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Mechanical Properties of *Bacillus subtilis* Cell Walls: Effects of Removing Residual Culture Medium

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Experiments are described in which the tensile strength, the initial (Youngs') modulus, and other mechanical properties of the bacterial cell wall were obtained as functions of relative humidity (RH) in the range of 20 to 95%. These properties were deduced from tensile tests on bacterial thread, a fiber consisting of many highly aligned cells of *Bacillus subtilis*, from which residual culture medium had been removed by immersion in water. Reasons are given to support the idea that the mechanical properties of bacterial thread relate directly to those of the cylinder wall and that they are not influenced by septa, cytoplasm, or the thread assembly. The data show that the cell wall, like many other heteropolymers, is visco-elastic. When dry, it behaves like a glassy polymer with a tensile strength of about 300 MPa and a modulus of about 13 GPa. When wet, its behavior is more like a rubbery polymer with a tensile strength of about 13 MPa and a modulus of about 30 MPa. Thus, the cell wall is stronger than previously reported. Walls of this strength would be able to bear a turgor pressure of 2.6 MPa (about 26 atm). The dynamic behavior suggests a wide range of relaxation times. The way in which mechanical behavior depends strongly on humidity is discussed in terms of possible hydrogen bond density and the ordering of water molecules. Cell walls in threads containing residual culture medium TB are, except at low RH, 10 times more flexible and about 4 times less strong. All of their mechanical properties appear to vary with change in RH in a manner similar to those of walls from which the culture medium has been washed, but with a downshift of about 18% RH.

The bacterial cell wall has a substantial mechanical role in addition to other functions. It must be strong enough to protect the cytoplasmic membrane from forces originating outside of the cell and to stabilize it against turgor. The wall must be stiff enough to maintain cell shape but ductile enough to allow growth. It must also be elastic enough to recover from environmentally induced changes. In vivo the wall is under stress and is also stretched (7, 17). Evidence for this has been obtained in several ways, including subjecting cells to electrochemical changes and osmotic shocks (1, 3, 5, 9, 18), but until recently estimates of cell wall mechanical properties have been largely qualitative. To understand how bacteria maintain their characteristic shape during growth and even during division, for example, to understand why rod shape is so stable, an investigation of the states of stress and deformation in the cell wall is necessary. For this, quantitative estimates of mechanical properties are required, but measurement, even indirectly, is difficult and often impossible in normal cultures. Bacterial thread is a fibrillar fiber consisting of many cellular filaments which lie parallel to the fiber axis and which adhere together very strongly. A thread contains hundreds of millions of highly aligned cells but closely resembles a textile fiber (whereas a macrofiber resembles a very small segment of twisted textile yarn). As such, it can be measured by a standard fiber technique such as the tensile test in which fiber tension is measured as a function of extension and time. Average cell wall properties in the axial direction can be readily deduced from such measurements. Although the individual cells in a bacterial thread are at different stages of development, with some alive and some dead and with varying degrees of septation, there are strong reasons for believing that the tensile properties of thread are directly related to those of the cylinder wall of an average cell. The existence of filament-forming mutants has so far confined such measurements to *Bacillus subtilis*, but there is no reason in principle why similar measurements could not be done for any rod-shaped bacterium or other filamentous microorganism such as fungi.

Previously reported measurements were made on threads drawn directly from the culture medium (15, 21), but there were indications that when the residual medium was removed, the properties changed (15). This was confirmed by the present work in which the residual culture medium was removed from the cellular filaments by immersing threads in water and redrawing them, thereby producing a more standard entity for testing. In addition to measuring mechanical properties, the swelling of bacterial thread was measured with various degrees of hydration.

The effects of further treatment of standard threads by equilibration in various concentrations of neutral salts and by partial digestion of lysozyme are reported in the accompanying paper.

