might eventually be corrected and/or completed. In any case, the sequences from *H. koreensis*, *H. maura* and *H. ventosae* are not very far from the quality standard defined above (Table 1).

As indicated above, at the time of writing, there are more than 600 16S rRNA gene sequences longer than 1400 nucleotides from representatives of the *Halomonadaceae*. Although many are environmental clones or have been obtained from unidentified isolates, they may be included during the preliminary stages of phylogenetic analyses. However, for the final presentation of the results, we recommend the inclusion of only those sequences from species whose names have been validly published. Furthermore, sequences from strains other than the type strain usually exhibit little divergence and can be removed from the final tree. Sequence duplicates – sequences derived from the same or an equivalent strain, but in different studies, and essentially identical – are unnecessary in final trees, and only those possessing the highest quality should be included (Table 1). Special care has to be taken during the alignment of the sequences. Manual inspection of the alignments generated with automatic aligners is recommended, taking into consideration the secondary structure and previous alignments.

Once a satisfactory multiple sequence alignment has been obtained, phylogenetic inference should be performed according to the state of the art. The most commonly used treeing methods are based on distance matrices, maximum-parsimony and maximum-likelihood (Ludwig *et al.*, 1998; Ludwig & Klenk, 2001). Topological differences are very likely to occur, since each treeing method relies on a different model of evolution. However, not all branches will be equally affected; those branches or groups of branches that remain unchanged regardless of the algorithm followed can be considered very stable. Other means to evaluate the statistical significance of the branching order are the application of filters and weighting masks and the use of resampling techniques (bootstrap analyses) (Ludwig *et al.*, 1998; Ludwig & Klenk, 2001). Such confidence tests are strongly recommended, especially when tree topologies are used to infer or support taxonomic conclusions.

Sequences accompanying a new species description have to be deposited in a public database and their accession numbers included in the new taxon description.

Publication

New taxonomic proposals should preferably be published in the IJSEM. However, an important part of the taxonomic work on the *Halomonadaceae* has been published elsewhere (mainly in the journals *Systematic and Applied Microbiology* and *Extremophiles*). As required by the *Bacteriological Code*, the IJSEM publishes Validation Lists containing new names and new combinations that were effectively, but not validly, published in other

Table 3. Standards for description of new taxa within the family *Halomonadaceae*

Feature
Required
Colonial morphology and pigmentation
Production of exopolysaccharides (EPS)
Cell morphology and size
Gram stain
Motility
Production of poly-β-hydroxyalkanoate
Salinity range and optimum
Specific ionic requirements
Temperature range and optimum
pH range and optimum
Oxidase and catalase activities
Facultatively anaerobic growth
Oxidation/fermentation of D-glucose
Respiration on fumarate, nitrate and nitrite
Production of acid from a range of compounds (see text)
Ability to grow on a range of single carbon (and nitrogen) sources (see text)
H_2S production from L-cysteine
Reduction of nitrate to nitrite
Gas from nitrate
Hydrolysis of gelatin, casein, starch, Tween 20, Tween 80 and DNA
o-Nitrophenyl-β-D-galactopyranosidase activity
Indole production
Methyl red test
Voges–Proskauer test
Urease activity
Phenylalanine deaminase
Lysine and ornithine decarboxylases
Sensitivity to different antimicrobial compounds (see text)
G+C content of DNA
16S rRNA gene sequence (>1400 nt, <0.5 % ambiguity) and phylogenetic analyses
DNA–DNA hybridization with closely related species (see text)
Recommended
Electron microscopy
Reduction of selenite
Hydrolysis of aesculin and tyrosine
Phosphatase activity
Haemolysis
Lecithovitellin test
Gluconate oxidation
Growth on modified selective media (MacConkey and cetrimide agar)
Miniaturized systems (biochemical and nutritional tests)
Major respiratory lipoquinone*
Fatty acid analysis*
Polar lipid patterns*
Phylogenetic analysis based on housekeeping protein-coding genes
DNA typing methods

*Required for the description of genera and higher taxa.

journals. For this purpose, authors should submit a covering letter and three copies of the published article or an electronic copy of the published paper to the Editorial Office of the IJSEM. The requirements for validation are identical to those for publication in IJSEM. They should provide evidence that the type strain of the novel species has been deposited in two recognized culture collections in two different countries (Tindall *et al.*, 2006). At the time of writing, and to the best of our knowledge, there are descriptions of three novel species whose names have not yet been validated: '*Halomonas glaciei*' (Reddy *et al.*, 2003), '*Halomonas alkaliphila*' (Romano *et al.*, 2006) and '*Halomonas alkaliantarctica*' (Poli *et al.*, 2007).

Minimal standards for the description of new taxa of the family *Halomonadaceae*

The subcommittee proposes the features described below and listed in Table 3 as minimum features to be included in the description of new taxa of the family *Halomonadaceae*. To stress their importance, some of them are listed as required and the rest as recommended.

