Mechanical Properties of *Bacillus subtilis* Cell Walls: Effects of Ions and Lysozyme

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Bacterial threads of *Bacillus subtilis* have been immersed in, and redrawn from, water of various pH values, in solutions of \((\text{NH}_4)_2\text{SO}_4\) and NaCl of various concentrations, and in lysozyme solutions. The changes in the tensile strength, elastic modulus, and other mechanical properties of the bacterial cell wall due to these treatments were obtained. The data show that change in pH has little effect but that as the salt concentration is increased, the cell walls become more ductile. A high salt concentration (1 M NaCl) can reduce the modulus by a factor of 26 to 13.5 MPa at 81% relative humidity and the strength by a factor of only 2.5. Despite attacking the septal-wall region of the cellular filaments, lysozyme has no effect on the mechanical properties. There is no significant change in the stress relaxation behavior due to any of the treatments. The dependence of mechanical properties on the salt concentration is discussed in terms of the polyelectrolyte nature of cell walls. The evidence presented in this and the accompanying paper (J. J. Thwaites and U. C. Surana, J. Bacteriol., 173:197–203, 1991) supports the idea that the peptidoglycan in bacterial cell wall is an entanglement network with a large degree of molecular flexibility, with some order but no regular structure.

The mechanical properties of bacterial cell wall are greatly influenced by the degree to which the walls are hydrated (25, 26). They are also affected by the presence of residual growth medium (18, 26). This growth medium (TB medium) contains NaCl. Salts and pH also have considerable influence over bacterial macrofiber conformation, not only during growth but also in immediate response to environmental change (16, 19). The geometry of macrofibers is thought to be mechanically determined, so that ion-induced changes in wall mechanical properties could be the cause. The walls of gram-positive bacteria are known to readily bind ions (3, 15, 20), and the chemistry of this action has received much attention. Both of the main constituents of the wall provide binding sites. The structural polymer peptidoglycan has both anionic (carboxyl) and positively charged (ammonium) groups. The covalently linked teichoic acid has anionic (phosphoryl) groups only (4, 6, 10). When the teichoic acid of *Bacillus subtilis* is replaced in phosphate-limited growth (1) by another anionic polymer, teichuronic acid, the charged groups are carboxyls. The net charge during growth is always negative, and counterions are supplied by the growth medium. The accessible binding sites are not uniformly distributed in the wall. Although the ionic polymer is present throughout the wall, there is evidence to show that in *B. subtilis* a large amount is at the outer surface (1, 2, 7). This could account for the greater proportion of binding sites there (23). Negatively charged groups are also more densely clustered at the poles than on the cylinder wall, but there appear to be none in the septa (22, 23).

When placed in distilled water, isolated cell walls need counterions only to the extent of the net negative charge. The elements of the wall are apparently flexible enough to arrange themselves so that all of the cationic groups of peptidoglycan act as counterions for anionic groups. In salt solutions the salt provides the counterions and, if the concentration is large, co-ions as well. A molecular rearrangement must accompany this change in counterion provision (11). For example, teichoic acid is rod shaped in distilled water but assumes a random coil shape in salt solution (8). Certainly, walls are made to contract by salt ions (13, 20). Such changes in the molecular arrangement ought to be accompanied by changes in the mechanical properties of the cell walls. The work reported here concerns the quantitative effects of ions on cell wall axial properties, measured directly by using the bacterial thread system (18, 24). The effects of increasing concentrations of NaCl and of \((\text{NH}_4)_2\text{SO}_4\), as well as the effects of changing pH, were measured.

Ions presumably exert influence via structural changes in the wall polymers. Other structural changes can be brought about by enzyme attack. These structural changes are thought to be the reason for the violent twisting reaction of macrofibers to lysozyme attack (9). Experiments are described in which the effects on cell wall mechanical properties of partial digestion by lysozyme have been measured in bacterial thread. Strong reasons for inferring these properties directly from those of bacterial thread are given in an accompanying paper (26). These reasons are equally valid for threads in which the cell walls have been modified by ions or by lysozyme attack.

