The Variable T Model for Gram-negative Morphology

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Gram-negative micro-organisms possess only a very thin murein sacculus to resist the stress caused by the internal hydrostatic pressure. The sacculus consists of at most one molecular layer of peptidoglycan in an extended conformation. It must grow by the insertion and cross-linking of new murein to the old before the selective cleavages of the stress-bearing murein are made which allow wall enlargement. Since insertion of new murein occurs all over the surface of *Escherichia coli* (even in completed poles), the internal pressure would tend to force the cells into a spherical shape and prevent both cylindrical elongation and cell division. Of course, Gram-negative bacteria do achieve a variety of shapes and do divide. Because prokaryote cells, unlike eukaryotic cells, do not have cytoskeletons and contractile proteins to transduce biochemical free energy into the mechanical work needed to achieve aspherical shapes and to divide, this paradox seems to be resolvable only by postulating that the details of the biochemical mechanism for wall growth vary in different regions of the surface, affecting the work required to enlarge the wall locally. Depending on the degree and rate of change in the biochemical energetics, it is possible to account for rod and the other more complex shapes of Gram-negative bacteria. Division occurs in Gram-negative organisms by the development of constrictions that progressively invade the cytoplasm. The work to cause these morphological processes must ultimately derive from the biochemical process of the stress-bearing wall formation. A biophysical basis for cell division in these prokaryotic organisms is proposed.

INTRODUCTION

We have developed the surface stress theory of bacterial morphogenesis. The theory has had some success in accounting for the shape of the poles of *Streptococcus* (Koch *et al.*, 1981*a*) and *Bacillus* (Koch *et al.*, 1981*b*, 1982*a*; Koch, 1983; Burdett & Koch, 1984) on the basis of narrow growth zones where the splitting septum is, respectively, reworked or stretched.

For Gram-negative enteric bacteria, the situation is more complicated. After considering a number of models (Koch, 1982*b*), only one general model seems to account for the important facts about *Escherichia coli*. This model, the variable T model, assumes that T , the analogue of surface tension, varies in different regions of the cell surface and varies in different stages of the cell cycle. This is in contradistinction to the situation in Gram-positive bacteria, where T appears to be constant both during pole formation and during cylindrical elongation of the side wall.

Previously (Koch, 1982*b*), four specific submodels within the general variable T model were considered. One of these (there designated model III) assumed that growth is narrowly zonal in the developing pole, but in addition there is a small amount of diffuse growth occurring elsewhere. For this mechanism to function, T must be different for the zonal and diffuse processes. With model simulations it was shown that the diffuse process would lead to a rounding of the sharp discontinuities that would arise where the growth zones had been initiated. It was also concluded that a small amount of diffuse growth would have little effect on the remainder of the cell. The second model proposed that both the sides and new poles grow by a diffuse mechanism,

but that T is exactly twofold different in the two regions so that both cylindrical elongation and fission into hemispherical poles would occur in the appropriate parts of the cell. The third model (designated model VIIc in Koch, 1982*b*) assumed that diffuse growth occurs and does lead to gradual widening of the cell. However, this widening is then compensated during cell division. These three models each have, we believe, elements of truth. In this paper we will consider a generalized variable T model for the morphology of enteric bacteria and other Gram-negative rods.

THICKNESS OF THE PEPTIDOGLYCAN LAYER IN *E. coli*

There are only two reports in the literature in which both the surface area was measured with the electron microscope and the amount of peptidoglycan determined chemically or radiochemically. Braun *et al.* (1973) found an average of 1.29 nm^2 per diaminopimelic acid (DAP) molecule. The later data of Zaritsky *et al.* (1979) can be re-expressed in the same units as 0.7 nm^2 per DAP for a culture doubling in 30 min. It can be shown with molecular models that the repeat distance along the glycan chain would be $1.0\text{--}1.1 \text{ nm}$ and the chains would be separated by $1\text{--}1.08 \text{ nm}$ when the murein is in the most compact, fully cross-linked conformation. Consequently, since there are two DAP molecules per cross bridge, the wall density would be $1 \times 1/2 = 0.5 \text{ nm}^2$ per DAP if the wall was one molecular layer thick. On the other hand, if the peptide bridges were in an extended conformation, the repeat distance of the glycan chain would change little but the interglycan chain distance would be almost doubled to 1.9 nm for the model of Oldmixon *et al.* (1974) or quadrupled (4.2 nm) for that of Braun *et al.* (1973). Consequently, in such extended conformations, the area covered would be $1\text{--}2 \text{ nm}^2$ per DAP.

