Cell wall assembly in *Staphylococcus aureus*: proposed absence of secondary crosslinking reactions

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The distribution of muropeptides formed by muramidase digestion of peptidoglycan from *Staphylococcus aureus* H was determined by gel-filtration HPLC. The observed crosslinking pattern supports the conclusion that incorporation of peptidoglycan in *S. aureus* proceeds by a similar mechanism to that proposed earlier for *Bacillus megaterium*. In this mechanism single glycan-peptide strands are incorporated into the sacculus by crosslinking reactions that take place only between the monomer muropeptide units of the incoming glycopeptide and muropeptides present in the innermost region of wall at the wall-membrane interface: such crosslinking reactions take place only during incorporation and no other crosslinking reactions occur. This assembly process has now been termed restricted monomer addition. The present analysis shows that the distribution of muropeptides in *S. aureus* peptidoglycan is in excellent agreement with that predicted by this mechanism. We propose that cell wall assembly in *S. aureus* proceeds via restricted monomer addition without any requirement for the secondary crosslinking reactions that have been suggested to occur in this organism. The high degree of crosslinking in *S. aureus*, 80% in this study, may result mainly from the freedom for crosslinking provided by the pentaglycine bridge peptide.

Introduction

Study of the maturation of crosslinking in bacterial peptidoglycans can give information on how bacteria safely increase in volume during growth. Oldmixon *et al.* (1976) recognized that the overall distribution of muropeptides in peptidoglycan can provide information on its mode of assembly. Polymerization reactions can proceed in different ways, for example by a stepwise addition of monomers to the growing oligomer or by a random mechanism in which oligomers can also combine with each other. Flory (1953) showed that different polymerization mechanisms lead to different size distributions of resultant polymer.

In the case of peptidoglycan assembly, two distinct polymerization reactions can be distinguished. The first is the formation of the glycan chains: this is known to proceed by a monomer addition mechanism that involves stepwise addition of new disaccharide-peptide monomer units to the growing end of the chain (Ward & Perkins,

1973). The second is the formation of crosslinked muropeptides by transpeptidation reactions between peptides resident on different glycan strands. It has not been clear whether this polymerization also proceeds by monomer addition. A recent study of crosslinking in *Staphylococcus aureus* (Snowden & Perkins, 1990) analysed the distribution of muropeptides formed upon digestion of cell wall with muramidase. The size distribution shown by gel-filtration HPLC was in close agreement with the distribution expected from a random polymerization mechanism. It was proposed that incorporation of new peptidoglycan into the wall proceeded by a monomer addition process in which peptides in the incoming strand formed linkages with peptides already present in the wall, but that secondary crosslinking reactions then occurred to produce the highly crosslinked peptidoglycan characteristic of these bacteria. Such secondary crosslinking reactions would presumably be effected by transpeptidases distinct from those acting during the initial incorporation.

The kinetic analysis described by Flory (1953) relates to reactions in which all oligomers retain an active terminus throughout the polymerization and all active termini are equally susceptible to reaction with monomer. We have noted (Gally, 1991; Gally *et al.*, 1991) that these conditions are unlikely to be met during peptido-

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glycan assembly in Gram-positive bacteria. The thickness of the wall in these bacteria is such that peptides in the outer regions will not be accessible at the wall-membrane interface where new material is incorporated. Furthermore, even those termini that are present at this interface may not be equally susceptible to reaction with incoming material since they may differ in orientation. Nevertheless, analysis of mucopeptide distributions can give valuable information on the assembly process. Thus the distribution of mucopeptides in *B. megaterium* is not that forecast by either the random or monomer addition mechanisms defined by Flory (1953). However, it is wholly consistent with a monomer addition mechanism in which crosslinking occurs only at the wall-membrane interface during the incorporation of single glycan-peptide strands (Gally *et al.*, 1991). This process can be termed restricted monomer addition to distinguish it from the monomer addition process defined by Flory. The distribution of mucopeptide oligomers in walls of these bacteria can be understood without any requirement for secondary crosslinking reactions. We now show that this is also the case with peptidoglycan in *S. aureus*. The terminology used here to describe peptidoglycan monomer and oligomer units is that defined in our previous paper (Gally *et al.*, 1991).

