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Model for the Structure of the Shape-Maintaining Layer of the *Escherichia coli* Cell Envelope

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The surface area per repeating murein unit (i.e. per molecule of diaminopimelate) has been determined for the cell envelopes of the *Escherichia coli* strains K-12 and W. This area was constantly found to be 1.3 nm^2. Using this value and other previously determined properties of *E. coli* murein, a three-dimensional model of murein is proposed. The model specifies a monomolecular layer in which disaccharide units are each 1.03 nm long, and the polysaccharide chains, all parallel, are 1.25 nm apart. The cross-linking peptide side-chains have the same atomic coordinates and are arranged above or below the polysaccharide chains.

Electron microscopy has shown (16, 20) that three layers are distinguishable in the cell envelope of *Escherichia coli* and in gram-negative bacteria in general: the inner cytoplasmic membrane, an outer membrane, and, sandwiched in between the two, the murein (25, 26). The chemistry of one basic building block of murein is shown in Fig. 1.

Since lysozyme or penicillin treatment converts rod-shaped cells to osmotically labile spheroplasts, it was more or less tacitly assumed that the murein also determines the shape of the bacterial cell. In a preceding communication (8) we, as well as others (23), provided some evidence that this does not appear to be so, since murein of one and the same composition can assume virtually any shape. However, a discussion of bacterial morphogenesis demands knowledge about the three-dimensional organization of this layer of the cell envelope. In *E. coli* and most other bacteria, almost nothing relevant is known. The results presented in this communication concerning the number of murein repeating units per cell envelope provide limits for the construction of three-dimensional models without, as expected, completely solving the problem.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains employed are listed in Table 1. Cells were grown, at the temperatures indicated, with vigorous aeration in antibiotic medium no. 3 (Difco). Where necessary, the medium was supplemented with diaminopimelate (10 μg/ml) and thymine (50 μg/ml). For mutant *lss*12 the medium in addition contained $MgCl_2$ (2×10^{-3} M) and 12% sucrose (8).

Preparation of cell envelopes. We have shown and discussed previously (8) that for surface measurements of murein sacculi it is considerably safer to use cell envelopes instead of sacculi. The present study was therefore carried out exclusively with envelopes. The following procedure was adopted to obtain preparations containing a maximum amount of complete, nonfragmented envelopes.

Cells were harvested while still growing exponentially (at about 10^8 cells/ml when very well aerated), suspended in water (1 g wet weight in 6 ml), and shaken with glass beads (0.17-0.18 mm, 5 g per 7 ml) for 20 min in a Mickle type shaker (22). Glass beads were removed by filtration through a coarse sintered glass, and the filtrate was diluted with water to a volume of 20 ml per g of cells. The filtrate then was centrifuged for 3 min at 4,500 × *g*. The pellet was shaken once more under the same conditions with glass beads for 10 min. After removal of the beads and centrifugation for 3 min at 4,500 × *g*, the two centrifuged supernatant fluids were combined and spun for 10 min at 4,500 × *g*. The pellet obtained from about 3 g of cells was suspended in 10 ml of water and subjected to two centrifugation cycles at 4,500 × *g*: envelopes sedimenting between 3 and 5 min of centrifugation were suspended with 2 to 3 ml of water and used immediately for electron microscopy, amino acid analyses, or determination of radioactivity.

Specimen preparation for electron microscopy. Counting of envelopes was performed by mixing a calibrated Latex sphere suspension (28) with the