In the paper of Braun *et al.* (1973) there was a tenfold error in converting units and the results were interpreted to indicate that there could be three layers of murein in the wall of *E. coli*. This was later corrected (Braun, 1975) with the statement that 30% of the area could be covered by the available peptidoglycan. We believe that the data available in the literature can be put together in a somewhat different, but consistent, way as follows.

The wall of a dead, fixed cell, or of a sacculus prepared from a Gram-negative bacterium in the presence of moderate concentrations of salt, is a single-layered network of peptidoglycan approximately in its most compact configuration. The electron microscopic data of Woldringh used by Zaritsky *et al.* (1979) probably represent an over estimate of the area because flattened cells result from the agar filtration (see Fig. 4C of Woldringh *et al.*, 1977). The correction for the flattening would bring the estimate derived from the data of Zaritsky *et al.* (1979) into excellent agreement with the theoretical 0.5 nm^2 per DAP for a compact monolayer. The data of Braun *et al.* (1973) were obtained with sacculi prepared with many washes with distilled water, then filtered and shadowed with Pt/Pd. As such, the repulsive electrostatic forces due to the predominance of negative charges on the peptides probably drove the structure into an expanded form. These workers' larger estimate of the surface covered by the DAP would be consistent with this, as is their finding of a surface area of 4-6-fold greater per cell than the results from Woldringh's laboratory. Of course this discrepancy could be due to strain and cultural conditions, but that is not too probable because we have used for the comparison in both cases data estimated for cells growing with a 30 min doubling time.

BIOCHEMICAL BASIS FOR THE ANALOGY WITH SURFACE TENSION

In the physics of surfaces, surface tension is defined as the work needed to increase the surface area by a unit amount. In the case of a surface of a fluid where there are attractive forces between the molecules, increasing the surface area means that those molecules entering the surface from the bulk phase must lose some of their previous attractive interactions. The work needed to break these interactions is the measure of surface tension. The work needed to increase the surface area of a bacterium arises in a quite different way, although pressure-volume work from cell growth is still transduced to force the wall enlargement process. The surface stress theory

holds that the wall area of the typical prokaryote can only be enlarged if stress-bearing covalent bonds are cleaved. However, mechanisms have been evolved that require that the splitting of such bonds be contingent on the prior insertion of new murein units linked in such a manner that the splitting does not weaken the wall. These new units, as originally integrated, would either have more highly coiled peptide bridges, or not be in the plane of stress, or both. When cleavage transfers stress to new units, they are pulled into the plane of stress, and in certain cases pulled into an extended conformation. Thus work is done to distort the peptidoglycan into a less probable configuration. Consequently pressure-volume work is converted into the work of extending the wall.

The only source of free energy for the enlargement other than the pressure-volume work comes from the transpeptidation reaction. Because the transpeptidation and the splitting of stress-bearing walls are mechanistically linked, the free energy of this reaction is also coupled to wall extension. The transpeptidation involved in murein synthesis trades an exo-peptide bond for an endo-peptide bond. Usually the latter bonds are a little more stable so the transpeptidation reactions proceed with a slightly negative free energy, but basically the reaction must be considered reversible and nearly energy free (see Borsook, 1953; Fruton, 1982). When the peptide bonds in the newly linked murein are pulled into an extended configuration by the cleavage of a stress-bearing bond, all the bonds which have now come to bear stress become thermodynamically less stable (Koch, 1983). However, the peptide bridges may be kinetically more stable at least with respect to cleavage by the transpeptidation enzyme that formed the bridge, since after the stress is applied the bond angles in the extended conformation of the peptide cross bridge are quite different. Depending on the properties of the hydrolytic autolysins, other bonds in the peptidoglycan could become more or less kinetically stable. Thus, on the reasonable assumption that the transpeptidation enzyme is tailored to the most probable conformation of the unstressed substrate, re-addition of D-alanine or a secondary transpeptidation event to other structural parts of the wall would be curtailed for parts of the sacculus under stress. This circumstance allows the free energy of transpeptidation and the pressure-volume work to be transferred to the work of enlarging the surface; that is, into the work of forcing the bonds of the murein material into an extended conformation. In the models presented previously (Koch *et al.*, 1981*a, b*; 1982*b*), it was tacitly assumed that the whole energetic component was pressure-volume work and that the *chemical* work of transpeptidation was essentially zero. However, these thermodynamic considerations show that this is a first approximation, albeit a good one.

A second problem associated with the wall growth of any organism is how the wall thickness is controlled. The surface stress theory proposes that Gram-negative bacteria solve this problem as follows. The new wall units become linked through glycoside and peptide bridges to the existing, stressed network from the cytoplasmic membrane side. This is followed by cleavages which transfer stress and pull the new units into the plane of the sacculus. The consequence of this sequence is that the stress-bearing parts of the wall can never become more than a single molecular layer thick. Obviously, quite different mechanisms of thickness control must operate for the poles of Gram-positive bacteria and different ones for the side walls of Gram-positive rods (see Koch, 1983).

