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Relationship between the Acceptor/Donor Radioactivity Ratio and Cross-Linking in Bacterial Peptidoglycan: Application to Surface Synthesis during the Division Cycle

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The relationship between the experimental measurement of the cross-linking of bacterial peptidoglycan and the mode of its insertion is analyzed. The cross-linking value, in practice and in theory, is independent of the pattern of strand insertion. Since the measure of the mode or pattern of insertion is the acceptor/donor radioactivity ratio (ADRR), no correction need be made for the ADRR. The measurement of cross-linking using radioactivity is independent of the labeling time, the specific activity of the label, and the mode of strand insertion. It is not concluded, however, that cross-linking does increase during the division cycle.

Two numerical values have been used to characterize peptidoglycan biosynthesis in bacteria. One is the cross-linking value, the fraction of the total available positions for cross-linking of glycan that are actually used for cross-linking. For example, if there were 100 potential sites for cross-linking and only 25% of these were used, the cross-linking fraction would be 0.25. The second numerical measure is the acceptor/donor radioactivity ratio (ADRR), which is a measure of the way in which new peptidoglycan strands are inserted into the preexisting cell wall (2). If new strands are inserted as single strands between resident unlabeled cell wall strands, then all of the new material will "donate" a peptide linker to an unlabeled "acceptor" molecule as cross-links are formed (3). This is due to the presence of pentapeptide side chains in the new material and the absence of pentapeptides in the older, unlabeled cell wall. Only the new material with pentapeptides can act as donors. Since it is possible to isolate dimers from the cell wall after muramidase digestion and determine the fraction of label in the donor and acceptor positions, one can measure the ADRR. The ADRR is 0.0 if all the acceptor positions are unlabeled and all of the dimer radioactivity is in the donor position. An ADRR value of 0.0 means that new strands are inserted as single strands. If new strands were inserted as pairs or larger sheets or if a new strand was inserted next to a radioactive strand during the labeling period, the ADRR would be greater than zero. This is because some radioactive peptidoglycan would now be in the acceptor form.

The cross-linking value and the ADRR are not constants but change depending on the labeling conditions and the age of the peptidoglycan. The cross-linking value increases slightly with time after insertion of new material into the peptidoglycan (1, 5, 8). An initial low ADRR found after a short pulse changes to higher values with further incubation (2, 3, 7).

In a recent analysis of the cross-linked fraction and the ADRR during the division cycle of exponentially growing *Escherichia coli*, De Jonge et al. (4) proposed that one must correct the measured cross-linking value for the ADRR. (Although the proposal was specifically that one must correct for the specific activity of the labeled fragments, in practice this is directly related to the ADRR. Therefore in the remainder of this paper we will discuss only the ADRR, although the arguments apply directly to specific activity.) De Jonge et al. (4) described a slight increase in radioactivity in the multimeric fraction during septum formation. Without any adjustment, this increase in radioactivity in the multimeric fraction would indicate that the degree of cross-linking increased during septum formation. The ADRR was also elevated in the septum-forming cells. De Jonge et al. argued, on the basis of proposals about the specific activity in molecules with either one or both positions labeled with radioactive diaminopimelic acid, that one must correct for the elevated ADRR. After making such a correction, De Jonge et al. concluded that there was no increase in cross-linking during septation.

Cross-linking will now be rigorously defined, and the relationship of the ADRR to the measurement and meaning of cross-linking will be examined. Irrespective of whether cross-linking is constant or varies during the division cycle, it will be demonstrated here that there is no relationship between the ADRR and the measurement of cross-linking.

