Patchiness of murein insertion into the sidewall of *Escherichia coli*

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This paper extends, with computer techniques, the authors’ previous work on the kinetics of pole wall and sidewall synthesis in *Escherichia coli*. These findings extend the conclusion that the nascent poles are made of entirely new material and that no new material is inserted into old poles. This requires re-evaluation of ideas in the literature about wall growth and cell division. Mechanisms of various types have been suggested for the growth of Gram-negative rod-shaped bacteria and these will also require major re-evaluation because of the finding, reported here, that the sidewall is made in several modes: patches of new murein, bands of new material largely going circumferentially around the cell, and areas of the sidewall that are enlarged by an intimate and regular admixture of new with the old muropeptides.

The present analysis is more definitive proof that the poles of the cells are inert and that the pole peptidoglycan does not exhibit turnover. This is support for the surface stress theory of Koch *et al*. (1981). This theory postulated that the poles were rigid structures and it was their inertness that formed the support for wall extension in a cylinder of constant radius (Koch, 1983, 2001), thus avoiding the necessity of postulating contracting proteins or rigid non-extensible constraining hoops or bands going around the cell (see discussion in Koch, 1998).

The work presented here also extends the findings of Schwarz *et al*. (1975), Woldringh *et al*. (1987), Wientjes & Nanninga (1989) and De Pedro *et al*. (1997) in showing that the region destined for pole formation is formed of entirely new material and is not an admixture of old and new murein. In a chase of one and two doubling times, there are regions of new (fully non-labelled) material inserted in the cylindrical sidewalls. These go nearly perpendicularly entirely around the cell and are, probably, the beginning of successive generations of division sites.

Although the present paper does not contain new experiments, it does use computer techniques to analyse photomicrographs obtained with the fluorescence microscope in a novel way and shows that as the sidewall grows, new material is mixed with old murein partially in a quite irregular patchwork or mosaic fashion, partially as bands that mark the next division sites, and partially as an intimate mixture with the old material in regions of sidewall extension. The implication of these findings for various proposed models of sidewall elongation is discussed.

INTRODUCTION

In previous work, two of us devised a way to follow the temporal formation and insertion of murein into the wall of growing bacteria by the incorporation of D-cysteine (D-Cys) (De Pedro *et al*., 1997). Growth in the presence of this abnormal amino acid causes the wall that has been laid down to exchange D-Cys for the terminal D-alanyl residue (D-Ala) of the muropeptides in a reaction catalysed by a periplasmic enzyme. This results in about 5% of the terminal alanine groups being replaced. The D-Cys content of the oligopeptidoglycan chains in different regions (pole, sidewall, division site) of the sacculi permits the pattern of temporal insertions into the cell wall to be deduced.

The D-Cys levels in the wall were followed by biotinylation of the SH groups and detection with fluorescent anti-biotin antibody. The pattern had been visualized with similar conclusions in the electron microscope by using antibody with attached gold beads, and in the fluorescence microscope with fluorescent antibodies. With the fluorescent label, the patchy incorporation in the sidewall was more evident than with the gold beads. The conclusion about the unevenness of incorporation in the sidewall was not emphasized in the 1997 paper. Although evident, it is much clearer when examined by computer analysis, as reported here.

The wall of Gram-negative bacteria is divided into three regions: poles, sidewall, and forming poles. By labelling and then chasing, the 1997 paper showed that extant poles were not appreciably diluted with new material during further growth, that the sidewalls were a mixture of new and old material, and that the wall of the division sites was made almost exclusively of new material.
METHODS