The surface stress theory as applied to Gram-positive rods and cocci made two basic suppositions. One was that the cellular hydrostatic pressure would be maintained constant. This, as indicated above, is because the cleavage-enzyme action is sensitive to the tension on its substrate (with the proviso that new units surrounding the bond to be cleaved were covalently pre-attached). The other assumption was that *T* remains constant because the chemistry of wall enlargement is assumed to be the same during all phases of the cell cycle. For Gram-negative bacteria it appears appropriate to retain the former (in slightly modified form), but reject outright the second assumption of the constancy of *T*. This change implies that the biochemical activity must vary qualitatively and quantitatively with time and cellular location.

For the energetics of a biochemical process that may potentially serve for wall enlargement there are two contributing terms, each composed of two factors: a chemical term obtained by multiplying the molar free energy of transpeptidation by the number of transpeptidation events

Fig. 1. One model for bacterial control of T . The thin stress-bearing murein overlies the cytoplasmic membrane. (a) A new oligopeptidoglycan unit with pentapeptide groups already attached is secreted and linked by transpeptidation to several glycan chains already in the fabric. Subsequent specific cleavages pull it into the stress-bearing plane and convert it into an extended conformation. (b) The same process with a group of pre-linked oligopeptidoglycan chains in compact configuration results in a larger increase in surface area. Consequently, less Pdv work is needed to increase the surface area, so T is effectively smaller. The where and when of changes in T lead to formation of wall with different shapes. In particular, this mode leads to invagination and cell division even when there is a positive pressure differential of the inside relative to the outside of the cell.

needed to make a unit area, and a statistical mechanical term obtained by multiplying the free energy needed for extending the normal conformation of unstressed bonds by their number in a unit area. The sum of these two terms must be divided by Avogadro's number. [There is an additional term when crosswall is split and converted to peripheral wall, but this is not applicable to the Gram-negative bacteria.] Consequently, T might be altered by changing: (1) the free energy of transpeptidation; (2) the number of transpeptidation events per unit area of created stressed wall; (3) the average extensibility of the peptide bridges; or (4) the number of bonds that will be extended by cleavage of stress-bearing bonds in creating a new unit of area.

Viewing the same problem from the point of view of the chemical events, the free energy of transpeptidation might be altered by the additional coupling of the hydrolysis of the second D-alanine from the acceptor peptide in the transpeptidation process. This would require an enzyme of the sophistication of a synthase that can drive a synthetic reaction by coupling the hydrolysis of ATP at a different site on the enzyme. Alternatively, the environment might be locally altered due to specially localized ion-currents, similar to processes well-known in higher organisms (see Harold, 1983). Thus, if the proton pump is more active in one region of the cell than in another, the pH and zeta potential will be altered in that locality and this could vary the local free energy of the transpeptidation reaction.

One of the most attractive proposals for the energetics of Gram-negative rod growth and division is that on the sides and at old poles single glycan chains are inserted as depicted in Fig. 1(a) of this paper or Fig. 9 of Koch *et al.* (1981 *a*), but that rows of chains formed by first linking a group of new oligomeric glycan chains are inserted into the stress-bearing wall in the same way that single oligomers are inserted elsewhere, e.g. the newly introduced material bridging adjacent regions of the stress-bearing wall (see Fig. 1 *b*). When single glycan chains are inserted, the bonds in two peptide bridges must be forced into an extended conformation, on average, for each one that is cleaved. However, if groups of glycan chains are first cross-bonded in peptide linkage and then this prefabricated unit inserted into the stress-bearing wall, more stress-bearing bonds and more new surface area are produced by cleavage of each older bond. This process would only lower the thermodynamic cost to the cell if the rows of chains formed a compact structure that then becomes greatly extended when stressed.

This model is consistent with the recent results of Goodell & Schwarz (1983) and Burman *et al.* (1983 *a, b*). They found that new murein units become bridged with old mureins. In our model some of the murein units at division sites would be initially bridged to other new murein units, although later, still younger murein would become inserted.

The experimental evidence at this time does not bear on the length of the nascent glycan chain. For clarity, we have depicted the insertion of fully formed glycan chains. It is possible that disaccharide units are added to extant glycan chains (Goodell *et al.*, 1983) and that subsequently, glycan chains are broken to increase the number of additional sites. Alternatively, new glycan chains may be started that become linked to the sacculus before they are completed.

