The Influence of Growth-limiting Substrate and Medium NaCl Concentration on the Synthesis of Magnesium-binding Sites in the Walls of *Bacillus subtilis* var. *niger*

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SUMMARY

A comparative study has been made of the influence of growth environment on the properties of the walls of *Bacillus subtilis* var. *niger* organisms, particularly with respect to ability to bind magnesium ions. The medium NaCl concentration and the nature of the growth-limiting substrate, in chemostat cultures, both affected markedly the subsequent affinity and capacity of the bacterial walls for Mg^{2+} adsorption. These changes correlated with differences in the phosphate content of the walls, which in turn related largely to changes in wall teichoic acid content. Thus it seemed that whenever a constraint to the adsorption of Mg^{2+} was applied (either by growing the organisms in a Mg^{2+} -limited environment, or in one containing high concentrations of a competing ion), walls were synthesized that had an increased teichoic acid content and an increased affinity for magnesium ions. These results support the thesis that anionic polymers in the walls of *Bacillus* organisms (i.e. teichoic acids and teichuronic acids) are involved in cation assimilation.

INTRODUCTION

We reported previously (Tempest, Dicks & Meers, 1967) that the affinities with which *Bacillus subtilis* and *Aerobacter aerogenes* cell walls bound magnesium ions were different, and that this difference correlated with the ability of the Gram-negative organisms to outgrow the bacilli in Mg^{2+} -limited mixed cultures in a chemostat. The above mentioned adsorption experiments were made with organisms grown in Mg^{2+} -limited culture only; but since the wall composition of organisms is known to vary with the chemical nature of the environment (Ellwood & Tempest, 1967; Tempest, Dicks & Ellwood, 1968), it was of interest to determine whether the affinity with which organisms bound Mg^{2+} at their surfaces also varied with changes in the nature of the growth limitation. In this connexion, Tempest & Meers (1968) reported that *A. aerogenes* had a lower tolerance for NaCl when its growth was limited by Mg^{2+} availability than when its growth was limited by the availability of any other essential nutrient. This suggested that sodium ions impeded Mg^{2+} assimilation, possibly by competing for adsorption sites on the bacterial surface. Since adsorbed Mg^{2+} probably has an important functional role in bacteria (Gray & Wilkinson, 1965; Asbell & Eagen, 1966; Kushner & Onishi, 1966; Tempest & Strange, 1966), it seemed possible that bacteria might adapt to environments containing low Mg^{2+} concentrations,

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or high Na^+ concentrations, either by increasing the number of sites available for Mg^{2+} adsorption or by changing the chemical nature of these sites such that their affinity for Mg^{2+} was increased. These possibilities have been investigated and the results are reported here. A preliminary account of this work has been published (Meers & Tempest, 1969).

METHODS

Organism. *Bacillus subtilis* var. *niger* (obtained from Fort Detrick, Maryland, U.S.A.; probably the same as ATCC 9372) and *Aerobacter aerogenes* (NCTC 418) were maintained by monthly subculture on tryptic-meat-digest agar slopes containing glucose (0.2%, w/v).

Growth conditions. Organisms were grown in 0.5 l. chemostats (Herbert, Phipps & Tempest, 1965) in the simple salts media described by Tempest & Meers (1968) and by Ellwood & Tempest (1969). The chemostat was operated at a dilution rate of 0.3 h^{-1} and at a temperature of 35° ; the pH value was automatically maintained throughout at $\text{pH } 7.0 \pm 0.05$.

