THE DIRECTION OF TRANSPEPTIDATION DURING CELL WALL PEPTIDOGLYCAN BIOSYNTHESIS IN *BACILLUS MEGATERIUM*

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1. Introduction

The biosynthesis of cell wall peptidoglycan from nucleotide-activated wall precursors has been studied in wall + membrane preparations [1–3], toluene-treated bacteria [4] and ether-treated bacteria [5]. Membrane-bound enzymes transfer the precursors onto a lipid intermediate (undecaprenyl phosphate) [6,7]. In Gram-positive organisms the disaccharide peptide is then transferred from the lipid intermediate to the growing glycan chain still attached to a lipid in the membrane to form nascent peptidoglycan. As yet no evidence of a growing nascent glycan chain has been found in Gram-negative bacteria [8]. The nascent peptidoglycan is transferred to the pre-existing wall either by transpeptidation [4] or by a combination of transpeptidation and transglycosidation [9]. Alternatively it may be lost to the surrounding medium if transpeptidation is blocked by a β-lactam antibiotic [10]. Protoplasts of *Streptococcus faecalis*, lacking cell wall peptidoglycan, secreted soluble peptidoglycan fragments suggesting that large fragments are normally transferred into the wall [11]. The transpeptidation reaction can be considered to be directional since there must be a donor and an acceptor molecule (fig.1). When transpeptidation occurs to attach the nascent peptidoglycan to the pre-existing

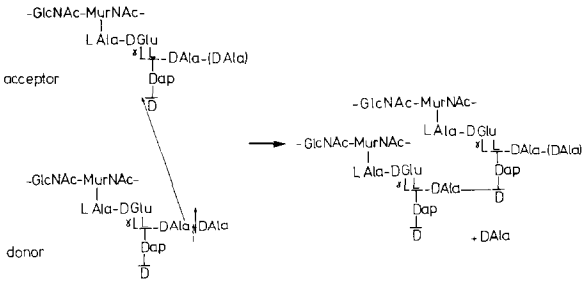

Fig.1. The transpeptidation reaction.

wall, the donor may be in the nascent peptidoglycan and the acceptor in the wall or vice versa. Hence there are two possible reaction directions at this stage of wall synthesis. After attachment of the nascent peptidoglycan to the pre-existing wall further transpeptidation may take place but here a direction of transpeptidation cannot be assigned since the donor and acceptor molecules are indistinguishable. In *Gaffkya homari* a unidirectional transpeptidation reaction has been reported with the donor (pentapeptide) in the pre-existing wall and the acceptor, necessarily having been converted to a tetrapeptide by a DD-carboxypeptidase enzyme in this particular organism, in the nascent peptidoglycan [12]. In a wall + membrane preparation of *Bacillus licheniformis* at least some transpeptidation occurred in the opposite direction [13]. This report describes studies on toluenised cells of *Bacillus megaterium* which indicate a unidirectional transpeptidation reaction in a direction opposite to that which occurs in *G. homari*.

Abbreviations: MurNAc, *N*-acetylmuramyl; Dap, 2,6-diaminopimelic acid; pentapeptide, L-Ala–D-Glu–meso-Dap–D-Ala–D-Ala; acetylpentapeptide, L-Ala–D-Glu–(D)-[*acetyl*-^{14}C]acetyl-meso-Dap–D-Ala–D-Ala; tetrapeptide, L-Ala–D-Glu–meso-Dap–D-Ala; TCA, trichloroacetic acid; SDS, sodium dodecyl sulphate; PBP, penicillin binding protein

2. Materials and methods

Bacillus megaterium KM was grown as in [1]. The preparation of toluenised cells was similar to that in [4] though a fluid pellet was never obtained. The toluenised cells were suspended in 50 mM Tris–HCl buffer (pH 8) containing 10 mM Mg^{2+}.

UDP–MurNAc–pentapeptide and UDP–MurNAc–L-Ala–D-Glu–meso-Dap–D-[^{14}C]Ala–D-[^{14}C]Ala were prepared as in [14]. UDP–MurNAc–tetrapeptide was generously provided by S. T. Shepherd. UDP–MurNAc–L-Ala–D-Glu–meso-Dap–D-[^{14}C]Ala was prepared by treatment of UDP–MurNAc–L-Ala–D-Glu–meso-Dap–D-[^{14}C]Ala–D-[^{14}C]Ala with a purified DD-carboxypeptidase (PBP 4) from *S. typhimurium* [15]. UDP–MurNAc–L-ALa–D-Glu–(D)-[*acetyl*-^{14}C]acetyl-meso-Dap–D-Ala–D-Ala was prepared as in [13].

