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The Permeability of the Wall Fabric of *Escherichia coli* and *Bacillus subtilis*

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To study the overall structure of the peptidoglycan fabric of the sacculi of gram-negative and gram-positive walls, actively growing cultures of *Escherichia coli* and *Bacillus subtilis* were treated with boiling sodium dodecyl sulfate solutions. The sacculi were then treated with enzymes to eliminate proteins and nucleic acids. These intact sacculi were probed with fluorescein-labeled dextrans with a range of known molecular weights. The penetration of the probes could be monitored by the negative-staining appearance in the fluorescence microscope. At several chosen times, the molecular weight fraction that allowed barely observable entry of the fluorescein-labeled probe and the molecular weight fraction that penetrated to achieve almost, but not quite, the concentration of probe in the solution external to the sacculi were determined. From three pairs of times and molecular weights that met one or the other of these two criteria, the effective pore size could be calculated. The minimum size of protein molecule that could diffuse through the pores was also calculated. Two mathematical models, which gave essentially the same results, were used to interpret the experimental data: one for the permeation of random coils through a surface containing holes and the other for rigid spheres diffusing through water-filled cylindrical pores. The mean estimate of the effective hole radius in walls from *E. coli* is 2.06 nm, and that of the effective hole size in walls from *B. subtilis* is 2.12 nm. These results are supported by experiments in which the loss of preloaded cells was monitored. Various fluorescein-labeled dextran samples were mixed with samples of intact cell walls, held for a long time, and then diluted. The efflux of the dextrans was monitored. Neither large nor small dextrans stained under these conditions. Only with dextran samples of a sufficiently small size were the sacculi filled during the preincubation period, and only with the largest of these could the probe not escape quickly. From the pore (or mesh) size, it can be concluded that the wall fabric of both organisms has few imperfections and that the major passageway is through the smallest possible pore, or "tessera," formed by the maximal cross-linking of the peptides from glycan chain to glycan chain compatible with the degree of rotational flexibility of the chains of repeating disaccharides of *N*-acetyl muramic acid and *N*-acetyl glucosamine. A tessera is composed of two chains of eight saccharides cross-linked by two octapeptides. The size of a globular hydrophilic molecule, if it did not bind to wall components, that could pass freely through the meshwork of an unstretched sacculus of either organism is roughly 25 kDa. We stress that this is only a rough estimate, and it may be possible for proteins of less than 50 kDa to pass through the native wall during normal growth conditions.

Little is known about the sieving properties of the murein layer covering the cytoplasmic layer of most eubacteria. On the other hand, a good deal is known from earlier work about the size of probe molecules that can diffuse into the wall (3, 21, 22). But entry into pores in the wall is an entirely different process from passing through the wall. It is knowledge about the latter that is relevant to the passage of proteins and oligosaccharides through the wall. We have studied the permeability of isolated sacculi, the bag-shaped macromolecules formed of peptidoglycan surrounding bacteria, by isolating these sacculi and measuring the sizes of fluorescein-labeled dextran probes which are capable of penetrating through the fabric of the intact sacculi at various predetermined times to one of two chosen degrees: either just slight entry or almost complete equilibration. The specified degrees of penetration are called the upper and lower criteria and are symbolized by the fluorescein concentration at the two criteria, designated $C_{<1}$ or $C_{>0}$. The key conclusion is that the fabric has very few rips, tears, or imperfections. The second conclusion is that large (>50-kDa) globular protein molecules cannot penetrate by simple diffusion through the wall.

Theoretical basis of the measurement of the effective pore size. Diffusion of molecules obeys the first-order process specified by Fick's law (9). For passive diffusion into the sacculus, entry will initially be directly proportional to the diffusion time. When back diffusion becomes important, the rate will slow and eventually the concentrations on both the inside and the outside of the sacculus will become identical. If the sacculi all have the same surface area and volume and if the distributions of pore size and number are uniform, the kinetic dependency is $C = C_O(1 - e^{-kt})$, where C is the fluorescein concentration inside at time t; C_O is the concentration outside in the bulk phase; and k is the first-order rate constant that applies to both the entry and exit processes. This is shown in Fig. 1 for four choices of the value of k. The first-order rate constant, k, depends on a number of parameters and can be determined from Fick's law for the relevant geometry (9), given D_E, the effective (model-dependent) diffusion constant through the wall; A, the surface area of the sacculus; τ, the wall thickness; and V, the volume of the sacculus, according to the equation $k = D_E A/\tau V$.

