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Cross-Linkage and Cross-Linking of Peptidoglycan in *Escherichia coli*: Definition, Determination, and Implications

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The glycan chains in peptidoglycan or murein are cross-linked by transpeptidation of the peptide side chains. To assess the fraction of side chains involved in cross-bridges, distinction has been made between cross-linkage and cross-linking. The first expression refers to the situation in unlabeled (or fully labeled) peptidoglycan, and the second refers to pulse-labeled peptidoglycan. It is argued that for the determination of the cross-linking value, the mode of insertion as denoted by the so-called acceptor/donor radioactivity ratio should be taken into account.

In an interesting paper, Cooper (3) discussed the definition and measurement of cross-linking in bacterial peptidoglycan. The main topic was whether determination of cross-linking in the peptidoglycan of pulse-labeled cells should include a correction for the distribution of radioactivity over donor and acceptor peptides, as was recently proposed by De Jonge et al. (4) and as was proposed earlier by others (10, 11). This distribution, characterized by the acceptor/donor radioactivity ratio (ADRR), reflects the mode of insertion of new peptidoglycan strands in the preexisting sacculus (1, 2). It was argued (3) that the measurement of cross-linking is independent of the mode of insertion and therefore no correction has to be made for the ADRR. The reason for this different viewpoint resides, we believe, in a different interpretation of the meaning of cross-linking, notably with respect to the differences between unlabeled (or fully labeled) and pulse-labeled material. In this paper, we continue the discussion on this topic.

We wish to show that for the correct determination of cross-links newly formed during labeling, the ADRR should be taken into account.

Definition and significance of cross-linkage. We will use the term cross-linkage for unlabeled peptidoglycan and cross-linking for pulse-labeled peptidoglycan.

Quantitation of cross-links in a sample of unlabeled peptidoglycan is based on the UV absorbance of the muropeptide compounds. By definition, every dimeric muropeptide contains one cross-link, every trimeric muropeptide contains two cross-links, and so on. The theoretical maximal (potential) number of cross-links in a sample of peptidoglycan is equal to the number of peptides (see below). Cross-linkage is then defined as follows (3, 9): cross-linkage = actual number of cross-links/potential number of cross-links. The mathematical formulation of this definition is as follows (for the sake of simplicity, it is presumed that multimers "higher" than trimers do not occur):

$$\text{cross-linkage} = \frac{D + 2T}{M + 2D + 3T} \quad (1)$$

where M, D, and T signify molar amounts of monomers, dimers, and trimers, respectively. Equation 1 can be rewritten as follows:

$$\text{cross-linkage} = \frac{0.5D' + 0.67T'}{M' + D' + T'} \quad (2)$$

where M', D', and T' signify the amounts of disaccharide peptide units occurring in monomers, dimers, and trimers, respectively. If multimers higher than trimers do occur in the peptidoglycan, this equation should be adapted. The general equation is

$$\text{cross-linkage} = \frac{\Sigma[(n-1)/n]N}{U} \quad (3)$$

where n signifies the number of disaccharide peptide units per n-mer ($n = 1$ for monomomers, $n = 2$ for dimers, etc.), N signifies the amount of disaccharide peptide units in the n-mer fraction, and U signifies the total amount of disaccharide peptide units in the sample. This equation was first formulated by Glauner (9).

In Fig. 1 (adapted from Cooper [3]), cross-linkage is visualized. Figure 1A shows a peptidoglycan fragment in which every disaccharide peptide unit acts as a donor. Consequently, every unit also acts as an acceptor. Thus, by definition, the cross-linking density is 100% (or 1.0). Digestion of this material with muramidase would yield no monomers or dimers but one huge multimer, kept together by peptide bonds only. However, such a fully cross-linked peptidoglycan probably does not exist in nature. Figure 1B shows a fragment with half as many cross-links (cross-linkage of 50%). Again, every peptide is involved in a cross-link, but now either as a donor or as an acceptor. Digestion of this material with muramidase would yield only dimers. Figure 1C shows fragments with a cross-linkage of 25%. Digestion with muramidase would yield 50% of the material as dimers. For comparison, according to equation 1 or 2, the cross-linkage of peptidoglycan in actively growing *Escherichia coli* cells is 23 to 26% (6, 7, 9).