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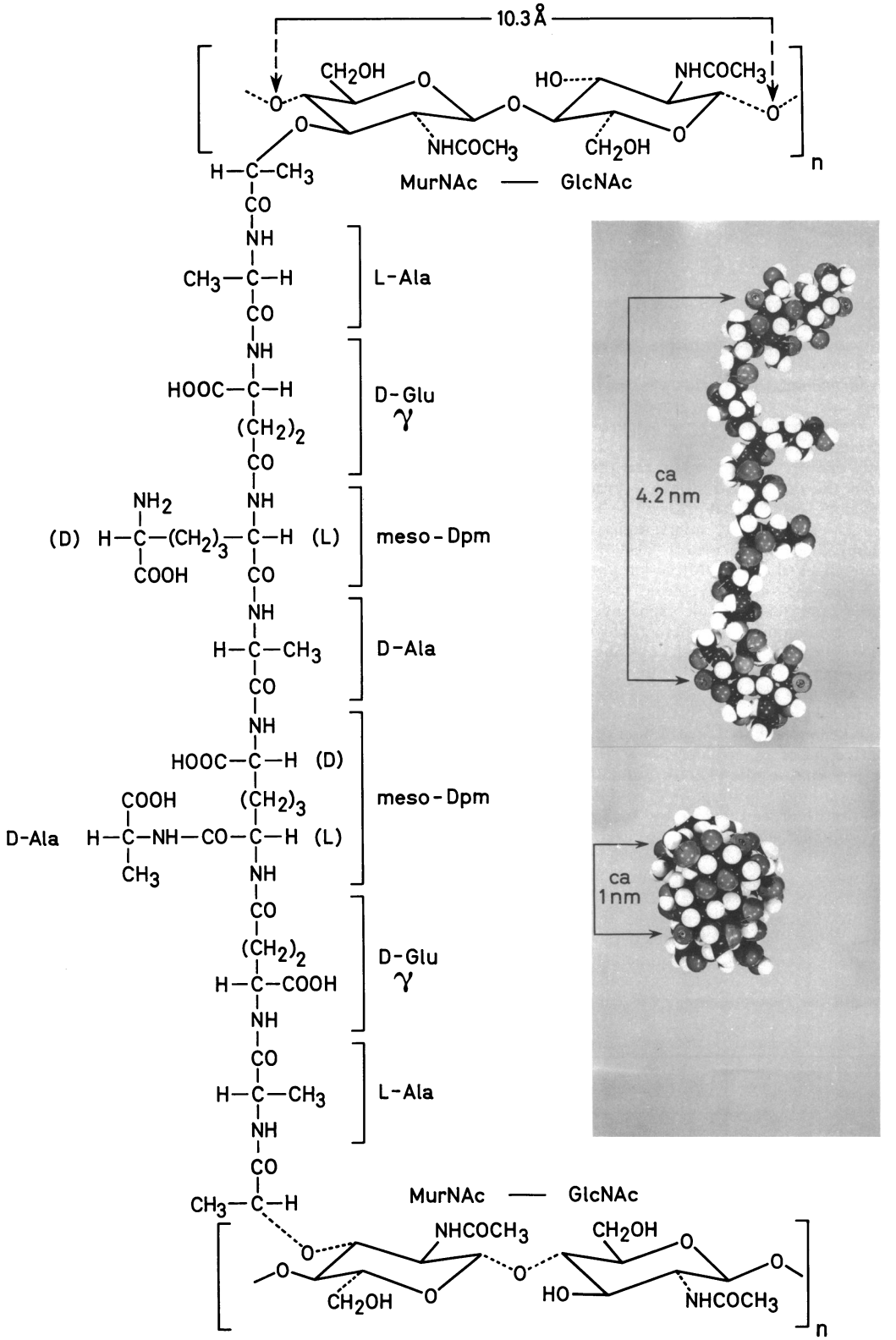

FIG. 1 *Basic building block of the E. coli murein (26) (cf. also Fig. 4). Disaccharide subunits of two neighboring polysaccharide chains and the cross-linking peptide bridge are shown. The same structure is also represented by space-filling models with the peptide side chain fully extended and maximally folded. Colors: white, hydrogen atoms; gray, oxygen atoms; black, carbon atoms; the darker gray of the nitrogen atoms is somewhat difficult to recognize. The two glycosidic oxygen atoms on both sides of the disaccharide unit to which the neighboring disaccharide units are linked (not drawn) are marked in the models by holes.*

envelope suspension by the method of Kellenberger and Arber (15). Spreading without aggregation of particles could only be achieved by the addition of sodium dodecyl sulfate.

