Streptococcus raffinolactis Orla-Jensen and Hansen, a Group N *Streptococcus* Found in Raw Milk

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The properties of the lactate dehydrogenases, percent guanine plus cytosine in the deoxyribonucleic acid (DNA), and DNA/DNA hybridization studies have shown that three strains of group N streptococci do not belong to either *Streptococcus lactis* or *Streptococcus cremoris*. The biochemical properties of the three strains were published about 25 years ago, and at that time the strains were not assigned to any species. The three strains are here identified as members of *Streptococcus raffinolactis* Orla-Jensen and Hansen.

Orla-Jensen and Hansen (11) studied the bacteriological flora of spontaneously soured milk and commercial butter starters. Besides *Streptococcus lactis* and *Streptococcus cremoris*, they named four other species among their isolates. These species have been largely overlooked since the original publication. However, Garvie (3) described some raw-milk group N streptococci which fermented raffinose and which were similar to strains of *Streptococcus raffinolactis* Orla-Jensen and Hansen. Swartling (12) included three of Garvie's strains in his work and was of the opinion that they were neither *S. lactis* nor *S. cremoris*. In the early 1950s it was felt that not enough was known about the strains to justify calling them *S. raffinolactis* as it was possible that they might prove to be subspecies of either *S. lactis* or *S. cremoris*.

Three of the strains isolated by Garvie were freeze-dried and kept in the National Collection of Dairy Organisms (NCDO). Recently they were examined for additional properties, and the results of these studies have shown that they belong to neither *S. lactis* nor *S. cremoris*.

MATERIALS AND METHODS

Strains. All of the strains studied are in the National Collection of Dairy Organisms. *S. raffinolactis* NCDO 617 (23.C.5), NCDO 618 (30.C.1), and NCDO 619 (33.C.1) were compared with *S. lactis* NCDO 712 and NCDO 2031, *S. lactis* subsp. *diacetylactis* NCDO 1007, and *S. cremoris* NCDO 924.

Cultures were grown in a yeast-glucose-phosphate broth (YGPB) containing (weight/volume): peptone (Oxoid), 1.0%; Lemco (Oxoid), 0.8%; glucose, 1.0%; NaCl, 0.5%; yeast extract (Oxoid), 0.3%; KH_2PO_4 , 0.15%; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02%; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.005%; pH 6.8.

All cultures were incubated at 30°C.

Preparation of lactate dehydrogenase (LDH) in cell extracts. Cultures were grown overnight at 30°C in YGPB. Cells were removed by centrifuging and were washed twice at 4°C with 0.85% NaCl. Cell

pellets were quick-frozen in CO_2 and stored, at least overnight, at -18°C . When cells were required, a chilled solution containing 0.425% NaCl in 0.01 M phosphate (pH 7.0) was added to the tube containing the frozen pellet; this material was kept at 4°C until the pellet was thawed. Approximately 5.0 ml of chilled solution was used for 0.5 to 0.7 g of wet-packed cells. The cell suspension was transferred to a 10-ml Kimex tube, and Ballotini beads (0.115 to 0.180 mm in diameter) were added to fill the tube to about 1 cm below the shoulder. The cells were broken on a Braun homogenizer for 6 min while kept cold with 2- to 3-s blasts of CO_2 every 30 s. After the cells were broken, the liquid was removed and the glass beads were washed three times with 3.0 ml of the sodium chloride-phosphate solution. The washings were added to the supernatant. Glass beads and cell debris were precipitated by centrifuging at $36,000 \times g$ for 45 min at 4°C.

The supernatant was dialyzed against 0.0425% NaCl in 0.01 M phosphate at pH 7.0, and the resulting liquid was freeze-dried and stored in a deep freeze.

For use, the freeze-dried material was dissolved in 0.01 M phosphate buffer (pH 7.0). Usually a concentration of 10 mg of freeze-dried powder per ml gave an active preparation.

Estimation of activity. LDH activity was assayed by following the decrease in absorption at 340 nm on a Unicam SP1700 spectrophotometer with a Philips XY recorder adjusted so that the x axis recorded time. A 0.02-ml volume of enzyme solution was added to 1.0 ml of assay solution to start the reaction. The assay solution contained 0.067 mM tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (pH 7.0) or 0.067 mM acetate buffer (pH 5.5) and 0.15 μM reduced nicotinamide adenine dinucleotide (NADH). The standard assay also contained 3.3 mM sodium pyruvate, 0.5 mM fructose 1,6-diphosphate (FDP), and no additional phosphate. When variations in these concentrations were used, they are shown in the tables. The decrease in optical density in 1 min was recorded. All reactions were done at 30°C.