If the chemical nature of the peptidoglycan is such that the peptide-chain conformation is so stable in a random-coil configuration that no extension is possible, then little pressure-volume work is converted into the work of forcing the molecule into a less probable conformation. However, if the normal conformation of the peptide chain is compact and the stress is sufficient to extend the cross bridge, then pressure-volume work can force extension to take place. As has been noted earlier (Koch, 1983), the extensibility of the murein of *Bacillus subtilis* and *E. coli* seems to be larger (about a twofold increase in area of stressed relative to unstressed), while it is about a tenth of this for *Streptococcus faecium*. These findings correlate with the difference between the charge on the peptide bridges at neutral pH due to different chemical structure (see Rogers *et al.*, 1980), the net negative charge being -4 for the former group and zero for *S. faecium*.

The charge on the wall may be altered after it matures. For example, new walls with the charges of the α -carboxyl group of glutamic acid residues neutralized by amide formation may be secreted and bound to old wall. Subsequently, the amino groups may be cleaved off, increasing the net negative charge. This would serve as a way to delay expansion until after the cleavage of the peptidoglycan. Such changes in wall charge would affect cell shape and should be considered for their possible role in morphological shape changes in bacterial development.

VARIABLE T IN CONTEXT OF A NARROW ZONE MODEL

Earlier (Koch *et al.*, 1981*a*; 1982*a*; Koch, 1982*a*), an equation for the slope of new wall produced by narrow zonal growth was developed. It can be easily derived on the assumption that $Pdv = TdA$; i.e. there is conservation of energy in the wall-enlargement process. In this expression, v is the volume and A is the surface area. The resultant equation for the slope of the growing region of the pole is:

$$S = \pm [(Pr/2T)^2 - 1]^{\frac{1}{2}} \quad (1)$$

where r is the radius of the cylindrically symmetric cell and where the slope is defined by $S = dr/dz$ (z is the axial distance).

When Pr/T is exactly 2, narrow zonal growth leads to cylindrical elongation. If, however, P or T changes, then S will no longer be zero. If P/T increases, growth can continue; and if the negative branch of the square root is the physically permissible solution, this would lead to constriction. [The other sign leads to expansion which, if not controlled, leads to catastrophic bulging and eventual rupture; this may be the consequence of the action of certain penicillins.] Here we assume that P remains constant and growth continues all over the cell surface as before, but that in a localized annular region, T decreases. The shape of the resultant structure as growth continued would depend upon how fast and to what degree T becomes smaller. Consequently, a variety of shapes of intermediates forms and the final poles could be produced. The results of T suddenly becoming $1/5$ the value that previously supported cylindrical extension are shown in Fig. 2. As growth continues, a V-shaped constriction develops in the cylindrical cell which does not continue far enough to lead to cell separation. Rather, the constriction only proceeds until the radius is $1/5$ the previous value, and then a tube of this new diameter elongates indefinitely. The change in slope would be less abrupt if T did not change discontinuously, but cell division could only occur if T became zero. This can be seen from equation (1): if r is to become zero, T must become zero also. Otherwise the slope would become imaginary.

Fig. 3 shows abortive attempts to devise ways for T to approach zero to yield shapes similar to the poles of typical prokaryotes. This figure and Figs 4, 5, 6, 10 and 11 show profiles of hypothetical cells that might be seen in median sections; if rotated around the x -axis they would generate the shape of the stress-bearing wall. Fig. 3 shows possible poles if T changes at the first arrow to $1/3$ the value supporting cylindrical extension, and at the second arrow changes in such a way that T is proportional to the n^{th} power of the radius. Note that the curves for $n \geq 1$ lead to closure; and that for $n = 1$, the shape produced is that of a conical pole. This figure makes the point that T must become zero almost discontinuously for a narrow zonal model. The requirement that T becomes zero when r becomes zero implies that the negative free energy for wall

Fig. 2. Progressive shape-change for narrow zonal growth when T is decreased fivefold. The shape of the central portion of a rod-shaped cell after a discontinuous change in T is shown from left to right. Progressive changes leading to a structure not unrelated to the shapes of *Hyphomicrobium* are shown. Cell division does not result from this growth mode unless T is decreased to zero.

Fig. 3. Pole formation by narrow zonal growth. In order for a narrow zonal mechanism to lead to division, T must become zero. The curves shown are profiles of portions of hypothetical bacteria. They represent various ways in which T may approach zero. See text for description.

formation would need to be paid entirely by the chemistry of wall formation and not at all by the pressure-volume work. This could be an unstable situation as indicated above. So we reject variable T narrow-zone models for *de novo* pole formation (with the possible exception of the case in which there is a completely different mechanism functioning for the final event of cell division that completes the pole and cleaves the final murein bonds that hold the two sister cells together).

