Purification and Characterization of PBP4a, a New Low-Molecular-Weight Penicillin-Binding Protein from *Bacillus subtilis*

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Penicillin-binding protein 4a (PBP4a) from *Bacillus subtilis* was overproduced and purified to homogeneity. It clearly exhibits DD-carboxypeptidase and thiolesterase activities in vitro. Although highly isologous to the *Actinomadura* sp. strain R39 DD-peptidase (B. Granier, C. Duez, S. Lepage, S. Englebert, J. Dusart, O. Dideberg, J. van Beeumen, J. M. Frère, and J. M. Ghuysen, Biochem. J. 282:781–788, 1992), which is rapidly inactivated by many β-lactams, PBP4a is only moderately sensitive to these compounds. The second-order rate constant (k_2/K) for the acylation of the essential serine by benzylpenicillin is 300,000 M^{-1} s^{-1} for the *Actinomadura* sp. strain R39 peptidase, 1,400 M^{-1} s^{-1} for *B. subtilis* PBP4a, and 7,000 M^{-1} s^{-1} for *Escherichia coli* PBP4, the third member of this class of PBPs. Cephaloridine, however, efficiently inactivates PBP4a ($k_2/K = 46,000$ M^{-1} s^{-1}). PBP4a is also much more thermostable than the R39 enzyme.

The *Bacillus subtilis* genome (16) contains a gene that encodes a putative 491-residue protein with sequence similarities to low-molecular-weight penicillin-binding proteins (PBPs). Successively referred to as *pbp* (25) and *dacC* (19), this gene was overexpressed in *Escherichia coli* by Pedersen et al. (19), who showed that *dacC* does indeed encode a membrane-bound PBP migrating between PBP4 and PBP5 of *B. subtilis* on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This protein is now referred to as PBP4a. The phenotypic effect of *dacC* mutations and the role of PBP4a in the synthesis of the *B. subtilis* spore peptidoglycan and its influence on the properties of the spore were investigated, but no physiological role or enzymatic activity could be assigned to the protein (20).

As already reported for *Actinomadura* sp. strain R39 PBP4 (in short, the R39 DD-peptidase) (14), *E. coli* PBP4 (17), and a *Haemophilus influenzae* putative PBP (4), the *B. subtilis* PBP4a possesses a large insert (175 residues) between the first and second active-site-defining motifs, and it can therefore be classified as a class C low-molecular-weight PBP (14). Importantly, although preliminary X-ray data for *E. coli* PBP4 are available (24), no three-dimensional structure of a protein belonging to this class of proteins has been presently established.

In this work, we describe the overexpression of PBP4a in *E. coli*, its purification, and its kinetic characterization as an in vitro DD-carboxypeptidase. Values for the parameter of inactivation (k_2/K, the second-order rate constant characterizing the acylation reaction) by some penicillins and cephalosporins were also determined and compared to those for the R39

DD-peptidase, to which PBP4a has the highest degree of sequence similarity (46% identity and 61% similarity) (8, 14).

MATERIALS AND METHODS

Recombinant DNA techniques, bacterial strains, plasmids, and growth conditions. *B. subtilis* 168 1A1 (Genetic Stock Center) was grown at 37°C in Luria-Bertani (LB) medium, and its genomic DNA was extracted with the Qiagen genomic tip 20/G kit. (Westburg, Leusden, The Netherlands).

The cloning and expression experiments were performed with *E. coli* TG1 (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), JM109, JM110 (Stratagene, Amsterdam Zuidoost, The Netherlands), Top 10, and LMG194 (Invitrogen, Groningen, The Netherlands) using pMK4 (22), a shuttle *Bacillus-E. coli* plasmid, or the pBAD/Myc-HisA (Invitrogen) vectors. The ampicillin resistance gene of the last plasmid was replaced by the kanamycin resistance gene using the following strategy. The vector (4.1 kb) was digested with *Bsp*HI, generating two fragments. The 3.1-kb fragment was purified from an agarose gel with the help of the Geneclean spin kit (Bio 101, Vista, Calif.), blunt-ended by filling with the Klenow DNA polymerase, and ligated to the 1.3-kb kanamycin resistance fragment isolated from the pYZ4 plasmid (3) by an *Nla*IV digestion, followed by the filling of the recessed 3′ ends. The resulting vector was named pBAD/Myc-HisAK^r.

