1   **Updating the taxonomic toolbox: classification of *Alteromonas* spp.**
2   **using Multilocus Phylogenetic Analysis and MALDI-TOF Mass**
3   **Spectrometry**

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15
16   **Abstract**

17   Bacteria of the genus *Alteromonas* are Gram-negative, strictly aerobic, motile,
18   heterotrophic marine bacteria, known for their versatile metabolic activities.
19   Identification and classification of novel species belonging to the genus *Alteromonas*
20   generally involves DNA-DNA hybridization (DDH) as distinct species often fail to be

resolved at the 97% threshold value of the 16S rRNA gene sequence similarity. In this study, the applicability of Multilocus Phylogenetic Analysis (MLPA) and Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) for the differentiation of *Alteromonas* species has been evaluated. Phylogenetic analysis incorporating five house-keeping genes (*dnaK*, *sucC*, *rpoB*, *gyrB*, and *rpoD*) revealed a threshold value of 98.9% that could be considered as the species cut-off value for the delineation of *Alteromonas* spp. MALDI-TOF MS data analysis reconfirmed the *Alteromonas* species clustering. MLPA and MALDI-TOF MS both generated data that were comparable to that of the 16S rRNA gene sequence analysis and may be considered as useful complementary techniques for the description of new *Alteromonas* species.

Keywords: *Alteromonas*, MLPA, MLSA, MALDI-TOF MS, Phylogeny, Taxonomy

Introduction

The genus *Alteromonas* (family *Alteromonadaceae*, order *Alteromonadales*, class *Gammaproteobacteria*) was first described by Baumann *et al.* for Gram-negative, strictly aerobic, motile, heterotrophic marine bacteria (Baumann *et al.* 1972). Bacteria of the genus *Alteromonas* have been studied widely due to their versatile metabolic activities, which include the production of enzymes, secondary metabolites and polysaccharides (Mikhailov *et al.* 2006). These bacteria are also known for their ability to degrade aromatic hydrocarbons (Cui *et al.* 2008). It has been suggested that this group of marine bacteria plays an important role in the global carbon cycle by

contributing to the dissolution of particulate organic matter (Ivars-Martinez et al. 2008; McCarren et al. 2010).

The taxonomy of this genus underwent a number of revisions and reclassifications, leaving *Alteromonas macleodii* as the single representative species of the genus for about a decade (Gauthier et al. 1995; Ivanova et al. 2004). Recently, the number of validly named species within the genus *Alteromonas* has increased dramatically and the genus now comprises nine species, namely, *A. macleodii* (Baumann et al. 1972), *Alteromonas marina* (Yoon et al. 2003), *Alteromonas stellipolaris* (Van Trappen et al. 2004), *Alteromonas litorea* (Yoon et al. 2004), *Alteromonas hispanica* (Martinez-Checa et al. 2005), *Alteromonas addita* (Ivanova et al. 2005), *Alteromonas simiduii* (Chiu et al. 2007), *Alteromonas tagae* (Chiu et al. 2007) and *Alteromonas genovensis* (Vandecandelaere et al. 2008) (List of Prokaryotic names with Standing in Nomenclature, <http://www.bacterio.cict.fr/a/alteromonas.html>).

Currently, 16S rRNA gene sequence analysis and DNA-DNA hybridization (DDH) are the primary tools for delineation of novel bacterial species. For a strain to be accepted as representing a new species it must typically share no more than 97% of its 16S rRNA gene sequence and 70% or less of its genome with any previously validly named species (Wayne et al. 1987; Stackebrandt and Goebel 1994). However, with the number of new species being identified, together with the increasing amount of sequencing data available, it is becoming apparent that the threshold value of 97% rRNA gene sequence similarity may not be as accurate as previously thought (Fox et al. 1992; Stackebrandt and Goebel 1994; Sutcliffe et al. 2012). As a result, a few alternative taxonomic tools such as Multilocus Phylogenetic Analysis (MLPA) and Matrix-Assisted Laser Desorption/ Ionization Time-of-flight Mass Spectrometry

(MALDI-TOF MS) have been suggested to be introduced into bacterial systematics (Stackebrandt et al. 2002; Figueras et al. 2011). MLPA, which is also often termed Multilocus Sequence Analysis (MLSA), has been shown to deliver a greater taxonomic resolution for classification of closely related bacteria at the species level when compared to that obtained using the 16S rRNA gene sequence analysis (Gevers et al. 2005; Figueras et al. 2011). MALDI-TOF MS has also been shown to be a rapid and reliable technique for the identification of bacteria at the genus, species and subspecies levels (Barbuddhe et al. 2008; Dieckmann et al. 2008; Ayyadurai et al. 2010; Murray 2010).