Isolation, maintenance and preservation methods

The methods for isolation, maintenance and preservation of the strains are not usually mentioned expressly in the taxon description, but they are part of the articles that contain such descriptions. A wide range of media and strategies for isolation, maintenance and preservation of species of the family *Halomonadaceae* have already been published and many are compiled and discussed elsewhere (Garriga *et al.*, 2005; Okamoto *et al.*, 2005; Ventosa, 2005; Vreeland, 2005; Arahal & Ventosa, 2006). In many cases, citation of an appropriate reference may therefore suffice. However, detailed description of the methodology is required when novel approaches are being introduced. The habitat and the geographical location (indicated by GPS data if possible) of the place of isolation should be described in as much detail as possible. Data about the physico-chemical composition of the samples, especially the salinity, pH and temperature, should be included.

Morphological and physiological characteristics

As discussed above, the delineation of physiological boundaries and the optimal conditions for growth within those ranges is of great importance, not only for the value they have per se as phenotypic traits but also because they have to be taken into account for further testing. In the case of the salt tolerance/requirement, it is important to give the salt composition of the media tested (or a suitable reference). Specific ionic requirements have to be tested when NaCl alone cannot support growth. Adjustment of pH has to be done with an electrode that can operate at high salinities, especially for alkaline media. Colonial appearance should be described using standard microbiological criteria and the culture medium and growth

conditions should be specified. Production of exopolysaccharides (EPS) is an important distinguishing feature according to Mata *et al.* (2002). Cell morphology should be examined in wet mounts by phase-contrast microscopy of exponentially growing liquid cultures. The possibility that the cell morphology could be affected by the salt concentration in the culture medium should be considered. These observations might be supplemented by electron microscopy. Transmission electron microscopy may supply information on flagellar arrangement in motile strains. Poly-β-hydroxyalkanoate (PHA) can be detected in most species of *Halomonas* and in *Cobetia marina* (Mata *et al.*, 2002); however, to date there is no report of this feature in *Chromohalobacter*, *Carnimonas* or *Zymobacter* species.

Nutritional tests

All members of the *Halomonadaceae* are aerobes, although some *Halomonas* species have been shown to grow anaerobically with nitrate, nitrite or fumarate as an electron acceptor and with glucose on solid media in sealed jars (Mata *et al.*, 2002). For determining the range of substrates used as carbon and energy sources, the classical medium of Koser (1923) as modified by Ventosa *et al.* (1982) is recommended: 75 g NaCl l^{-1}, 2 g KCl l^{-1}, 0.2 g $MgSO_4.7H_2O$ l^{-1}, 1 g KNO_3 l^{-1}, 1 g $(NH_4)_2HPO_4$ l^{-1} and 0.5 g KH_2PO_4 l^{-1}. Substrates are added as filter-sterilized solutions to give a final concentration of 1 g l^{-1}, except for carbohydrates, which are used at 2 g l^{-1}. When the substrate is an amino acid, it should be tested as a nitrogen source, and the basal medium is therefore prepared without KNO_3 and $(NH_4)_2HPO_4$. Mata *et al.* (2002) employed 39 different sources including carbohydrates (aesculin, L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, D-melezitose, D-raffinose, L-rhamnose, ribose, D-salicin, starch, trehalose, D-xylose), organic acids (acetate, citrate, formate, fumarate, gluconate, malonate, propionate, succinate), alcohols (adonitol, ethanol, glycerol, *myo*-inositol, D-mannitol, sorbitol) and amino acids (L-alanine, L-cysteine, L-histidine, L-isoleucine, L-lysine, L-methionine, L-serine, L-valine). Considering the large number of strains used in their study, it can be assumed that this is the set of substrates for which most information is available. However, many other substrates have been employed by other authors and data are recorded for various species. Among them the following can be considered of certain relevance: *N*-acetylglucosamine, L-arabinose, L-arginine, L-aspartate, benzoate, caprylate, creatine, L-glutamate, hippurate, DL-β-hydroxybutyrate, lactate, L-leucine, malate, melibiose, L-ornithine, L-phenylalanine, L-proline, 1-propanol, pyruvate, tartrate, L-threonine and L-tryptophan.

Regarding commercial miniaturized systems, the use of Biolog GN in this group was first reported by Mata *et al.* (2002), with satisfactory results. Generally, miniaturized systems are not designed for halophilic or marine microorganisms, so it is important that the authors mention what variations have been introduced in the manufacturer's protocols (for instance, the replacement of the inoculation fluid with an appropriate saline solution) to improve the performance of the system. It is also important to remember that the principle is the detection of substrate oxidation and not turbidity or mass growth, as in other approaches. Therefore, results from different methodologies have to be presented separately both in the text and tables. Other miniaturized kits that have been tested on some members of the *Halomonadaceae* are API 20NE and API 50CH (bioMérieux). Again, the considerations that have been pointed out above for the use of Biolog plates apply to all miniaturized kits.

