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Automated Systems for Identification of Heterotrophic Marine Bacteria on the Basis of Their Fatty Acid Composition

S. BERTONE,[1]* M. GIACOMINI,[2] C. RUGGIERO,[2] C. PICCAROLO,[3] AND L. CALEGARI[1]

Institute of Microbiology[1] and DIST,[2] University of Genoa, and ICMM, Consiglio Nazionale delle Recerche,[3] Genoa, Italy

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The fatty acid methyl ester composition of a total of 71 marine strains representing the genera *Alteromonas*, *Deleya*, *Oceanospirillum*, and *Vibrio* was determined by gas-liquid chromatographic analysis. Over 70 different fatty acids were found. The predominant fatty acids were 16:0, 16:1 *cis* 9, summed-in-feature (SIF) 4 (15:0 iso 2OH and/or 16:1 *trans* 9) and SIF 7 (18:1 *cis* 11, 18:1 *trans* 9, and/or 18:1 *trans* 6) for all the strains considered, but minor quantitative variations could be used to distinguish the different genera. In addition to a conventional statistical processing method to analyze the data and draw comparison between species and genera, an approach involving neutral network-based elaboration is applied. The statistical analysis and dendrogram representation gave a comparison of the species considered, while the neural network computation provided a more accurate assignment of species to their genera. Moreover, by using neural networks, it was possible to conclude that only 22 fatty acids were important for the identification of the marine genera considered. A database of *Alteromonas*, *Deleya*, *Oceanospirillum*, and *Vibrio* fatty acid methyl ester profiles was generated and is now routinely used to identify fresh marine isolates.

In the last 10 years, interest in marine bacteria has grown considerably. However, the identification of fresh marine isolates, a preliminary step in microbiological research, is a continuing problem for marine microbiologists. The identification of marine bacteria is commonly based on a wide range of biochemical and physiological tests. The major difficulties found with this traditional approach are the need for an easily cultivable strain and the time required for the preparation of cultures. The availability of miniaturized multitest systems that allow the simultaneous determination of numerous phenotypic characters has overcome the previous difficulties. However, the identification of environmental bacteria remains problematic because there is often a lack of agreement between the biochemical tests reported in the literature for the type strain and the results obtained in the laboratory with fresh isolates (2, 4, 9). The reason stems from the similar physiological and biochemical characters shared by aerobic marine bacteria (2) and from the differences in the biochemical features of fresh and stored isolates. In fact, not only may plasmid DNA be lost during storage, but also fresh isolates may have greater enzymatic activity than their counterparts which have been stored on laboratory media (5).

More sensitive and reliable techniques such as genomic fingerprinting hybridization analysis based on specific cleavage of DNA by restriction endonucleases and all PCR-based variants are generally difficult to perform, require many expensive reagents, and do not reduce the problem of long culture preparation times; therefore, they are not of practical use in routine analyses. Recent advances in the biochemistry of microorganisms revealed that analysis of cell components, such as proteins and fatty acids, can be effectively applied to bacterial identification, providing the basis for chemotaxonomy (13, 22).

The use of fatty acid analysis by gas chromatography for the identification of bacteria since its initial introduction of Abel et al. (1) has given results in agreement with DNA-DNA hybridization data (16, 29, 35) and is currently widely used for the identification of both clinical and environmental isolates (8, 14, 15, 27, 32, 33). This technique has practical advantages, such as the simplicity of the analytical method, the speed of analysis, and the low cost of materials. Moreover, the whole-cell fatty acid content is a direct and stable expression of the cellular genome. In fact, the cellular fatty acid pattern is a phenotypic character that is not affected by mutations or acquisition or loss of plasmids.

A method for the rapid and reliable identification of marine bacteria has yet to be developed. In the present study, we describe a method for identification at the genus level of marine bacteria belonging to the genera *Alteromonas*, *Deleya*, *Oceanospirillum*, and *Vibrio* by using gas-chromatographic profiles of fatty acid methyl esters (FAMEs). Two approaches to the elaboration of FAME data were evaluated. The first is a classical numerical analysis with a similarity coefficient chosen according to its suitability for fatty acid correlation. The second is an elaboration of fatty acid patterns based on an artificial neural network. Neural computing is the study of networks of adaptable nodes which, through a process of learning from task examples, store experimental knowledge and make it available for use (3). These networks seek to reproduce the style of computing of the brain and can be regarded as computer models in which the nodes correspond to neurons and the connections between nodes correspond to synapses. Neural networks have the ability to identify unknown patterns after a training phase during which known patterns are shown to the net together with the expected identification.

The aim of this study was to evaluate whether fatty acid analysis, according to a suitable computerized procedure, can be used as a rapid and reliable identification key, at least at the genus level, for marine bacteria.

MATERIALS AND METHODS

Strains and culture conditions. A total of 71 strains belonging to the genera *Alteromonas*, *Deleya*, *Oceanospirillum*, and *Vibrio* were used (Table 1). Since the cellular fatty acid composition varies significantly with culture age, growth tem-

* Corresponding author. Mailing address: Institute of Microbiology, University of Genoa, Largo Rosanna Benzi 10, 16132 Genoa, Italy. Phone: 39-10-3537649. Fax: 39-10-504837.

TABLE 1. List of the marine strains used for the study

Species	Strain source[a]
A. aurantia	NCIMB 2033
A. citrea	LMG 2863^T
A. haloplanktis	LMG 2874, LMG 2867, NCIMB 2084^T
A. luteoviolacea	NCIMB 1942, LMG 2871^T
A. macleodii	NCIMB 1963^T
A. nigrifaciens	LMG 2227T, LMG 2228
A. rubra	LMG 2876^T
A. undina	NCIMB 2128^T
D. aquamarina	LMG 6797, NCIMB 557^T, NCIMB 1980
D. cupida	NCIMB 1978^T, LMG 6841, LMG 6794
D. halophila	LMG 6456^T
D. marina	LMG 6798, LMG 2218, NCIMB 1877^T
D. pacifica	NCIMB 1977^T, LMG 6843, LMG 6795
D. venusta	NCIMB 1979^T, LMG 6793, LMG 6844
O. jannaschii	NCIMB 2044^T
O. japonicum	LMG 5215^T
O. kriegii	NCIMB 2042^T, LMG 7638, LMG 7639
O. linum	NCIMB 56^T, LMG 5332
O. maris	LMG 5213^T, LMG 5305^T
O. multiglobiferum	NCIMB 2227^T
V. alginolyticus	LMG 4408^T, LMG 4409, LMG 4407
V. anguillarum	NCIMB 6^T
V. carchariae	LMG 7890^T, LMG 11754, LMG 11755
V. costicola	LMG 11651, NCIMB 701^T, LMG 11756
V. fischeri	LMG 10945, NCIMB 1281^T
V. harveyi	LMG 11658, NCIMB 1280^T, LMG 11226
V. logei	LMG 4415
V. mediterranei	LMG 11259, LMG 11258^T, LMG 11663
V. natriegens	LMG 10950, DSM 759^T, LMG 11227
V. nereis	LMG 11669, LMG 3895^T, LMG 11668
V. nigrapulchrituda	LMG 3896^T
V. parahaemolyticus	LMG 4424, LMG 2850^T, LMG 4423
V. pelagicus	LMG 3897^T
V. proteolyticus	LMG 3772^T
V. splendidus	LMG 4042^T, LMG 10952

[a] T, type strain; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Brunswick, Germany; LMG, Laboratorium Microbiologie Rijksuniversiteit, Gent, Belgium; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

perature, and cultivation medium, the culture conditions of the tested strains were strictly standardized. Bacteria were grown on marine agar (Difco 2216) at 25°C: one single colony was inoculated in 200 ml of marine broth (3 g of yeast extract per liter, 5 g of Difco peptone per liter, 750 ml of seawater, 250 ml of distilled water) and incubated at 25°C until the beginning of the stationary phase (24). The bacteria were collected by centrifugation at about 4,000 × *g* for 20 min, washed twice with deionized water and freeze-dried.

