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Effect of Culture pH on the D-Alanine Ester Content of Lipoteichoic Acid in *Staphylococcus aureus*

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The lipoteichoic acid in *Staphylococcus aureus* growing at high pH values contained very little alanine ester, showing that high overall levels of substitution were not essential for growth. The low alanine content could have resulted from a progressive loss due to base-catalyzed hydrolysis of the labile ester linkages.

The widespread distribution of lipoteichoic acid (membrane teichoic acid) in gram-positive bacteria has led to the idea that this acid plays an important role in the physiology of these bacteria. An early suggestion (11) was that both wall and membrane teichoic acids are involved in cation binding and in maintaining an optimum ionic environment at the membrane-bound sites of wall polymer synthesis. It was suggested that teichoic acids might also regulate the action of autolysins in cell division and that the presence of alanine ester or another basic substituent could be important in both of these suggested functions (11). More recent studies support this regulatory function and suggest that fluctuations in the alanine ester content of lipoteichoic acid could form an efficient basis for the regulation of autolytic activity in growing bacteria (10). Such regulation would require that the alanine ester content could vary in teichoic acid molecules in different cellular locations or at different times in the cell cycle, but at present little is known of the processes by which such alterations could be brought about. In contrast to reports (2, 7) that the alanine ester content of wall teichoic acid varied in bacteria grown at different pH values in chemostat culture, Fischer and his colleagues (8, 9) found that the alanine ester content of lipoteichoic acid in staphylococci did not alter significantly with decreasing pH during growth under batch conditions. Differences in the control of alanine ester content of wall and membrane teichoic acids could have an important relationship to function, and we have therefore examined the effect of growth pH on the alanine ester content of both wall teichoic acid and lipoteichoic acid in *Staphylococcus aureus* grown under balanced conditions in chemostat culture.

Our "trained" derivative of *S. aureus* H was grown in a 3-liter chemostat essentially as described previously (5) at a dilution rate of 0.12/h in fully defined glucose-limiting medium. The pH was maintained at the set value (pH 6, 7, or 8) by the automatic addition of sterile 2 M ammonium hydroxide. After equilibration for 10 generation times, bacteria were collected from the overflow line into ice-cooled receivers and harvested by centrifugation at 11,000 × *g* for 10 min at 2°C. The bacterial pellet was washed with 0.1 M sodium acetate buffer, pH 4.5, suspended in buffer, and disrupted in a Braun disintegrator. Walls were prepared by adding disrupted bacteria to an equal volume of boiling 4% sodium dodecyl sulphate, pH 5.0. The mixture was stirred for 4 h until it was cold and then walls were recovered by centrifugation and washed with water as previously described (5). Lipoteichoic acid was isolated from the disrupted bacteria essentially as described by Fischer et al. (8). Crude lipoteichoic acid was analyzed for phosphate (4), D-alanine ester (12), and hexose (6) and then was purified by column chromatography on Sepharose 6B (8). In each case this method gave one major peak of material, representing about 85% of the total phosphate. Fractions constituting this peak were combined to give a purified lipoteichoic acid fraction that was analyzed for D-alanine and phosphate. Typically, the lipoteichoic acid from 5 g (wet weight) of bacteria yielded ca. 65 μmol of phosphate.

Crude lipoteichoic acid from staphylococci grown at pH 6.07 contained phosphate, alanine, and hexose in the molar proportions 1.0:0.70:0.13. The optical extinction at 260 nm indicated that nucleic acid could represent up to 10% of the phosphate that was present. This nucleic acid was removed by chromatography on Sepharose, which gave a purified lipoteichoic acid in which less than 1% of the phosphorous was due to nucleic acid. A sample of this material (containing ca. 2 mg of lipoteichoic acid) was passed through a column containing Dowex 50 (NH_4^+-form) resin (5 ml) to remove Na^+ ions, taken to dryness, and then hydrolyzed for 3 h at 100°C in M HCl. The hydrolysate was taken to dryness over KOH pellets and then examined chromatographically by using the solvent systems and chromatographic reagents and standards previously described (1) for the characterization of teichoic acids. As expected of lipoteichoic acid, the main products were glycerol and its mono- and diphosphates together with some hexose and a substantial amount of alanine, which was the only ninhydrin-positive compound present. Crude lipoteichoic acid from staphylococci grown at pH 7.05 contained only traces of UV-absorbing material, and less than 2% of the phosphate was present as nucleic acid. Phosphate, alanine, and hexose were present in the molar proportions 1.0:0.45:0.12. Acid hydrolysis of the purified material gave products similar to those described above. The crude lipoteichoic acid fraction from bacteria grown at pH 8.1 contained phosphate, alanine, and hexose in the molar proportions 1.0:0.05:0.09 but was more heavily contaminated with nucleic acid, which accounted for up to 15% of the total phosphate. This nucleic acid was not removed by passage through Sepharose, and the teichoic acid fraction showed distinct charring upon acid hydrolysis although the main products were again glycerol and its phosphates. The chromatograms were similar to those obtained with the other lipoteichoic acid preparations except that a greatly diminished proportion of alanine was present, although this was again the only ninhydrin-positive material formed.

