

## Diversity of Lactic Acid Bacteria Associated with Fish and the Fish Farm Environment, Established by Amplified rRNA Gene Restriction Analysis<sup>∇</sup>

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Lactic acid bacteria have become a major source of concern for aquaculture in recent decades. In addition to true pathogenic species of worldwide significance, such as *Streptococcus iniae* and *Lactococcus garvieae*, several species have been reported to produce occasional fish mortalities in limited geographic areas, and many unidentifiable or ill-defined isolates are regularly isolated from fish or fish products. To clarify the nature and prevalence of different fish-associated bacteria belonging to the lactic acid bacterium group, a collection of 57 isolates of different origins was studied and compared with a set of 22 type strains, using amplified rRNA gene restriction analysis (ARDRA). Twelve distinct clusters were delineated on the basis of ARDRA profiles and were confirmed by sequencing of *sodA* and 16S rRNA genes. These clusters included the following: *Lactococcus raffinolactis*, *L. garvieae*, *Lactococcus l.*, *S. iniae*, *S. dysgalactiae*, *S. parauberis*, *S. agalactiae*, *Carnobacterium* spp., the *Enterococcus* “faecium” group, a heterogeneous *Enterococcus*-like cluster comprising indiscernible representatives of *Vagococcus fluvialis* or the recently recognized *V. carniphilus*, *V. salmoninarum*, and *Aerococcus* spp. Interestingly, the *L. lactis* and *L. raffinolactis* clusters appeared to include many commensals of fish, so opportunistic infections caused by these species cannot be disregarded. The significance for fish populations and fish food processing of three or four genetic clusters of uncertain or complex definition, namely, *Aerococcus* and *Enterococcus* clusters, should be established more accurately.

Several species of lactic acid bacteria account for major infectious diseases in aquaculture. Although clinical cases involving serogroup B streptococci were reported in the United States as early as the beginning of the 1960s (34), they were at first considered a minor source of trouble. A notable exception was *Enterococcus seriolicida* (20), a junior synonym of *Lactococcus garvieae* (11), which caused considerable economic losses in Japanese marine-cage aquaculture long before it spread worldwide. In the late 1980s, other species became threatening for the intensive culture of several fish species in various environments and geographic areas. Even though major advances in taxonomic knowledge and characterization procedures have probably favored the identification of new or still unsuspected agents, the concurrent development of international trade, changes in managing procedures, and diversification of domesticated fish species have also been critical in the increasing numbers of lactic acid bacterial infections reported to affect fish (12).

Besides *L. garvieae*, now firmly established in many European salmonid farms, other illustrative examples are *Strepto-*

*coccus iniae*, regularly involved in tilapia and striped bass mortalities in the United States and Israel but widely distributed, in fact, in both fresh and saline waters in all warm areas, and *Streptococcus difficilis*, a variant form of *Streptococcus agalactiae* (43). The more opportunistic species *Carnobacterium maltaromaticum* (formerly called *Carnobacterium piscicola*) is normally associated with the intestinal microflora of fish. Other representatives with less common occurrence or more limited impact are *Streptococcus parauberis* in turbot and *Vagococcus salmoninarum* in salmonids; these may, nonetheless, cause serious damage on a local scale (10, 38).

In France, the involvement of lactic acid bacteria in fish infectious diseases was discovered progressively: *C. maltaromaticum* was isolated in clinical cases from several fish species (26), and *V. salmoninarum* was isolated from southwest rainbow trout hatcheries (27). The first cases of *L. garvieae* infection were experienced concurrently in Brittany and Aquitaine in 1999, more than 5 years after the agent had been identified in Italy and Spain (12). Since chemotherapy appeared to be poorly effective, early detection of new cases, based on rapid and specific diagnostic methods, was the only alternative for preventing further spread of lactococcosis. Unfortunately, identification methods based on phenotypic characterization fall short of basic requirements when applied to bacteria of aquatic origin. Although rapid systems (API 20 Strep, API Rapid ID 32 Strep, and API 50 galleries) may be used, com-

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prehensive tests require several steps and can only provide results after several days or, sometimes, several weeks. Due to the lack of specific documentation and well-defined numerical profiles, result interpretation is especially time-consuming. For these reasons, molecular diagnostic methods based on PCR amplification and/or sequencing of the 16S rRNA gene or other genes of taxonomic interest have been proposed for characterizing most of the newly described agents (1–4, 22, 23, 47). The performance of these techniques, however, has been tested with a limited number of fish-associated bacteria, generally chosen among well-characterized pathogens.

Performing routine examination on increasing numbers of infected fish and tissue samples has also helped researchers to realize that, in fact, recognized pathogens are not the only lactic acid bacteria occurring in fish. A variety of related but different organisms are frequently isolated, rising intriguing questions about their identity, their relationship to pathogens they otherwise closely resemble, like the case with *Lactococcus lactis* and *L. garvieae*, and their possible interference in identification tests. To obviate the risk of drawing inaccurate conclusions about closely related and sometimes poorly characterized bacteria, restriction fragment length polymorphism analysis appeared more convenient than PCR amplification based on species-specific primers. Preliminary assays conducted in one of our laboratories had shown that amplified 16S rRNA gene restriction analysis (ARDRA) could be adapted reliably to the specific diagnosis of *L. garvieae* infection (8). The diversity of the lactic acid bacteria isolated from fish, however, made it difficult to immediately consider extensive use of the method. A cooperative program set up to resolve this issue provided the opportunity to screen and clarify the status of as yet poorly identified species associated with fish. The work was based on the application of ARDRA to a large part (16S-23S) of the rRNA operon; this had already been reported as a reliable technique for identification of lactic acid bacteria (6, 37).