Methods used in the previous paper and for the analysis presented here. A culture of *Escherichia coli* strain MC6RP1 that had been exponentially growing for 3-5 doublings (45 min doubling time) in the presence of d-Cys was chased for one or two mass doubling times; then sacculi were prepared, purified, reduced with NaH\(_4\)B\(_4\) (to regenerate the thiol groups quantitatively) and biotinylated with N-\((6\text{-[biotin-amido]hexyl})\text{-3\text{-'}}(2\text{-pyridyldithio})\text{propionamide}\). The biotinylated sacculi were visualized by treating with rabbit anti-biotin antiserum followed by Cy3-labelled goat anti-rabbit antibody. The chasing was done in the presence of aztreonam; this monolactam antibiotic blocks the action of PBP 3 (Sykes et al., 1986), which is involved in the constriction and cell division process and thus leads to filamentous growth. Portions of a single picture of many cells obtained with the fluorescence microscope after either a one- or two-doubling-time chase, published as Fig. 5B and 5C by De Pedro et al. (1986), which is involved in the constriction and cell division process and thus leads to filamentous growth. Portions of a single picture of many cells obtained with the fluorescence microscope after either a one- or two-doubling-time chase, published as Fig. 5B and 5C by De Pedro et al. (1986), were examined here with the NIH Image program (see below). For the present work, the entire pictures showing hundreds of bacteria in both positive and negative form were scanned to make two electronic files that were then examined with the computer program.

Computer methods. The images were analysed on a Macintosh model G3 using the public domain NIH Image program version 1.62 (written by Wayne Rasband at the US National Institutes of Health and available from the internet by anonymous FTP from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port, Royal Rd, Springfield, VA 22161, USA; part number PB93-504868). This program was used to produce pictures of single cells. These could be viewed both in original form and with the images inverted so that light regions become dark. For presentation, they were cropped, rotated, analysed, and presented in various formats. After the file image is loaded into the NIH Image program a chosen cell is outlined and the image copied to a new field. This field can be rotated and again boxed and transferred to another new field. This detail is mentioned because it is important that all of these operations be carried out without changing the magnification, the contrast, or the density of the image. The images can be transferred to another program, such as Canvas or Photoshop, or printed directly. Within the NIH Image program the object was analysed in a number of ways that served as controls. For some purposes the contrast and density were made temporarily extreme, to determine how little old material is present in a band of new material that corresponds to a cell division site. The image was temporarily magnified to examine its graininess and the sharpness of the boundaries between regions. Other types of presentations, used below, include plots of the density of the image either longitudinally or across the cell. Yet another representation of considerable use is the three-dimensional reconstruction of the density map called a wire frame surface plot. It is necessary to emphasize that in all the representations presented here the contrast and density were maintained at their original values. It should be noted that fluorescent microscopy is not limited by the Heisenberg uncertainty principle in the same way as phase or light microscopy.

Controls from the previous report and from the present computer data analysis. The chases were carried out under conditions that blocked cell division and led to filament formation. The action of aztreonam leading to filament formation is the inhibition of PBP 3 (Sykes et al., 1986). If some divisions had completed during the chase then the sacculi from such cells should have been observed with a d-Cys-labelled old pole and a partially unlabelled new pole. However, out of 657 cells photographed after the one-doubling-time chase, only eight such cells (1-22%) were observed. These had a single, and quite small, white area at the end of one of the poles. Note that because the labelling with d-Cys is by exchange in the cytoplasm and is not dependent on growth, the 3-5 generations of growth in the presence of d-Cys would completely label all the cells including the poles. No cells were observed with white areas on both poles. This means that only half of these eight cells divided before the blockade from aztreonam became effective.

If some d-Cys continued to be incorporated during the chase period or if the incorporation process were to intersperse new oligoglycopeptides with old in the division process, then the regions in the centre of the cell (septal regions) would still have been labelled to some degree. However, the regions where constrictions and division sites would have ordinarily developed had only a very low background level of density, comparable to that in regions at an extended distance away from the cells. Because the image contrast and density can be adjusted by the NIH Image program over a large range, it can be asserted that extremely little d-Cys (<0.5%) could have been incorporated de novo or existed in the murein already present in these polar regions during the chase. At an extreme setting of the density parameter, a few grey specks were visualized in the presumptive septal region, but only close to the sidewall regions. The basis of these pixels, be it optical artifact or mixing of old and new murein, is not clear, but it is evident that this is an extremely minor process.