MATERIALS AND METHODS

Bacterial cultures. Cultures were produced from FJ7, a cell separation-suppressed (*lyt*) mutant derived from *B. subtilis* 168 that was used in previous studies of mechanical properties (15, 20) and of macrofibers (13, 14). They were grown in the rich medium TB which consists of 10 g of tryptose, 3 g of beef extract (Difco), and 5 g of NaCl, each per liter of water. Cultures were grown at 20°C in plastic petri dishes containing 8 ml of TB medium. They were inoculated by transferring a small amount of "seed" from a previous culture of the same kind. The inoculum, which consisted of filaments, was

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first broken up by using a sterile toothpick so that no aggregations remained. It was found that the optimum seed size lay in the range of 2×10^5 to 5×10^5 CFU/ml. After overnight growth without agitation, the cultures consisted of a web of cellular filaments each containing several hundred cells.

Thread production. A thread was drawn from a culture by inserting a small hook into the web and raising it at a constant speed (approximately 20 mm/min) by means of a clock motor. Filaments in the web were drawn radially into the point of thread formation, where surface tension appeared to act like a die, for the filaments were compressed into a compact, highly aligned, fibrillar fiber. Standard technique produced threads with diameters in the range of 40 to 80 μm and with lengths in the range of 200 to 400 mm. For convenience, threads were drawn into the laboratory atmosphere. Previous studies (15, 21) have shown that threads made this way and subsequently tested at a different relative humidity (RH) had properties that were no different from those of threads drawn at and maintained at the test RH. The residual culture medium was removed from newly drawn threads by immersion in 2 liters of deionized water at pH 4.5. The threads were lowered into the water, where the filaments separated to a substantial extent, so the individual filaments could be seen and the culture medium could diffuse away. Despite the separation, the filaments maintained some cohesion, and after a short period the filaments could be redrawn into a standard, washed thread. This process, which took 15 min, could be repeated several times before thread integrity was lost. It was found that although the mechanical properties of threads immersed once were different from those of threads drawn directly from the culture medium, those of threads immersed several times showed no further difference. It was concluded that all of the culture medium could be removed in one wash. Washed threads had the same compact and highly aligned structure as did threads drawn from culture medium (see Fig. 4A of the accompanying paper [23] and also Fig. 4 (lower) of reference 21) but lacked the coating of TB medium residue (clearly visible in Fig. 4 (upper) of reference 21).

Diameter and length measurement. Variations in thread length and diameter due to changes in RH were measured. Diameter was measured by use of a light microscope at 225× magnification with a calibrated eyepiece graticule. The samples were mounted on cards (see below) and equilibrated for at least 30 min, at each value of RH, in an atmospheric enclosure (Atmosbag) through which the microscope eyepiece projected. Length was measured, also after a 30-min equilibration period, by suspending 25- to 73-mm lengths of thread close to a vertical rule in the Atmosbag and observing changes directly.

The humidity inside the Atmosbag was maintained by mixing dry and saturated air supplies. The humidity was measured by using an electronic hygrometer (Lee-Integer type DHL40). Variations in diameter with change in length along threads were initially measured in the same way, but it was later found to be less tedious and just as accurate for 30-mm lengths to take a series of 10 photographs at 10× magnification with slightly overlapping frames. Maximum and minimum diameters were then readily measured by projecting the images from a movable stage onto a screen at a further magnification of 20×. Although washed threads were not as uniform as those drawn directly from the culture medium, an average ratio of maximum to minimum diameter of 1.23 was achieved for the standard specimen length of 30 mm.

Tensile tests. Tensile tests were done by using an instrument of standard form, i.e., similar to those made by Instron, but with a lower load-measuring capability (21). Thread extension could be determined to ±2.5 μm and tension could be determined to 0.5% full scale in the range of 0.1 down to 1 mN (9.81 N equals 1 kg weight). The instrument was put in the Atmosbag, and specimens were left for 4 h at the test RH before being tested. Some specimens were left overnight, but no difference in mechanical properties could be detected. Because of their fragility, threads were mounted on cards with central slots which were then fixed in the jaws of the tester and cut away immediately before a test. Since the determination of elastic modulus depended on the use of an average diameter, it was desirable to keep the specimen lengths short. However, slight misalignments in mounting and the stiffening effects of glue near the ends made it desirable to have long specimens. A specimen length of 30 mm was found to provide a reasonable compromise.

