D-Glutamic acid and amino sugars as cell wall constituents in lactic acid bacteria

D-Alanine is localized in high concentration in the cell walls of *Streptococcus faecalis* and *Lactobacillus casei*[1]. None of the other amino acids present in hydrolysates are attacked by D-amino acid oxidase. However, both glutamic acid and lysine are prominent components of these walls[1], and since neither of their D-isomers is attacked by D-amino acid oxidase[2], and since D-glutamic acid is reported[3,4] to occur in lactic acid bacteria, further investigations of the optical form of these amino acids from cell walls were made.

Cell wall preparations were those described previously[1] from a culture of *L. casei** and one of *S. faecalis* Dunn. These were hydrolyzed by sealing 45–75 mg of the dry material with 2.5 ml of 3*N* HCl and heating at 110° for 24 hours. Hydrolysates were evaporated to dryness in a vacuum desiccator, taken up in a small amount of water, humins removed by centrifugation, and aliquots of the supernatant liquids chromatographed sequentially on Dowex 1 and on Dowex 50 as described by HIRS, MOORE AND STEIN[5]. Excellent separations were achieved, as shown for the cell wall material from *S. faecalis* in Fig. 1.

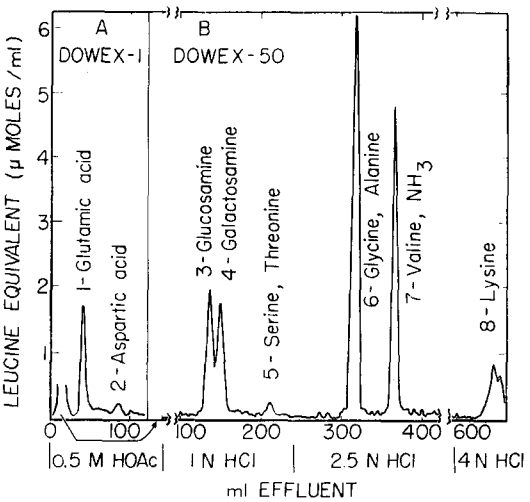

Fig. 1. Chromatography of 45 mg of *S. faecalis* cell wall hydrolysate. (A) Dowex 1-X 10 (acetate form, 10 × 350 mm). (B) First zone from Dowex-1 column re-chromatographed on Dowex-50 (acid form, 10 × 950 mm).

Quantitative assays of certain ninhydrin-positive fractions are given in Table I. Of special interest is the high figure for D- plus L-glutamic acid, as opposed to the low figure for L-glutamic acid. Total glutamic acid was determined both microbiologically and colorimetrically; the validity of the specific microbiological method for L-glutamic acid in the presence of varied amounts of D-glutamic acid was repeatedly confirmed. Approximately 80–90% of the total glutamic acid is D-glutamic acid**. The high proportion of total alanine comprised by D-alanine confirms previous findings[1] that much of the D-alanine of lactic acid bacteria is present in the cell wall.

In *S. faecalis* cell walls, all of the lysine is present in the L-configuration, as indicated by agreement in the colorimetrically determined figure for total lysine and the optically specific (for the L-isomer) microbiological determinations. In the *L. casei* cell walls the possible occurrence of some D-lysine has not been eliminated.

Each of the other amino acids from the Dowex column (diaminopimelic acid excepted) was determined by microbiological methods[6,7] specific for the L-isomer, and checked for presence of the D-amino acid with D-amino acid oxidase. In a few cases up to 3 to 5% of the total appeared present in the D-form; however, similar amounts of these same D-amino acids were found in a control experiment where casein was hydrolyzed with starch; they were assumed to arise by racemization during hydrolysis and column separation. Such racemization does not account for the large amounts of D-alanine and D-glutamic acid found in the cell walls. Analytical figures (g per 100 g of cell wall) for these amino acids in the cell walls of *L. casei* and *S. faecalis*, respectively, were: aspartic acid, 3.1, 0.8; diaminopimelic acid (by ninhydrin color), 6.7, 0; glycine 1.0, 0.2; isoleucine 1.4, 0.4; leucine, 1.4, 0.4; serine, 0.6, 0.2; threonine 1.1, 0.2; valine 1.4, 0.24; ammonia, 2.0, 1.7. Some of these amino acids may arise from contaminating protein incompletely removed during preparation of the cell walls (*cf.* [10]).