In light of the recent developments (e.g., Schleifer 2009; Sutcliffe et al. 2012), the aim of this study was to select the most suitable set of housekeeping genes for *Alteromonas* spp., design and test *Alteromonas* spp.-specific primers and evaluate the possible application of MLPA and MALDI-TOF MS techniques for the delineation of the *Alteromonas* species. Among majority of the species of *Alteromonas*, e.g., *A. macleodii*, *A. marina*, *A. stellipolaris*, *A. litorea*, *A. hispanica*, *A. addita*, and *A. genovensis*, the 16S rRNA gene sequence similarities are greater than 97%, and therefore the introduction of MLPA and MALDI-TOF MS may be a suitable alternative to avoid time consuming and laborious DDH experiments for discrimination and delineation of *Alteromonas* species.

Materials and methods

Bacterial strains and growth conditions

Type strains of all nine validly named species of *Alteromonas* were used in this study (Table 1). The type strains of *A. macleodii* LMG 2843^T, *A. stellipolaris* LMG 21861^T, *A. marina* LMG 22057^T, *A. litorea* LMG 23846^T, *A. hispanica* LMG 22958^T, *A. addita* LMG 22532^T and *A. genovensis* LMG 24078^T were obtained from the BCCM/-LMG culture collection; *A. simiduii* BCRC 17572^T was obtained from the BCRC culture collection and *A. tagae* JCM 13895^T was obtained from the JCM culture collection. In addition to *Alteromonas* strains, six type strains representing the closely related taxa were used for comparison studies. *Shewanella colwelliana* ATCC 39565^T was obtained from the ATCC culture collection; *Pseudoalteromonas translucida* KMM 520^T and *Glaciecola mesophila* KMM 241^T were obtained from the KMM culture collection; *Marinomonas communis* LMG 2864^T and *Aestuariibacter aggregatus* LMG 25283^T were obtained from the BCCM/-LMG culture collection; and *Salinimonas chungwhensis* KCTC 12239^T was obtained from the KCTC culture collection. All bacterial strains were grown on marine agar/broth 2216 (BD, USA), at 25°C. For long-term storage, bacterial strains were maintained in marine broth 2216 supplemented with 20% glycerol and stored at -80°C.

DNA extraction

Genomic DNA was extracted from 1 mL of an overnight culture by using the Wizard® Genomic DNA Purification Kit (Promega, USA) according to manufacturer's instructions. The quality of the DNA was checked on 1% agarose gels and subsequently stored at -20°C.

Genes and primers for MLPA

MLPA was initially carried out using the genes and primers that have been previously reported in the study of *Alteromonas* (Ivars-Martínez et al. 2008) and some other genera of *Proteobacteria* (Yamamoto and Harayama 1995, 1998; Thompson et al. 2004; Martens et al. 2008; Menna et al. 2009). The usefulness of the genes (*dnaK*, *rpoB*, *sucC*, *glyA*, *pmg*, *gyrB*, *metG*, *recA*, *atpD*, *gap* and *rpoD*) and the specificity of the primers were tested by polymerase chain reaction (PCR) amplification on the nine *Alteromonas* strains. Genes and primers that gave a single band for all of the *Alteromonas* strains were selected directly. The primer pairs which gave single amplification products for at least five *Alteromonas* strains were sent for sequencing at the Australian Genome Research Facility (AGRF). The resulting sequencing data were then used to redesign the primers which were subsequently reassessed. In total five genes (*dnaK*, *sucC*, *rpoD*, *rpoB*, *gyrB*) were selected for inclusion in the final MLPA experiments. The primer pairs used to amplify/sequence each gene are summarized in Table 2.

