Differentiation of *Streptococcus faecalis* Andrewes and Horder and *Streptococcus faecium* Orla-Jensen Based on the Amino Acid Composition of Their Murein

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The amino acid composition of the murein (peptidoglycan) of 75 strains of enterococci was investigated. All strains designated *Streptococcus faecalis* according to their physiological properties contained the lysine-alanine type of murein; all strains classified as *S. faecium* contained the lysine-aspartic acid type of murein.

The studies of Deibel et al. (3, 4) and of Whittenbury (18) have shown that two physiologically different types of enterococci may be distinguished, namely, *Streptococcus faecalis* Andrewes and Horder (including the variety *liquefaciens*) and *S. faecium* Orla-Jensen (including the variety *durans*). (*S. bovis* and *S. equinus*, which also belong to serological group D, are usually excluded.) Raj and Colwell (11) came to a similar conclusion, when they applied the methods of numerical taxonomy.

During our investigations on the composition of the murein (peptidoglycan) of streptococci (13, 14), we found two different types of murein in various strains of enterococci. In the strains designated as *S. faecalis*, a murein with an interpeptide chain consisting of lysine (Lys) and 3 moles of L-alanine (Ala) [Lys-(Ala)₃ type; Fig. 1] was found, whereas the murein of *S. faecium* contains D-aspartic acid (Asp) as the crosslinking amino acid (Lys-Asp type; Fig. 2). The latter type was found in most lactobacilli (10) and in *S. lactis* (14). On the other hand, Ghuyssen et al. (6) found a Lys-Asp type of murein in *S. faecalis* (ATCC 9790). Therefore, one would have to assume that two different types of murein occur in one and the same species.

To check this assumption, the murein composition and the most important physiological properties of 75 strains of *S. faecalis* and *S. faecium* were studied. An excellent correlation between murein structure and classification based on other criteria was found.

MATERIALS AND METHODS

The various strains were sent to us by the American Type Culture Collection (ATCC), the Streptokokken-

zentrale Kiel (Germany), and R. Whittenbury (Edinburgh). In addition to the strains listed in Table 1, the following strains were available: from the Streptokokkenzentrale Kiel (Germany), *S. durans* strains 11,507, 11,534, 11,553, 11,555, 11,965, and 12,005; from R. Whittenbury, *S. faecalis* strains black, H₆₉D₆, 585, and 2707, and *S. faecium* strains S32, HGH511, 2703, and S43.

The organisms were grown in yeast extract-dextrose medium (5 g of yeast extract, 10 g of peptone from casein, 5 g of dextrose, 1,000 ml of water; pH 7.2). Growth with pyruvate as the sole source of energy was tested by the method of Deibel and Niven (5); resistance to tellurite, as well as the fermentation of carbohydrates in a semiliquid medium, was determined by the method of Whittenbury (18).

Cell walls were isolated from cells in the early stationary phase of growth, disintegrated with glass beads, incubated with trypsin, and extracted with 5% trichloroacetic acid at 100 C for 10 min (13).

Paper chromatography of the amino sugars and amino acids was performed on Schleicher and Schuell (2043b) paper, with the following solvent systems: isopropanol:acetic acid:water (75:10:15; v/v); α-picoline:concentrated NH₄OH:water (70:2:28, v/v). To distinguish between lysine and ornithine, which have the same *R_F* values in these solvent systems, samples of all hydrolysates were separated by one-dimensional paper chromatography in the solvent system methanol:pyridine:HCl:water (32:4:1:7; v/v), as described by Rhuland et al. (12).

RESULTS

Physiological properties. Using the strains listed in Table 1, we studied a number of physiological properties which have been regarded (4, 18) to be especially convenient and determinative for distinction between the two species. In addition to the characteristics listed (Table 2), all strains showed the following properties and thus proved

FIG. 1. Amino acid sequence of the murein of *S. faecalis*. MurNAc, N-acetylmuramic acid; GlcNAc, N-acetylglucosamine; Glu(NH₂), glutamine.

FIG. 2. Amino acid sequence of the murein of *S. faecium*. Asp(NH₂), asparagine.

to be enterococci: growth in alkaline media at pH 9.6 (2), growth in 6.5% NaCl, and failure to produce catalase. Microscopically, all cultures proved to contain only chain-forming cocci.