* This culture of unidentified history was obtained in Cambridge, England. It does not have an absolute requirement for vitamin B_6, and contains diaminopimelic acid. In both these respects it resembles *Lactobacillus arabinosus* 8014 and differs from *L. casei* 7469, and may be identical with the former organism.

** These walls are high in carbohydrate[9,10]. Hydrolysis of casein with acid in the presence of an equal amount of starch under the above conditions racemizes from 12 to 20% of the L-glutamic acid. If racemization occurs to a similar extent during hydrolysis of the wall, essentially all of the glutamic acid in the unhydrolyzed walls must be present in the D-configuration.

TABLE I

ALANINE, GLUTAMIC ACID, LYSINE, AND AMINOSUGAR CONTENT OF CELL WALL HYDROLYSATES

Component*	*L. casei*		*S. faecalis*	
	L-	*Total*	L-	*Total*
	g per 100 g cell wall			
Alanine	3.3[a]	7.1[b], 8.4[c]	9.1[a]	12.0[b]
Glutamic acid	2.2[d], 2.0[b]	6.2[e], 7.2[c]	0.7[d]	4.9[e], 5.4[c]
Lysine	1.3[f], 1.5[d]	2.3[c]	4.3[f], 4.3[d]	4.5[c]
Glucosamine		8.9[c], 6.0[g]		7.6[c], 6.2[g]
Galactosamine		0		7.4[c], 7.0[g]

* Superscript letters by figures indicate analytical methods as follows: a, by difference, with D-alanine determined by D-amino acid oxidase method[1]; b, microbiological with *L. citrovorum*[6]; c, colorimetric (ninhydrin) against same amino acid as standard; d, microbiological[7] with *S. faecalis* as test organism; e, microbiological[7] with *Lactobacillus pentosus* 124-2, which responds to both D- and L-glutamic acid, as test organism; f, microbiological with *Leuconostoc mesenteroides*; g, colorimetric (ELSON-MORGAN)[8]. All microbiological assays were checked for optical specificity.

Two ninhydrin-reactive amino sugars were separated on the Dowex 50 column from hydrolysates of *S. faecalis* walls. These were identified as *glucosamine* and *galactosamine* by direct chromatographic comparisons with authentic samples and by chromatography of their ninhydrin-degradation products, arabinose and lyxose, respectively (Table II). The amounts found are minimal, since some destruction occurs on hydrolysis. Only glucosamine was present in walls of *L. casei*. Non-amino sugars also occur in wall preparations of related species[9,10].

TABLE II

CHROMATOGRAPHIC IDENTIFICATION OF AMINO SUGARS FROM CELL WALLS

Solvent system*	Glucosamine hydrochloride	(1) Fraction 3 (Fig. 1) *S. faecalis*	(2) Fraction 3 *L. casei*	Galactosamine hydrochloride	(3) Fraction 4 (Fig. 1) *S. faecalis*	Arabinose	Degraded (1)**	Degraded (2)**	Degraded (3)**	Lyxose
	RF values									
1	0.12	0.12	0.13	0.11	0.10					
2	0.41	0.40	0.41	0.35	0.36	0.47			0.54	0.54
3	0.09	0.09	0.09	0.07	0.08	0.19	0.18	0.18	0.24	0.23
4						0.24			0.27	0.27
5						0.50			0.46	0.46

* Solvent systems: 1, *n*-butanol saturated with 0.45 *M* NH_4OH; 2, lutidine–H_2O (65:35); 3, *n*-butanol–ethanol–water (4:1:1); 4, *n*-butanol–acetic acid–water (4:1:1); 5, water-saturated phenol. Dr. SAUL ROSEMAN kindly supplied an authentic sample of galactosamine.