Dow Latex suspension (0.234-μm diameter spheres, at 1.5×10^{13} particles/ml [Serva Entwicklungslabor, Heidelberg]) was diluted 1,000-fold with water. A 0.10-ml sample of this suspension was mixed with four different amounts of cell envelope suspension (from 0.01 to 0.08 ml), and the final volume was adjusted to 0.18 ml with water. Immediately after addition of 0.02 ml of 0.4% sodium dodecyl sulfate, the mixtures were spread onto three agar plates covered with a thin film of nitrocellulose (E. F. Fullam, Schenectady, N.Y.). From each plate five grids were collected and shadow-cast with platinum alloy (for details see reference 8).

Electron microscopy. For particle counting, performed at a magnification of about 4,000 diameters, only one photograph was taken from each grid, and from the different dilutions a total of 15 grids per envelope preparation was used. For size measurements of envelopes, micrographs were taken, at a nominal magnification of 20,000 diameters, which were checked with a diffraction grating replica (Fullam) on the same day. From five different grids 60 plates were exposed for each envelope preparation. The randomness of micrographs was assured by photographing every tenth envelope appearing in view while moving the stage. Photographed envelopes were cut out and weighed. The weight was related to the surface by the square of the final magnification; the conversion unit of 1 g is 1.5 nm^2, corresponding to both sides of an envelope.

The final results (Tables 2 and 3) were obtained as follows. For determination of envelope concentrations the ratio of cell envelopes to Latex spheres was determined separately from each micrograph and averaged for each dilution. The four values (four dilutions, see above) obtained for each envelope preparation were then averaged to yield the final result. Figure 2 demonstrates this procedure. For measuring the size of envelopes, all cut-out photographic images of one preparation were weighed together to reduce weighing errors, and the average weight per envelope image was calculated. The standard error was derived from the distribution (Fig. 3) that resulted from weighing each image separately. It turned out that this precaution was unnecessary since variations in the average values obtained by the two methods showed up only in the third decimal of the result.

TABLE 1. *Bacterial strains*

Strain (*E. coli* type)	Properties	Source or reference
HfrH (K-12)	Prototroph	H. Kneser
W945T1 (K-12)	*thi thr leu trp str*^{R}, F$^-$	5
W945T3282[a] (K-12)	*thi thr leu trp his thy lys dap str*^{R}, F$^-$	8
*lss*12 (K-12)	*rod* mutant derived from W945T3282	8
W7 (W)	*lys dap* (derived from W173-25 [6])	7

[a] For brevity's sake strain W945T3282 is designated in this communication "3282."

The standard errors of the ratios of cell envelopes to Latex spheres were increased by 1% for the Latex sphere diameter (as indicated by the manufacturer) and by another 1% for the Latex sphere concentration (as found by dry weight determination). A total error of about 2% has to be taken into account for the determination of envelope sizes stemming from the combined errors of weighing and microscope magnification. These errors are included in Tables 2 and 3.

Amino acid analyses. Usually 0.2 ml of cell envelope suspensions prepared for electron microscopy were oxidized with performic acid (10) for separation of diaminopimelate and methionine. Diaminopimelic and muramic acids were determined with a Unichrom amino acid analyzer (Beckman, Munich) calibrated with diaminopimelic and muramic acids (1, 2). A 0.1-μmol amount of taurine and 0.1 μmol of β-thienyl-alanine were added to each run as internal standards.