The significance of these results and those of earlier experiments (18, 25, 26) is discussed in relation to the molecular organization of the bacterial cell wall. These results are not in conflict with the presently accepted picture. However, because they are quantitative, they will in due course be important in the study of bacterial shape in various environments.

MATERIALS AND METHODS

**Bacterial cultures and thread production.** Cultures of the cell separation-suppressed (lyt) mutant FJ7 were grown at 20°C in TB medium which consists of 10 g of tryptose, 3 g of...
beef extract (Difco), and 5 g of NaCl, each per liter of water. Bacterial threads were produced by using the methods described in the accompanying paper and previously (18, 24, 26). Standard threads were obtained by washing off the residual culture medium in deionized water and then redrawing the threads (26).

Treatments. Standard threads were washed a second time in 2 liters of water containing either (i) small quantities of NaOH, in order to alter pH (meter used, Kent type EIEL3055), (ii) NaCl or (NH₄)₂SO₄ to various concentrations, or (iii) lysozyme in a 66 mM phosphate buffer at pH 6.2. The lysozyme-treated threads were then washed a third time in deionized water to prevent the enzyme reaction from continuing. In all washing and redrawing operations, the cellular filaments separated to a marked degree so that they could be discerned individually. Thus, it is reasonable to suppose that all of the cells in any thread were exposed to the same pH, NaCl concentration, or lysozyme attack. Although in general, despite this separation, there was sufficient cohesion for threads to be redrawn, their quality was not uniformly as good as that of standard threads. Drawing threads from solutions of different pH presented no problems, but obtaining them from salt solutions of high concentration (up to 1 M) proved to be extremely difficult and those that were made were often so irregular in diameter as to be useless for tensile tests. The lysozyme concentration used (50 μg/ml for 15 min with just a few at 100 μg/ml) was as high as possible before it became impossible to redraw threads; indeed, much material could be observed being shed at a concentration of 100 μg/ml, so that similar problems of nonuniformity were encountered. A further difficulty was that, as was observed earlier by Mendelson (15a), threads hydrated in lysozyme twisted slowly but noticeably. This presumably was the same effect as that observed in microfibers before dissolution in higher lysozyme concentrations (9). When redrawn from the lysozyme, the threads twisted in the opposite sense, and it is believed that the individual filaments were finally axially aligned to the same high degree as standard threads, but this belief rests on evidence from only a few scanning electron micrographs.

Tensile tests. Treated specimens were mounted on cards (30-mm gauge length), and their diameters were measured photographically. They were then tested by being extended to the breaking point at a constant rate of extension (1 mm/min) in atmospheres of various humidity as described in the accompanying paper (26). Some were first rapidly extended a small amount, and the initial part of their stress-relaxation curve was obtained. Complete stress versus strain curves were obtained, and from them and the data on diameter, the tensile strength, extensibility, and the initial Young’s modulus were calculated. Testing over the whole humidity range at various pH values or salt concentrations would have involved an inordinate number of specimens. Tests were therefore restricted to approximately 81% relative humidity (RH) for pH, to approximately 46, 65, and 81% RH for salt, and to approximately 65 and 81% RH for lysozyme. Control tests on standard specimens were interspersed at all three RH values.

SEM pictures. Scanning electron micrographs were obtained of standard, salt-washed, and lysozyme-treated thread specimens after tensile testing. The specimens were coated with gold and examined with a Cambridge Stereoscan 100 microscope.

RESULTS

Surface appearance of treated threads. Scanning electron micrographs of standard (i.e., water-washed), 1 M NaCl-treated, and lysozyme-treated threads are shown in Fig. 1. Apart from the absence of a layer of culture medium, the surface of standard threads appears, even after tensile testing, to be no different from that of threads drawn from TB medium (see also Fig. 4 of reference 18). In particular, the individual filaments are seen to be highly aligned and there is no suggestion of cells being pulled apart at cross walls. By contrast, although the filaments are highly aligned, indicating that the twist induced by lysozyme immersion had been removed upon redrawing, there are indications in the lysozyme-treated thread of cracks linking the ends of cells in different filaments. Even where there is no crack, there are signs that lysozyme has attacked the cell wall preferentially near the septa because the shapes of individual cells are clearly visible. In micrographs of threads treated at the higher concentration of lysozyme (100 μg/ml), there is "smearing," a loss in definition due presumably to fragments of hydrolyzed cell wall that have not diffused away in the wash adhering to the thread surface.