It must be noted that when a hypothetical sacculus of a rod-shaped cell with a hemispherical pole is collapsed to make a flat image a wrinkle is formed in the pole region. If a hemispherical shell is collapsed with no wrinkling and no shrinking, it would appear as an ellipse with a semi-major axis of \(\pi r^2\) and a semi-minor axis of \(r\), where \(r\) is the radius of the cell. Sometimes, but not always, wrinkles develop such as can be seen in some of the electron micrographs shown in De Pedro et al. (1997). In experiments where there had been no chase of labelled cells, the sacculi of cells were uniformly dark with only a very occasional density increase, evidently due to wrinkles in the polar region.

RESULTS

Images from the one-doubling-time chase

Images were available from the two computer files of scanned photographs from the fluorescence micrographs of more than 600 cells for each chase, and two more from the density-inverted images. The NIH Image files of the positive images that were inverted allowed the clearest visual distinctions to be made, and this mode was used for all the figures included here. Fig. 1 shows the three-dimensional representation of the images of four cells. These cells (i-iv) are progressively longer and thus represent cells that were progressively longer at the time of the chase. Of course, all cells then grew for one doubling time in the chase in the presence of aztreonam, preventing division. The shortest one, Fig. 1(i), would have had the minimal amount of labelled sidewall at the time of chase, and it exhibits a broad region with little or no old murein in the cell middle. This cell would have just become ready to divide by the end of the one-doubling-time chase (if there had been no PBP 3 inhibitor) and the white part must be a combination of new sidewall and new wall that would have been part of a division site. The cell in Fig. 1(iv) on the other hand had a good deal of labelled sidewall murein at the beginning of the chase and probably would have undergone cell division if aztreonam had not been present. The central region consisting of almost entirely new wall is,
no doubt, where the cell would have divided in the absence of inhibitor of PBP 3. Fig. 1 (ii and iii) shows cells of intermediate length that had a good deal of sidewall murein at the beginning of the chase and during the chase also formed new non-central regions with essentially no old murein. These several regions may have been anticipatory of the subsequent division process two doubling times yet to come. The beginnings of the next division sites are evident. It is apparent that the new murein, intimately admixed with the old murein, is present in the sidewall regions. This admixture was both uniform and intimate as shown by the murein in the presumptive sidewall area being a uniform light grey and the height of the surface plot being lower than at the poles.

Another representation of very short cells is shown in Fig. 2, a montage of the eight shortest cells from the field of the one-doubling-time chase. These were selected, cropped, and rotated. It can be seen that most of the white (new material) is centrally located, but not all. These cells show that the
sidewall present at the beginning of the chase is separated both from old poles and from the new wall laid down in the centre of the cells that would have become both new sidewall and new pole wall (in the absence of antibiotic). Because of their short length these probably had divided almost immediately before the chase was started and at the time when the PBP 3 was blocked.

Fig. 3 shows three representations of a cell chased for one doubling time. This cell is longer and was presumably older at the time of the chase than that in Fig. 1(i). Fig. 3 shows a sacculus from a single cell, well separated from its neighbours. This cell was one of the 657 cells on the film negative. It was typical and randomly selected among the several hundred suitable cells in the photograph. These met the following criteria: the cell was not overlapped by another cell, the cell was essentially straight, and the field was not contaminated with observable dirt. Like the cells of Figs 1 and 2 this image was manipulated by computer: it was copied, rotated so that its longitudinal axis was essentially horizontal, and cropped. The cell in Fig. 3 is represented in three ways. The main image is a wire frame surface plot as in Fig. 1. The microscopic image itself is shown on the upper right, where the D-Cys stain in the sacculus is recorded as dark and the murein laid down during the chase as white. On the bottom right a graph of the transect of the photographic density is shown. This graph is the summation of the vertical densities at the given longitudinal position and shows that the sidewall is not of a uniform mixture of old and new wall, but on the other hand, the wall is not as ‘patchy’ as will be evident in the two-doubling time chase, below. As in Figs 1 and 2, it is clear that the new wall regions near the poles are not as perpendicular to the cell axis as is the central one.