Analysis of peptidoglycan structure in Gram-positive bacteria is complicated by the presence of covalently attached anionic polymers. In previous studies these negatively charged polymers have usually been removed from muramidase digests by anion-exchange chromatography (Tipper & Strominger, 1968; Oldmixon *et al.*, 1976; Snowden *et al.*, 1989). It has been reported (Snowden & Perkins, 1990) that the size distribution in mucopeptides associated with anionic polymer in *S. aureus* is similar to that in mucopeptides that are not linked to anionic polymer (and thus not retained on anion-exchange resin). Comparison of attached and non-attached mucopeptides is significant since it relates to the way in which anionic polymer is distributed in the cell wall. Thus if anionic polymer were randomly attached to muramic acid residues at different locations along the glycan chain, the larger mucopeptides (containing more muramic acid residues) would have greater probabilities of being attached to anionic polymer so that differing size distributions would be seen in mucopeptide fractions containing and not containing anionic polymer. The similar distribution in such fractions shown in the previous study (Snowden & Perkins, 1990) would therefore indicate a non-random location of anionic polymer. For this reason, and also because the kinetic analysis can legitimately be applied only to the total distribution, the present study has further explored the effects of fractionation procedures on mucopeptide distributions. We find that anion-exchange chromatography

preferentially removes higher mucopeptide oligomers so that the eluted material is biased towards smaller oligomers. Prior removal of anionic polymers by digestion with hydrofluoric acid, used previously in our study of crosslinking in *B. megaterium* (Gally *et al.*, 1991), gives more reliable results.

Methods

Organism and growth conditions. *Staphylococcus aureus* H (NCIB 6571) was grown in double-strength nutrient broth (25 g l⁻¹; Difco) at 37 °C in a rotary shaker.

Isolation of cell walls. Cell walls were prepared as described by Gally *et al.* (1991). A large-scale preparation was carried out from 15 litres of bacterial culture grown in Bacto yeast extract (10 g l⁻¹). The cell paste was harvested in a continuous flow centrifuge and then disrupted by vigorous agitation with glass beads in a Braun disintegrator. The cell wall fraction was isolated by centrifugation at 48 000 g.

Preparation of peptidoglycan. The cell wall fraction was suspended in water and added to an equal volume of boiling sodium dodecyl sulphate (8%, w/v) and boiled for 30 min. Insoluble material was recovered by centrifugation (48 000 g) and then washed five times with hot (90–95 °C) water. Covalently attached protein was removed by treatment with Pronase (type XIV from *Streptomyces griseus*; Sigma), pre-treated at 60 °C to remove any contaminating mureinolytic activity. The cell wall preparation was suspended in 50 mM-Tris/HCl, pH 7.0 (1–3 mg ml⁻¹), the Pronase added to a final concentration of 100 µg ml⁻¹, and the mixture incubated for 1 h at 60 °C.

Removal of anionic polymers. Anionic polymers must be removed prior to fractionation of mucopeptides by HPLC. Anionic polymers were removed from SDS- and Pronase-treated cell walls by two alternative methods. In the first method cell wall samples were digested with *Chalaropsis* muramidase B so producing a mixture of mucopeptides with and without attached anionic polymer. These two sets of mucopeptides were then separated by anion exchange as described by Snowden *et al.* (1989). In the alternative procedure teichoic acid was removed from walls before digestion with muramidase. The linkage between teichoic acid and peptidoglycan is labile to both alkali and acid, though neither treatment was satisfactory for the present purpose since alkali hydrolyses pentaglycine crosslinkages (Archibald *et al.*, 1970) and treatment with TCA or mineral acids gave variable results. Treatment with HF (Gally *et al.*, 1991) removed all of the phosphate from the wall and gave the most reproducible HPLC profile. Treatment with HF did not appear to cause any depolymerization of the mucopeptides even when incubation was extended for periods up to 5 d.