The surfaces of all salt-washed threads were to some extent distorted. This was particularly obvious for NaCl (Fig. 1), where the surface was distinctly wrinkled and the wrinkles were in no particular orientation. As far as can be judged, the average direction of the filaments was, despite this deformation, closely axially aligned. It was noticeable that threads shrank substantially when immersed in NaCl even at the 50 mM concentration. This is in agreement with the observations of Marquis and Ou (13, 20). A feature that was not noticed until after mechanical testing was the adherence of the salt crystals to the surface. These salt crystals no doubt could have been removed by a third (water) wash, but this was not done because it would presumably remove the effects of the salt wash.

Variation in mechanical properties with change in pH. As the pH of a second water wash was varied in the range from 3.3 to 9.0, the threads became more ductile (Fig. 2) but there was no change in tensile strength. There appeared to be a slight decrease in initial modulus, but it is doubtful whether it is statistically significant (Fig. 3). Twenty specimens were used in this test at approximately 81% RH. The early parts of the stress relaxation curves of these specimens were in all respects the same as those of standard specimens.

Effects of salt treatment. Treatment of bacterial threads with (NH₄)₂SO₄ in concentrations up to 250 mM had a negligible effect on mechanical properties with humidity in the range of 46 to 47% RH, but there were observable effects on all properties with humidity in the range of 65 to 66% RH. The walls became more ductile, and both strength and modulus decreased significantly. These effects increased in magnitude for even wetter threads tested in the range of 80 to 82% RH. (Fig. 4 and 5). The extensibility of these threads was as great as that measured for any standard thread (with an overall maximum of 88% at 250 mM concentration; Fig. 2 shows average behavior in this respect). The data at 80 to 82% RH could indicate that both strength and modulus vary as some power of salt concentration, but the number of data points is too small to establish this. There is no evidence of saturation in the effects at 250 mM even though the strength has been reduced by a factor of 2.5 and the modulus has been reduced by a factor of 8.6 from the corresponding values for walls from standard.
threads. Treatment with NaCl was done mainly at a high concentration (0.5 and 1 M). The effects at both 65 to 66% and 80 to 82% RH were to increase the ductility (with extensibility as high as 80% even at the lower humidity level) and to reduce both modulus and strength to lower levels, in general, than those achieved by 250 mM (NH₄)₂SO₄ (Fig. 4 and 5). It is possible that the modulus (at the 80 to 82% RH level only) obeys the same (conjectured) relationship to NaCl concentration as it does to (NH₄)₂SO₄ concentration, but the data for strength clearly suggest some form of saturation (Fig. 4).

Included in Fig. 4 and 5 are the corresponding data at the three humidity levels for cell walls in threads drawn from TB medium and not washed in any way (25). The concentration of NaCl in TB medium is 83 mM. The data indicate much greater differences in mechanical properties than would be achieved by this concentration of salt. It could be argued that the effective concentration of residual medium rehydrated on the threads is quite different, but there is no reasonable means of estimating this. There are also, undoubtedly, other ions in TB medium, in the beef extract for example. However, it seems unlikely that salt is the sole cause of the differences between the walls in standard threads and in those drawn from TB medium because the effects of salt in these experiments cannot at any given concentration be summarized, as can the effects of residual