Teichoic acid was not isolated from cell walls, but walls

TABLE 1. Effect of growth pH on the D-alanine ester content of the wall teichoic acid and lipoteichoic acid in *S. aureus* H[a]

Growth pH	D-alanine ester content (moles of D-alanine per mole of phosphate) of:	
	Wall teichoic acid	Lipoteichoic acid
6.07	0.65	0.75
7.05	0.40	0.54
8.10	0.02	0.07

[a] Values for lipoteichoic acid were obtained by analysis of material purified by Sepharose gel chromatography. Values for wall teichoic acid were obtained by analysis of isolated cell walls.

were analyzed for phosphate and for base-labile (i.e. ester) alanine. The results (Table 1) confirm the previous (2) demonstration that growth pH affects the alanine ester content. However, there appeared to be little variation in the total content of teichoic acid in the walls; those from bacteria grown at pH 6, 7, and 8 contained 4.3, 3.9, and 4.2% phosphorus (wt/wt), respectively.

The results (Table 1) also show that culture pH affects the alanine ester content of lipoteichoic acid nearly as much as it affects wall teichoic acid in staphylococci. The very low content of alanine ester in bacteria grown at pH 8 appears to be incompatible with suggestions that alanylation has a regulatory function that is important in the growth of these bacteria. However, it is possible that newly synthesized teichoic acid is fairly highly substituted and that the alanine ester groups are lost by base-catalyzed hydrolysis of the labile ester linkages. Those functions that depend on the presence of alanine could be fulfilled by the most recently synthesized molecules while older and dealanylated molecules may be excreted or concentrated in surface regions where alanylation is not functionally required.

The control of alanine incorporation into teichoic acid is not yet fully understood (3) although studies of the distribution of alanine residues in lipoteichoic acid (9) have led to the suggestion that incorporation proceeds in a random but nevertheless precise manner. We think, however, that the content and distribution of alanine residues in lipoteichoic acid could also be affected by removal of the alanine residues after their incorporation. We have shown previously that hydrolysis of the base-labile ester linkages can proceed rapidly enough to account for the low alanine content of wall teichoic acid in bacteria grown at high pH values in chemostat culture (2). A similar explanation could apply to lipoteichoic acid; consequently, there could be a relationship between the alanine content and age (and perhaps cellular location) of the lipoteichoic acid chains in the bacteria. There could also be a relationship between growth rate and alanine ester content in bacteria growing at high pH values, and this may be why little pH-dependant variation was seen in batch-grown staphylococci (9).

Regardless of whether hydrolysis is the sole cause of the low alanine content in bacteria grown at high pH values, the observed differences in alanine content may have important consequences. For example the tolerant (13) response to oxacillin shown by various clinical isolates of *S. aureus* is greatly dependent on the conditions used in growing the bacteria before testing their sensitivity. Tolerance was shown (14) by bacteria that had been grown overnight in media that gave a final pH of 6.2 or less. The same bacteria grown in media giving a final pH of 7.1 or above did not show tolerance when tested under otherwise identical conditions. This difference is of obvious importance in considering the action of antibiotics on bacteria growing under acidic conditions in infected tissue, and a suggested explanation for this effect of pH (14) is that it results in some alteration in the composition of the bacteria. One clear possibility indicated by the present study is that the different growth conditions cause differences in the alanine ester contents of wall and membrane teichoic acids and that these are involved in the expression of tolerance.

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