All specimens were tested by being extended at a constant speed (standard, 1 mm/min) until they broke, during which time the tension was recorded continuously. In addition, about half of the specimens were first extended rapidly, and the stress relaxation (at constant extension) that followed was recorded over a period of 30 min. The tester jaws were then returned to the zero-extension position, and the specimen was allowed to recover for a further 30 min. Residual extension was then measured by noting the point at which tension first developed during the ensuing period of extension to the breaking point. Calculations were then made of the tensile strength and extensibility, i.e., the stress in the wall and the proportional extension (i.e., the strain) at break. The initial (Young's) modulus was obtained from the slope of the stress versus strain graph. A relaxed modulus was also calculated, based on the final stress after relaxation and the strain at which relaxation was recorded. The wall cross-sectional areas were, for modulus calculations, based on the geometric mean of maximum and minimum diameters and, for strength, based on the diameter of the broken cross section. In all cases, the ratio of wall area to thread area was taken to be 0.2, corresponding to a wall thickness of 40 nm (19). This may seem to be a somewhat arbitrary figure given the range of humidity in the tests, but it represents the same amount of wall under different conditions. It also does not invalidate important conclusions concerning turgor pressure (see below).

RESULTS

Swelling of thread. The diameters of 15 thread samples and the lengths of 19 were measured in the range of 10 to 90% RH. Measurements above 80% RH were difficult and fully hydrated measurements of diameter were impossible because the filaments separated to such an extent from each other. A crude estimate of fully hydrated length could be made. This agreed with an earlier estimate made by Mendelson (12). The 15 initial diameters were in the range of 21 to 83 μm, and the 19 initial lengths were in the range of 26 to 74 mm. Typical curves of diameter and length versus RH are shown in Fig. 1, but considerable spread was observed. To examine average behavior, the proportional increases in diameter and length between 20 and 80% RH were plotted as functions of initial diameter and length, respectively. There was some suggestion that the increases in both diameter and length increased as initial diameter or length decreased, but the spread, quite naturally, was also much greater for these

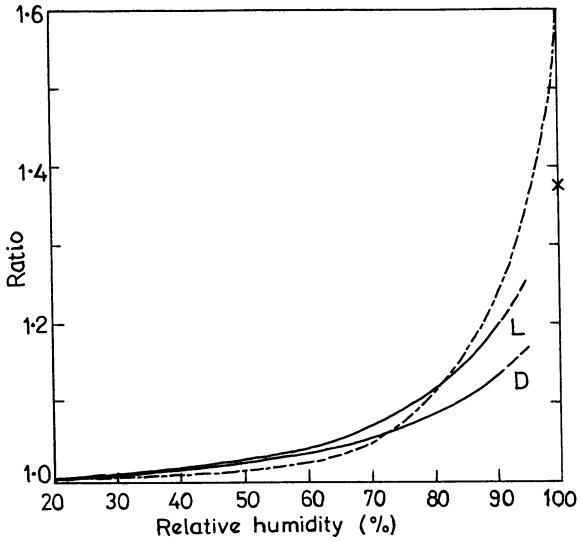

FIG. 1. Typical variation in length and diameter of bacterial thread with change in RH. The single point at 100% RH is for fully hydrated length. Variation in the linear dimension for *B. megaterium* derived from sorption isotherm (8) is shown (— — —).

measurements. The average increases in length and diameter were 10.5% (coefficient of variation, 0.17) and 8.55% (coefficient of variation, 0.175), respectively. No sorption isotherms have so far been obtained for bacterial thread nor are any known to the authors for normal *B. subtilis* cells, but measurements exist for whole cells of *Bacillus megaterium* (8). These measurements have been used to derive an approximate curve for linear dimension versus RH to be used in comparison with the swelling curves of Fig. 1. In the derivation it has been assumed that volume ratio (wet to dry) equals the cube of the length ratio and that variations in cell density with RH are negligible.