FIG. 1. Scanning electron micrographs of bacterial threads: (A) standard, i.e., water washed, tested at 56.3% RH, strength of 143 MPa, extensibility of 2.7%; (B) treated with 1 M NaCl, untested; (C) treated with 50 μg of lysozyme per ml, tested at 65.5% RH, strength of 94 MPa, extensibility of 4.2%. Bars, 10 μm (A and C) and 5 μm (B).
TB medium, by a simple shift in RH (26). If that were so, the modulus and the strength would change, for a given salt concentration, from the control values by factors which would be the same at the 65 to 66% RH and 80 to 82% RH levels. This is clearly not the case for 250 mM (NH₄)₂SO₄ nor for either of the high concentrations of NaCl. Nonetheless, the failure properties of all salt-treated walls show in one respect the same behavior as the standard walls do. The tensile strength of salt-treated walls bears the same relationship to extensibility as that for walls from standard thread (Fig. 6); this is also true for walls in thread drawn from TB medium (see Fig. 7 of the accompanying paper [26]). Stress relaxation curves were obtained only for threads treated with up to 250 mM salts. There was no qualitative difference between their behavior and that of standard threads. Relaxation was, for lack of time, not continued long enough for accurate estimates of relaxed modulus (at, for example, 30 min) or characteristic times to be made.

**Effect of treatment with lysozyme.** Fifteen specimens were redrawn after the cells had been partially digested by lysozyme. They were tested at 65 to 66% RH and 80 to 82% RH (Table 1). Two further specimens were tested at 20.5% RH. There is no evidence for differences between any of the mechanical properties measured in the lysozyme-treated thread, including relaxation behavior, and the corresponding

![FIG. 1—Continued.](image)

![FIG. 2. Nominal stress versus strain curves of bacterial cell wall at approximately 81% RH either water washed at pH 4.55 (A), water washed at pH 9.0 (B), or washed in 250 mM (NH₄)₂SO₄ (C). Results with water washed control tested at 94.8% RH are shown in curve D (from Fig. 2 of the accompanying paper [26]).](image)

![FIG. 3. Initial modulus ($E_t$) and strength ($σ_b$) of bacterial cell wall versus pH at approximately 81% RH. The number of specimens in each test is shown in parentheses; the mean values plus or minus standard deviations are logarithmic averages.](image)
properties of cell walls from standard thread. It is not surprising, therefore, that the data points for strength as a function of extensibility lie on the same failure locus as those for standard (and indeed all other) specimens thus far measured (Fig. 6).

FIG. 4. Tensile strength of treated bacterial cell wall as a function of salt concentration at three RH values. Symbols: V, treatment with (NH₄)₂SO₄; O, treatment with NaCl; x, standard cell wall i.e., water washed; □, cell wall drawn from TB. The number of specimens in each test is shown in parentheses; the mean values plus or minus standard deviations are logarithmic averages. Note that only those controls which were contemporary are included. A complete set is shown in Fig. 3 of the accompanying paper (26).

FIG. 5. Initial (Young’s) modulus of treated bacterial cell wall as a function of salt concentration at three RH values. The symbols are described in the legend to Fig. 4.

DISCUSSION

In view of the dimensional changes in cell walls due to salt ions, it is most likely as Marquis and Ou argue (13, 20) that a major effect of ions is to change the polymer conformation and therefore to change, in particular, the stiffness of the network. The extent to which ions influence cell wall mechanical properties should then depend upon the degree of hydration, which is as we observed. It is known that increasing the salt concentration diminishes the electrostatic forces between charged groups in the wall (13, 20), and it has been estimated that for a salt concentration greater than 0.2 M, the charges are completely screened (5). The wall polymers should then behave as if they were uncharged, and there should be no further change in mechanical properties as the salt concentration is increased. It may be that the results indicate such a saturation effect, but if so this saturation effect would be at a higher concentration than 0.2 M, i.e., more like 1 M, which is comparable with the concentration required for minimum wall volume in experiments on other bacteria (13, 20). Furthermore, the greatest changes produced by treatment with salt ions are much less than those produced by increase in degree of hydration. For example, at 81% RH, the maximum reduction in modulus is by a factor of 26 and the reduction by salt ions is only a factor of 2.5. The ratio of the modulus of dry walls to that of wet walls is about 430, and the ratio of strength of dry walls to that of wet walls is about 23 (26). Hydrogen bonding appears to have a much greater influence on mechanical properties than does ion binding.

