Binding of Magnesium Ions to Cell Walls of *Bacillus subtilis* W23 containing Teichoic Acid or Teichuronic Acid

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When grown in a chemostat under various nutritional conditions, cells of *Bacillus subtilis* W23 produce walls containing teichoic acid or teichuronic acid. The binding of Mg^{2+} to these walls and to the isolated anionic polymers in solution was measured by equilibrium dialysis. In solution the ribitol teichoic acid bound Mg^{2+} in the molar ratio $Mg^{2+}/P = 1:1$ with an apparent association constant ($K_{assoc.}$) of $0.61 \times 10^3 M^{-1}$, and the teichuronic acid bound Mg^{2+} in the ratio $Mg^{2+}/CO_2^- = 1:1$, $K_{assoc.} = 0.3 \times 10^3 M^{-1}$. Cell walls containing teichuronic acid exhibited closely similar binding properties to those containing teichoic acid; in both cases Mg^{2+} was bound in the ratio Mg/P or Mg/CO_2^- of 0.5:1 and with a greater affinity than displayed by the isolated polymers in solution. It was concluded that Mg^{2+} ions are bound bivalently between anionic centres in the walls and that the incorporation of teichoic acid or teichuronic acid into the walls gives rise to similar ion-binding and charged properties. The results are discussed in relation to the possible functions of anionic polymers in cell walls.

There is now considerable evidence supporting the suggestion (Archibald *et al.*, 1961) that the primary role of teichoic acids in the cell walls and membranes of Gram-positive bacteria is concerned with the assimilation of bivalent cations, especially Mg^{2+} , and their maintenance at relatively high concentration in the region of the cytoplasmic membrane. Thus selective removal of teichoic acid from the cell walls of *Staphylococcus aureus* H leads to a substantial decrease in their ability to bind Mg^{2+} (Heptinstall *et al.*, 1970), and the increase in Mg^{2+} -binding capacity of *Bacillus subtilis* var. *niger* in response to changes in the environment in which it is grown has been correlated with an increase in the teichoic acid content of its cell wall (Meers & Tempest, 1970). Further, the activity of membrane-bound enzymes involved in the biosynthesis of *Bacillus licheniformis* cell walls is dependent on Mg^{2+} , which can be supplied to the cytoplasmic membrane by wall and membrane teichoic acids (Hughes *et al.*, 1973). In fact, the membrane enzymes show a marked preference for Mg^{2+} bound to teichoic acid over that supplied in solution in the suspending medium.

The importance of anionic cell wall polymers has also been demonstrated by changes in cell-wall composition of bacteria grown under controlled

conditions in a chemostat (Ellwood & Tempest, 1972a). Cell walls of *B. subtilis* var. *niger* contained a glycerol teichoic acid when growth was limited by the availability of magnesium, potassium, carbon, nitrogen or sulphur, but this was replaced by teichuronic acid, which does not contain phosphorus, when phosphate was the growth-limiting nutrient (Ellwood, 1970). Teichuronic acid thus replaced teichoic acid when the availability of phosphate was limited (Meers & Tempest, 1970). Later work has utilized equilibrium dialysis under controlled conditions of ionic strength to obtain association constants for binding of Mg^{2+} to teichoic acids, and has provided information on the number and nature of the binding sites (Lambert *et al.*, 1975a,b). These studies showed that Mg^{2+} was bound less strongly to isolated ribitol wall teichoic acid than to glycerol wall teichoic acid and that the molar ratio Mg^{2+} bound/P was 1:1 with the ribitol polymer but 0.5:1 with the glycerol polymer. It was concluded that with the ribitol polymer Mg^{2+} shares its valencies between phosphate and counter ions, whereas Mg^{2+} binds to the glycerol polymer only through phosphate groups. However, with intact cell walls binding affinities were high regardless of the type of teichoic acid, and in either case there was 0.5 Mg^{2+} site/phosphate group. It was thus suggested that in intact cell walls bivalent binding of Mg^{2+} occurs between adjacent teichoic acid chains, a mechanism which is likely to be important in the transfer of ions from the environment to the cytoplasmic membrane.