What is cross-linking, and how is it measured? Peptidoglycan is composed of many monomeric units that are made up of a disaccharide with an attached peptide chain. The peptide chain of the monomer has both the acceptor and the donor portions that produce or allow cross-linking. In Fig. 1 a monomer is schematically drawn to illustrate this basic situation. (A monomer is the minimal unit released by lysozyme digestion of peptidoglycan; the monomeric unit contains a disaccharide with an appended peptide chain. Dimers, trimers, and tetramers are molecules with two, three, or four of these monomeric units, respectively.) An arrow point is the donor of the monomer (normally the carboxyl end of the penultimate D-alanine), and the circle is the acceptor end (the free amino end of diaminopimelic acid). By definition, each monomer contains the material for one cross-link; this is because each cross-link is composed of an acceptor and a donor. If we now consider dimers, trimers, and tetramers, we may see how the number of potential cross-links (equal to the number of subunits) is expressed in actual cross-links. The number of potential cross-links in any fragment is equal to the number of subunits in the fragment.

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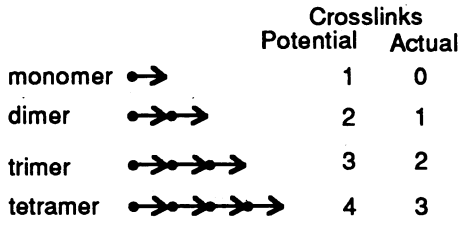


FIG. 1. Potential and actual cross-links in different peptidoglycan fragments. The numbers of actual cross-links in the monomer, dimer, trimer, and tetramer illustrated here are equal to the subunit numbers (1, 2, 3, and 4, respectively). The actual number of cross-links formed is one less than the potential number because at the ends there is material equivalent to one cross-link.

The actual number of cross-links is one less than the number of subunits in the fragment because at the ends there are an acceptor and a donor, which are equivalent to one cross-link. The general equation for the cross-linking fraction is as follows: cross-linking = actual cross-links formed/potential cross-links. The number of cross-links in each fragment may be determined by considering that in each fragment there are a fixed number of cross-links formed which is one less than the possible number of cross-links. The resulting equation (6) is as follows:

$$\text{Cross-linking} = \frac{0.5 \times \text{dimers} + 0.66 \times \text{trimers} + 0.75 \times \text{tetramers} + \ldots [(n-1)/n] \times n\text{-mers}}{\text{monomers} + \text{dimers} + \text{trimers} + \text{tetramers} + \ldots n\text{-mers}}$$

where each of the values (monomers, trimers, tetramers, etc.) signifies the amount of material in terms of the number of monomeric subunits present in that fraction.

The same reasoning can be applied to peptidoglycan. A peptidoglycan section is drawn with 100% cross-linking in Fig. 2a; every available position for cross-linking is used. Each D-alanine in the tetrapeptide is linked to a neighboring diaminopimelic acid, and each free amino group of diaminopimelic acid is involved in cross-linking. If this material is digested with muramidase, no monomers or dimers will be found; all of the material will be in high multimers. (The value of 100% is strictly correct if the sheet is infinitely long; if there are, for example, 100 strands linked as in Fig. 2a, then the cross-linking value would be only 0.99.) In Fig. 2b, half of the cross-links are removed, giving 50% cross-linking. If this peptidoglycan is digested with muramidase, all of the material (100%) will be found in the dimer fraction and it can be determined that the cross-linking fraction is 0.50. Each monomer has two available sites, and in Fig. 2b each monomer is shown using one of its sites for cross-linking; this is what is meant by 50% cross-linking. If one now removes half of the remaining cross-links, we find the situation shown in Fig. 2c, with 25% cross-linking. In this case half of the total material is in dimers and half is in monomers; the basic equation again gives the right result, with a cross-linking fraction of 0.25.