DIFFUSE WALL GROWTH

Although the mathematics of this case is much more difficult than that for the wall growth of a narrowly restricted region, the basic tenets of the surface stress theory are applicable to diffuse growth as well as zonal growth. Wall expansion in this case too is an essentially constant-pressure process in which the Pdv work becomes translated into TdA work. Because of the equality of Pdv and TdA , the same formalism applies to the growing wall as to the surface film of an ordinary soap bubble. We previously derived an equation for numerically computing cylindrical bubble shapes (Koch *et al.*, 1982*a*). Now we need a version that can take into account

the possibility that T changes. On going over the derivation given previously, it could be shown that taking T as a variable leads to no change in the mathematics. Consequently, if T is a function of radius (or time) or anything but a function of distance in the direction of the cylindrical axis, then T can be taken as a variable. Therefore, equations (3) through (9) of Koch *et al.* (1982a) can still be used. Subsequently (Koch, 1982b), two equations were combined so as to eliminate a constant of integration, but we have since found a better way to formulate the equation for numerical analysis and now have it programmed for an advanced programmable pocket calculator (Hewlett Packard 41 CV). This version, like the earlier ones, takes a great deal of time to execute. This program calculates a new slope, S , from the old slope, S_0 , at an adjacent point where the radius is r_0 according to:

$$1 + S^2 = [1 + S_0^2] \left[\frac{1 + (\Delta S_0/r_0)}{T_0/T + P\Delta S (1 + S_0^2)^{1/2}/T} \right]^2 \quad (2)$$

where Δ is the incremental interval in the axial distance z .

Equation (2) can be rearranged to give:

$$S = \sqrt{S_0^2 + [1 + S_0^2] \left[\left(\frac{1 + (\Delta S_0/r_0)}{1 + P\Delta S_0(1 + S_0^2)^{1/2}/T_0} \right)^2 - 1 + \left(\frac{T - T_0}{T} \right)^2 \right]} \quad (3)$$

The program adjusts Δ so that $\Delta S_0/r_0$ is kept within specified bounds. This allows the computer to proceed rapidly when the slope is shallow, and slowly when it is great. When the slope is sufficiently shallow, the computer's ability to take square roots becomes limiting to its accuracy and the program branches to a form derived by expanding the powers and roots by the binomial theorem. Expansion of equation (3) yields:

$$S = \left[S_0^2 + (1 + S_0^2) (1/r_0 - P(1 + S_0^2)^{1/2}/T) 2\Delta S_0 + \left(\frac{T - T_0}{T} \right)^2 \right]^{1/2} \quad (4)$$

and then further expansion gives:

$$S = S_0 + (1 + S_0^2)[1/r_0 - P(1 + S_0^2)^{1/2}/T_0]\Delta \quad (5)$$

For many of the purposes of this paper, T can be taken as a constant, in which case the term T_0/T in equation (2) can be replaced by 1 and $(T - T_0)/T$ can be dropped from equations (3) and (4).

A necessary prerequisite to cell division for all prokaryotes, except fusiforms, is that the slope becomes vertical at the instant of division; for S to become minus infinity, the fraction on the right-hand side of equation (2) must become and remain greater than one. This implies, dropping the zero subscripts on S and r ,

$$1 + S\Delta/r > T_0/T + P\Delta S(1 + S^2)^{1/2}/T \quad (6)$$

or

$$1/r > P(1 + S^2)^{1/2}/T - (T - T_0)/(T\Delta S) \quad (7)$$

This can be rearranged to give:

$$1 > [(1 + S^2)^{1/2} - (T - T_0)/(P\Delta S)]Pr/T \quad (8)$$

or if T is constant, to:

$$1 > (1 + S^2)^{1/2}Pr/T \quad (9)$$

The implication of the last relationship is that S can go to infinity as r gets smaller, even though T remains greater than zero. This is an important conclusion: if growth is diffuse, cell division can result from a decrease in T to a smaller positive value; but if growth only occurs in a narrow zone, T must become zero.

