IN VITRO PEPTIDOGLYCAN POLYMERIZATION CATALYSED BY PENICILLIN BINDING PROTEIN 1b OF *ESCHERICHIA COLI* K-12

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1. Introduction

The discovery of penicillin binding proteins (PBP) in bacteria [1,2] and the isolation of mutants defective in these proteins [3,4] have provided a new approach to the study of the β-lactam-sensitive enzymes involved in the biosynthesis of peptidoglycan and of their correlation with cell elongation and cell division. In *Escherichia coli*, 7 PBPs have been described and their possible physiological roles speculated [3–6]. In particular, it was suggested that PBP-1b is directly involved in the polymerization steps of the biosynthesis of peptidoglycan [4,5,7,8]. This polymerization is known [9,10] to proceed at the expense of the lipid intermediate *N*-acetylglucosaminyl-*N*-acetylmuramyl-(pentapeptide)-pyrophosphoryl-undecaprenol by formation of the linear glycan strands (transglycosylation step) and crosslinking of the peptide subunits (transpeptidation step). The data presented here clearly substantiate the fact that PBP-1b can catalyse polymerization from the purified specifically radiolabelled lipid intermediate. This was established in two different ways:

(1) The polymerizing activity of particulate fractions from strains of *E. coli* defective in PBP-1b were investigated by using the assay for transglycosylation in [11].
(2) Purified PBP-1b was tested in a similar way to determine whether it possessed polymerizing activity.

2. Materials and methods

2.1. *Strains and growth conditions*

The following strains of *E. coli* K-12 were used: JE 7319 *ponB 704 dacA 1191 dacB 12 ponA^{ts} 1104 F$^-$ leuA str lac^{del} metB tonA;* JE 5784 *ponB 704 F$^-$ leu thi metA ile argG his trp purE str ara mtl xyl gal tonA;* JE 5605 *dacA 1191 dacB 12 F$^-$ aroB xyl str;* and JE 5770 *strA 120 ponA 980 ftsI 730 dacA 1191 dacB 12* pLC 19-19. Plasmid pLC 19-19 is a Col E1 plasmid synthesized in [12]. We found that pLC 19-19 carries a gene coding for PBP-1b and leads to the host cell overproducing PBP 1b at ~10-times the level of wild-type (in preparation). All strains were grown in L-broth (1% Difco Bactotrypton, 0.5% Difco-Yeast Extract, 0.5% NaCl and 0.1% glucose). In the case of strain JE 7319 the growth medium was supplemented with 0.5% NaCl, 12% sucrose and 0.2% $MgSO_4 \cdot 7\ H_2O$, because this strain lyses at 42°C with double mutation *ponA^{ts}* and *ponB 704* present [4]. Cells were grown at 30°C (JE 5770 and JE 7319) or at 37°C (JE 5605 and JE 5784) and harvested at 1/3rd maximal growth (JE 5605, JE 5784 and JE 7319) or at the late log phase (JE 5770).

2.2. *Preparation of PBP-1b, lipid intermediate and particulate fractions*

PBP-1b was purified from strain JE 5770/pLC 19-19 as in [13] with some modification [14]. In this way it was possible to obtain PBP-1b with nearly 90% purity (fig.1) in a single step by using affinity chromatography on β-lactam Sepharose gels. The lipid intermediate *N*-acetylglucosaminyl-*N*-acetylmuramyl-(pentapeptide)-pyrophosphoryl-undecaprenol containing D-[^{14}C]alanine residues and the particulate fractions from strains JE 7319, JE 5784 and JE 5605 were prepared as in [11].

2.3. *Analysis of reaction products*

Digestion of the in vitro polymerized material by

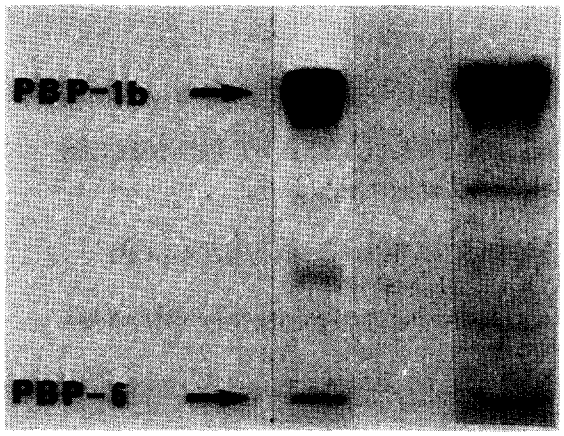

Fig.1. SDS–gel electrophoresis of isolated PBP-1b. Electrophoreses were performed as in [4]. (i) ~5 μg PBP-1b were treated with benzyl [^{14}C]penicillin and detected by fluorography. (ii) 50 μg PBP-1b were applied to the gel and revealed by staining with Coomassie blue.

