Some Slime-Forming Heterofermentative Species of the Genus Lactobacillus

M. ELISABETH SHARPE, ELLEN I. GARVIE, AND R. H. TILBURY

National Institute for Research in Dairying, University of Reading, RG2 9AT, England, and Tute & Lyle Ltd., Research Centre, Keston, Kent BR2 6HJ, England

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Lactobacillus coprophilus subsp. confusus (NCDO 1586) and 18 other strains of slime-forming heterofermentative lactobacilli obtained from diverse sources are considered to form a new species on the basis of their physiological characteristics and similarities of their pyruvate reductases and lactate dehydrogenases. It is suggested that these strains should be named Lactobacillus confusus (Holzapfel & Kandler) comb. nov. (L. coprophilus subsp. confusus Holzapfel & Kandler), reasons being given. The type strain is NCDO 1586 (NCIB 9311, ATCC 10881). Strains of Lactobacillus vermiciforme and Lactobacillus viridescens also form slime. However, L. vermiciforme was readily separated from the other two by the criteria used. Although physiological characteristics separated L. viridescens from L. confusus, a relationship between these two species and also between them and the leuconostocs was indicated by the properties of the pyruvate reductases and lactate dehydrogenases. The slime produced by all species was found to be a glucan, probably a dextran, containing primarily α-1-6-glycosidic linkages.

Over a period of 15 years, occasional strains of bacteria sent to the National Institute for Research in Dairying as leuconostocs have on examination proved to be gram-positive heterofermentative rods, forming DL-lactic acid and gas from the fermentation of glucose, and producing ammonia from arginine. Thus these strains were considered to be lactobacilli and not leuconostocs. A recent investigation of several such strains isolated from spoiled sugar cane (R. H. Tilbury, Ph.D. Thesis, University of Aston in Birmingham, 1970) led us to examine these organisms in more detail. As they were heterofermentative slime-forming strains, a comparison was made with other slime-forming and non-slime-forming species of heterofermentative lactobacilli. The slime-forming species examined included Lactobacillus vermiciforme (17, 20, 25), Lactobacillus viridescens (19), an unclassified heterofermentative lactobacillus obtained 22 years ago from the National Collection of Type Cultures (NCTC) and found by us to ferment cellobiose, a strain from grass silage (15), and a strain from rory beer (18) later identified in this laboratory as Lactobacillus brevis.

One of our slime-producing strains, NCDO 1586, was recently studied by O. Kandler and named Lactobacillus coprophilus var. confusus (9). Three other strains of this new species received from him were included in the present work.

MATERIALS AND METHODS

Strains examined. The source of the organisms is given in Table 1. All strains were cultured at 30°C.

Physiological tests. The methods used were those previously described (22, 23). Production of gas from gluconate was observed by transferring the organisms three times into MRS broth (16) in which the glucose was replaced by 4% sodium gluconate (21).

Estimation of lactic acids. Strains were grown in dilute tomato juice broth, and lactic acid was estimated by the method used previously (6).

Enzyme activities. The methods for obtaining crude enzyme preparations were those of Garvie (7).

Presence of aldolase and phosphofructokinase. Some lactic acid bacteria have a reduced nicotinamide adenine dinucleotide (NADH) oxidase (EC 1.6.99) (7). Other strains such as Lactobacillus casei and Lactobacillus fermenti destroy NADH in the absence of added substrate although a specific oxidase has not been found. Such reactions interfere with the estimation of phosphofructokinase and aldolase when linked to the oxidation of NADH. For this reason the bacterial enzymes were first allowed to react with their respective substrates, and the NADH and excess of commercial enzymes (purchased from Boehringer Mannheim) were added.
Lactobacillus sp.

| Date deposited | NCDO no. | Other identification | Original source | Donor       |
|----------------|----------|----------------------|----------------|-------------|
| 1949           | 233      | NCTC 4037; NCIB 4037 | U.S.A.         | NCTC        |
| Sept 1956      | 889      | WH25                 | Raw milk, U.K. | M. E. Sharpe|
| Oct 1956       | 930      | 55G/2                | Saliva, Italy  | A. C. Hayward|
| Feb 1962       | 1586c    | NCIB 9311; ATCC 10881| Sugar cane, Jamaica | R. H. Tilbury | |
| Feb 1968       | 1937     | 65b                  | Sugar cane, Jamaica | R. H. Tilbury | |
| Oct 1970       |          | J4, J57, J65         | Sugar cane, Jamaica | R. H. Tilbury | |
| Jan 1970       | 1969     | ATCC 14434           | Grass silage, U.S.A. | ATCC (Langston and Bouma) | |
| Feb 1970       | 1975     | L. coprophilus var. con- | Carrot juice, Germany | O. Kandler | |
| Dec 1970       |          | fusus 12, 13, 22     |                |             | |