Determination of diaminopimelate in envelopes by radioactive labeling. For the experiment shown in Table 3, both strains were grown at 42 C (*lss*12 growing as sphere) in complete medium supplemented with thymine (50 μg/ml), $MgCl_2$ (2×10^{-3} M), 12% sucrose, ^{3}H-diaminopimelate (The Radiochemical Centre, Amersham, England; 1.25 μg/ml), and unlabeled diaminopimelate (Sigma Chemical Co., St. Louis, 5 μg/ml); the final specific radioactivity was 39.3 Ci/mol. Envelopes were prepared as described above. According to the manufacturer, all three diastereomers of the ^{3}H-diaminopimelate must have the same specific radioactivity, but the proportion of the diastereomers is not known; the latter is also true for the nonlabeled acid. We have therefore separated meso-plus D,D- from L,L-diaminopimelate by the method of Hoare and Work (11). In the radioactive as well as nonradioactive material the L,L-acid amounted to about $^1/_3$ of the meso-plus D,D-mixture, and we have assumed that the three diastereomers are present in equal quantities.

Radioactivity measurements were performed in a Tri-Carb liquid scintillation spectrometer model 3002 (Packard). Radioactive material was placed onto filter paper discs (Whatman 3 MM, 2.3 cm diameter), dried, and measured in a 0.4% solution of Omnifluor (New England Nuclear Corp., Boston). The counting efficiency of this method was determined with ^{3}H-diaminopimelate, ^{3}H-leucine, and ^{3}H-thymine (New England Nuclear Corp., Boston). It was found for all these substances (based on the amount of radioactivity in the preparations indicated by the different manufacturers) to be $6.35 \pm 0.15\%$.

Radioactivity measurements of the envelope suspension are useless because internal quench controls are impossible. Therefore, the envelopes were hydrolyzed (4 N HCl, 15 h at 105 C). Radioactivity was then measured in these hydrolysates with and without the addition of known quantities of ^{3}H-diaminopimelate.

TABLE 2. *Chemical determination of diaminopimelate concentration*

E. coli strain	No. of envelopes counted	No. of envelopes used for surface determination	Envelopes/ml	Surface/envelope	Moles diaminopimelate/ml	Molecules diaminopimelate/envelope	Surface per molecule diaminopimelate (nm^2)
3282	5,936	118	$4.78 \times 10^{10} \pm 1\%$	$3.89 \times 10^7\ nm^2 \pm 3\%$	0.215	2.71×10^6	14.3
HfrH	9,694	118	$2.65 \times 10^{10} \pm 6\%$	$3.07 \times 10^7\ nm^2 \pm 3\%$	0.120	2.65×10^6	11.6

TABLE 3. *Determination of diaminopimelate concentration via radioactivity*

E. coli strain	No. of envelopes counted	No. of envelopes used for surface determination	Envelopes/ml	Surface/envelope	Radioactivity (dpm/ml)	Moles diaminopimelate/ml	Molecules diaminopimelate envelope	Surface per molecule diaminopimelate (nm^2)
3282	2,703	89	$5.22 \times 10^{10} \pm 6\%$	$3.31 \times 10^7\ nm^2 \pm 2\%$	1.93×10^7	0.22	2.65×10^6	12.5
*lss*12	1,373	84	$2.54 \times 10^{10} \pm 9\%$	$3.68 \times 10^7\ nm^2 \pm 3\%$	1.01×10^7	0.115	2.77×10^6	13.3

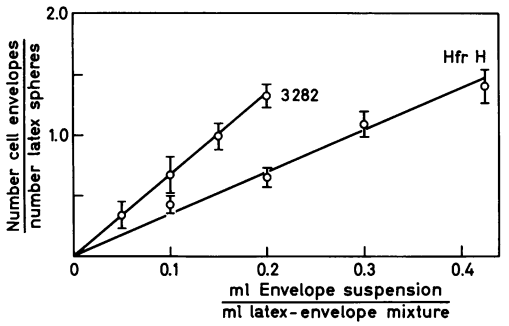

FIG. 2 *Determination of particle concentration. The results obtained for two different strains are shown; for detail see text.*