The evidence in Figs 1–3 demonstrates that insertion of new material (white murein) occurs at the 1/2, and also at the presumptive 1/4 and 3/4 regions. These latter insertion events occur at the appropriate positions for subsequent divisions. They grew, however, as an extension of the sidewall at the same diameter without any evidence of constriction. The central and subsidiary sites acquired the amount of purely new wall that would have been adequate and sufficient such that if aztreonam had not been present enough new wall would have been present for cell division and new pole wall formation to occur. Even in the presence of this antibiotic, blocking the PBP 3 that is normally

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**Fig. 2.** Sacculi from the shortest cells chased for one doubling time. This figure shows eight of the shortest cells in the one-doubling-time chase. Images from the file were selected and rotated. By comparing the images before and after rotation it was concluded that little additional resolution was lost. These images are those of the fluorescent microphotographs shown in the same format as Fig. 6 for cells from a two-doubling-time chase. Beside the dark polar and white septal region, some regions of intermediate density are shown. These vary in location from cell to cell, but indicate that insertion of new material occurs in clustered regions and isolated regions of older wall.

**Fig. 3.** A single cell chased for one doubling time. This cell is represented in three ways. The large image was generated by the NIH Image program as a wire frame surface plot. The image itself is shown at the upper right and its longitudinal density profile below. In the sidewall region it is clear that mixing of old and new wall has occurred. In the central region, where division would occur in the absence of the PBP 3 inhibitor, the murein is almost entirely new. The poles are dark because the polar wall does not turn over and the murein does not become diluted. There is a possibility that the saccular layer on the poles may have a wrinkle. However, any effect on the images is slight because only the upper surface should have been stained with the fluorescent antibody as the labelling technique was applied to cells adsorbed on a solid glass surface. The evidence (not shown) for this is the uniform labelling of the sacculi from cells that have been labelled but not chased.
involved in constriction and division, the cell continued to
grow and form new peptidoglycan in the normal amount
associated with the cell division process. The elongation
process of the cell, probably due to PBP 2, presumably
forms this wall, although we cannot exclude some other of
the various PBPs.

**Images from the two-doubling-time chase**

With a longer chase, the proportion of old sidewall
compared to new sidewall decreases and the proportion
of the filamentous cell composed of wall that in the absence
of PBP 3 inhibitor would be new poles also increases.
Therefore the exposure time was increased to see the
history of the wall development in this photograph of the
two-doubling-time chase.

The cell filaments in the two-doubling-time chase in the
presence of aztreonam were longer and showed many more
additional growth sites. A single, but typical, cell is shown
in both Figs 4 and 5. In Fig. 4 the irregular nature of the
patches of remnants of old wall is evident. In Fig. 5, the cell
appears to have 15–18 bands or hoops containing new
wall going around the cell. This was a consistent pattern of
many cells, and (as an additional control) was apparent no
matter how the sacculus was oriented before the surface
plot was generated. Both Fig. 4 and Fig. 5 show that the
bands are approximately at right angles to the cell axis.
This pattern was seen in many of the sacculi in the original
photomicrograph and was noted by De Pedro *et al.* (1997).
Such bands are apparent in the samples of cells chased
for two generations that are shown in Fig. 6. In contrast to
the figures derived from the one-doubling-time chase in
Figs 1–3, where the partially complete division sites were
not perpendicular to the cell axis angles, in the longer chase
many of the new bands, apparently completed, appear to be
normal to the cell axis. Typically, in the two-generation-time
chase there are three complete and possibly four generations
of times of division sites in various states of development.