Fatty acid analysis. FAMEs were prepared by the standardized procedure described by Miller and Berger (25). Approximately 10 to 15 mg of a freeze-dried sample was incubated for 30 min at 100°C after the addition of 1 ml of 15% (wt/vol) NaOH in 50% aqueous methanol. The samples were then acidified to pH 2 by adding 2 ml of 6 N aqueous HCl in CH_3OH, and the methylated fatty acids were further extracted with 1.25 ml of a 1:1 (vol/vol) solution of methyl-terbutyl ether and exane.

FAMEs were analyzed by gas-liquid-chromatography on an HP5890A gas chromatograph (Hewlett-Packard) equipped with a flame ionization detector. A fused-silica capillary column (0.2 mm by 25 m; cross-linked 5% methyl phenyl silicone [Hewlett-Packard]) with ultrahigh-purity hydrogen as the carrier gas was used. The gas-liquid chromatography settings were as follows: injector temperature, 250°C; detector temperature, 300°C; initial column temperature, 170°C, increasing by 5°C/min to 270°C in 20 min; carrier gas flow rate, 50 ml/min; total analysis time, 25 min; sample volume, 1 μl (0.01 μl of the sample is split into the column). The peak retention time and peak area values were recorded with an HP3392A integrator (Hewlett-Packard). The retention time data were used to calculate equivalent chain length data, and FAMEs were identified by the Microbial Identification System software package (MIS version 3.2, MIDI; Microbial ID, Inc., Newark, Del.). The results are expressed as percentages relative to the total peak area. Summed-in-feature values (SIFs) represent groups of fatty acids which could not be separated by gas-liquid chromatography with the MIDI system: SIF 3 = 16:1 iso I and/or 14:0 3OH; SIF 4 = 15:0 iso 2OH and/or 16:1 *trans* 9; SIF 6 = 18:2 *cis* 9 and/or 18:0 anteiso; SIF 7 = 18:1 *cis* 11, 18:1 *trans* 11, and/or 18:1 *trans* 6; SIF 9 = unknown 18.846, unknown 18.858 and/or 19:0 cyclo. The unknown fatty acids have no name listed in the Peak Library File of the MIDI system and therefore can be identified only by their equivalent chain lengths. The double-bond position indicated by a capital letter (i.e., 15:1 A, 15:1 B, 16:1 *iso* E, 17:1 B, and 17:1 C) is unknown.

FAME data management. Fatty acid patterns of the standard strains were elaborated by two approaches: a classical numerical analysis with dendrogram representation and a neural network-based elaboration. Because of the originality of the neural network application to the identification of marine bacteria, this approach has been described in more detail.

Numerical analysis. To evaluate the similarity between the species, a numerical analysis of fatty acid data was performed, using the "overlap" coefficient, which is based on the degree of overlapping of two superimposed traces (8). Similarities were calculated from FAME mean values of each species. The percentages obtained were used to establish a phenetic dendrogram by using the unweighted pair group method of association (31) performed with an automatic program developed in DIST Laboratories on a Macintosh II fx computer with 8 megabytes of random access memory (RAM) (12).

Neural network-based elaboration. A feed-forward multilayer supervised artificial neural network was used. These nets act between input data presented at some of the nodes of the network (input neurons) and output data presented at other nodes of the network (output neurons). The networks learn to associate patterns at the former with patterns at the latter. The term "multilayer" refers to the fact that the overall input neurons are not directly connected to the output neurons but are connected through a set of hidden neurons. We chose this particular network architecture because, as stated by the Kolmogorov theorem (6), it is capable of separating any data classes.

In this study, we used a two-level network with the following features (Fig. 1): 72 input neurons (one for each fatty acid considered), 50 hidden neurons (a compromise between precision and training speed), and 4 output neurons (one for each genus considered). To achieve an artificial neural network capable of separating classes, the network has to be trained so that the weights that connect input to hidden neurons and hidden to output neurons reach values which allow the formation of the desired output values at the output neurons (output patterns). In the case considered here, training was performed with 56 fatty acid analyses of standard strains: 16 *Alteromonas*, 12 *Deleya*, 12 *Oceanospirillum*, and 16 *Vibrio* strains. For each strain analysis, the percentages of all 72 fatty acids considered were given to the net as the input pattern, the corresponding desired output pattern was a set of four numbers (one for each genus considered) in which only one number was equal to 1 (the number corresponding to the genus to which the strain belongs) and all the other numbers were 0. With this type of desired output pattern, when a set of fatty acid percentages is presented to the input neurons, the output neurons respond with four numbers (all belonging to the 0–1 range); the highest number indicates the genus in which the neural network classifies the input strain.

The training process consists of a cycle during which all input and desired output patterns are presented to the net; the cycle ends when the mean square error between all real and desired output patterns goes below a defined threshold (training threshold).

In the present work, we used a supervised back-propagation network (30) whose training algorithm was enhanced as regards training speed by the SuperSAB algorithm (34). The implementation was carried out in DIST Laboratories, and the final version which produced the results presented here is running (for dissemination purposes) on a Macintosh II fx with 8 megabytes of RAM. The training phase took about 1 1/2 h, the training threshold was set to 0.005, and 20,000 training steps were necessary to reach this threshold.

Another aim of the present work was the evaluation of the fatty acids which can be regarded as important for the identification of each marine genus considered. Because the training process consists mainly of changes in neuron connection weights, we chose to monitor these changes to determine which were

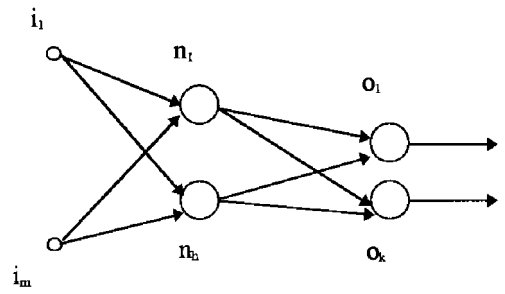

FIG. 1. Model of the two-layer neural network used. Abbreviations: i, input neuron (m = 72); n, hidden neuron (h = 50); o, output neuron (k = 4).

