Lactobacillus piscicola, a New Species from Salmonid Fish†

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The name *Lactobacillus piscicola* sp. nov. is proposed for a group of 17 bacterial strains that were isolated from diseased rainbow trout (*Salmo gairdneri*), cutthroat trout (*Salmo clarki*), and chinook salmon (*Oncorhynchus tshawytscha*). This bacterium was found most frequently in infected fish which had suffered some form of stress, such as that which occurs at spawning. Occasionally, pathological signs in the internal organs or skin were observed. Phenotypically, *L. piscicola* belongs to the family *Lactobacillaceae* and can be distinguished from other species of *Lactobacillus* by its morphology and physiological characteristics. DL-Lactic acid was produced homotermentatively from glucose. Diaminopimelic acid was present in the cell wall peptidoglycan. The 17 isolates were closely related genetically, as demonstrated by similar percent guanine-plus-cytosine contents (35 mol%) and high deoxyribonucleic acid reassociation values, both characteristics of a single species. The isolates exhibited less than 10% deoxyribonucleic acid reassociation with other reference *Lactobacillus* strains with similar guanine-plus-cytosine contents. Strain B270 (= ATCC 35586), which was isolated in 1970 from diseased cutthroat brood trout at Bandon Hatchery in Oregon, is designated the type strain of this new species of *Lactobacillus*.

A bacterium with characteristics resembling those of a *Lactobacillus* has been isolated by workers in our laboratory from rainbow trout (*Salmo gairdneri*) and cutthroat trout (*Salmo clarki*) for more than 25 years at various hatcheries in Oregon. These isolations have occurred most frequently from fish 1 year old and older which may have experienced stress, such as that associated with handling and spawning. The pathological signs have been varied and include septicemia, distention of the abdomen, splenomegaly, accumulation of ascites fluid, large muscular abscesses, internal hemorrhaging, and blood cavities or blisters under the skin. Various combinations of these disease signs have been observed in chronically infected trout. *Lactobacilli* have been isolated from a variety of salmonid (14) and nonsalmonid species (43). Two strains isolated in our laboratory from diseased brood trout were examined previously, but the taxonomic status of the isolates was never determined (N. Sriranganathan, Ph.D. thesis, Oregon State University, Corvallis, 1974).

Lactobacilli are commonly found in fermenting plant and animal products and also in the oral cavities and intestinal tracts of warm-blooded animals, including humans (4). Pathogenicity in warm-blooded animals, although rare, has been reported (2, 20, 50). The possible role of *lactobacilli* as the cause of disease in salmonid fish has been described previously (8, 40, 41), but the bacterium associated with the condition has not been identified. In this study, biochemical tests and deoxyribonucleic acid (DNA) hybridization data were used to compare and classify the *Lactobacillus* strains found in salmonid fish. Our results indicate that this organism represents a new species of *Lactobacillus*.

MATERIALS AND METHODS

Bacterial strains. A total of 16 *Lactobacillus* strains isolated from diseased fish at selected hatcheries in Oregon were

obtained by pathologists of the Oregon Department of Fish and Wildlife over a period of 15 years; 13 of these strains

were isolated from rainbow trout, two were isolated from cutthroat trout, and one was isolated from spring chinook salmon (*Oncorhynchus tshawytscha*). The bacterium was isolated from fish by streaking kidney tissue onto brain heart infusion agar or tryptic soy agar (TSA; Difco Laboratories, Detroit, Mich.). Strain GH17 was obtained from P. Ghittino, Centro Studio Malattie Pesci, Torino, Italy. Reference strains *Lactobacillus acidophilus* ATCC 4356^T (T = type strain), *Lactobacillus salivarius* ATCC 11742^T, *Lactobacillus jensenii* ATCC 25258^T, and *Lactobacillus yamanashiensis* subsp. *yamanashiensis* ATCC 27304^T, as well as *Erysipelothrix rhusiopathiae* ATCC 19414^T and *Brochothrix thermosphacta* ATCC 11509^T, were obtained from the American Type Culture Collection, Rockville, Md. *Lactobacillus* sp. strains VPI 7635, VPI 1754, VPI 1294, and VPI 7960, which represent *L. acidophilus* groups A2, A3, A4, and B2, respectively (23), were obtained from J. L. Johnson, Virginia Polytechnic Institute and State University, Blacksburg. Recently, group A2 was found to be synonymous with *Lactobacillus crispatus* (7).

