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Site-directed mutagenesis of dicarboxylic acid residues of the penicillin-binding module of the *Escherichia coli* penicillin-binding protein 3

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Abstract: The glutamic acid E396, aspartic acid D409 and glutamic acid E411 residues of the *Escherichia coli* penicillin-binding protein 3 were each converted into an alanine residue. As deduced from penicillin-binding and complementation experiments, none of these dicarboxylic acid residues is involved in the mechanism of acylation by penicillin and none of them is essential for the in vivo functioning of the PBP. The mutation E396, however, causes an increased thermolability of the protein.

Key words: Escherichia coli penicillin-binding protein 3; Catalytic mechanism; Site-directed mutagenesis

Introduction

The penicilloyl serine transferases fall into two major groups [1]. Upon reaction with penicillin, the β-lactamases perform a double proton shuttle which leads to the formation of a serine ester-linked penicilloyl enzyme and, from this, to the hydrolytic release of penicilloate and concomitant enzyme recovery. The penicillin-binding proteins (PBPs) perform only the first part of the cycle and the reaction stops at the abortive level of the penicilloyl enzyme. In the β-lactamases of class A (and using the Ambler amino acid numbering), the motifs S*70XXK (where S* is the active-site serine), S130DN and K234T(S)G which are common to all the penicilloyl serine transferases, and the pentapeptide E166XELN form the immediate boundary of the active site. E166 is an essential component of the catalytic machinery but its exact role is still a matter of controversy. According to Lamotte-Brasseur et al. [2], the acylation and deacylation steps are mirror images involving the same general base catalyst, E166. According to Strynadka et al. [3], acylation is accomplished by K73N^{ζ} acting as a general base in abstracting the proton from S*70γOH while deacylation is accomplished by a water molecule assisted by the general base E166.

The high-M_r PBPs 2 and 3 of *Escherichia coli* are involved in cell shape and cell septation,

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respectively. They are of similar modular design [4,5]. A membrane anchor bears on the outer face of the membrane, an N-terminal module (assumed to be a transglycosylase) itself fused to a C-terminal penicillin-binding module (assumed to be a penicillin-sensitive DD-transpeptidase). The C-terminal module possesses at the expected places, the motifs S*330TVK (PBP2)/S*307TVK (PBP3), S387AD/S359SN, K544SGT/K494TGT of the serine penicilloyl transferases and, located between the two latter motifs, several dicarboxylic amino acid residues that might be the equivalents of E166 of the E166XELN motif of the class A β-lactamases [5].

On the basis that the D447E and D447N mutants of the *E. coli* PBP2 bind penicillin while only the D447E mutant performs complementation activity, Adachi et al. [6] suggested that D447 is not required for acylation of the active-site serine by penicillin but might be essential for acylation by D-alanyl-D-alanine-terminated peptidoglycan precursors, the carbonyl donor substrates of the presumed DD-transpeptidase/penicillin-binding module. However, at variance with this hypothesis, the D447A protein mutant not only lacks complementation activity but also fails to bind penicillin.

As derived from similarity searches and hydrophobic cluster analysis, E396, D409 or E411 of the penicillin-binding module of the *E. coli* PBP3 may be equivalent to D447 of the penicillin-binding module of the *E. coli* PBP2. The dicarboxylic acid residues of PBP3 have each been converted into an alanine residue and the protein mutants have been analysed functionally by genetic complementation and chemically in terms of thermostability and penicillin binding.

Materials and Methods

Recombinant DNA techniques

Most of the techniques and manipulations involving the phages M13 were as described [7,8]. The restriction endonucleases were from Boehringer (Mannheim, FRG) and Bethesda Laboratories (Gaithesburg, MD) and the synthetic oligonucleotides were from Eurogentec (Liège, Belgium). The sequencing primers were synthesized with an AB1380B DNA synthesizer. Sequencing was performed using [^{35}S]dATP and the Pharmacia sequencing kit.

E. coli strains

E. coli TG1 [9] was used for the penicillin-binding experiments. *E. coli* RP41 [10], a derivative of strain MC6RP1, contains the *fts*I 2158 allele encoding a thermosensitive PBP3 bearing two mutations G191D and D266N in the N-terminal module. *E. coli* RP41 was used for the complementation experiments.

Site-directed mutagenesis

The 2.7-kb *Pvu*II DNA fragment carrying the *fts*I gene was inserted into the *Sma*I sites of the replicative form of M13mp9. Mutagenesis was carried out as described [11] using the *mut*L *E. coli* strain BMH7118. The [^{32}P]oligonucleotides 5′CCACTGCGT<u>G</u>CTCCGAC (for the mutation E396A), 5′CCCTCTCTATGG<u>C</u>AGACCAC (for the mutation D409A), and 5′GGCCCTC<u>G</u>CTATGTCAGA (for the mutation E411A) were used to screen the mutated clones by hybridization [7] and the accuracy of the mutations was checked by DNA sequencing. The underlined nucleotides directed the desired mutations.

