A novel glycan polymerase that synthesizes uncross-linked peptidoglycan in *Escherichia coli*

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A simple and efficient procedure to assay peptidoglycan synthesis in vitro was established. By this procedure, a novel activity for glycan polymerization in *Escherichia coli* was found in the fraction containing no detectable penicillin-binding protein (PBP). This polymerase activity was relatively insensitive to moenomycin, showed requirement for Ca^{2+} or Mn^{2+} but not for Mg^{2+}, and led to production of uncross-linked glycan chains. These properties distinguished the glycan polymerase from the activities shown by the fractions containing PBPs. The glycan polymerase catalyzing polymerization of glycan units from lipid intermediates was purified and identified as a protein of 34 kDa.

In vitro peptidoglycan synthesis *Lipid intermediate* *Glycan polymerase*
Peptidoglycan cross-linking *Penicillin-binding protein* *Moenomycin*

1. INTRODUCTION

The last stage of peptidoglycan synthesis in bacteria involves polymerization of disaccharide units from lipid intermediates and transpeptidation of peptide chains to cross-link adjacent glycan chains, the latter reaction being sensitive to penicillins [1]. In *Escherichia coli*, 7 major penicillin-binding proteins (PBPs) were described [2–5], and those with higher molecular masses, first PBP-1b [6–8] and subsequently PBP-1a [9] and PBP-3 [10], were identified as bifunctional enzymes that carry out glycan polymerization as well as transpeptidation. PBP-1b appeared to be a major peptidoglycan synthetase in *E. coli*, because the membrane fractions of mutants defective in this PBP failed to support in vitro peptidoglycan synthesis and accumulated lipid intermediates [4,5].

A part of the work was presented at the International FEMS Symposium on the Murein Sacculus of Bacterial Cell Walls, Berlin, March 13–18, 1983 [11]

Abbreviations: PBP, penicillin-binding protein; DMSO, dimethyl sulfoxide; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); DTT, dithiothreitol

We found, however, that the activity for peptidoglycan synthesis was detectable even in the membrane fractions defective in PBP-1b on addition of aprotic polar substances like glycerol to the reaction mixture [11]. By improving the assay system for peptidoglycan synthesis by solubilized protein fractions, we identified a glycan polymerase which did not bind benzylpenicillin and had no transpeptidase activity.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and growth conditions*

JE5782 is a mutant defective in PBP-1b (*ponB1085* [8]) and JE5783 (F^- *str tonA thi his met ile trp leu argG purE*) is its isogenic strain of wild type for PBP-1b. EJ801 defective in PBP-1b is a derivative of JE5702 (F^- *lac strA tonA metB proA tsx ponA1104 ponB1085 dacA1191 dacB12* [8]) and has the same genetic background for PBPs but can grow well in an ordinary medium without an extra addition of magnesium and sucrose. All strains were grown at 30°C in an enriched medium containing buffered salts [12].

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2.2. *Measurement of activity for peptidoglycan synthesis*

^{14}C-labeled lipid intermediates (103 Ci/mol) were allowed to accumulate in vitro in the membrane fraction prepared from EJ801 [8]. UDP-*N*-acetylmuramyl-pentapeptide and UDP-*N*-acetyl-[^{14}C]glucosamine (Amersham) were the substrates. The lipid intermediates were extracted with either 10 vols of chloroform/methanol (1:1, v/v) [8] or 3 vols of *n*-butanol/6 M pyridinium acetate (pH 4.2) (2:1, v/v) [13], dried and dissolved in *n*-octanol at a concentration of 12–18 μM. A 40 μl reaction mixture contained 5 μl of the lipid intermediate solution, 10 μl DMSO, 50 mM Pipes–NaOH buffer (pH 6.5), 0.2 mM DTT, 10 mM $CaCl_2$ (where indicated) and an appropriate amount of enzyme preparation. The mixture was incubated at 30°C for 15–90 min and subjected to paper chromatography with isobutyric acid/1 M ammonia (5:3, v/v).