Electrophoresis of LDH. The composition of the gels was as given previously (4) except that cylindrical gels were prepared with 0.2 M Tris-phosphate buffer (pH 7.0) (final concentration in the gel was 0.05 mM). A Quickfit electrophoresis module was used with 0.05

M Tris-phosphate buffer (pH 7.0) as tank buffer. The buffer surrounding the gel tubes was cooled by running tap water. Gels were developed in 0.1 M Tris-chloride buffer (pH 7.0) or 0.1 M acetate buffer (pH 5.5), containing (weight per volume per milliliter): sodium pyruvate, 2.75 mg; sodium FDP, 0.4 mg; NADH, 1.0 mg. After 30 min of incubation at 30°C the gels were stained in a solution containing (weight per volume per milliliter): nitroblue tetrazolium, 0.5 mg; phenazine methosulfate, 0.05 mg, in 0.1 M Tris-chloride buffer (pH 8.1) (4).

Preparation of DNA. Cultures were grown in YGPB, cells were harvested, and deoxyribonucleic acid (DNA) was purified as described by Garvie (5). The melting curves of the DNA dissolved in standard saline citrate (8) were followed by using a Unicam SP1700 spectrophotometer with an SP876 (Mark 2) temperature program and Phillips XY recorder.

DNA/DNA hybridization studies. The relationship of *S. raffinolactis* to other serological group N streptococci was estimated by DNA renaturation rates according to the method of De Ley et al. (2). DNA was sheared before denaturation by passing it 10 times through a 1-inch (ca. 2.54-cm), 26-gauge syringe needle. Renaturation was done at 65°C, and the change in optical density was followed on a Unicam SP1700 spectrophotometer with an AR55 recorder attached.

Phenotypic characteristics. The strains were checked by the technique described in 1953 by Garvie (3) for a number of the properties previously described.

RESULTS AND DISCUSSION

The melting temperatures of the DNA of *S. raffinolactis* were found to be 1.5 to 2°C higher than those of strains of either *S. lactis* or *S. cremoris* (Table 1). It follows that there is a difference of 4 to 6% between the guanine-plus-cytosine (G+C) content of the DNA of *S. raffinolactis* and that of the DNA of other group N streptococci. This difference is indicative of a species distinction (10).

A second property considered to be of significance concerned the LDHs of *S. raffinolactis*. On electrophoresis this enzyme activity was detected in two distinct bands. The slower-moving band of activity travelled the same distance as the single LDH detected in preparations of *S. lactis* (Fig. 1). Gels of the LDH of *S. cremoris* were indistinguishable from those of *S. lactis* LDH.

The LDHs of strains NCDO 617, NCDO 618, and NCDO 619 were examined several times. The cell extracts were found to remain stable for up to 2 years. The LDH patterns of the three strains were not identical (Fig. 1). In the original gels two areas of activity were detected visually in extracts of NCDO 618, and of these the slower was very faint and did not reproduce. The faster-moving component in NCDO 618 was always more distinct than either component in extracts

TABLE 1. Percent guanine plus cytosine (% G+C) in the DNA of some group N streptococci

Species	NCDO no.	T_m (°C)	% G+C ^a
<i>S. raffinolactis</i>	617	87.4	41.5
	618	87.4	41.5
	619	86.9	40.3
<i>S. lactis</i>	C2 (Lac ⁺)	85.3	36.3
	C2 (Lac ⁻)	85.4	36.7
<i>S. cremoris</i>	Fast variant	84.7	34.9
	Slow variant	84.9	35.4
<i>L. mesenteroides</i> 39	768	86.6	39.6
<i>E. coli</i> K-12	1984	91.75	52.1

^a % G+C = $[(T_m - 70.4)/2.44]$. Since the melting temperature (T_m) of strains 39 and K-12 measured in this laboratory are consistently 1°C higher than the figures given by Marmur and Doty (9) and De Ley (1) for these same strains, the formula used for calculating % G+C from T_m has been modified to take account of this experimental difference between laboratories.