Biochemical tests

A valuable starting point, containing many methodological details or references, is the list of biochemical tests conducted in the study of Mata *et al.* (2002). These included catalase, oxidase, acid production from carbohydrates and alcohols (adonitol, L-arabinose, D-fructose, D-galactose, D-glucose, *myo*-inositol, lactose, maltose, D-mannitol, D-mannose, D-melezitose, L-rhamnose, sucrose, D-salicin, D-sorbitol, sorbose, trehalose), oxidation/fermentation of glucose, respiration on fumarate, nitrate and nitrite, H_2S production from L-cysteine, reduction of nitrate to nitrite, reduction of selenite, hydrolysis of gelatin, casein, starch, Tween 20, Tween 80, aesculin, tyrosine and DNA, phosphatase production, haemolysis, lecithovitellin test, growth on modified selective media (MacConkey and cetrimide agar), gluconate oxidation, *o*-nitrophenyl-β-D-galactopyranosidase activity, indole production, methyl red and Voges–Proskauer tests, urease activity, phenylalanine deaminase and lysine and ornithine decarboxylases.

Sensitivity to antimicrobial agents

The following antimicrobial compounds are recommended for testing: amoxicillin, ampicillin, carbenicillin, cefoxitin, cefotaxime, chloramphenicol, erythromycin, kanamycin, nalidixic acid, nitrofurantoin, polymyxin B, rifampicin, streptomycin, sulphamide, tobramycin and trimetoprim-sulphametoxazol. All of them were included in the study of Mata *et al.* (2002) and in other previous and later studies. However, for some of them, the concentration at which they are employed varies across studies. Of course, this might affect the results and thus their comparability. To avoid this, we recommend following the concentrations given in the article by Mata *et al.* (2002), since it is the most extensive phenotypic study within the family *Halomonadaceae*.

Chemotaxonomic characterization

After the chemotaxonomic study of members of the family *Halomonadaceae* by Franzmann & Tindall (1990), other authors have included these methodologies in the descriptions of new taxa within this group of organisms. For about

60 % of the current species, the major respiratory lipoquinone has been determined and found to be ubiquinone 9 (Q9), except that *Halomonas campaniensis* possesses only Q8 (Romano *et al.*, 2005). Fatty acid analysis has been reported more often (for about 75 % of current species), but the exact composition is dependent on the medium and incubation conditions. Therefore, standardization is again essential. Finally, polar lipid patterns are available for almost half of the current species of the family *Halomonadaceae*. For the purpose of classification at the genus level, analysis of fatty acids and polar lipids is highly recommended.

Nucleic acid characterization

Information on the G+C content of the DNA is available for all members of the *Halomonadaceae* and, although it has little differential importance, it is recommended to include this standard feature in new species descriptions with indication of the methodology employed [HPLC, thermal denaturation (T_m), buoyant density (Bd)].

As discussed above, the 16S rRNA gene is the most commonly used molecular marker, and it has had a profound impact in the current taxonomic classification and delineation of the genera of the family *Halomonadaceae*. The use of alternative phylogenetic markers is encouraged, since they may provide novel insights into the evolutionary relationships of the definable taxa. However, it has to be kept in mind that a phylogeny based on an alternative gene is not necessarily more reliable than the one that is inferred from the 16S rRNA gene: indeed, many attempts have proven just the opposite (Ludwig & Schleifer, 1999; Ludwig & Klenk, 2001). One phylogenetic marker with an information content greater than the 16S rRNA gene is the gene encoding the large-subunit ribosomal RNA (23S), which was further analysed by Arahal *et al.* (2002b) for 18 *Halomonas* and *Chromohalobacter* species. The use of protein-coding genes as phylogenetic markers among species of the family *Halomonadaceae* has had little impact so far (Okamoto *et al.*, 2004).

Determination of inter- and intraspecies relatedness by rapid DNA typing methods (AFLP, RAPD, rep-PCR, PFGE, ribotyping of ribosomal ribonucleic operons, ARDRA) has not been widely applied to the *Halomonadaceae* except for the studies by Mellado *et al.* (1998) and Llamas *et al.* (2002), who applied PFGE, Garriga *et al.* (1998), who used RAPD, and Heyrman *et al.* (2002), who reported rep-PCR genomic fingerprinting.

Finally, DNA–DNA hybridization studies remain essential when novel species are described, in order (i) to test the genomic coherence between the strains that form the species and (ii) to determine that they are genomically different from closely related species. Again, the methodology followed should be clearly stated. A recent review on DNA–DNA reassociation methods is that of Rosselló-Mora (2006).

Final remarks

It has to be stated that the distinction between required and recommended minimal standards is by no means intended to limit the characterization of new isolates to the characteristics that are indicated as required. Moreover, current or yet to be described species may show new and interesting features not listed in Table 3, and such features may prove to be of taxonomic importance.

Acknowledgements

We thank A. Gambacorta and R. Montalvo-Rodríguez for their critical reading of the manuscript. D. R. A. has a contract with the University of Valencia under the Ramón y Cajal program (Ministerio de Educación y Ciencia).

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