Characterization tests. For biochemical testing, the bacteria were grown in 10 ml of *Lactobacillus* MRS broth (12) (Difco) for 24 h at 27°C. The cells were harvested by centrifugation, washed in phosphate-buffered saline (0.01 M sodium phosphate, pH 7.2), and suspended in 10 ml of MRS identification broth (MRS with glucose and beef extract omitted). API 50L test strips (Analytab Products, Plainville, N.Y.) were inoculated with each bacterial suspension. Test strips inoculated with *Lactobacillus* isolates from fish were incubated at 27°C, whereas test strips containing known *lactobacilli* were incubated at 37°C. The test strips were observed at 24 and 48 h of incubation. Supplementary tests for carbohydrate utilization were conducted by using phenol red broth base (Difco). Stock solutions of each carbohydrate were sterilized by filtration and added to the basal medium to a final concentration of 1%. Tubes were examined at 24 and 48 h of incubation.

Morphology and motility of the bacterium were determined by microscopic examination of Gram stains and wet mounts. In addition, electron microscopy was used to exam-

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ine cells for the presence of flagella and to measure cell size. Cells were grown in brain heart infusion broth (Difco) at 15 and 23°C to log phase and prepared for examination by electron microscopy as described by O'Leary et al. (34). Cytochrome oxidase test strips (General Diagnostics, Warner-Lambert Co., Morris Plains, N.J.) were used to detect the presence of this enzyme. Hydrogen peroxide (3%) was used to determine catalase activity. Hydrogen sulfide production was detected in triple sugar-iron agar (TSI; Difco) slants.

The effect of temperature on growth rate was determined by using a temperature gradient incubator (American Scientific Products, McGraw Park, Ill.). Test tubes containing 7 ml of tryptic soy broth (TSB; Difco) were preincubated at 5°C increments between 5 and 50°C and inoculated with 0.1 ml of a 24-h TSB culture of the test organism. Absorbance at 520 nm was recorded every hour.

The effect of pH on growth rate was determined by inoculating nephelo culture flasks (Belco Glass, Inc., Vineland, N.J.) with 1 ml of a 24-h culture grown in TSB. Each flask contained 50 ml of TSB buffered with 0.067 M phosphate at 0.5-pH unit increments from pH 5.0 to 8.0. The cultures were placed on a rotary shaker at 27°C, and absorbance at 520 nm was recorded every hour. Generation times were calculated by plotting absorbance versus time.

Vitamin requirements. Vitamin requirements were determined by the method of Rogosa et al. (39). A mixture containing vitamin-free acid-hydrolyzed casein and a pancreatic digest of casein was prepared by the method of Ford et al. (15). Medium without vitamins and basal medium which contained all of the vitamins tested were used as controls. Strains of bacteria to be tested were grown at 27°C for 24 h in 10 ml of TSB, harvested by centrifugation, washed twice in sterile phosphate-buffered saline, and resuspended in 10 ml of phosphate-buffered saline. Test tubes containing the vitamin test mixture were inoculated with 0.1 ml of the washed suspension and incubated at 27°C. The tubes were examined for turbidity at 24 and 48 h.

Lactic acid production. Lactic acid production was determined by analyzing spent glucose phosphate broth (1% glucose, 1% Proteose Peptone, 0.1% beef extract, 0.5% NaCl in 0.1 M potassium phosphate buffer, pH 7.4). Nephelo culture flasks containing 100 ml of glucose phosphate broth were inoculated with 1 ml of culture grown at 27°C for 24 h in the same medium. The flasks were placed on a rotary shaker to study aerobic growth and in a GasPak jar (BBL Microbiology Systems, Cockeysville, Md.) containing a hydrogen-generating envelope to study anaerobic growth. Samples of culture grown aerobically were aseptically removed before inoculation and at different times during the fermentation to quantify glucose utilization and acid production. The cultures incubated anaerobically were sampled at 60 h. Samples of cultures collected for lactic acid and glucose analysis were deproteinized and diluted 1:10 (30).

Glucose concentration was assayed colorimetrically as described in *Sigma Technical Bulletin 510* (49). Test tubes containing the reaction mixture were incubated at 37°C in a water bath for exactly 30 min; 1 drop of 2 N HCl was added to each tube after 30 min to stop the reaction.

The total lactic acid content of the deproteinized filtrate was determined by the colorimetric method of Barker and Summerson (1). For this assay, the lactate standards or test solutions were diluted 1:10 by the deproteinization step and were further diluted 1:100 in distilled water. The concentration of L-(+)-lactic acid was determined by the enzymatic procedure described by Mattsson (30). The percentage of L-

(+)-lactic acid was calculated as described by Cato and Moore (6).

