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THE ALANINE ESTER CONTENT AND MAGNESIUM BINDING CAPACITY OF WALLS OF *STAPHYLOCOCCUS AUREUS* H GROWN AT DIFFERENT pH VALUES

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SUMMARY

Recent studies have shown that teichoic acids are functionally involved in the uptake of Mg^{2+} and that their ability to bind Mg is reduced by the presence of alanine ester substituents. Walls of *Staphylococcus aureaus* H grown at pH 5 contain more ester alanine and bind less magnesium than do walls of *S. aureus* grown under similar conditions at pH 6 and 7. The differences in alanine content are largely due to its removal by base catalysed hydrolysis of the labile ester linkages during growth in media of pH 6 and 7 and the incorporation of alanine esters into the walls does not appear to be influenced by the pH of the medium in which the bacteria are grown under the conditions described. The decreased magnesium binding capacity of walls of *S. aureus* grown at pH 5 correlates with their increased alanine ester content but there is no proportional relationship between these two quantities.

INTRODUCTION

Teichoic acids[1] make important contributions to the surface charge[2] of the cell of Gram-positive bacteria. The early suggestion[3] that they are involved in ion exchange and in the control of access of ions to the inner regions of the cell has been supported by recent studies. Thus it has been shown that the ability of certain isolated walls to bind cations is due almost entirely to their teichoic acid components[2] and that Mg so bound to wall-membrane preparations is available to certain Mg-requiring multienzyme complexes in the membrane, the activity of which in such preparations is then independent of the Mg content of the suspending buffer[4]. Growth of various Gram-positive bacteria under conditions of phosphate limitation results in synthesis of walls that contain phosphate-free acidic polysaccharides in place of teichoic acid[5,6]. Such walls have a decreased affinity for Mg, and when uptake of this cation is constrained by the presence of excess of NaCl in the medium the cells synthesize teichoic acid even when grown under phosphate limitation[7,8]; these results support the conclusion that teichoic acids are involved in the assimilation of cations. We have shown[2] that the capacity of walls to combine with Mg is substantially influenced by the presence of D-alanine ester substituents on the teichoic

acid and that removal of these groups leads to an increase in the amount of combined magnesium, so that it is possible that the alanine ester residues of the teichoic acid function in regulation surface charge and the uptake of cations. The proportion of D-alanine ester substituents on teichoic acid has been reported[6] to vary markedly on growth of certain bacilli at different pH values. We have therefore extended our studies on *Staphylococcus aureus* H and have examined the alanine ester content and Mg binding capacity of walls of this organism grown at different pH values. We have also studied the hydrolytic removal of alanine ester residues from walls suspended in media of the pH values used for growth of cells in order to determine whether the differing amounts of alanine ester in isolated walls are caused by different levels of incorporation or by hydrolytic losses during growth.

METHODS

S. aureus H was grown in a 700 ml chemostat in medium containing Difco tryptone, 5 g; K_2HPO_4, 2.5 g; $(NH_4)_2\ SO_4$, 2 g; K_2SO_4, 100 mg; thiamine HCl, 0.5 mg; nicotinic acid, 2.5 mg; biotin, 0.05 mg; 3,4 dihydroxybenzoic acid, 7.5 mg; cysteine·HCl, 0.5 mg; glucose, 15 g and demineralized water, 1 l. The vitamins and glucose were sterilized separately at 105 °C for 10 min and then added aseptically to a previously sterilized (121 °C for 30 min) solution of the other constituents which had been adjusted to pH 7.0 by the addition of H_2SO_4. The only source of Mg in this medium was found to be the Difco tryptone which provided 0.12 mequiv Mg/l. Chemostat cultures were established by inocculating medium (500 ml) in the growth chamber with a batch culture (10 ml) of *S. aureus* H which had been grown in the same medium at 37 °C for 16 h. The dilution rate was 0.12 h^{-1} throughout the experiment. The temperature was held at 37 °C and the culture was stirred and aerated (flow rate 2 l/h^{-1}). The pH of the culture was maintained at either 7.0 or 6.0 by the automatic addition of 2 M NH_4OH or at pH 5.0 by similar addition of 1M HCl. When the density of the culture was constant, usually after 2–3 days, cells were collected in a cooled reservoir at 2 °C and harvested by centrifugation at intervals of up to 24 h. The cells were then washed with water and freeze-dried. The yields (dry weight) obtained on growth at pH 7, 6 and 5 were 690, 440 and 280 mg/l, respectively.