The patchy light or white sites had to be of almost
completely new material and well separated from older wall

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**Fig. 4.** A two-doubling-time chase in several
representations. The image itself is shown at
the top, with its longitudinal density profile
immediately below. At the bottom are a
central cross-sectional image and an equiva-
 lent profile. Another type of view of the
sacculus from the same cell is shown in
Fig. 5. Because of the dilution of the old
murein by new wall growth, the exposure
was increased, and no doubt saturated the
photographic image in the pole regions. This
image shows that the old sidewall murein is
distributed not at random, but in patches
surrounded by new sidewall.

**Fig. 5.** A three-dimensional representation (a wire frame sur-
face plot similar to that shown in Fig. 1) of a cell chased for
two doubling times. This image is of the same sacculus as
shown in Fig. 4. It appears that the new material is inserted in
bands that are at about right angles to the cell axis. Because
of their colour and height, the bands of primarily old wall must
be formed of intimate mixtures of newer and older (presump-
tively sidewall).
that had been formed before the chase. At present there is little understanding of how intercalation at the molecular level takes place either as bands or intimately mixed in presumptive sidewall. For the bands, some admixture of single new murein cannot be common, but cannot be fully excluded in the growth process.

The width throughout the entire length of the cylindrical region of these long sacculi was without indentations in both the one- and two-doubling-time chases, even though there are hooplike regions of new material. Interestingly, in regions where there were large or numerous mixtures of light and dark patches, the saccular diameter was constant.

DISCUSSION

Wall growth and cell division in E. coli

During wall growth in the presence of the aztreonam at least three types of wall growth occur. In the cylindrical part of the cell in those regions not programmed to divide in the current cell cycle, new wall is also inserted mixed intimately with the old. This addition is necessary since the sidewall region is getting progressively longer. This can be seen in Fig. 1 by the coloration and height of the surface plot in frames (ii–iv). It is shown more evidently, if indirectly, in Figs 4–6 for the two-doubling time chase. The sidewall had become diluted with new murein, the exposure was increased and the residual old wall is mixed intimately, in many places, with new wall.

The second growth mode is of new wall scheduled for division sites. This is evident in all the figures. In later divisions, it is first noticeable as lighter regions at the 1/4 and 3/4 positions in the one-doubling-time chase (Fig. 1). These regions appear to be the beginnings of the cell’s next attempts at division. It has been suggested by many workers that new sites start before the developing poles are completed. The new finding is clear evidence that they do. However, initially these bands are not regular and are not formed as hoops perpendicular to the axis of the cell (this is shown more clearly in Fig. 2). They are depicted clearly in Fig. 3. As shown in Fig. 5, in the two-doubling-time chase there are many in addition to the primary division site: there are two secondary, four tertiary and eight quaternary sites. This large number of bands was observed

Fig. 6. Seven sacculi from cells chased for two doubling times. A group of sacculi from the same field as used to select the sacculus chosen for Figs 4 and 5 is shown. That cell is the second from the top of this figure. They are marked with numbers indicating their position on the original computer file. Using NIH Image the photographs of the cells were excised and rotated to become oriented horizontally. There was little loss of resolution in the rotation process. The two processes of forming swathes at sites of cell divisions and of inserting patches of murein throughout the cylindrical part of the cell can be seen. The insertion of new patches appears to be quite variable. Note that the fluorescent micrograph was exposed longer because the old wall in the sidewall area away from the original poles had become diluted by growth and therefore had less density.
most clearly in the surface plot format with any of the sacculi from the two-generation chase. In the image format (Fig. 6) the primary and secondary sites show most clearly.

In part by inserting additional non-central circumferential bands of new wall the cell continues its cylindrical elongation. The bands as originally inserted are not always exactly normal to the cell axis. Comparing many cells from the two sampling times, it may be concluded that the bands gradually come to be more precisely at right angles to the cell axis with time. While the idea that cell division sites are somehow ‘marked’ before constriction actually occurs has been in the literature for a long time, the present data are clear evidence of this process. In the chase periods, the region in the central parts and subsidiary parts of the sacculi appear to be made entirely of new with very little or none of the older D-Cys-containing murein.