#### MATERIALS AND METHODS

**Isolates.** Fifty-seven field isolates, revived from the Jouy-en-Josas strain collection (JIP) or obtained from different fish disease laboratories, were selected after preliminary tests conducted to establish phenotypic characteristics and/or PCR-restriction fragment length polymorphism profiles according to the method of De Buzon et al. (8). Table 1 summarizes the information available for these strains, together with the results of provisional identification. Twenty-two type strains purchased from different international or national culture collections were also used in various steps of the research (Table 2). All strains were cultured on solid or in liquid brain heart infusion medium enriched with 5% defibrinated horse serum, and aliquots were suspended in 10% glycerol-supplemented medium for freezing preservation at  $-70^{\circ}\text{C}$ .

**DNA extraction.** After verification of the purity and morphological characteristics of the bacteria, 2 ml of a 24- or 48-h broth culture was collected for DNA extraction. DNA extraction was performed using a Wizard genomic DNA purification kit (Promega, Charbonnières-les-Bains, France) according to supplier specifications, with an additional step of lysozyme treatment (1 mg/ml in 40 mM EDTA for 60 min at  $37^{\circ}\text{C}$ ) before extraction. DNA quality and quantity were controlled in 1% agarose minigels run in Tris-borate-EDTA buffer (45 mM; pH 7.8 [1 mM EDTA]) at 100 V/cm, stained with ethidium bromide, and observed under UV light. All DNAs were stored at  $4^{\circ}\text{C}$  until further use.

**ARDRA.** The technique developed by Gurtler et al. (16) was followed to amplify the extracted DNAs and to prepare the rRNA gene for ARDRA. The primers used were P1 (5' AGA GTT TGA TCM TGG CTC 3') and P2 (5' ACA TCG AGG TGC CAA AC 3'). Amplification was performed in a PTC 100 cycler (MJ Research Inc., Tahoe, NV), using mixes incorporating primers at 20  $\mu\text{M}$ , deoxynucleoside triphosphates at 200  $\mu\text{M}$ , 0.5  $\mu\text{l}$  (2.5 U) of Taqara ExTaq

polymerase (Cambrex Bio Science Paris), 5  $\mu\text{l}$  of  $10\times$  Taq buffer, and 4  $\mu\text{l}$  of template DNA into distilled water adjusted to a 50- $\mu\text{l}$  final volume. DNA denaturation was performed for 5 min at  $94^{\circ}\text{C}$ , followed by 30 cycles of amplification (30 s at  $94^{\circ}\text{C}$ , 1 min at  $57^{\circ}\text{C}$ , and 6 min at  $72^{\circ}\text{C}$ ) and a final elongation step of 7 min at  $72^{\circ}\text{C}$ . The size of the expected 16S-23S fragment, about 5,000 bp, was checked by 1% agarose gel electrophoresis and staining with ethidium bromide. Digestion of the amplified products was tested with several restriction enzymes, including CfoI (Promega), Sau3A (Gibco BRL, Cergy-Pontoise, France), HaeIII, HpaII, MboII, DraI, and SmaI (all from MBI Fermentas, distributed by Coger, Paris, France). It required incubation for 3 h at  $37^{\circ}\text{C}$  of a mixture of enzyme (5 U) and amplified product (4  $\mu\text{l}$ ) prepared in the specific buffer supplied by the manufacturer in a final volume of 20  $\mu\text{l}$ . Electrophoretic migration was performed in 1% Seakem GTG agarose (Tebu, Le Perray-en-Yvelines, France) gels in Tris-borate-EDTA buffer at 65 V/cm. The reference marker was Jules (QBiogen), which covers a size range of 100 to 2,000 bp.

**Gel analysis.** After preliminary assays aimed at testing seven endonucleases to digest the complete 16S-23S fragment on type strains and comparing the resulting electrophoresis profiles, CfoI and Sau3A were considered to be the most suitable for further analysis of 78 strains, including 21 type strains (*Lactococcus raffinolactis* type strain DNA was rejected in this step, following quality control) and 57 representative fish isolates (Tables 1 and 2). Numerized pictures of migration gels run under standardized conditions were taken and processed with GelCompar software (Applied-Maths, Sint-Martens-Latem, Belgium). This made it possible to calibrate, analyze, and combine the results obtained with the different enzymes.

**Sequencing.** In order to confirm or determine the identities of dubious species, sequencing was carried out on representative bacterial strains. We chose to use two different genes, the 16S rRNA gene and *sodA*, depending on the suspected identities of the bacteria and the availability of published information. Amplification and sequencing of the 16S rRNA gene were performed according to the method described by Cibik et al. (7). For the *sodA* gene, the method of Poyart et al. (30) was applied, modifying only the annealing temperature ( $50^{\circ}\text{C}$  instead of  $45^{\circ}\text{C}$ ) in order to ensure more stringent conditions. Our sequences, together with those of related species listed in GenBank, were used for comparison and phylogenetic analysis.

#### RESULTS

A comparison of the profiles resulting from restriction with Sau3A and CfoI is presented in Fig. 1. Most of the strains clustered in distant but clear-cut groups, some of which could be identified immediately; the others were numbered until additional tests could be performed. Strains that occupied isolated positions in the dendrogram corresponded to unrelated type strains or misidentified non-lactic-acid bacteria and were not considered in the subsequent steps of the study.

Sequencing was carried out on representative isolates of the different ARDRA clusters to confirm their relationship to representative type strains or to tentatively identify those that could not be related to any type strain. The choice between sequencing of the 16S rRNA and *sodA* genes was made based on the information available from libraries on reference strains and the recognized limits of 16S rRNA gene sequence discrimination within certain bacterial genera (14, 42). In some cases, both genes were sequenced in order to obtain independent series of data and strengthen the ARDRA results. Figures 2 and 3 show that strong similarity was established between fish isolates and reference strains and that, in every case, the classification of the strains was consistent for all three techniques used in this study.