Muramidase digestion. The peptidoglycan fraction was suspended in 30 mM-triethanolamine acetate buffer (pH 4.7) to 1 mg ml⁻¹. *Chalaropsis* muramidase B (EC 3.2.1.17) was added to a final concentration of 10 µg ml⁻¹, and the mixture incubated at 37 °C for 18 h. The enzyme was inactivated by placing the samples in a boiling water bath for 3 min. Any insoluble material was removed by centrifugation (30 000 g, 5 min at room temperature). The supernatant containing the mucopeptides was freeze-dried. Control experiments showed that, as previously (Gally *et al.*, 1991), digestion to disaccharide units was complete under the conditions used.

Fractionation of mucopeptides by HPLC. The mucopeptides were separated by HPLC as described by Snowden *et al.* (1989). Samples (200–700 µg) were dissolved in 20 µl water (HPLC grade, Rathburn) and injected onto a TSK2000 SW gel-filtration column (7.5 mm × 60 cm; Beckman) fitted with a Spherogel TSK SW guard

Table 1. Muropeptide distribution of *S. aureus* H cell walls subjected to anion exchange or HF treatment to remove anionic polymer

Muropeptide	Percentage of peptidoglycan (w/w)				
	HF treatment only*	Anion exchange†			
		Untreated cell walls		HF-treated cell walls‡	
		pH 3	pH 7	pH 3	pH 7
Monomer	5.2	8.7	8.6	7.1	7.6
Dimer	7.4	10.3	10.2	9.4	9.4
Trimer	7.2	11.1	10.4	9.7	9.7
Tetramer	7.1	10.6	9.4	9.3	9.2
Pentamer	6.7	9.6	8.7	8.4	8.3
Hexamer	6.3	8.4	7.7	7.5	7.6
Octomer	5.6	6.1	5.9	6.1	5.9
Oligomer (9+)	48.4	28	32.3	35.8	35.6
Percentage crosslinking	80	73	74	75	75

* The muropeptide distribution was determined as described in Methods. The figures shown are the means of three separate determinations, the standard deviations not exceeding 0.5%.

† Cell wall samples were digested with muramidase before removal of teichoic acid-peptidoglycan complexes by anion exchange. In one set of experiments the uncomplexed muropeptides were eluted with distilled water (pH 7), in another they were eluted with 0.001 M-HCl (pH 3). The resulting muropeptides were separated by HPLC. The figures shown are the means of three separate determinations, the individual standard deviations not exceeding 0.3%.

‡ Cell wall samples giving the distribution shown in the first column, i.e. treated with HF and digested with muramidase, were then passed through the anion-exchange column and eluted as described above. The figures are the means of three separate determinations, the standard deviations not exceeding 0.5%.

column (7.5 mm × 10 cm; Beckman). The muropeptides were separated in a running buffer of 50 mM-sodium phosphate (pH 7.0) with a flow rate of 0.3 ml min⁻¹ and were detected at 214 nm.

Results

Distribution of muropeptides in S. aureus walls

The distribution of muropeptides in muramidase digests of walls of *S. aureus* is shown in Table 1. Gel-filtration HPLC of the muropeptide fraction washed off the anion-exchange column with water, as described by Snowden *et al.* (1989), gave a muropeptide distribution similar to that reported by those authors. This distribution was shown by Snowden and his colleagues to be consistent with that expected of a random polymerization process. The muropeptide distribution in digests of walls from which anionic polymers had been removed by treatment with HF prior to incubation with muramidase is also shown in Table 1. This distribution differs substantially from that obtained by fractionation after muramidase digestion, particularly in that it contains a substantially increased proportion of higher oligomers. It appears from this that the use of anion exchange to remove muropeptides bound to anionic polymer imposes a bias on the distribution of the remaining muropeptides relative to the overall muropeptide distribution in the wall such that the larger muropeptides are under-represented.