TABLE 2. FAME composition of the marine genera studied[a]

Genus	Percentage of following fatty acid in genus:															
	10:0	10:0 3OH	12:0	11:0 iso 3OH	11:0 3OH	12:0 iso 3OH	12:0 3OH	14:0	15:0 anteiso	15:1 A	15:1 B	15:0	16:1 iso E	SIF 3	16:0 iso	16:1 B
Alteromonas																
Mean	0.21	1.13	2.02	0.17	1.37	0.41	5.76	1.60	0.17	**2.01**	0.49	**3.35**	0.04	0.33	0.51	0.63
SD	0.40	0.92	1.06	0.26	1.23	0.51	2.38	0.76	0.35	**1.91**	0.73	**2.91**	0.15	1.10	0.56	0.41
Minimum	0.00	0.00	0.62	0.00	0.00	0.00	1.55	0.43	0.00	**0.30**	0.00	**0.59**	0.00	0.00	0.00	0.00
Maximum	1.44	1.79	3.73	0.80	4.37	1.46	9.42	3.41	1.17	**6.20**	2.46	**11.10**	0.52	3.81	1.69	1.36
Deleya																
Mean	2.29	0.16	2.24	0.54	0.00	0.00	7.66	1.08	0.04	**0.00**	0.00	**0.01**	0.00	0.00	0.00	0.00
SD	1.03	0.15	1.58	1.15	0.01	0.00	1.20	1.23	0.13	**0.00**	0.00	**0.03**	0.00	0.00	0.00	0.00
Minimum	0.62	0.00	0.36	0.00	0.00	0.00	5.70	0.00	0.00	**0.00**	0.00	**0.00**	0.00	0.00	0.00	0.00
Maximum	4.13	0.39	4.76	4.05	0.02	0.00	9.69	3.01	0.51	**0.00**	0.00	**0.12**	0.00	0.00	0.00	0.00
Oceanospirillum																
Mean	0.57	3.83	3.94	0.00	0.02	0.00	1.89	0.50	0.01	**0.00**	0.01	**0.20**	0.00	0.00	0.11	0.00
SD	0.40	2.25	1.58	0.00	0.03	0.00	2.07	0.14	0.03	**0.00**	0.02	**0.24**	0.00	0.00	0.23	0.00
Minimum	0.00	0.30	1.94	0.00	0.00	0.00	0.00	0.22	0.00	**0.00**	0.00	**0.00**	0.00	0.00	0.00	0.00
Maximum	1.32	6.21	6.37	0.00	0.10	0.00	4.76	0.68	0.08	**0.00**	0.07	**0.71**	0.00	0.00	0.55	0.00
Vibrio																
Mean	0.00	0.03	4.83	0.17	0.03	0.01	**2.47**	4.76	0.01	0.06	0.02	**0.47**	0.15	3.60	0.68	0.53
SD	0.01	0.07	2.76	0.65	0.06	0.02	**0.69**	1.60	0.02	0.09	0.04	**0.45**	0.72	0.86	1.38	0.69
Minimum	0.00	0.00	0.00	0.00	0.00	0.00	**1.27**	1.49	0.00	0.00	0.00	**0.00**	0.00	1.60	0.00	0.00
Maximum	0.04	0.29	14.26	3.71	0.24	0.08	**3.88**	8.63	0.08	0.33	0.14	**1.76**	4.13	5.27	6.53	2.36

[a] Values in boldface type indicate the most important FAMEs according to the neural network elaboration (see the text).

the most important fatty acids for strain classification. This monitoring process was done by using the Karnin algorithm (20), which measures the sensitivity of each weight, i.e., the influence of a change in a weight onto all output values. The sensitivity coefficients were calculated according to a formula (equation A1 in the appendix) modified from the Karnin algorithm to take into account the features of the SuperSAB algorithm. The higher the sensitivity of a connection weight, the greater its importance for classification. The sensitivity coefficients in the present application were spread in a range from 1^{-20} to 1^{+4}. To validate the hypothesis that the connection weights with low sensitivity had little influence on the classification, we cut the connections with weights below a threshold and performed another classification on the same test set. Making the threshold variable with steps of 1 magnitude (from 1^{-20} to 1^{+4}), we found thresholds which caused the change of the classification of all strains belonging to a genus; therefore, we considered all weights with sensitivity coefficients greater than the preceding threshold to be important for the classification of that genus. To evaluate the importance of the input fatty acids for the classification of that genus, we calculated a relevance degree (RD) for each fatty acid-genus pair. RD is an integration of all information obtained from the sensitivity coefficients of the important weights which connect input fatty acids with the output neuron devoted to representing the considered genus. The RD formula is in the appendix (equation A2).

RESULTS

Reproducibility of the fatty acid analysis. To evaluate the reproducibility of the preparation and analysis of fatty acid samples, each strain was cultured and analyzed at least three times under the same standardized conditions, showing a consistently reproducible profile both qualitatively and quantitatively. The coefficient of variation was calculated within each strain as (standard deviation/mean) $\times$ 100 for each fatty acid representing >4% of the total fatty acid content. For most fatty acids, the coefficient of variation was <15%.

Fatty acid composition. Seventy-two different fatty acids with 10 to 20 carbon atoms were detected in the bacterial samples and were used in both the statistical analysis and the neural network elaboration. Mean percentages, standard deviations, and ranges for each genus are listed in Table 2. The fatty acids that were found in trace quantities (less than 1.00%) in all the strains tested or were not found in all the strains of each species are not shown.

Alteromonas spp. contained 31 fatty acids. The most abundant were the saturated 16:0, SIF 4, and the unsaturated 16:1 *cis* 9 (53.8% in all) followed by SIF 7 (10.79%), 12:0 3OH (5.76%), and 17:1B (5.33%). *Deleya* spp. possessed 26 kinds of fatty acids, with a predominance of the monounsaturated SIF 7 (43.13%). Moderate quantities of the monounsaturated 16:1 *cis* 9 (19.69%) and of the saturated 16:0 and 12:0 3OH were found as well (15.59 and 7.66%, respectively). For *Oceanospirillum* spp., 25 different fatty acids were detected. The predominant were the monounsaturated 16:1 *cis* 9 and SIF 7 (38.55 and 18.30%, respectively), and abundant amounts of 16:0 (16.58%) were found. Discrete quantities of SIF 4 (6.47%) were found as well. For *Vibrio* spp., 33 different fatty acids were found. The major fatty acid was the unsaturated 16:1 *cis* 9 (30.46%) followed by SIF 7 (18.12%), 16:0 (16.72%), and SIF 4 (9.91%).

Numerical analysis. Similarities between the marine species considered were calculated by using the overlap coefficient and were represented in a phenetic dendrogram (Fig. 2). Cluster analysis revealed four major fatty acid clusters, each including a genus. The genera *Vibrio* and *Deleya* each formed one separate cluster at about 77% similarity (CL2 and CL3). The genus *Alteromonas* converged at 70% similarity (CL4), including one *Oceanospirillum* species, *O. kriegii*, which clustered with *A. rubra* at 81% similarity. The *Oceanospirillum* species, with the above-mentioned exception, constituted one cluster (CL1) at about 77% similarity.

