

## Biomechanics of bacterial walls: Studies of bacterial thread made from *Bacillus subtilis*

(Young's modulus/breaking stress/effect of relative humidity/viscoelastic properties/charge effects on stress)

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**ABSTRACT** Bacterial threads of up to 1 m in length have been produced from filaments of separation-suppressed mutants of *Bacillus subtilis*. Individual threads may contain 20,000 cellular filaments in parallel alignment. The tensile properties of bacterial threads have been examined by using conventional textile engineering techniques. The kinetics of elongation at constant load are indicative of a viscoelastic material. Both Young's modulus and breaking stress are highly dependent upon relative humidity. By extrapolation to 100% relative humidity, it appears that cell walls may be able to bear only internal osmotic pressures of about 2 atmospheres ( $2.03 \times 10^5$  Pa) in living cells. Similarly, the strength of wall material limits the amount of cell-surface charge permissible to only a small fraction of that known to be carried by the negatively charged wall polymers.

A fundamental understanding of the relationship between growth and form in bacterial cells requires, in addition to details concerning genetic and physiological regulation of the cell cycle, knowledge of the mechanical properties of cells. The interplay of forces within the cell wall is of particular importance; consequently, the material properties of the wall must be known. Such information is difficult to obtain from individual cells because of their minute dimensions. Nevertheless, it has been shown that bacterial cells can be stretched appreciably (1) and that the strength-bearing wall polymer, peptidoglycan, is elastic (2, 3). The role of stress in the cell surface also has been recognized as a factor in cell-shape determination (4, 5).

Direct measurement of the mechanical properties of bacterial cells has hitherto proved impossible. To circumvent this difficulty, we have developed a means to produce thread-like structures of *Bacillus subtilis* that are suitable for direct measurement by techniques used in the study of textile threads. This communication describes the construction, structure, and properties of bacterial thread, and the results of initial studies of their mechanics.

Basically, the cell walls behave as viscoelastic materials. Both the initial elastic modulus and the breaking stress are highly dependent upon the degree of hydration of the bacterial thread. The strength of cell walls determined in this manner places limitations on the degree of internal osmotic pressure that can be withstood by growing cells. Permissible values are appreciably lower than those currently accepted on the basis of indirect measurements. Similarly, the electrostatic structure of cell walls must be compatible with the strength of wall material as measured in bacterial thread. The results indicate that nearly all of the cell-wall teichoic acid phosphates must be neutralized in order to maintain cell wall integrity. Therefore, bacterial thread appears to provide a unique way in which to study the mechanics of bacteria.

## MATERIALS AND METHODS

Bacterial threads have so far been produced only from cultures of cell-separation-suppressed (*lyt*) mutants of the 168 strain of *B. subtilis*. The FJ7 strain previously studied in the context of macrofiber formation (6, 7) and a derivative called 7/12 that carries the *tms-12* mutation (8) in the FJ7 background have been used in the studies reported here. Cultures from which thread was produced were grown at 20°C in TB medium (9). Under these conditions, the *tms-12* mutation is not expressed. Cultures (8 ml) were maintained by daily serial passage. A 24-hr culture grown statically in a 100 × 15 mm plastic Petri dish contained sufficient bacterial filaments to yield a thread of up to 1 m in length. Threads were produced as shown in Fig. 1 by lifting a portion of the culture, attached to a sterile toothpick, from the culture dish. Surface tension forces acting at the culture fluid/air interface apparently served to compress the individual cellular filaments into a close-packed thread. As the thread was drawn out, the cellular filaments remaining in solution became aligned, and the cone created at the fluid/air interface migrated around the Petri dish until, finally, all of the filaments were taken into the drying thread. The uniformity of thread so produced was primarily dependent upon the uniformity of distribution of the cell filaments in the initial culture.

Air-dried thread was stored at room temperature ( $\approx 24^\circ\text{C}$  and  $\approx 45\%$  relative humidity), suspended from the toothpick used to produce it. For mechanical measurements, sections of thread were glued to support cards as shown in Fig. 2. Card-mounted samples were transferred to a controlled atmosphere (Atmosbag, Aldrich) and allowed to come to equilibrium over a 24-hr period. Various relative humidities were obtained by passing air through solutions of  $\text{H}_2\text{SO}_4$  according to formulas given in ref. 10 before introduction into the chamber. The actual relative humidity achieved was determined by use of a hygrometer (Markson, certified  $\pm 3\%$ ). Threads were stretched by using loads consisting of fine wire of known density. The degree of elongation (or shrinkage) was measured by using a compound microscope containing an ocular micrometer and sighting along the upper edge of the triangular portion of the card to which the loads were attached. Protocols for individual experiments are given in the figure legends. For scanning electron microscopy, thread samples were fixed to aluminium stubs by using double-sided tape and were coated with gold.