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The cell walls of *B. subtilis* W23 contain teichoic acid when grown in a chemostat with excess of phosphate; under phosphate limitation, however, teichuronic acid is the only anionic polymer (Ellwood & Tempest, 1972a). The reason for the apparent preference for teichoic acid is not known, but might be related to a possible superiority over teichuronic acid in Mg^{2+} binding (Meers & Tempest, 1970). To gain further information on this point, we have used equilibrium dialysis to compare the Mg^{2+} -binding properties of cell walls of *B. subtilis* W23, containing either teichoic acid or teichuronic acid, and the isolated polymers themselves.

Teichoic acid and teichuronic acid are also responsible for maintaining the negative surface charge carried by the cells. This is important to their survival in suspension, since it prevents aggregation with other cells or with other particulate matter (Heptinstall *et al.*, 1970). The magnitude of the surface charge is presumably controlled by the nature of the charged groups and their number at or near the cell surface. In this respect the configuration of the polymer chains in the wall matrix is likely to be an important factor. We have used electrophoretic-mobility measurements on whole cells of *B. subtilis* W23 to investigate the effects of variation in the composition of the walls on their surface charge.

Experimental

Growth conditions

Bacillus subtilis W23 was grown in a 0.5-litre Porton-type chemostat at pH 7.0 and 37°C. Foaming was suppressed by continuous addition of sterile poly(ethylene glycol) at approx. 50 μ l/h and aeration was maintained at 2 litres/min. The media used were the potassium-limited and phosphorus-limited media described by Tempest *et al.* (1968) and the magnesium-limited medium of Tempest *et al.* (1967). Cells were collected overnight in a chilled reservoir and cell walls were prepared and purified as described previously (Wright & Heckels, 1975).

Analytical methods

Phosphate, amino acids and hexosamine were determined as described previously (Heckels *et al.*, 1975). Uronic acid was determined by the method of Bitter & Muir (1962). Glucose was determined by the method of Dubois *et al.* (1956) and with glucostat reagent (Worthington Biochemical Corp., Freehold, NJ, U.S.A.).

Extraction of cell-wall polymers

Teichuronic acid was extracted from cell walls with trichloroacetic acid and purified by chromato-

graphy on DEAE-cellulose as described previously (Wright & Heckels, 1975).

Teichoic acid was extracted into trichloroacetic acid and purified by passage through a stacked column of Sephadex G-25 and G-75 (Slabyj & Panos, 1973).

Chromatography

Cell-wall and polymer samples were examined by chromatography after hydrolysis as described previously (Wright & Heckels, 1975).

Measurement of Mg^{2+} binding

The binding of Mg^{2+} to cell walls and to purified teichoic acid and teichuronic acid was measured by equilibrium dialysis as described by Lambert *et al.* (1975a,b). All measurements were made at 20°C in buffer solutions containing 10 mM-NaCl and sufficient 3,3'-dimethylglutaric acid (0.1 mM) and NaOH (0.18 mM) to maintain pH 7.0. Cell walls, prepared and purified as described above, were washed twice in buffer and finally suspended in buffer at 1 mg dry wt./ml. The suspensions contained no Mg^{2+} detectable by atomic absorption spectroscopy after wet digestion (Eagon, 1969). In one experiment Mg^{2+} binding to walls was measured after removal of the ribitol teichoic acid by periodate oxidation (Heptinstall *et al.*, 1970). Walls (1 mg/ml), prepared from cells grown under magnesium limitation at a dilution rate of 0.3 h⁻¹, were treated with sodium metaperiodate (0.01 M) in sodium acetate buffer (0.1 M, pH 4.55) at 20°C for 18 h. The walls were washed with 3 × 50 ml of water and treated with aq. 0.1 M-KBH₄ for 4 h at 20°C. After removal of excess of borohydride with acetic acid the walls were washed with 3 × 50 ml of water, with 3 × 5 ml of equilibrium-dialysis buffer and finally suspended to the original volume in buffer for measurement of Mg^{2+} binding. Periodate oxidation decreased the phosphorus content of the walls by 92% from 1.35 to 0.1 μ mol of P/mg.

Teichoic acid and teichuronic acid were converted into the respective sodium salts by passage through a column of Dowex 50 (Na⁺ form) resin (AG 50W-X8; 50–100 mesh; Bio-Rad Laboratories, Richmond, CA, U.S.A.). Solutions of teichoic acid (1 μ mol of P/ml) or teichuronic acid (1 μ mol of uronic acid/ml) were then prepared in buffer for measurement of Mg^{2+} binding by equilibrium dialysis.