2.1. *Assay of transpeptidation*

Toluenised cells (20 mg dry wt/ml) were suspended in: 0.33 mM UDP–[^{14}C]GlcNAc (1.32 Ci/mol); 1.6 mM UDP–MurNAc–pentapeptide; and 174 mM Tris–HCl buffer (pH 8). The sample volume was 30 μl for time courses and 90–120 μl for analysis of reaction products. The incubation temperature was 23°C. For time courses the reaction was terminated by the addition of 0.5 ml cold 5% TCA. After centrifugation the pellet was treated with 4% SDS at 100°C for 20 min to solubilise radioactive material not bound covalently to the cell wall peptidoglycan (unpublished data). The insoluble material was obtained by filtration using glass fibre discs (Whatman GF/C).

Reaction products were analysed after 90 min incubation. Assay mixtures were centrifuged and the supernatant liquid chromatographed. The pellet was washed in 0.5 ml 0.01 M sodium acetate buffer (pH 4.6), resuspended in 50 μl of the same buffer and placed in a boiling water bath for 1 min. After cooling, a further 50 μl buffer containing 20 μg Chalaropsis lysozyme was added, and the samples were incubated at 37°C for 24 h. After digestion and centrifugation the supernatant liquid and pellet were chromatographed and the chromatogram subjected to autoradiography.

In order to estimate the ability to synthesise nascent peptidoglycan using different substrates a membrane preparation was used. Exponentially growing *B. megaterium* was harvested at 0.4 mg dry wt/ml and broken by shaking with polystyrene beads as in [1]. The membrane fraction was separated from the wall + membrane fraction by differential centrifugation. The membrane preparation was then used in place of toluenised cells in transpeptidation assay mixtures supplemented with 10 mM Mg^{2+}. After incubation at 23°C for 60 min the incubation mixture was centrifuged and the supernatant liquid and pellet were chromatographed. Material in the supernatant liquid that remained at the origin was found to contain nascent peptidoglycan since digestion with Chalaropsis lysozyme gave rise only to disaccharide peptide monomer (unpublished data).

Radioactivity on glass fibre filters and on paper was determined in a Packard Tricarb liquid scintillation spectrometer using 2,5-bis-(2-(5-*tert*-butyl-benzoxazolyl))-thiophene in toluene (4 g/l) as scintillant. Descending paper chromatography was performed on Whatman no. 1 paper in isobutyric acid/1 M ammonia (5:3, v/v) for 48 h. Chromatograms were dried and left in contact with Kodak Blue Brand X-ray film for 2–3 weeks.

UDP–GlcNAc was obtained from Boehringer Mannheim GmbH, UDP–[^{14}C]GlcNAc (300 Ci/mol) from the Radiochemical Centre, Amersham, and Chalaropsis lysozyme was the generous gift of Dr Hash. All other chemicals were of Analar reagent quality whenever these were available.

3. Results and discussion

Peptidoglycan precursors were incorporated linearly for ⩾1 h (fig.2). Incorporation was dependent on the presence of UDP–MurNAc–pentapeptide and was sensitive to β-lactam antibiotics. These observations suggested that genuine peptidoglycan synthesis was occurring and that this involved the covalent attachment of nascent peptidoglycan to pre-existing wall. The complete sensitivity to low levels of cloxacillin indicated that the attachment of the nascent peptidoglycan to wall was by transpeptidation only. UDP–MurNAc–tetrapeptide was not incorporated after an initial 5–10 min period.

The most likely reason for the lack of incorporation of UDP–MurNAc–tetrapeptide is that a