These results are consistent with a random attachment of teichoic acid to peptidoglycan but control experiments suggest that the effect of the anion-exchange column is not solely due to the presence of attached anionic polymer. Thus the muropeptide fraction isolated from HF-digested walls was also fractionated on passage through the anion exchanger so that the eluate contained diminished proportions of higher oligomers (Table 1). Retention of the higher oligomers could not in this case be due to their attachment to anionic polymer and is presumably a consequence of the carboxyl groups present. Attempts to reduce this by elution with 0.001 M-HCl instead of water were not successful (Table 1). These results show that analysis of muropeptide distribution is most reliably carried out with samples prepared by treatment with HF rather than chromatographed on the anion-exchange column. From the results so obtained the percentage of crosslinking in the wall can be estimated (Martin & Gmeiner, 1979) at 80%. This figure is slightly higher than reported by Snowden & Perkins (1990) but is consistent with other estimates (Dobson & Archibald, 1978).

A monomer additional model for peptidoglycan assembly

The two equations derived by Flory (1953) for random and monomer addition polymerization in solution are shown in Appendix 1. We here describe a restricted

Fig. 1. Incorporation of new peptidoglycan at the wall-membrane interface. The wall is shown diagrammatically as a layered structure. Incorporation of nascent peptidoglycan is accomplished by transpeptidation reactions in which the terminal D-alanine is removed from the incoming donor peptide while its penultimate D-alanine becomes linked to the free amino group of the bridge peptide in an acceptor peptide. Only those acceptor peptides (a, b and c) in the wall layers immediately adjacent to the cytoplasmic membrane are accessible to incoming material. An incoming monomer mureopeptide unit on a new glycopeptide strand can be incorporated without forming a crosslinkage and may be held in the wall by crosslinkages involving peptides at other locations along the glycopeptide chain. Alternatively the new monomer mureopeptide may form a crosslink to a mureopeptide present at the inner wall surface. Three possibilities are shown in illustration. Linkage to mureopeptide a would leave mureopeptides b and c free to link to further new material whereas linkage to mureopeptide c would remove mureopeptide b from the wall-membrane interface so that it would be unable to take part in subsequent crosslinking events. In this case mureopeptide b would remain as a monomer and mureopeptide c would become a trimer having a unit still present at the inner surface. It should be noted that the wall may not have a regularly layered structure, nor need the disposition of crosslinkages within and between layers be as shown in this diagrammatic representation.

monomer addition mechanism and derive an equation that models this assembly process, dependent on the degree of crosslinking. In the proposed mechanism single glycopeptide strands are incorporated into the sacculus by crosslinking reactions that take place only between the monomer mureopeptide units of the incoming glyco-

peptide and mureopeptides present in the innermost wall layer at the wall-membrane interface. Incoming glycopeptide strands can be considered to be strings of linked monomer mureopeptide units and these may link to accessible acceptor peptides in the wall. This is illustrated in Fig. 1, where the wall is depicted as a layered structure in which new material underlies existing wall, so removing it from the wall-membrane interface and from the possibility of direct participation in crosslink formation. Crosslinks are formed by membrane-associated transpeptidases that attach the sub-terminal D-alanyl residue of the donor pentapeptide moiety of the incoming mureopeptide unit to the amino terminus of the acceptor pentaglycine side chain of a mureopeptide already linked into the wall and present at the wall-membrane interface. Such crosslinks may attach the incoming mureopeptide to an adjacent glycopeptide chain within the same layer (e.g. attachment at 'c' in Fig. 1) or in an immediately overlying layer (e.g. attachment at 'b' or 'a' in Fig. 1). Wall mureopeptides that form crosslinkages with incoming units will grow in size by one unit but those that do not form such crosslinkages before being underlaid by new material will be terminated since they will become inaccessible to incoming mureopeptides. Thus in Fig. 1, linkage of incoming monomer to bridge peptide 'c' will have the effect of removing bridge peptide 'b' from the interface so that its associated mureopeptide will remain as a monomer throughout subsequent wall growth.