It eventually became clear that most of our isolates could be assigned to 12 species or groups of interest. An unexpected abundance of isolates (group 1) roughly related to a large branch encompassing *Lactococcus plantarum* and the type strain supplied as *Lactococcus piscium* was evidenced by ARDRA. Further sequence comparison showed that all group

TABLE 1. Bacteria isolated or collected during this study, with available information about their origins and presumptive identifications, based on phenotypic characteristics in API systems, and the accession numbers of deposited sequences<sup>a</sup>

Isolate	Presumptive identification	Origin	Original area or country	Definitive identification	EMBL library accession no.	
					16S rRNA gene	sodA
<b>LD40 isolates</b>						
C6	<i>L. lactis</i> / <i>E. faecalis</i>	?	Poitou-Charentes			
C22	<i>L. lactis</i> / <i>E. faecalis</i>	?	Poitou-Charentes	<i>L. lactis</i>	AM490366	
1609	<i>E. faecalis</i>	?	Poitou-Charentes			
5076 H	<i>S. uberis</i> ?	?	?	<i>S. dysgalactiae</i> subsp. <i>equisimilis</i>		AM490331
5076 NH	<i>L. garvieae</i> ?	?	?	<i>Aerococcus</i> sp.		AM490332
28565-2	<i>Aerococcus</i>	?	?	<i>Aerococcus</i> sp.		AM490327
6491-93	?	Natural environment	Pyrenees			
2725-99	<i>L. garvieae</i>	<i>Oncorhynchus mykiss</i>	?			
1798-00	<i>L. lactis</i>	<i>O. mykiss</i>	Aquitaine			
2188-00	<i>E. faecium</i>	<i>O. mykiss</i>	Aquitaine	<i>E. casseliflavus</i>		AM490321
3362-01	<i>A. viridans</i>	<i>O. mykiss</i>	Poitou-Charentes	<i>V. fluvialis/carniphilus</i>	AM490373	
3363-01	<i>S. acidominimus</i>	<i>O. mykiss</i>	Poitou-Charentes			
3364-01	<i>E. faecalis</i>	<i>O. mykiss</i>	Poitou-Charentes	<i>C. maltaromaticum</i>		Am490329
3365-01	<i>E. faecalis</i>	<i>O. mykiss</i>	Poitou-Charentes			
3366-01	<i>S. anginosus</i>	<i>O. mykiss</i>	Poitou-Charentes	<i>L. lactis cremoris</i>	AM490365	
3525-01	<i>S. dysgalactiae</i> / <i>S. equisimilis</i>	<i>Acipenser baeri</i>	Aquitaine	<i>S. dysgalactiae</i> subsp. <i>dysgalactiae</i>		AM490330
3667-01	<i>E. faecium</i> / <i>S. bovis</i>	<i>O. mykiss</i>	Brittany			
2514-02	<i>L. garvieae</i>	<i>O. mykiss</i>	Aquitaine			
952-03	?	<i>O. mykiss</i>	Aquitaine			
<b>JIP collection</b>						
112-82 (2)	<i>Streptococcus</i> sp.	<i>Paracheirodon innesi</i>	Ile-de-France			
108-83 (4)	<i>Streptococcus</i> sp.	<i>Channa striata</i>	Thailand	<i>S. iniae</i>		AM490314
20-85 (3)	<i>C. piscicola</i>	<i>O. mykiss</i>	Picardy			
CII-5b-88	<i>Streptococcus</i> sp.	<i>Channa striata</i>	Bangkok			
31-90 (2)	<i>Streptococcus</i> sp.	Tilapia	Belgium	<i>L. garvieae</i>		AM490328
08-92 (1)	<i>S. parauberis</i> ?	Tilapia	Lorraine			
18-93 (2)	<i>V. salmoninarum</i>	<i>O. mykiss</i>	Aquitaine	<i>V. salmoninarum</i>		AM490319
18-93 (4)	<i>V. salmoninarum</i>	<i>O. mykiss</i>	Aquitaine			
10-94 (4)	<i>L. lactis</i>	<i>Pterophyllum scalare</i>	Alsace			
12-94 (2)	<i>L. piscium</i> ?	<i>Betta splendens</i>	MNHN, Paris			
21-96 (1)	<i>Streptococcus</i> sp.	<i>O. mykiss</i>	Réunion Island			
21-96 (2)	<i>Streptococcus</i> sp.	<i>O. mykiss</i>	Réunion Island			
22-96 (2)	<i>L. piscium</i> ?	Koi carp	Jura	<i>L. raffinolactis</i>		AM490322
23-98 (C11a)	<i>L. lactis</i>	<i>O. mykiss</i>	Aquitaine			
31-98 (3)	<i>L. lactis</i> ?	Tilapia	Brittany	<i>L. lactis</i> subsp. <i>lactis</i>	AM490364	
25-99	<i>S. mundtii</i> ?	Trout diet	Brittany	<i>E. faecium</i>		AM490323
26-99	<i>Streptococcus</i> sp.	Fish farm water	Brittany	<i>E. faecium</i>		AM490325
29-99	<i>L. garvieae</i>	<i>O. mykiss</i>	Brittany			
19-00(2)	<i>C. piscicola</i>	<i>P. innesi</i>	Hong Kong			
20-00	<i>Leuconostoc</i> ?	<i>Carassius carassius</i>	Jura	<i>V. salmoninarum</i>	AM490375	AM490320
38-00 (3)	<i>Lactococcus</i> sp.	<i>Symphysodon discus</i>	Ile-de-France			
10-01	<i>Leuconostoc</i> ?	Koi carp	Jura	<i>L. raffinolactis</i>	AM490367	AM490311
14-01	<i>V. salmoninarum</i> ?	<i>C. carassius</i>	Jura	<i>V. fluvialis/carniphilus</i>	AM490371	
Me I.5-02	<i>C. piscicola</i>	<i>O. mykiss</i>	Brittany			
<b>Other origins</b>						
<b>France</b>						
1525A	<i>S. parauberis</i>	<i>Dicentrarchus labrax</i>	SAVU			
1525E	<i>S. parauberis</i>	<i>D. labrax</i>	SAVU	<i>S. parauberis</i>		AM490317
1534E	<i>S. parauberis</i>	<i>D. labrax</i>	SAVU			
<b>Israel</b>						
KF 173	<i>S. iniae</i>	<i>O. mykiss</i>	A. Eldar			
KFP 404	<i>S. iniae</i>	<i>O. mykiss</i>	A. Eldar			
<b>Spain</b>						
17-02	<i>Enterococcus</i> sp.	<i>O. mykiss</i>	M. Blanco	<i>E. gallinarum</i>		AM490318
18-02	<i>Enterococcus</i> sp.	<i>O. mykiss</i>	M. Blanco	<i>V. fluvialis/carniphilus</i>	AM490372	
24-01	<i>S. mutans</i> ?	<i>O. mykiss</i>	Trouw, Spain			
26-01	<i>S. mutans</i> ?	<i>O. mykiss</i>	Trouw, Spain	<i>Lactococcus</i> sp.	AM490370	AM490324
27-01 (2)	<i>Lactococcus</i> sp./ <i>Gemella</i>	<i>O. mykiss</i>	Trouw, Spain	<i>V. salmoninarum</i>	AM490374	AM490326
28-01	<i>S. mutans</i> ?	<i>O. mykiss</i>	Trouw, Spain	<i>L. raffinolactis</i>	AM490368	
<b>United Kingdom</b>						
CF 00021	<i>L. garvieae</i>	<i>O. mykiss</i>	CEFAS			
CF 01144	<i>L. garvieae</i>	<i>O. mykiss</i>	CEFAS			
CF 01173	<i>S. agalactiae</i>	<i>O. mykiss</i>	CEFAS			