PCR amplification and sequencing

PCR amplifications were performed using a MyCycler™ Thermal Cycler (Bio-Rad, USA). Each reaction was performed in a final volume of 50 µL, containing 25 µL MangoMix™ (Bioline, USA), 0.2 µM of each of the primers and 4 µL of genomic DNA. PCR amplifications of the *gyrB* and *rpoD* genes were carried out as previously described (Yamamoto and Harayama 1995, 1998) while the remaining genes (*dnaK*, *sucC* and *rpoB*) were subjected to an initial denaturation step at 94°C for 4 min, followed by 35 cycles of repetitive DNA denaturation (94°C for 2 min), primer hybridization (T_a for 1 min) and primer extension (72°C for 2 min), and a final extension step at 72°C for 10 min. The annealing temperature (T_a) for each primer pair

is listed in Table 2. PCR products were analysed by electrophoresis on 1% agarose gels, stained with 0.5 $\mu\text{g mL}^{-1}$ ethidium bromide (Promega, USA) and bands were visualized under UV light. The bands corresponding to the amplified products were excised from the gels and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's instructions. Purified products were then sent to AGRF for purified DNA (PD) sequencing service using the AB 3730xl platform. Nucleotide sequences generated from this study have been submitted to the National Center for Biotechnology Information (NCBI) GenBank with the accession numbers as listed in Table 1.

Phylogenetic analysis

Several phylogenetic trees were constructed from different datasets of the *Alteromonas* strain sequences. These include the 16S rRNA gene sequences (retrieved from GenBank), each of the five house-keeping genes sequences, concatenated sequences of the five house-keeping genes and concatenated peptide sequences translated from the five house-keeping genes. Sequences generated from the MLPA studies were edited using BioEdit version 7.0.9.0 software (Hall 1999) and the 'supergene' was generated by concatenating the sequences of the five genes (*dnak*, *sucC*, *rpoD*, *rpoB*, *gyrB*) for a given strain. Peptide sequences were translated using ExPASy (SIB Swiss Institute of Bioinformatics) (Gasteiger et al. 2003). The resulting nucleotide and peptide sequences were aligned using the CLUSTAL W program (Thompson et al. 1994). Evolutionary phylogenetic trees were constructed using the neighbour-joining (NJ) (Saitou and Nei 1987), maximum-parsimony (MP) and maximum-likelihood (ML) algorithms. Genetic distances were calculated using Kimura's two-parameter model (Kimura 1980) for nucleotide sequences and the P-

distance model for amino acid sequences, using MEGA 5 software (Tamura et al. 2011).

A matrix of gene similarity between each species of *Alteromonas* was also constructed, based on the distance matrices calculated as part of the generation of neighbour-joining trees. For each individual gene included in the MLPA analysis, the concatenated sequences of all five genes, and the 16S rRNA gene, the most similar sequences to each of the nine species of *Alteromonas* were identified.

MALDI-TOF MS analysis

Bacterial colonies were grown overnight, transferred with 5 µL loops into 1.5 mL extraction tubes and subjected to protein extraction with ethanol and formic acid according to the Bruker Daltonics protocol. One µL of the supernatant was transferred onto the MALDI target plate and air dried at room temperature. Samples were then overlaid with 2 µL of matrix solution (saturated solution of α -cyano-4-hydroxycinnamic acid (HCCA) in a mixture of 47.5% ultra-pure water, 2.5% trifluoroacetic acid, and 50% acetonitrile) and again air dried at room temperature until crystal formation was observed visually. Samples were then subjected to analysis using a Microflex MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Leipzig, Germany) equipped with a 60 Hz nitrogen laser. Spectra were recorded in the positive linear mode for the mass range of 2000 – 20000 Da at maximum laser frequency. Raw spectra were analysed using the MALDI Biotyper 3.0 software package (Bruker Daltonik GmbH, Bremen, Germany) with default settings. Measurements were performed automatically without any user intervention.