Lactobacillus vermiciforme

| Date deposited | NCDO no. | Other identification | Original source | Donor       |
|----------------|----------|----------------------|----------------|-------------|
| 1955           | 1965     | BV                   | Tibi grains     | Delft       |
| Jan 1957       | 961      | NRRL B1127           | Sugar beet      | NRRL        |
| 1957           | 962      | NRRL B1139           |                | NRRL        |

Other strains

| NCDO no. | Other identification | Original source | Donor       |
|----------|----------------------|----------------|-------------|
| 1655     | L. viridescens S38A  |                 | NCIB        |
| 1179     | L. viridescens WB14A |                 | NCIB        |
|          | L. brevis NCIB 8664  |                 | NCIB        |

Any reaction with NADH due to the bacterial enzyme preparation was negligible under these conditions. Heating to destroy the NADH oxidase before addition of the commercial enzymes also destroyed some of the products of aldolase action.

Detection of aldolase. A mixed commercial enzyme preparation (Boehringer Mannheim) was used in this assay. It contained glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) and triosephosphate isomerase (EC 5.3.1.1). The pipettes used for adding solutions drop-wise delivered approximately 0.02 ml/drop.

The reaction mixture (A) contained 0.15 ml of sodium fructose-1,6-diphosphate (0.1 M), 1.5 ml of triethanolamine buffer (0.2 M), pH 7.6 adjusted with HCl, and water to 3.0 ml. Crude bacterial enzyme preparations for testing were diluted 10-fold with water, and one drop of the resultant solution was added to 0.5 ml of solution A. The mixture was held at 30 C for 30 min, and then one drop of a recently prepared aqueous sixfold dilution of the commercial enzyme mixture and one drop of NADH (2.0 mg/ml) were added. The optical density was read at 340 nm within 20 to 30 sec.

Detection of phosphofructokinase. This assay was similar to that just described for aldolase, with two modifications. In the first place, the reaction mixture A was changed to 0.1 ml of adenosine triphosphate (ATP; 10 mg/ml), 0.1 ml of sodium fructose-6-phosphate (0.2 M), 0.1 ml of MgSO4·7H2O-KCl mixture (both 10 mg/ml 1.5 ml of triethanolamine buffer (as used above), and water to 3.0 ml.

The second modification came after incubation of the solution A with the bacterial enzyme preparations. In this assay, one drop of commercial aldolase (10 mg/ml) diluted sixfold with water was added as well as the commercial enzyme mixture used previously and NADH.

Pyruvate reductase and lactate dehydrogenase. In lactic acid bacteria NADH-linked enzymes con-
vert pyruvate to either D(−) (EC 1.1.1.28) or L(+) (EC 1.1.1.27) lactic acid. These enzymes may or may not be reversible and will be called D(−) or L(+) pyruvate reductases according to the product formed. Oxidation of NAD, convert lactate to pyruvate and are not known to be reversible. These enzymes will be called D or L lactate dehydrogenase (EC 1.1.99) according to their substrate. Methods for estimating activity of the enzymes and for electrophoresis have been previously described (7). For the present work tris(hydroxymethyl)aminomethane (Tris; 0.1 M) plus maleate buffer was used throughout when developing the gels after electrophoresis.

$K_m$ values were obtained after following enzyme reaction rates on a Unicam SP700C recording spectrophotometer.