The examples depicted in Fig. 1 illustrate the linear relationship between cross-linkage according to the defini-

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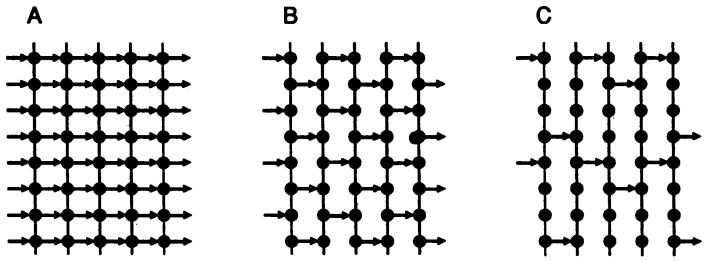

	A	B	C
Cross-linkage	100 %	50 %	25 %
Dimers	0	100	50
Monomers	0	0	50

FIG. 1. Peptidoglycan fragments with different cross-linkage values (adapted from Cooper [3] by permission). Glycan chains are schematically drawn as interconnected circles. Arrows represent disaccharide peptide units involved in cross-links. Cross-linkage values are 100% (A), 50% (B), and 25% (C). These values can be obtained by applying equation 1 or 2, with the restriction that one takes account of the edge effect. If larger sheets are drawn, this effect becomes smaller.

tion given above and the quantity of cross-links in a peptidoglycan sample. According to an alternative definition of cross-linkage which has been frequently used in previous studies, there is a different relationship. In the study of De Pedro and Schwarz (5), for example, cross-linkage was defined as the molar fraction of dimers (trimers were not detected). It follows in that case that

$$\text{cross-linkage} = \frac{D}{M + D} \tag{4}$$

where M and D signify molar amounts of monomers and dimers. Note that this equation fundamentally differs from equation 1, which in the absence of trimers equals $D/(M + 2D)$. By using equation 4, a cross-linkage in *E. coli* peptidoglycan of 30% was found (5). By using equation 1, the cross-link density would have been 23%. This dissimilarity in the two definitions of cross-linkage has been the cause of some confusion and complicates the comparison of results of different studies. Note that our equation of cross-linkage of unlabeled peptidoglycan (equation 3) is the same as that of Cooper (3).

Mode of insertion. A numerical value that is particularly relevant to the mechanism of insertion of newly synthesized peptidoglycan strands into the sacculus is the ADRR of cross-linked muropeptides.

If newly synthesized (labeled) peptidoglycan is inserted as single strands between preexisting (unlabeled) strands, the radioactivity will be exclusively in donor peptides. The reason for this is that only new peptidoglycan can provide the pentapeptide residues that act as donor peptides in cross-linking. Thus, in this situation, new cross-links will be made up of donors originating from new peptidoglycan and acceptors originating from preexisting peptidoglycan. This type of cross-link will be referred to as a "new-old" cross-link (ADRR = 0). If, on the other hand, new strands are inserted as larger fragments (pairs, for instance) or if a new strand is inserted next to another new strand, some of the radioactivity will be in acceptor peptides (ADRR > 0). In this situation, there will be cross-links of the new-old type but also cross-links where donor and acceptor originate from new peptidoglycan. The latter are "new-new" cross-links (ADRR = 1.0). Experimental data have indicated that in pulse-labeled *E. coli* cells, the ADRR in the major dimer (bisdisaccharide tetrapeptide) is about 0.25 (2, 4, 8). This means that 80% of the radioactivity is in donor positions (60% in new-old and 20% in new-new cross-links) and 20% is in acceptor positions.

Definition of cross-linking. Determination of the composition of pulse-labeled peptidoglycan is based on quantitation of radioactivity in the different muropeptides, with the assumption that every count represents one disaccharide peptide unit. However, now there arises a problem in defining the fraction of cross-bridges in relation to the equations given in the previous section. An essential presupposition of these equations is that there is one cross-link for every two detected disaccharide peptide units in a dimer, two cross-links for every three detected disaccharide peptide units in a trimer, etc. In the case of labeled peptidoglycan, this is true only if the radioactivity is equally distributed between donor and acceptor positions, i.e., if the ADRR is 1.0. Experimental data, however, have indicated that the ADRR in the peptidoglycan of pulse-labeled, exponentially grown *E. coli* cells is about 0.25 (2, 4, 8).