Experimental procedures. To determine the capacity and affinity of binding of Mg^{2+} to bacteria, the following procedure was adopted: 2 ml. volumes of culture, each containing about 3 mg. equivalent dry weight of organisms, were centrifuged at 3000 g for 5 min. Usually the bacterial pellet was then sufficiently compacted for the supernatant fluid to be removed by decantation; otherwise it was carefully removed using a Pasteur pipette. The bacterial pellet was then dispersed in 4 ml. of 0.85% (w/v) NaCl solution, using a 'Whirlimixer' (Fisons Scientific Apparatus Ltd, Loughborough, Leicestershire), recentrifuged, the supernatant fluid again discarded and the pellet retained. Washing the pellets in saline removed the surface-adsorbed Mg^{2+} that was associated with organisms grown in the presence of an excess of this cation (Strange & Shon, 1964). The pelleted organisms were then suspended in various 2 ml. volumes of 0.85% (w/v) NaCl solution, and 0.017% (w/v) NaCl solution, that contained concentrations of Mg^{2+} between 1 and $256 \mu\text{g./ml.}$, and left to equilibrate for 2 to 3 min. at room temperature before being separated by centrifugation. Changing the contact time did not alter the extent of Mg^{2+} adsorption, indicating that equilibrium was established rapidly. Occluded Mg^{2+} was removed by washing the organisms, once, in a small volume (4 to 5 ml.) of water. Finally, samples were 'wet-ashed' (with 1 ml. volumes of 60% (w/v) HClO_4), diluted to an appropriate volume with water and analysed for Mg^{2+} by using an E.E.L. Atomic Adsorption Spectrophotometer (model 140). From these data, plus determinations of the dry weight of organisms in the original suspension (Tempest, Hunter & Sykes, 1965), the adsorbed Mg^{2+} (per mg. dry weight of organisms) could be calculated along with the equilibrium extracellular Mg^{2+} concentration.

The above method was also used to determine the capacity and affinity for Mg^{2+} of bacterial wall preparations which had been obtained by the method of Tempest *et al.* (1968). With bacterial wall preparations the starting suspensions contained 1 mg. freeze-dried walls/ml.; each centrifugation step was of 15 min. duration (at 3000 g).

Potentiometric titration curves were obtained by the method of Brown (1965) using 50 mg. quantities of freeze-dried bacterial walls suspended in 5 ml. volumes of water, or of solutions of various salts. These suspensions could be repeatedly titrated between values of pH 2.0 and 7.0, giving reproducible results. However, at pH values greater than 8.0 the walls changed irreversibly.

Isolation and examination of wall teichoic acids and teichuronic acids were carried out as described previously (Tempest *et al.* 1968).

RESULTS

The Mg^{2+} adsorbed by saline-washed suspensions of *Bacillus subtilis* and *Aerobacter aerogenes* organisms (from Mg^{2+} -limited and PO_4^{3-} -limited cultures) when suspended in 0.017% (w/v) NaCl solutions containing graded concentrations of MgCl_2 are shown in Fig. 1 *a, b*. This low concentration of NaCl was chosen in order to simulate the salts concentration normally present in the various growth media. It is clear (Fig. 1) that the maximum amount of Mg^{2+} that each sample could adsorb (that is, its Mg^{2+} -binding capacity) varied with the growth environment. Thus Mg^{2+} -limited *B. subtilis* organisms had a greater capacity for Mg^{2+} adsorption than had the organisms from a PO_4^{3-} -limited culture. And similar, though quantitatively less marked, results were obtained with the organisms from *A. aerogenes* chemostat cultures.

Fig. 1. Magnesium ion-binding capacity of (a) *Bacillus subtilis* var. *niger* organisms and (b) *Aerobacter aerogenes* organisms that had been grown in chemostat cultures ($D = 0.3 \text{ h.}^{-1}$, 35° , pH 7.0) in simple salts environments with either Mg^{2+} availability limiting growth (open symbols) or PO_4^{3-} availability limiting growth (blocked symbols). Data were obtained as described in Methods; solutions of MgCl_2 each contained 0.017% (w/v) NaCl.

Although *Aerobacter aerogenes* organisms generally had a much lower capacity for Mg^{2+} adsorption than had *Bacillus subtilis* organisms, they nevertheless bound Mg^{2+} more avidly (Tempest *et al.* 1967). Adsorption of Mg^{2+} was competitively inhibited by Na^+ ; therefore, by measuring the Mg^{2+} bound by the variously grown samples of organisms (or isolated bacterial walls) when suspended in solutions of NaCl (0.85%, w/v) containing graded Mg^{2+} concentrations, the specific affinity of each of the cell surfaces for Mg^{2+} could be assessed. When organisms from variously limited *B. subtilis* chemostat cultures were thus compared (Fig. 2) it was evident that Mg^{2+} -limited organisms had a much greater affinity for Mg^{2+} than had organisms from other *B. subtilis* cultures. But, in almost every case, addition of 4% (w/v) NaCl to the medium

in which the organisms were growing caused the synthesis of bacteria whose walls had an increased affinity for Mg^{2+} adsorption (Fig. 2). Thus it seemed that whenever a constraint was applied to the uptake of Mg^{2+} (either by limiting growth by the availability of Mg^{2+} or by adding substantial amounts of Na^+ to the growth environment), bacterial walls were synthesized that had an improved affinity for Mg^{2+} binding.