FIG. 1. Electrophoresis gels showing the LDHs of four strains of streptococci. Left to right: *S. lactis* NCDO 712, *S. raffinolactis* NCDO 617, NCDO 618, and NCDO 619.

of NCDO 617. Extracts of NCDO 619 always showed two equally distinct components.

Although the intensity of the two LDH components varied between the strains, the migration distances were the same. When FDP was omitted from the developing solution there was no LDH activity, and therefore both components are FDP activated. It is not known

whether these strains contain two different LDHs or whether the two components are different molecular forms of the same enzyme. Double banding was not observed after electrophoresis of extracts of strains of other species of group N streptococci.

Other differences between the LDHs of the various species were found when enzyme activity in relation to several variables was examined. The LDHs of *S. lactis* and *S. cremoris* were active at both pH 7.0 and 5.5. Earlier, Jonas et al. (7) observed the broad pH range at which the LDH of *S. cremoris* was active. The LDHs of *S. lactis* and *S. cremoris* are also characterized by having a low requirement for FDP, and enzyme activity was not greater with 500 μ M than with 25 μ M even at low concentrations of pyruvate (0.5 mM) (Table 2). On the other hand, the LDH of *S. raffinolactis* had low activity at pH 7.0, which activity more than doubled at pH 5.5. At pH 7.0 the LDH activity faded quickly when the concentration of either pyruvate or FDP was reduced. At pH 5.5 the LDH of *S. raffinolactis* was more sensitive to a decrease in pyruvate concentration than the LDH of the other species of group N streptococci. At low pyruvate concentrations a high level of FDP was required.

The LDH of *S. raffinolactis* was inhibited by lower concentrations of phosphate than the LDH of the other species of group N streptococci (Table 3).

The properties of the LDHs of *S. lactis* and *S. cremoris* suggest that these species may have the same LDH but that the enzyme in *S. raffinolactis* is significantly different. Similar differences between FDP-activated LDHs of two spe-

cies have been found by Hensel et al. (6) when working with *Lactobacillus casei* and *Lactobacillus curvatus*.

The relationship between *S. raffinolactis* and other streptococci as estimated by DNA homology is shown in Table 4. De Ley et al. (2) found that the reduction in optical density of DNA renaturation curves was straight for some 40 min after the reaction started. In the present experiments, even with DNA sheared in a French pressure cell as recommended by De Ley, renaturation curves were not straight and the renaturation rate was very slow. Needle-sheared DNA seemed more satisfactory, and the tech-

TABLE 3. Effect of phosphate on the activity of the LDHs of some group N streptococci^a

NCDO no.	Δ OD at 1 min (%)							
	pH 7.0				pH 5.5			
	500 ^b		50		500		50	
	20 ^c	10	10	5	20	10	10	5
712	83	88	28	66	35	85	8	42
1007	68	83	14	66	47	85	13	66
924	57	70	20	74	42	78	14	64
617	0	27	0		0	52	8	19
618	31	49			20	56	5	36
619	7	34	25	33	7	42	3	15

^a Conditions of the assay were the same as for Table 2. Phosphate (PO_4) was added to the assay solution as potassium phosphate buffer at the concentrations shown. Activity in the absence of phosphate was considered 100%.

^b FDP concentration (micromolar).

^c Phosphate concentration (millimolar).

TABLE 2. Effect of pyruvate and FDP on the activity of the lactate dehydrogenase of some group N streptococci^a

Species	NCDO no.	[(Δ OD pH 7.0)/(Δ OD pH 5.5)] $\times$ 100	Δ OD at 1 min	Δ OD at 1 min (%)							
				pH 7.0				pH 5.5			
				3.3 ^b		3.3		3.3		0.5	
				500 ^c	500	500	25	500	25	500	25
<i>S. lactis</i>	712	115	0.337	100	89	38	24	100	108	43	45
<i>S. lactis</i> subsp. <i>diacetylactis</i>	1007	127	0.276	100	109	32	30	100	102	38	36
<i>S. cremoris</i>	924	106	0.338	100	98	37	36	100	92	41	34
<i>S. raffinolactis</i>	617	234	0.191	100	0	0	0	100	96	31	14
	618		0.215	100	11	8	0	100	87	13	5
	619	240	0.153	100	3	0	0	100	100	10	8

^a LDH activity was measured by the decrease in optical density (Δ OD) at 340 nm in 1 min at 30°C. The assay solution contained 0.067 M Tris-chloride buffer (pH 7.0) or 0.067 M acetate buffer (pH 5.5) and 0.15 μ M NADH. Pyruvate and FDP were used at the concentrations shown. The reaction was started by adding 0.02 ml of a solution of freeze-dried cell extract to 1.0 ml of assay solution.