Complementation

The *Eco*RI-*Bam*HI DNA fragments encoding the wild-type and mutated *fts*I genes were isolated from the relevant recombinant M13mp9 phages and inserted into the corresponding sites of the high copy number pUCBM20 (Boehringer, Mannheim, FRG). Each gene was under the control of its own promoter. The mutations were checked by double-stranded DNA sequencing of the recombinant plasmids, obtained from both strains TG1 and RP41.

Thermostability and penicillin-binding

E. coli TG1 cells grown in LB medium at 37°C to an absorbance of a 0.3 at 600 nm, were infected with the M13mp9 phages carrying the wild-type and mutated *fts*I genes using a phage to bacteria ratio of 1 to 10. Overexpression from the *lac* promoter was induced by 1 mM IPTG

and the cultures were maintained for one additional hour at 37°C. The cells were collected, suspended in 10 mM sodium phosphate buffer pH 7.5/0.25 M NaCl/1 mM EDTA and disrupted by freezing and thawing. Penicillin binding using [^{3}H]benzylpenicillin (5 Ci/mmol; Radiochemical Centre, Amersham, Bucks, UK) was carried out as described [12].

Results and Discussion

At 42°C, *E. coli* RP41 produces a thermosensitive PBP3. At this temperature, PBP3 is not detectable and the strain grows as filaments in liquid medium and fails to form colonies on agar plates. Precultures (made at 30°C in LB medium/0.2% glucose/50 μg ampicillin per ml) of *E. coli* RP41, *E. coli* RP41/pUCBM20, and *E. coli* RP41/pUCBM20 carrying either the wild-type *fts*I gene or each of the mutated *fts*I genes (and overexpressing the corresponding PBP3s) were used to inoculate ampicillin-free LB liquid media and agar plates. At 42°C, the cells grew as rods in liquid medium (after 3 h) and formed colonies on agar plates (after 16 h), demonstrating that the mutations E396A, D409A and E411A in PBP3 did not prevent genetic complementation (Fig. 1).

E. coli TG1 cells infected with M13mp9 phages overexpressed (after induction with IPTG) the wild-type or the mutated PBP3s. The cells were frozen and thawed and the PBPs were analysed by radioactive benzylpenicillin labelling and SDS-PAGE. As derived from saturation experiments carried out at 30°C, the value of the second-order rate constant of penicilloylation was the same, 40–50 M^{-1} s^{-1} for the wild-type PBP3 and the E396A, D409A and E411A PBP3 mutants. Note, however, that this value was 10-fold smaller than that determined on the isolated membranes of the *E. coli* wild-type strain [13].

Incubation of the disrupted cells in phosphate/NaCl/EDTA, for 10 min at 30°C, 42°C, 47°C and 52°C, respectively, followed by reaction with radioactive penicillin led to the following