Direct comparison of the Mg^{2+} -binding properties of the various cell-wall suspensions is possible when the results are expressed in terms of μ mol of Mg^{2+} bound/mg dry wt. of cell wall. For the isolated teichoic acid and teichuronic acid the results are expressed as binding isotherms, i.e. $\bar{r}$ versus A , where $\bar{r}$ is the number of Mg^{2+} ions bound/phosphate or uronic acid group and A is the equilibrium concentration of

Table 1. *Composition of walls of B. subtilis W23 grown in a chemostat*

Cell walls were prepared and analysed as described in the text. The content of D-alanine was determined on cell walls, which had not been purified by trypsin/phenol treatment, from the amount released by incubation in 0.5M-NaOH at 25°C for 1 h. Results are expressed as $\mu\text{mol/g}$ of cell walls; —, absent; n.d., not determined.

Limitation	Dilution rate (h^{-1})	Phosphorus	Glucose	Glucuronic	Galactosamine	Molar ratio P/Ala
K ⁺	0.1	1354	1310	—	—	1:0.01
K ⁺	0.3	1419	1380	—	—	1:0.01
Mg ²⁺	0.1	1290	1370	—	—	1:0.05
Mg ²⁺	0.3	1483	1334	—	—	n.d.
P	0.1	280	112	1040	583	n.d.
P	0.3	1935	n.d.	trace	25	n.d.

Mg²⁺ in solution (mM), and as Scatchard plots, i.e. $\bar{r}/A$ versus $\bar{r}$ (Lambert *et al.*, 1975a).

Electrophoretic mobility of cells

Measurements were made with a particle micro-electrophoresis instrument (mk II, Rank Bros., Bottisham, Cambridge, U.K.). The instrument was operated with a cylindrical cell fitted with reversible blacked platinum electrodes and viewed under dark-field illumination. The apparatus was calibrated by using washed human erythrocytes (Heard & Seaman, 1960). The procedure described by Gittens & James (1960) was followed, particular attention being given to the precautions described by these authors. Mobilities of whole cells were measured at 25°C over the pH range 2.5–8.0 by using buffer solutions prepared from sodium barbiturate, sodium acetate and HCl (Michaelis, 1931), with the addition of NaCl to constant ionic strength (I , 0.02). The cells were washed twice in the appropriate buffer before measurement of mobility. All the cell populations were electrokinetically homogeneous, and so mean values of readings on 30 individual cells are quoted at each pH.

Results

Walls of *B. subtilis* W23 from cells grown under potassium and magnesium limitation at dilution rates of 0.1 h^{-1} and 0.3 h^{-1} contained products characteristic of a glucosylribitol teichoic acid (Table 1). The D-alanyl substituents in crude cell walls were lost during the purification procedure, which involved incubation with trypsin and extraction with phenol (Archibald & Stafford, 1972). Although the presence of alanyl ester residues in the cell wall of *S. aureus* influences the binding of Mg²⁺ (Lambert *et al.*, 1975b), their loss in this study was unimportant, since they were originally present in only small amounts. Walls of cells grown under phosphorus limitation contained teichuronic acid when the dilution rate was 0.1 h^{-1} , but as reported previously this was replaced

Fig. 1. Binding of Mg²⁺ ions to walls of cells grown under phosphorus, magnesium or potassium limitation at dilution rates of 0.1 h^{-1} and 0.3 h^{-1} .

The binding of Mg²⁺ to cell walls of *B. subtilis* W23 was measured by equilibrium dialysis (see the Experimental section) at 20°C in the presence of 10mM-NaCl at pH 7.0. Cell-wall suspensions (1 mg/ml) were prepared from cells grown under phosphorus (○), potassium (●) or magnesium (□) limitation at dilution rates $D = 0.1 \text{ h}^{-1}$ (a) and $D = 0.3 \text{ h}^{-1}$ (b).

by teichoic acid at the higher dilution rate (Wright & Heckels, 1975).