Cell wall analysis. Cell wall mucopeptides were isolated and purified by the method of Park and Hancock (35). Cells were harvested by centrifugation at 5,858 × g for 10 min, washed twice in sterile distilled water, and lyophilized before the purification procedure. The trypsin solution described by Schleifer and Kandler (42), which contained 12 mg of crystalline trypsin (Sigma Chemical, St. Louis, Mo.) per ml and 0.1 N sodium phosphate buffer (pH 7.9), was used for the enzymatic digestion step. Two-dimensional descending paper chromatography (42) was used to separate the amino acid components. A known mixture of amino acids containing alanine, diaminopimelic acid, aspartic acid, glutamic acid, lysine, glycine, and serine was used as a control. The relative migration distances compared with alanine were calculated, and amino acids identified by comparison with the standard mixture.

DNA isolation. A representative *Lactobacillus* strain isolated from diseased fish was selected at each of the eight hatcheries where the bacterium was known to occur. The strains from fish and *Brochothrix* were grown in TSB for 24 h at 25°C. Reference *Lactobacillus* strains were grown in MRS broth for 24 h at 37°C. *Erysipelothrix* was grown in brain heart infusion broth supplemented with 1% (vol/vol) calf serum and was incubated at 37°C for 24 h. Cells were harvested by centrifugation, washed twice in sterile distilled water, and frozen. For the preparation of tritium-labeled *Lactobacillus* DNA, strain B270⁺ was grown in 200 ml of TSB supplemented with 0.1% yeast extract and 2 ml of [*methyl*-³H]thymidine (10 µCi/ml of TSB; 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.).

Cells were lysed by a modification of the Garvie technique for heterofermentative lactic acid bacteria (17). Wet packed cells (1 to 2 g) were suspended in 4.5 ml of distilled water and lysed with 0.25 ml of Promase-CB (40 mg/ml in 0.15 M NaCl, 0.01 M ethylenediaminetetraacetate, pH 7.0; Calbiochem-Behring, La Jolla, Calif.) and 5 mg of crystalline lysozyme (Sigma) at 40°C for 4 h. Sodium dodecyl sulfate (0.5 ml; 20%, wt/vol) was added to facilitate lysis. The mixture was sheared by two passes through a French pressure cell at 16,000 lb/in². Ribonuclease (0.5 ml; 1 mg/ml in 0.15 M NaCl, pH 5.0; Sigma) was added, and the mixture was incubated at 40°C for 1.5 h. Saline-ethylenediaminetetraacetate buffer (0.15 M NaCl, 0.01 M disodium ethylenediaminetetraacetate, pH 8.0) was added to a final volume of 25 ml. Protein was removed from the lysed culture by extraction with water-saturated redistilled phenol. DNA was purified by the hydroxylapatite procedure of Johnson (22).

G+C determination. DNA preparations were dialyzed against 0.1× SSC (0.015 M NaCl, 0.0015 M trisodium citrate), and the absorbance at 260 nm was recorded at 1.0°C increments between 50 and 90°C with a Beckman model DU-8 computing spectrophotometer equipped with a Tm Computer Module. The guanine-plus-cytosine (G+C) content of *Lactobacillus* DNA was calculated by using the equation of Mandel et al. (29). DNA extracted and purified from *Escherichia coli* strain WP2 (46) (51.0 mol% G+C) was used as a standard.

DNA hybridization. DNA hybridization experiments were performed by the hydroxylapatite free solution method described by Johnson (22). Polypropylene microtubes (capacity, 0.5 ml; Sarstedt, Inc., Princeton, N.J.) were used as reaction vials. These were incubated in a water bath (Forma Scientific, Marietta, Ohio) at 58.0°C (melting temperature [T_m] – 25°C or 68.0°C (T_m – 15°C) for 22 h.

FIG. 1. Electron micrograph of strain B270^T grown in TSB at 15°C. Bead diameter = 0.264 μ m.

Precipitated DNA was collected on 0.45- μ m nitrocellulose filters (type HAWP-025; Millipore Corp., Bedford, Mass.). The filters were dried, placed into scintillation vials containing 10 ml of Omnifluor (New England Nuclear Corp.), and counted for 10 min with a Beckman model LS 8000 liquid scintillation counter.