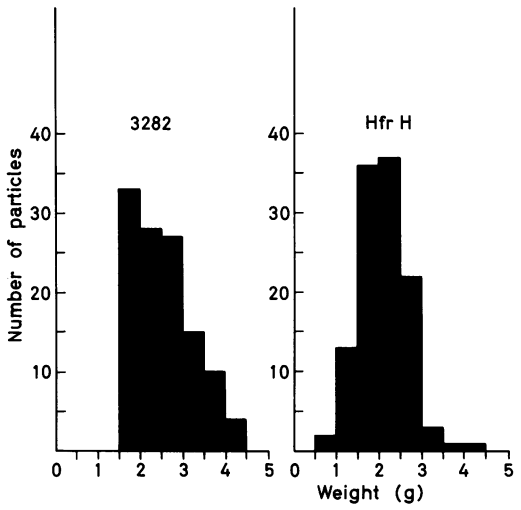

FIG. 3 *Size distribution of cell envelopes. Size is expressed in terms of weight since the determination was performed by weighing prints of electron micrographs; for details see text.*

The counts found always corresponded in a perfectly linear fashion to the amounts of hydrolysate or hydrolysate plus ^{3}H-diaminopimelate placed onto the filter. Quench due to material in the hydrolysates was found to vary, from one preparation to another, between 12 to 17%. From counting efficiency and quench the values for disintegrations per minute in Table 3 were calculated.

Model building. Dimensions of murein repeating units were deduced from Dreiding models or from space filling models (CPK Atomic Models from Ealing Scientific Ltd., London, England).

RESULTS AND DISCUSSION

The experimental design was simple. Diaminopimelate is present in no cellular material other than in murein. A double mutant we isolated is blocked in the synthesis of diaminopimelate and is lacking diaminopimelate decarboxylase activity completely (6, 8). Therefore, label of diaminopimelate from the growth medium will not appear in lysine, and it will be present in the murein with the same specific radioactivity as that of the amino acid added to the medium.

In isolated cell envelopes the amount of diaminopimelate per volume of the suspension was measured. This was done by radioactivity

measurements as well as by amino acid analyses of acid hydrolysates. The number of sacculi per volume and the mean surface area per sacculus were determined by electron microscopy. From these data it was possible to calculate the number of diaminopimelate molecules per unit surface, a parameter which then had to fit into one or the other model of murein organization.

The results obtained are summarized in Tables 2 and 3. For four different preparations from three different strains the values found for the surface per diaminopimelate molecule were very nearly the same (between 11.6 nm^2 and 14.3 nm^2). Excellent agreement was obtained between the diaminopimelate determinations by amino acid analysis and by ^3H-diaminopimelate incorporation. The correctness of the evaluation of the radioactivity data rests on the assumption (see Materials and Methods) that the specific radioactivity of each enantiomorph of diaminopimelate is the same, and we could not strictly prove this assumption. We feel, however, that the very close agreement mentioned justifies the use of the radioactivity data (although this justification is clearly based on circular reasoning).

For these measurements we have included mutant *lss*12 which is a temperature-sensitive *rod* mutant derived from strain W945 T3282 (8). In confirmation and extension of results presented previously (8), the mutant, when grown as a sphere (Table 3), possesses the same surface area per molecule diaminopimelate in its murein as does the rod-shaped parent.

Towards a model. The chemical structure of the main repeating unit is known, but one should keep in mind that the structure of a whole series of minor split products which are obtained by lysozyme degradation of murein has remained unknown (21). In some cases the thickness has been approximated by electron microscopy (see below). We have added here another parameter for the *E. coli* murein, the surface area per molecule diaminopimelate. Although this information obviously does not allow construction of a complete murein model, it does provide fairly restrictive conditions for any such model building. In the following, we apply these restrictions to considerations concerning murein structure. It appears justified to proceed from the simplest assumptions as long as experimental evidence does not demand more complicated structures.

(i) Murein principally has a regular structure. Regular, highly symmetrical structures are found in all biological systems where some sort of repeating subunits (chemical or morphological) are present, e.g., cellulose or chitin (3), surface protein of *Bacillus polymyxa* (19) or *Spirillum* sp. (12), viruses (4), etc. (ii) The polysaccharide chains run in parallel (see below). (iii) The atomic coordinates of the peptide side chains are the same from one chain to another within the murein of a given species. The peptide side chains very probably must have a stable conformation because of a steric requirement in murein biosynthesis. It appears likely that the amino group of the optical D-center of diaminopimelic acid and the carboxyl group of the D-alanine of the neighboring peptide side chain have to come in close contact automatically to allow the transpeptidation reaction, cross-linking the polysaccharide chains, to occur.