The formation of crosslinkages will be subject to a number of steric constraints determined by the angle at which the donor peptide emerges from the glycan chain, the length and flexibility of its stem and bridge peptides and by the accessibility of acceptor groups in the wall. The latter will be affected by the structure, disposition and flexibility of the acceptor peptide and by the nature of any crosslinkage in which it is already engaged. The specificity and activity of the transpeptidases will also affect crosslinking and all of these factors will together lead to the probability that any particular crosslink will be formed. The central proposal of the restricted monomer addition mechanism is that distribution of mureopeptides in the mature wall is determined solely by the various probabilities that monomer, dimer and oligomer peptides located in the innermost wall layer will form crosslinkages with incoming mureopeptides incorporated at the wall-membrane interface.

The content of any particular mureopeptide oligomer in the mature peptidoglycan can be calculated from the probabilities of such crosslink formation. The equations described here are derived in terms of the probability that a mureopeptide is either elongated or terminated when incoming material is incorporated. This is illustrated in Fig. 2. If the probability that a monomer

Fig. 2. Restricted monomer addition: a diagrammatic representation of the increase in crosslinking associated with peptidoglycan maturation. The diagram illustrates monomer, dimer and trimer muropeptide formation as nascent monomers are incorporated at the membrane-cell wall interface. Peptidoglycan assembly proceeds through a repetitive pattern of incorporation of layers of new material. In the Figure, the probability that a muropeptide monomer at the inner surface of the wall will accept an incoming monomer to form a dimer is shown as 3/6. The probability that a muropeptide dimer at the inner surface of the wall will accept an incoming monomer to form a trimer is shown as 1/3. The probability that a muropeptide trimer, accessible at the inner surface of the wall, will accept an incoming monomer is 0. When a muropeptide is displaced from the membrane-wall interface (by underlying new material) it can no longer crosslink a nascent monomer and so has matured. This distinguishes restricted monomer addition from the monomer addition process described by Flory (1953).

muropeptide present at the interface (i.e. at insertion interval 1) will become crosslinked to an incoming muropeptide is P_1 , the probability that it will not become crosslinked is $1 - P_1$. If it does not become crosslinked as it is underlaid by new material it will remain as monomer in the mature wall since it will have become inaccessible to further incoming material. Thus the probability that a monomer will remain as such in the mature peptidoglycan is:

$$P(\text{monomer in mature peptidoglycan}) = 1 - P_1$$

where P_1 is the probability that a monomer present at the wall-membrane interface will form a crosslinkage with an incoming muropeptide to form a dimer.

Similarly the probability that a monomer at the interface will become a dimer as the next layer is

incorporated and will then remain as a dimer when the subsequent layer is incorporated is:

$$P(\text{dimer in mature peptidoglycan}) = P_1 \times (1 - P_2)$$

where P_2 is the probability that a dimer accepts an incoming monomer to form a trimer.

Similarly the probability that a trimer is formed and then remains as such in the mature peptidoglycan is:

$$P(\text{trimer in mature peptidoglycan}) = P_1 \times P_2 \times (1 - P_3)$$

where P_3 is the probability that a trimer accepts an incoming monomer to form a tetramer. The equations can be extended to calculate the proportions of higher oligomers present.

Figure 2 illustrates an example of the consequences of a restricted monomer addition process in which: (a) the probability (P_1) that a monomer unit (an uncrosslinked disaccharide peptide) located at the inner surface of the wall will crosslink to incoming monomer unit is 0.5 (i.e. 3/6, as shown); (b) the probability (P_2) that a dimer located at the inner surface of the wall will accept an incoming monomer to form a trimer is 0.333 (i.e. 1/3, as shown); (c) the probability (P_3) that a trimer located at the inner surface of the wall will accept an incoming monomer unit is 0, as shown – there being no tetramers.