Models for the permeation kinetics. The effective diffusion constant, D_E, for passage through the wall depends on the

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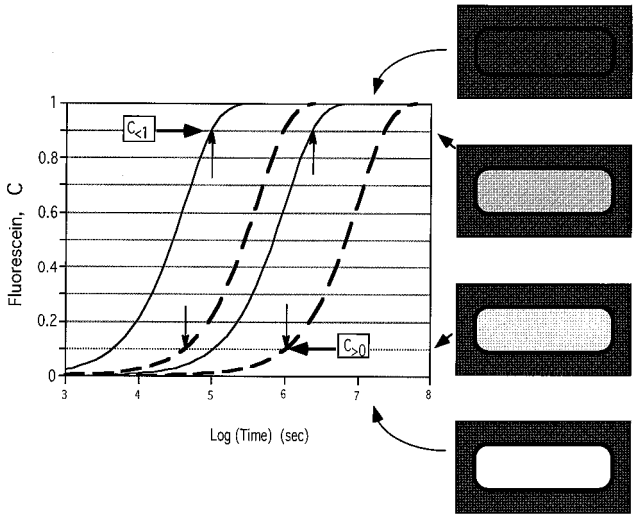

FIG. 1. Theoretical time course of the filling of sacculi. The graph shows time courses for the entry of four different sizes of labeled dextrans into sacculi. Initially, the sacculi appear to be negatively stained. See lower right-hand image. At a later time, the fluorescein-labeled dextran penetrates enough to just slightly decrease the negative staining. This condition is the lower criterion, $C_{>0}$. Subsequently, enough penetration occurs, as in the second image from the top, so that one can judge that the negative staining has been almost lost. This is the upper criterion, $C_{<1}$. Finally, after a long time, equilibration is complete and the sacculi are no longer negatively stained.

details of the diffusion process and the molecular geometry of the probe and the wall. Two different models for the diffusion process have been developed (Fig. 2). The model shown at the top is for the case of a random coil (2). It depends on the reasonable assumption that the dextran behaves as a perfect random coil. Dextran is largely, but not completely, a linear chain of β-1,6-linked glucose residues, with 5% of the glucose residues as very short chain side branches, linked β-1,3 to the main chain (see the review in reference 2). The other, quite different model applies to a rigid spherical probe passing through water-filled cylindrical holes (12). This is relevant to the degree that the internal hydrogen bonds are formed in the dextran, making its solution structure closer to a sphere. Probably the former model is somewhat more realistic, but the key result of fitting data to these models is that there is very little difference in the estimate of pore size between these dissimilar models.

For the formulation that assumes that the probe is a random coil, the molecule has a chance of penetrating only if one end enters the circular thin pore and if none of the remainder of the flexible probe molecules collide with the edges of the hole (Fig. 2A). For this model, the value of k is not determined, but a value proportional to it was derived by a Monte Carlo simulation dependent only on the number of glucose residues in the dextran chain and the radius of the circular hole. The details are given in reference 2. The simulation showed that the probability that the chain will pass through the pore is given by $P = \exp(-0.186xr^{-1.43})$. In this expression, x is the number of glucose residues in the random coil and r is the radius of the pore measured in nanometers. D_E would be proportional to the fraction of the surface area that is pores, f. Thus, $D_E = Qf\exp(-0.186xr^{-1.43})$, where Q is an unknown proportionality constant. In the actual simulation, the dimensions of the pore were expressed in terms of the number of glucose residues in the chain that could stretch from the center of the pore to its edge.