**Chemical composition of the slime.** Organisms were grown in MRS broth modified by the addition of 5% (w/v) sucrose and a lower glucose content of 0.2% (w/v). Cells were removed by centrifugation, and soluble polysaccharides were precipitated from the supernatant fluid with three volumes of absolute ethanol. This precipitate was purified by the method of James (11). In the earlier part of the work, polysaccharide for paper chromatography was hydrolyzed with 1 N HCl (1), and that for gas liquid chromatography with 45% (v/v) formic acid. In later work, for both methods of analysis, 20 mg of polysaccharide was hydrolyzed with 2 ml of 1 N H$_2$SO$_4$ in a sealed tube at 100°C overnight. After neutralization with silver carbonate, the hydrolysates were evaporated under vacuum over P$_2$O$_5$ at 40°C. For descending paper chromatography the solvent was ethyl acetate-pyridine-water (10:4:3), and the chromatogram was developed with 1% p-anisidine-hydrochloride in butan-1-ol. The trimethylsilyl derivatives of the hydrolysates were estimated by the gas-liquid chromatographic (GLC) method of Sweeney et al. (24).

**Effect of dextranase on polysaccharides of slime-producing lactobacilli.** A pharmaceutical-grade enzyme preparation of dextranase-(endo-a-1,6 glucanase, EC 3.2.1.11) from *Penicillium lilacinum* was used (Swiss Ferment Co. Ltd., Basle). The enzyme was dissolved in 0.2 M acetate buffer, pH 5.0, to give a stock solution containing 330 units/ml.

The purified polysaccharides (0.2%, w/v) were used as substrates. Duplicate 10-ml samples of each polysaccharide solution were incubated in a shaking water bath at 40°C. One sample was treated with 1 ml of enzyme solution, the other was treated with 1 ml of acetate buffer only. After 30 min, the enzyme was inactivated by immersion of the tubes in a boiling water bath for 5 min. After cooling, the polysaccharide content was determined. For this a modification of the “haze analysis” technique (14) was used. A sample (5 ml) of test solution was mixed with an equal volume of absolute ethanol and allowed to stand at room temperature for 20 min. The absorbance was then measured in a spectrophotometer at 720 nm against a corresponding blank treated with distilled water. The instrument was calibrated with standard solutions of *Leuconostoc mesenteroides* dextran (Dextran 2000; A.B. Pharmacia, Sweden).

**RESULTS**

**Physiological characteristics.** The 27 strains examined were differentiated into six groups based on their physiological characteristics (Table 2). Three of these six groups contained slime-forming strains. Group 1 comprised 19 strains, and 14 of these had been received as presumptive leuconostocs; one was a previously unclassified heterofermentative lactobacillus (NCDO 233); four were received as *L. coprophilus* var. *confusus* from Kandler. The Group 1 strains differed from all the other strains examined, except *Lactobacillus cellobiosus*, in fermenting both cellulose and amygdalin. Production of slime and inability to ferment trehalose (89.5% of the strains examined) and raffinose (84%) distinguished Group 1 from *L. cellobiosus*. Furthermore *L. cellobiosus* may or may not ferment salicin and xylose (22), whereas the Group 1 strains gave consistently positive and negative responses, respectively.

The other slime-forming species *L. vermiciforme* and *L. viridescens* (not all strains of the latter form slime) were clearly differentiated from Group 1 and from each other: unlike Group 1, neither of these species produced ammonia from arginine, hydrolyzed esculin, or fermented melibiose, mannose, or salicin. They were, however, distinguished from each other by fermentation of xylose, arabinose, galactose, and trehalose. It is of interest that, with strains of *L. vermiciforme*, slime formation was only observed, and fermentation of carbohydrate was more active in an atmosphere of 90% H$_2$ plus 10% CO$_2$.

A comparison of the characteristics of *L. mesenteroides* previously obtained by Garvie (6) with those of Group 1 showed that the Group 1 strains differed from *L. mesenteroides* in the formation of D(-)-lactic acid instead of D(+)-lactic acid, usually in the failure to ferment trehalose, and usually by producing ammonia from arginine (Table 2).

Table 3 shows in detail some variable physiological characteristics of the Group 1 strains. Fermentation of arabinose and growth at 45°C were the most variable, being positive in 42% and 37% of the strains, respectively. Fermentation of melibiose, lactose, or raffinose occurred infrequently. Final pH in MRS broth varied from 4.1 to 4.6. Three strains did not hydrolyze arginine, and two of these, NCDO 1969 and 1975, fermented trehalose.