## RESULTS

When viewed with a low-power stereoscopic microscope, bacterial thread has the appearance of glass fiber. Scanning electron micrographs (Fig. 3), showing surface features and broken ends, indicate that individual cellular filaments are aligned parallel to the thread axis and are closely packed with cross-sections deformed into approximately hexagonal shape. Uniform threads of diameter in the range of 24-115

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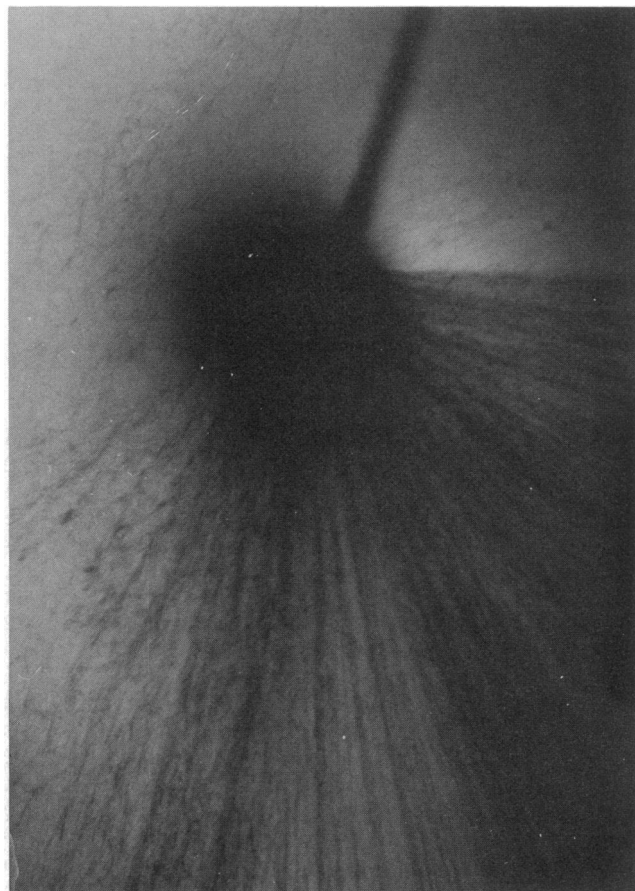


FIG. 1. Micrograph showing the drawing of bacterial thread from a culture. The dark mass near the center is the cone produced as the thread, which appears as a solid dark line at the top of the figure, is drawn through the surface tension of the growth medium at the fluid/air boundary. The individual bacterial filaments being drawn into the thread can be seen in the fluid below, radiating from the cone.

$\mu\text{m}$  have been produced. Calculations based on a cell diameter of  $0.8 \mu\text{m}$  indicate that there are up to 20,000 filaments in a thread cross section, with an average of  $\approx 7000$ . These figures were confirmed by counting the fila-

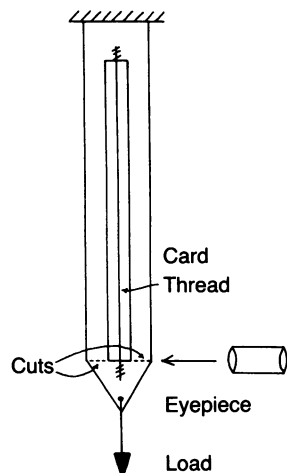


FIG. 2. Bacterial thread mounted on a support card for tensile testing. Thread length is 68 mm. The triangular portion of the card is cut away from the rest prior to loading; it contains a hole through which incremental loads are attached via an S hook.