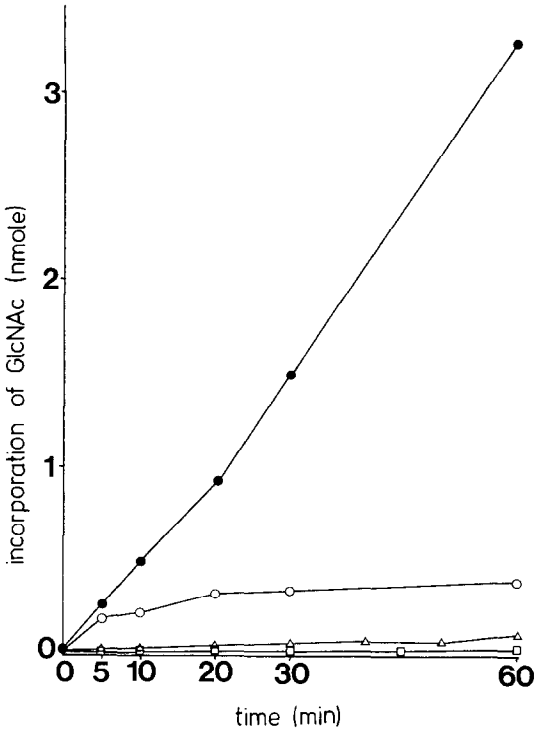

Fig.2. Incorporation of peptidoglycan precursors into toluenised cells. Transpeptidation assay mixtures were incubated at 23°C and the reaction terminated by addition of cold TCA. After centrifugation the resulting pellets were treated with 4% SDS at 100°C for 20 min before filtration on glass fibre discs and determination of radioactivity incorporated. In order to detect low levels of incorporation of MurNAc–tetrapeptide the specific activity of UDP–GlcNAc was increased 2.5-fold for this incubation. All assay tubes contained UDP–[^{14}C]GlcNAc. The nucleotide sugar peptide present was: (•) UDP–MurNAc–pentapeptide; (○) UDP–MurNAc–tetrapeptide; (△) UDP–MurNAc–pentapeptide in the presence of cloxacillin (1 μg/ml); and (□) none.

unidirectional transpeptidation reaction was occurring which required a donor MurNAc-pentapeptide in the nascent peptidoglycan. (The alternative explanation that the lack of incorporation is due to the nascent peptidoglycan synthesising enzymes not recognising the UDP–MurNAc–tetrapeptide is considered below.)

The incorporation of MurNAc–tetrapeptide in the first 5–10 min may have been due either to addition of the substrate onto incomplete nascent peptidoglycan chains previously containing some MurNAc–pentapeptide or to the presence of some endogenous UDP–MurNAc–pentapeptide in the toluenised preparation, which could then function as the donor, since it only takes one transpeptidation event to result in attachment of the whole nascent peptidoglycan chain to the wall. In order to investigate this possibility the reaction products were analysed after incubation of the toluenised cells with various potential peptidoglycan precursors as detailed in table 1. Little dimer was formed in the presence of UDP–MurNAc–tetrapeptide although more was formed when the label was in the UDP–GlcNAc rather than in the UDP MurNAc–tetrapeptide. This would be

Table 1
Analysis of reaction products after incubation of toluenised cells with potential peptidoglycan precursors

Peptidoglycan precursors		Disaccharide peptide dimer (nmol)[a]
Labelled	Unlabelled	
UDP–MurNAc–pentapeptide	UDP–GlcNAc	4.080
UDP–GlcNAc	UDP–MurNAc–tetrapeptide	0.235
UDP–MurNAc–tetrapeptide	UDP–GlcNAc	0.130

[a] These are maximum values calculated by assuming that all the disaccharide peptide dimer has been formed by crosslinking labelled nascent peptidoglycan to unlabelled wall

Toluenised cells were incubated for 90 min at 23°C with the peptidoglycan precursors indicated above followed by investigation of the reaction products after digestion with Chalaropsis lysozyme as described in the text

expected if the above explanation for the initial incorporation of UDP–MurNAc–tetrapeptide were correct since labelled UDP–GlcNAc could form dimers by transpeptidation into wall with endogenous UDP–MurNAc–pentapeptide or MurNAc–pentapeptide attached to a lipid intermediate. However this does not account for the fact that some dimer formation has occurred with the label in UDP–MurNAc–tetrapeptide. This can be explained since some MurNAc–tetrapeptide will enter the wall by the mechanism outlined above which can then act as an acceptor in transpeptidation reactions occurring in the wall using pre-existing wall MurNAc–pentapeptide as donor. It has already been demonstrated in vivo that transpeptidation proceeds for ⩾25 min after the initial incorporation into wall [16].

An alternative method of investigating the direction of transpeptidation has been used [13]. This involved blocking the free amino group of UDP–MurNAc–pentapeptide by acetylation, thus making the substrate usable only as a donor and not as an acceptor (which requires a free amino group on Dap). If transpeptidation in *B. megaterium* proceeds unidirectionally by transfer of a MurNAc pentapeptide donor in the nascent peptidoglycan to an acceptor amino group on Dap in the wall, this acetylated substrate should be incorporated by toluenised preparations of the organism. However the results in fig.3 indicate that the substrate was not incorporated. This could be explained by the transpeptidase(s) not recognising the acetylated pentapeptide since it is an unnatural substrate. Indeed it was found [13] that incorporation into *B. licheniformis* was reduced by 77% with this substrate but whether this was due to reduced efficiency with the unnatural substrate or by some transpeptidation occurring in the opposite direction was not ascertained.