Tensile behavior. Typical curves of stress versus strain are shown for tests in the range of 65 to 95% RH (Fig. 2). The stress is equal to the load divided by the average cross-sectional area before the test (nominal stress); the strain is equal to the extension divided by the initial length. At lower humidity (less than about 50% RH), the stress versus strain curves were approximately straight lines with much steeper slopes and smaller extensibilities (in the range of 1 to 1.8%). At low humidity, the strength of cell wall as measured was about 300 MPa and its initial modulus was about 13 GPa. These properties remained fairly constant as RH was increased to about 50% and then fell dramatically with a further increase in RH (extrapolated to 100% RH) to values of about 13 MPa and 30 MPa, respectively. (Fig. 3 and 4). The stress versus strain curve behavior at lower RH and the high values of strength and modulus are typical of the behavior and properties of a glassy polymer. At high RH, the form of the stress versus strain curve, the ductile nature of the cell wall, and the low values of strength and modulus are all characteristic of polymers above their glass transition temperatures.

There is no obvious curve that could be fitted to the 85 data points for strength (Fig. 3), but the data for modulus can be fitted by two straight lines: for the points below 52% RH, a line of constant modulus of 12.6 GPa, and for points above 55% RH, the line $ln\ E = 9.28 - 0.128\ H$, where E is the

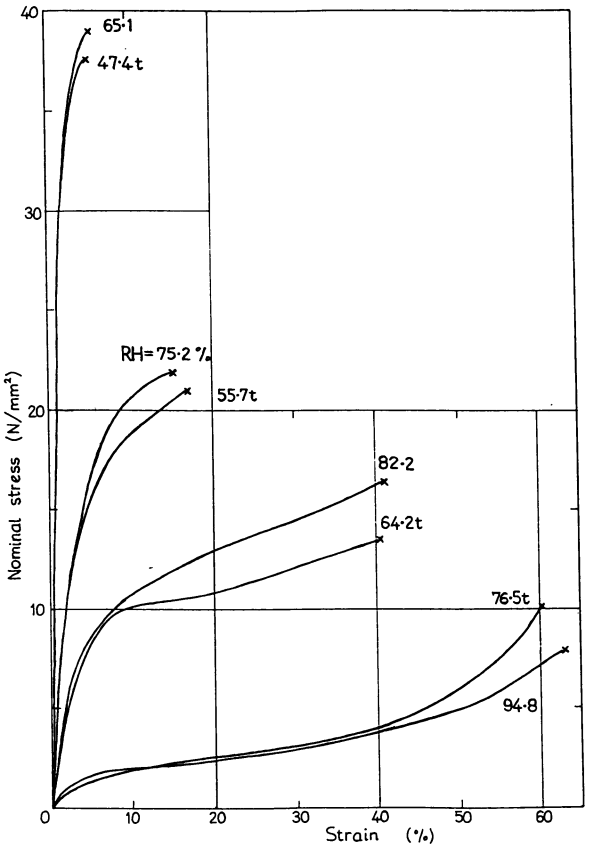

FIG. 2. Nominal stress versus strain curves for bacterial cell wall after removal of residual TB medium. Tests were done at the four values of RH indicated. Corresponding curves for thread drawn from TB medium at approximately 18% lower RH are indicated by t. Nominal stress is based on an average diameter. The strain is extension divided by initial length; gauge length, ≅30 mm; rate of extension, 1 mm/min.

modulus in gigapascals and H is percent RH. The fittings are of the logarithms of the modulus. On this basis, the plus or minus one standard deviation values are 16.0 and 9.9 GPa for the points below 52% RH and, for the others, 1.55 E and 0.645 E. The correlation coefficient is 0.955. Although this curve fitting is entirely empirical, it is of use when making comparisons with other results.

A stress relaxation curve superficially resembles an exponential decay, from a stress that is equal to the initial modulus multiplied by the suddenly imposed strain towards one equal to the relaxed modulus multiplied by the strain. However, none of those measured for thread could be described by a single time constant. The data in fact suggest a very wide distribution of relaxation times, but it would be inappropriate to attempt such analysis here. Some idea of the time scale of relaxation can be obtained by measuring the time that would be taken to reach the final (equilibrium) stress at the initial rate of decay. For an exponential decay, this would of course be equal to the time constant. For the specimens for which stress versus strain curves are shown in Fig. 2 and also for a dry specimen, the times are given in Table 1. There appears to be a minimum time at about 75% RH. This corresponds approximately to a minimum of 0.2 in the ratio of the relaxed modulus after 30 min to the initial modulus. The relaxed modulus is shown in Fig. 5 as a