**Aldolase and phosphofructokinase.** Prepa-
TABLE 2. Physiological characteristics of slime-producing and some other heterofermentative species of lactobacilli

| Characteristic | Group 1: L. coprophilus var. confluens and Lactobacillus sp. | L. vermi-forme | L. cellobiosus NCDO 928 | L. brevis NCDO 1749 | L. viridescens | L. pastorianus NCIB 8664 | L. mesenteroides |
|---------------|---------------------------------------------------------------|----------------|------------------------|---------------------|----------------|------------------------|----------------|
| No. of strains | 19 | 3 | 1 | 1 | 2 | 1 | 31 |
| Fermentation of: | | | | | | | |
| Xylose | + | + | - | - | - | + | + (77.5) |
| Arabinose | - (58) | + | - | - | - | + | + (97) |
| Mannose | + | - | - | + | - | + | + (92) |
| Galactose | + | - | + | + | - | + | + (92) |
| Sucrose | + | + | + | - | + | + | + (92) |
| Trehalose | - (89.5) | - | + | - | + | + | + (92) |
| Maltose | + | - | + | + | - | + | + (92) |
| Cellobiose | + | - | - | + | - | + | + (92) |
| Melibiose | - (74) | - | + | - | + | + | + (92) |
| Lactose | - (68.5) | - | + | - | + | + | + (92) |
| Raffinose | - (84) | - | + | - | + | + | + (92) |
| Salicin | + | - | - | + | - | + | + (92) |
| Amygdalin | + | - | - | + | - | + | + (92) |
| Arginine hydrolysis | + (84) | - | - | + | - | + | + (92) |
| Slime from sucrose | + | + (66) | - | - | + (50) | - | + |
| Esculin hydrolysis | - | - | + | - | + | + | + (92) |
| Growth at 45°C | + | - | + | - | - | - | - |
| Lactic acid configuration | DL | DL | DL | DL | DL | DL | DL |

* All are catalase-negative, growing at 15°C and forming gas from glucose, and all except strains 22 and NCDO 233 form gas from sodium gluconate. All ferment fructose and glucose, and all except strain NCDO 233 ferment ribose. None ferments rhamnose, melezitose, inulin, dextrin, glycerol, sorbitol, mannitol, or inositol.
* Including type strain.
* Data taken from previous work (5).
* Figures in parentheses indicate per cent of strains positive or negative.
* Two strains positive only under anaerobic conditions.

TABLE 3. Variable physiological characteristics in slime-forming Group 1 strains of heterofermentative lactobacilli

| Characteristic | L. coprophilus var. confluens NCDO 889, NCDO 1937, 123a, 69b, J4, J57 | L. cellobiosus NCDO 928 | L. brevis NCDO 930, 931 | L. viridescens NCDO 168, J65 | L. pastorianus NCDO 233 | L. mesenteroides NCDO 1969, NCDO 1975 |
|---------------|---------------------------------------------------------------|----------------|------------------------|---------------------|----------------|------------------------|
| No. of strains | 5 | 6 | 1 | 2 | 1 | 1 | 1 |
| Growth at 45°C | + | - | - | - | - | - | - |
| NH₃ from arginine | + | + | + | + | + | - | - |
| Final pH MRSB | 4.2-4.4 | 4.1-4.3 | 4.4 | 4.6 | 4.2 | 4.5 | 4.3 |
| Fermentation of: | | | | | | | |
| Arabinose | - | + | - | - | - | - | - |
| Trehalose | - | - | - | - | - | - | - |
| Melibiose | - | - | - | - | - | - | - |
| Lactose | - | - | - | - | - | - | - |
| Raffinose | - | - | - | - | - | - | - |

* Weak positive reaction.
rations were examined under one set of conditions only. Thus, failure to detect enzyme activity may not mean complete absence, but may mean that conditions were unfavorable.

Neither aldolase nor phosphofructokinase was detected in crude enzyme preparations of three strains of Group 1, one strain of L. vermiciforme, and one strain of L. viridescens. Both enzymes were shown to be present in similar preparations of five strains of homofermentative lactobacilli belonging to four different species. The results with the fructose-1,6-diphosphate aldolase are in agreement with those of Rogosa (21).