Table 2 gives the properties which proved to be completely uniform within each species, e.g., resistance to 0.04% tellurite, a property first used for classification by Skadhauge (15). Also, pyruvate as the sole source of energy proved to be a very good criterion, as suggested by Deibel and Niven (5). This test yielded clear quantitative differences; after 48 hr of growth, the cultures with pyruvate either showed the same optical density value (about 1.5) at 600 nm as cultures with glucose or only one-third of this value (about 0.5). Among the fermentation tests, only mannitol gave uniform results within each species. In the "classical" test, i.e., anaerobic glycerol fermentation, as well as the fermentation of sorbitol, melibiose, and melezitose, some exceptions occurred. The percentage of atypical strains was about the same as in the investigations of Deibel et al. (4) and Whittenbury (18). The fermentation of arabinose, important for the separation of *S. durans* from *S. faecium*, was tested with only three strains designated as *S. faecium* var. *durans* as well as with four strains each of the other species and varieties. Arabinose was fermented by all four strains of *S. faecium* var. *faecium*, whereas

the three strains of *S. faecium* var. *durans* and *S. faecalis* var. *faecalis* gave negative results. Table 2 shows the possible variations within the two species of enterococci. It is obvious that most of the strains exhibit those combinations (A or a) which are generally thought to be typical of the two species.

Comparison with previous classifications. In Table 1, the strains are listed according to the combinations of properties presented in Table 2. In addition, Table 1 gives the designation under which the strains were obtained. Only one strain (11756) from the Streptokokkenzentrale in Kiel is differently classified. At first, this strain yielded varying results and had to be purified by isolating single colonies after dilution on plates. The strain obtained after purification proved to be *S. faecium*.

Several strains from the ATCC had to be classified differently. Strain ATCC 882 was not called *S. faecalis*, inasmuch as it had been previously identified as *S. bovis* by Raj and Colwell (11); it differed from all of the other strains in our experiments and we too designated it as *S. bovis*. Strains ATCC 1295, 349, 6057, and 12,755, originally called *S. faecalis*, proved to be *S. faecium* var. *faecium*, in our experiments, and strains ATCC 9790 and 10,541 proved to be *S. faecium* var. *durans*. Strain ATCC 9790 merits

TABLE 1. *Designations of strains of Streptococcus employed*

Designation suggested by us	Type ^a	Strains	Designation under which the strains were obtained
<i>S. faecalis</i>	A	ATCC 828, 4082, 4200, 10741, 11420, 11700, 14506, 14507, 14508, 12953, 13398, 14428, 14429, 14433, 6055, 12958, Kiel 11724, 11727, 11732, 11746, 11747, 11755, 11789, 11794, 11797	<i>S. faecalis</i> <i>S. faecalis</i> var. <i>liquefaciens</i> <i>S. faecalis</i> var. <i>zymogenes</i> <i>S. faecalis</i>
	B	ATCC 4079, 13399	<i>S. faecalis</i>
	C	Kiel 11798, 11800	<i>S. faecalis</i>
	D	ATCC 12984, Kiel 11788	<i>S. faecalis</i>
	E	ATCC 4083, 7080	<i>S. faecalis</i>
<i>S. bovis</i>	F	ATCC 882	<i>S. faecalis</i>
<i>S. faecium</i> var. <i>faecium</i>	a	ATCC 12952, Kiel 11717, 11739, 11749, 11750, 11771, 11774, 11775, 11776, 11777, 11786, 11787, 11791, 11793, 11796	<i>S. faecalis</i> <i>S. faecium</i>
	b	ATCC 14432, 14436	<i>S. faecium</i> var. <i>mobilis</i>
	c	ATCC 349, 6057, 12755	<i>S. faecalis</i>
	d	Kiel 11735	<i>S. faecium</i>
		Kiel 11756	<i>S. faecalis</i>
<i>S. faecium</i> var. <i>durans</i>	e	ATCC 8043, 9790	<i>S. faecalis</i>
	f	ATCC 10541	<i>S. faecalis</i>

^a Each type represents a combination of characters, as listed in Table 2.

TABLE 2. *Physiological properties of the investigated strains^a*

Property	<i>S. faecalis</i>					<i>S. bovis</i>	<i>S. faecium</i>				<i>S. faecium</i> var. <i>durans</i>	
	A	B	C	D	E	F	a	b	c	d	e	f
Combination of characters	26	3	2	2	2	1	15	2	3	2	2	1
No. of strains	+	+	+	+	+	+	—	—	—	—	—	—
Tolerance to potassium tellurite	+	+	+	+	+	+	—	—	—	—	—	—
Growth with sodium pyruvate	+	+	+	+	+	+	—	—	—	—	—	—
Acid production from												
Glycerol + sodium fumarate	+	+	+	+	—	—	—	—	—	+	—	—
Sorbitol	+	—	+	+	+	—	—	—	—	—	—	—
Mannitol	+	+	+	+	+	+	+	+	+	+	—	—
Melibiose	—	—	—	+	—	+	+	+	—	+	+	—
Melezitose	+	+	—	+	+	—	—	+	—	—	—	—

^a The murein type of *S. faecalis* and *S. bovis* is Lys-(Ala)₃; the murein type of *S. faecium* and *S. faecium* var. *durans* is Lys-Asp.

further mention, since it was used by Ghuyssen et al. (6) and called *S. faecalis*. It contains murein of the Lys-Asp type (Fig. 2).