Determination of cross-linking by using radioactivity. When one attempts to analyze the cross-linking fraction in radioactively labeled peptidoglycan, there arises a problem with the definition of cross-linking. How does one "count" cross-links? What happens when there is a cross-link between a radioactive monomer and an unlabeled monomer? Is that cross-link, formed with unlabeled material, to be considered a single, whole cross-link? It is proposed here that when one considers material made up of labeled and unlabeled material it is necessary to think of each cross-link as being made up of two "half"-cross-links. When a cross-link is between a radioactively labeled strand and an unlabeled strand, one need only consider the half of the cross-link from the radioactive strand. Consider the two labeled peptidoglycan sheets at the bottom of Fig. 3. Each sheet has two labeled strands, one in which the strands are separated and one in which the strands are adjacent. When the labeled two strands are separate, the number of half-cross-links to the radioactive strands (open circles) can be easily counted; each strand has eight half-cross-links to the unlabeled material (closed circles). Since the number of potential or available half-cross-links in the labeled material is 64 per strand, one gets a cross-linking fraction of 1/8 or 0.125. When the radioactive strands are next to each other, as in the bottom right of Fig. 3, one may count the half-cross-links in the same manner and get, again, a cross-linking value of 0.125.

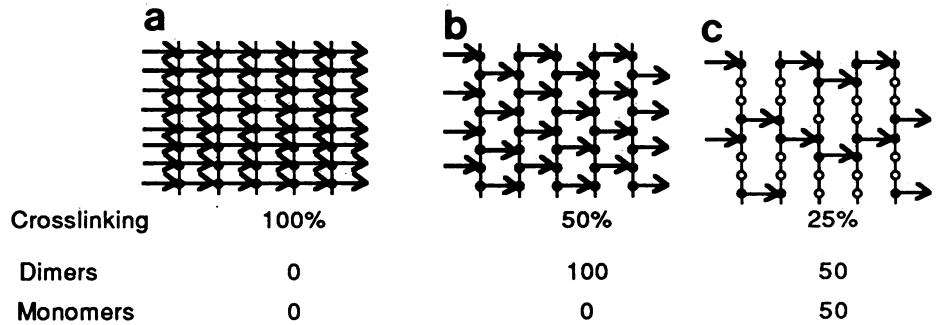
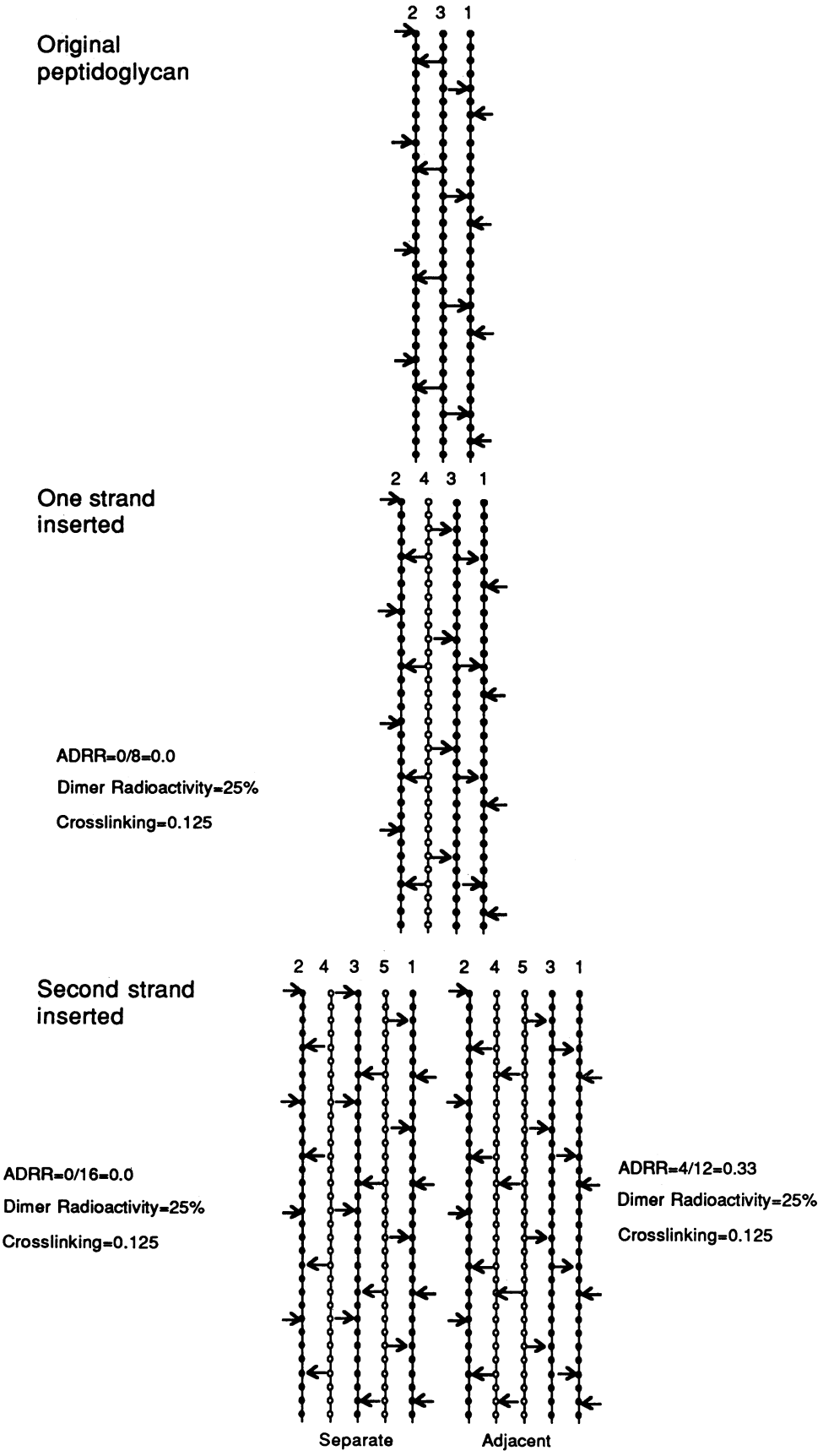