The binding of Mg²⁺ to suspensions of cell walls (1 mg/ml) prepared from cells of *B. subtilis* W23

grown under conditions of potassium, magnesium or phosphorus limitation at dilution rate $D = 0.1 \text{ h}^{-1}$ is shown in Fig. 1(a). The walls of cells grown under phosphorus limitation, which contained teichuronic acid in place of teichoic acid, exhibited binding properties closely similar to those of the walls that contained teichoic acid. At a higher dilution rate ($D = 0.3 \text{ h}^{-1}$) walls from cells grown under phosphorus limitation, which contained teichoic acid, exhibited a lower binding capacity than did the walls from cells grown under magnesium or potassium limitation (Fig. 1b).

Fig. 2 shows the effect of removal of the poly-

Fig. 2. Effect of removal of teichoic acid on binding of Mg^{2+} ions to cell walls

The binding of Mg^{2+} to walls (1 mg/ml) prepared from cells grown under magnesium limitation at a dilution rate $D = 0.1 \text{ h}^{-1}$ as shown before (○) and after treatment with periodate (●) to remove teichoic acid. See legend to Fig. 1 and the Experimental section for details.

(ribitol phosphate) teichoic acid chain from the cell walls of magnesium-limited cells ($D = 0.3 \text{ h}^{-1}$) on their binding ability. Removal of the teichoic acid by periodate oxidation results in a very large decrease in the amount of Mg^{2+} bound by the walls. The low residual binding capacity of the walls might be due to the small number of phosphate groups remaining in the walls after oxidation; these arise from the periodate-stable glycerol phosphate-containing linkage unit between the teichoic acid and peptidoglycan (Heckels *et al.*, 1975; Coley *et al.*, 1976; R. R. Archibald & E. Tarelli, unpublished work). Alternatively, the residual binding could occur at sites on the peptidoglycan, possibly between un-cross-linked peptides as described by Rayman & MacLeod (1975). The degree of cross-linking of peptidoglycan in bacilli is between 35% (Warth & Strominger, 1971) and 46% (Hughes, 1971), so carboxyl groups in the peptidoglycan could contribute to the Mg^{2+} binding by the walls. However, the results in Fig. 2 indicate that teichoic acid is the major binding component of the walls; a similar conclusion was reached by Heptinstall *et al.* (1970) for walls of *S. aureus* H.

The similarity in binding properties of walls containing either teichoic acid or teichuronic acid (Fig. 1) suggests that under certain nutritional conditions teichuronic acid can functionally replace teichoic acid in the walls. Nevertheless, the intrinsic Mg^{2+} -binding properties of the two isolated polymers in solution are not the same; Fig. 3(a) shows that over a given range of Mg^{2+} concentration teichoic acid at a concentration of $1 \mu\text{mol}$ of P/ml binds more Mg^{2+} than does teichuronic acid at an equivalent concentration of $1 \mu\text{mol}$ of uronic acid/ml. The respective Scatchard plots (Fig. 3b) indicate that Mg^{2+} binds to the teichoic acid in the ratio Mg^{2+}/P of 1:0.97 and to the teichuronic acid in the ratio $\text{Mg}^{2+}/\text{uronic}$

Fig. 3. Binding of Mg^{2+} ions to isolated teichoic acid and teichuronic acid

The binding of Mg^{2+} to teichoic acid (●) and teichuronic acid (○) are expressed as isotherms (a) and as Scatchard plots (b), where $\bar{r}$ is the number of Mg^{2+} ions bound/phosphate group in teichoic acid or carboxyl group in teichuronic acid and A is the concentration of free Mg^{2+} (mM). Binding was measured by equilibrium dialysis (see the Experimental section) in the presence of 10 mM-NaCl at pH 7.0 with a teichoic acid concentration of $1.0 \mu\text{mol}$ of P/ml and a teichuronic acid concentration of $1.0 \mu\text{mol}$ of uronic acid/ml.