Despite the strong evidence for an entirely ionic explanation for the observed changes in behavior, it remains possible that they are in part due to changes in water ordering. A twist in macrofibers, which is thought to be a mechanical response depending on polymer conformation, changes with exposure to various ions as if they were arranged in a lyotropic series (19). It is possible that this is true of cell wall properties, but an inordinate amount of time would be required to demonstrate this. A twist in macrofibers also changes (reversibly) when the pH is reduced to 4 or less (19). The lack of measured change in cell wall stiffness due to washing threads of low pH might indicate that twist is not a mechanical response, but these results are for the axial modulus only. It is likely that twist depends primarily on the wall shear modulus, which has yet to be measured (26).

Macrofiber twist also changes, rapidly for left-hand fibers, as a result of lysozyme attack (9). This, too, is thought to be a mechanical response due to changes in mechanical properties of the wall. The enzyme hydrolyzes the glycosidic bonds of the peptidoglycan backbone, although admittedly a very small proportion of them. In so doing, the enzyme provides new binding sites for ions within the cell wall (7, 23). The enzyme is also itself charged. Nonetheless, the evidence provided by these experiments and also by those in which lysozyme was added to the culture medium before the drawing of thread (18), that there is no change in cell wall axial properties, supports the supposition that the backbone is oriented on average in approximately the circumferential direction. It could be argued that the extent of lysozyme damage is unknown, but it must be substantial because, as remarked above, much material was observed being shed from the cellular filaments before the threads were redrawn. An assay of the number of reducing sugars would settle the matter, but it would require a substantial amount of purified peptidoglycan which would be difficult to obtain from bacterial thread. There is other strong evidence for cell wall
properties being anisotropic. The bacterium contrives to maintain its diameter remarkably constant in size while it extends longitudinally. This argues for a much higher modulus in the circumferential (hoop) direction. The fact that the hoop stress in a cylinder is twice the longitudinal stress (when these stresses are due solely to internal pressure) suggests that the wall is also stronger in the hoop direction. The twisting with elongation which has been established as the normal growth pattern for *B. subtilis* (17) strongly suggests that the anisotropy is helical rather than orthotropic.

Lysozyme is known to attack preferentially the septal-wall region of cells. There is some evidence for this in the thread in Fig. 1C where there are signs of cell separation in surface filaments. The extent of such damage, if any, could undoubt-
edly be better seen in electron micrographs of longitudinally sectioned threads. However, the significant observation is that lysozyme attack does not alter the tensile properties of bacterial thread. This emphasizes just how strongly the filaments adhere to each other laterally. It also indicates that the mechanical properties of the cylinder wall of cells in the axial direction are not changed. The strong reasons for directly relating properties of the cylinder wall to those of the thread are the same as for standard threads (26). There is, for example, no sign in fracture cross sections, even in those of threads treated with lysozyme, of anything other than fracture of the cylinder wall of cells (Fig. 1; see also Fig. 3 of reference 24 and Fig. 4 of reference 18).

Although the results support the idea that the average backbone direction of peptidoglycan is approximately circumferential, the observed wall mechanical properties cannot be used to support a regular molecular structure model of any kind. Order is a feature of all polymers including amorphous ones. Regular structures must surely be crystalline, but there is much evidence to show that peptidoglycan in bacteria is not (12, 21). All of the evidence from thread measurements (18, 25, 26) shows the cell wall to be in no way different from other amorphous polymers. There is no reason to suppose that the molecular arrangement in peptidoglycan is other than an entanglement network. Peptidoglycan differs from other polymers in being a polyelectrolyte with a large amount of interaction between charged groups which can be changed, e.g., by ions (14). To allow such changes, there must be a substantial amount of molecular flexibility, something which is not possible even in a semi-crystalline polymer.
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REFERENCES