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Lack of Murein in a Formamide-Insoluble Fraction from the Stable L-Form of *Streptococcus faecium*

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Formamide-insoluble material was isolated from the L-form of *Streptococcus faecium* strain F24, but this material was not murein.

Group A streptococcal L-forms have been shown by Edwards and Panos (2) to contain the nucleotide precursors for murein (mucopeptide) synthesis. Although unconfirmed chemically, the nucleotide precursors for murein were found by paper chromatography (King and Altenbern, *unpublished data*) in extracts from the stable L-form of *Streptococcus faecium* strain F24 (ATCC 19635). James, Hill, and Maxted (4) reported the isolation of murein from L-forms of *S. pyogenes* by a method utilizing hot formamide. This report described the chemical analyses of formamide-insoluble residues isolated from the *S. faecium* L-form. Our results differ from those of James et al. and suggest that murein is lacking in the enterococcal L-form.

Our methods were adapted from those of James et al. (4). The organisms were grown overnight at 37 C from a 1% (v/v) inoculum added to fresh Brain Heart Infusion broth containing 0.43 M NH_4Cl and 0.5% additional glucose. The L-forms were harvested by centrifugation for 15 min at 5,860 × *g* and washed three times with physiological saline containing enough deoxyribonuclease to disperse clumps of the organisms. The saline-washed organisms were washed three times with distilled water by centrifugation for 30 min at 100,500 × *g*. The final pellet was lyophilized and weighed. After overnight chloroform-methanol (2:1) extraction, the solubilized lipids were separated from insoluble material by centrifugation, dried, and weighed. The chloroform-methanol-insoluble material was dried, weighed, and treated overnight at 37 C with phosphate-buffered saline (PBS, *p*H 7.5) containing trypsin (0.1 mg/ml). The trypsin-treated material was centrifuged for 10 min at 12,100 × *g* and washed three times with PBS and three times with distilled water. After the pellet was lyophilized and weighed, the material was treated twice at 170 C with formamide. After each treatment, the insoluble material was collected by centriugation for 30 min at 34,800 × *g*. For final gravimetric analysis, the formamide-insoluble material was washed three times with PBS and three times with distilled water, resuspended in distilled water, and transferred quantitatively to tared weighing vessels for gravimetric analysis. The dry weight of each pellet obtained in the procedure may be seen in Table 1. The amount of formamide-insoluble material found in the enterococcal L-form was less than 1% of the dry weight compared to 6 to 9% found in the *S. pyogenes* L-form (4).

In the experiments shown in Table 1, the organisms were harvested from 2 liters of growth medium. The final yield was inadequate for further chemical analysis, and so much larger quantities of formamide-insoluble material were prepared and analyzed.

Samples of the formamide-insoluble material were hydrolyzed with 6 N HCl at 120 to 130 C for 18 hr. Amino acid analyses were performed on the hydrolysates by the method of Wolfe (9), substituting the ninhydrin reagent of Barrollier (1). The amino acids found in formamide-insoluble material are given in Table 2. We anticipated primarily finding hexosamines and the major amino acids normally associated with *S. faecium* (5) murein (alanine, aspartic acid, glutamic acid, and lysine). However, no hexosamines were detectable by paper chromatography with the colorimetric method of Levvy and McAllen (6). Furthermore, a large array of amino acids were present in addition to the amino acids normally found in murein. These chemical analyses suggested that the formamide-insoluble material was not pure murein.

The formamide-insoluble fraction was subjected to lysozyme digestion. Over 90% of the formamide-insoluble material was lysozyme-insoluble, indicating the relative paucity of murein if it were indeed present. The formamide-insoluble material was also treated with hot phenol by the method of Wheat, et al. (8) to

TABLE 1. *Dry weights of samples after indicated treatment*

Substance	Wt of sample no.		
	1	2	3
	mg	*mg*	*mg*
Original washed L-form pellets	258.4	269.1	257.8
Lipids	18.2	19.9	17.0
Defatted L-form material	243.5	251.2	245.0
Trypsinized organisms	13.8	15.5	16.4
Formamide-insoluble material	2.4	2.6	2.9

purify any existing murein. However, the formamide-insoluble material was soluble in hot phenol. These additional results suggest that murein is lacking from the L-form of *S. faecium* F24.

The analyses presented in this report were carried out to determine whether this enterococcal L-form contained murein. Although a formamide-insoluble material was found, it did not fit the usual criteria for murein (mucopeptide), as given by Rogers and Perkins (7). Thus, the experiments shown in this report characterize the *S. faecium* strain F24 L-form as devoid of murein. These observations are important because the enterococcal L-form used in this study is the only L-form presently known to contain a large deletion in the genome after being subcultured under relatively nonselective conditions (3).

TABLE 2. *Results from analyses performed on formamide-insoluble fractions*

Amino acids	Per cent of amino acid in batch no.			
	1	2	3	4
Alanine	2.0	1.8	2.7	2.2
Aspartic acid	3.5	5.0	3.3	2.9
Arginine	2.5	2.1	1.9	1.5
Glutamic acid	3.2	3.2	ND[a]	3.3
Glycine	4.4	2.2	1.6	2.4
Isoleucine	3.0	2.9	2.7	2.4
Leucine	3.1	3.2	4.6	3.1
Lysine	2.1	2.1	2.7	2.2
Methionine	ND	0.4	ND	0.4
Phenylalanine	2.5	0.5	2.6	3.1
Proline	ND	Trace	1.9	1.7
Serine	1.3	1.4	1.5	1.4
Threonine	1.9	1.9	1.8	1.6
Tyrosine	1.4	0.9	1.6	1.2
Valine	2.0	2.5	2.5	2.2
Histidine	0.3	ND	ND	0.4
Hexosamines	ND	ND	ND	ND

[a] Not detectable.

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