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Site-directed mutagenesis of penicillin-binding protein 3 of *Escherichia coli*: Role of Val-545

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Abstract: Val545 of the *Escherichia coli* penicillin-binding protein 3 is essential to the acyl transfer mechanism through which the active-site serine 307 is acylated by benzylpenicillin and cephalexin and to the mechanism through which the protein allows rapidly growing cells to divide.

Key words: Penicillin-binding protein; *Escherichia coli* cell septation; Site-directed mutagenesis

Introduction

The cell-cycle penicillin-binding protein 3 (PBP3) of *Escherichia coli* is a component of the wall peptidoglycan assembly machinery involved in cell septation. PBP3 is constructed in a modular fashion. After elimination of the C-terminal I578-S588 peptide from the precursor, the mature PBP3 (M1-V577) consists of a membrane anchor (M1-A36) fused to a non-penicillin-binding module (F37-S259), itself fused to a penicillin-binding module (G260-V577). The penicillin-binding module possesses the three motifs of the penicilloyl serine transferases: S*307TVK, where S* is the active-site serine residue, S359SN and K494TG [1]. Penicilloylation of S*307 causes cell filamentation and cell death.

On the basis of published reports, the carboxy end of the mature PBP3 appears to be important both for penicillin binding and for ensuring the cells to undergo septation. The M1-R559 protein binds penicillin, functions in vivo but is unstable [2]. Proteins truncated farther than R559 are inert in both respects [3]. These inert proteins, however, may be toxic for the cell. Expression of the M1-A544 protein in a thermosensitive PBP3-producing *E. coli* prevents growth at the permissive temperature [2]. Amino acid changes have also shown that the polypeptide segment 542–547 plays a critical role. Proteins altered at position 542 or 547 still bind penicillin but are not functional in vivo, and the V545I PBP3 mutant has a decreased affinity for cephalexin but remains functional in vivo [4,5].

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The work described below was aimed at clarifying the role of Val545 of the stretch 542–547 of the *E. coli* PBP3. Val-545 was changed by site-directed mutagenesis into Ala, Leu and Glu, and the mutated PBP3s were compared to the wild-type PBP3 with respect to their affinity for penicillin and cephalexin and their ability to achieve cell septation in a thermosensitive PBP3-producing *E. coli* strain grown at the non-permissive temperature.

Materials and Methods

E. coli strains and growth media

E. coli XL1-Blue (used for the penicillin-binding experiments) was from Stratagene (La Jolla, CA). *E. coli* RP41 [6] (used for the complementation experiments) was a *leu*$^+$, *recA*$^-$ derivative of strain MC6RP1 (K12, F$^-$, *thrA leuA*, *proA*, *lysA*, *dra*, *drm*) that contains the *ftsI* 2158 gene encoding a thermosensitive PBP3 bearing the mutations Gly191Asp and Asp266Asn (in short, the D191, N266 PBP3) [7]. Growth was carried out on LB (Luria-Bertani) or 2XYT (Bacto trypton 16 g, yeast extract 10 g, NaCl 5 g, water 1 l, pH 7.0) media.

Recombinant DNA techniques and site-directed mutagenesis

Most of the techniques and manipulations involving phage M13mp9 were as described [8]. The restriction endonucleases were from Boehringer (Mannheim, FRG) and Bethesda Laboratories (Gaithesburg, MD). The mutant and primer oligonucleotides were synthesized using an AB391 PCR-MATE™ DNA synthesizer. Sequencing was performed using [^{35}S]dATP and the Pharmacia sequencing kit.

The 2.7-kb *Pvu*II DNA fragment carrying the *fts*I gene [9] was inserted into the *Sma*I sites of the replicative form of M13mp9. Mutagenesis was carried out as described using the *mutL E. coli* strain BMH7118 and the following oligonucleotides:

5′-CGG CGC GGA AGC GGC GCC GCC for the mutation V545A;

5′-CGG CGC GGA AAG GGC GCC GCC for the mutation V545L;

5′-CGG CGC GGA TTC GGC GCC GCC for the mutation V545E.

The underlined nucleotides directed the desired mutations. The ^{32}P-labelled oligonucleotides were used to screen the mutated clones by hybridization. The accuracy of the mutations was checked by DNA sequencing.

Cell membranes and penicillin-binding

Membranes prepared from *E. coli* strains as described [10] were suspended in 10 mM Tris · HCl (pH 7.5)/10 mM $MgCl_2$ and stored at −20°C at a concentration of 20 mg protein per ml. Binding of radioactive penicillin and non-radioactive cephalexin was carried under the conditions described [11].