Neural network elaboration. The neural net was able to identify all marine standard strains used for the validation phase: it succeeded in assigning to the correct genus all 154 samples analysed as unknown. Table 3 shows the values of all four output neurons (since the input values which have been provided are the observed of the percentages of the 72 fatty

TABLE 2—*Continued*

Percentage of following fatty acid in genus:																	
16:1 *cis* 9	SIF 4	16:0	17:0 iso	17:1 B	17:1 C	17:0 cyclo	17:0	18:3 *cis* 6,12,14	SIF 6	18:1 *cis* 9	SIF 7	18:1 *trans* 11	18:0	19:1 *trans* 7	SIF 9	19:0 cyclo	20:0
17.16	**18.17**	**18.47**	0.14	**5.33**	0.06	0.00	**2.64**	0.00	0.35	0.75	**10.79**	1.22	1.57	0.06	0.15	0.00	0.01
4.45	**7.35**	**4.53**	0.29	**3.77**	0.19	0.00	**2.03**	0.00	0.55	0.42	**8.63**	1.12	0.95	0.17	0.45	0.00	0.03
8.18	**5.02**	**12.40**	0.00	**0.73**	0.00	0.00	**0.33**	0.00	0.00	0.23	**1.30**	0.00	0.56	0.00	0.00	0.00	0.00
22.21	**28.53**	**25.76**	1.03	**11.24**	0.66	0.00	**5.78**	0.00	1.95	1.50	**33.71**	3.15	3.44	0.57	1.56	0.00	0.09
19.69	2.40	**15.59**	0.00	0.01	0.00	0.07	0.02	0.12	0.37	0.76	**43.13**	0.45	1.51	0.89	0.09	0.15	0.26
3.78	6.65	**3.52**	0.01	0.03	0.00	0.15	0.04	0.45	0.57	0.56	**12.77**	1.79	1.07	1.19	0.29	0.39	0.49
12.22	0.00	**9.43**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**15.22**	0.00	0.45	0.00	0.00	0.00	0.00
25.31	22.23	**21.08**	0.03	0.10	0.00	0.43	0.13	1.80	2.22	2.43	**58.50**	7.17	4.29	3.27	1.18	1.39	1.54
38.55	6.47	16.58	0.00	**0.16**	0.02	0.01	0.15	0.01	0.21	0.47	**18.30**	3.85	0.68	0.06	0.02	0.00	0.00
16.24	11.43	2.89	0.00	**0.25**	0.05	0.02	0.18	0.03	0.15	0.29	**6.90**	7.34	0.41	0.13	0.04	0.00	0.00
6.38	0.00	12.05	0.00	**0.00**	0.00	0.00	0.00	0.00	0.04	0.06	**6.17**	0.00	0.16	0.00	0.00	0.00	0.00
54.99	33.02	22.48	0.00	**0.59**	0.16	0.05	0.54	0.08	0.57	0.96	**28.67**	22.41	1.52	0.41	0.13	0.00	0.00
30.46	9.91	**16.72**	0.02	**0.58**	0.20	0.10	0.36	0.04	0.24	0.79	**18.12**	0.57	1.28	0.17	0.05	0.01	0.00
4.95	6.29	**4.65**	0.06	**0.65**	0.34	0.44	0.52	0.16	0.34	0.50	**4.66**	1.04	0.60	0.50	0.13	0.03	0.00
20.96	0.00	**9.75**	0.00	**0.00**	0.00	0.00	0.00	0.00	0.00	0.00	**8.44**	0.00	0.60	0.00	0.00	0.00	0.00
40.01	19.08	**27.15**	0.22	**2.45**	1.65	2.50	2.49	0.85	1.80	2.07	**28.90**	3.74	3.72	2.35	0.64	0.12	0.00

acids in the strain shown in the source column); the highest value indicates the genus in which the network classifies the input strain.

To assess the significance of each fatty acid for a correct classification, we eliminated an increasingly large number of connections, checking the consequences on the classification for each elimination. When all weights with sensitivity below 1^{-13} were eliminated, no change in the classification occurred. When weights with sensitivity between 1^{-13} and 1^{-1} were eliminated, only a few incorrect identifications occurred. Therefore, all weights with sensitivity below 1^{-1} are regarded as having no influence for the classification and have been neglected in the calculation of the RDs.

When weights with sensitivity between 1^{-1} and 1^{0} were eliminated, neither *Oceanospirillum* nor *Vibrio* strains could be classified. Therefore, the weights between 1^{-1} and 1^{0} are regarded as important for the classification of *Oceanospirillum* and *Vibrio* strains. These weights have contributed to the calculation of RDs shown in Fig. 3C and D. When weights with sensitivity between 1^{0} and 1^{+1} were eliminated, no *Deleya* strains could be classified. Therefore, the weights between 1^{0} and 1^{+1} are regarded as important for the classification of *Deleya* strains. These weights have contributed to the calculation of RDs shown in Fig. 3B. When all weights with sensitivity below 1^{+1} were eliminated, *Alteromonas* strains were still correctly classified. It could therefore be concluded that the important weights for *Alteromonas* classification were those with sensitivity above 1^{+1}. These weights have contributed to the calculation of RDs shown in Fig. 3A.

DISCUSSION

The marine bacteria analyzed in the present study are all gram-negative rods, coccal rods, or spirilla and are heterotrophic, aerobic, or facultatively anaerobic. The chosen microbial population is representative of the most common heterotrophic bacteria from seawater samples (5). To date, only three major studies (7, 23, 26) describing the fatty acid composition of some marine strains have been published; these mostly concern the genus *Vibrio*. Oliver and Colwell (26) reported that 16:1, 16:0, and 18:1 were the major fatty acids in the *Vibrio* species they tested, with 16:1 being predominant. We confirm these results. Moreover, we found very similar values for the other fatty acids. These authors analyzed two further strains, *Achromobacter aquamarinus* (now *Deleya aquamarina*) and *Oceanospirillum linum*, which are included in the list of standard strains we studied. Our results also confirm the data obtained with these strains; in fact, their fatty acid composition is included in the value ranges of the *Deleya* and *Oceanospirillum* strains we tested.

As far as the results reported by Bøe and Gjerde for *Vibrio parahaemolyticus* are concerned (7), our results for the genus *Vibrio* did not agree. We detected smaller amounts of 16:0 iso and 15:0 anteiso, and we did not detect 13:0, 20:0, 17:0 anteiso, and 18:0 iso or anteiso. Our results are similar to those reported by Lambert et al. (23) concerning the marine *Vibrio* strains, although we found slightly smaller amounts of 16:1Δ11 and 18:0 and small amounts or traces of 11:0 iso 3OH, 16:1 iso, and 19:1 *trans* 7, which they did not detect.

The differences in FAME data can be explained by the different culture conditions and different strains used by the previous authors; moreover, Bøe and Gjerde (7) expressed the fatty acid composition as percentages relative to hexadecanoate rather than to the total peak area, which makes it more difficult to compare the results. In general, it is rather difficult to compare FAME data from different authors, since a standard method for strain cultivation and FAME extraction has yet to be defined when data have to be used in species differentiation.

The overlap coefficient used to calculate the similarities between the standard strains attempts to mimic the way in which fatty acid profiles might be compared visually. Chromatograms which could be superimposed exactly can be regarded as completely similar, whereas those showing no overlap can be re-