acid of 1:0.91. Hence the Mg^{2+} binds univalently to each phosphate or uronic acid residue and presumably univalently to a counterion. A similar stoichiometry of binding was reported for the poly(ribitol phosphate) wall teichoic acid from *S. aureus* H (Lambert *et al.*, 1975b), where the construction of molecular models showed that it is not sterically feasible for one Mg^{2+} ion to bind bivalently to two adjacent phosphate groups in the teichoic acid chain. The same reasoning can be applied for the poly(ribitol phosphate) teichoic acid in *B. subtilis* W23, where adjacent phosphate groups are too far apart to permit bivalent binding of the Mg^{2+} . Similarly, the construction of accurately scaled molecular models (Catalin Ltd., Waltham Abbey, Essex, U.K.) shows that it is not possible for one Mg^{2+} ion to bind bivalently to two adjacent uronic acid groups in the isolated teichuronic acid polymer without producing considerable steric strain. The apparent association constants ($K_{\text{assoc.}}$) for Mg^{2+} binding, which are obtained from the slope of the Scatchard plots, are $0.61 \times 10^3 \text{ M}^{-1}$ for teichoic acid and $0.3 \times 10^3 \text{ M}^{-1}$ for teichuronic acid. These values reflect the superior binding ability of the phosphate groups in isolated teichoic acid molecules over the carboxyl groups in teichuronic acid. However, the results in Fig. 1(a) show that walls containing teichuronic acid, from cells grown at a dilution rate $D = 0.1 \text{ h}^{-1}$, are almost indistinguishable from those containing teichoic acid as far as binding is concerned. Further, consideration of the initial slopes of the binding curves in Figs. 1(a) and 3(a) indicates that both teichoic acid- and teichuronic acid-containing walls exhibit much higher affinities for the ions than might be expected from the affinities of the isolated anionic polymers. A possible explanation of the high affinity of the walls is that the Mg^{2+} is bivalently bound between anionic centres, either phosphate or carboxylate, from adjacent polymer chains in the walls. Support for this view, which has also been used to explain a similar effect with teichoic acid in walls of *S. aureus* H (Lambert *et al.*, 1975b), is obtained by extrapolation of the results to give the total amount of Mg^{2+} bound by the walls. Thus, when the data in Fig. 1(a) are plotted in terms of μmol of Mg^{2+} bound/ μmol of phosphate or uronic acid against free Mg^{2+} concentration, the curves approach maximum extents of binding equivalent to about $0.5 \mu\text{mol}$ of Mg^{2+} / μmol of P or uronic acid, suggesting that the Mg^{2+} is bound bivalently in the walls.

Measurement of the electrophoretic mobility of bacterial cells gives an indication of the net charge on the cell surface, and variation in the mobility accompanying changes in pH gives information about the nature of the ionizable groups on the cell surface. The pH-mobility curves for whole cells of *B. subtilis* W23 grown under various conditions and measured at constant ionic strength (0.02) are

Fig. 4. Effect of pH on electrophoretic mobility of whole cells

pH-electrophoretic mobility curves of whole cells grown under phosphorus limitation ($\bullet$) and potassium limitation ($\circ$) are shown at dilution rates $D = 0.1 \text{ h}^{-1}$ and under magnesium limitation ($\square$) at a dilution rate $D = 0.3 \text{ h}^{-1}$. See the Experimental section for details.

shown in Fig. 4. The mobility of cells grown under magnesium limitation at $D = 0.3 \text{ h}^{-1}$ has a maximum value between pH 3 and 5. A less marked maximum is also shown by cells grown under potassium limitation at $D = 0.1 \text{ h}^{-1}$. Similar maxima in pH-mobility curves have been found in *B. subtilis* var. *niger* (Neihof & Echols, 1973) and in certain strains of *S. aureus* (James & Brewer, 1968), and have been attributed to a pH-dependent reversible rearrangement of teichoic acid molecules on the cell surface. The presence of strongly acidic groups on the surface of the *B. subtilis* W23 walls containing teichoic acid is indicated by their retention of high mobility, and hence of negative charge, at pH 2.5. This is in marked contrast with the teichuronic acid-containing cells grown under phosphorus limitation at $D = 0.1 \text{ h}^{-1}$, which have zero mobility and hence no surface charge at pH 2.5, indicating the presence of weakly acid carboxyl groups on the cell surface. However, it is significant that over the pH range 6–8 the cells have the same mobility as those containing teichoic acid, and therefore the teichuronic acid produces a surface charge on the cell that is closely similar to that produced by teichoic acid over the physiological pH range.

Discussion

We have shown that, in response to variation in growth conditions, cells of *B. subtilis* W23 produce walls containing teichoic acid or teichuronic acid, anionic polymers of widely different chemical composition. Despite these chemical differences the walls are remarkably similar in two properties that are thought to depend on the anionic polymers, i.e. surface charge and binding of bivalent cations.