It thus appears relevant to have a cross-linkage estimate that is a measure of the number of newly formed cross-links during the labeling period. We propose the following definition for this value and we furthermore propose to call it cross-linking to distinguish it from cross-linkage: cross-linking = actual number of new cross-links/potential number of new cross-links. The potential number of new cross-links is equal to the number of new disaccharide peptide units, which is represented by the total amount of radioactivity in the sample. The actual number of new cross-links is equal to the number of new donor peptides, which is represented by the amount of radioactivity in donor positions. This yields the following: cross-linking = radioactivity in donors/total radioactivity. For the sake of simplicity, we consider peptidoglycan to consist of monomers and dimers only. The equation then becomes

$$\text{cross-linking} = \frac{[d/(d + a)]D}{M + D} \tag{5}$$

where M and D signify the amounts of radioactivity in monomers and dimers and d and a signify the amounts of radioactivity in donor and acceptor positions, respectively.

Significance of cross-linking. To further illustrate the meaning of cross-linking and the role of the ADRR, two theoretical schemes of peptidoglycan insertion will be discussed (Fig. 2). For the sake of comparison, we use again the figures originally put forward by Cooper (3). (At that time they were

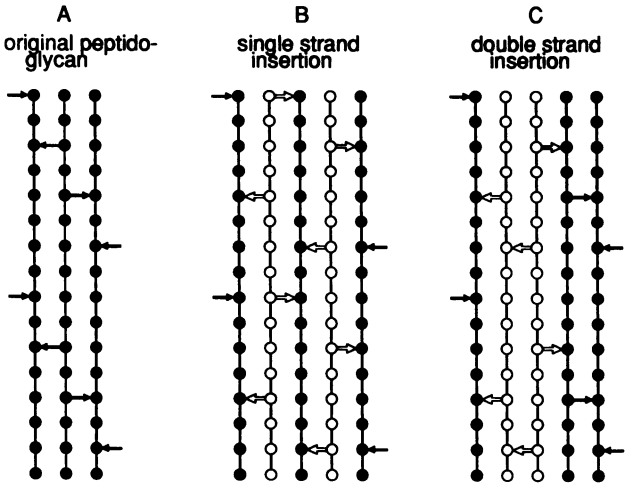

FIG. 2. Relationship between mode of insertion of new peptidoglycan strand, ADRR, and cross-linkage and cross-linking values (adapted from Cooper [3] by permission). Three strands of unlabeled peptidoglycan are shown at the left (A). Each strand consists of 16 disaccharide peptide units, of which 4 units are dimers; the cross-linkage value of the material is 12.5%. Next, two newly labeled peptidoglycan strands are inserted (open circles), either as single strands (B) or as a pair (C). The strands are drawn so that one-fourth of the units form part of a dimer (as in the original unlabeled peptidoglycan). Newly formed cross-links are represented by open arrows.

used to substantiate the view that determination of cross-linking in pulse-labeled peptidoglycan is independent of the mode of insertion.) Pattern A (Fig. 2) represents unlabeled peptidoglycan. Each strand consists of 16 disaccharide peptide units and has 4 units in dimers. If this were a much larger sheet, making the edge effect negligible, one would find (in agreement with Cooper) that the cross-linkage value is 12.5% (equation 2). Next, two modes of insertion of new strands are considered.

In pattern B (Fig. 2), two new, radioactively labeled strands are inserted as single strands between preexisting, unlabeled ones. Each of these two new strands "donates" four cross-links, two to the left and two to the right, making a total of eight new cross-links. All of these cross-links are of the new-old type, meaning that all of the radioactivity in dimers is in donor positions; i.e., the ADRR is 0.0.

In pattern C, two new strands are inserted as a pair; six new cross-links are formed, four of the new-old and two of the new-new type. Consequently, 75% of the radioactivity in dimers is in donor positions; i.e., the ADRR is 0.33. In pattern B as well as in pattern C, 25% of the radioactivity is in dimers. Thus, in both patterns the cross-linkage value of the labeled peptidoglycan is 12.5% (equation 2). This is compatible with what one can observe by eye also: the distribution of cross-links is the same.

What about the cross-linking value? This value is 25% for pattern B and 18.75% for pattern C (equation 5). These values correspond to the respective fractions of newly formed cross-links: eight new cross-links per 32 new units in pattern B and six new cross-links per 32 new units in pattern C. This example clearly illustrates that the cross-linking value is a correct measure of the relative activity of the cross-linking system and that it is dependent on the mode of peptidoglycan insertion, i.e., whether it is single stranded (pattern B) or multistranded (pattern C). The cross-linkage value, on the other hand, measures the quantity of cross-links in the final product and is independent of the mode of how it is achieved, i.e., insertion. The difference between 25% and 18.75% in this example is explained by the fact that before an insertion event can take place, preexisting cross-links have to be hydrolyzed. The insertion of two new single strands with eight new cross-links in pattern B was preceded by the hydrolysis of four preexisting cross-links per 32 units. The same net result is obtained in pattern C: six cross-links formed and two cross-links hydrolyzed.