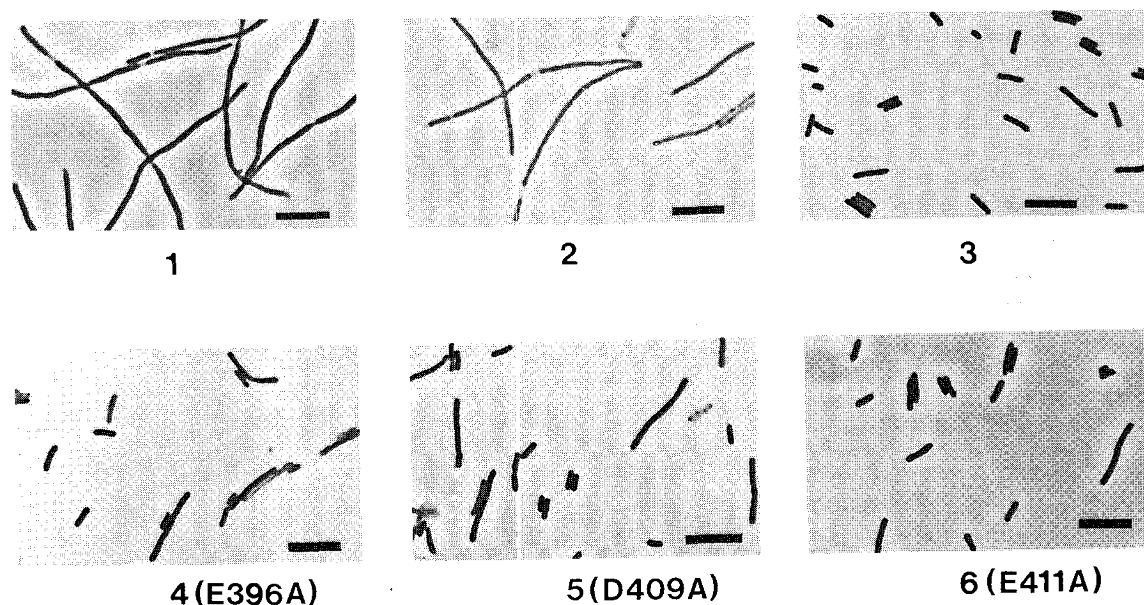

Fig. 1. Genetic complementation activity of the E396A, D409A and E411A PBP3 mutants, at 42°C and in liquid medium. 1) *E. coli* RP41; 2) *E. coli* RP41 harbouring the high copy number pUCBM20; 3) *E. coli* RP41 harbouring pUCBM20 containing the wild-type *fts*I gene; 4, 5 and 6) *E. coli* RP41 harbouring pUCBM20 containing the altered *fts*I genes coding for the mutations E396A, D409A and E411A, respectively. Bars correspond to 3.0 μm.

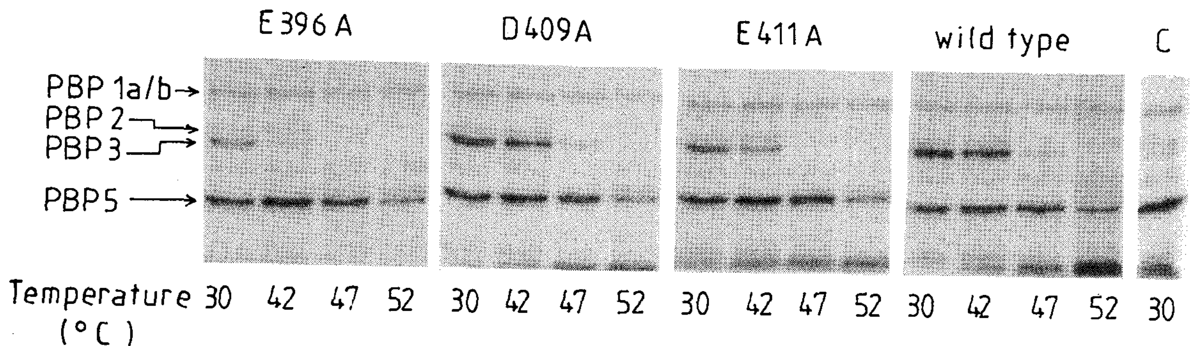

Fig. 2. Thermostability of the wild-type PBP3 and the E396A, D409A and E411A PBP3 mutants. Preincubation of the samples before labelling with radioactive penicillin was carried out for 10 min at the indicated temperature. *E. coli* TG1 infected with the relevant M13mp9 phages overexpressed the wild-type PBP3 and the desired PBP3 mutants. C (control): the original *E. coli* TGI strain showing the low basal level of PBP3 synthesis. Each sample corresponded to 0.4 ml of cell culture at an absorbance (600 nm) of 0.8.

observations (Fig. 2). By reference to PBP1B, which is the most thermostable PBP of *E. coli*, the wild-type PBP3 and the D409A and E411A PBP3 mutants showed comparable low thermostability. As a result of exposure to increasing temperatures, the wild-type and the mutated PBP3s progressively disappeared and, in parallel to this, increasing amounts of low-M_r, penicillin-binding polypeptides were formed. These polypeptides had a molecular mass of about 26 kDa, compatible with that of the penicillin-binding module of PBP3. In contrast, the E396A PBP3 mutant was much more thermosensitive than the wild-type PBP3. In all likelihood, degradation gave rise to small polypeptide fragments devoid of penicillin-binding capacity.

From the above, it follows that none of the dicarboxylic acids E396, D409 or E411 is involved in the proton shuttle leading to penicilloylation of the *E. coli* PBP3 and is essential for the in vivo functioning of the PBP at least in terms of complementation activity. As a corollary, none of these dicarboxylic amino acid residues is the equivalent of D447 of *E. coli* PBP2 or E166 of the β-lactamases of class A. In analogy with the β-lactamases of class C which lacks the pentapeptide EXELN [1,14], both penicilloylation and in vivo functioning of the *E. coli* PBP3 may not depend on a dicarboxylic acid residue as general base catalyst. The mutation E396A significantly increases the thermolability of PBP3. Given that by reference to the wild-type chromosomal PBP3, the E396A PBP3 is largely overexpressed from the replicative form of the recombinant M13mp9 or plasmid, the rate of synthesis probably largely compensates the rate of decay allowing genetic complementation to occur and cell septation to take place.

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