RESULTS

Morphology and growth. All isolates of *Lactobacillus* from fish were gram-positive, nonmotile, nonsporeforming rods which became gram variable within 24 h on TSA. The cell size in log phase was approximately 1.1 to 1.4 by 0.5 to 0.6 μ m. Electron micrographs of the type strain from broth cultures (Fig. 1) revealed short chains of two or three nonflagellated cells. In Gram-stained smears prepared from the kidneys of infected fish, the cells were generally smaller and occurred singly more often. Colonies on TSA were white, round, entire, nonpigmented, and less than 2 mm in diameter after 48 h of incubation at 25°C.

Strain B270^T grew best at 30°C, with a doubling time of 55 min. The growth rate decreased rapidly at temperatures below 15°C, and no growth occurred at temperatures above 40°C after 56 h of incubation. The optimum pH range for strain B270^T was from 6.0 to 7.0. At pH 7.0, the minimum generation time was 65 min at 27°C.

Biochemical tests. The biochemical test results for the *Lactobacillus* isolates from fish are shown in Table 1. All of

the isolates were catalase and oxidase negative, did not reduce nitrate, and did not produce H₂S on TSI slants. Some variations were observed in the carbohydrate fermentation patterns of the 17 fish isolates. Except for the inability to ferment gluconate with the production of CO₂, the isolates

TABLE 1. Biochemical reactions of *Lactobacillus* sp. isolated from diseased salmonid fish

Test	<i>Lactobacillus piscicola</i>		<i>Lactobacillus</i> sp. isolated by:	
	No. of strains positive/no. of strains tested	Reaction of type strain B270	Ross and Toth ^a	Cone ^b
Glycerol	14/17	+	NR	NR
Erythritol	0/17	—	NR	NR
Arabinose	0/17	—	NR	+
Ribose	17/17	+	NR	NR
<i>d</i> (+)-Xylose	0/17	—	NR	NR
<i>l</i> (-)-Xylose	0/17	—	NR	NR
Adonitol	0/17	—	NR	NR
Methyl-xyloside	0/17	—	NR	NR
Galactose	11/17	+	NR	NR
<i>d</i> (+)-Glucose	17/17	+	+	+
<i>d</i> (-)-Fructose	17/17	+	NR	NR
<i>d</i> (+)-Mannose	17/17	+	NR	NR
<i>l</i> (-)-Sorbitose	0/17	—	NR	NR
Rhamnose	0/17	—	NR	—
Dulcitol	0/17	—	NR	—
Inositol	0/17	—	NR	—
Mannitol	15/17	+	NR	+
Sorbitol	0/17	—	NR	+
Methyl-D-mannoside	0/17	—	NR	NR
Methyl-D-glucoside	9/17	w	NR	NR
N-Acetyl glucosamine	17/17	+	NR	NR
Amygdalin	17/17	+	NR	NR
Arbutine	17/17	+	NR	NR
Esculin	17/17	+	NR	—
Salicin	17/17	+	NR	+
<i>d</i> (+)-Cellobiose	17/17	+	NR	NR
Maltose	16/17	+	+	+
Lactose	6/17	—	+	—
<i>d</i> (+)-Melibiose	5/17	w	NR	NR
Sucrose	17/17	+	+	+
<i>d</i> (+)-Trehalose	17/17	+	NR	NR
Inulin	0/17	—	NR	NR
<i>d</i> (+)-Melezitose	2/17	—	NR	NR
<i>d</i> (+)-Raffinose	5/17	w	NR	NR
Dextrin	0/17	—	NR	NR
Amylose	0/17	—	NR	NR
Starch	0/17	—	NR	NR
Glycogen	17/17	—	NR	NR
NH ₃ from arginine	0/17	—	NR	—
Gas from glucose	16/17	+	NR	NR
Teepol (0.4%)	16/17	+	NR	NR
Teepol (0.6%)	16/17	+	NR	NR
Gluconate	0/17	—	NR	NR
Urease	0/17	—	NR	—
ONPG ^d	10/17	+	NR	NR
Catalase	0/17	—	—	—
Cytochrome oxidase	0/17	—	—	—
Nitrate reduction	0/17	—	NR	NR
Voges-Proskauer	15/17	+	—	—

^a Data from reference 40.

^b Data from reference 8.

c, +, Positive reaction; —, negative reaction; w, weak positive reaction; NR, not reported.

^d ONPG, *o*-Nitrophenyl- β -D-galactopyranoside.

FIG. 2. Aerobic growth of strain B270^T and lactic acid production in 0.1 M phosphate-buffered glucose broth (pH 7.5). $A_{525\text{nm}}$, Absorbance at 525 nm.

most closely resemble the streptobacterium group of the genus *Lactobacillus* (37).