Results and discussion

House-keeping genes selection and analyses

In this work, five house-keeping genes, i.e. *dnaK* (chaperone protein DnaK), *sucC* (succinyl-CoA synthetase), *rpoB* (RNA polymerase, β subunit), *gyrB* (DNA gyrase subunit B) and *rpoD* (RNA polymerase, sigma 70 factor) were selected and employed in the MLPA study. Initially, a set of eleven house-keeping genes (*dnaK*, *rpoB*, *sucC*, *glyA*, *pmg*, *gyrB*, *metG*, *recA*, *atpD*, *gap* and *rpoD*) and their associated primers that were used in the previous MLSA study as described in Materials and Methods were tested for their applicability to the type strains of the nine validly named *Alteromonas*. Among these, two genes, *gyrB* and *rpoD*, with the previously reported primer pairs (Yamamoto and Harayama 1995, 1998) were successfully applied to all *Alteromonas* species, and thus were adopted for this study. A new set of PCR and sequencing primers were designed for *dnaK*, *sucC* and *rpoB* based on the conserved regions of sequences from at least 5 *Alteromonas* spp. since previously reported primer pairs were unable to generate a single band across all *Alteromonas* species. The newly re-designed primers were tested for each of the nine *Alteromonas* species and were found to successfully amplify their corresponding genes in all nine species. The developed primer sets were also tested on the type strains representing a number of genera closely related to *Alteromonas*, namely, *Salinimonas*, *Aestuariibacter*, *Glaciecola*, *Pseudoalteromonas*, *Marinomonas* and *Shewanella* (Supplementary Fig. S1) and they appeared to be specific to the *Alteromonas* species.

Sequence divergence based on the nucleotide sequences of the five house-keeping genes was found to be less conservative when compared to the 16S rRNA

sequences (Fig. 1), with the overall mean distances of 0.2153 for *rpoD*, 0.2150 for *gyrB*, 0.1641 for *rpoB*, 0.1168 for *sucC*, 0.1062 for *dnaK* and 0.0166 for 16S rRNA. The performances of the individual house-keeping genes were also analysed using phylogenetic tree topologies constructed from the sequences of each of the house-keeping genes (Supplementary Fig. S2). From each of the individual trees, it appeared that the five house-keeping genes indeed could provide a higher evolutionary rate than the 16S rRNA gene, as indicated by the evolutionary distance scale bars. Of note, three genes *rpoD*, *gyrB*, and *rpoB* demonstrated higher confidence bootstrap values, resolving power and topological stability when compared to the trees reconstructed from the *dnaK* and *sucC* gene sequences. These observations are in agreement with earlier reports (Konstantinidis et al. 2006; Zeigler 2003) and suggested that *rpoD*, *gyrB* and *rpoB* have the best capability to be practically used as the marker genes for effective identification and classification of *Alteromonas* species.

Comparative MLPA

A supergene that contained 3356 nucleotides from partial sequences of the five house-keeping genes was reconstructed for the MLPA study. A concatenated phylogenetic tree was constructed, based on the resulting supergene, with bootstrap analysis of 1000 replications using the three tree-making algorithms (the NJ tree is presented in Fig. 2b). Phylogenetic trees based on the MP and ML also showed a robust phylogeny with identical topologies and well supported branching that agreed with the NJ tree.

Comparison of the MLPA phylogenetic tree and 16S rRNA phylogenetic tree (Fig. 2) revealed that both trees had similar topologies; however the phylogenetic tree

constructed from the five concatenated partial sequences was better supported with higher bootstrap values. Also, the phylogenetic tree from the MLPA demonstrated a higher resolving power as indicated by the scale bars (Fig. 2). This finding confirmed previous observations that multiple genes could compensate the difference between each gene in order to improve the phylogenetic reconstruction (Cantarel et al. 2006), by increasing the phylogenetic signal and minimising the effects of horizontal gene transfer and recombination of single loci (Gevers et al. 2005; Schleifer 2009). Another tree based on the concatenated amino acid sequences (Supplementary Fig. S3) showed an identical topology to the concatenated nucleotide tree, however with a lower resolution than the nucleotide tree. This finding is also in agreement with the results reported by Simmons et al. (2002) and thus suggested that nucleotide sequences should be used for MLPA in order to obtain a higher resolution and sequence diversity.