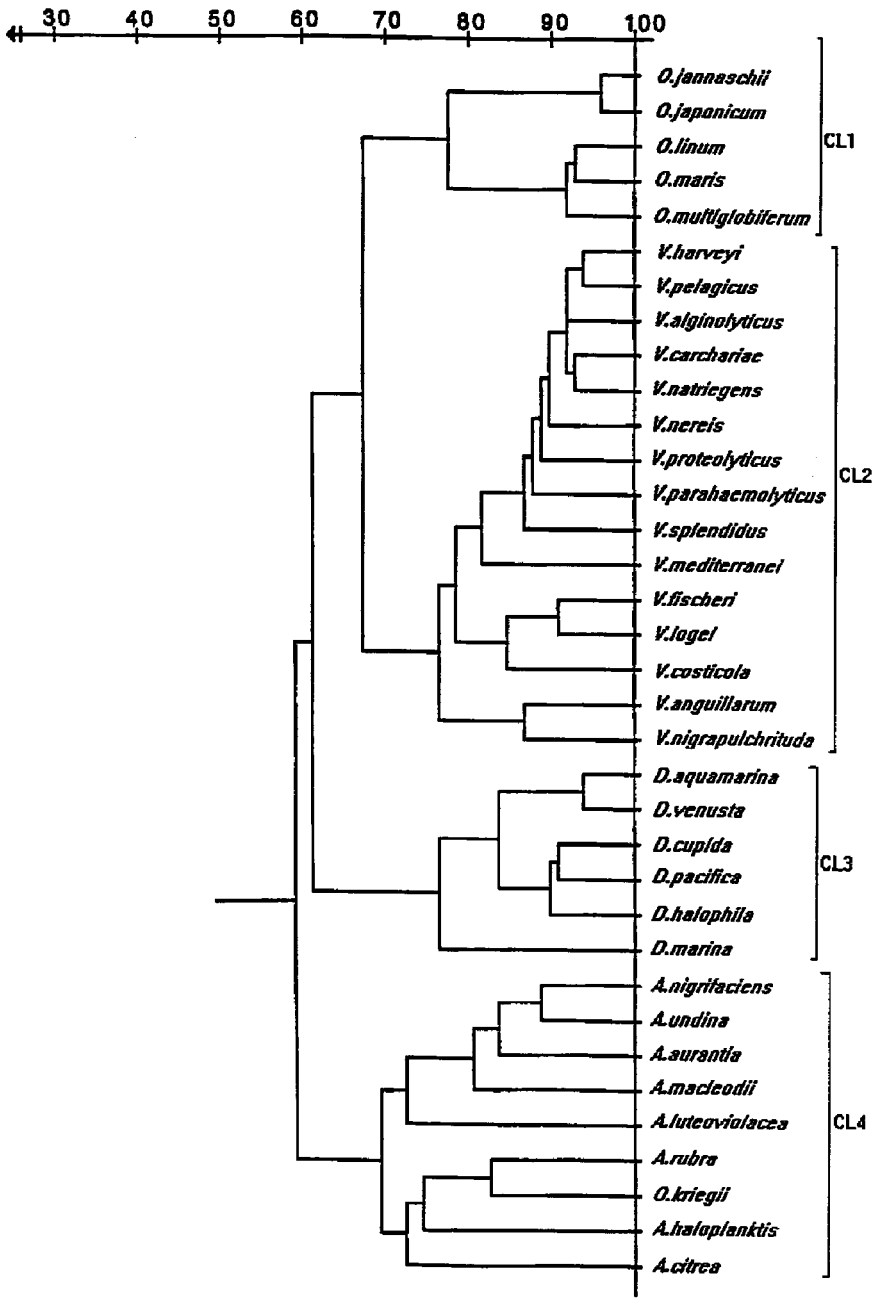

FIG. 2. Phenetic dendrogram showing the relationships of marine species according to numerical analysis.

garded as completely dissimilar. Since its introduction by Bousfield et al. (8), other authors (11) have reported that this coefficient is one of the most satisfactory, when applied to fatty acid data, compared with the correlation coefficient (10, 18, 19) and the coefficient based on angular separation of vectors (10, 17). As shown in the phenetic dendrogram (Fig. 2), it was possible to clearly separate the genera by this approach. The only exception was the genus *Oceanospirillum*. In fact, the statistical analysis incorrectly allocated the species *O. kriegii* within the *Alteromonas* cluster. Previous studies on DNA-rRNA hybridization data for the genus *Oceanospirillum* (28) reported that the species *O. kriegii* and *O. jannaschii* are not to be considered to belong to the genus *Oceanospirillum* sensu strictu and that they should be redefined. We confirm that *O. kriegii* did not show a high FAME relatedness to the other *Oceanospirillum* species, whereas the *O. jannaschii* had the typical FAME composition of the *Oceanospirillum* strains we tested.

The neural network was able to correctly identify all 154 input strains, including 10 analyses of the *O. kriegii* strains (Table 3), although the output values were slightly lower than for the *O. linum* and *O. maris* strains.

The above results suggest that the neural network is a more flexible and reliable data elaboration method than the numerical analysis, allowing the actual identification of the *O. kriegii* species wrongly assigned in the dendrogram (Fig. 2).

However, the statistical elaboration involving the overlap

TABLE 3. Neural network identification of standard strains used as unknown samples

Species	Source	Output values for[a]:				Identification
		A	D	O	V	
A. haloplanktis	NCIMB 2084	0.98	0.03	0.01	−0.06	A
A. haloplanktis	NCIMB 2084	0.75	0.40	0.32	0.38	A
A. haloplanktis	NCIMB 2084	0.98	0.08	0.03	−0.02	A
A. haloplanktis	NCIMB 2084	0.97	0.14	0.10	0.02	A
A. haloplanktis	NCIMB 2084	0.87	0.32	0.24	0.31	A
A. haloplanktis	LMG 2867	0.95	0.07	0.05	0.35	A
A. haloplanktis	LMG 2867	0.98	0.04	0.06	0.15	A
A. haloplanktis	LMG 2867	0.99	0.01	0.06	0.07	A
A. luteoviolacea	LMG 2871	0.99	−0.04	−0.16	−0.01	A
A. luteoviolacea	LMG 2871	0.99	−0.06	−0.06	−0.04	A
A. luteoviolacea	LMG 2871	0.99	−0.03	−0.08	−0.06	A
A. luteoviolacea	LMG 2871	0.99	−0.02	−0.15	−0.05	A
A. nigrifaciens	LMG 2228	0.99	0.03	0.06	−0.01	A
A. nigrifaciens	LMG 2228	0.99	0.04	0.05	−0.02	A
A. nigrifaciens	LMG 2228	0.98	0.03	0.05	0.00	A
A. nigrifaciens	LMG 2228	0.98	0.02	0.07	−0.01	A
A. nigrifaciens	LMG 2228	0.99	0.03	0.03	0.00	A
A. nigrifaciens	LMG 2228	0.99	0.03	0.02	0.00	A
D. aquamarina	NCIMB 1980	0.25	0.99	0.00	−0.09	D
D. aquamarina	NCIMB 1980	0.05	0.99	0.00	−0.08	D
D. aquamarina	NCIMB 1980	0.00	0.99	−0.06	0.05	D
D. aquamarina	NCIMB 1980	−0.13	0.99	−0.02	0.08	D
D. cupida	LMG 6841	0.03	0.99	−0.08	0.24	D
D. cupida	LMG 6841	0.09	0.99	−0.13	0.20	D
D. cupida	LMG 6841	0.06	0.99	0.00	0.01	D
D. cupida	LMG 6794	0.33	0.99	0.03	−0.09	D
D. cupida	LMG 6794	0.33	0.98	0.10	−0.04	D
D. cupida	LMG 6794	0.31	0.98	0.04	−0.05	D
D. cupida	LMG 6794	0.25	0.99	0.02	−0.10	D
D. cupida	LMG 6794	0.44	0.98	0.11	−0.16	D
D. marina	NCIMB 1877	0.04	0.99	−0.01	0.01	D
D. marina	NCIMB 1877	0.01	0.99	−0.01	0.00	D
D. marina	NCIMB 1877	0.04	0.99	−0.01	0.00	D
D. marina	NCIMB 1877	0.05	0.99	−0.01	−0.01	D
D. marina	LMG 2218	0.39	0.81	0.14	0.46	D
D. marina	LMG 2218	0.14	0.98	−0.19	0.39	D
D. marina	LMG 2218	0.40	0.76	0.30	0.54	D
D. pacifica	LMG 6843	0.01	0.99	0.03	−0.04	D
D. pacifica	LMG 6843	0.15	0.99	−0.01	−0.04	D
D. pacifica	LMG 6795	0.16	0.99	−0.03	−0.05	D
D. pacifica	LMG 6795	0.32	0.87	0.62	−0.14	D
D. pacifica	LMG 6795	0.08	0.99	0.02	−0.10	D
D. pacifica	LMG 6795	0.12	0.99	−0.01	−0.02	D
D. pacifica	LMG 6795	0.04	0.99	−0.02	−0.06	D
D. pacifica	LMG 6843	0.08	0.99	0.01	−0.03	D
D. pacifica	LMG 6843	0.06	0.99	0.01	−0.03	D
D. pacifica	LMG 6843	0.07	0.99	0.00	−0.02	D
D. venusta	LMG 6844	−0.08	0.99	0.02	0.06	D
D. venusta	LMG 6844	−0.01	0.99	−0.01	−0.01	D
D. venusta	LMG 6844	0.01	0.99	0.00	0.00	D
D. venusta	LMG 6793	0.08	0.99	0.01	0.00	D
D. venusta	LMG 6793	0.09	0.99	0.01	−0.05	D
O. kriegii	LMG 7638	0.49	0.13	0.90	0.28	O
O. kriegii	LMG 7638	0.49	0.13	0.90	0.28	O
O. kriegii	LMG 7638	0.54	0.13	0.88	0.25	O
O. kriegii	LMG 7638	0.59	0.12	0.87	0.25	O
O. kriegii	LMG 7638	0.45	0.11	0.91	0.35	O
O. kriegii	LMG 7638	0.41	0.11	0.86	0.36	O
O. kriegii	LMG 7639	0.42	−0.02	0.85	0.58	O
O. kriegii	LMG 7639	0.43	−0.01	0.86	0.55	O
O. kriegii	LMG 7639	0.54	0.05	0.87	0.32	O
O. kriegii	LMG 7639	0.45	0.10	0.88	0.37	O
O. linum	LMG 5332	0.06	0.10	0.99	0.08	O
O. linum	LMG 5332	0.07	0.06	0.99	0.09	O
O. linum	LMG 5332	0.09	0.05	0.99	0.09	O