Composition of the cell walls. Cell walls of all strains listed in Table 1, as well as of the six strains of *S. faecium* var. *durans* from the Streptokokkenzentrale Kiel and of the four strains each of *S. faecalis* and *S. faecium* kindly supplied by R. Whittenbury, were analyzed. A total hydrolysate of all of the cell walls (4 N HCl, 16 hr, 100 C) was prepared and separated by paper chroma-

tography. Only two different patterns of amino acid composition were found. In one case only, Lys, Glu, and Ala, in addition to the two amino sugars, were located. Ala, however, yielded a much larger spot than did the other amino acids, indicating that more than 2 moles of Ala per mole of Glu was present. This was confirmed in some samples by quantitative determination with an amino acid analyzer (Table 3). The partial hydrolysate of such cell walls (Fig. 3) contained the same peptides as previously described in more

TABLE 3. Amino acid composition of trichloroacetic acid-extracted cell walls of four strains of *S. faecalis*

Strain	Glu		Ala		Lys		Mur		GlcNH ₂	
	Amt	Ratio	Amt	Ratio	Amt	Ratio	Amt	Ratio	Amt	Ratio
4079	0.595	1.0	2.405	4.04	0.640	1.07	0.590	0.99	0.58	0.97
828	0.250	1.0	1.01	4.04	ND ^b	ND	0.255	1.02	ND	ND
4200	0.640	1.0	2.540	4.05	ND	ND	0.69	1.08	ND	ND
12984	0.625	1.0	2.430	3.95	ND	ND	0.66	1.06	ND	ND

^a The amounts of amino acids are expressed as micromoles per milligram. Glu, glutamic acid; Mur, muramic acid; GlcNH₂, glucosamine.

^b Not determined.

detail in investigations on the cell wall of *S. thermophilus* and *S. faecalis* (13). This suggests that the probable amino acid sequence is that shown in Fig. 1.

In the other case, Asp and the slowly hydrolyzing ϵ -(aminosuccinyl)-lysine formed during hydrolysis from N⁶-Asp-L-Lys (16) were found in addition to the amino acids mentioned above. Thus, it is evident that the murein is of the Lys-Asp type (Fig. 2), as in *S. lactis* (14), most of the lactobacilli (10), and *S. faecalis* ATCC 9790 described by Ghuysen et al. (6). As shown in Table 2, all strains designated *S. faecalis*, as well as the strain of *S. bovis*, belong to the Lys-(Ala)₃ type. The strains designated *S. faecium* var. *faecium* and *S. faecium* var. *durans* belong to the Lys-Asp type. In addition, all the eight strains of *S. faecium* var. *durans* obtained from the Streptokokkenzentrale Kiel were shown to contain the Lys-Asp type. Among the strains received from Whittenbury, all strains designated *S. faecalis* contained the Lys(Ala)₃ type, and those designated *S. faecium* contained the Lys-Asp type.

DISCUSSION

Complete correlation was found between tellurite resistance and growth with pyruvate as the sole source of energy, on the one hand, and murein composition, on the other hand. The correlation between murein composition and other traditional criteria used for the distinction of *S. faecalis* and *S. faecium* was also very good. Thus, it seemed justified to subdivide the enterococci into two physiologically different species and to classify *S. durans* as a variety of *S. faecium*, as suggested by previous authors and recently by William and Bowden (19) on the basis of triose phosphate dehydrogenase properties.

In the future, it will be important to determine the amino acid composition of the cell wall for the identification of "intermediate strains." The occurrence of Asp in the cell wall might be significant for the identification of enterococci, as is the occurrence of diaminopimelic acid for the

FIG. 3. Chromatogram of partial hydrolysate (4 N HCl, 1 hr, 100 C) of cell walls of *S. faecalis* (1, Lys; 2, Glu; 3, Ala; 4, Mur; 5, Glc(NH₂); 6, D-Glu-Lys; 7, L-Lys-D-Ala; 8, N⁶-L-Ala-L-Lys; 9, N⁶-(L-Ala-L-Ala)-L-Lys; 10, N⁶-L-Ala-L-Lys-D-Ala; 11, L-Ala-D-Glu; 12, D-Ala-L-Ala; 13, L-Ala-L-Ala).

separation of *Lactobacillus plantarum* from the other species of the subgenus *Streptobacterium* (1, 10).

