POLYMERIZATION BY TRANSGLYCOSYLATION IN THE BIOSYNTHESIS OF THE PEPTIDOGLYCAN OF *ESCHERICHIA COLI* K 12 AND ITS INHIBITION BY ANTIBIOTICS

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1. Introduction

The biosynthesis of bacterial cell wall peptidoglycan is a complex process involving cytoplasmic and membrane steps [1]. *N*-Acetylglucosaminyl-*N*-acetylmuramyl-(pentapeptide)-pyrophosphoryl-undecaprenol is the last membrane precursor prior to polymerization which proceeds by transglycosylation (formation of the linear glycan strands) and transpeptidation (crosslinking of the peptide subunits) [1]. This membrane intermediate has been utilized in cell-free systems for the formation of peptidoglycan [2–4], but the difficulty in conveniently isolating it in adequate amounts has greatly limited the direct investigation of the polymerizing enzymes. Most commonly, the in vitro synthesis of peptidoglycan is carried out by incubating the cytoplasmic precursors, UDP-*N*-acetylmuramyl-pentapeptide and UDP-*N*-acetylglucosamine, with appropriate particulate preparations, crude cell walls or treated cells [3–10] (see [1] for ref. before 1972). Under these conditions only the over-all course of the different membrane reactions is considered. However, a study of each membrane step in itself is essential for the understanding of the mode of action of certain antibiotics, for the development of the genetic analysis of these reactions and for the determination of the mechanisms involved in their regulation. Recently, the transpeptidation step, uncoupled from the other membrane reactions, has been successfully studied with artificial systems of donor and acceptor peptides [1,11]. As far as we are aware, the transglycosylation step has not yet been directly investigated to any extent.

The present paper describes a convenient in vitro assay specific for the transglycosylation reaction which promotes the formation of uncrosslinked peptidoglycan. The main features of the assay were the use of *N*-acetylglucosaminyl-*N*-acetylmuramyl-(pentapeptide)-pyrophosphoryl-undecaprenol as substrate, the use of particulate fraction from *E. coli* K12 in the presence of penicillin G, the high stimulating effect of 0.1% sodium deoxycholate and the inhibition by antibiotics such as 11837 R.P. and moenomycin.

2. Materials and methods

2.1. *Preparation of the membrane precursor and of the particulate fraction*

N-Acetylglucosaminyl-*N*-acetylmuramyl-(pentapeptide)-pyrophosphoryl-undecaprenol containing D-[^{14}C]alanine residues was prepared as in [12], slightly modified. The amino acid and hexosamine composition of the purified product (2.1 mCi/mmol) was: glutamic acid, 1; alanine, 2.7; diaminopimelic acid, 1; muramic acid, 0.9; glucosamine, 0.9.

E. coli K12 HfrH was grown under strong aeration at 37°C in a 20 liter fermentor in the medium in [13]. Cells were harvested at 1/3rd maximal growth, washed with 0.02 M Tris–HCl buffer, pH 8, and disrupted by grinding with alumina as in [14]. The cell–alumina mixture was suspended in 0.05 M Tris–HCl buffer, pH 7.5, containing 10^{-3} M 2-mercaptoethanol and 10^{-4} M $MgCl_2$, centrifuged for 5 min at 10 000 × *g*. The supernatant was centrifuged for 1 h at 100 000 × *g*. The pellet was washed once with the same buffer, suspended at a protein concentration of 30 mg/ml and used as source of enzyme.

2.2. *Enzymatic assay*

The assay for transglycosidase activity was performed by incubating at 30°C for 30 min 5 nmol ^{14}C-labeled precursor (23 000 dpm) with 10 μl 0.5 M Tris–HCl buffer, pH 7.5, 3 μl 0.1 M $MgCl_2$, 5 μl 0.25% penicillin G solution, 5 μl 0.5% sodium deoxycholate solution and 5 μl enzyme. The reaction products were separated by chromatography on Whatman no. 1 filter paper run overnight in isobutyric acid–1 M ammonia (5:3). After localization with a 4π Tracerlab scanner the radioactive spots were cut out and converted into $^{14}CO_2$ by combustion in an Oxymat apparatus (Intertechnique, Plaisir, 78, France). $^{14}CO_2$ was trapped in 18 ml solvent containing 7 g biphenyl-4-butyl-PBD[(biphenylyl-4)-2-(*tert*-butyl-4-phenyl)-5-oxadiazole-1,3,4] for 100 ml water, 220 ml methanol, 330 ml phenethylamine and 400 ml toluene. Samples were counted by liquid scintillation spectrometry.

2.3. *Digestion of the in vitro polymerized material by egg-white lysozyme*

The assay was scaled up 5-fold and the incubation prolonged for 1 h. After paper chromatography the polymerized material remaining at the origin was soaked with 0.1 M ammonium acetate (pH 6.5) containing 0.15% egg-white lysozyme and incubated overnight at 37°C in a screwed tube. By elution with water 90% of the radioactivity was recovered. The resulting degradation products were chromatographed on Whatman 3 MM filter paper run 3 days in *n*-butanol-acetic acid-water (4:1:5; upper phase) and compared with monomer and dimer peptidoglycan fragments of known structure[15]. R_F values were expressed with disaccharide tetrapeptide C6 taken as reference.