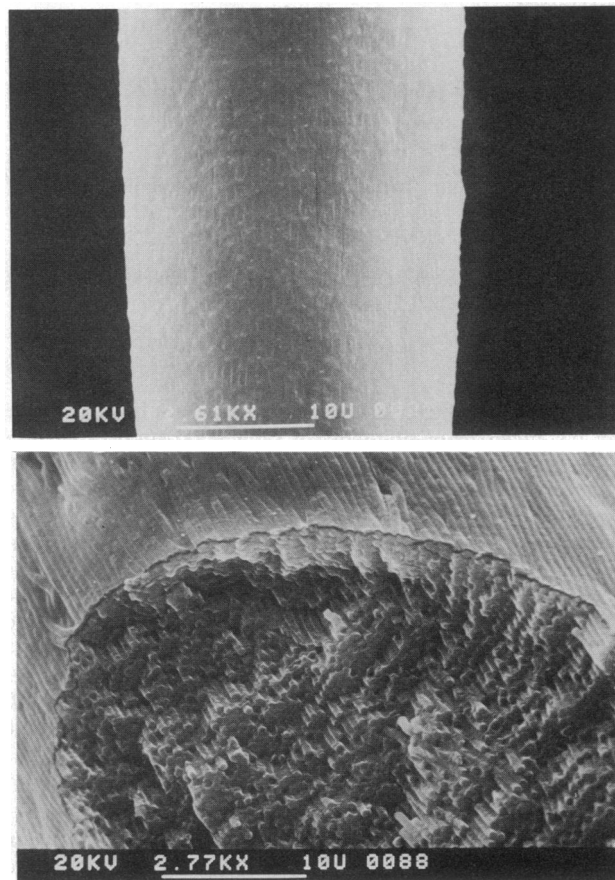


FIG. 3. Scanning electron micrographs showing the surface of a dry bacterial thread (*Upper*) and a fracture cross section (*Lower*). The parallel alignment of cellular filaments along the thread axis is evident. Cell septa also can be discerned as short lines running perpendicular to each filament. The packing of the filaments can be resolved; individual cells appear not to have collapsed during drying but have been deformed into approximately hexagonal shape. (Bars =  $10 \mu\text{m}$ .)

ments in micrographs such as that shown in Fig. 3 *Lower*. Thus, in a 1-m length of thread, the number of cells approaches  $10^{10}$ .

In all, 60 threads were given tensile tests, at room temperature, over the range of 15–93% relative humidity. Some were tested to break in one steadily increasing loading process. Others were subjected to cycles of increasing and decreasing load in order to investigate elastic recovery. The deformed shape of the filament cross sections indicates that, during the shrinkage in length observed on drying, there was a corresponding transverse shrinkage accompanied by substantial transverse compressive stress. The existence of this stress, combined with the gluing action of the residual amount of dried growth medium, suggested that the thread might remain integral under tensile tests. This was confirmed for most of the samples for which an incremental load produced an elongation response typical of a viscoelastic material—i.e., an instantaneous change followed by an approximately first-order asymptotic increase in elongation with time (Fig. 4). For some samples, generally at high relative humidity, we obtained a response that is typical of the *assemblage* behavior in a textile sliver, for example, when filaments slip past each other and the thread breaks by “pulling out” without significant *material* fracture. The properties reported below were all obtained from samples for which it was clear that the material behavior only was being observed.

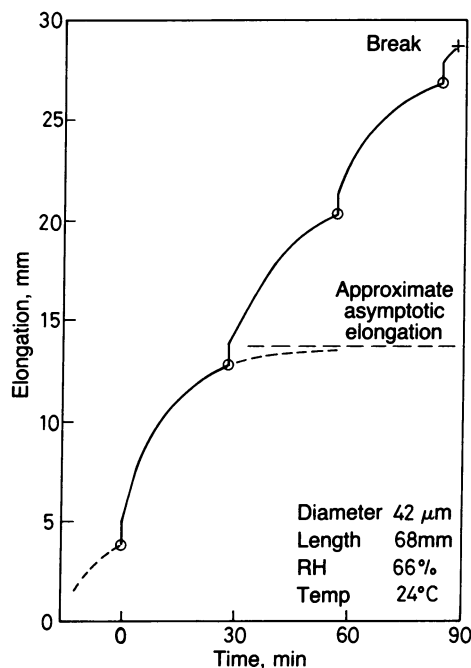


FIG. 4. Elongation of bacterial thread under an increasing series of constant loads. A thread (of strain 7/12) of 42- $\mu\text{m}$  diameter and 68-mm length was tested at 66% relative humidity at 24°C. The elongation was recorded as a function of time under a series of loads increased by 0.28 mN (0.20 N/mm<sup>2</sup>) at each step. The times of load increment are indicated by circles. Total stress at break was 1.10 N/mm<sup>2</sup>. The observed behavior is typical of a viscoelastic solid.