At least for the *E. coli* murein, a covalent long-range fixation of the structure in both dimensions of the plane is also necessary since, even in hot dodecyl sulfate or in phenol, isolated sacculi retain their shape.

For building a murein model only one distance can be assumed to be applicable. It is the length of one disacchraide unit. *N*-acetyl-muramic acid and *N*-acetyl-glucosamine are linked by β-1,4-glycosidic bonds (13, and see Fig. 1). For equivalent disaccharide units, glucosyl-β-1,4-glucose in cellulose and *N*-acetyl-glucosaminyl-β-1,4-*N*-acetyl-glucosamine in chitin, this length has been found by X-ray analysis to be 1.03 nm (3). From the data of Tables 2 and 3 it can be calculated that the average surface unit per one diaminopimelate residue, i.e., per one murein repeating unit, is 12.9 nm^2. Therefore, 1.25 nm are left for the distance between centers of neighboring polysaccharide chains. Considering the bulky amino acetyl groups and the lactyl residue of the muramic acid, the diameter of the polysaccharide chain in the plane of the sugars is about 1.1 nm. In a monolayered murein, where the polysaccharide chains are arranged such that the plane of the sugar residues is parallel to the cell surface, these chains then must almost touch each other. The peptide side chains obviously have to be arranged above or below the polysaccharide plane. In fact, a regular murein structure, with all corresponding bond angles of each repeating unit being the same (and where a covalently closed sacculus exists), can only be constructed with the peptide side chains in a plane outside the polysaccharide chains. Within the polysaccharide chain no rotation of the repeating units is possible. In a β-1,4-linked polysaccharide chain, all the peptide side chains extend towards the same direction (Fig. 1). As can be seen from Fig. 4, from such an arrangement it is also highly probable that the