Vitamin requirements. The isolates from fish required folic acid, riboflavin, pantothenate, and niacin for growth. There was no requirement for vitamin B₁₂, biotin, thiamine, or pyridoxal. These isolates were most similar to *L. acidophilus* with respect to vitamin requirements.

Lactic acid production. Growth and lactic acid production by strain B270^T in glucose phosphate broth demonstrated that the lactate concentration continued to increase when the cells were in stationary phase and was accompanied with a corresponding decrease in pH (Fig. 2). Lactic acid produc-

tion was enhanced when cells were grown anaerobically with 88 μmol of lactate per ml of broth (measured after 60 h of incubation at 27°C). A glucose analysis showed that 42 μmol of glucose per ml of broth was fermented in the same period. Homofermentative utilization of glucose would theoretically yield 2.0 μmol of lactate per μmol of glucose fermented. With fish *Lactobacillus* sp. strain B270^T 2.1 μmol of lactate was produced per μmol of glucose (88/42), indicating that all of the glucose was converted homofermentatively to lactic acid. The L-(+)-lactate concentration was 52 μmol/ml, indicating that 59% of the lactate (52/88) was of the L-(+) isomer and that DL-lactate was produced.

Cell wall analysis. Diaminopimelic acid, alanine, and glutamic acid were found in the cell wall peptidoglycan from each tested strain isolated from fish. No lysine was associated with the cell wall peptidoglycan of any of these strains. Among the reference strains, diaminopimelic acid was present in cell walls of *L. plantarum*, and lysine was present in *Lactobacillus casei* and *Erysipelothrix rhusiopathiae*.

DNA base composition and hybridization. The DNAs of the fish *Lactobacillus* strains melted at relatively low temperatures (approximately 66°C in 0.1 × SSC). The G+C contents of these strains ranged from 33.7 to 35.6 mol%. These values are similar to those of the group of lactobacilli having low G+C contents (37) which were included in this study (Table 2).

The reassocia-tion values obtained with DNAs from strain B270^T and other strains from fish ranged from 73 to 92% at $T_m - 25^\circ\text{C}$ and 70 to 93% at $T_m - 15^\circ\text{C}$. No more than 7%

TABLE 2. DNA relatedness to type strain B270 to eight strains isolated from diseased salmonid fish and to selected reference strains

Strain	G+C content (mol%)	% Reassociation at:	
		$T_m - 25^\circ\text{C}$	$T_m - 15^\circ\text{C}$
Strains from fish			
B270 ^T	35.6	100	100
LRPK1	35.5	92	93
CC1-83	35.3	92	93
K180	33.7	87	89
GHIT	35.0	91	90
OS3-68	35.3	87	89
RR2T-79	35.2	87	88
WH4	34.9	73	70
Reference strains			
<i>L. acidophilus</i> ATCC 4356 ^T	37.0 ^a	0	1
<i>L. acidophilus</i> VPI 1754 (group A3) ^b	37.4	0	0
<i>L. acidophilus</i> VPI 1294 (group A4) ^b	34.2	0	0
<i>L. acidophilus</i> VPI 7960 (group B2) ^b	32.8	0	0
<i>L. crispatus</i> VPI 7635 ^c	35.7	0	0
<i>L. salivarius</i> ATCC 11742 ^T	35.0 ^a	2	1
<i>L. jensenii</i> ATCC 25258 ^T	36.0 ^a	0	3
<i>L. yamanashiensis</i>	33.2 ^d	7	ND ^e
subsp. <i>yamanashiensis</i>			
ATCC 27304 ^T			
<i>Erysipelothrix rhusiopathiae</i>	36.0 ^a	2	0
ATCC 19414 ^T			
<i>B. thermosphacta</i>	37.4	2	4
ATCC 11509 ^T			

^a Data from reference 4.

^b Strains obtained from J. L. Johnson, Virginia Polytechnic Institute and State University, Blacksburg.

^c Synonymous with *L. acidophilus* group A2 (7).

^d Data from reference 32.

^e ND, Not determined.

reassociation at $T_m - 25^\circ\text{C}$ or 4% reassociation at $T_m - 15^\circ\text{C}$ was observed with DNA from strain B270^T and DNA from *Brochothrix*, *Erysipelothrix*, or any reference *Lactobacillus* strain (Table 2).