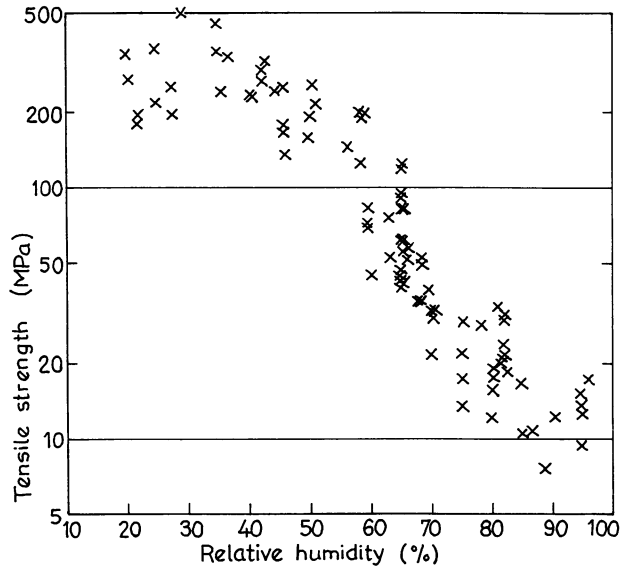

FIG. 3. Tensile strength of bacterial cell wall in standard threads as a function of RH.

function of the initial modulus over the range of 60 to 95% RH. The ratio of the relaxed modulus to the initial modulus is about 0.25 at 60% RH and about 0.34 at 95% RH.

Properties compared with those of threads drawn from TB medium. In a previous paper (14), some evidence was given of different properties before and after removal of the residual culture medium. This was applicable to only a small

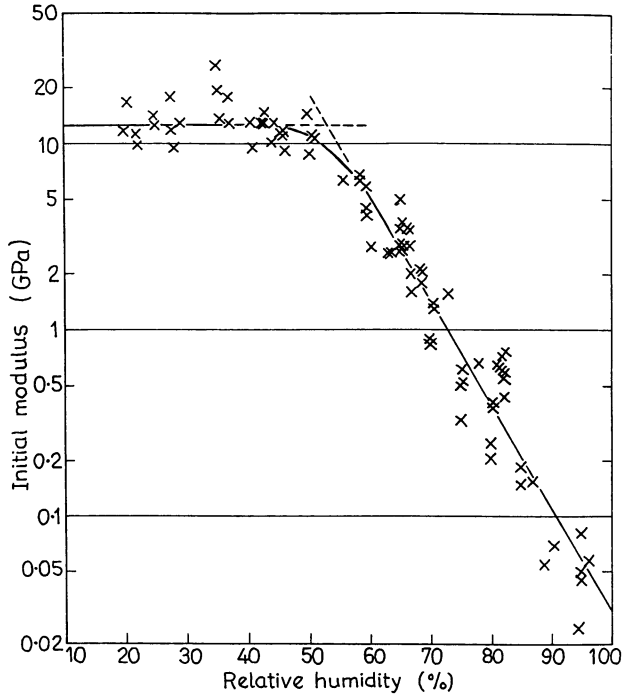

FIG. 4. Initial (Young's) modulus of bacterial cell wall in standard threads as a function of RH. The modulus is derived from the tangent to the stress versus strain curve at the origin. The straight lines are least-mean-square fits to the data below 52% and above 55%.

TABLE 1. Approximate time scale for stress relaxation of bacterial cell wall

Cell wall threads	RH (%)	τ^a (s)
Standard	24.9	72
	65.1	34
	75.2	21
	82.2	26
	94.8	53
Drawn from TB medium	10.8	84
	47.4	44
	55.7	34
	64.2	36
	76.5	54

[a] τ is the time needed, at the initial relaxation rate, for the stress to fall to that corresponding to the 30-min relaxed modulus.