The lack of transpeptidation into wall with UDP–MurNAc–tetrapeptide or UDP–MurNAc–Acetylpentapeptide due to the inability to synthesise nascent peptidoglycan was investigated in the following manner. The effect of these unnatural nucleotide substrates on synthesis of nascent peptidoglycan was tested using a purified membrane preparation rather than a toluenised preparation. Significant quantities of nascent peptidoglycan were produced with these unnatural nucleotide substrates (60% and 80%, respectively, of the rate obtained using the two

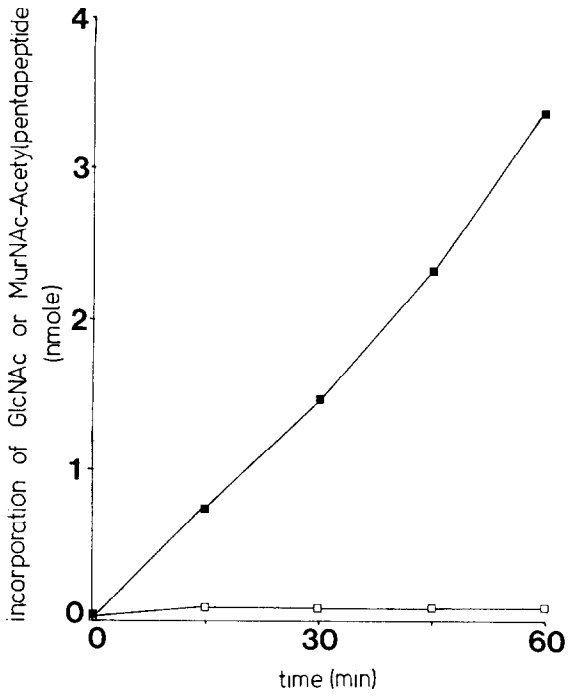

Fig.3. Incorporation of UDP–MurNAc–acetylpentapeptide into toluenised cells. Details as for fig.2 except that the label was in UDP–MurNAc–acetylpentapeptide for (□). (■) Incorporation from UDP–MurNAc–pentapeptide and UDP–[^{14}C]-GlcNAc. (○) Incorporation from UDP–MurNAc–L-Ala–D-Glu–(D)-[*acetyl*-^{14}C]acetyl-meso-Dap–D-Ala–D-Ala and UDP–GlcNAc.

natural precursors which were incorporated at 10 nmol/mg protein/h). Therefore the lack of incorporation into wall from labelled UDP–MurNAc–tetrapeptide and UDP–MurNAc–acetylpentapeptide was the result of specificity at the level of the transpeptidase step. The production of nascent peptidoglycan from UDP–MurNAc–tetrapeptide would result in the nascent peptidoglycan containing MurNAc–tetrapeptides. These MurNAc–tetrapeptides would be natural substrates for the transpeptidase step if any transpeptidation was occurring with acceptor in the nascent peptidoglycan and donor in the pre-existing wall as shown for *G. homari*. Since no incorporation of this substrate occurred all the transpeptidation must occur in the opposite direction. The lack of incorporation of UDP–MurNAc–acetylpentapeptide is probably due to the transpeptidase(s) not

recognising this unnatural substrate as a donor. However there is not even an initial incorporation period of 5–10 min as for UDP MurNAc–tetra-peptide. This can be explained by the transpeptidase(s) not recognising the natural donor (MurNAc–penta-peptide) when the unnatural donor (MurNAc–acetylpentapeptide) is also present in the nascent peptidoglycan. Such extraordinary specificity of a transpeptidase has been postulated in *G. homari* where MurNAc–L-Ala–D-Glu–L-Lys–D-Ala–D-Ala must be converted to MurNAc–L-Ala–D-Glu–L-Lys–D-Ala in order to act as an acceptor and also the nascent peptidoglycan must contain MurNAc–L-Ala–D-Glu–L-Lys [17].

Hence we report a unidirectional transpeptidation reaction in toluenised cells of *B. megaterium* that is opposite in direction to that reported in wall + membrane preparations of *G. homari* [12]. Preliminary studies with toluenised cells of *G. homari* confirm the findings of Hammes and Kandler (unpublished data).

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