The mathematics for calculating the shapes of cylindrical bubbles with various physical

Fig. 4. 'Hunt and shoot' cylindrical profiles. Suppose we wish to calculate the possible profiles of a soap bubble - or the side of a bacterial cell - from radius of a on one edge to a radius b at a distance l (in this case set arbitrarily where $z = 200$ and b 10% greater than a). By choosing the indicated angles, various trajectories can be calculated from equation (3) (see text). It can be seen that none of the choices of angles works exactly, but by interpolation, an initial angle between 0° and 10° or a second equally valid solution between -10° and -45° would be acceptable solutions. Also shown (broken lines) are the desired solutions found by an iterative procedure connecting the dots indicating the desired boundary conditions. The unit of axial distance is percentage of the initial radius on the left-hand side.

shapes does not lead to analytical solutions, nor does it lead to a direct way to calculate the shape connecting given boundary points. Given P , T , and a and b , the radius of the cylindrical structure at the two ends, and the axial distance, l , between them, we must try various values for initial slopes until a value is found that leads to a curve that connects the endpoints. For example, we wished to calculate the curve going from one dot to the other in Fig. 4. The curves for several choices of slope are shown. In the example of Fig. 4, an initial slope somewhere between 0° and 10° or one between -10° and -45° would actually connect with the right-hand dot. Thus sometimes several initial choices of slope give an acceptable solution, sometimes there is a unique solution, and sometimes there is none. Iterative computer methods are needed to find the curves connecting two specified points. Fig. 4 also shows the two curves satisfying the boundary conditions found by an efficient search procedure based on a modified regulo falsi method (Dowell & Jarratt, 1972). [The programs will be sent to those desiring them.]

Consider what happens when the initial conditions are such that $Pa/T = 1$ and that $a = b$. Now an initial choice of the slope of zero leads to a horizontal straight line connecting a and b and predicts a right cylinder shape. Thus, for every value of l , the condition that a equals b leads to a zero slope and to a cylindrical shape. If any other small initial slope, either positive or negative, is chosen corresponding to chance fluctuation during growth of a living cell, curves are generated that return to the original radius in a distance of nearly $2\pi a$ (see Thompson, 1942). This means that no solution but a right cylinder is acceptable if the length is less than $2\pi a$ (i.e. cylindrical growth is the only possible mode of diffuse insertion on the growing wall and slight fluctuations during growth will be compensated during further growth). This mathematical fact is the source of the stability for cylindrical elongation that diffuse growth engenders. On the other hand, narrow zonal growth is inherently unstable (see Koch, 1983).

If the length is near or greater than $2\pi a$, non-zero initial slopes also give valid solutions. Physically, this corresponds to the fact that a cylindrical soap bubble is stable until it is elongated to this length; then it becomes metastable and quivers and finally separates into a larger and a smaller bubble. The claim of the surface stress theory is that this mathematical fact is the basis of cell division in Gram-negative organisms. But since the lengths of newborn bacteria are not always equal to their circumference, we must assume that the cell locally changes T and hence Pa/T .

If the value of Pa/T is discontinuously changed from 1 to 3 (for Fig. 5a) or to 10 (for Fig. 5b),

Fig. 5. Diffuse growth giving rise to cell division. The hypothetical profiles of poles were calculated assuming that to the left of the vertical axis diffuse growth was and is occurring such that cylindrical extension takes place. The profiles indicate the pole shapes that develop if, to the right of the origin, the value of T is threefold (a) or tenfold (b) smaller. Curves are shown for various initial angles. Only if the angle is exactly zero would division not take place, but any minute disturbance, whether inward or outward, would lead to cell division. The unit of axial distance is percentage of the initial radius on the left-hand side.

then the curves are quite different. The only stable mathematical solutions between two rigid rings of equal radius are obtained when they are separated by a small distance. [Of course, if the slope is initially zero, the metastable solution leading to cylindrical growth is still valid.] Otherwise, no initial slope gives a curve that hits the other end. Slopes of large outward initial slopes lead to structures that correspond to blowouts, i.e. no mathematical equilibrium solution is possible. Fig. 5 also shows that either negative slopes or small positive slopes lead to constrictions that progressively lead to division. Thus, changes in T can lead to cell division but at the risk of possible bulges and then blowouts, such as are observed with agents that partially block murein synthesis.

Formation of poles by discontinuous changes in T

In the earlier section on zonal growth, we concluded that while a decrease in T would lead to constriction, it would not lead to division unless T actually become zero (and very rapidly if a pointed pole was not to result). In contrast, in the last section it is shown that when growth is diffuse, a quite small decrease in T of only threefold and tenfold leads to constrictions that lead to divisions. Fig. 6 shows the profiles developed if T is decreased by factors ranging from 1.33-fold to tenfold below the value of T that gives cylindrical elongation. It can be seen that a change in T of at least a twofold decrease is enough to cause division.

As noted above, the shape of the curves depends on the initial slope of the surface at the region of the discontinuity in T . If S_0 is zero, then the system is metastable and cylindrical extension will continue, but a slight perturbation is enough to lead to division. The curves shown in Fig. 6 are for the case where the initial inward slope was only 10^{-6} degrees. If it had been 1° , then the curves would be marginally shifted to the left. If the initial angle had been outward by 10^{-6} degrees, then the curves would be shifted infinitesimally to the right, and the diameter would increase infinitesimally before it then decreased. Consequently, it can be concluded from these calculations that the surface stress theory can explain constriction and division by proposing a modest degree of a few-fold change in T ; special mechanisms to start the inward growth need not be postulated since random fluctuations would suffice.