** Degraded to corresponding pentose with ninhydrin. R_F of pentose spot given. R_F values for ribose in solvent systems 2–5, respectively, were 0.55, 0.25, 0.29, 0.56; the corresponding R_F values for xylose were 0.51, 0.23, 0.27, and 0.43.

The occurrence of both D-alanine and D-glutamic acid together with amino sugars as prominent components of these cell walls recalls presence of these same amino acids in the peptide component of a uridine-5′-phosphate-aminosugar-peptide complex produced by penicillin-treated *Staphylococcus aureus*[11], the presence of D-glutamic acid in capsular polypeptides of several bacilli[12], and the nutritional requirement of some strains of *Lactobacillus bifidus* for glucosamine derivatives[13]. Whether these varied findings represent independent facets of related processes is not yet known.

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Prodigiosin synthesis in *Serratia marcescens*: isolation of a pyrrole-containing precursor*

Serratia marcescens produces a distinctive red pigment, prodigiosin, which has been characterized[1] as a tripyrrylmethene. Evidence has recently been obtained by RIZKI[2] and by WILLIAMS AND GREEN[3] that some stable prodigiosin-deficient mutants of this species accumulate substances capable of causing the appearance of pigment in certain other mutants impaired in prodigiosin synthesis. A pair of such mutants, not previously reported to exhibit cross-feeding, has been examined in some detail, since one of the two mutants (9-3-3) was found to excrete a relatively stable substance that permitted pigment formation in the other (W-1). The excreted substance has now been isolated in pure crystalline form; the progress of the isolation was followed by bioassay with strain W-1. This assay detects about 0.1 μg of the pure material.

For the isolation of the excreted substance, strain 9-3-3 was grown at 30° for four days in aerated peptone-glycerol medium[4]. The culture was centrifuged, and the supernatant liquid was extracted with chloroform. The chloroform layer was washed successively with 1 *N* hydrochloric acid, 1 *N* sodium hydroxide, and distilled water, and was then taken to dryness *in vacuo*. The residue was taken up in hexane (in which the active substance is sparingly soluble) and transferred to an alumina (Fisher Scientific Co., for chromatographic analysis) column. The latter was washed successively with benzene, and benzene-ether, and the active substance was then eluted with ether and ether-chloroform. After recrystallization from ethanol, the substance was obtained as colorless needles that do not melt at 250°, but decompose on further heating. The yield was about 1 mg per liter of culture. Elementary analysis agrees with the formula $C_{10}H_{10}O_2N_2$ (calculated: C, 63.2; H, 5.3; O, 16.8; N, 14.7; CH_3O, 16.3; found: C, 63.0; H, 5.3; O, 17.0; N, 14.7; CH_3O, 16.6)**. The isolated substance has neither pronounced basic nor pronounced acidic properties. It is sparingly soluble in such solvents as ethanol, chloroform, ethyl ether, benzene, or tetrahydrofuran; it is very sparingly soluble in water. The substance absorbs strongly in the ultra-violet; in ethanol solution it gives absorption maxima at 363 and 254 mμ with molar extinction coefficients of about $3.5 \cdot 10^4$ and $1.3 \cdot 10^4$, respectively. It rapidly forms a red color at 25° with *p*-dimethylaminobenzaldehyde in ethanolic hydrochloric acid; therefore, it presumably contains a pyrrole ring with a free alpha-position.

The excretion of the isolated material by one mutant strain and its utilization by another indicate that this compound is an intermediate in the synthesis of prodigiosin. This indication was supported by tracer experiments. Strain 9-3-3 was cultivated in the usual medium supplemented with glycine-2-^{14}C (*cf.* HUBBARD AND RIMINGTON[5]). Crystalline material was isolated as

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** The value for oxygen was obtained by direct analysis. Molecular weight determinations are complicated by the low solubility of the substance in common solvents; the formula given agrees with one methoxyl group per formula weight. This formula weight was used in the computation of the molar extinction coefficients and the specific activity of the substance. Microanalyses were performed by the Schwarzkopf Microanalytical Laboratory, by the Microchemical Laboratory of New York University, and by Mr. STANLEY MILLS.