The sequence similarities of *dnaK*, *sucC*, *rpoB*, *gyrB*, and *rpoD* among *Alteromonas* species were found to be in the range of 77.9% - 98.9% while the 16S rRNA gene sequence similarities were in the range of 95.9% - 99.8% (Table 3). *A. stellipolaris* and *A. addita* showed the highest degree of sequence similarities, which was also reflected in the trees based on each individual gene (Supplementary Fig. S2). Since six of the nine validly named species of the genus *Alteromonas* are described based on single strains, assessment of the MLPA intraspecies variations was not possible at this time. However, with the growing number of newly isolated bacteria, this issue may be addressed. The evolutionary rate of the concatenated sequences was higher than that of the 16S rRNA sequences (Fig. 1), with the overall mean distances calculated to be 0.1693 (concatenated sequences) and 0.0166 (16S rRNA sequences).

The lower sequence similarity and the higher evolutionary rate determined from the MLPA study highlighted the increased resolving power of MLPA.

MLPA as possible alternative to DDH

DDH, the gold standard technique that was introduced several decades ago into bacterial systematics (Brenner et al. 1969), has been broadly discussed and studied for its consistency with the results obtained from 16S rRNA, MLPA and complete genome sequences analysis (Konstantinidis and Tiedje 2007; Richter and Rossello-Mora 2009; Martens et al. 2008; Rong and Huang 2012). 16S rRNA gene sequence analysis is easy to perform; however, due to the increasing number of 16S rRNA gene sequencing data, a significant number of distinct species have been documented to have their 16S rRNA gene sequence similarity >97%. This phenomenon is frequently reported for different bacterial taxa including *Pseudoalteromonas*, *Acinetobacter* and *Pseudomonas*, where the 16S rRNA gene sequence similarity within these genera have been found to be in the range of 90 – 99.9%, 93.5 – 99.1%, and 92.9 – 100% respectively. Complete genome sequences analysis is an alternative to determine overall taxonomic relatedness, however, it is still not routinely practically available. Hence, MLPA may serve as the affordable alternative technique to DDH when 16S rRNA gene sequence similarity fails to give the satisfactory threshold value. Earlier studies on the genera *Xanthomonas* (Young et al. 2008), *Ensifer* (Martens et al. 2008), *Bradyrhizobium* (Rivas et al. 2009), *Gluconacetobacter* (Cleenwerck et al. 2010) and *Streptomyces* (Rong and Huang 2012) have shown that MLPA is a suitable alternative to DDH. In the latest study, the proposed MLSA cut-off value for *Streptomyces* spp. was reported to be 99.3% (evolutionary distance of 0.007) (Rong and Huang 2012). The results of the present study indicated that MLPA sequence similarities of the five gene-set for *Alteromonas*

spp. was up to 98.9%, with the highest percentage was found for two species, *A. addita* and *A. stellipolaris* (the reported DDH value for these species is 49%; Ivanova et al. 2005). Thus, by considering the DDH value together with the MLPA percentage sequence similarity, 98.9% (evolutionary distance of 0.011) can be suggested to be the cut-off value for the differentiation of *Alteromonas* species.

MALDI-TOF MS analysis

In order to enhance the classification of *Alteromonas* spp., MALDI-TOF MS was used as a complementary taxonomic technique for species differentiation within the genus *Alteromonas*. This technique has been broadly employed in clinical microbiology where fast, accurate and rapid identification of potential pathogenic bacteria is crucially important (Murray 2010). The potential of this technique in the taxonomic identification and classification of environmental bacteria has, however, not been extensively studied. In this study, a main spectra library (MSP) dendrogram was constructed by the MALDI Biotyper, based on the species-specific profiles generated by the MALDI-TOF MS (Fig. 3). The topology of the dendrogram correlated well with both the 16S rRNA and the MLPA phylogenetic trees. To confirm that the natural variability in each of the spectra did not affect the discrimination between the *Alteromonas* species, a Principle Component Analysis (PCA) plot with spectra derived from four technical replicates for each species was generated (Fig. 4). From the cluster analysis, it could be seen that each of the *Alteromonas* species formed a unique protein profile, resulting in the close clustering of the replicates, allowing discrimination of the *Alteromonas* species. The applicability of MALDI-TOF MS for the differentiation of genus *Alteromonas* from six closely related taxa was also tested (supplementary Fig. S4). The result clearly demonstrated that each of the six closely related strains formed separate clusters to

Alteromonas. MALDI-TOF MS approach for the detection of protein mass fingerprinting is independent to the genomic approach, however generating results which are comparable to the 16S rRNA gene analysis and MLPA. Thus, the results from this study highlight the applicability of MALDI-TOF MS as a quick and affordable tool aiding in the classification and identification of studied bacteria.