Fig. 6. Effect of a discontinuous decrease in T during diffuse growth. The fold decrease of T is shown on each curve. It was assumed that the initial slope was 10^{-6} degrees inward when the discontinuous change in T occurred. For factors less than twofold a U-shaped constriction develops. The figure shows only half of such constrictions and the resultant cell would not divide but possess undulations. Division would ensue for greater decreases of T than twofold. Only the profile of one of the two identical poles is shown. Note that the poles initially produced have nearly flat ends. The unit of the abscissa is percentage of the initial cell radius.

REQUIREMENTS FOR GRAM-NEGATIVE DIVISION

Prokaryotes exhibit three different modes of division. First, Gram-positive cocci and rods form thick cross-walls that are then split. The criterion for this type is the existence of a T-like structure. Second, Gram-negative rods and cocci develop constrictions that progressively invaginate forming deep clefts that eventually separate the cell into two (Schwarz *et al.*, 1975; Mirelman, 1978; Nanninga *et al.*, 1981). The criterion for this type is that the stress-bearing part (the murein) never forms a T-like structure, but appears to be of constant thickness throughout. The third type is that found in budding bacteria where a bud forms on a stalk, or in budding yeasts where it forms as an outpouching of the wall. After the bud grows sufficiently large, the bud abscisses. The distinction between the first and second type is blurred if a splitting of the crosswall follows very closely the inward growth of the septum, but considering the thinness of the Gram-negative peptidoglycan it is difficult to imagine how a double thick wall could be made and then split. Certainly the electron microscopic evidence (Burdett & Murray, 1974*a, b*) shows an electron-transparent gap in the dividing regions of *E. coli*. Frequently, the outer membrane does not invaginate as quickly, although it must complete coverage of a new pole very shortly after it is formed.

Fig. 6 shows that T must decrease at least twofold for diffuse intercalative growth to lead to division, assuming that fluctuations occur which give rise to slight positive or negative deviation from metastable elongation. If, however, some special mechanism exists that gives the wall a momentary inward growth, then even a smaller change in T will suffice. Fig. 7 shows the range of possibilities if T decreases 1.75-fold. For small angles of initial constriction (the dash-dotted line), the growth process does not lead to a V-shaped constriction but to a U-shaped one with no division resulting. Above a critical angle (30°), the constriction develops a V-shape and ultimately leads to division with a rounded pole tip (i.e. the slope approaches infinity as the radius becomes zero). Fig. 8 shows the consequence of decrease in T up to tenfold, assuming an initial inward angle of 10^{-6} degrees. Below a twofold change in T , a U-shaped constriction develops, but infinitesimally above twofold, a V-shaped constriction leading to division develops. These alternatives of division vs U-shaped constriction may be the explanation for the formation of 'blunt constrictions' with furazlocillin (Olijhoek *et al.*, 1982). This penicillin binds relatively specifically to penicillin binding protein 3, which appears to be involved in cell division (Spratt, 1975).

Division in rod-shaped Gram-negative organisms

What is the magnitude of the shift needed in T for division of *E. coli* to ensue? Examination of many electron photomicrographs shows that the poles, as initially formed, are flatter,

Fig. 7

Fig. 8

Fig. 7. Effect of initial angle on the occurrence of division. For the small factor of decrease in T of only 1.75-fold below the value that leads to cylindrical extension, division only ensues if the initial inward angle is sufficiently great. The curve drawn as a solid line indicates initial angles such that V-shaped constrictions would lead to division. The dash-dotted line extension corresponds to angles producing U-shaped constrictions that would result in an undulating filament. The unit of the abscissa is percentage of the initial cell radius.

Fig. 8. Range of division behaviour as a function of the factor of T reduction. For reductions of T less than twofold, U-shaped constrictions develop whose half length and minimum radius are shown by the solid lines. It was assumed that initially there was an infinitesimal deviation from zero slope. For T decreases greater than twofold, division ensues; the half-length of constriction is shown by the dash-dotted line. This will be the axial height of the pole as initially formed. The unit of the ordinate is percentage of the initial cell radius.

progressively approach a hemispherical shape and then become slightly more pointed (Koch, 1983; I. D. J. Burdett, C. L. Woldringh & A. L. Koch, unpublished). Electron micrographs of a median thin section of a cell nearing separation are shown in Fig. 9(a). A fit to the electron microscope morphology of this dividing cell is shown in Fig. 9(b). Iterative calculation showed that the pole was best fitted if the constriction was initiated by a localized discontinuous decrease in T of 2.1-fold. This fitting of a single picture, although satisfactory, does not indicate the dynamics of the process. There are a number of possibilities which will only be resolved when many more median sections of nearly dividing bacteria are analysed. It could be that T varies at different parts of the constricting region (see below) or that a different value of T applies in the constricting region than its value in the sidewall region. At the start of constriction, T becomes smaller and then returns to that value after the division cycle. In the upper model in Fig. 10, a gradual return to T occurs as the new pole is forming, and in the lower, the return is subsequent to the actual separation.