<sup>a</sup> Abbreviations: CEFAS, Center for Environment, Fisheries and Aquaculture Science, Weymouth, United Kingdom; JIP, Jouy Ichthyopathology collection, INRA, Jouy-en-Josas, France; LV40, Laboratoire Départemental des Landes, Mont-de-Marsan, France; MNHN, Museum National d'Histoire Naturelle, Paris, France; and SAVU, Service d'Assistance Vétérinaire Aquacole d'Urgence, Saint Jean de Vedas, France.

1 isolates were closely related to *L. raffinolactis* (Fig. 2 and 3). While the “type strain” of *L. piscium* was unexpectedly found to be questionable (Fig. 1), the isolate 26-01 appeared to be roughly related to this species according to published 16S

rRNA sequences (Fig. 2). Sequencing results established the unique character of both *L. garvieae* and *L. lactis* isolates, especially the former, which split into quite remote subgroups (2a and 2b) in the ARDRA dendrogram. Conversely, the other

TABLE 2. Type strains used in the study (accession numbers of the genes sequenced during the study are indicated)

Species	Collection no. <sup>a</sup>	Other no.	EMBL accession no.	
			16S rRNA gene	<i>sodA</i> gene
<i>Aerococcus viridans</i>	CIP 54.145 <sup>T</sup>			AM490333
<i>Carnobacterium maltaromaticum</i>	NCIMB 2264 <sup>T</sup>			AM490310
<i>C. inhibens</i>	CIP 106863 <sup>T</sup>			AM490313
<i>Enterococcus durans</i>	NCIMB 700596 <sup>T</sup>			
<i>Lactococcus garvieae</i>	CNRZ 1323 <sup>T</sup>			
<i>L. lactis</i> subsp. <i>cremoris</i>	CNRZ 105 <sup>T</sup>			
<i>L. lactis</i> subsp. <i>lactis</i>	CNRZ 142 <sup>T</sup>			
<i>L. lactis</i> subsp. <i>lactis</i>	CNRZ 144 <sup>T</sup>			
<i>L. piscium</i>	CIP 104371 <sup>T</sup>	NCIMB 13196 <sup>T</sup>		
<i>L. plantarum</i>	CNRZ 1322 <sup>T</sup>			AM490316
<i>L. raffinolactis</i>	CNRZ 1214 <sup>T</sup>		AM490369	AM490315
<i>Streptococcus acidominimus</i>	CIP 82.4 <sup>T</sup>			
<i>S. agalactiae</i>	CIP 103227 <sup>T</sup>			
<i>S. anginosus</i>	CIP 102921 <sup>T</sup>			
<i>S. bovis</i>	CIP 102302 <sup>T</sup>			
<i>S. dysgalactiae</i> subsp. <i>dysgalactiae</i>	CIP 102914 <sup>T</sup>			
<i>S. dysgalactiae</i> subsp. <i>equisimilis</i>	CIP 105120 <sup>T</sup>			AM490312
<i>S. mitis</i>	CIP 103335 <sup>T</sup>			
<i>S. parauberis</i>	NCIMB 702020 <sup>T</sup>			
<i>S. shiloi</i>	ATCC 51499 <sup>T</sup>	= <i>S. iniae</i>		
<i>S. uberis</i>	NCIMB 702038 <sup>T</sup>			
<i>V. salmoninarum</i>	NCIMB 13133 <sup>T</sup>	formerly NCFB 2777 <sup>T</sup>		