RH range. More complete sets of data for threads drawn from TB medium have appeared elsewhere (20, 21) and are given, for the initial modulus only, in Fig. 6. The way in which the modulus varies with change in RH is distinctly similar to that of walls from standard threads; indeed, they can be superimposed with a shift of about 18% RH. To avoid too many data points, the fitted straight lines are used in Fig. 6 to represent the modulus data derived from experiments on standard threads. This same shift in RH can be used to superimpose data on strength and extensibility, with remarkable agreement. All features of stress versus strain curve behavior for the two sets of experiments appear to be related in the same way (Fig. 2). One way of presenting the strength and extensibility data is to plot one against the other in what would, if it were not for the scatter, be called a failure locus (Fig. 7). Both sets of data fall, within the scatter, on the same locus. The relaxed modulus also appears to be related to the initial modulus in the same way for both sets of experiments (Fig. 5). The time scale for relaxation always appears to be shorter for standard threads than for those drawn from TB

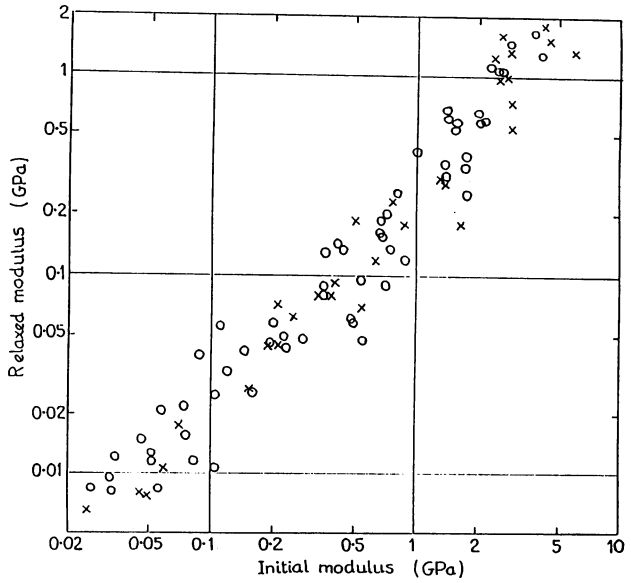

FIG. 5. Relaxed modulus after 30 min as a function of initial modulus of bacterial cell wall. Symbols: ×, standard cell wall (60 to 95% RH); ○, cell wall drawn from TB medium (45 to 90% RH).

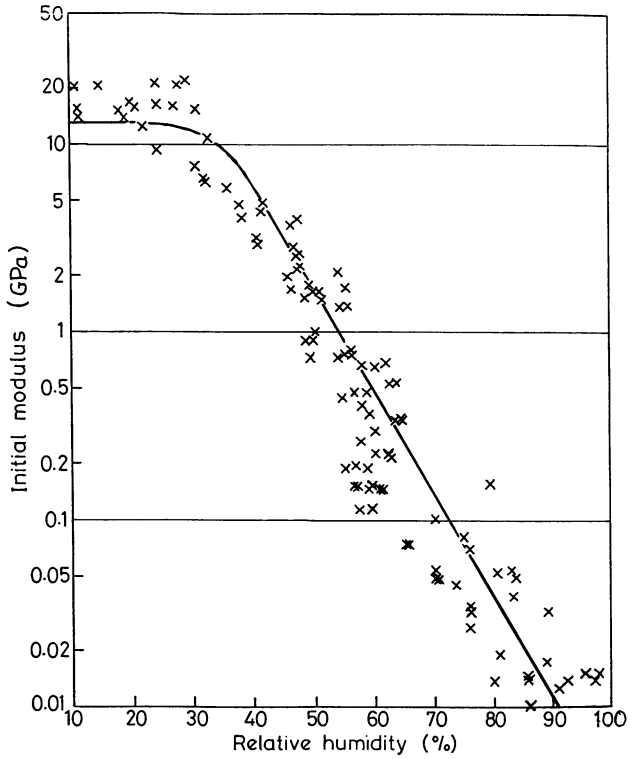

FIG. 6. Initial (Young's) modulus of bacterial cell wall in threads drawn from TB medium as a function of RH (21, 22). The straight lines are those which fit the data for washed cell walls (Fig. 5) downshifted by 18% RH.

medium at the corresponding (shifted) RH, but the numbers in Table 1 are no more than crude estimates of what is a very complicated process. Few data were recorded about recovery from imposed strain, but qualitatively they appear to be the same for the two experiments (21).