Application of these probability values in the above equations gives forecast (molar) proportions of monomer, dimer and trimer of 0.5:0.33:0.167, i.e. 3:2:1, which is the distribution in the mature wall shown in Fig. 2. As illustrated in Fig. 2 this result is achieved by a repetitive pattern of incorporation. Of every 10 units incorporated, on average 6 are incorporated initially as monomers, 3 link to monomers present in the overlying layer and 1 links to a dimer present in the overlying layer. Inspection of models constructed with other probability values illustrates the validity of such forecasts. For example the values $P_1 = 0.67$, $P_2 = P_3 = 0.5$, and $P_4 = 0$ describe an incorporation process in which of every 13 new units incorporated, on average 6 are present initially as monomers, 4 link to monomers in the overlying layer, 2 link to dimers in the overlying layer and 1 links to trimer in the overlying layer. Mature wall so formed will contain monomer, dimer, trimer and tetramer muropeptides in the (molar) proportions 2:2:1:1.

In the above equations the probabilities of extending monomer, dimer and trimer muropeptides are different. In order to model peptidoglycan assembly in *B. megaterium* KM it was necessary (Gally *et al.*, 1991 and unpublished) to consider only two different probabilities, one (P_1) being the probability that a muropeptide monomer in the innermost wall layer would crosslink to an incoming monomer and the other the probability (P_2) that a dimer, trimer or higher oligomer would crosslink

Table 2. A comparison of calculated theoretical muropeptide distributions with the experimentally-obtained muropeptide distribution from *S. aureus* H

Muropeptide	Percentage of peptidoglycan (w/w)		
	HF treated experimental distribution*	Restricted monomer addition†	Flory monomer addition‡
Monomer	5.2	4.0	0.4
Dimer	7.4	6.4	2.9
Trimer	7.2	7.7	8.8
Tetramer	7.1	8.2	15.6
Pentamer	6.7	8.2	19.5
Hexamer	6.3	7.8	18.7
Heptamer	6.1	7.3	14.6
Octomer	5.6	6.7	9.5
Oligomer (9+)	48.4	43.6	10.0

* The experimentally obtained muropeptide distribution, using HF to remove covalently attached anionic polymers.

† The predicted distribution of muropeptides using the equation derived for restricted monomer addition. The distribution is based on a crosslinking probability of 0.8. The distribution is identical to that predicted for random addition (equation 1, Appendix 1).

‡ The distribution was calculated using equation 2, Appendix 1, with a crosslinking probability of 0.8.

to an incoming monomer. The difference in probabilities may reflect a greater accessibility of monomer muropeptide to incoming peptidoglycan (Gally *et al.*, 1991). In the case where only these two probabilities apply the probability of any particular oligomer (n -mer) is:

$$P(n\text{-mer in mature peptidoglycan}) = P_1 \times (P_2)^{n-2} \times (1 - P_2)$$

It is proposed here that for *S. aureus* the probability equation can be simplified even further. The steric constraints applicable to the accessibility of muropeptide oligomers in A1 γ peptidoglycans may not apply to the A3 α peptidoglycan present in *S. aureus* since the pentaglycine bridges in the latter will provide a greater degree of flexibility and increased freedom for crosslinking reactions. This extra flexibility may overcome the diminished accessibility (Gally *et al.*, 1991) of muropeptide oligomers in A1 γ peptidoglycans so that peptidoglycan assembly in *S. aureus* may be modelled with only one crosslinking probability (P). In this case the probability of a particular oligomer in the mature wall is:

$$P(n\text{-mer}) = P^{(n-1)} \times (1 - P)$$

It can be seen that in this special case of the restricted monomer addition model, the final oligomer distribution is the same as that expected from the random polymerization mechanism analysed by Flory (1953). The equations derived by Flory (Appendix 1) predict the proportions of given oligomer on a weight basis in the

wall. Conversion of the above equation to the same basis gives:

$$P(n\text{-mer}) = nP^{(n-1)}(1 - P)^2$$

which is identical to the relation derived by Flory.