2.3. *Analysis of reaction products for cross-linking*

The synthesized peptidoglycan remaining at the paper-chromatographic origin was digested with egg white lysozyme (more than 75% of the radioactivity was recovered in the digest) and separated into disaccharide-peptide monomers and cross-linked dimers by paper electrophoresis with 7% formic acid [14].

2.4. *Purification of glycan polymerase*

The solubilized membrane proteins that passed through a DEAE–cellulose column were applied to a CM–agarose (CM Bio-Gel A) column (see legend to table 1). Tris–maleate–NaOH buffer at 10 mM (pH 5.6 or 6.8) containing 0.1% Triton X-100 and 2 mM 2-mercaptoethanol was used throughout the column operations detailed below. The adsorbed proteins were eluted from the CM–agarose column with a linear NaCl gradient from 50 to 350 mM at pH 5.6. The active materials for glycan synthesis were eluted at about 150 mM NaCl. After adjustment of the pH to 6.8 and NaCl concentration to 100 mM, the eluate containing the active materials was applied to a hydroxyapatite (Bio-Gel HT) column prewashed with 100 mM NaCl at pH 6.8. The fraction eluted stepwise with 1 M NaCl was dialyzed against a pH 5.6 buffer containing 50 mM NaCl and chromatographed on a P–cellulose (Bio-Rad Cellex P) column at pH 5.6 with an NaCl gradient from 50 mM to 3 M. The active material was eluted at about 0.8 M. The enzyme was finally purified by rechromatography on P–cellulose. The purified enzyme was concentrated on P–cellulose and dialyzed against 10 mM Tris–HCl buffer (pH 7.4) containing 50% glycerol, 0.5 M NaCl and 0.2 mM DTT.

3. RESULTS AND DISCUSSION

3.1. *Detection of glycan polymerase activity other than PBP-1b*

An aprotic polar substance such as glycerol was found to stimulate peptidoglycan synthesis by the membrane fraction of *E. coli* defective in PBP-1b [11]. However, glycerol was not efficient for peptidoglycan synthesis by solubilized protein fractions. The assay system for solubilized enzymes depends on lipid intermediates as a substrate and the insolubility of the substrate in an aqueous reaction mixture appeared to be a major obstacle to activity measurement. Among the aprotic polar substances, DMSO was thought to be more suitable for dissolving hydrophobic materials but it was a rather poor solvent for lipids. In search of a better solvent, we found *n*-octanol to be excellent for dissolving lipids without impairment of enzymatic activities for glycan polymerization.

The improved simple procedure to assay activity for peptidoglycan synthesis in vitro is described in section 2. This procedure allowed the detection and quantification of active components for peptidoglycan synthesis in solubilized fractions devoid of PBP-1b.

3.2. *Separation of glycan polymerase activities into fractions with and without penicillin-binding activity*

From the membrane fractions of strain JE5782 defective in PBP-1b (PBP-1b$^-$) and its isogenic strain JE5783, wild type for PBP-1b (PBP-1b$^+$), two protein fractions that were adsorbed on DEAE–cellulose (DE-fraction) and on CM–cellulose (CM-fraction) were prepared as described in the legend to table 1, and examined for penicillin-binding and peptidoglycan-synthesizing activities. The CM-fraction did not contain any detectable PBP, while the DE-fraction contained PBP-1a, -1b, -2, -3 and -4, except for the absence