Old poles and side walls do incorporate murein precursors (Koch *et al.*, 1982*b*; Burman *et al.*, 1983*a*). If the growth process in the pole returned to the same value as that of the side walls, the pole would tend toward a flattened shape since its curvature must become twice that of the cylindrical point (Koch, 1982*b*). Since the growth process slows but does not stop as measured by autoradiography, it seems likely that additional insertion is responsible for the growth of the poles toward a hemispherical shape.

Gradual change in T

The remaining case to be treated is that in which the value of T changes with distance from the cylindrical regions. The pole shape depends a great deal on the way that T changes almost more than the amount it changes. Fig. 11 shows the shape that the pole of a cell would have if a three-fold reduction in T occurred in the central region of a cylindrically diffuse growing cell at different rates. The dash-dotted line shows the pole if the slope initially, for some special reason,

Fig. 9. Pole shape of *E. coli*. (a) Electron micrograph of a longitudinal section of *E. coli* B/r A (ATCC 12407) taken from a culture grown at 37 °C in M9 + glucose medium (doubling time, 45 min). The organisms were fixed with paraformaldehyde/glutaraldehyde/OsO₄. Bar marker, 0.5 μm . (b) The central portion of this dividing cell was enlarged photographically so that measurements could be made of the nascent poles and the data used to plot a curve fitting of the pole shape. Graph paper was aligned on the photograph in such a way that the side walls were parallel to the grid markings and equidistant to a meridian line. Then the vertical r distances were measured. The four values for a given pole height, z , were averaged to form the co-ordinates of each one of the points shown. The smooth curve was calculated with the computer procedure described in the text, fixing the curve so that it went through the point marked with the arrow. Then various values of Pa_0/T were tested and critical shapes giving the best fit through a_0 found by an iterative search. The finally chosen value of Pa_0/T was 2.1 and the slope corresponded to an angle of -5.05° . The slope in the opposite direction was set at $+5.05^\circ$ and the value of Pa_0/T at 1. This fitting is only presented for illustration. The tentative conclusion is that for this deeply constricted cell, Pa_0/T appears to be only slightly greater than 2.

became 45° inward. The upper dotted line shows the failure to constrict and the metastable cylindrical elongation if no deviation from zero slope ever occurs. The remaining dotted line shows the pole shape if the initial slope was -10^{-6} (-5.7×10^{-5} degrees). The solid curves also correspond to this initial inward slope but relate to cases where T only gradually became smaller, although eventually becoming threefold less. The numbers marked on the curve correspond to the linear rate of change of P/T . The exact significance is irrelevant here, but at the 10^{-3} rate, the change would only have been accomplished when z became 1. Obviously the point of the figure is not the detailed shape of the curves, because there is no way to measure T directly, much less its variation over a distance of about 0.1 μm , but several conclusions emerge from consideration of the solid curves. A very gradual decrease in T leads to an elongated pole; a more rapid decrease to a flatter one. In fact, a more rapid rate of change than 10^{-3} gives a blunter pole than a discontinuous change. This latter result will probably be important in future investigations and is, indeed, surprising. It implies that the gradation from one biochemical mechanism to another may, along the surface, have a more profound effect on the pole shape than the ultimate change in T .

Fig. 10. Theoretical shapes for developing pole for the discontinuously changed T growth model. Curves are calculated assuming a fivefold decrease in the value of T below its value in the cylindrical region. Eventually, T returns to the initial value. Two scenarios are shown for the course of increase of T back to its original value.

Fig. 11. Pole shapes produced by a gradual change in T . The axial unit of distance is percentage of the initial radius on the left-hand side. See text for details.

CONCLUSIONS

How do Gram-negative bacteria divide? Here we have presented an energetically possible hypothesis. However, it requires that the organisms be able to alter the details of the polymerization of peptidoglycan and the autolytic processes in specific regions of the cell. Then surface tension-like forces power the constriction. This mechanism can be tested via biophysical and ultrastructural studies, but we hope that others will exploit the obvious implication in regard to possible roles for penicillin binding proteins, etc., to test this variable T hypothesis.

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