Saturation of the PBP3s with [^{3}H]benzylpenicillin and cephalexin

Cell envelope samples were supplemented with 60 nM cloxacillin (to inhibit residual AmpC β-lactamase) and incubated for 10 min at 30°C with increasing concentrations of [^{3}H]benzylpenicillin (5 Ci mmol^{-1}; Radiochemical Centre, Amersham, UK). The amounts of radioactive PBP3 formed were estimated by gel fluorography after SDS-PAGE (10%). The amounts of PBP3 left in a free form after reaction with increasing concentrations of non-radioactive cephalexin were estimated by subsequent binding with [^{3}H] benzylpenicillin.

Thermostability of the PBP3s

Cell envelope samples were first maintained for 10 min at 30°C,37°C,42°C,47°C and 52°C in 30 mM Tris · HCl (pH 8.0)/20% (w/v) sucrose/4 mM EDTA/14 mM $MgCl_2$/14 mM $CaCl_2$. The amounts of PBP3s left in an active form were estimated by [^{3}H]benzylpenicillin binding.

Half-life of the [^{3}H]benzylpenicilloyl-PBP3s

After reaction with the radioactive penicillin and hydrolysis of the penicillin in excess with the *Bacillus cereus* β-lactamase II (0.03 mg per ml; final concentration), the decay of the radioactive penicilloyl derivative was followed at 37°C as a function of time up to 3 h.

Second-order rate constants ($M^{-1}s^{-1}$) of acylation of the PBP3s

Saturation of the PBP3s with [^{3}H]benzylpenicillin or cephalexin was carried out at 30°C. In most cases, a 25-fold increase of the β-lactam compound concentration caused a change in the extent of enzyme acylation from 10% to 90%. Under these conditions, the use of equation:

$$\text{Second-order rate constant} = 0.69/[D] \cdot t$$

with $[D]$ = the antibiotic concentration (M) required to achieve 50% saturation and t = time of incubation (600 s), was justified [11].

Results

In vitro penicillin-binding

In order to overexpress the wild-type and mutated PBP3s, *E. coli* XL1-Blue cells (which contain 50 copies of the membrane-bound PBP3 per cell) [12] were grown in LB medium at 37°C to an absorbance of 0.3 at 550 nm; infected with M13mp9 phages containing the wild-type or the mutated *ftsI* genes (phage to bacteria ratio: 1 to 10); induced with 1 mM IPTG and maintained at 37°C for one additional hour. The phage-encoded PBP3s were 50-fold overexpressed with respect to the host PBP3. The cells underwent varying morphological abnormalities but they did not lyse at least within 1 h after induction.

Analysis of the membrane-bound PBP3s led to the following conclusions. The Ala545, Leu545 and Glu545 PBP3s each were as thermostable as the wild-type Val545 PBP3 (0%, 15%, 30% and 100% denaturation after 10 min of incubation at 37°C, 42°C, 47°C and 52°C, respectively). The PBP3 mutants each formed [^{3}H]benzylpenicilloyl derivatives which were as stable as that formed with the Val545 PBP3 (half-life at 37°C > 3 h). The values of the second-order rate constant of protein acylation by benzylpenicillin and cephalexin were much decreased in the order: Val545 > Ala545 > Leu545 > Glu545 (Table 1). The change of Val545 into Glu was especially damaging: the Glu545 PBP3 was not saturated by 250 μM benzylpenicillin and was not sensitive to 12 mM cephalexin. Note also that the value of the second-order rate constant of acylation of the Ala545 PBP3 was comparable to that of the Leu545 PBP3 (approx. 100 M^{-1} s^{-1} for benzylpenicillin and approx. 1 M^{-1} s^{-1} for cephalexin, as derived from published data [5]; see Introduction).

Table 1

Mutation of Val545 of the penicillin-binding protein 3 of *Escherichia coli*

PBP3	Rate constant value ($M^{-1}s^{-1}$)	
	Benzylpenicillin	Cephalexin
Val545	500	17
Ala545	50	0.8
Leu545	8	0.2
Glu545	< 8 [a]	< 0.2 [b]

Second-order rate constant of acylation of the Val545 (wild-type), Ala545, Leu545 and Glu545 PBP3s by benzylpenicillin and cephalexin at 30°C.

[a] Not saturated at 250 μM benzylpenicillin.

[b] Not sensitive to 12 mM cephalexin.

In vivo complementation

*Eco*RI–*Bam*HI double-stranded DNA fragments carrying the wild-type and mutated *ftsI* genes were excised from the relevant recombinant M13mp9 phages and inserted into the corresponding sites of pUCBM20; transformants, using *E. coli* RP41 as hosts, were isolated at 30°C on 2XYT plates containing 50 μg ampicillin per ml, and the mutations were checked by double-stranded DNA sequencing of the recombinant plasmids. The plasmid-borne (wild-type and mutated) *ftsI* genes were under the control of their own promoters.