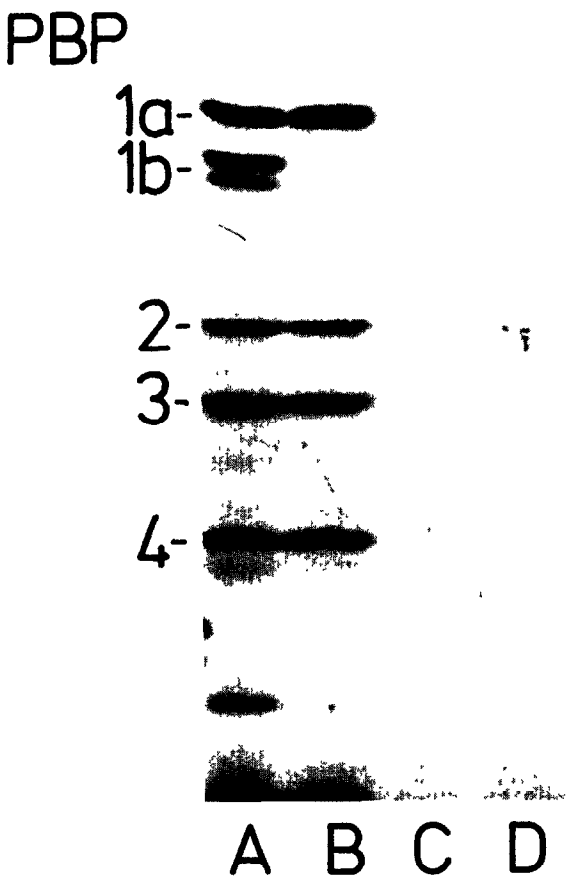

Fig.1. Electrophoretic analysis of PBPs in fractions separated by ion-exchange celluloses. A portion of each fraction was treated with benzyl[^{14}C]penicillin (59.5 Ci/mol, Amersham) and analyzed by SDS-gel electrophoresis and fluorography as in [2,5]. Lanes A (JE5783) and B (JE5782), DE-fractions; C (JE5783) and D (JE5782), CM-fractions.

of PBP-1b in the mutant JE5782 (fig.1). PBP-5 and -6 were found in the fraction adsorbed neither to DEAE–cellulose nor to CM–cellulose. Activity for glycan synthesis was measured for each fraction in the linear range of reaction rate with respect to both reaction time and amounts of the active components in each fraction. The CM-fractions showed striking activity for glycan synthesis from lipid-linked precursors. The level of activity in the CM-fraction was almost the same in the PBP-1b$^-$ mutant and in its isogenic PBP-1b$^+$ strain. In the PBP-1b$^-$ mutant, synthetic activity in the CM-fraction amounted to 77% of the total activity (table 1). The products by the CM-fraction were susceptible to lysozyme digestion; monomers but no dimer were detected in the digests, whereas lysozyme digestion of the products by the DE-fractions released both monomers and dimers in proportions representing about 20% (or higher in other experiments) cross-linking (table 1).

Table 1

Fractionation of glycan polymerase activities with ion-exchange celluloses

Strain	Fraction[a]	Protein[b]	Polymerase activity[c]	Cross-linking		
				Monomer (M) (cpm)	Dimer (D) (cpm)	D/(M+D) (%)
JE5782 (PBP-1b$^-$)	DE	43.0	2.43	651	184	22
	CM	10.3	7.95	974	ND	–
JE5783 (PBP-1b$^+$)	DE	45.9	6.91[d]	902	227	20
	CM	10.5	7.82	1022	ND	–

[a] Membrane proteins were solubilized with 2% Triton X-100 and 1 M NaCl [8] and fractionated in the presence of 50 mM NaCl. The DE-fraction was first separated as materials adsorbed on DEAE–cellulose (Whatman DE52) at pH 8 (10 mM Tris–HCl); and from the fraction unadsorbed on DEAE–cellulose, the CM-fraction was recovered as materials adsorbed on CM–cellulose (Whatman CM52) at pH 5.6 (20 mM Tris–maleate–NaOH). The materials adsorbed on the ion-exchangers were eluted stepwise at 2 M NaCl with steps to reduce Triton concentration to 0.1%

[b] Percent of solubilized membrane proteins

[c] Total activity in each fraction was normalized for the total amount of solubilized protein in the starting material and expressed in pmol/min per mg protein

[d] Underestimated because the assay condition was not optimum for the activity contributed by PBP-1b

ND, not detected

The results indicate that glycan polymerase in the CM-fraction can contribute to the major part of glycan synthesis in the PBP-1b$^-$ mutant and that almost the same level of polymerase activity is present in a cell, irrespective of the defect in PBP-1b. It is unlikely that the activity in the CM-fraction may result from the mutant type of PBP-1b defective in penicillin-binding activity but still active for glycan polymerization.