3. Results and discussion

3.1. *In vitro formation of peptidoglycan*

When *N*-acetylglucosaminyl-*N*-acetylmuramyl-(pentapeptide)-pyrophosphoryl-undecaprenol was incubated with the particulate preparation from *E. coli* K12 HfrH in the absence of detergent and the mixture was analysed by paper chromatography, it appeared that about 5% of the membrane precursor had been converted into immobile polymerized material (table 1). In all cases penicillin G was added to the assay so as to inhibit the transpeptidation step of polymerisation and the D–D-carboxypeptidase activity associated with the particulate fractions, which could catalyse the release of alanine (R_F 0.60) from the membrane precursor or from the newly-formed peptidoglycan. The addition of detergents such as sodium deoxycholate or Triton X-100 greatly enhanced the amount of in vitro synthesized material (table 1). In pacticular, sodium deoxycholate at a final conc. 0.1% led to a 10-fold stimulation of the reaction. Under these conditions, the time course of formation of peptidoglycan was essentially linear for the first 30 min. After 1 h the formation of side-products still remained low and up to 65% of the membrane precursor could be converted into polymerized material.

Table 1
Effect of sodium deoxycholate and Triton X-100 on the transglycosylation reaction catalysing the formation of peptidoglycan

Detergent added (final conc.)		Membrane precursor (%)	Peptidoglycan (%)
None		95	5
Sodium deoxycholate	(0.01%)	90	10
Sodium deoxycholate	(0.1%)	46	54
Sodium deoxycholate	(1%)	88	12
Triton X-100	(0.01%)	86	14
Triton X-100	(0.1%)	73	27

Assays were carried out as in section 2. The appropriate amounts of detergent were added prior to the addition of enzyme. Values are expressed as % each compound as compared to the sum of both

3.2. *Characterization of the in vitro synthesized peptidoglycan*

When the in vitro polymerized material was digested with lysozyme and the degradation products analysed by paper chromatography, essentially one component (R_F 1.07) accounting for 90% of the digested material was encountered. No well defined fragment of lower R_F value (dimer or oligomer) was detectable and the residual radioactivity in this region was estimated at 3% of the solubilized material. A faster moving component (R_F 1.50) was detected and accounted for 5% of all the radioactivity.

The main component (R_F 1.07) was further puri-

fied by paper electrophoresis and its composition was determined: alanine, 3; glutamic acid, 1; diaminopimelic acid, 1; muramic acid, 1; glucosamine, 1. Furthermore, it had the same electrophoretic mobility at pH 2 as monomer C6 [15]. These results showed that it was presumably the disaccharide pentapeptide monomer. Since lysozyme digestion yielded mainly this fragment, it was concluded that uncrosslinked peptidoglycan was formed and that the assay was specific for the transglycosylation reaction. Our material thus resembled the soluble peptidoglycan synthesized from the nucleotide precursors under other conditions [3,8,9,16]. The length of the glycan strands and the nature of the reducing ends are now being examined.

3.3. *Effect of antibiotics*

Several antibiotics known to interfere with the biosynthesis of peptidoglycan were tested (table 2).

Table 2
Effects of antibiotics on the transglycosylation reaction catalysing the formation of peptidoglycan

Antibiotic added	Final conc. (μg/ml)	% Inhibition
Bacitracin[a]	100	20
	10	0
Novobiocin[b]	100	10
	10	0
Ristocetin[c]	100	87
	10	8
	1	5
Vancomycin[d]	100	100
	10	38
	1	3
11837 R.P.	1	100
	0.1	95
	0.01	5
Moenomycin	1	100
	0.1	100
	0.01	39

[a] Purchased from Sigma, St Louis, MO
[b] Purchased from Théraplix, 45500 Gien
[c] Gift from Abbott Lab. Chicago, IL
[d] Gift from Eli Lilly Co., Indianapolis, IN

The results obtained with ristocetin and vancomycin were similar to [3] for another system from *E. coli*.

Compound 11837 R.P. [17] and moenomycin [18] were the best inhibitor of the reaction since they had ID_{50} values from 0.01–0.1 μg/ml. They thus most probably interact with the transglycosidase itself and not with the substrate. They interfered with the biosynthesis of peptidoglycan [18–20] and it had been speculated [21] that moenomycin might inhibit the elongation of the glycan chains. MIC values from 8–250 μg/ml were reported when moenomycin and 11837 R.P. were tested on *E. coli* [22]. These high values are presumably due to difficulties of penetration. With gram-positive bacteria MIC values from 0.005–0.3 μg/ml were found [22]. The inhibition of the transglycosylation reaction by these antibiotics is thus probably the main mechanism responsible for their antibacterial activity. Moenomycin and 11837 R.P. will undoubtly be useful tools for the further investigation of the transglycosylation step and of the other membrane reactions involved in the biosynthesis of peptidoglycan.

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