FIG. 2. Peptidoglycan with different cross-linking values. (a) Peptidoglycan with 100% cross-linking; (b) peptidoglycan with 50% cross-linking; (c) peptidoglycan with 25% cross-linking. The 100% cross-linking pattern is probably never achieved in practice and is presented here as a theoretical construct to illustrate the idea of cross-linking as the use of all available cross-linking elements in the peptidoglycan.


Original peptidoglycan
2 3 1
One strand inserted
2 4 3 1
ADRR=0/8=0.0
Dimer Radioactivity=25%
Crosslinking=0.125
Second strand inserted
2 4 3 5 1
2 4 5 3 1
ADRR=0/16=0.0
Dimer Radioactivity=25%
Crosslinking=0.125
ADRR=4/12=0.33
Dimer Radioactivity=25%
Crosslinking=0.125
Separate
Adjacent

The problem with labeled material adjacent to unlabeled material is that one may also count the physical cross-links differently depending on whether strands are adjacent or separate. For example, in the lower left we may count 16 "cross-links" in the radioactive material, while the total number at the lower right is only 12. This would lead to a different calculation of cross-linking depending on how one counted the cross-links. The difference between the two determinations is determined by the mode of insertion of strands. The ADRR value for the two single strands is 0.0, while the ADRR value for the adjacent labeled strands is 0.33. De Jonge et al. (4) have proposed that one must correct for the fact that when a dimer has both of the monomers labeled (Fig. 3, lower right) there is only one cross-link. In the view of De Jonge et al. (4), one must take account of the specific activity of the monomers and correct for the ADRR.

This view may be refuted by considering a distinction between the "process" of cross-linking and the "product" of cross-linking. If we look only at the final result of strand insertion and do not consider the way in which this final product is produced, then the number of cross-links is more consistently described by considering the number of half-links rather than the number of physical links. For example, if the labeled adjacent strands were linked together with four cross-links and then inserted together with eight cross-links to the unlabeled resident material, we could see that there would be only 12 cross-links produced in the process of strand insertion. In contrast, the two single strands would have required 16 new cross-links. The final products, in both cases, would be the same with regard to the number of half-links involved in the newly inserted material. This analysis deals only with the final product of strand insertion and does not depend on the actual mode of strand insertion.