3.3. *Properties of glycan polymerase in the CM-fraction*

Properties of glycan polymerases in the CM-fraction were investigated in comparison with glycan polymerases in the DE-fractions.

Benzylpenicillin was neither inhibitory nor stimulatory to glycan synthesis by the CM-fraction (table 2). Slight stimulation in glycan synthesis was observed for the DE-fraction of wild type. The stimulated activity may be ascribed to PBP-1b [7]. Glycan synthesis by the DE-fraction of PBP-1b$^-$ mutant was slightly reduced in the presence of benzylpenicillin.

Vancomycin inhibited glycan polymerase activity in the CM-fraction as well as in the DE-fractions. However, moenomycin was far less effective on the activity in the CM-fraction: Almost 90% of the activity remained at 1 μg/ml in the CM-fraction, whereas about 80% of the activities in DE-fractions were inhibited even at 0.1 μg/ml (table 2). Vancomycin is a glycopeptide that binds to the peptide moiety of substrates for peptidoglycan synthesis [15]; moenomycin is a phosphoglycolipid antibiotic [16] and probably interacts with enzymes. Such a difference in the mechanism of action may be a cause of different effects on glycan polymerases.

Effects of divalent cations are shown in fig.2. Addition of EDTA at 5 mM to the reaction mixture strongly inhibited glycan polymerase activity in the CM-fraction. Further addition of $CaCl_2$ or $MnCl_2$ at 10 mM not only reversed the effect but enhanced glycan synthesis. $CoCl_2$ showed a similar effect but restoration of activity was only partial. $MgCl_2$ was not effective at all; $CdCl_2$, $ZnSO_4$ and $CuSO_4$ were all ineffective. Glycan synthesis was not significantly affected by addition of EDTA in the DE-fraction of the PBP-1b$^-$ mutant but was slightly stimulated in the DE-fraction of the wild type, although EDTA sequestered Mg^{2+} which was reported to stimulate in vitro glycan synthesis performed with a membrane fraction [17].

The pH optimum for glycan synthesis was 6.0–6.5 in the CM-fraction, around 6.5 in the PBP-1b$^-$ DE-fraction and 7.5–8.5 in the PBP-1b$^+$ DE-fraction.

These results show that glycan polymerase contained in the CM-fraction has properties different

Table 2

Effects of antibiotics on the glycan polymerase activities of CM- and DE-fractions

Antibiotic added	Final conc. (μg/ml)	Relative activity (% of control)		
		CM-fraction	DE-fraction (PBP-1b$^+$)	DE-fraction (PBP-1b$^-$)
Benzylpenicillin	10	105	111	89
	100	99	141	85
Vancomycin	10	7	39	24
	100	9	25	25
Moenomycin	0.1	93	22	23
	1	89	16	25

Assays were performed in the linear range of reaction rate. Activities in the absence of antibiotics (100%) were: 90.9 pmol/mg protein per min in the experiments with benzylpenicillin (Sigma) and moenomycin (Hoechst) and 50.2 pmol/mg protein per min in the experiment with vancomycin (Sigma) for the CM-fraction (JE5783); 8.38 pmol/mg protein per min for the PBP-1b$^+$ DE-fraction (JE5783); 3.42 pmol/mg protein per min for the PBP-1b$^-$ DE-fraction (JE5782)