TABLE 1. ADRR, percentage and cross-linking value of the bisdisaccharide tetrapeptide compound (Tet-Tet), and cross-linkage value in pulse-labeled peptidoglycan in relation to the percentage of constricting cells[a]

% of constricted cells	ADRR	% of Tet-Tet	Cross-linking (%)	Cross-linkage (%)
4	0.18	26.4	22.4	18.9
5	0.15	26.0	22.6	19.0
14	0.23	29.9	24.3	19.3
30	0.27	29.2	23.0	18.6
37	0.32	29.4	22.3	19.5
40	0.35	30.2	22.4	19.6
42	0.28	28.5	22.3	19.0
42	0.35	29.8	22.0	19.8
52	0.43	32.2	22.5	20.0
56	0.39	31.6	22.8	19.8
60	0.47	30.9	21.0	19.5
66	0.41	32.0	22.7	19.9

[a] Values are based on data of De Jonge et al. (4). Synchronized cultures or asynchronous cultures were pulse-labeled with [^{3}H]diaminopimelic acid for 4 min. The percentage of constricted cells was determined by electron microscopy. The ADRRs given here were determined for the major dimer, bisdisaccharide tetrapeptide. By using these values and the detected amounts of the compound, cross-linking values were determined according to equation 5. Cross-linkage values were determined according to equation 2; the calculation included the amounts of 19 different muropeptides (five monomers, eight dimers, five trimers, and one tetramer).

Cross-linking and cross-linkage during the cell cycle. De Jonge et al. (4) described the composition of pulse-labeled peptidoglycan during the cell cycle of *E. coli* by using synchronously growing cultures. It was proposed that for determination of the cross-linking value, one should correct for the specific activity of the labeled material. It was argued that one should correct, first, for the ADRR and, secondly, for endogenous peptidoglycan synthesis. As pointed out by Cooper (3), the latter argument is incorrect. However, the proposed correction for the ADRR is indeed required, at least if one wants the cross-linking value to be a measure of the activity of the cross-linking system.

Using the data of De Jonge et al. (4), we have calculated the ADRR and the cross-linking value for the major dimeric compound, bisdisaccharide tetrapeptide, and the cross-linkage in pulse-labeled peptidoglycan from synchronous cultures containing 4 to 66% constricting cells (Table 1). The ADRR showed a considerable increase with increasing percentages of constricting cells. The cross-linking value, on the other hand, was found to remain more or less constant. If we

included two additional dimers in the calculation of cross-linking value, namely, bisdisaccharide tetrapeptide-pentapeptide and bisdisaccharide tetrapeptide-tripeptide, the values still remained constant (not shown). Together, these three dimers represent more than 85% of the cross-linked compounds. These findings indicate that the relative activity of the cross-linking system is constant throughout the cell cycle. If one uses the equation for cross-linkage, which according to Cooper is the correct equation for labeled and unlabeled peptidoglycan, one also finds that the cross-linkage value remains more or less the same during the division cycle. However, as pointed out above, calculation of cross-linkage is in our opinion not meaningful in the case of pulse-labeled peptidoglycan.

Also, with respect to other structural parameters such as the average glycan chain length, the fraction of trimers and tetramers, and the fraction of bound lipoprotein, no significant differences during the cell cycle were found (4). Taken together, the data indicate that, within the present technical limitations, the muropeptide composition of new peptidoglycan synthesized during elongation is indistinguishable from that of peptidoglycan synthesized during constriction.

Conclusions. We have made a distinction between cross-linkage and cross-linking. Cross-linkage is a measure of the quantity of cross-links in peptidoglycan and is independent of the ADRR. The interpretation of this value in unlabeled or fully labeled peptidoglycan is straightforward but needs modification for pulse-labeled peptidoglycan. The reason is that pulse-labeled muropeptides always carry a certain amount of unlabeled material (due to new-old cross-links).

The term cross-linking has been used to describe the situation in pulse-labeled peptidoglycan. We have shown that there is a relationship between cross-linking and ADRR. The higher the ADRR, the more new-new cross-links are formed. In our view, a consequent distinction has to be made between cross-linkage (unlabeled or fully labeled peptidoglycan) and cross-linking (pulse-labeled peptidoglycan). In fact, a basic rationale of pulse-labeling is to investigate cross-linking activity.

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