At 30°C, *E. coli* RP41 produces less than 50 copies of the membrane-bound, thermosensitive (D191, N266) Val545 PBP3 per cell (see Materials and Methods). At 42°C, the host chromosome-encoded PBP3 is not detectable (by penicillin binding or by immunoreaction) and the strain grows as filaments in liquid media and fails to form colonies on agar plates. The only *E. coli* RP41 transformants which grew at 42°C on agar plates were those that contained the recombinant pUCBM20 plasmid carrying the wild-type *ftsI* gene.

E. coli RP41 strains harbouring the recombinant plasmids were grown in liquid LB medium at 42°C. At this temperature the plasmid-encoded Val545 (wild-type), Ala545, Leu545 or Glu545 were expressed but not the chromosome-encoded D191,N266 PBP3 of the host. Culture samples were removed during the exponential phase of growth (A) and 6 h after the stationary phase was reached (B). Stationary phase cultures were then diluted into fresh media, growth was resumed at 42°C and samples were removed 3 h later (C). *E. coli*/pUCBM20 (control) grew as filaments in A, B and C. As shown in Fig. 1, *E. coli* producing the wild-type Val545 PBP3 grew as rods in A, B and C. *E. coli* producing the Ala545, Leu545 or Glu545 PBP3 mutant grew almost exclusively as filaments during the initial exponential phase (> 95%; samples A); as rods during the station-