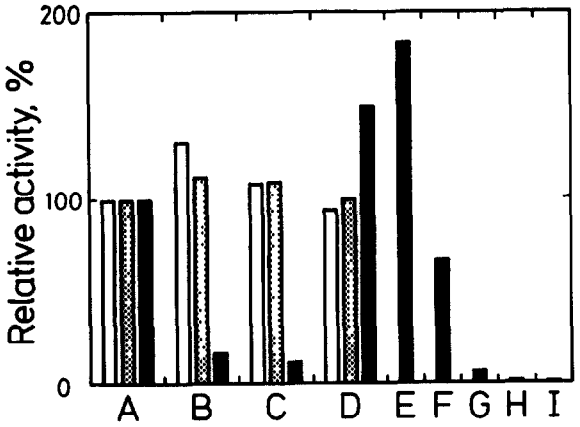

Fig.2. Effects of EDTA and divalent cations on glycan polymerase activities of DE- and CM-fractions. A, no addition; B, 5 mM EDTA added. C–I, 5 mM EDTA and 10 mM divalent cations added in: C, $MgCl_2$; D, $CaCl_2$; E, $MnCl_2$; F, $CoCl_2$; G, $CdCl_2$; H, $ZnSO_4$; I, $CuSO_4$. Assays were performed in the linear range of reaction rate. Open bars, PBP-1b$^+$ DE-fraction (JE5783); shaded bars, PBP-1b$^-$ DE-fraction (JE5782); closed bars, CM-fraction (JE5783). Activities without any addition (100%) were: 8.38 pmol/mg protein per min for the PBP-1b$^+$ DE-fraction, 3.42 pmol/mg protein per min for the PBP-1b$^-$ DE-fraction and 50.2 pmol/mg protein per min for the CM-fraction.

from those found in the DE-fractions in which glycan polymerase activities are presumably contributed by PBPs.

3.4. *Identification of glycan polymerase*

Glycan polymerase found in the CM-fraction was purified from cells of JE5783, wild type for PBPs, as described in section 2. At the final step of purification on P–cellulose column chromatography, the peak of activity for glycan polymerization corresponded to the intensity peak in the electrophoretic band of protein as shown in fig.3.