For the other model, i.e., the case of diffusion of rigid spherically shaped particles through cylindrical pores, the expression for k can be written down from Fick's diffusion law on the basis of the assumptions that the sphere must be smaller than the hole and that it must enter without colliding with the external wall (Fig. 2B). This introduces a factor, $(1 - R/r)^2$, where R is the radius of a spherical probe. However, it must be assumed that the diffusion constant while the probe is inside the water-filled pore depends not only on the diffusion constant in bulk solution but also on the hydrodynamic resistance to movement in a narrow channel. Therefore, D_E depends on the ratio of the radius of the sphere to the radius of the pore according to the Stokes equation. When the Stokes relationship is combined with the chance of hitting the wall (9, 12, 20), Renkin showed that $D_E = D(1 - R/r)^2[1 - 2.104R/r + 2.09(R/r)^3 - 0.95(R/r)^5]$. Thus, k can be rewritten from the general form to depend, as before, on the volume of the cell, V, and the wall thickness, τ, but now is expressed in terms of the number of pores per cell, P; the radius of the individual pore, r; and the radius of the spherical probe molecule, R. Consequently (12), $k = D(1 - R/r)^2[1 - 2.104R/r + 2.09(R/r)^3 - 0.95(R/r)^5]fAP2\pi r^2/V\tau$. It can be assumed that f, A, P, V, and τ are constant for a particular preparation of sacculi and that these values will cancel out during the calculation.

Determining the pore size. It is possible to estimate the pore's radius with any given set of assumptions about the geometry of the probe and the pore. This can be done even though there are a number of unknowns in the mathematical expressions for the k's. The estimation of the pore radius can be obtained by comparison of pairs of times and the corresponding probe sizes that yield the same internal criterion fluorescein concentration, designated either $C_{<1}$ or $C_{>0}$. Figure 1 shows upper and lower choices for the criterion. One "critical" concentration of fluorescein inside the cell, $C_{<1}$, corresponds to the case in which the probes are just large enough to be only slightly impeded from entry, and the other, $C_{>0}$, corresponds to the case in which the probes are just small enough to barely enter by the end of the experimental period. Although we do not know the values of $C_{<1}$ or $C_{>0}$, for illus-

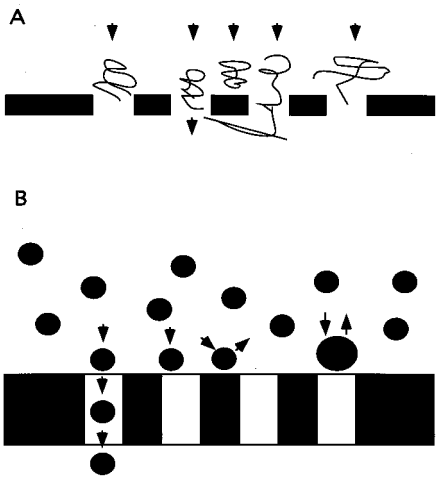

FIG. 2. Models for permeation through bacterial walls. (A) Penetration of random coils through thin circular holes. Only if the coils pass without colliding with the wall of the pore can they diffuse through. (B) A quite different model, in which the probe particles have rigid spherical shapes. If they are smaller than the hole and enter into it without colliding with the walls, then they may continue through. However, in passing through the water-filled pore, the particle's effective diffusion constant is reduced by hydrodynamic flow according to the Stokes equation.

trative purposes in Fig. 1 it is assumed that $C_{<1}$ is 90% of C_O and $C_{>0}$ is 10% of C_O. Because the value of C is the same for the three datum points collected for each criterion, it follows that

$$C_{<1} = C_O[1 - \exp(-k_1t_1)] = C_O[1 - \exp(-k_2t_2)] = C_O[1 - \exp(-k_3t_3)]$$

and

$$C_{>0} = C_O[1 - \exp(-k_4t_4)] = C_O[1 - \exp(-k_5t_5)] = C_O[1 - \exp(-k_6t_6)]$$

where the first three subscripts apply for the three chosen times for the $C_{<1}$ criterion and the second three apply for the $C_{>0}$ criterion. Then, by taking these relationships pairwise for both the $C_{<1}$ and the $C_{>0}$ cases, canceling C_O and 1 from both sides of each equation, and finally taking logarithms, we obtain $k_1t_1 = k_2t_2$ and five similar additional equations with suitable combinations of subscripts. All the common parameters in the expressions for the k's cancel out. Because the value of k depends on the pore radius, six estimates of R can be calculated from the six equations.

Calculation of the equivalent size of globular protein that could just pass through the wall. The radius of a globular protein with a molecular weight of M and a density of 1.3 g/cm^3 was calculated from the following equation: $r = \{3M/[4\pi(6.023 \times 10^{23})(10^{-21})(1.3)]\}^{1/3}$.