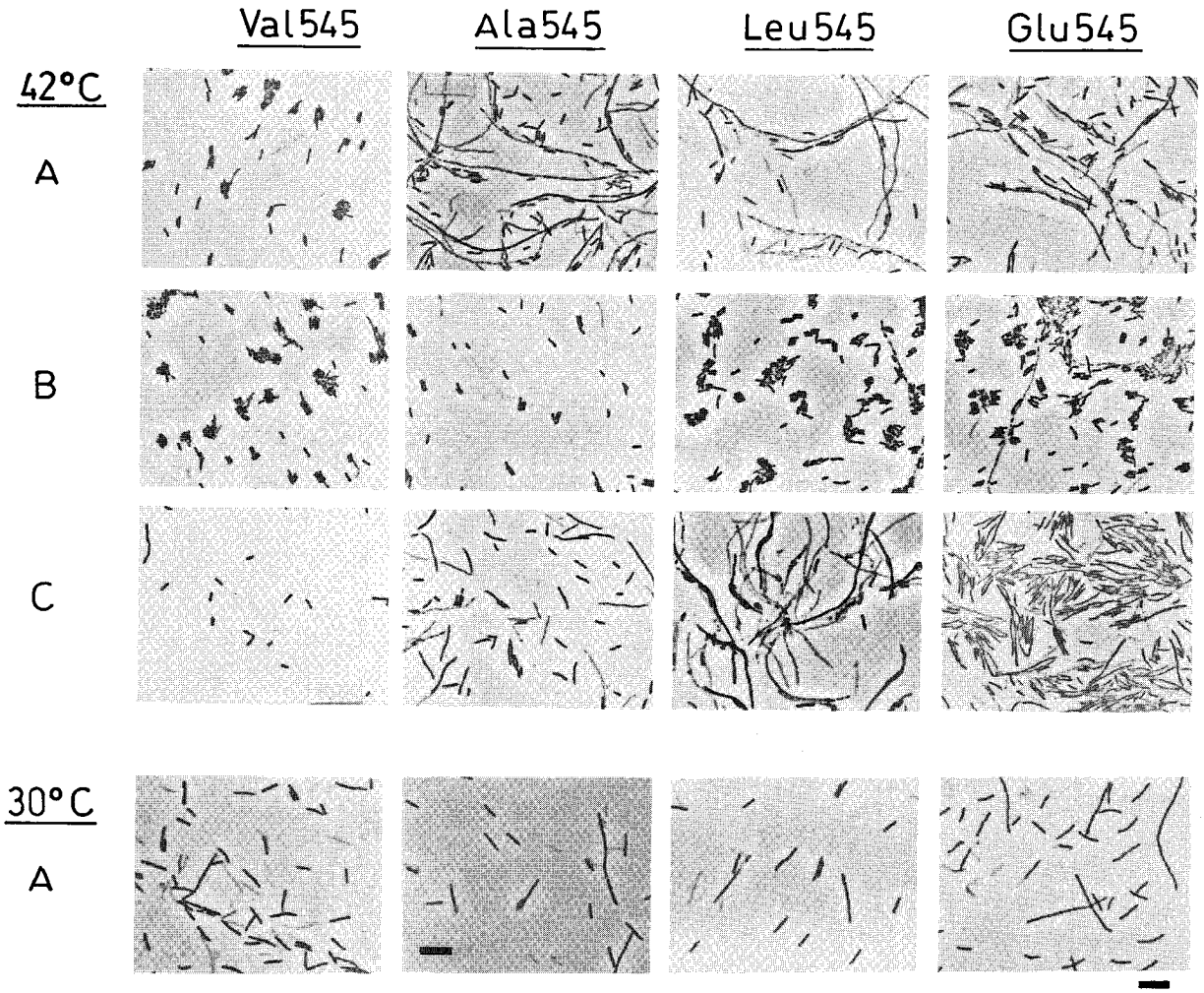

Fig. 1. Mutation of Val545 penicillin-binding protein 3 of *Escherichia coli*. Morphology of RP41 cells expressing the plasmid-encoded Val545 (wild-type), Ala545, Leu545 or Glu545 PBP3 during the exponential phase of growth (**A**), stationary phase of growth (**B**) and re-initiation of growth (**C**). The chromosome-encoded thermosensitive Val545 PBP3 of the host is not expressed at 42°C and it is expressed at 30°C. Bar represents 10 μm.

ary phase (> 90%; samples B); and as a mixture of normal and aberrant cells during re-initiation of growth (samples C).

The same experiment was carried out at 30°C. At this temperature, the chromosome-encoded (D191,N266) Val545 PBP3 of the host was expressed together with the plasmid-encoded Val545, Ala545, Leu545 or Glu545 PBP3. All the *E. coli* transformants including that co-expressing the host and the plasmid-encoded Val545 PBP3s, grew as mixtures of rod-shaped and elongated cells in samples A (Fig. 1). Normal cells occurred predominantly in samples B and C (not shown).

Discussion

Replacement of Val545 by Ala, Leu or Glu of the *E. coli* PBP3 has no detectable effect on the thermostability of the protein nor on the rate of breakdown of the penicilloyl enzyme derivative, but it decreases the value of the second-order rate constant of acylation by penicillin and cephalexin – i.e. the affinity of the PBP for the β-lactam compounds – by 90%, 98% and more than 98%, respectively. Amino acid residues occurring 50 residues or more downstream from the conserved KTG motif (see Introduction) do not form the immediate boundary of the active-site of the penicilloyl serine transferases of known three-dimensional structure [1]. Val545 and, most likely, other amino acid residues of the polypeptide segment 542–547 (see Introduction) probably control the spatial disposition of the active-site defining amino acid residues of the penicillin-binding module of PBP3 through hydrogen bonding interactions.

E. coli cells expressing, at 42°C, the plasmid-encoded low penicillin affinity Ala545, Leu545 or Glu545 PBP3 but not the chromosome-encoded, thermosensitive (D191,N266) Val545 PBP3, grow as filaments during the exponential phase but re-acquire a normal rod-shaped morphology during the stationary phase. The life-cycle PBP3 is one of the many components of the *E. coli* cell-septum synthesizing machinery [7]. It is thought to incorporate disaccharide-tripeptide units of lipid II precursors into pentapeptide stems of the wall peptidoglycan by trans-peptidation via a Ser307 ester-linked acyl enzyme [13,14]. Assuming a direct relationship between the catalytic efficacy of the PBP3 on the natural substrates and the rate of acylation of Ser307 of the PBP by penicillin, the above observations suggest that, when compared to the wild-type Val545 PBP3, a 10% residual activity (the Ala545 PBP3) is not sufficient to allow a coordinated synthesis of the septum to take place in rapidly growing cells. The observations also suggest that a less than 2% residual activity (the Glu545 PBP3) is sufficient to match a very slow growth rate.

E. coli cells co-expressing, at 30°C, the chromosome-encoded (D191,N266) Val545 PBP3 and plasmid-encoded Val545 PBP3 – both as membrane-bound proteins – grow as a mixture of rod-shaped and elongated cells during the exponential phase of growth. Co-expression of the chromosome-encoded (D191,N266) Val545 PBP3 and the plasmid-encoded low penicillin affinity Ala545, Leu545 or Glu545 PBP3 has the same overall limited deleterious effects. Hence, in these competing situations, synthesis of the septum appears to be mainly under the control of the Val545 PBP3 of the host, but interactions between the host PBP3 and the plasmid-encoded PBP3s somehow disturb the process.

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