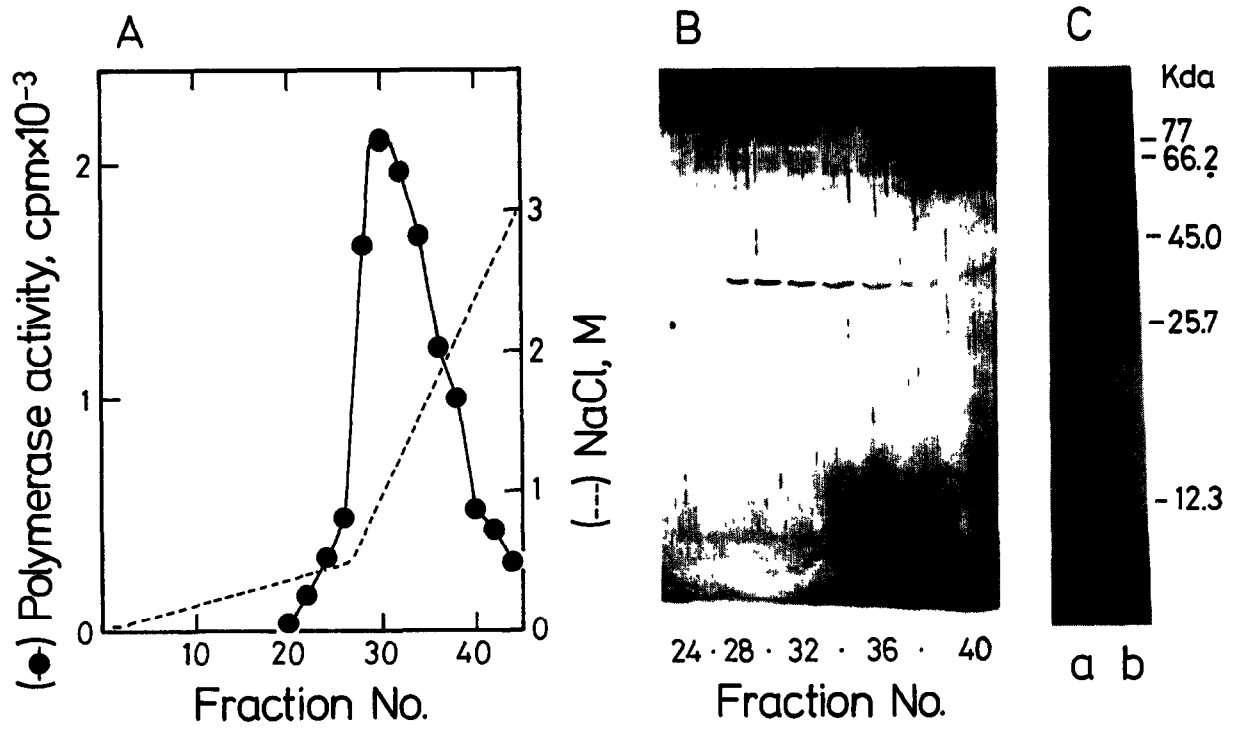

Fig.3. Purification of glycan polymerase on a P-cellulose column. (A) The activities in the fractions eluted from the final P–cellulose column were measured in the presence of 10 mM $CaCl_2$. (B) Proteins in the fractions in A were analyzed by SDS-gel electrophoresis on 12% polyacrylamide and by silver-staining. (C) Purified enzyme preparation (a) and standard proteins for molecular mass calibration [(b) ovotransferrin, 77 kDa; serum albumin, 66.2 kDa; ovalbumin, 45.0 kDa; chymotrypsinogen, 25.7 kDa; cytochrome *c*, 12.3 kDa] were electrophoresed on a 12% gel and stained with Coomassie brilliant blue.

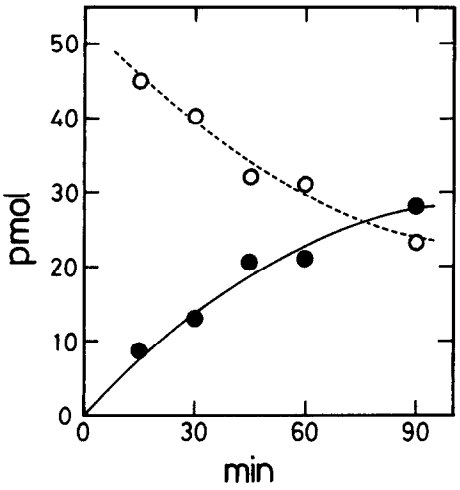

Fig.4. Glycan polymerization by purified enzyme preparation. The activity of 6.2 ng of the enzyme was measured in the presence of 10 mM $CaCl_2$. Closed circles, polymerized glycan; open circles, lipid intermediates

Thus, the glycan polymerase was identified as this protein of molecular mass 34 kDa. The purified enzyme catalyzed polymerization of disaccharide units from lipid intermediates (fig.4). The initial velocity of polymerization was about 95 nmol/mg protein per min, or 3.2 mol/mol enzyme per min. This rate is much higher than the V_{max} of 15.4 nmol/mg protein per min determined for purified PBP-1b (unpublished). The glycan polymerase showed no transpeptidase activity and its products were not cross-linked.

A not insignificant part of the peptidoglycan synthesis in *E. coli* may be sustained by this glycan polymerase in cooperation with an as yet unelucidated cross-linking enzyme. If sacculi are synthesized via macromolecular intermediates [18,19], the polymerase might be involved in synthesis of nascent linear chains; or [20,21], the polymerase and the cross-linking enzyme might function in a coupled fashion.

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