Limitations on the estimates of pore size and number. There are significant, although minor, limitations to this fitting method. One arises because we have so far assumed that there is only one size of hole in the cell wall. If there is a range of sizes, then the larger-sized probes will only penetrate the largest pore, while the smaller-sized probes will pass through the largest pore and also through some pores of smaller sizes and, thus, will achieve either criterion $C_{<1}$ or $C_{>0}$ faster. This corresponds to the calculated radius being biased towards the larger-sized pores. Consequently, the radii listed in Table 1 are overestimates of the mean pore radius but underestimates of the upper limit of the maximum pore radius. Other parameters, as well, could be variable within the population and might cause errors in the simple procedure of fitting that assumes homogeneity in all parameters. Consequently, a computer program that allows the summation of many first-order curves with different values of k due to a Gaussian variation in any or all of the six parameters that determine the first-order rate constant—volume, pores per cell, particle radius, diffusion constant, pore radius, and wall thickness—was constructed. Experimentation with this program showed that unless the variation of a parameter was very large, there was little effect on the calculated mean radius of pores. Specifically, a simulation of a range of pores of different values showed that although the effect is significant, it is small.

There is, however, an effect acting in the opposite way. If any enzymatic autolysis or mechanical injury during the preparation of the sacculi created a minor rupture, it would lead to an overestimate of the average radius of pores in the native wall. Note that a large rupture would not generate any error, because the sacculi would not be negatively stained even with a dextran of large size. However, essentially all the sacculi of both species did stain negatively, and therefore, major ruptures which would allow dextrans of molecular masses greater than 100,000 kDa to enter were very rare.

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MATERIALS AND METHODS

Preparation of fluorescein-labeled dextrans. Dextrans were labeled with fluorescein by a modification of the method of De Belder and Granath (1), which is not much different from the techniques commonly used to label protein (19). Portions of dextran fractions obtained from Sigma Chemical Co. (St. Louis, Mo.) were dissolved in dimethyl sulfoxide (0.1 g of dextran per ml). Fluorescein isothiocyanate (isomer I; Sigma) was added (0.01 g of fluorescein isothiocyanate per ml of dimethyl sulfoxide). The mixture was heated at 96°C for 2 h. After cooling, 3 ml of methanol per ml of dimethyl sulfoxide was slowly added with stirring. The material was subjected to four cycles of methanol precipitation, and the pellet was dried in vacuo at 55 ± 10°C.

Fractionation of fluorescein-labeled dextrans. Five grams of fluorescein-labeled dextran was fractionated on a Sephacryl column according to a protocol similar to that of Granath and Kuist (3a). The column running buffer contained 0.3% (wt/vol) NaCl, 10 mM 3-[*N*-morpholino]propanesulfonic acid (MOPS), 5 mM NaOH, and 0.02% sodium azide. An XK-type column (Pharmacia), 70 cm long with a 2.6-cm diameter, was packed according to the manufacturer's instructions with Sephacryl S-300HR (Pharmacia). Even though we started with a fractionated product, we obtained 45 fractions for which the molecular mass ranged from 161 to 19 kDa.

Assays for reducing sugars. Determinations were done essentially by the method of Somogyi (23).

Determinations of total carbohydrate. The method of Hanson and Phillips (4) was modified as follows: 1 ml of 5% (wt/vol) phenol that had recently been purified by glass distillation, 1 ml of sample, and 5 ml of concentrated sulfuric acid were rapidly mixed. Four minutes after the sulfuric acid addition, the reaction mixture was again mixed and placed in a 30°C water bath, and between 25 and 35 min later the optical density at 535 nm was measured. The resulting color intensity was greatly affected by small differences in the final percentage of sulfuric acid. Quality control was exerted by weighing each tube at different stages to reject deviant samples.

Fluorescence determinations. The fluorescence of each fraction was determined with an Aminco model SPF-1255 spectrofluorometer at pH 7.8, using an excitation wavelength of 495 nm and an emission wavelength of 525 nm.

Light absorbance determinations. The A_{527} was determined for each fraction.

End group analysis. The average chain length of the dextran fractions was computed by dividing the moles of glucose residues by the moles of reducing sugar, there being only one reducing end per chain. The molecular weight was directly calculated from this.