DISCUSSION

Bacterial thread is a multifilament fiber. Bacterial thread might be supposed to deform by interfilament slippage, but there is no sign of this in any experimental stress versus strain curve, even at the highest RH. Also, there is almost complete recovery from imposed strain up to about the 2% level. This behavior is no different from that of other polymer materials and indicates that the measured properties are those of the individual cellular filaments. They in turn consist of many cells in different stages of development. The cylinder wall appears to be continuous. Micrographs of longitudinal sections of FJ6, a very similar filament-forming *lyt* mutant, show this (see, for example, reference 6). Nonetheless, it might be argued that extension could result from pulling apart of cross walls. If so, it would be difficult to explain the recovery from imposed extension. Also, cross walls occupy only a small proportion of a filament length so that the pulling apart of one in a given thread cross section would be attended by the extension of many cylinder walls. There is, therefore, good reason to take the strain to be that of the cylinder wall of an average cell. Micrographs of fracture cross sections also show areas in which many adjacent filaments are broken in precisely the same plane. This would not be possible if they broke at septa.

It has been assumed that the cell wall bears the whole tension and that the effect of the cytoplasm is negligible. It is easy to show that in the tensile test any bulk material constrained within a closed cylinder experiences tensile stress. The lack of a cohesive mechanical structure in the cytoplasm, even if the cytoplasm adheres to the cell wall, means that it can bear negligible tensile stress. In effect, its bulk modulus is very small, so the assumption is reasonable. This is true irrespective of the degree of hydration or whether the cells are dead or alive. This is so because, in the unlikely event that at very high RH some cells maintained turgor pressure, the compressive stress in the cytoplasm would remain constant and so have no effect on the test.

Polar solvents, in particular water, influence the mechanical properties of most polymers, but the degree of influence varies greatly, even for biopolymers. The modulus of cellulose, for example, decreases by a factor of only 4 from the dry to the wet state (16), whereas that of bacterial cell wall (basically peptidoglycan) decreases by about 400 times (indeed more than this if we consider the properties without water washing). Cellulose is of course a very different polymer from peptidoglycan, with backbone chains perhaps 100 times as long but with very short side chains. Elastin is more comparable to bacterial cell wall in this respect, although it has greater ductility. Its modulus decreases by a factor of 1,000 from the dry state to a water content of 60% (2). If we take the sorption curve of Maeda et al. for *B. megaterium* as a guide (8), the water content of cell wall is 60% at approximately 92% RH. There is the possibility of many hydrogen bonds between peptides in both elastin and peptidoglycan when dry. This is undoubtedly why the polymer networks are stiff when dry. As the degree of hydration is increased, water must compete for hydrogen bond sites, introducing more flexibility into the networks. In the cell wall, water associates not only with peptidoglycan but also with the accessory polymers which have ionizable and other hydrophilic groups.

Water also in general affects the dynamic behavior of polymers. The glass transition temperature of elastin, which is approximately 20°C at 35% water content, changes markedly (2, 4). The glass transition is sharply defined for elastin, but it seems unlikely that it is for cell wall, because the results suggest a very wide range of relaxation times. Although there are some differences in relaxation behavior between standard cell walls and those with remnant TB medium (Table 1), preliminary analysis of the stress relaxation curves shows them to be very similar in general shape (see Fig. 5 of reference 21). For the cell walls in threads drawn from TB medium, it has been suggested that there is a humidity–time equivalence corresponding to the well-known temperature–time equivalence (21). An approximate (and tentative) master curve for relaxation modulus extends over about 22 decades in time (22). There is no reason to suppose that removal of residual TB medium makes any difference in this conclusion.