Radioactivity and cross-linking values. The growth of peptidoglycan in the presence of radioactive diaminopimelic acid, a specific marker for peptidoglycan, is illustrated in Fig. 3. The topmost pattern represents the preexisting, unlabeled peptidoglycan. Each strand is 32 subunits long, and each strand has 25% of its subunits in dimers. If this were an infinite sheet extending in all directions so that the edge effect did not have to be considered, one would find that 25% of the total material was in dimers. From the formula for cross-linking given above, the calculated cross-linking fraction is 0.125. Let this peptidoglycan grow so that one new strand is inserted (age 4). This new strand donates eight half-cross-links to its adjacent neighbors, four to the left (to strand age 2) and four to the right (to strand age 3). The ADRR is 0.0. The cross-linking fraction, when one considers either the radioactivity or the total chemical material, is 0.125; there are eight half-cross-links made out of a total of 64 potential half-cross-links. Experimentally, we would find that 25% of the total radioactivity is in the dimer fraction. Now consider two different situations with the addition of another radioactive strand. In the first case the second strand is inserted between two unlabeled strands (ages 1 and 3), and in the second case the second strand is inserted between a labeled (age 4) and an unlabeled (age 3) strand. The cross-linking density is the same in both cases. The two final products differ in the ADRR. In the case in which the two radioactive strands are not adjacent, the ADRR is still 0.0. In the case in which the new radioactive strand is adjacent to the initially inserted radioactive strand, the ADRR is 0.33. In the second case, some of the initial radioactive subunits (strand age 4) are now acceptors for the newly inserted radioactive donor strand (strand age 5). In both cases the fraction of radioactivity in the dimer fraction is the same, and the calculated, as well as expected, cross-linking fraction is identical. Thus, we see that the ADRR does not affect the cross-linking fraction. The cross-linking fraction is determined by the relative rates of monomer insertion and cross-link formation. The cross-linking fraction is solely a result of the chemistry of peptidoglycan synthesis and is independent of the labeling pattern. The ADRR is a reflection of the insertion of new strands between older strands or relatively young strands. It is concluded from this analysis that no correction is necessary for variable ADRRs when calculating the cross-linking fraction.

The view proposed here is a consistent one. If one grew peptidoglycan with either single- or double-strand insertion until all of the peptidoglycan was radioactive, one would have a final product with a 0.125 cross-linking value made from inserted material with a cross-linking value of 0.125. In contrast, if one corrects for the ADRR, one would have, in the case of double-strand insertion, a final product with a cross-linking value of 0.125 which was produced by the continuous insertion of material with a cross-linking value of 0.94.

Cross-linking and the ADRR during exponential growth. Consider that newly made peptidoglycan is synthesized with a given cross-linking fraction and that there is no further change in the cross-linking as the peptidoglycan gets older. Now consider two cultures, one labeled for 1 min and one labeled continuously for 1,000 min. Assuming that the radioactivity is not limiting in either case, one has 1,000 times as much radioactivity in the long-term incorporation experiment as in the short-term experiment. The fraction of radioactivity in the multimeric fraction would be the same in both cultures. This can be understood by realizing that the long-term label culture is made up of 1,000 1-min labeling periods. The only difference between the long-term label and the short-term label is the amount of label, since each 1-min labeling period in the long-term culture can be imagined as qualitatively and quantitatively the same as the single initial short 1-min labeling period. The distribution of the label between monomers and multimers in the two cultures would be the same, as would the calculated cross-linking value. When one considers the ADRR, however, there is a difference. In the case of the short-term label, the ADRR is relatively low. In the case of the long-term label, the peptidoglycan may be considered totally labeled, with acceptors and donors equally labeled, and thus the ADRR is 1.0. Since we have shown that the cross-linking fraction is