Continued on following page

TABLE 3—*Continued*

Species	Source	Output values for[a]:				Identification
		A	D	O	V	
O. linum	LMG 5332	0.09	0.10	0.99	0.07	O
O. linum	LMG 5332	0.11	0.10	0.99	0.12	O
O. maris	LMG 5305	−0.02	0.03	0.99	−0.04	O
O. maris	LMG 5305	−0.02	0.01	0.99	0.04	O
O. maris	LMG 5305	−0.01	−0.01	0.99	0.01	O
O. maris	LMG 5305	−0.05	−0.05	0.99	−0.01	O
V. alginolyticus	LMG 4408	0.03	−0.01	0.01	0.99	V
V. alginolyticus	LMG 4408	0.05	0.00	0.01	0.99	V
V. alginolyticus	LMG 4408	−0.03	0.01	−0.02	0.99	V
V. alginolyticus	LMG 4408	0.04	0.00	0.02	0.99	V
V. alginolyticus	LMG 4407	−0.03	−0.03	0.00	0.99	V
V. alginolyticus	LMG 4407	−0.02	−0.04	0.03	0.99	V
V. alginolyticus	LMG 4407	−0.03	−0.02	0.00	0.99	V
V. alginolyticus	LMG 4407	−0.02	−0.02	0.00	0.99	V
V. alginolyticus	LMG 4407	0.01	−0.01	0.01	0.99	V
V. carchariae	LMG 11755	−0.01	0.00	0.00	0.99	V
V. carchariae	LMG 11755	0.01	0.00	0.01	0.99	V
V. carchariae	LMG 11755	−0.01	0.00	−0.01	0.99	V
V. carchariae	LMG 11754	0.12	−0.04	−0.04	0.99	V
V. carchariae	LMG 11754	0.14	−0.06	−0.03	0.99	V
V. carchariae	LMG 11754	0.06	−0.01	−0.01	0.99	V
V. carchariae	LMG 11754	0.06	−0.03	0.00	0.99	V
V. carchariae	LMG 11754	0.06	−0.03	0.00	0.99	V
V. carchariae	LMG 11754	0.07	−0.01	0.00	0.99	V
V. costicola	NCIMB 701	0.06	0.02	0.05	0.99	V
V. costicola	NCIMB 701	0.05	0.00	0.05	0.99	V
V. costicola	NCIMB 701	0.06	0.00	0.07	0.99	V
V. costicola	NCIMB 701	0.02	0.00	0.01	0.99	V
V. costicola	NCIMB 701	0.02	0.00	0.01	0.99	V
V. costicola	NCIMB 701	0.05	0.01	0.04	0.99	V
V. costicola	NCIMB 701	0.04	0.00	0.03	0.99	V
V. costicola	NCIMB 701	0.05	0.02	0.09	0.99	V
V. costicola	LMG 11756	0.04	0.01	0.09	0.99	V
V. costicola	LMG 11756	0.04	−0.01	0.12	0.99	V
V. costicola	LMG 11756	0.07	0.01	0.13	0.99	V
V. fischeri	NCIMB 1281	0.02	−0.03	0.12	0.99	V
V. fischeri	NCIMB 1281	0.00	−0.03	0.08	0.99	V
V. fischeri	NCIMB 1281	0.01	−0.03	0.08	0.99	V
V. fischeri	NCIMB 1281	0.01	−0.02	0.07	0.99	V
V. fischeri	NCIMB 1281	0.01	−0.03	0.11	0.99	V
V. fischeri	NCIMB 1281	0.00	−0.02	0.05	0.99	V
V. harveyi	NCIMB 1280	−0.02	−0.04	0.00	0.99	V
V. harveyi	NCIMB 1280	−0.07	−0.11	0.04	0.98	V
V. harveyi	NCIMB 1280	0.01	0.01	−0.04	0.99	V
V. harveyi	NCIMB 1280	0.01	0.01	−0.04	0.99	V
V. harveyi	LMG 11226	0.00	0.01	0.00	0.99	V
V. harveyi	LMG 11226	−0.03	0.01	−0.03	0.99	V
V. harveyi	LMG 11226	−0.02	0.01	−0.03	0.99	V
V. harveyi	LMG 11226	−0.03	0.01	−0.03	0.99	V
V. harveyi	LMG 11226	0.01	0.01	0.01	0.99	V
V. mediterranei	LMG 11258	−0.12	−0.02	0.10	0.99	V
V. mediterranei	LMG 11258	−0.16	−0.05	0.17	0.99	V
V. mediterranei	LMG 11258	−0.02	−0.01	−0.04	0.99	V
V. mediterranei	LMG 11258	−0.04	−0.02	−0.06	0.99	V
V. mediterranei	LMG 11663	−0.03	0.02	−0.04	0.99	V
V. mediterranei	LMG 11663	−0.01	0.01	−0.04	0.99	V
V. mediterranei	LMG 11663	−0.01	0.02	−0.03	0.99	V
V. mediterranei	LMG 11663	−0.02	0.01	−0.03	0.99	V
V. mediterranei	LMG 11663	−0.03	0.02	−0.04	0.99	V
V. natriegens	DSM 759	−0.01	0.01	−0.01	0.99	V
V. natriegens	DSM 759	0.05	−0.02	−0.03	0.99	V
V. natriegens	DSM 759	0.32	−0.08	−0.10	0.98	V
V. natriegens	DSM 759	0.18	−0.08	−0.07	0.99	V
V. natriegens	DSM 759	0.00	0.00	−0.02	0.99	V
V. natriegens	LMG 11227	0.00	0.00	0.02	0.99	V
V. natriegens	LMG 11227	0.00	−0.01	0.06	0.99	V