The similarity in water-induced variations in behavior between walls in standard threads and those in threads drawn from TB medium is quite striking because of the 18% RH shift. A possible explanation is that there is only one curve of each property versus water content and that the water content in the two situations is different at any given RH because of some conformational differences in the walls. The latter could be caused directly by the ions in TB medium or by changes in water ordering. A more reasonable alternative is that, since all the materials in TB medium are hygroscopic, the cell walls in unwashed threads, which are

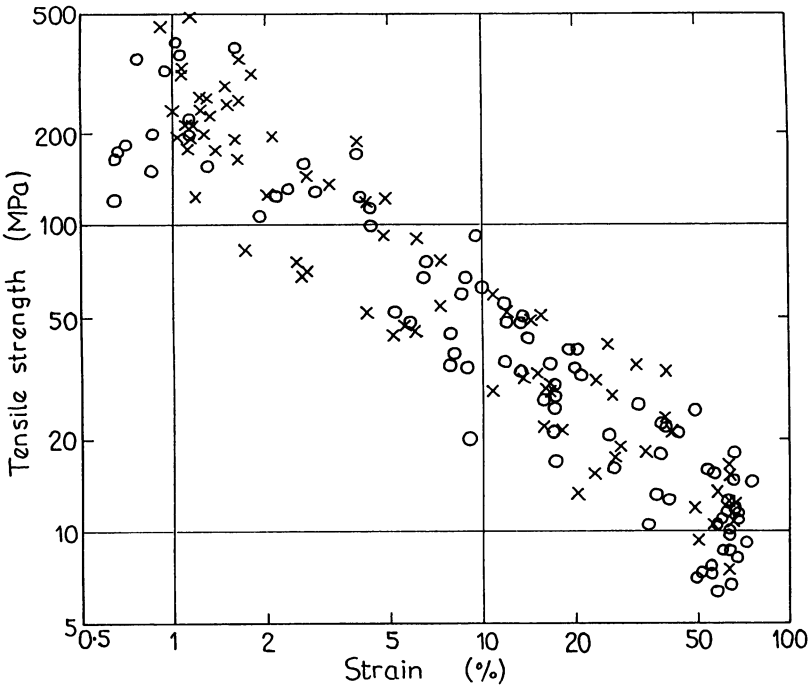

FIG. 7. Tensile strength of bacterial cell wall as a function of extensibility (breaking strain). Symbols ×, standard cell wall; O, cell wall drawn from TB medium.

surrounded by TB medium, are in an "atmosphere" of higher water activity than that indicated by the RH of the bulk atmosphere. Certainly, unwashed threads feel wetter and are more difficult to glue to the cards used to mount them in the tensile tester. The difficulty with both of these explanations is that the property differences extend right up to 100% RH or as close as the experimental system allows. This suggests differences between properties obtained by extrapolation to 100% RH and those in the fully hydrated state. Measurements of water content by sorption isotherm are not in very good agreement with those obtained on fully hydrated cells (10), but more pertinent here is the fact that diffusion is possible only when fully hydrated. Indeed, this is how the culture medium is removed in these experiments. Unfortunately it is not possible to measure the properties in this state because the filaments separate when threads are hydrated.

This difficulty affects deductions about mechanical properties in vivo. However, if we accept the extrapolated value, the tensile strength of bacterial cell wall is more than four times greater than previously reported (15, 21). The average for the five specimens tested at about 95% RH is 13.2 MPa. With this strength, cells should be able to withstand a turgor pressure of 2.64 MPa (about 26 atm). This is in line with previous estimates derived by quantitative plasmolysis and other techniques (11, 17). This conclusion does not depend on the assumption of a particular ratio for wall thickness to cell diameter, since the same ratio appears in calculations of wall stress from turgor pressure and from tension in these experiments. Nonetheless, there are reasons for viewing the conclusion with some caution. Firstly, the wall stress in the circumferential (hoop) direction due to a particular turgor pressure is twice that in the longitudinal direction. The wall is clearly stiffer in that direction, since the bacterium maintains a remarkably constant diameter while extending axially. It may also be much stronger. Secondly, because the walls of gram-positive bacteria are relatively thick, the stress should not be distributed evenly through the wall so that, for a given turgor pressure, the stress in the outer part of the wall should be greater than that calculated.

There are limitations to the use of bacterial thread. Only the axial properties of the cylinder wall have been deduced thus far. To attack the problems of cell shape, the transverse (hoopwise) and the shear properties of the cylinder wall and the properties of polar wall need to be found. The shear modulus can be found by means of a torsional test on a thread, and this is the subject of a current experiment, but the others will require a more sophisticated technique. Much can be inferred by theoretical modeling, by using the techniques of engineering stress analysis, which is at present being applied to the problem of stress distribution in the wall during growth.

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