The ductility and elasticity of cell wall were measured by observing the extensibility (geometrical strain at break), the tensile strength (stress at break), and the initial (Young's) modulus ( $E$ ; ratio of stress to immediately occurring strain) of thread. We assumed that the cell wall bears virtually the whole tensile load. In the absence of measurements of cell-wall thickness over the whole range of humidity, an average figure of 40 nM was used in the calculation of wall stress—i.e., the ratio of the cross-sectional area of the wall to that of the thread was taken to be 0.2. The results did not indicate any significant differences in tensile properties between the two bacterial strains.

Our findings are that, at 65% relative humidity, which is a standard condition for textile testing, the extensibility of bacterial thread was  $\approx 20\%$ . It rose to  $\approx 50\%$  at 88% relative humidity and fell to  $<1\%$  as the relative humidity decreased. Except at low humidity, this behavior is comparable to that of viscose rayon (a cellulose) and of wool (11), but the extensibility is much less than that of other biological materials such as elastins. The tensile strength of cell walls at 65% relative humidity was  $\approx 7 \text{ N/mm}^2$  ( $1 \text{ N/m}^2 = 1 \text{ Pa}$ ) but varied greatly with humidity (Fig. 5 Upper). The data shown may indicate a maximum as relative humidity varies, but the spread at low relative humidity is much more likely to be the result of the difficulty in handling very brittle threads, in which case there is a variation in tensile strength over the humidity range of  $\approx 2$  orders of magnitude ( $\approx \times 100$ ). This is much greater than that for natural fiber textiles. For example, the strength of rayon when dry is  $\approx 3$  times its value when wet (11). Over the range of 65–100% relative humidity, the strength of cell walls changed by a similar factor to that of chitin, but chitin is, overall,  $\approx 14$  times stronger (12). Textile fibers are also much stronger than cell walls at high humidity levels (270 N/mm<sup>2</sup> for rayon at 65% relative humidity), but when dry they are comparable in strength.

Cell-wall stiffness, as indicated by initial (Young's) modulus, showed even greater variation ( $\approx \times 1000$ ) with humidity

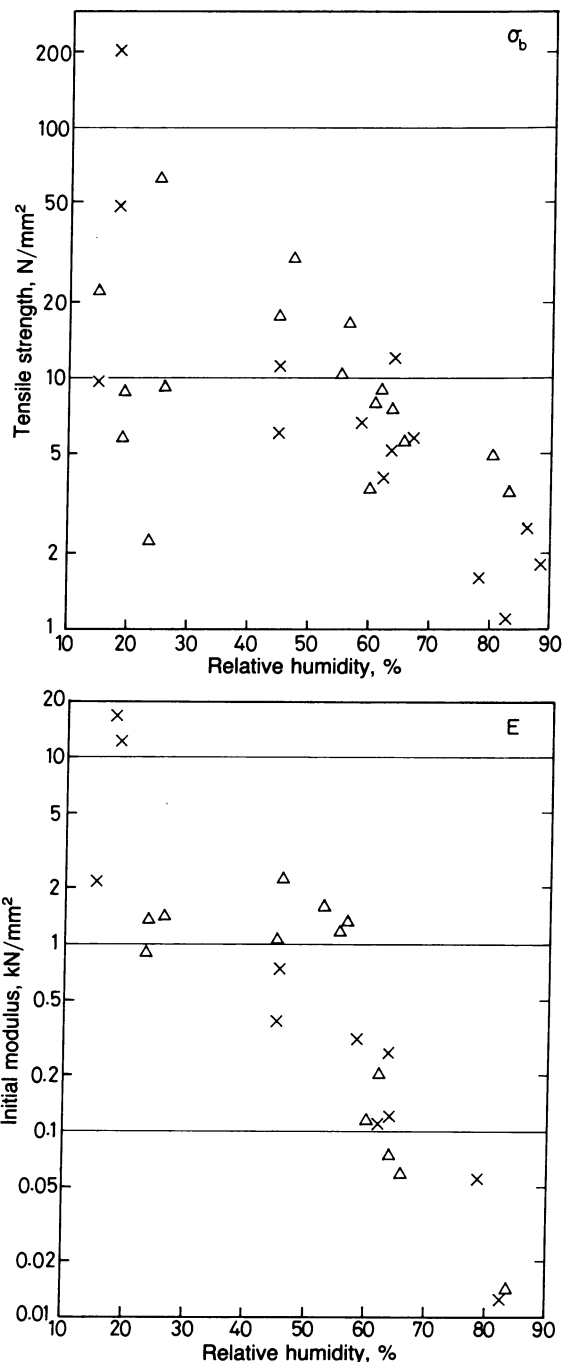


FIG. 5. Tensile strength ( $\sigma_b$ ) (Upper) and initial (Young's) modulus ( $E$ ) (Lower) of cell-wall material as a function of relative humidity.  $\times$ , Threads of bacterial strain 7;  $\Delta$ , threads of bacterial strain 7/12. Tensile strength is stress at break. Initial modulus is measured from a graph of instantaneous incremental geometrical strain versus incremental stress (see Fig. 4). The slope of this (straight line) graph is  $1/E$ .