<sup>a</sup> ATCC, American Type Culture Collection, Manassas, VA; CIP, Collection de l'Institut Pasteur, Institut Pasteur, Paris, France; CNRZ, Collection CNRZ de Bactéries Lactiques et Propioniques, Unité de Recherches Laitières et de Génétique Appliquée, INRA, Jouy-en-Josas, France; NCFB, National Collection of Food Bacteria; NCIMB, National Collections of Industrial, Food and Marine Bacteria, Aberdeen, Scotland.

major fish pathogen, *Streptococcus iniae* (cluster 4), proved noticeably homogenous in its restriction patterns. Clusters 5 to 7, representing other *Streptococcus* species, and cluster 8, corresponding to *Carnobacterium maltaromaticum*, also displayed strong homogeneity and were easily recognized in ARDRA. In the cases of *Streptococcus dysgalactiae* and *S. agalactiae*, however, only one or two isolates were available.

The next two clusters were not so easily defined. They included typical enterococci that could not be characterized at the specific level by ARDRA, although some isolates were representative of the "faecium" group (cluster 9). Group 10 strains shared high rRNA gene sequence homology with *Enterococcus* or *Vagococcus* spp. Some could be characterized as *Enterococcus casseliflavus* and *Enterococcus gallinarum* by *sodA* gene sequences only (Fig. 3), whereas 16S rRNA analysis eventually revealed several others to be *Vagococcus fluvialis* or *Vagococcus carniphilus*, a species recently reported to be in beef meat (39). This was the only case, indeed, in which ARDRA did not enable the assignment of isolates to the correct genus. All group 11 strains were closely related to the *V. salmoninarum* type strain. All group 12 isolates belonged to the *Aerococcus* genus, although no further conclusions could be derived regarding the species.

## DISCUSSION

The considerable diversity of lactic acid bacteria associated with fish may explain the difficulties encountered when identification procedures are based only on phenotypic characteristics. While *Carnobacterium maltaromaticum*, *Lactococcus lactis*, *L. garvieae*, and *Vagococcus salmoninarum* were already documented in French fish farm environments, the use of

ARDRA made it possible (i) to detect the occurrence of *Streptococcus dysgalactiae* and *S. parauberis* in France, (ii) to assign past cases from tropical environments to *S. iniae* and *S. parauberis*, and (iii) to establish repeated associations of *L. raffinolactis*, *Aerococcus* spp., *Enterococcus* spp., and *Vagococcus* spp. with apparently healthy fish. The practical importance of these different clusters for fish health management is worth considering.

All of the isolated streptococci were found to belong to fish pathogenic species and were easily characterized in ARDRA (groups 4 to 7), as demonstrated with *Streptococcus iniae*. Depending on the diagnosis methods used, confusion is possible with *Streptococcus uberis*, an agent that may be misidentified as several different organisms (25) but that seems to occur rarely in fish farm environments. A single strain of *S. agalactiae*, CF 01173, originating from an English trout farm, was recognized in this study. More interesting is the independent isolation of two *S. dysgalactiae* strains, one of them from diseased sturgeons. The pathogenicity of this species had been reported only once, in Japan (28). The same remark may be made for *S. parauberis*, whose detrimental impact, until now, seemed to be restricted to turbot (10) in Spanish sea farms. The present isolates were all from French sea bass farms, except for one, *Streptococcus* sp. strain 08-92 (1), which was isolated from diseased tilapia in 1992.

In most of the groups delineated in Fig. 1 (groups 1, 2, 3, 8, and 11), both pathogenic and nonpathogenic species are represented. *Lactococcus raffinolactis* has been reported as a contaminant of food products (36), but a debate on possible confusion with the fish pathogen *L. piscium* has arisen in more recent studies (35). Such confusion cannot be ruled out, as suggested in Fig. 1, where the two species cluster together, and