egg-white lysozyme and analysis of the resulting products by paper chromatography was done as in [11]. After paper chromatography of the reaction products (fig.2) [^{14}C]alanine was not readily quantified owing to low amounts, to the low specific radioactivity of the substrate used and to a slight overlapping with the substrate which trailed. Therefore the [^{14}C]alanine region (R_F 0.5–0.7) was eluted, [^{3}H]alanine used as a marker was added (10^6 dpm, 50 nmol) and the mixture was rechromatographed on Whatman no. 1 filter paper (solvent system: *n*-butanol/acetic acid/water, 4:1:1). After localization with a 4 π Tracerlab scanner, the alanine spot was cut out and converted into $^{14}CO_2$ and ^3H_2O by combustion in an Oxymat apparatus. $^{14}CO_2$ was quantified as in [11]. Under these conditions a [^{14}C]alanine background of 400–500 dpm was observed when no polymerized material was formed.

3. Results and discussion

3.1. *Polymerase activity of strains defective in PBP-1b*

The polymerizing activity of strains JE 5784 and JE 7319 defective in the penicillin binding property of PBP-1b was compared to that of strain JE 5605 which had a normal PBP-1b content (table 1). No activity was found in particulate fractions from strain JE 5784 at 30°C or 37°C nor in those from strain

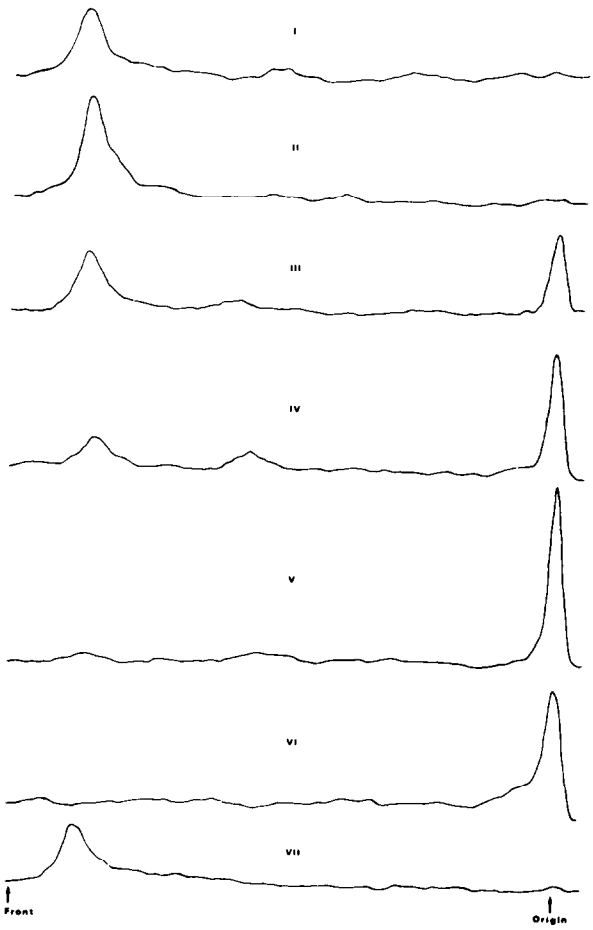

Fig.2. Separation of the products of the polymerization reaction by paper chromatography. Tracings were made with a 4 π Tracerlab scanner and correspond to experiments described in table 2.

JE 7319 at 30°C. However, a low level of activity was detected at 37°C in particulate fractions from strain JE 7319, but it was 10-fold less than that observed under the same conditions with preparations from strain JE 5605. It can thus be concluded that PBP-1b plays an essential role in catalysing the in vitro formation of peptidoglycan material from the lipid intermediate. These results are in agreement with observations showing that particulate fractions from mutants defective in PBP-1b failed to support peptidoglycan synthesis which was assayed for in vitro with the uridine precursors [4,5,7].

The polymerization assay performed with particulate fractions from strain JE 5784 could only be carried out in the presence of penicillin, since this

Table 1
In vitro peptidoglycan synthesis catalysed by particulate fractions from mutants defective in PBP-1b

Origin of the particulate fractions	Defect in PBPs	Incubation temp.	Penicillin G	Labelled reaction products (dpm)	
				Lipid intermediate	Peptidoglycan
JE 5605	1b$^+$/*dacA*$^-$/*dacB*$^-$/	30°C	–	3080	1035
		37°C	–	2220	1930
		37°C	+	1830	2910
		37°C (without) DOC	–	4430	305
JE 5784	1b$^-$/*dacA*$^+$/*dacB*$^+$/	30°C	+	5535	100
		37°C	+	4810	115
JE 7319	1b$^-$/1a^{ts}/*dacA*$^-$/ *dacB*$^-$/	30°C	–	5640	60
		30°C	+	4670	75
		37°C	–	4680	285
		37°C	+	4675	210

Assays with the particulate fractions and the lipid intermediate were done as in [11]. Each assay contained 150 μg protein and the incubation was for 30 min at 30°C or 37°C. When added, penicillin G was at 400 μg/ml final conc. DOC, sodium deoxycholate