Isolation of cells walls

Cells were disrupted and walls were isolated as previously described[2]. The walls were washed repeatedly with 0.9% (w/v) aqueous NaCl which removed all bound Mg; they were then washed with deionised water and freeze dried. Except where otherwise stated such walls were used without further treatment. For some experiments walls were heated to inactivate autolytic enzymes. Aqueous suspensions (5mg/ml) of walls were heated quickly to 100 °C and maintained at that temperature for 10 min. The walls were then washed with water and freeze dried.

Analysis of walls

Phosphate was determined by the method of Chen *et al.*[9]. Alanine ester was determined as follows: walls (10 mg) were suspended in 0.1 M-NaOH (10 ml) and kept with occasional shaking at 22 °C for 1 h. The walls were then removed by centrifugation (10000 × *g*, 15 min), washed with water (10 ml) and the supernatant

solutions were combined, mixed with 1 M HCl (1 ml) and adjusted to 25 ml with water. Analysis of this solution using a Jeol amino acid analyser showed that alanine was the only amino acid present. Further extraction of the walls with 1 M NaOH did not yield more alanine. The amounts of alanine in the extracts were determined either by autoanalysis or by conversion to dinitrophenylalanine which was then extracted into ether and determined colorimetrically[10]. Both methods gave similar results.

Determination of the cation binding capacity of walls

Walls were suspended in buffers containing varying amounts of metal chlorides under the conditions previously described[2]. Bound cations were removed from the walls by extraction into $HClO_4$ (ref. 11) and analysed by atomic absorption spectrophotometry. Control experiments showed that this procedure gave quantitative recovery of bound cations.

Preliminary studies showed that trivalent cations were much more strongly bound than divalent cations which were in turn more strongly bound than monovalent cations. The walls had equal affinities for Ca and Mg which were bound in the same relative proportions as those present in buffers containing both cations. In subsequent studies only the Mg binding capacity of walls was determined. Walls (25 mg) were suspended in 50 mM sodium acetate buffer (pH 5.0), (10 ml) 50 mM sodium citrate buffer (pH 6.0) or 50 mM Tris–HCl buffer (pH 7.0), each containing 25 mM $MgCl_2$. After incubation for 15 min at 25 °C the walls were washed and analysed for Mg and phosphate.

Removal of alanine ester residues from walls in media of various pH values

Medium similar to that used for growth of bacteria but containing no tryptone or amino acids was adjusted by the addition of H_2SO_4 to pH values 7.0, 6.0 and 5.0. Heat-treated walls (10 mg) were suspended in batches (10 ml) of medium at these pH values and stirred at 37 °C. At intervals the walls were recovered by centrifugation and analysed for alanine ester residues. In repeat experiments it was found that alanine was lost from isolated teichoic acid at the same rate as from the walls. It was also found that the phosphate and alanine contents of walls did not change when they were treated under the conditions employed in their initial isolation. However, the heat treatment used to inactivate autolytic enzymes removed both alanine and phosphorus and reduced the alanine ester/phosphate ratio of the walls examined from 0.52:1 to 0.44:1 and the phosphate content from 2.8 to 2.0%.

RESULTS AND DISCUSSION

Walls isolated from *S. aureus* H that had been grown in a chemostat at pH 5, 6 and 7 differed in their content of alanine ester residues and also in their capacity to combine with Mg (Table I). Walls of cells grown at pH 7 bound more Mg when incubated with $MgCl_2$ in a buffer at pH 7 than did those of cells grown at the lower pH values; they also bound more Mg than did the walls of the other cells when incubated with $MgCl_2$ at pH 5 and 6. This difference correlates with the increased amount of alanine ester residues in walls of cells grown at the lower pH values and is in agreement with our earlier observation[2] that chemical removal of alanine ester

residues from isolated walls increases their capacity to bind Mg. It is, however, noteworthy that the increases in Mg-binding observed in the earlier study were not proportional to the amounts of alanine removed. The present results (Table I) also show that there is no proportional relationship between the amount of alanine ester and capacity to bind Mg of walls of *S. aureus* grown at different pH values. We conclude that although the presence of alanine affects the capacity of walls to bind Mg, other, at present unknown, factors are also important. It is possible that the increased amount of alanine ester in walls of cells grown at low pH values reflects a greater incorporation of alanine into the teichoic acid under these conditions. This could be of functional and physiological significance and it is noteworthy that greatly enhanced proportions of aminoacyl phospholipids are found in bacteria grown at low pH values[12,13,14]. An alternative explanation might be that the incorporation of alanine ester residues into the wall is not influenced by the pH at which the cells are grown but that many of these residues are removed from the wall by base-catalysed hydrolysis during growth at the higher pH values. It is known that alanine ester substituents of many teichoic acids are highly susceptible to base-catalysed hydrolysis[15] because[16] of the presence of neighbouring phosphate and hydroxyl groups. We have therefore studied the removal of alanine ester residues from walls suspended in media of the pH values used in the growth experiments. For these studies heat-treated walls were used; these contained 2.0% phosphorus and 0.44 mole of alanine ester per mole of phosphate. Little or no phosphate was removed from the walls during incubation with $MgCl_2$ at the different pH values, but in the media of higher pH values substantial amounts of alanine were lost (Table II). Thus after 24 h in medium of pH 7 the alanine ester: phosphate ratio was reduced from 0.44:1 to 0.19:1. At the dilution rate used for growth of the bacteria the mean residence time of each cell in the chemostat is approx. 6 h. During this period alanine will be removed by hydrolysis from walls of cells growing in media of pH 6 and 7. It is likely that loss of alanine from whole cells will occur at a rate similar to that observed with isolated walls. The latter lose 16% of their alanine in 6 h at pH 6 and 36% of their alanine in 6 h at pH 7. Walls from cells grown at pH 6 have an