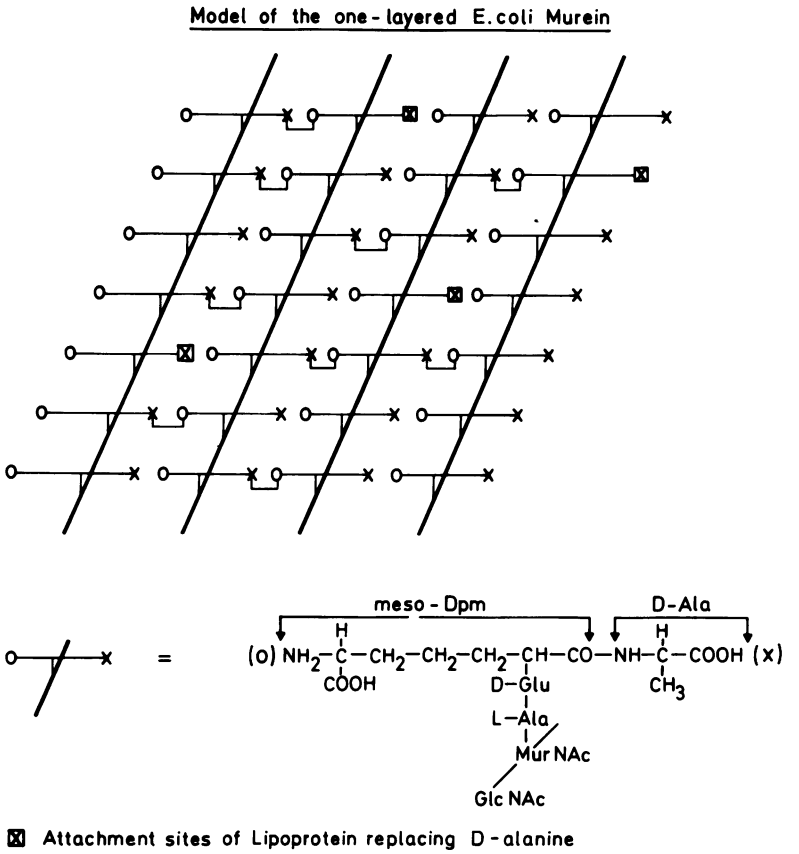

FIG. 4 *Model of a one-layered E. coli murein. A murein section consisting of four parallel polysaccharide chains (heavy lines), all running in the same direction (1 → 4) with T-shaped peptide side chains (faint lines) is illustrated. The plane of the sugar residues can be parallel or perpendicular to the cell surface. All peptide side chains have the same steric configuration, and they can be folded (e.g., as suggested in references 9, 24) with quite a number of stabilizing hydrogen bonds. The extent of crosslinkage between the amino group (O) of diaminopimelate residues and the carboxyl group (×) of D-alanine residues depends on the growth phase (23, approximately 15–30%). The distance between peptide side chains within one polysaccharide chain is probably 1.03 nm. The distance between polysaccharide chains was deduced to be 1.25 nm (see text). The attachment sites of the lipoprotein molecules (1, 2) are, on the average, at every 10th to 12th repeating unit, replacing the D-alanine at the carboxyl group of the optical L-center of diaminopimelate. This scheme does not imply that such a microcrystalline structure need exist over the whole surface of the cell. Areas of the type shown may be interrupted by areas with a less dense arrangement of polysaccharide chains.*

polysaccharide chains run in the same direction, i.e., parallel.

Localization of peptide chains between polysaccharide chains would therefore require different folding of peptide side chains. Not only is it difficult to imagine what forces should lead to such a different folding but also, as mentioned before, it is very difficult to imagine how the cross-linking enzyme should react unless it is always confronted by the same stereochemistry. The basic murein structure resulting from these considerations is shown in Fig. 4.

It can be calculated from the data of Tables 2 and 3 how much space per sacculus surface is not occupied by murein constituents when the sugar planes are oriented parallel or perpendicular to the cell surface. In the first case this space amounts to 3×10^6 nm^2 and in the latter case to 2×10^7 nm^2. In other words, the overall "packing density" is not so high in either case as to exclude the passing through of macromolecules, perhaps at discontinuous areas of the murein (see legend to Fig. 4).

We have so far assumed that the *E. coli* murein constitutes a monolayer. Regardless as to whether the plane of the sugar residues is parallel or perpendicular to the plane of the cell surface, the polysaccharide chains plus the

peptide side chains (maximally folded) contribute roughly 1 nm or 1.5 nm, respectively, to the thickness of the wall. Electron microscopy revealed a thickness of 1.5 to 3 nm (18, 20). A strict comparison of these values is difficult because it is not known how much the lipoprotein (1, 2) is contributing to this thickness, since its nonglobular conformation and its deep penetration into the cell wall may prevent its observation by electron microscopy.

The upper limit which our data allow is a three-layered murein. A three-layered murein would demand that the peptide side chains be completely unfolded. Cross-links would only be possible between two adjacent antiparallel polysaccharide chains, and such a murein would fall apart outside the cell envelope. An experimental decision as to whether the *E. coli* murein constitutes a mono- or double layer is not yet possible.

From the data of White, Dworkin, and Tipper (27) on *Myxococcus xanthus*, it appears that an amount less than about 0.9% murein per cell dry weight is no longer compatible with the existence of a closed sacculus. The presence of 1 to 1.2% (17) murein in *E. coli* would therefore argue for a monolayered murein.

Finally, an interesting (and somewhat reassuring) comparison can be made with *Bacillus subtilis* 168 *ind*$^-$. It contains the same repeating murein unit but 40 times more such units per cell surface unit than *E. coli* (14). If the *E. coli* murein constitutes a monolayer, then *B. subtilis* murein should be about "40-layered." Forty layers (our model) would yield a thickness of about 40 nm, and 30 to 38 nm have been measured (14).

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