DISCUSSION

The morphology and physiological characteristics of the bacteria isolated from diseased salmonid fish suggest that these organisms belong to the family *Lactobacillaceae* (4). All of the isolates were gram-positive, facultatively anaerobic, nonmotile, nonsporeforming, short rods occurring in short chains. They did not produce cytochrome oxidase or catalase, did not reduce nitrate, did not produce H_2S on TSI slants, fermented a variety of carbohydrates with the production of lactic acid, and required a number of vitamins. These features are typical of the genus *Lactobacillus* (38) and differ from the characteristics of the other genera in the family *Lactobacillaceae* and the lactic acid bacteria belonging to the family *Streptococcaceae*.

Exponential phase cultures of *Brochothrix* produce rods in long, filamentous, knotted masses, which give rise to coccoid forms in stationary phase cultures (51). The species of the *Listeriaceae* are coccoidal, are motile by means of peritrichous flagella, and are usually catalase positive (45, 52). *Erysipelothrix* produces H_2S when grown on TSI agar, is aerobic, and tends to form long filaments which thicken and show characteristic granules (44). The cell walls of *Erysipelothrix* contain lysine, which is absent in the cell walls of the isolates from fish. *Caryophanon* forms large rods divided by cross walls into numerous disk-shaped cells and is strictly aerobic (18).

The new isolates from fish were also distinct from the lactic acid bacteria belonging to the family *Streptococcaceae* (9). Within this family, *Aerococcus* and *Gemella* show poor anaerobic growth (13, 36), whereas *Streptococcus* and *Pediococcus* are coccoidal (10, 26). *Leuconostoc* is spherical or lenticular, produces D(-)-lactic acid, and is heterofermentative (16).

The bacterium from fish, represented by strain B270^T, produced lactic acid homofermentatively from glucose without gas formation, did not require thiamine for growth, grew at 15°C but not at 45°C , and fermented ribose; these are features of the streptobacterium group of the genus *Lactobacillus* (47). However, the inability of the isolates to ferment gluconate with the production of CO_2 is not typical of members of this group. The low G+C contents of the isolates, although different from the G+C contents of the majority of the streptobacteria, are similar to the G+C content of *Lactobacillus yamanashiensis*, which recently has been placed in the streptobacterium group (47).

The species of *Lactobacillus* with low G+C DNA contents (33 to 39 mol%) differ from each other in fermentation pattern, the type of fermentation (homo- or heterofermentative), the type of lactic acid produced [L-(+), DL-, or D(-)], and the type of diamino acid in the cell wall peptidoglycans (Table 3). The cell wall type and G+C contents of the isolates from diseased fish are similar to those of *Lactobacillus vaccinostrercus* (33), *Lactobacillus malvaronicus* (31), *Lactobacillus vitulinus* (48), *L. yamanashiensis* subsp. *yamanashiensis*, and *L. yamanashiensis* subsp. *mal* (5, 32). *L. vaccinostrercus* is clearly distinguished by its fermentation pattern and heterofermentative production of DL-lactic acid. *L. malvaronicus* produces L-(+)-lactic acid and is characterized by the production of volatile aldehydes, which give a malty aroma in milk and TSB (31). *L. vitulinus* is anaerobic, produces only D(-)-lactic acid, and grows at 45°C but not at

15°C (48). In contrast, the isolates from fish are homofermentative, produce DL-lactic acid, are facultative anaerobes, and grow at temperatures ranging from 6 to 40°C .

Other species of *Lactobacillus* with cell walls containing diaminopimelic acid include *Lactobacillus plantarum*, *Lactobacillus ruminis*, *Lactobacillus agilis*, and *Lactobacillus shарае* (38, 48, 53). These species differ from the isolates from fish by their higher G+C contents (44 to 53 mol% G+C). The isolates examined in this study are similar to *L. plantarum* in cell wall peptidoglycan type and carbohydrate pattern, but may be differentiated by their inability to ferment gluconate and sorbitol and their lower G+C content (35 mol%). *L. plantarum* ferments both gluconate and sorbitol and has a G+C content that is 10 mol% greater (38). Studies have shown that bacteria having G+C ratios which vary by more than 4 mol% usually belong to different species (3, 11, 24).