Etched microscope slide depressions. Standard microscope slides were cleaned with 5% ammonium hydroxide, rinsed with water, and thoroughly dried. The narrow ends of plastic Eppendorf-type pipette tips were cut off. The uncut base of each pipette tip was coated with silicone grease. Each tip was then pressed greased side to glass onto the surface of a microscope slide. Care was taken not to smear the grease across the surface of the slide to make a seal. The etching solution contained 10 g of H_2SO_4, 10 g of NH_4F, and 50 g of water. Each pipette tip was loaded approximately half full with etching solution. Etching was continued for 50 to 250 s at 25°C to produce different depths. The depression chambers used for the experiments reported were all very nearly 4 μm deep.

Methods used to determine the depth of etching. One method used to determine the depth of etching was to measure the diameter of a small droplet of mercury on a dry glass surface with a dissecting microscope and then place the droplet in the etched microscope slide depression. Then a drop of water was added and a cover glass (no. 2 thickness) was pressed down as described below. The depth of the etched slide was calculated from the diameter of the disk of mercury by assuming that the mercury was a sphere on the dry glass surface and was a right cylinder when under the cover glass. The second method for determining the depth of the etched region was the standard dye dilution method, using the automatic exposure unit of the microscope as the equivalent of a spectrophotometer. By setting the ASA dial to 1600, the automatic exposure time could be measured, and different dilutions of the dye were tested to find that which had the same apparent time of exposure as when using a very deep depression slide whose depth could be measured with a micrometer and a very much more diluted dye.

Media. Nutrient agar (Difco) and nutrient broth (Difco) were used. Luria-Bertani (LB) medium (a modification of that used by Miller [18]) was made by dissolving 10 g of tryptone (Bacto), 5 g of yeast extract (Bacto), 1.5 ml of 1 M NaOH, and 10 g of NaCl per final liter of broth.

Strains and growth conditions. Strains from the American Type Culture Collection used were *Escherichia coli* ATCC 11775 and *Bacillus subtilis* ATCC 6051. Each culture was aerated by bubbling with humidified filtered air, so that the culture was not at any time oxygen limited. The *E. coli* cultures were grown and monitored (11) in Luria-Bertani medium at 37°C, and *B. subtilis* cultures were grown in nutrient broth at 37°C.

Preparation of sacculi. Liquid cultures were grown into the stationary phase. The culture was diluted 1:10^8 into fresh prewarmed medium and incubated with aeration at 37°C. The biomass of the cultures was monitored at 660 nm. When the optical density reached 0.100 ± 0.007, a portion of the culture was diluted 1:20 into a boiling solution containing 0.1% sodium dodecyl sulfate (SDS) and 10 mM MOPS and adjusted to pH 7.00 ± 0.06 (at 25°C) with NaOH. The mixture, under continued heating, reached 99°C within 4 min and was held within 1°C for

TABLE 1. Dextran sizes satisfying the two criteria and the calculated pore radii[a]

Organism and incubation time (min)	Probe size (kDa) meeting criterion:		Calculated pore radius (nm)[b]			
			Random coil model[c]		Rigid sphere model[d]	
	$C_{>0}$	$C_{<1}$	$C_{>0}$	$C_{<1}$	$C_{>0}$	$C_{<1}$
E. coli						
3	46.6	26.8	2.82[e]	1.93[e]	1.96[e]	1.61[e]
60	57.0	32.8	2.67[f]	1.83[f]	1.84[f]	1.53[f]
1,440	69.6	40.1	2.95[g]	2.01[g] (2.37)	1.89[g]	1.63[g] (1.74)
B. subtilis						
3	29.7	20.8	2.51[e]	2.72[e]	1.71[e]	1.62[e]
60	40.1	31.2	2.73[f]	2.74[f]	1.60[f]	1.47[f]
1,440	49.0	42.2	2.32[g]	2.70[g] (2.62)	1.71[g]	1.62[g] (1.62)

[a] Two criteria were used: $C_{>0}$ is the minimally detectable level of internal fluorescence, and $C_{<1}$ is the fluorescence level that is barely lower than that of the surrounding medium.
[b] Values in parentheses are averages of the values for the indicated model in that row. Values for the grand average (the average of the two averages in a given row) are 2.06 and 2.12 for *E. coli* and *B. subtilis*, respectively.
[c] The model that assumes that the dextran is an ideal random coil.
[d] The model that assumes that the dextran hydrogen bonds to itself to act as a hard rigid sphere.
[e] Calculated from the 3- and 1,440-min observations.
[f] Calculated from the 3- and 60-min observations.
[g] Calculated from the 60- and 1,440-min observations.