Table 2
In vitro peptidoglycan synthesis catalysed by purified PBP-1b

Assay no.	PBP 1b	Particulate fraction	Sodium deoxycholate	Labelled reaction products (dpm)			Dimer/monomer ratio
				Lipid intermediate	Alanine	Peptidoglycan	
I	+	–	+	5640	510	220	
II	–	+	+	5340	450	220	
III	+	–	–	4060	630	1755	0.1–0.3
IV	+	+	–	1970	1270	2955	0.53
V	+	+	+	1050	1785	3635	0.55
VI	+	+ Penicillin G added	+	715	480	5250	
VII	+	+ Moenomycin added	+	5350	500	150	
VIII	+	Supernatant after heating	+	5820	560	155	
IX	+	Pellet after heating	+	4760	1010	980	

Experiments were carried out by incubating for 30 min at 30°C mixtures containing in 30 μl final vol. 0.2 M Tris–HCl buffer (pH 7.5), 0.01 M $MgCl_2$, 0.1% sodium deoxycholate and 0.1 mM lipid intermediate (8000 dpm). In expt I–VII either PBP-1b (2 μg protein) or particulate fraction (150 μg protein) from strain JE 7319 or both were added. In expt VIII, IX the supernatants and pellets added were obtained from the particulate fraction of strain JE 7319 after heat inactivation for 10 min at 100°C, sonication and centrifugation at 130 000 × g. When added penicillin G and moenomycin were at a final concentration of 100 μg/ml and 1 μg/ml, respectively. Separation of the reaction products by paper chromatography and their quantification were performed as in [11].

strain contains normal levels of penicillin-sensitive D-alanine carboxypeptidase activities which, when not inhibited, catalyse the degradation of the lipid intermediate and subsequently lead to low yields of polymerized material [10,11], independently of any PBP-1b deficiency. The importance of the deleterious effect of the D-alanine carboxypeptidase activities was stressed by the fact that particulate fractions from strain JE 5605, defective in these activities, catalysed well the polymerization reaction in the absence of penicillin, although to a slightly lesser extent than in the presence of the antibiotic. Furthermore, it was found in this case that the products of the lysozyme digestion of the polymerized material contained predominantly monomer and dimer fragments (dimer/monomer ratio = 0.47). Thus polymerization had proceeded both by transglycosylation and transpeptidation. Peptidoglycan material formed under the conditions of the assay in the presence of penicillin was uncrosslinked [11]. These results further substantiated the observation [4] that transpeptidation can occur in the absence of D-alanine carboxypeptidases 1A and 1B. It is also noteworthy that sodium deoxycholate was found to be necessary for the polymerization reaction even in the absence of penicillin (table 1).

3.2. *Polymerization catalysed by purified PBP-1b*

The role of PBP-1b in the in vitro formation of peptidoglycan material from the lipid intermediate was examined under different conditions as reported in table 2 and fig.2. The results showed that PBP 1b alone can catalyse a polymerization reaction. However, the addition of the particulate fraction from strain JE 7319 (*ponB*$^-$ *dacA*$^-$ *dacB*$^-$) to the PBP 1b preparation had a strong stimulating effect on the reaction. Sodium deoxycholate had an inhibiting effect in the absence of the particulate fraction, but a stimulating one in its presence. As observed with the particulate fraction from strain JE 5605, the polymerization reaction catalyzed by PBP-1b was stimulated by penicillin (table 2). Moenomycin, which was shown to inhibit the transglycosylation step of polymerisation [11], strongly inhibited the activity of PBP-1b. Furthermore, when the polymerized material formed in expt III–V (table 2) was digested by lysozyme, in each case ~90% of the radioactivity was recovered as soluble products containing predominantly monomer and dimer fragments. From these results it can be concluded that PBP-1b catalyses in vitro a transglycosylation reaction at the expense of the lipid intermediate.

When the polymerization reaction was carried out with PBP-1b in the presence of the particulate fraction (expt IV,V) the release of important amounts of [^{14}C]alanine and high ratios of dimer to monomer fragments in the lysozyme digestion products (table 2) clearly suggested that some transpeptidation had occurred. A critical point was to appreciate the extent of transpeptidation when PBP-1b alone (expt III) was used. Preparations of PBP-1b were found to possess both transglycosylation and transpeptidation activities [8]. From the results here it was difficult to draw a clear conclusion as to the occurrence or not of transpeptidation in expt III. The amounts of alanine detected were not much higher than the background level and different determinations of the dimer/monomer ratio yielded values of 0.10–0.30. Furthermore, it should be cautioned that our PBP-1b preparations were ~90% pure and thus the presence of some transpeptidation activity may be merely due to contamination with small amounts of other PBP possessing this activity. In particular, PBP-6 is still detectable in our preparations (fig.1). Further work will require purification to homogeneity of PBP-1b, a more detailed investigation of its enzymatic properties and of the effects of membrane, detergents and antibiotics.

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