The results obtained in this study indicated that MLPA and MALDI-TOF MS are useful and viable techniques for addition to the suite of tools to be used routinely in taxonomic studies. Five house-keeping genes, *dnaK*, *sucC*, *rpoB*, *gyrB*, and *rpoD*, could be used in MLPA studies for the identification and classification of *Alteromonas* spp., where strains showing sequences with the percentage sequence similarity $\leq 98.9\%$ can be considered as distinct species. Overall, our data demonstrated that MLPA provided a reliable classification and grouping of *Alteromonas* spp., and in some cases may eliminate the necessity of performing time-consuming and labour-intensive DDH experiments. MALDI-TOF MS can also be used as a supporting/complementary technique for the classification of new strains. The addition of new techniques and improvement of existing procedures in prokaryotic systematics is of considerable importance to ensure that the field does not stagnate and is continually improving and progressing.

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Supplementary data

Supplementary data associated with this article can be found in the online version.

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Table 1 *Alteromonas* strains and their corresponding GenBank accession number reported in this study.

Species Name	GenBank Accession Numbers					Isolation Source
	<i>dnaK</i>	<i>sucC</i>	<i>rpoD</i>	<i>rpoB</i>	<i>gyrB</i>	
<i>Alteromonas macleodii</i> LMG 2843 ^T (= Baumann 107 ^T , ATCC 27126 ^T , DSM 6062 ^T)	JQ407010	JQ411701	JQ411711	JQ411691	JQ406999	Sea water, Hawaii
<i>A. marina</i> SW-47 ^T (= LMG 22057 ^T , JCM 11804 ^T , KCCM 41638 ^T)	JQ407011	JQ411702	JQ411712	JQ411692	JQ407000	Sea water, East Sea, Korea
<i>A. stellipolaris</i> LMG 21861 ^T (= ANT 69a ^T , R-15466 ^T , R-9875 ^T , DSM 15691 ^T)	JQ407013	JQ411704	JQ411714	JQ411694	JQ407002	Sea water, Antarctic
<i>A. litorea</i> TF-22 ^T (= JCM 12188 ^T , LMG 23846 ^T , KCCM 41775 ^T)	JQ407009	JQ411700	JQ411710	JQ411690	JQ406998	Intertidal sediment, Yellow Sea, Korea
<i>A. hispanica</i> F-32 ^T (= CECT 7067 ^T , LMG 22958 ^T)	JQ407008	JQ411699	JQ411709	JQ411689	JQ406997	Hypersaline water, Fuente de Piedra, Spain
<i>A. addita</i> R10SW13 ^T (= KCTC 12195 ^T , LMG 22532 ^T , CIP 108794 ^T)	JQ407006	JQ411697	JQ411707	JQ411687	JQ406995	Sea water, Chazhma Bay, Russia
<i>A. simiduii</i> BCRC 17572 ^T (= AS1 ^T , JCM 13896 ^T)	JQ407012	JQ411703	JQ411713	JQ411693	JQ407001	Sea water, Er-Jen River estuary, Taiwan
<i>A. tagae</i> JCM 13895 ^T (= AT1 ^T , BCRC 17571 ^T)	JQ407014	JQ411705	JQ411715	JQ411695	JQ407003	Sea water, Er-Jen River estuary, Taiwan
<i>A. genovensis</i> LMG 24078 ^T (= R-28792 ^T , CCUG 55340 ^T)	JQ407007	JQ411698	JQ411708	JQ411688	JQ406996	Sea water electroactive biofilm, port of Genoa, Italy

Table 2 Genes and the corresponding PCR/ sequencing primers used in the MLSA study.