20 min. The culture was cooled by pouring it into a glass flask with a large surface area and agitated in an ice-water slurry; then it was centrifuged. The mixture was washed eight times by centrifugation and resuspended in RB buffer (10 mM MOPS, 5 mM NaOH, 0.02% sodium azide [pH 7.1]).

To the resuspension, RNase A (Worthington Biochemical Company, Freehold, N.J.), DNase I (Worthington), and a concentrated $MgSO_4$ solution were added so that the final concentrations were as follows: RNase, 1,000 U/ml; DNase, 100 U/ml; Mg^{2+}, 10 mM. After 3 h of incubation at 37°C, the mixture was pelleted and the process was repeated. Then, trypsin (Sigma) was added to a final concentration of 30 μg/ml. Incubation was continued with hourly additions of 30 μg of trypsin per ml for 4.5 h. Then the sample was washed by pelleting three times. The entire enzyme treatment was repeated, and intact-wall preparations were held at 5°C until use.

Fluorescence microscopy. Microscopy was done with a Zeiss Photomicroscope III with epifluorescence attachment (Zeiss III RS) and an oil immersion planoapo 63× objective lens (numerical aperture, 1.4) with the appropriate filters. All materials were at room temperature before the start of the experiments. For each microscopic observation, one of a series of the fluorescein-labeled dextran fractions of known molecular weight was mixed with column running buffer so that the fluorescein-labeled dextran concentration was either 5 ± 1 or 1 ± 0.3 mg/ml. The diluted fluorescein-labeled dextran sample was then mixed 1:1 with an intact-cell wall sample and incubated. Shortly before a 3-, 60- or 1,440-min period elapsed, the sample was placed in an etched microscope slide depression and covered with a no. 2 cover glass. Care was taken to achieve close contact between the cover glass and the slide. The slide was then quickly placed on the stage and viewed. Focus was accomplished by closing the iris diaphragm on the fluorescence attachment as much as possible and then focusing on the edge of its image. After focusing was completed, the iris was opened until the shadow was visible near the edge of the field of view. Its presence allowed continuous checking of the focus. At the chosen times, observations were made. Because of the subjective nature of the observations, the probe samples of different sizes were coded and randomized so that the experimenter operated blindly. Then, the fractions that had dextrans that were almost fully penetrant or those that were almost fully excluded were noted. The molecular weight of these fractions that had met the upper and lower criteria were then used to estimate the pore size.

Positive staining with fluorescein-labeled dextrans. Liquids used for these procedures were held at 5°C unless otherwise indicated. For each microscopic observation, a fluorescein-labeled dextran sample was mixed with column running buffer so that the fluorescein-labeled dextran concentration was 0.8 ± 0.2 mg/ml. The diluted fluorescein-labeled dextran fraction was then mixed 1:1 with the cell wall sample. The mixed samples were held at 4°C for 24 ± 3 h. Then the sample was brought to room temperature, and the sample was diluted 1:50 into column running buffer. The diluted sample was placed in an etched microscope slide depression and covered with a no. 2 cover glass. Although some care was taken to minimize the distance between the cover glass and the slide, minimizing the time to viewing was a higher priority. The slide was then placed under the stage, and the sample was viewed under the same conditions used for the negative staining. At a known time after the samples were diluted, observations were recorded as given in Table 2.