Continued on following page

TABLE 3—*Continued*

Species	Source	Output values for[a]:				Identification
		A	D	O	V	
V. natriegens	LMG 11227	−0.01	0.01	−0.02	0.99	V
V. natriegens	LMG 11227	−0.01	0.01	−0.01	0.99	V
V. natriegens	LMG 11227	0.00	0.00	0.02	0.99	V
V. nereis	LMG 3895	−0.01	0.01	−0.01	0.99	V
V. nereis	LMG 3895	0.02	0.03	−0.01	0.99	V
V. nereis	LMG 3895	0.00	0.02	0.00	0.99	V
V. nereis	LMG 3895	−0.01	0.02	−0.01	0.99	V
V. nereis	LMG 3895	0.03	0.05	−0.01	0.99	V
V. nereis	LMG 11668	0.02	0.00	−0.01	0.99	V
V. nereis	LMG 11668	−0.01	0.00	−0.01	0.99	V
V. nereis	LMG 11668	0.02	0.00	−0.01	0.99	V
V. nereis	LMG 11668	0.02	0.00	−0.02	0.99	V
V. parahaemolyticus	LMG 2850	0.02	−0.03	−0.05	0.99	V
V. parahaemolyticus	LMG 2850	0.07	0.04	−0.07	0.99	V
V. parahaemolyticus	LMG 2850	0.07	−0.01	−0.06	0.99	V
V. parahaemolyticus	LMG 2850	0.17	0.02	−0.06	0.99	V
V. parahaemolyticus	LMG 4423	0.00	−0.03	−0.04	0.99	V
V. parahaemolyticus	LMG 4423	−0.01	−0.03	−0.03	0.99	V
V. splendidus	LMG 10952	−0.02	−0.01	0.01	0.99	V
V. splendidus	LMG 10952	−0.01	−0.03	0.04	0.99	V
V. splendidus	LMG 10952	−0.03	−0.01	0.00	0.99	V
V. splendidus	LMG 10952	−0.02	−0.01	0.00	0.99	V
V. splendidus	LMG 10952	−0.02	−0.01	0.00	0.99	V

[a] Columns A, D, O, and V contain the output values of the network output neurons corresponding to genera *Alteromonas* (A), *Deleya* (D), *Oceanospirillum* (O), and *Vibrio* (V). The maximum value corresponds to the identification reported in the right-hand column.

similarity coefficient gives the relative comparison of species and, consequently, genera in the phenetic dendrogram, allowing the identification of an unknown isolate by clustering within its genus, while the neural network gives the identification of the unknown strain directly, without any comparison of the different species used in the training phase. The different information obtained reflects the different data elaboration approach, suggesting the usefulness of the neural network mostly for identification purposes, while the numerical analysis, which in the present work does not itself involve classification, could also be considered worthwhile from a taxonomy point of view, of course joined with other features. Moreover, it was possible, with the calculation of RD (see equation A2 in the appendix), to assess the importance of particular fatty acids in discriminating between genera. Although the important fatty acids for all genera were essentially the same, 22 of the total 72 have different RDs according to the considered genus.

Figure 3 shows ordered histograms of the most important fatty acids for each genus. The most relevant fatty acids for the genus *Alteromonas* were 15:0, 17:1B, 16:0, 15:1A, 16:1 *cis* 9, SIF 7, 17:0, and SIF 4 (Fig. 3A). Analysis of the *Alteromonas* FAME composition (Table 2) shows that the mean values of the first four fatty acids were always higher than in the other genera, 16:1 *cis* 9 and SIF 7 were lower than in the other genera, and, finally, 17:0 and SIF 4 were higher than in the other genera.

For the genus *Deleya*, the most important fatty acids were 15:0, 15:1A, 16:0, and SIF 7 (Fig. 3B). The mean value of 15:0 was close to zero (0.01%), and that of 15:1A was zero, which can be considered important distinguishing characters relative to *Alteromonas*. The mean value of 16:0 was slightly lower than in the other genera. Finally, the mean value of SIF 7 was always higher than in the other genera (Table 2).

As far as the genus *Oceanospirillum* is concerned, the acids 15:0, 16:0, 15:1A, SIF 7, 17:1B, and 16:1 *cis* 9 were defined as the most relevant (Fig. 3C). The mean values corresponding to the first three fatty acids and 17:1B were lower than in the genus *Alteromonas*, SIF 7 was lower than in the genus *Deleya*, and 16:1 *cis* 9 was always higher than in the other genera (Table 2).

The most important fatty acids for the genus *Vibrio* were 17:1B, 16:1 *cis* 9, 16:0, 15:0, SIF 7, and 12:0 3OH (Fig. 3D). The mean values of the acids 17:1B, 16:0, and 15:0 were lower than in the genus *Alteromonas*, that of 16:1 *cis* 9 was higher than in *Deleya* and *Alteromonas* and lower than in *Oceanospirillum*, that of SIF 7 was lower than in *Deleya* and higher than in *Alteromonas*, and, finally, that of 12:0 3OH was higher than in *Oceanospirillum* and lower than in *Alteromonas* and *Deleya* (Table 2).

The results show the plain correspondence between the most relevant fatty acids defined by the neural network elaboration and the actual difference in the relative quantities of these fatty acids in the genera studied.

It may be noticed that since RDs are calculated from the weights and sensitivity of the connections between neurons, they do not directly depend on the prevalence of fatty acids in the analyses, but the absence or presence of some fatty acids in trace amounts may be particularly significant for genus identification by neural networks. Moreover, it is interesting that only 22 fatty acids of the 72 used were considered important, suggesting that the detection of only those acids by the neural network elaboration would have been sufficient for the correct identification.

In conclusion, our results show that the fatty acid composition, according to a suitable data elaboration approach, could be a useful, rapid, and reproducible means of identification of marine bacteria once a proper database has been constructed. Our results were achieved with strictly standardized cultivation and extraction conditions and specialized software.