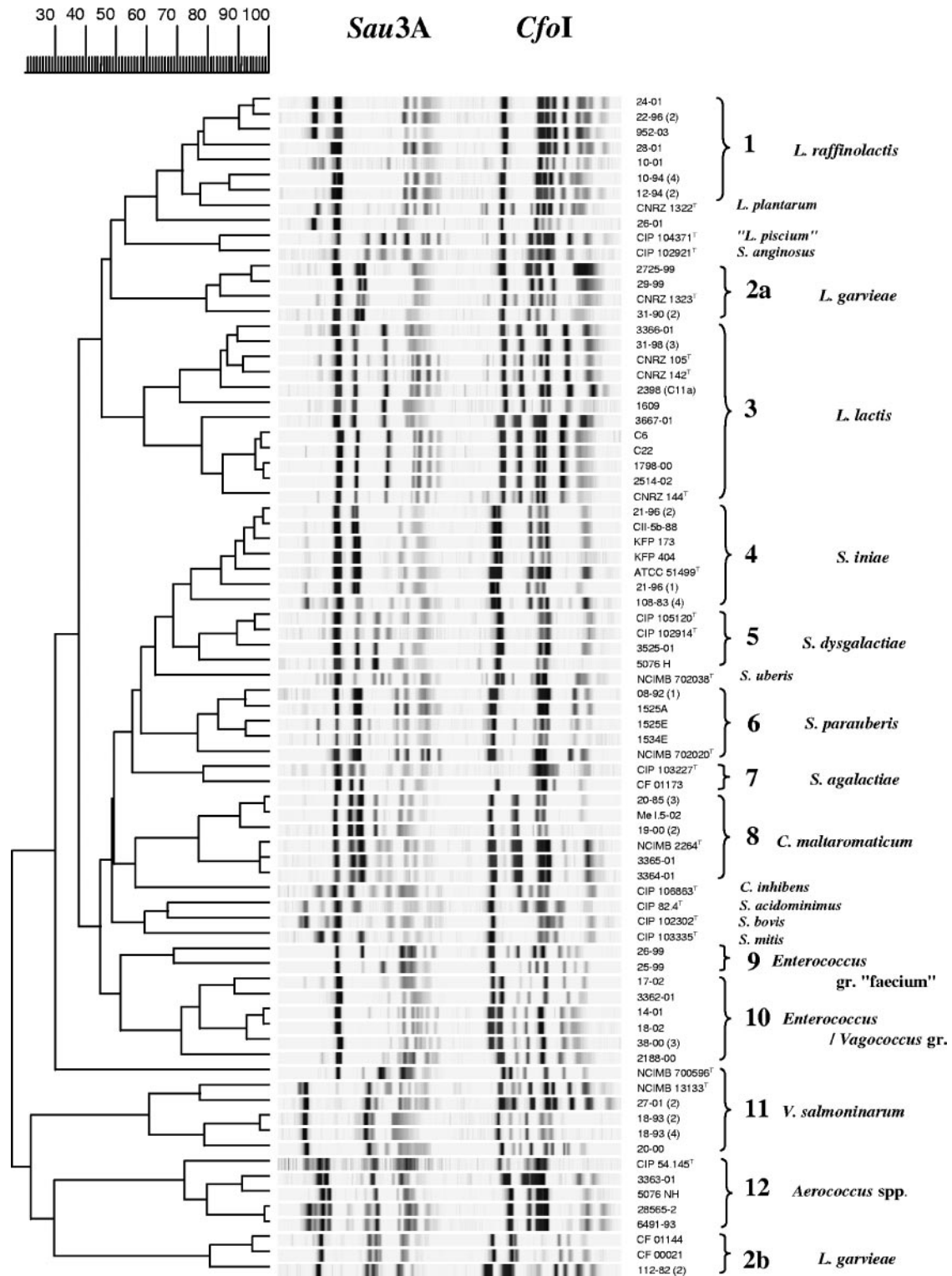


FIG. 1. Comparison of ARDRA profiles resulting from the restriction of 5,000-bp 16S-23S fragments with *Sau3A* and *CfoI*, using the Pearson similarity coefficient and the unweighted-pair group method using average linkages program included in GelCompar software (Applied-Maths, Belgium).

by the presumptive identification of our isolates 12-94 (2) and 22-96 (2) as *L. piscium* by API systems (Table 1). Whereas additional research easily showed that the ARDRA profile resemblance with *Streptococcus anginosus* (Fig. 1) was coinci-

dental, it was more difficult to analyze the relationship between *L. piscium* and *L. raffinolactis*. Following further 16S rRNA gene sequence checks conducted at the CIP (D. Clermont, personal communication), doubts have arisen with regard to