There are also patches of mainly old wall that are both regular and irregular in shape and orientation. These are most clear in Figs 2, 4 and 6. This third mode is the irregular insertion of new murein separating regions containing older murein sidewall. This mode is not regular and may be the early form of what will subsequently become organized to a band or hoop. This patchiness was clear in the fluorescent microphotographs of De Pedro et al. (1997), but was not stressed in that paper. In part this was because the patchiness was not as evident in the electron microscopic studies with gold beads used in the same study. Probably the two types of study would have been consistent if many more gold beads could have been attached to the sacculi. The difference is simply due to random statistics of affixing the labelled gold beads.

These patches or bands of new murein are clear in these fluorescent micrographs, and stand in contradiction to earlier ideas that the cell wall was inserted as chains (one strand or two strands: Cooper et al., 1988; Park, 1996; as well as suggestions by several other groups). The new finding is that the older material as well as the new appears to be in aggregates that have fairly discrete edges as if the new patches are imbedded as all-or-none arrays in various sites in the enlarging sidewall.

The filaments produced in the presence of aztreonam are uniform cylinders and of the same diameter as the normal cylindrical part of cells either in the patchy region of sideward or in the region of potential division. It appears that the insertion of murein occurs in sites where division would have occurred but had not because of the presence of an antibiotic that blocks PBP 3. This murein insertion was probably carried out by PBP 2 even though it was murein incorporated at a site where division should have occurred and in which PBP 3 should have been involved. The cylindrical structure is consistent with the biophysical model derived previously (Koch et al., 1983; Koch, 2002).

This analysis of published photographs led to an unexpected finding: the sidewalls do not elongate by uniform random insertion over the entire cylindrical sidewall region by insertion of single penta-muropeptide units. In some other regions of the sidewall the enlargement is an intimate mixture of patches of old and of new material. However, in other parts, it occurs in part by the insertion of groups of many oligopeptidoglycan chains as units called here patches or bands. These patches are hundreds of oligopeptidoglycan chains across and long. The patches observed here are wider and much longer than the mean length of 14 glycan residues per chain observed by Obermann & Hölter (1994) and Ishidate et al. (1998).

Helical wall growth

The new material inserted in the sidewall as patches, bands or hoops is mainly normal to the cell axis. On the other hand, there appears to be no evidence of helical structure and therefore these are not the phenomena found in recent work with Bacillus subtilis and Caulobacter crescentus, described below.

The proposal that wall growth of B. subtilis occurs by helical insertion was made by Mendelson (1976), who studied multicellular structures of filamentous mutants. Later work (Koch, 1989, 1990) showed that a B. subtilis filament does indeed rotate as it grows, but this was because of the way the wall on the outer surface was autolysed. More recently workers in Errington’s laboratory (Jones et al., 2001) presented critical evidence that a closed helical protein structure formed under the wall of this organism. Subcellular localization of the MreB and Mbl proteins revealed that each forms a distinct kind of helical structure lying close to the cytoplasmic membrane surface. If these structures are important in the growth and division of B. subtilis, then a closed helical structure must become converted into two helical structures as part of the division process.

The E. coli pictures presented here were closely inspected to see if the wall formed during the chases could possibly grow to be part of helices, but this does not appear to be so. We must mention the unpublished finding from Gober’s laboratory; see England & Gober (2001). They reported in a poster that helical structures form under the wall of C. crescentus. Obviously, the mode of wall insertion is fundamental to bacterial growth.

Consequence of our observations for previously proposed theories

These results bear on the mode of Gram-negative wall growth. There are three kinds of models currently proposed to explain the phenomenon of cylindrical growth of bacteria; these are summarized in Koch (1998).