A *Lactobacillus* species identified as *L. plantarum* has been isolated from the intestinal tract of saithe (*Gadus virens*) (43). This isolate differs from the type strain of *L. plantarum* by producing L-(+)-lactic acid from glucose and by its inability to ferment sorbitol. The cell wall type of the isolate of Schröder, its ability to grow at low temperatures, its inability to ferment sorbitol, and its isolation from fish are characteristics which are similar to characteristics of the isolates described in this paper. However, the limited number of biochemical tests reported in the Schröder study and the lack of any genetic information, such as G+C content, precludes further comparison. The isolates from fish were most similar to *L. yamanashiensis* subsp. *yamanashiensis* with respect to G+C content, type of fermentation, type of lactic acid isomer produced, and fermentation pattern. However, *L. yamanashiensis* differs greatly from the fish isolates because it produces dextran from sucrose, does not ferment mannitol, maltose, or ribose, does not hydrolyze arginine, and exhibits only 7% DNA reassociation with the fish isolates. Furthermore, *L. yamanashiensis* may be weakly motile by means of a few peritrichous flagella (32). None of the isolates from fish was motile. Electron micrographs showed that flagella were absent at both 15 and 23°C .

The biochemical results for the *Lactobacillus* species isolated by Ross and Toth (40) from rainbow brood trout are similar to the results obtained in this study. However, the limited number of observations in the study of these authors precludes a precise comparison (Table 1). The *Lactobacillus* species described by Cone (8) differed from the fish isolates in this study in a number of important biochemical characteristics (Table 1). The precise taxonomic status of the isolates of Cone remains undetermined.

DNA reassociation experiments were used to assess the genetic relationships among the strains from fish, reference strains of *Lactobacillus*, and strains of other genera belonging to the family *Lactobacillaceae* which have G+C contents similar to the G+C content of the bacterium isolated from diseased fish (Table 2). Whenever possible, each species was represented by its type strain. The reassociation values indicate a lack of genetic relationship between the fish strains, represented by strain B270^T, and any of the reference strains tested (Table 2).

Although the 17 strains of lactobacilli from fish showed some variation in their carbohydrate fermentation patterns, they were closely related genetically, as demonstrated by similar G+C contents and relatively high DNA reassociation values (73 to 92% at $T_m - 25^\circ\text{C}$). Studies have shown that bacteria having reassociation values exceeding 70% generally belong to the same species (21). DNA reassociations at T_m

TABLE 3. Characteristics for differentiating *L. piscicola* from *Brochothrix*, *Erysipelothrix*, and *Lactobacillus* spp. having low G+C contents

Characteristic or test	<i>L. piscicola</i> B270 ^T	<i>L. acidophilus</i> ^a	<i>L. salivarius</i> ^a	<i>L. jensenii</i> ^a	<i>L. kandleri</i> ^b	<i>L. gasseri</i> ^c	<i>L. vaccinostercus</i> ^d	<i>L. vitulinus</i> ^e	<i>L. yamanashiensis</i> subsp. <i>yamanashiensis</i> ^f	<i>L. yamanashiensis</i> subsp. <i>malii</i> ^g	<i>L. maltaromicus</i> ^h	<i>L. hordniae</i> ⁱ	<i>B. thermosphacta</i> ^j	<i>Erysipelothrix rhusiopathiae</i> ^a
Lactic acid isomer	DL	DL	L-(+)	D-(-)	DL	DL	DL	D-(-)	DL	NR	L-(+)	L-(+)	L-(+)	NR
Fermentation type	Homo	Homo	Homo	Homo	Hetero	Homo	Hetero	Homo	Homo	Homo	Homo	Homo	Homo	NR
Peptidoglycan type	DAP ^k	Lys	Lys	Lys	Lys	Lys	DAP	DAP	DAP	DAP	DAP ^l	Lys ^m	DAP	Lys
DNA G+C content (mol%)	35	37	35	36	39	34	36	36	33	33	36	33	36	36
Growth at 15°C	+	+	+	+	NR	+	+	+	+	+	+	+	+	+
Growth at 37°C	+	+	+	+	NR	+	+	+	NR	NR	+	+	+	+
Growth at 45°C	+	+	+	+	NR	+	+	+	NR	NR	+	+	+	+
Esculin hydrolysis	+	+	+/–	+	NR	NR	NR	+	+	+	NR	NR	NR	NR
NH ₃ from arginine	+	+	+	NR	+	+	NR	+	+	+	NR	+	NR	NR
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	NR
Cellobiose	+	+	+	+	+	+	+	+	NR	+/–	+	+	+	NR
Galactose	+	+	+	+	+	NR	+/–	+	+	+/–	+	+	+/–	+
Inulin	+	NR	NR	NR	NR	+	+	+/–	NR	+	NR	+	NR	+
Lactose	+	+	+	+	+	+	+/–	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+/–	+	+	+	+	+	+	NR
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	NR
Mannose	+	+	+	+	+	NR	NR	+	+	+	+	+	+	NR
Melezitose	+	+	+	+	+	NR	NR	+	+	+	+	NR	+	NR
Melibiose	+/–	+/–	+	+	+	+/–	+	+	NR	+	+	+	+/–	+/–
Raffinose	+/–	+/–	+	+	+	+/–	+	+	+	+	+	+	NR	+
Rhamnose	+	+	+/–	+	NR	+	+	+	+	+	+	+	+	NR
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	NR	NR
Salicin	+	+	+/–	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+/–	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Dextran from sucrose	+	NR	NR	NR	+	NR	NR	NR	+	NR	NR	+	NR	NR
Trehalose	+	+	+	+	+	+/–	+	+/–	NR	+	+	+	NR	+
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+/–
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Habitat	Diseased fish	Mouth, feces, gut	Mouth, feces, gut	Human vaginal discharge	Desert spring, plants	Mouth, feces, gut	Cow dung	Bovine rumen	Wine must	Cider	Milk, butter	Insect body	Pork sausage	Mammals, birds, fish