It is also evident (Fig. 2) that PO_4^{3-} -limited *Bacillus subtilis* organisms had a particularly low affinity for Mg^{2+} . Addition of 4% (w/v) NaCl to these PO_4^{3-} -limited cultures led to a particularly marked increase in Mg^{2+} binding affinity. Since Mg^{2+} -limited and PO_4^{3-} -limited *B. subtilis* organisms had walls that were substantially different chemically (Tempest *et al.* 1968), an attempt was made to relate these changes in magnesium-binding affinity to changes in wall macromolecule content and composition.

Fig. 2. Magnesium ion-binding affinity of *Bacillus subtilis* var. *niger* organisms, and wall preparations. The open symbols refer to organisms grown in the absence of added NaCl and the closed symbols to those grown in the presence of 4% (w/v) NaCl. Organisms were grown at a dilution rate of 0.3 h^{-1} (35° , pH 7.0) in (a) PO_4^{3-} -limited environments, (b) Mg^{2+} -limited environments, and (c) K^+ -limited environments. Data were obtained as described in Methods; solutions of $MgCl_2$ each contained 0.85% (w/v) NaCl. Similar data were obtained with isolated bacterial wall preparations (d); samples were prepared from Mg^{2+} -limited organisms ($\circ$), PO_4^{3-} -limited organisms ($\triangle$), and from PO_4^{3-} -limited organisms grown in the presence of 4% (w/v) NaCl ($\blacktriangle$).

In order to investigate further the nature of the anionic Mg^{2+} -binding sites, potentiometric titration experiments were undertaken on preparations of isolated *Bacillus subtilis* walls. With walls of Mg^{2+} -limited *B. subtilis* organisms, the pH titration curve showed three prominent areas of buffering (Fig. 3a); one in the region of pH 2.2 (due to primary phosphate groups), one at pH 3.6 (due to carboxyl groups) and one at pH 5.8 (also possibly due to phosphate) (see Galdiero, 1968; James & Brewer, 1968). In contrast, the walls of PO_4^{3-} -limited *B. subtilis* organisms lacked buffering at pH 2.2 and 5.8 but had a greatly increased buffering capacity at pH 3.4 (Fig. 3b). These findings were consistent with the presence of much teichoic acid in the walls of Mg^{2+} -limited organisms and much teichuronic acid in the walls of PO_4^{3-} -limited organisms.

Fig. 3. Buffering capacity of wall preparations from *Bacillus subtilis* var. *niger* organisms grown in (a) a Mg²⁺-limited environment and (b) a PO₄³⁻-limited environment that contained either no added NaCl (solid line) or 4% (w/v) NaCl (broken line). Suspensions of walls (50 to 100 mg. in 5 ml. water) were titrated with 0.1 N-HCl or 0.1 N-NaOH, using an Agla micrometer syringe (Burroughs Wellcome & Co., London). The wall suspensions were continuously mixed during the course of the titration by sparging with oxygen-free nitrogen gas. In order to derive the data shown above, blank values, obtained by titrating 5 ml. volumes of water, were subtracted from the values obtained with the wall suspensions.

Table 1. The influence of growth environment on the phosphate content of isolated walls of *Bacillus subtilis* var. *niger*, and on their capacity and affinity of binding of Mg²⁺

To measure the Mg²⁺-binding capacity, wall preparations were suspended in 100 mM-MgCl₂ solution and then washed repeatedly with deionized water until the bound Mg²⁺ content was constant. To measure the Mg²⁺-binding affinity, wall preparations were suspended in a solution of MgCl₂ (0.5 mM) in 0.85% (w/v) NaCl. The walls were separated by centrifugation, washed once with deionized water and the adsorbed Mg²⁺ determined by atomic adsorption spectrophotometry. The values for Mg²⁺-binding affinity (below) are expressed as ratios of Mg²⁺ adsorbed by wall preparation to Mg²⁺ adsorbed by an equivalent weight of Mg²⁺-limited *B. subtilis* walls.