New units of muropeptide are inserted as the cell’s cytoplasm grows, and the murein is subsequently stretched in a plastic fashion. It has been shown that if supported by rigid poles at each end of the cell cylindrical extension with no bulging out or in occurs. Because of its plasticity the sidewall is a non-rigid structure until stretched; however the sacculus can maintain a constant diameter under the appropriate physical and metabolic restrictions. The key condition is that the poles are rigid and support the cylinder region as it grows (Koch et al., 1981; Koch, 1983, 2002).

**Non-elastic models.** Although not emphasized in their presentation of their models, both Hülte (1993) and Park (1996) (see Koch, 1988, 2001) tacitly assumed that the linked chains are non-elastic and are connected such that they surround the circumference of the cell. This is required to prevent the wall from bulging and becoming wider each generation. By considering the organic chemistry and possible conformations of the muropeptide it can be concluded that this cannot be so.

Note that as new air is pumped into an unconstrained soap bubble, it would enlarge all over. Similarly, as a bacterium is grown in the presence of a low concentration of an antibiotic of the penicillin type, swelling occurs in the middle and the bacterium bulges outward in the cell centre at the site where a constriction would normally develop in the absence of the antibiotic. For this second class of model to serve, the non-elastic new chains going around the cell would have to be duplicated in exact length and be subject to tension as inserted into the wall.

Thus, in order to prevent the chains from bulging, newly inserted chains would have to be under tension from the time of their formation. (There is an alternative version that could apply if the enlargement of the wall depended on a mechanism that could copy a template essentially counting the number of hexose residues.)

**The mechano-protein model.** There is a third class of models that assume the existence of mechano-proteins that could exert forces, causing extension over the length of the cell or contraction over the width of a cell (Norris et al., 1994). This is in spite of the fact that there is no evidence of force-generating proteins in bacteria (Koch, 1991, 1998) and in addition although clearly FtsZ and FtsA have a homology to tubulin and actin and are involved in cell division they cannot have a role in constraining the diameter of the cell because of their small numbers and their distribution within the bulk of the cell throughout the cell cycle.

**Future models**

For none of these three classes of models is there any direct evidence. All three will need to be modified or discarded to cope with the observations presented here. Previous versions of the surface stress theory assume that new disaccharide peptidoglycan units are inserted at random in the cylindrical wall depending on chance random events. Now it will be necessary to make the new assumption that the cell wall grows by insertion, in part, of single chains into the stress-bearing wall and, in part, by groups of peptidoglycan chains inserted en masse.

The new results present difficulties for all contending models. They place grave difficulties on the ‘three-for-one’ model of Hülte (1993, 1996, 1998), which proposes that a chain in the existing wall is replaced in the same place by a raft of three disaccharide oligopeptide chains linked together in parallel exactly the same length. In a later version of his model, these chains are constructed on the template strand as it is removed and they are of exactly the same length as was the template or docking strand. Such new rafts would increase by a factor of two the surface area of the wall covered previously by a template strand. If replacement template strands were installed at random, a uniform mixture of new and old murein would be produced. If this were the case then because of the distance scale of the fluorescence microscope images in the pictures shown here such an intimate mixture would appear as a grey region as in Fig. 1(i), and not as a mottled or patchy region. Finally, models postulating insertion of stress-bearing constraining bands going around the cell whose lengths are precisely the same as existing stress-bearing hoops will have particularly great difficulty in dealing with the patchwork nature of the sidewall murein reported here.

Now that the old poles have been critically shown to be metabolically inactive (De Pedro et al., 1997; Koch, 2002; the present work) and therefore able to form a rigid structure on which the elongating sidewall and the developing division site can develop, it can be plausibly assumed that it is that the old completed poles that have the role of determining the radius of the cylindrical cell and the new poles. A significant new finding further supporting this idea is that the region of the cell that would have been part of a constricting region (if the antibiotic had not inhibited the PBP 3 function) retains the same diameter as the rod portions.

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