^a Data from reference 4.^b Data from reference 19.^c Data from reference 28.^d Data from reference 33.^e Data from reference 48.^f Data from reference 32.^g Data from reference 5.^h Data from reference 31.ⁱ Data from reference 27.^j Data from reference 51.^k DAP, Diaminopimelic acid; Lys, lysine.^l Data from reference 53.^m Data from reference 25.ⁿ +, Positive; –, negative; +/–, variable; NR, not reported.

– 15°C revealed no further nucleotide divergence among the isolates from fish.

The habitat of the new isolates and their association with disease in salmonid fish are unique among the genus *Lactobacillus*. Species of *Lactobacillus* with similar G+C contents are mainly associated with dairy products, with the oral and digestive tracts of animals and insects, or with fermentation products, such as wine and cider (Table 3). The isolates from fish best match the description of the genus *Lactobacillus*. They are, however, distinct from other members of the genus genetically, in their fermentation patterns, and by their association with disease in fish, and thus should be recognized as a new species, for which we propose the name *Lactobacillus piscicola* (pis.ci' co.la. L.n. piscis fish; L. suff. -cola dweller; M.L. n. piscicola fish (dweller). The designated type strain, strain B270, was isolated in 1970 at the Bandon Trout Hatchery (Coos County, Oreg.) from a diseased adult cutthroat trout which was part of a post-spawning loss of brood fish.

Description of *Lactobacillus piscicola* type strain B270. Gram-positive, nonmotile, nonsporeforming rods which occur singly and in short chains.

Grows well on many standard laboratory media, including TSA and brain heart infusion agar, and in MRS broth and thioglycolate broth.

Colonies are pinpoint, convex, white, circular, entire, and nonpigmented when grown at 25°C for 24 h on TSA.

Temperature range for growth is 6 to 40°C. Optimum temperature is approximately 30°C.

Optimum pH range is from 6.0 to 7.0.

Facultatively anaerobic. DL-Lactate is produced homofermentatively. Lactic acid production is enhanced under anaerobic growth conditions.

Folic acid, riboflavin, panthothenate, and niacin are required for growth. Vitamin B₁₂, biotin, thiamine, and pyridoxal are not required.

Catalase and oxidase are not produced.

Nitrate is not reduced to nitrite.

No gas is produced from glucose or from gluconate.

Acid is produced from glycerol, ribose, galactose, glucose, fructose, mannose, mannitol, N-acetyl glucosamine, amygdalin, arbutine, salicin, cellobiose, sucrose, and trehalose. Acid is not produced from arabinose, xylose, sorbose, rhamnose, dulcitol, inositol, methyl-D-mannoside, inulin, or melzitose.

Arginine and esculin are hydrolyzed.

H₂S not detected in TSI slants.

Resistant to 0.4 and 0.6% Teepol.

Cell wall peptidoglycan contains diaminopimelic acid. DNA G+C content is 34 to 36 mol%.

Host: isolation associated with salmonid fish, but the organism may occur among nonsalmonid species.

Disease: lactobacillosis of fish, a septicemic disease of salmonid fish producing varied pathology.

Distribution: North America, Europe, and perhaps other areas where salmonid fish occur.

The type strain is strain B270 (= ATCC 35586), which was isolated in 1970 from a diseased adult cutthroat trout reared at Bandon Trout Hatchery in Coos County, Oreg.

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