This is the first report of the usefulness of fatty acid data for differentiating marine bacteria belonging to the genera *Alteromonas*, *Deleya*, *Oceanospirillum* and *Vibrio*. This work has fo-

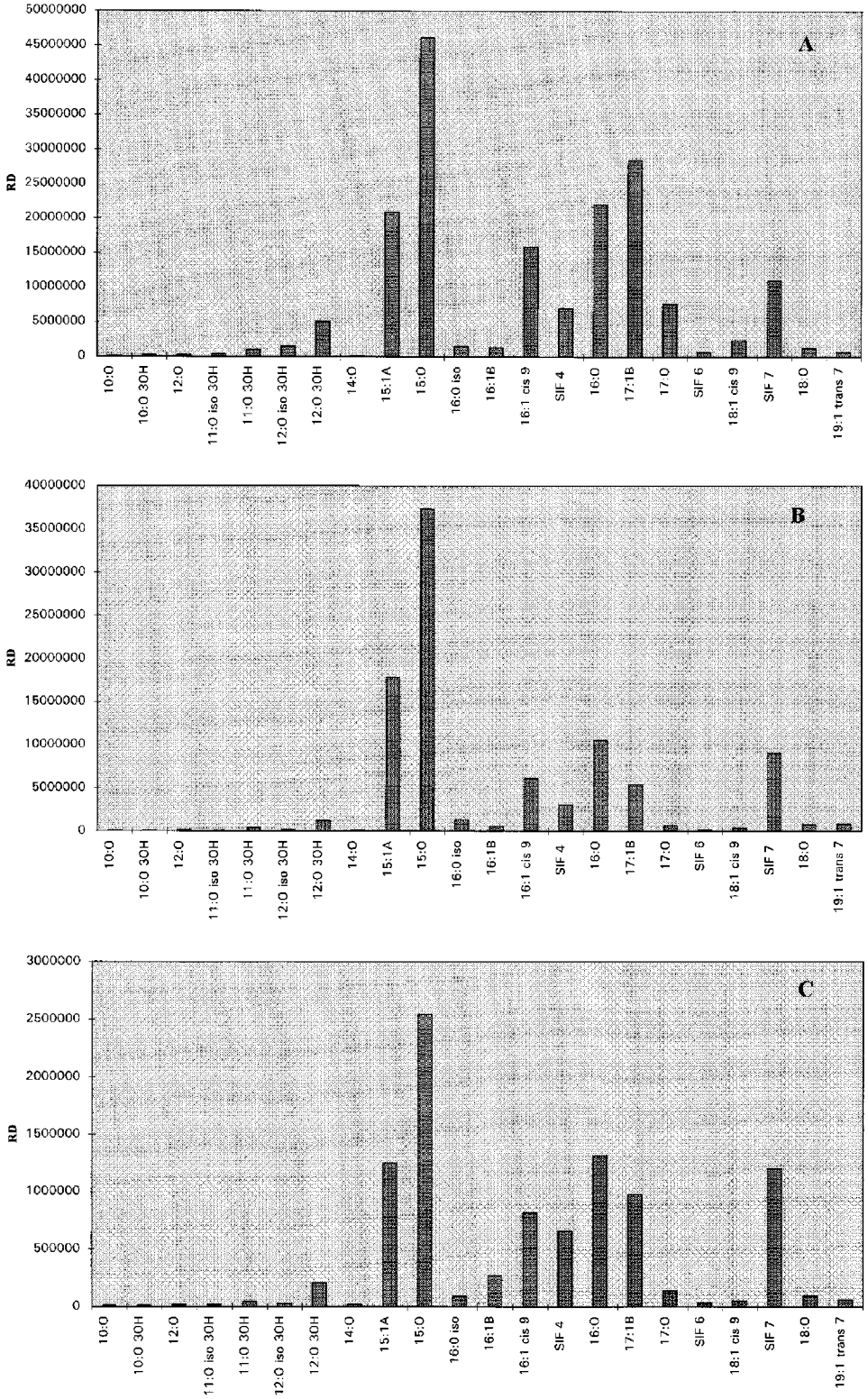

FIG. 3. Important fatty acids according to the neural net elaboration. (A) *Alteromonas*; (B) *Deleya*; (C) *Oceanospirillum*; (D) *Vibrio*. RD was calculated from equation A2.

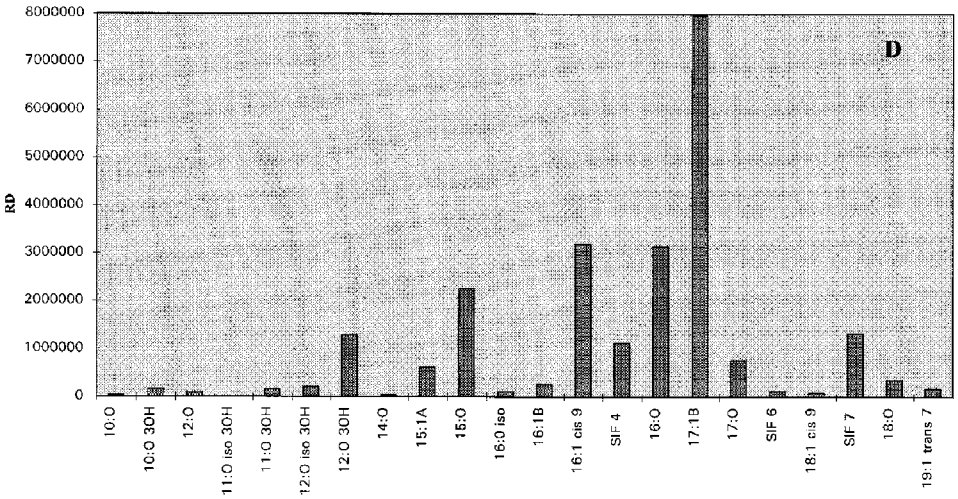

FIG. 3—*Continued.*

cused on the identification of marine bacteria at the genus level because, although whole-cell fatty acid analysis is a useful method for identification at both the genus and species levels and is routinely used in our laboratory to identify fresh isolates, we have recently begun to study more sensitive and discriminating chemotaxonomic characters for the species and subspecies levels, such as the whole-cell protein content (21).

Our future aim is to develop a polyphasic approach that combines identification techniques with different discrimination powers, e.g., FAME compositions for the genus level and protein patterns for more specific levels, to identify any fresh isolates at the appropriate level. The finding of the usefulness of the neural network approach suggests further applications of neural nets trained at the genus, species, and subspecies levels, working stepwise and using different data.

APPENDIX

The sensitivity to the removal of connection w_{ij}, in the case of use of the SuperSAB back-propagation method in the neural network elaboration, can be calculated as follows:

$$S_{ij} = \left\{ \sum_{n=0}^{N-1} [\Delta w_{ij}(n)]^2 \right\} \frac{w_{ij}^{\,f}}{n_{ij}(w_{ij}^{\,f} - w_{ij}^{\,i})} \qquad \text{(A1)}$$

where S_{ij} is the sensitivity coefficient for the weight ij, N is the number of training steps (in the present work, $N = 20{,}000$), $\Delta w_{ij}(n)$ is the change for the weight ij at training step n, $w_{ij}^{\,f}$ is the value of the connection weight between neuron i of a level and neuron j of the following level at the end of the training phase, $w_{ij}^{\,i}$ is the value of the connection weight between neuron i of a level and neuron j of the following level at the beginning of the training phase, and n_{ij} is the final training step size for the weight ij. Formulas for calculating all these quantities in the case of the SuperSAB algorithm can be found in reference 34.

The RD for each fatty acid-genus pair was calculated as follows:

$$\mathrm{RD}_{ag} = \sum_{j=1}^{K} S_{aj} \cdot S_{jg} \qquad \text{(A2)}$$

where a is the fatty acid, j is the hidden neuron, g is the genus, and K is the number of all hidden neurons (in this case 50). The sum is extended to all hidden neurons, but the product is added only if both S_{aj} and S_{jg} (calculated as in equation A1) overcome the relevance threshold (defined in the last paragraph of the "Neural network-based elaboration" section of Materials and Methods).

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