The disagreement between the findings of Ghuysen et al. (6) and our own is explained by the fact that the strain (ATCC 9790) used by Ghuysen et al. (6) should be classified as *S. faecium* var. *durans* and not as *S. faecalis*. Another disagreement in the literature may be explained in a similar way: Ikawa and Snell (9) and Ikawa (8) found murein of the Lys-Asp type in *S. faecalis* ATCC 8043. Originally, this strain had been designated *S. lactis* R, but it was assigned to *S. faecalis* by Gunsalus et al. (7). Deibel et al. (4) also found that this strain belongs to *S. faecium* var. *durans*. This is in agreement with our own findings. Thus, there remains no exception

to the parallelism between the Lys-(Ala)₃ type and *S. faecalis* and between the Lys-Asp type and *S. faecium*.

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LITERATURE CITED

1. Abo-Elnaga, I. G., and O. Kandler. 1965. Zur Taxonomie der Gattung *Lactobacillus* Beijerinck. I. Das Subgenus *Streptobacterium* Orla-Jensen. Zentr. Bakteriell. Parasitenk. Abt. II. **119**:1-36.
2. Chesbro, W. R., and J. B. Evans. 1959. Factors affecting the growth of enterococci in highly alkaline media. J. Bacteriol. **78**:858-862.
3. Deibel, R. H. 1964. The group D streptococci. Bacteriol. Rev. **28**:330-366.
4. Deibel, R. H., D. E. Lake, and C. F. Niven, Jr. 1963. Physiology of the enterococci as related to their taxonomy. J. Bacteriol. **86**:1275-1282.
5. Deibel, R. H., and C. F. Niven, Jr. 1964. Pyruvate fermentation by *Streptococcus faecalis*. J. Bacteriol. **88**:4-10.
6. Ghuyssen, J. M., E. Bricas, M. Leyh-Bouille, M. Lache, and G. D. Shockman. 1961. The peptide *N*^ε-(L-Alanyl-D-isoglutaminyl)-*N*^ε-(D-isoasparaginyl)-L-lysyl-D-alanine and the disaccharide *N*-acetylglucosaminyl-β-1,4-*N*-acetylmuramic acid in cell wall peptidoglycan of *Streptococcus faecalis* strain ATCC 9790. Biochemistry **6**:2607-2619.
7. Gunsalus, I. C., C. F. Niven, Jr., and J. M. Sherman. 1944. The identification of "Streptococcus lactis R" as a strain of *Streptococcus faecalis*. J. Bacteriol. **48**:611.
8. Ikawa, M. 1961. The partial chemical degradation of the cell walls of *Lactobacillus plantarum*, *S. faecalis* and *Lactobacillus casei*. J. Biol. Chem. **236**:1087-1092.
9. Ikawa, M., and E. E. Snell. 1960. Cell wall composition of lactic acid bacteria. J. Biol. Chem. **235**:1376-1382.
10. Kandler, O. 1967. Taxonomie und technologische Bedeutung der Gattung *Lactobacillus* Beijerinck. Zentr. Bakteriell. Parasitenk. Abt. I. Suppl. 2, p. 139-164.
11. Raj, H., and R. R. Colwell. 1966. Taxonomy of enterococci by computer analysis. Can. J. Microbiol. **12**:353-362.
12. Ruhland, L. E., E. Work, R. F. Deham, and D. S. Hoare. 1955. The behavior of the isomers of α,ε-diaminopimelic acid on paper chromatograms. J. Am. Chem. Soc. **77**:4844-4846.
13. Schleifer, K. H., and O. Kandler. 1961. Zur chemischen Zusammensetzung der Zellwand der Streptokokken. I. Die Aminosäuresequenz des Mureins von *S. thermophilus* und *S. faecalis*. Arch. Mikrobiol. **57**:335-364.
14. Schleifer, K. H., and O. Kandler. 1961. Zur chemischen Zusammensetzung der Zellwand der Streptokokken. II. Die Aminosäuresequenz des Mureins von *S. lactis* und *cremoris*. Arch. Mikrobiol. **57**:364-381.
15. Skadhauge, K. 1950. Studies on enterococci with special reference to the serological properties. Ejnar Munksgaard Forlag, Copenhagen.
16. Swallow, D. L., and E. P. Abraham. 1958. Formation of ε-(aminosuccinyl)-lysine from ε-aspartyl-lysine from bacitracin A and from the cell walls of lactobacilli. Biochem. J. **70**:364-373.
17. Whittenbury, R. 1963. The use of soft agar in the study of conditions affecting the utilization of fermentable substrates by lactic acid bacteria. J. Gen. Microbiol. **32**:375-384.
18. Whittenbury, R. 1965. The differentiation of *S. faecalis* and *S. faecium*. J. Gen. Microbiol. **38**:279-287.
19. Williams, R. A. D., and E. Bowden. 1968. The starch-gel electrophoresis of glucose-6-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus faecalis*, *S. faecium* and *S. durans*. J. Gen. Microbiol. **50**:329-336.