FIG. 3. Variation of the ADRR with constant cross-linking during peptidoglycan growth. Three strands of unlabeled peptidoglycan are shown at the top. The numbers (1 to 5) describe the relative age of each strand of peptidoglycan, with the lower numbers being the older strands. An older strand is drawn as an acceptor of cross-links with arrows drawn toward the older strand. When a new strand of age 4 is inserted between strands 2 and 3 (middle panel), it donates four cross-links to each of the adjacent strands. In all cases the peptidoglycan strands are drawn so that one-fourth of the newly inserted glycan subunits form a cross-link. Three-fourths of the newly inserted subunits are not cross-linked. After one strand is inserted, 25% of the total radioactivity is in dimers. This gives a 0.125 cross-linking value. The ADRR is 0.0. In the bottom panel, two different positions for the insertion of the next strand are shown. At the left, the new strand is inserted, as before, between two unlabeled strands. The ADRR for both strands is still 0.0, and the cross-linking fraction is 0.125. At the right, the second strand is inserted between a radioactive and a nonradioactive strand. Again, the cross-linking fraction is 0.125, although the ADRR is 0.33.

the same in both cultures and the fraction of radioactivity yielding the cross-linking fraction is the same, one would not want to adjust the experimentally measured cross-linking for any difference in ADRR. We again conclude that the ADRR should not be used to adjust the cross-linking measurement.

Caveats and comments. The original proof that one must correct the measured cross-linking ratio for the ADRR was mathematical in form (4). The derivation was based on a concept that there is a difference between dimers in which both subunits are labeled and dimers in which only one is labeled. The experimental measurement of the acceptors and donors is done on hydrolyzed material. There is no separate analysis or determination of dimers with only one labeled monomer and dimers with both monomers labeled. The original derivation also introduced the concept of specific activity. At no time is there any measurement of specific activity since the radioactivity per chemical unit is not determined. Only the ratio of the amount of radioactivity in different fractions is used for both the ADRR and the cross-linking determinations. The derivation of De Jonge et al. (4) introduced a factor of 0.5 because of the fact that half of the diaminopimelic acid residues inserted during the labeling period came from endogenously synthesized material. This argument means that if one had more or less endogenous synthesis, i.e., the specific activity of the inserted material was different in different bacteria, one would have to correct for specific activity of the incorporated diaminopimelic acid. Unfortunately, this argument is incorrect. If one dilutes radioactive diaminopimelic acid with unlabeled diaminopimelic acid and changes the specific activity, there is no change in the cross-linking measurement or the ADRR determination.

In the original study by De Jonge et al. (4) an elevated cross-linking was observed during septation. When the proposed ADRR correction was applied, there was no cross-linking increase, and so De Jonge et al. proposed that cross-linking is constant during the division cycle. I do not propose that cross-linking varies during the division cycle. There are problems with synchronization as an approach to the analysis of the division cycle (S. Cooper, *Bacterial Growth and Division*, in press), and therefore it is not clear that the data can be used to support a cycle-specific pattern of peptidoglycan synthesis. This problem is left for further experimental analysis. What is proposed here is that one does not need to correct cross-linking for the ADRR.

Meaning of cross-linking. In the discussion above we noted that the ADRR is a measure of where, in relation to other radioactive strands, new radioactive strands are inserted. What is the meaning of cross-linking? What does cross-linking measure? Cross-linking is a measure of the relative efficiency of the cross-linking activity compared with the activity involved in inserting new glycan subunits into the peptidoglycan. This relative activity is independent of the actual mode of insertion of strands, i.e., whether strands are inserted as single strands or as two or more strands at a time. A higher cross-linking value means that a cell has, during the time of strand insertion, a greater ability to form cross-links from the newly inserted material. When cross-linking is viewed from this perspective, it is clear that there is no relationship of cross-linking to the ADRR value.

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