**Pyruvate reductases.** All the lactobacilli examined formed D(-)-lactic acid. The amount of L(+) lactic acid formed by strains of L. viridescens was low (between 10 and 30%), but with strains of other species the amounts of L(+) lactic acid were nearer to 50%. From these results it was anticipated that the lactobacilli would contain both D(-) and L(+) pyruvate reductases, whereas in leuconostoc strains which form only D(-)-lactic acid, only a D(-) pyruvate reductase has been demonstrated (7).

**D(-) Pyruvate reductases.** These enzymes were readily reversible in all the strains examined and could therefore be located after electrophoresis using D(-)-lactate as substrate. The reaction lactate → pyruvate was more active at pH 8.3 than at 7.5 (Table 4).

The relative positions of the enzymes after electrophoresis are shown in Fig. 1, and it can be seen that the enzymes of Group 1, L. viridescens, and L. mesenteroides ran to the same position. L. fermenti and L. ceblobiosus, while moving to the same position as each other, lay close to but were slightly less mobile than the leuconostoc D(-) pyruvate reductase. L. vermiciforme was markedly different from all other strains examined.

Other properties are shared by the D(-) pyruvate reductases of L. mesenteroides, L. viridescens, and Group 1 strains. K_m values were estimated for NCDO 768, 930 and 1655 (lactate → pyruvate, pH 8.3 in 0.1 M Tris plus maleate buffer), and were 0.066 M, 0.066 M, and 0.078 M, respectively. Furthermore, none of these enzymes was inhibited by 20 mM potassium oxamate.

**L(+) Pyruvate reductase.** In Group 1 strains, the balance of the reaction of the L(+) pyruvate reductase was strongly in favor of pyruvate → lactate. The pH optimum at which lactate was used was lower than for the D(-) enzymes (Table 4), and also lower than for the L(+)-pyruvate reductase of L. viridescens and L. vermiciforme.

Of the L(+) pyruvate reductases shown in Fig. 1, only that of L. vermiciforme was readily detected with lactate. Those of Group 1 and L. viridescens were only detected with pyruvate. All the Group 1 strains examined were found to have two pyruvate reductases linked to NADH. The fastest moving enzyme could be equated with the D(-) pyruvate reductase. The

| Table 4. Times of reduction (min) of nitro-blue-tetrazolium by pyruvate reductases of enzyme preparations of different strains using calcium lactate as substrate |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Strain           | 6.3  | 7.0  | 7.5  | 8.3  | 6.3  | 7.0  | 7.5  | 8.3  |
| Lactobacillus viridescens |      |      |      |      |      |      |      |      |
| NCDO 1655        | NT   | NT   | NT   | 1    | NT   | 40   | 40   | 26   |
| L. fermenti      |      |      |      |      |      |      |      |      |
| NCDO 215         | NT   | 35   | 7    | 4    | 13   | 6    | 7    | 37   |
| L. ceblobiosus   |      |      |      |      |      |      |      |      |
| NCDO 924         | NT   | 15   | 6    | 2    | NT   | 60+  | 45+  | 45+  |
| Leuconostoc mesenteroides |      |      |      |      |      |      |      |      |
| NCDO 768         | 75   | 29   | 8    | 2    |      |      |      |      |

a D(-) and L(+) reactions were examined with the same concentration for any one enzyme preparation. No attempt was made to standardize rates between preparations.

* NT, Not tested; SI, slight reaction.

* Sodium salt, as calcium inhibitory.
slower moving enzyme showed maximum intensity when the pH of the developing solution was 5.5. In some strains, notably NCDO 930, this band was sometimes absent when gels were developed at pH 7.0. This enzyme band is assumed to be the d(-) pyruvate reductase. An intermediate band was seen in some strains and not always detected in any one strain. Its nature is uncertain. It was first thought to be pyruvate-linked, but further work has indicated that this may not be the case.

The weak pyruvate reductase band in _L. viridescens_, moving to the same position as the slowest moving enzyme of Group 1, is believed to be the l(+) pyruvate reductase. This band was difficult to detect because a strong NADH oxidase (not pyruvate-linked) lay between the l(+) and d(-) pyruvate reductase. If the NADH oxidase was too strong it masked the weak l(+) pyruvate reductase, but when preparations were diluted the latter enzyme was often missing.

Since the l(+) pyruvate reductase of _L. viridescens_ oxidized lactate faster at pH 8.3 than 7.5 it may be different from that of the Group 1 strains.