RESULTS

Experimental results are tabulated in Table 1 for the negative-staining experiments and in Table 2 for the positive-staining experiments in which the sacculi were allowed to take up dextrans for a long time and then allowed only a brief period for efflux. With very small dextrans, diffusion occurred so fast that no negative staining could be detected, equilibration was complete at the earliest observation time possible, and C equaled C_O. With very large dextrans, negative staining persisted for many days. From the results given in Table 1, the molecular mass of a globular object of the density of protein that made no interactions with the wall was calculated from the following formula: $M = R^3[4\pi(6.023 \times 10^{23})(10^{-21})(1.3)]/3$. This yielded 22 and 24 kDa, respectively, for *E. coli* and *B. subtilis* for the grand average (three incubation times, two criteria, two diffusion models) for each species. Because the third power of the probe radius is involved, the molecular

TABLE 2. Positive staining of sacculi with fluorescein-labeled dextrans[a]

Fraction no.	Size (kDa)	Observation at indicated time after dilution (min):			
		2		60	
		E. coli	*B. subtilis*	*E. coli*	*B. subtilis*
5		+	+	−	−
10		+	+	−	−
15		++	++	−	−
20		++	++	+	+
25		++	++	+	++
30	70	++	+++	++	++
35	48	++	+++	+	++
40	38	+++	+++	+	+
45	29	+++	++	+	+
50		++	+	+	+
55		++	+	+	+

[a] −, no observed fluorescence above background; +, ++, and +++, increasingly intense fluorescence above background. Every attempt was made to be consistent in the intensity estimations; within the *E. coli* experiment and within the *B. subtilis* experiment, they were nearly the same.

weight is greatly affected by small variations in R. For example, if the 2.62-nm radius appropriate for the coil model for *B. subtilis* is chosen, then the molecular mass of a protein, if it had no charge interaction with the walls of the pore, that could just pass through the wall is 59 kDa. At the other extreme of 1.62 nm for the rigid sphere model, this figure would be 14 kDa.

For the positive-staining experiment whose results are presented in Table 2, in theory the fluorescein concentration inside the sacculi should fall as $C = C_O e^{-kt}$. We made no attempt to measure the kinetics and made observations at only two time points. With small probes the dextran had escaped even after 2 min, and with large ones it had never entered. One other possibility is that the walls bind the labeled dextrans. This possibility was excluded, because then cells prelabeled with dextrans of at least some size would be bound to the walls. In this second type of experiment, such a cell should fluoresce at the edges where a thicker layer of wall is being viewed much more than at the center of the cells, but no such cells were seen.

DISCUSSION

Earlier work to measure the porosity of bacterial cell walls has come mainly from the laboratory of Gerhardt (3, 21, 22). These studies measured the ability of probe molecules to enter wall fragments, but they did not measure the ability of probe molecules to go across from one side to the other as did our studies. The interesting studies of Hughes et al. (5) measured the range of protein sizes diffusing out of cells treated with *n*-butanol to make their lipid bilayers permeable. These experiments were based on the expectation that the *n*-butanol would not damage the walls. Because of denaturation, proteolysis, and the effects of charge on permeability, however, this interpretation is difficult.

Regardless of whether the probe molecules are random coils or rigid bodies or have other shapes, as long as the sacculi are identical, the fluorescein concentration inside the sacculus will rise according to $C = C_O(1 - e^{-kt})$ as shown in Fig. 1, where the time course depends only on k. Even if there is a range of pore sizes or shapes, then C, initially zero, will approach the outside concentration, C_O, after a long time. Of course, if the constant k is equal to zero, no dye enters and then C will remain at zero. We found that this latter prediction applied to exposure to very large dextrans and that the sacculi remained negatively staining for days. This demonstrates that almost none of the sacculi had been ripped or torn during their isolation. It also suggests that the large dextrans rarely achieve a conformation that allows them to pass into the sacculi.

This work was carried out for two purposes: to assess the ability of biomolecules of different sizes to cross the walls of these two species and, equally important, to assess the intactness of the wall fabric of *E. coli* and *B. subtilis* organisms. This laboratory has long been interested in the physical properties of the bacterial wall (6–8, 10, 15, 16). The implication of the results shown in Table 1 is that only small uncharged hydrophilic molecules were able to pass: i.e., ones with a radius in the range of 1.47 to 2.95 nm, with a mean of 2.06 nm for *E. coli* and a mean of 2.12 nm for *B. subtilis*. These figures correspond to 22 to 24 kDa of a globular uncharged protein molecule being able to pass through the sacculus of either species while in the unextended globular state. Although our results make it clear that much larger proteins could not penetrate our prepared sacculi, there are several limitations to our current method. The tension that normally stretches the wall fabric should increase the size of the pores in the walls of cells. So we must extrapolate from the in vivo situation to the in vitro one. We found previously (15) that the linear dimension of the fabric in *E. coli* organisms is 17% longer during normal growth than when the turgor pressure, and hence the tension, is removed by disaggregation of the cytoplasmic membrane of filaments of cells in vivo with SDS. This 17% enlargement would increase the size of a probe that could pass through the wall, and the normal extension of the fabric under growth may not be enough to allow a 55-kDa protein to pass through the wall of *B. subtilis*. This can be concluded because we found that levansucrase, a protein of this size, could not diffuse through the wall but could only pass to the external medium via the inside-to-outside growth process used by the gram-positive organism (6).