(Fig. 5 Lower). By far the greatest variation for textile fibers, that of rayon, is a mere  $\times 60$  between wet and dry conditions. Cell walls, though comparable in stiffness to textile fibers when dry, are much less stiff when wet ( $E = 100 \text{ N/mm}^2$  at 65% relative humidity compared with 13 kN/mm<sup>2</sup> for rayon), and their stiffness approaches that of such materials as resilin and elastin (13).

Viscoelastic behavior can be characterized by, in addition to the initial modulus, a "final" modulus, based upon the asymptotic strain instead of the initial strain (Fig. 4) and by

time constants describing the kinetic behavior. For cell wall at 66% relative humidity, the ratio of final to initial modulus was  $\approx 0.15$  (Fig. 4) and at 45% relative humidity was  $\approx 0.4$ . There was little sign of viscoelastic behavior at 20% relative humidity. Too few suitable curves were obtained for the variation in time constants with relative humidity to be established, but the rises shown in Fig. 4 are approximately first order, with a time constant of  $\approx 16$  min.

Firm statements about elastic recovery cannot yet be made. This is largely because of sample-to-sample variation under similar test conditions. Nonetheless, total recovery was observed at a relative humidity as high as 53%, with a maximum applied stress of just less than average tensile strength. A possible cause of less-than-total thread recovery, other than that it is a feature of material behavior, is that there is interfilament slip at an early stage of elongation, which does not show in the final stages near break.

All of the results given above show substantial sample-to-sample variation under similar test conditions. The handling difficulties at low humidity have been referred to. Another possible source of variation, which might explain why some threads pulled out whereas others that were tested under the same conditions yielded material properties, pertains to the average filament length in the culture, before thread formation. One would expect, as for textile assemblies, those threads containing shorter filaments to pull out more readily than those with longer filaments.

## DISCUSSION

The experimental results presented here confirm quantitatively Marquis' supposition that peptidoglycan, because of its shorter chain and noncrystalline structure, is much less stiff than either cellulose or chitin (2, 3). This is certainly true in the hydrated state. The very strong dependence of wall mechanical properties on humidity suggests that, as with proteins and other macromolecules where structure is influenced by water (14, 15), the state of the cell-wall polymers may likewise be influenced and that there might be ordering of water molecules in the cell wall. Recent experiments on macrofibers (to be reported) indicate that the effect of neutral salts and of changes in pH on cell-wall structure and mechanical properties may not be only a question of ion-exchange as suggested by Marquis (2, 3).

A major conclusion of the work reported here is that extrapolation to 100% relative humidity (Fig. 5 *Upper*) indicates that cell walls should be able to bear a longitudinal stress of about  $1 \text{ N/mm}^2$ ; as a consequence, a cylindrical cell with this strength of wall should only be able to bear an internal osmotic pressure of 2 atmospheres ( $1 \text{ atm} = 1.013 \times 10^5 \text{ Pa}$ ). This is much less than the figures generally accepted for Gram-positive organisms on the basis of indirect measurement. Mitchell and Moyle, having used an equilibrium-vapor technique, report 20 atmospheres for *Micrococcus lysodeikticus* and 20–30 for *Staphylococcus aureus* (16). Similar figures can be deduced from the work of Marquis and Carstensen, who used a quantitative plasmolysis technique (17). If the turgor pressure for *Bacillus subtilis* were 20 atmospheres, the longitudinal stress in the wall would, on the basis of a 40-nm thickness, be about  $10 \text{ N/mm}^2$ , a stress supportable in our experiments only at 60% relative humidity or lower (Fig. 5 *Upper*). It should be noted here that the assumption of a particular wall thickness does not affect this conclusion because the same factor ( $\times 4$  thickness per diameter) appears in the calculation of wall stress both from

internal pressure *in vivo* and from thread stress in our experiments.