The l(+) pyruvate reductases of _L. fermenti_ and _L. cellobiosus_ were located readily with pyruvate at both pH 7.0 and 5.5 and were sometimes detected with lactate. The l(+) pyruvate reductase of _L. vermiciforme_ was located with lactate at pH 8.1 and with pyruvate at pH 7.0, but not at pH 5.5.

**Lactate dehydrogenases.** Many of the lactate dehydrogenases were weak and could not be detected after electrophoresis. The results available suggest the same relationships between species as found with the pyruvate reductases.

The d(-) lactate dehydrogenases of Group 1, _L. mesenteroides_, and _L. viridescens_ were stronger than the l(+). None of these enzymes moved on electrophoresis at pH 7.0. An l(+) lactate dehydrogenase different from that of any other strain was found in NCDO 1586 (Fig. 1). The significance of this enzyme is not known at the present time. The l(+) lactate dehydrogenase of _L. vermiciforme_ migrated at pH 7.0. Lactate dehydrogenases have been found in three other species of lactobacilli comprising _L. plantarum_, _L. casei_, and _L. lactis_; all migrated at pH 7.0.

The d(-) and l(+) lactate dehydrogenases of NCDO 768, 930, and 1655 had several properties in common. They were slightly inhibited...
by a concentration of 0.625 mM potassium oxamate and markedly by 20 mM (lactate concentration, 0.125 mM). The $K_m$ values at pH 6.3 in Tris plus maleate buffer for the $d(-)$ lactate dehydrogenases of these strains using a concentration of 0.1 mM dichlorophenol indophenol as hydrogen acceptor were found to be 66 mM, and, for the $d(-)$ enzymes, 6.6 to 9.9 mM.

Chemical composition of the slime. Five strains of Group 1 (NCDO 889, 930, 1937, and 123a and 69b), one strain of $L$. vermiforme (NCDO 1965), and two strains of $L$. viridescens (NCDO 1655 and 1179) were examined. The type strain of $L$. viridescens (NCDO 1655) did not produce any slime in the sucrose medium. The slime from the other six strains yielded only glucose when they were examined by both paper chromatography and GLC. It was concluded that glucose was the sole constituent sugar of all these polysaccharides.

Effect of dextranase on purified polysaccharides of slime-producing lactobacilli. Pure polysaccharides from the same seven organisms were examined. After treatment with dextranase, all the solutions showed zero absorbance, indicating hydrolysis to oligosaccharides which do not form haze in 50% alcohol. Since dextranase is specific for $\alpha$-1-6-glucosidic linkages, and no significant amounts of other enzymes hydrolyzing glucosidic linkages were present, the polysaccharides contained high proportions of $\alpha$-1:6 glycosidic linkages and therefore were probably dextrans.

DISCUSSION

Of the three groups of slime-forming heterofermentative lactobacilli studied, $L$. vermiforme is readily separated from the other two. The fermentation patterns and other characteristics of the three strains examined agree with those reported by Perquin (20), except that his strains fermented raffinose while ours did not. $L$. vermiforme differs from the other slime-forming species in that growth is stimulated and slime is formed on incubation in an atmosphere of 90% $H_2$ plus 10% $CO_2$ rather than in air. This agrees with the earlier observations of Ward (25). Possibly other non-slime-forming heterofermentative lactobacilli with apparently weak fermentation ability under aerobic conditions might exhibit more positive characteristics if incubated under an atmosphere of $H_2$ plus $CO_2$.

The fermentation pattern of $L$. vermiforme is similar to that of some non-slime-forming strains of $L$. brevis. However $L$. vermiforme does not hydrolyze arginine whereas $L$. brevis does. A comparison of the electrophoretic patterns of the pyruvate reductases with the results given by Gasser (8) suggests that these enzymes are different in the two species. More information is required to establish the relationship between $L$. vermiforme and $L$. brevis, but at present they are regarded as separate species, and NCDO 1965 is designated as the neotype strain of $L$. vermiforme.

$L$. viridescens has been described in detail (19). Garvie (5) discussed the similarity between the phenotypic characters of $L$. viridescens and $L$. dextranicum and stated that $L$. dextranicum could be short rods. It is now known that this observation was based on a strain forming DL-lactic acid, i.e., a slime-forming strain of $L$. viridescens.