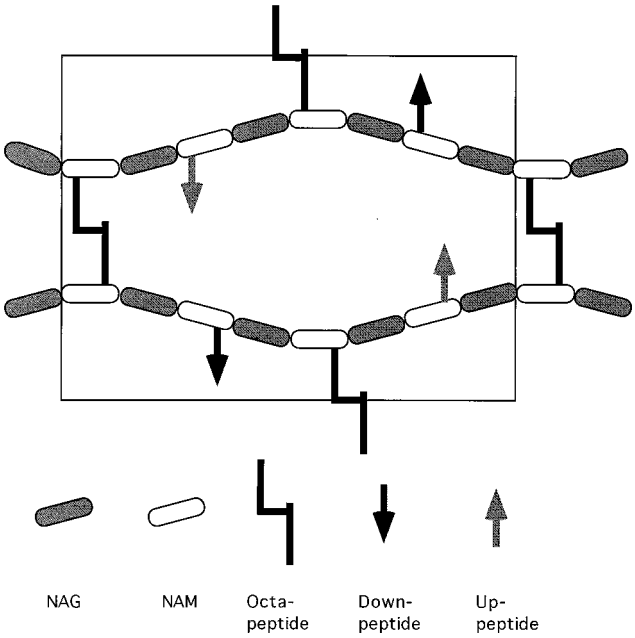

FIG. 3. The strongest unit of wall fabric: the smallest tessera. The function of the peptidoglycan is to cover the cytoplasmic membrane. As such, it is a fabric; the basic unit is displayed inside the rectangle. The parts protruding outside the rectangle are in the same plane but are parts of other tesserae. The arrows correspond to peptides protruding up or down. These may connect to other layers, as in the case of gram-positive organisms. The space through which diffusion could occur is not yet well defined by computer models or experimental measurements but probably is about 2.2 nm (radius), comparable to the pore sizes reported here. NAG, *N*-acetyglucosamine; NAM, *N*-acetylmuraminic acid.

Our methods could be improved. One way would be to use probes of a defined rigid spherical shape. But it would be necessary to choose ones (like our dextrans) that are hydrophilic but do not have charged groups that might affect the ability to permeate the wall. This approach may be followed in the future. Other ways include measuring the permeability of sacculi other than those with "clean" walls, to include some of the normal constituents that may decrease the pore size, and culturing in growth states other than exponential growth.

The geometrical area of pores formed by the wall network is called the tessera (10) or mesh size (17a). When additional peptides between the two tail-to-tail tetrapeptides in the cross-bridges are not present, as is the case with *E. coli* or *B. subtilis*, the most compact planar tessera that can be formed when every fourth disaccharide links two oligosaccharide chains with peptide cross-bridges is shown in Fig. 3. The peptides on the two sets of three intervening disaccharide moieties point in some other direction than ones that allow the formation of a unit of fabric. Both the ones pointing upward and the ones

pointing downward cannot be linked by tetrapeptides to glycan chains lying in the same surface plane. The third set points outward, and these peptide chains in the plane of the fabric are part of other tesserae. They do not form, however, part of the unit tessera under consideration. When maximally stretched in all directions, the pore produced is slightly oblong but presents a passageway that is approximately 2 nm in radius (13, 16). The best estimate of the radius opening of the bulk of the tesserae from this study is not much bigger than this. Because a few larger pores can dominate the kinetics of the diffusion process, we estimate that the results are consistent with there being only a few holes somewhat greater than the pore of the smallest possible tessera. The role of bound water influencing the effective size at this point is not at all clear. This leads to the important conclusion that the actual bacterial fabric is formed in a quite complete but regular way, with few imperfections, even those engendered by the formation and turnover of the wall.

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