Growth limitation	Medium NaCl content (g./l.)	Wall phosphorus content (g./100 g. dry walls)	Mg ²⁺ -binding capacity (μg./mg. dry wt)	Mg ²⁺ -binding affinity
Mg ²⁺	0.1	5.8	15.6	1.00
Mg ²⁺	40.0	6.4	19.0	1.30
PO ₄ ³⁻	0.1	0.3	13.3	0.54
PO ₄ ³⁻	40.0	3.0	17.5	0.73

Addition of 100 mM-MgCl₂ to the bacterial wall suspensions caused a marked shift in the titration curves showing that this cation was bound by both the phosphate and carboxyl groups (see Galdiero, Lembo & Tufano, 1968).

Addition of 4% (w/v) NaCl to the PO₄³⁻-limited medium in which *Bacillus subtilis* was grown caused an appreciable change in the wall phosphate content and Mg²⁺-binding capacity (Table 1) and a marked change in the pH titration curve (Fig. 3*b*) and Mg²⁺-binding affinity (Table 1). The most obvious explanation for these observations was that the presence of NaCl in the growth medium induced, in some way, the synthesis of some wall-bound teichoic acid in the PO₄³⁻-limited organisms. When the walls of these organisms were isolated, hydrolysed with 6 N-HCl (3 h., 100°) and analysed chromatographically (Tempest *et al.* 1968), amounts of glucose and glycerol monophosphate, and traces of glycerol diphosphate, were detected. These substances, which are characteristic of a glycerol teichoic acid, were absent from hydrolysed wall preparations of PO₄³⁻-limited organisms grown in the absence of NaCl. Clearly (Table 1), the phosphorus content, the Mg²⁺-binding affinity and the buffering capacity at pH 2.2, of wall preparations each increased with addition of 4% (w/v) NaCl to the growth environment but the three factors did not change proportionately.

DISCUSSION

The data given in this paper show unequivocally that the walls of bacteria (*Bacillus subtilis* var. *niger* and *Aerobacter aerogenes*) changed markedly in response to changes in the environment in which they were grown. In particular, when the environment was adjusted so that a constraint was applied to the adsorption of Mg²⁺ (that is, by growing the organisms in a medium that contained low concentrations of Mg²⁺ or high concentrations of Na⁺), walls were synthesized that had an increased affinity for magnesium ions. With cultures of *B. subtilis* var. *niger*, the increased affinity for Mg²⁺ correlated with an increased teichoic acid content of the walls and it is reasonable to conclude, therefore, that teichoic acids are involved in the process of Mg²⁺ assimilation, particularly when this nutrient is not readily accessible. In this context, it has been suggested by Archibald *et al.* (1961) that teichoic acids, being strongly anionic, may be functionally involved in the movement of ions between the organism and its environment; the results reported here accord fully with this hypothesis.

The fact that the walls of PO₄³⁻-limited *Bacillus subtilis* var. *niger* organisms, though lacking teichoic acid, contained an alternative anionic polymer adds further support to the suggestion that these polymers play an essential role in the flux of charged metabolites across the cell wall and plasma membrane. However, the Mg²⁺-binding affinity of teichuronic acid, although not negligible, was clearly much less than of teichoic acid (Table 1) and presumably was functionally inadequate when the growth environment contained much NaCl. Addition of 4% (w/v) NaCl to PO₄³⁻-limited cultures of *B. subtilis* caused the synthesis of walls containing both teichoic acid and teichuronic acid, and these walls had a greatly increased Mg²⁺-binding affinity.

It has been reported (Kushner & Onishi, 1966) that extremely halophilic Gram-negative bacteria require a high concentration of Mg²⁺ for growth. Furthermore, the proteins in their envelopes are, compared with mesophilic cell walls, extremely acidic (Brown, 1963). It would be of interest to know whether the walls of these halophilic organisms have a strong affinity for magnesium ions; if our thesis is correct, this should be so.

The results reported here reinforce our previous conclusion (Tempest & Ellwood, 1969) that the walls of bacteria are highly variable entities which can change structurally (and, presumably, functionally) in such a way as to fit the organism for life in a wide variety of environments.

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