Furthermore, by extrapolating the results for initial modulus to 100% relative humidity (Fig. 5 *Lower*), a figure of  $\approx 2 \text{ N/mm}^2$  was obtained. The initial strain due to a stress of  $10 \text{ N/mm}^2$  would be 500%, and the final strain after the viscoelastic response would be several times greater. The membranes of isolated protoplasts of *Bacillus megaterium* have been shown to be capable of linear strain of 100% (18), but this larger figure seems inconceivable for walls. There may be a very large difference between the condition of cell walls *in vivo* and in our experiments but, except perhaps in one respect, we believe not. First, threads held at room temperature remain viable for at least a month, whereas most of the specimens were tested within 2 weeks of thread manufacture. We have not yet established the upper limit for viability, nor do we know what proportion of cells remain viable, but rehydration of thread in growth medium suggests that it is a substantial one, and the rehydrated cells when viewed under the microscope appear not to have been degraded.

Second, no dependence of mechanical properties on storage time has been observed. The retention of viability for extended periods without change in mechanical properties and the apparent normality of rehydrated cells argue against possible weakening of the cell wall during thread production or storage, due to, for example, residual activity by autolytic enzymes. Such activity would in any case be expected to be slight, since the threads are made from *lyt* mutants that are deficient in both amidase and glucosaminidase.

Concerning the *lyt* mutation itself, there is no reason to suppose that the cell walls made by such strains are inherently weaker than those made by wild-type strains. On the contrary, one might expect that, with reduced levels of autolytic enzyme activity, the integrity of the peptidoglycan would be preserved and, consequently, its strength enhanced compared to that found in wild-type cells.

Third, the average rate of straining in the tests was comparable with the rate of normal cell growth. For example, at the average rate of elongation shown in Fig. 4, the cell length would double in about 120 min. At this rate of extension, even though the load was applied in discrete increments, it is most unlikely that the relatively small instantaneous increments in length could have the effect of reducing the tensile strength by a factor of 10. That is, the lower material strength found in our experiments, when compared to that inferred from indirect measurements, is unlikely to be an artefact, resulting from too rapid extension.

Where the cell-wall conditions might be different is that the stress distribution *in vivo* might not be uniform throughout the wall thickness, whereas this is the most probable situation in our experiments. Newly created wall material on the inner surface is likely to bear little stress, so that the outer parts must bear greater stress than average. Of course, this makes the maximum stress that wall material is required to bear for a given pressure even greater than that calculated above.

In the same way as they do for turgor pressure, our findings concerning tensile strength place limitations on the unneutralized net electrostatic charge that cell walls can support. The density of charged groups in Gram-positive bacterial walls has been carefully investigated (2, 3, 19), particularly in relation to cell wall structure, and the net charge, due to different numbers of positively charged and negatively charged groups, has been shown to be the important factor in wall conductivity (20). Most cell walls have a net negative charge due to an excess of phosphate and carboxyl groups over amino groups. In *Bacillus subtilis* the phosphate groups on the teichoic acid dominate, and the resulting net (negative) charge calculated by R. E. Marquis

(private communication) on the basis of the analysis reported in ref. 19 is about 0.21 equivalents (eq)/liter. This is an immense charge in electrostatic terms; clearly much of it is neutralized by cations, either bound or mobile, within the open structure of the wall. The figure for wall tensile strength allows a quantitative estimate of the extent of this neutralization.

A spherical shell, which bears a uniformly distributed charge of surface density  $q$ , is subject, due to electrostatic repulsion, to the tensile stress that would result from an internal pressure of  $q^2/2\epsilon$ , where  $\epsilon$  is the permittivity of the surrounding medium. By taking the latter to have the value for water, a surface charge density of 0.017 C/m<sup>2</sup> would have the same effect as a pressure of 2 atmospheres. For a wall of thickness 40 nm, this represents a uniform charge density of 4.2 meq/liter. Not much is known about the charge distribution on the surface of *Bacillus subtilis*, nor is the cell spherical. But in terms of its electrostatic effects, the charge distribution is unlikely to be very different from uniform. Also, although the stress produced in a cylindrical shell by uniform charge density is not the equivalent of a uniform internal pressure, it is sufficiently close for the above calculation to give a fairly correct result. We must conclude, therefore, that no more than  $\approx 2\%$  of the charged groups in the isolated cell wall are not neutralized. *In vivo*, the distribution of charge in the cytoplasm complicates the problem. Even less is known about this distribution, but there is no reason to suppose that the general conclusion is invalid.

The results reported here make clear the utility of highly ordered bacterial thread in the direct measurement of the mechanical properties of cell walls.

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