Investigations with *B. subtilis* var. *niger* have shown that when cells are grown under conditions that impose a constraint on the availability of Mg^{2+} , i.e. under conditions of magnesium limitation (Ellwood, 1970) or in a high concentration of competing Na^+ (Meers & Tempest, 1970), they increase their Mg^{2+} -binding capacity by incorporating increased amounts of teichoic acid into their walls. When grown under conditions of phosphorus limitation with an adequate supply of Mg^{2+} , teichoic acid in the wall is replaced by a teichuronic acid, which acts as the cation-binding and surface-charge-regulating component (Meers & Tempest, 1970). However, under conditions of phosphorus limitation and high Na^+ concentration the cells revert to incorporating teichoic acid in the walls (Ellwood, 1971). This suggests that teichoic acid-containing walls are more efficient at assimilating Mg^{2+} from a limited supply than are walls containing teichuronic acid. Evidence supporting this view has been given by Meers & Tempest (1970), who measured the amount of Mg^{2+} retained by walls after exposure to various concentrations of Mg^{2+} in the presence of 0.85% (w/v) NaCl, followed by washing with water. The results showed that teichoic acid-containing walls bound more Mg^{2+} than did teichuronic acid-containing walls. However, these results are not strictly comparable with our measurements of cation binding to walls of *B. subtilis* W23, since the teichoic acids are different: *B. subtilis* W23 contains a poly-(ribitol phosphate) teichoic acid and *B. subtilis* var. *niger* contains a mixture of teichoic acids, the predominant one being a poly(glucosylglycerol phosphate) polymer (Ellwood & Tempest, 1972b), and probably more importantly the methods of measurement of cation binding were different. The walls from cells of *B. subtilis* W23 grown under phosphorus limitation at a relatively high dilution rate (0.3 h^{-1}) contain teichoic acid. Although the amount of this polymer is greater than that found under magnesium or potassium limitation, its Mg^{2+} -binding efficiency appears to be lower than it is in walls of cells grown under the other limiting conditions. The reason for this has not been examined, but it could reflect a difference in the arrangement of polymers in the wall.

The binding properties of the isolated teichoic acid and teichuronic acid from *B. subtilis* W23 are clearly different from each other; the teichoic acid binds more Mg^{2+} than does the teichuronic acid over the concentration range 0.1–1.0 mM- Mg^{2+} . When the polymers are incorporated into the walls the difference is no longer apparent and the cells exhibit closely similar binding and surface-charge properties.

The close similarity in cation affinities of cell walls containing the two polymers demonstrates the ability of teichuronic acid to substitute for teichoic acid in binding of cations when the supply of phosphate is

limited, but the reason for the preference for synthesis of teichoic acid under the other limitations is unclear. However, it might be related to the nature of the control process that regulates change-over from teichoic acid synthesis to teichuronic acid synthesis. The nature of this process is not known, but it has been suggested that teichuronic acid synthesis is repressed by a phosphate-containing precursor of teichoic acid, and only under conditions of phosphate limitation is it present in a sufficiently low concentration to allow teichuronic acid synthesis to occur (Ellwood & Tempest, 1969). Relative energy requirements for synthesis of the two polymers, and the advantage of control of cation binding by alanyl ester residues, might also be important.

On the other hand, the predominance of teichoic acids might be related to properties other than cation binding. For example, in some organisms wall teichoic acids appear to be involved in the regulation of autolytic activity, a process which is essential for normal cell-wall growth and the separation of cells after division (Herbold & Glaser, 1975). In *B. licheniformis*, however, it seems that teichuronic acid controls lytic activity. Cells of this organism grown under glucose limitation, or in batch culture, contain both teichoic acid and teichuronic acid in their walls. Removal of teichoic acid or protein from isolated walls has no effect on autolysin sensitivity, whereas removal of teichuronic acid made the walls resistant to autolysin; the autolytic activity was related to the amount of teichuronic acid in the wall preparations. Moreover, a novobiocin-resistant mutant of this organism (nov 12) lacked teichuronic acid; it was defective in its cell morphology because of increased resistance of its walls to autolysin and the absence of such autolysin from the isolated walls. It was concluded that teichuronic acid is necessary for both the binding of the autolysin to the wall and for the bound enzyme to hydrolyse the peptidoglycan (Robson & Baddiley, 1977a,b). Thus both anionic polymers can be important in functions other than cation binding, and the factors deciding the relative importance of these in a given organism are obviously complex.

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