TABLE I

PHOSPHATE, ALANINE ESTER CONTENT AND MAGNESIUM BINDING CAPACITY OF WALLS OF *S. AUREUS* GROWN AT pH VALUES 5, 6 AND 7.

Analytical procedures were as described in the text. All wall samples were incubated with $MgCl_2$ in buffer of the same pH as the medium in which the cells were grown.

pH at which bacteria were grown	*Phosphorus content of walls (%)*	*Alanine ester content of walls (moles/mole of P)*	*pH of $MgCl_2$ solution in which walls were suspended*	*Mg content of walls after suspension in buffer containing $MgCl_2$ (equiv. Mg/mole P)*
7.0	2.8	0.52	7.0	0.26
6.0	2.6	0.71	7.0	0.20
6.0	2.6	0.71	6.0	0.14
5.0	2.3	0.83	7.0	0.16
5.0	2.3	0.83	5.0	0.12

TABLE II

ALANINE ESTER CONTENTS OF WALLS OF *S. AUREUS* H AFTER INCUBATION IN SOLUTIONS OF DIFFERENT pH VALUES

At the times shown walls were recovered by centrifugation and analysed for alanine ester and phosphate as described in the text. Results are quoted as moles alanine ester per mole of phosphate. Under these conditions less than 5% of the phosphate was extracted.

Time	*Moles of alanine ester per mole of phosphate after suspension in solution of pH value*			
(h)	*5.0*	*6.0*	*7.0*	*7.8*
0	0.44	0.44	0.44	0.44
1	0.44	0.42	0.39	0.39
4	0.42	0.41	0.38	0.31
6	0.44	0.37	0.28	0.16
24	0.45	0.34	0.19	0.05

alanine ester: phosphate ratio that is 14% lower than that of walls from cells grown at pH 5; in walls grown at pH 7 the difference is 39%. There is no obvious reason why alanine should be removed less readily from growing cells than from isolated walls and we conclude that the differences in alanine content of walls of cells grown at different pH values is due to removal by base-catalysed hydrolysis and not to differences in incorporation of alanine by the teichoic acid-synthesizing enzyme system. The differing capacities of the various walls to bind Mg are probably due in part to these differences in their alanine content. Ellwood and Tempest[6] have also observed differences in alanine ester content of walls of *Bacillus subtilis* grown at different pH values. With nitrogen-limited *B. subtilis* var. *niger* the alanine ester/phosphate ratios in the teichoic acid isolated from cells grown at pH 7 was 0.06:1; from those grown at pH 5 the ratio was 0.20:1. This is a much greater difference than that observed by us in Mg limited *S. aureus* and, especially since the bacilli were grown at a much faster dilution rate, it is probable that hydrolytic removal of alanine does not fully account for the differences observed by Ellwood and Tempest; under the growth conditions used by these authors incorporation of alanine esters may thus be pH dependent. It should be noted that the amount of alanine observed in the teichoic acids from the bacilli grown at pH 5 is less than that commonly[1,17] found in this laboratory in cells that have been grown in batch culture and also less than that found in the present study of *S. aureus*. This may be a species difference or may be due to the differing growth conditions employed; but differences in the conditions of storage of bacteria prior to harvesting, and in the procedures used for isolation of walls, could lead to substantial differences in the alanine content of walls because of the ease with which such residues are hydrolysed. It is not known whether the accumulation of aminoacyl phospholipids observed[12–14] during growth of several bacteria at low pH values is due to increased synthesis, but it is important to note that the alkali-lability of the aminoacyl ester residues in these compounds is similar to that of the alanine ester residues in teichoic acid.

The lability of these aminoacyl substituents towards alkali and their consequent

removal, at least from the walls of *S. aureus*, during growth at high pH values may be of physiological importance since it will increase the overall negative charge on the cell surface.

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