The 19 strains of Group 1 form a homologous group with similar physiological and fermentation characteristics and identical electrophoretic patterns of their $d(-)$ and $l(+)$ pyruvate reductases. Strains NCDO 233, 1969, and 1975 differ from the others of the group because they do not hydrolyze arginine or ferment trehalose. However, these differences are not considered sufficient at this time to justify a separation from the remaining 16 strains.

The extracellular slime produced by strains of Group 1 was shown to be a glucan, probably a dextran. Glucans are also formed by the leuconostocs and by four strains of $L$. brevis ($L$. pastorianus) (4). Other strains of lactobacilli examined by Duncan and Seeley (4) formed a heteropolysaccharide which was probably a lytic product. This was similar to the material described by Williamson (26) and by Carr (3) when working with $L$. pastorianus NC1B 8664. In the current work it was found that this last strain did not form an extracellular polysaccharide. Non-slime-forming mutant colonies were formed in three strains of $L$. pastorianus (4), and one of the three strains of $L$. vermiforme examined was found to have lost the ability to form slime during storage in this laboratory. Variable slime formation has not been observed in the strains of Group 1.

The amino acid composition and sequence of the cell wall mucopeptide are characteristic of each species and are therefore useful in species differentiation (12). Group 1 strain NCDO 889 has the same sequence as ATCC 10881 (NCDO 1586) (Kandler, personal communication), i.e., L-lys-L-ala. $L$. viridescens and $L$. mesenteroides have slightly different sequences from these Group 1 strains, but both species contain either lysine and alanine, or lysine, alanine, and serine. All these species are different from
L. cellobiosus, in which the linkage consists of ornithine and asparagine.

L. coprophilus was described and named by Kandler and Abo-Elnaga (13). Subsequently Holzapfel and Kandler (9) considered L. mesenteroides ATCC 10881 (NCDO 1586) to be a subspecies of this organism and named it L. coprophilus subsp. confusus. The latter was described as differing from the former in producing dextran from sucrose, in forming peroxide, and in failing to ferment arabinose. Unfortunately no strain of L. coprophilus subsp. coprophilus is available for study at present. The original strains are no longer viable and no new strains are known to have yet been isolated. We consider that NCDO 1586 and the other 18 strains of Group 1 belong to the same species on the basis of physiological characteristics and the similarities of their pyruvate reductases and lactate dehydrogenases. They form a cluster of strains readily recognizable physiologically and are differentiated from other heterofermentative species. They are widely distributed in different countries and in different habitats. It is therefore suggested that L. coprophilus subsp. confusus should be raised to the rank of species. The species named proposed is Lactobacillus confusus (Holzapfel and Kandler) comb. nov. (L. coprophilus subsp. confusus (Holzapfel and Kandler]). The type strain by monotypy is NCDO 1586, NCIB 9311, ATCC 10881.

An examination of the early literature indicates that L. confusus may have been examined previously but classified as L. mesenteroides. Barendrecht (2) observed that slime-forming organisms which he considered to be similar to L. mesenteroides formed Dl-lactic acid. He suggested this organism should be called Leuconostoc agglutinans. Later workers (10) found that five strains of L. mesenteroides isolated from slimy sugar solutions formed inactive lactic acid.

The difficulty of separating L. confusus and L. mesenteroides has persisted over the years, as is shown by the source of many of the strains examined in the current work. In fact, the difference between the two species appears less than between L. confusus and other heterofermentative lactobacilli. The similarity was emphasized by two strains appearing to have characteristics intermediate between the two species. Strains NCDO 1969 and NCDO 1975 are morphologically more rod-shaped than cocoid and form Dl-lactic acid, but neither hydrolyzes arginine or ferments trehalose. Before these strains were found, it was thought that all four of these characteristics separated the two species.

The difficulty in separating L. dextranicum and L. viridescens, and the same problem with L. mesenteroides and L. confusus, raises important systematic questions. By present definition, the rods and cocci in the family Lactobacillaceae fall into two different genera, Lactobacillus and Leuconostoc, respectively, but this may not be justified. In the opinion of one of us (E.I.G.), on the evidence available at present, there is little justification for separating them. The logical extension of this idea would be that the heterofermentative lactobacilli and the leuconostocs should be reclassified to form a single genus leaving the homofermentative lactobacilli on their own.

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