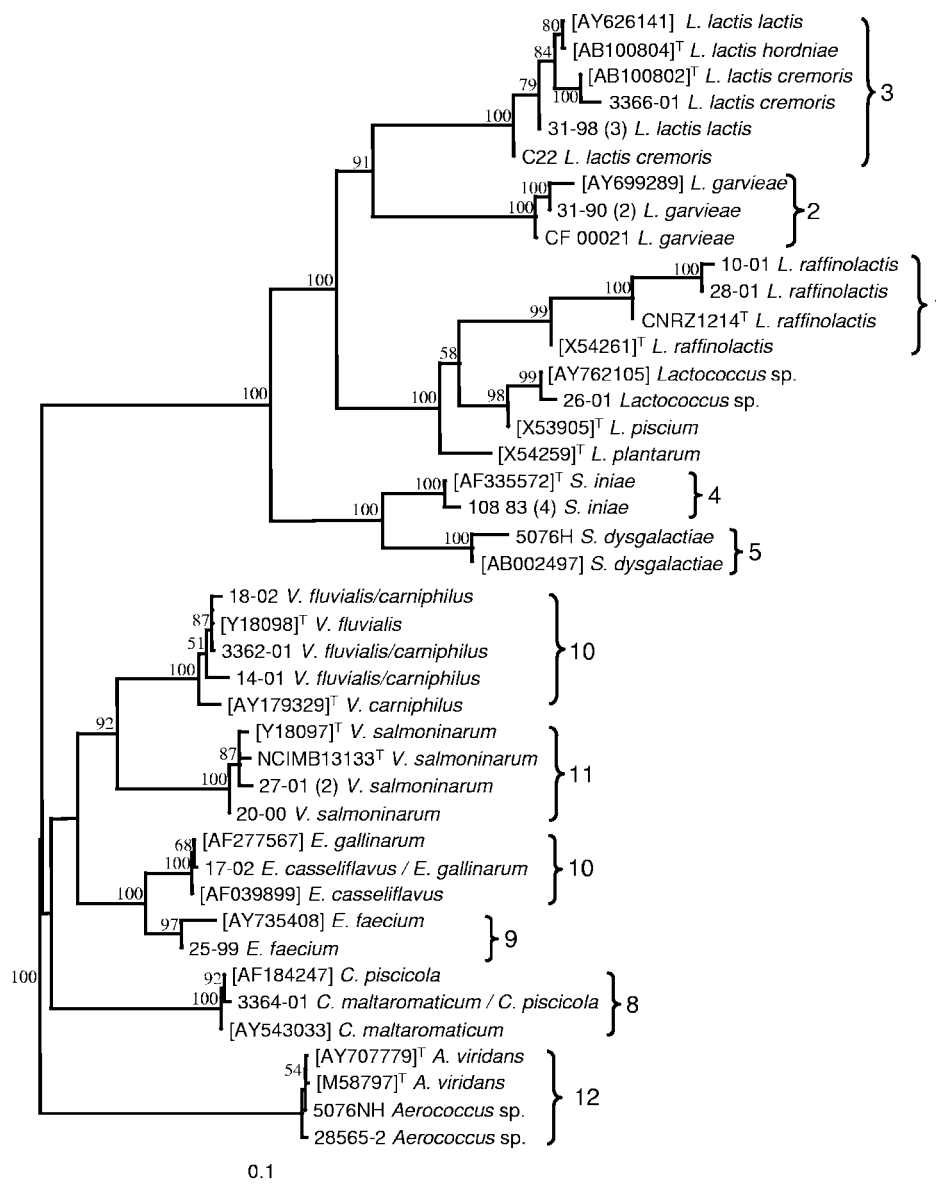


FIG. 2. Distance tree resulting from comparison of 16S rRNA gene sequences of selected isolates with reference sequences extracted from the GenBank database. ARDRA groupings are indicated. The tree was constructed using the model of Jukes and Cantor (18) and the neighbor-joining method included in PAUP software (40). Bootstrap values (percentages for 1,000 replicates) of >50% are indicated at the nodes. GenBank accession numbers are shown in square brackets, and "T" indicates type strains. The *Aerococcus* group was used as an outgroup. Bar, 10% divergence.

the conformity of CIP 104371<sup>T</sup> and other type strain cultures maintained in several collections to ATCC HRIA 68, the first culture that was deposited and the most representative of the original description of *L. piscium* (46). Unlike the isolates described by Sakala et al. (35), the type strain is a fastidious organism and is probably the only one ever to have been isolated from diseased fish (46). Our tentative conclusion is that *L. raffinolactis* and *L. piscium* are closely related bacteria regularly isolated from fish and food products, sometimes in the clinical context, although any detrimental effect on health is still far from being demonstrated.

Since close relationships have repeatedly been found in the phylogenetic comparison of *L. lactis* and *L. garvieae*, these two

species may be considered to represent a large cluster of bacteria frequently associated with the aquatic environment and its inhabitants and characterized by wide variability. This makes clear characterization difficult. *L. garvieae* represents a threat mainly under intensive farming conditions but may be isolated from almost all fish species (21). In fact, two of our isolates were from ornamental fish. It thus appears to be as ubiquitous as *L. lactis*, which is not an actual pathogen but may proliferate in weakened or moribund fish, thereby highlighting the importance of differential diagnosis.

*Carnobacterium* species (group 8) are commonly associated with food processing problems, and some of them (*C. maltaromaticum*, *Carnobacterium divergens*, and *Carnobacterium inhi-*