In the special case where the crosslinking probability is the same for all muropeptide oligomer sizes, the muropeptide distribution resulting from restricted monomer addition is thus indistinguishable from that forecast for random polymerization (Flory, 1953). The muropeptide distribution obtained from HF-treated cell walls (Table 2) agrees closely with that calculated (using a crosslinking probability of 0.8) from the restricted monomer addition mechanism. We conclude that it is unnecessary to postulate secondary crosslinking reactions during wall assembly in *S. aureus*.

Discussion

Analysis of the peptidoglycan structure in Gram-positive bacteria is complicated by the presence of covalently attached anionic polymers. The present study has shown that analysis of muropeptide size distribution following anion-exchange chromatography is unsatisfactory, as it preferentially removes the larger muropeptide oligomers. More reliable results are obtained by using hydrofluoric acid (HF) to remove anionic polymer from intact wall before analysis of muropeptide distribution. This application of HF has previously been used in a study of crosslinking in *B. megaterium* (Gally *et al.*, 1991) and in the reverse-phase HPLC analysis of *S. aureus* peptidoglycan (Oshida & Tomasz, 1992; De Jonge *et al.*, 1992). The crosslinking values determined by this method are higher than those obtained by anion exchange but agree with earlier results based on end group analysis (Dobson & Archibald, 1978).

The distribution of muropeptides in *S. aureus* H is consistent with a peptidoglycan assembly process in which single glycopeptide chains are crosslinked into the wall at the wall-membrane interface, with crosslinks being formed only at the time of incorporation. This is a simpler process than proposed in earlier studies and the mechanism proposed here accounts for the experimental results without requiring the secondary crosslinking reactions proposed by other workers (Tipper & Strominger, 1968; Snowden & Perkins, 1990, 1991). One of the considerations leading to proposals that secondary crosslinking reactions are involved in wall assembly is the presence of multiple penicillin-binding proteins. Thus it has been suggested that penicillin-binding protein 4 in *S. aureus* acts as a secondary transpeptidase so that in the absence of that activity *S. aureus* has a considerably reduced crosslinking (Wyke *et al.*, 1981). However, recent work has shown that inhibition by various

antibiotics of penicillin-binding proteins 2, 3 and 4 yields similar reductions in crosslinking percentages and remarkably similar muropeptide profiles (Snowden & Perkins, 1991). These muropeptide distributions are all consistent with the predictions based on Flory's random polymerization mechanism, and therefore with the restricted monomer addition mechanism proposed here. It appears possible therefore that the various penicillin-binding proteins are all concerned with transpeptidation reactions effecting the incorporation of new glycopeptide into the wall. Support for an assembly process requiring only one type of transpeptidation reaction (effecting initial incorporation) comes from the demonstration that methicillin-resistant staphylococci can produce highly crosslinked peptidoglycan while containing only one functional penicillin-binding protein (De Jonge *et al.*, 1992).

We conclude that the high degree of crosslinking found in *S. aureus* (80%, this study) can be explained without invoking secondary crosslinking reactions. It is possible that this highly crosslinked material reflects the increased freedom for crosslinking provided by the penta-glycine bridge in staphylococcal peptidoglycan (Gally *et al.*, 1991; Labischinski *et al.*, 1985). While multiple penicillin-binding proteins in *S. aureus* may carry out essentially the same transpeptidation reaction, it is possible that they act at different times or sites, such as in the initial morphogenesis or the subsequent thickening of the septum.

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Appendix 1

The equations derived by Flory (1953) are as follows:
Random addition:

$$\%(w/w) n\text{-mers} = np^{n-1}(1-p)^2 \times 100 \quad (1)$$

Monomer addition:

$$\%(w/w) n\text{-mers} = \frac{nw^{n-1}}{(v+1)e^v(n-1)!} \times 100 \quad (2)$$

$$\text{where } v = \frac{1}{(1-p)} - 1,$$

n is the number of disaccharide peptides in the muropeptide, and p is the fraction of monomers that are crosslinked, i.e. the degree of crosslinking.

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