Locus	Product	Primers	Sequence (5' → 3')	<i>Ta</i>	Reference
<i>dnaK</i>	Chaperone protein DnaK	JdnaK-F JdnaK-R	GCGTTTTTCGCTTCRATKTCWGC ATTCCAACGAAGAAGTCGCAAAC	61.0	This study
<i>sucC</i>	Succinyl-CoA synthetase	JsucC-F JsucC-R	GCACCGTTACCATACAACCTAC TTGGTGACKTTYCAGACTGAC	54.3	This study
<i>rpoD</i>	RNA polymerase sigma factor	rpoD 70F rpoD 70Fs rpoD 70R rpoD 70Rs	ACGACTGACCCGGTACGCATGTAYATGMGNGARATGGGNACNGT ACGACTGACCCGGTACGCATGTA ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTCYTTYTT ATAGAAATAACCAGACGTAAGTT	58.0	Yamamoto and Harayama, 1998
<i>rpoB</i>	RNA polymerase β subunit	JrpoB-F JrpoB-R	AAAGTGCTTTATAAYGCACG GRTTYTCWGCCATTTTCRCC	51.0	This study
<i>gyrB</i>	DNA gyrase subunit B	UP-1 UP-2r UP-1S UP-2Sr	GAAGTCATCATGACCGTTCTGCAYGCNNGGNGNAARTTYGA AGCAGGGTACGGATGTGCGAGCCRTCACRTCNGCRTCNGTCAT GAAGTCATCATGACCGTTCTGCA AGCAGGGTACGGATGTGCGAGCC	60.0	Yamamoto and Harayama, 1995

Table 3 Interspecies similarity of the 16S rRNA gene sequences and the concatenated sequences of *dnaK*, *sucC*, *rpoB*, *gyrB*, and *rpoD* genes.

		Percentage similarity of 16S rRNA/concatenated house-keeping genes (%)								
		1	2	3	4	5	6	7	8	9
1	<i>A. macleodii</i> LMG 2843 ^T	100/100								
2	<i>A. marina</i> SW-47 ^T	98.7/87.4	100/100							
3	<i>A. stellipolaris</i> LMG 21861 ^T	98.1/80.5	98.1/79.7	100/100						
4	<i>A. litorea</i> TF-22 ^T	98.0/86.0	98.7/87.6	97.8/78.1	100/100					
5	<i>A. hispanica</i> F-32 ^T	97.3/81.4	97.7/80.1	97.8/77.9	98.4/79.7	100/100				
6	<i>A. addita</i> R10SW13 ^T	98.1/80.9	98.2/79.7	99.8/98.9	97.8/78.2	98.0/77.9	100/100			
7	<i>A. simiduii</i> BCRC 17572 ^T	97.5/97.9	97.7/87.0	96.7/80.5	96.8/86.1	96.1/81.2	96.7/80.9	100/100		
8	<i>A. tagae</i> JCM 13895 ^T	97.3/87.0	98.3/85.3	97.3/80.4	97.9/84.0	97.0/80.8	97.4/80.7	96.2/87.1	100/100	
9	<i>A. genovensis</i> LMG 24078 ^T	97.2/80.5	97.6/79.8	97.3/79.4	98.4/79.6	99.1/86.6	97.5/79.4	95.9/80.5	96.9/79.6	100/100

Figure captions

Fig. 1 Genetic similarity matrix for *Alteromonas* type strains. For each species, the corresponding species with the most similar DNA sequence for the given gene or group of genes is presented.

Fig. 2 Comparative phylogenetic analysis of *Alteromonas* species based on (a) 16S rRNA gene sequences obtained from NCBI GenBank and (b) Multi-locus sequence analysis of concatenated sequences of *dnaK*, *sucC*, *rpoB*, *gyrB*, and *rpoD* genes. Numbers at branching points are percentage bootstrap values based on 1000 replications, with only values > 50% shown. Scale bars represent 0.002/0.02 substitutions per nucleotide position.

Fig. 3 Main Spectra Library (MSP) dendrogram of MALDI-TOF mass spectral profiles from nine *Alteromonas* species generated by the MALDI Biotyper 3.0 software. Distance is displayed in relative units.

Fig. 4 Three-dimensional Principle Component Analysis (PCA) plot of the nine *Alteromonas* species; *A. stellipolaris* (1), *A. addita* (2), *A. hispanica* (3), *A. genovensis* (4), *A. litorea* (5), *A. tagae* (6), *A. macleodii* (7), *A. simiduii* (8), *A. marina* (9). Each dot represents a single technical replicate.