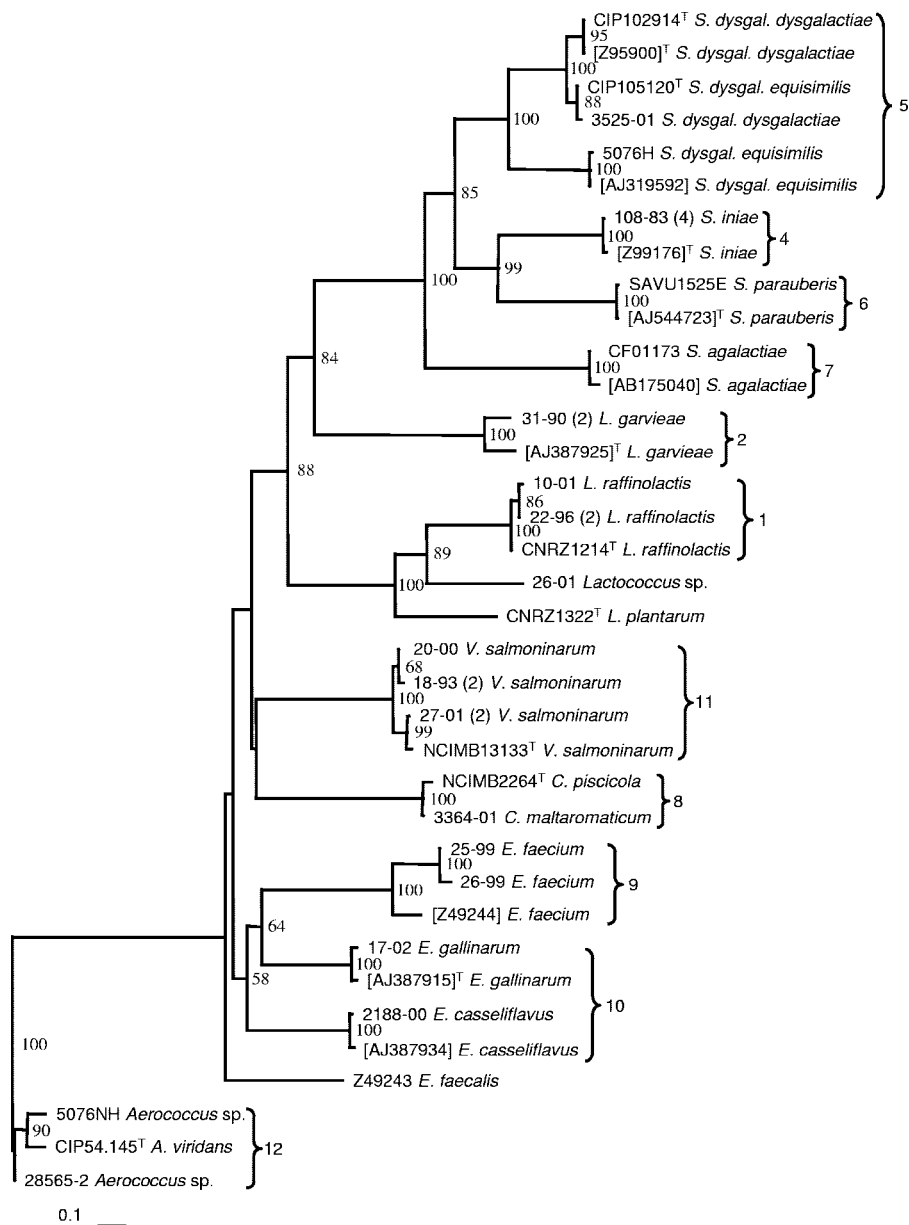


FIG. 3. Distance tree resulting from comparison of *sodA* gene sequences of selected isolates with reference sequences extracted from the GenBank database. ADRA groupings are indicated. The tree was constructed using the model of Jukes and Cantor (18) and the neighbor-joining method included in PAUP software (40). Bootstrap values (percentages for 1,000 replicates) of >50% are indicated at the nodes. GenBank accession numbers are shown in square brackets, and "T" indicates type strains. The *Aerococcus* group was used as an outgroup. Bar, 10% divergence.

*bens*) are normal components of the fish integument and fish intestinal microflora (17, 33). In addition, *C. maltaromaticum* may act as a common opportunistic pathogen and be likely to produce chronic infection in different species of fish under detrimental conditions (26, 41). The ARDRA approach ascertained the identities of the isolates tested; this is consistent with previous reports which concluded that rRNA molecular techniques were particularly well suited for the specific diagnosis or genotyping of these species (6, 32).

*Vagococcus salmoninarum*, which is rare but well documented and of serious consequence in salmonid farms where it occurs, is the last pathogen of note among the bacteria iden-

tified during this study (27, 38). Until strain 20-00 was isolated from a crucian carp, *Carassius carassius*, this agent had been reported only from salmonids. The detection of strains (group 10) related to the *V. fluvialis/carniphilus* cluster (Fig. 2) is too new to form an opinion on their practical significance, but because one of them (14-01) was first misidentified as *V. salmoninarum* in API systems and as ARDRA did not separate them from enterococci, it is important to be aware of this species interference in current diagnostic procedures.

Up to now, none of the species included in groups 9, 10, and 12 has been demonstrated to threaten fish health. While isolation of *Aerococcus* sp. strains from freshwater fish on several

occasions is an original finding, the occurrence of enterococci in fish or fish environments has been mentioned in rare microflora analyses and surveys (5, 19, 29) and could reasonably have been expected in routine diagnostic results. Enterococcus identification, however, has always been confusing, so among the isolates assigned to these groups, definitive identification was only achieved through ARDRA and sequencing data. In spite of the unexpected position of *Enterococcus durans* in the dendrogram (apparently due to experimental flaws), strains 25-99 and 26-99, which came from salmonid farm environments, clearly belonged to the "faecium" group recently defined by Poyart et al. (31). While some of the other isolates were consistent with the *E. casseliflavus/flavescens* (a single species according to Poyart et al. [31]) and *E. gallinarum* definitions, the identification of others was deferred until 16S rRNA gene sequencing revealed that they were *V. fluvialis* and *V. carniphilus* relatives.

It appears that live fish either regularly or transiently host a variety of as yet unsuspected or poorly documented lactic acid bacteria. Since there is little reason to suspect fish pathogenic properties in the new species identified in this study, the main inconvenience for aquaculturists is the risk of misidentification between true pathogens and environmental bacteria. It is therefore important to reconsider the suitability of the techniques employed in routine diagnosis. Although conclusive identification and typing methods have been proposed to improve the diagnosis of lactic acid bacteria in fish, they generally are designed for limited ranges of species of recognized significance (*L. garvieae*, *S. iniae*, and *Carnobacterium* spp.). For more global approaches, ARDRA offers interesting possibilities, provided that the procedure can be simplified and made less cumbersome. Improvement of the already mentioned technique (8) is now in progress, taking into account the issues of the present work.

Another consequence of the presence of such a variety of bacteria is far removed from the aquaculturist's concerns. One cannot help being puzzled by the similarities frequently observed in the lactic acid bacteria isolated from fish and fish products and those that are more generally encountered in the food processing industry (e.g., *Lactococcus*, *Carnobacterium*, and *Enterococcus*) and, sometimes, in human infections (e.g., *Streptococcus* and *Enterococcus*). With regard to fish products, the idea that the microbial populations that develop after slaughtering result from terrestrial environments (24) should probably be partly revised, although the responsibility of lactic acid bacteria in food spoilage is generally questioned. Such severe fish pathogens as *L. garvieae* and *S. iniae* are sometimes isolated from human or other mammalian clinical cases, and *S. iniae* has even been suspected of being a zoonotic agent (13, 45). The presence of other pyogenic streptococci and "faecium" group enterococci in fish could give rise to similar conjectures. Indeed, direct contact between fish and consumers does not appear likely to be frequent compared to other epidemiological sources; the relevant question is whether fish and mammal strains are strictly equivalent and share common virulence properties. Rare typing studies conducted with *S. iniae* (9, 15) and *L. garvieae* (44) isolates of human and fish origin were able to detect some biochemical and genetic differences, respectively, suggesting that fish isolates are special forms

closely adapted to aquatic habitats. Other bacterial species could be investigated in the same way.

To conclude, this research has made it possible to confirm the abundance of lactic acid bacteria in fish and fish farm environments and to assess for the first time the presence or unsuspected frequency of several groups or species. It has added to our knowledge of lactic acid bacteria and to the ways that they can be differentiated using diagnostic procedures and has addressed the refinement of rapid and reliable molecular methods for this purpose. This study paves the way to a better understanding of the species regularly or occasionally encountered in fish health monitoring and to further investigations into their relationships with similar bacteria involved in fish and other animal food processing and in human health.

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