^b Pyruvate concentration (millimolar).

^c FDP concentration (micromolar).

TABLE 4. Relationship between strains of streptococci calculated from DNA renaturation rates

Species	NCDO no.	Hybridization of DNA (%)
<i>S. lactis</i> × <i>S. cremoris</i>	2031 × 924	91
<i>S. lactis</i> × <i>S. raffinolactis</i>	2031 × 617	36
<i>S. lactis</i> × <i>S. raffinolactis</i>	2031 × 619	43
<i>S. cremoris</i> × <i>S. raffinolactis</i>	924 × 617	36
<i>S. raffinolactis</i> × <i>S. raffinolactis</i>	619 × 617	98

nique was tested by using DNA from *Leuconostoc* strains that had been used in previous hybridization studies (5). It was found that the larger DNA fragments obtained by using a syringe needle gave a reasonable change in optical density, and by using the initial rate of renaturation the relationships could be calculated. Test runs with several strains gave results in agreement with those obtained earlier with labeled DNA.

The results in Table 4 show that *S. raffinolactis* is not closely related to either *S. lactis* or *S. cremoris*. The close relationship between the last two species was especially noteworthy. This observation needs confirmation with other strains before any conclusions can be drawn.

The newly discovered properties of NCDO 617, NCDO 618, and NCDO 619 confirm the earlier belief that these strains are not members of either *S. lactis* or *S. cremoris*. None of the strains examined by Orla-Jensen and Hansen was available for direct comparison. The properties that they give and the sources of their strains show that their strains might well be related to NCDO 617, NCDO 618, and NCDO 619. The properties of several more strains were given by Garvie in 1953 (3). Not all of these ferment raffinose, but at the time it was felt that

all the strains belonged to a single species. The only three strains to be kept all fermented raffinose.

REPRINT REQUESTS

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LITERATURE CITED

1. De Ley, J. 1969. Reexamination of the association between melting point, buoyant density, and chemical base composition of deoxyribonucleic acid. *J. Bacteriol.* 101:733-754.
2. De Ley, J., H. Cattori, and A. Reynearts. 1970. The quantitative measurement of DNA hybridization from renaturation rates. *Eur. J. Biochem.* 12:329-372.
3. Garvie, E. I. 1953. Some Group N streptococci isolated from raw milk. *J. Dairy Res.* 20:41-44.
4. Garvie, E. I. 1969. Lactic dehydrogenases of strains of the genus *Leuconostoc*. *J. Gen. Microbiol.* 58:85-94.
5. Garvie, E. I. 1976. Hybridization between the deoxyribonucleic acids of some strains of heterofermentative lactic acid bacteria. *Int. J. Syst. Bacteriol.* 26:116-122.
6. Hensel, R., U. Mayer, K. O. Stetter, and O. Kandler. 1977. Comparative studies of lactic acid bacteria. 1. Purification and kinetics of the allosteric L-lactate dehydrogenase from *Lactobacillus casei* ssp. *casei* and *Lactobacillus curvatus*. *Arch. Microbiol.* 112:81-93.
7. Jonas, H. A., R. F. Anders, and G. R. Jago. 1972. Factors affecting the activity of the lactate dehydrogenase of *Streptococcus cremoris*. *J. Bacteriol.* 111:397-403.
8. Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J. Mol. Biol.* 3:208-218.
9. Marmur, J., and P. Doty. 1962. Determination of the base composition of deoxyribonucleic acid from its denaturation temperature. *J. Mol. Biol.* 5:109-118.
10. Marmur, J., S. Falkow, and M. Mandel. 1963. New approaches to bacterial taxonomy. *Annu. Rev. Microbiol.* 17:329-372.
11. Orla-Jensen, A. D., and P. A. Hansen. 1932. The bacteriological flora of spontaneously soured milk and of commercial starters for butter making. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 2* 86:6-29.
12. Swarthling, P. F. 1951. Biochemical and serological properties of some citric acid fermenting streptococci from milk and dairy products. *J. Dairy Res.* 18:256-267.