The expression experiments with *B. subtilis* were performed with partly protease-deficient strain 1A751 (from the *Bacillus* Genetic Stock Center) grown at 37°C in LB medium supplemented with 7 μg of chloramphenicol ml^{-1}. Cultures of *E. coli* TG1, JM109, or JM110 transformed by the recombinant plasmid derived from pMK4 were grown at 30°C in LB medium supplemented with 10 μg of chloramphenicol ml^{-1}. *E. coli* LMG194 cells transformed with the plasmid derived from pBAD/Myc-HisAK^r were grown in minimal RM medium (Invitrogen) supplemented with 0.2% glucose and 50 μg/of kanamycin ml^{-1}.

The oligonucleotides were purchased from Eurogentec (Seraing, Belgium) or from Amersham Pharmacia Biotech.

PCR amplifications were carried out with a DNA thermal cycler (Biometra-Trio Thermoblock; Westburg, Leusden, The Netherlands) using Vent DNA polymerase (New England Biolabs, Beverly, Mass.) or Platinum DNA polymerase (Gibco BRL, Merelbeke, Belgium). The PCR products were cloned into pUCBM20 (Boehringer, Mannheim, Germany), and their sequences were completely verified before insertion in the expression vectors.

Purification of PBP4a. *E. coli* JM110 cells transformed with the expression vector derived from pMK4 were grown overnight at 30°C in 1 liter of LB medium. The cells were collected by centrifugation and resuspended in 30 mM Tris-HCl, pH 8.0, containing 27% (wt/vol) sucrose. The periplasmic content was
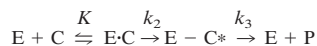
isolated as described previously (9), dialyzed against 100 mM Tris-HCl, pH 7.8, and loaded onto a DEAE-cellulose column equilibrated with the same buffer. The PBP4a was eluted with a curvilinear gradient of NaCl (the mixing flask held a constant volume of 300 ml; the added solution was 175 mM NaCl in the same buffer). The fractions exhibiting the highest activity against N^α-acetyl-L-Lys-D-Ala-D-Ala (AcKAA) were concentrated by ultrafiltration on an Amicon PM-30 membrane and loaded onto a molecular-sieve Sephacryl S100 column equilibrated in 50 mM Tris-HCl, pH 7.8–300 mM KCl–10% ethyleneglycol. The latter step was repeated to obtain homogeneous PBP4a. The active fractions were collected and concentrated by centrifugation in a Millipore Ultrafree-15 filter.

Since the enzyme was sensitive to freeze-and-thaw sequences, it was stored at 4°C in the presence of 0.02% sodium azide or at −20°C in 100 mM Tris, pH 7.8, containing 20% ethyleneglycol or 30% glycerol.

Substrates. AcKAA was a gift from UCB Bioproducts (Braine-l'Alleud, Belgium). N^α-Acetyl-L-Lys-D-Ala-D-thiolactate (AcKAThl) was a gift from Hoechst Marion Roussel (Romainville, France). The thiolesters benzoyl-Gly-thioglycolate (Bz-Gly-Thg) and benzoyl-D-Ala-thioglycolate (Bz-D-Ala-Thg) were obtained as previously described (1, 2). Benzylpenicillin, ampicillin, and cephaloridin were from Sigma (Bornem, Belgium). Cefuroxime was a gift from Glaxo Wellcome (Verona, Italy). β-Iodopenicillanate was obtained as described previously (6, 11). Ceftiofur (also named Exenel) was from Upjohn (Crawley, United Kingdom).

Kinetic measurements. Hydrolysis of AcKAA was followed by monitoring the amount of D-alanine released using the D-amino acid oxidase method (12). Hydrolysis of Bz-Gly-Thg and Bz-D-Ala-Thg was monitored at 250 nm on a Uvikon 860 apparatus using a $\Delta\varepsilon$ value of $-2,000$ M^{-1} cm^{-1}. k_{cat} and K_m values were derived from the complete time courses of hydrolysis of the substrate as described by De Meester et al. (7). Hydrolysis of AcKAThl was monitored at 412 nm in the presence of 1.2 mM 5,5′-dithiobis(2-nitrobenzoic acid) using a $\Delta\varepsilon$ value of 13,600 M^{-1} cm^{-1}. k_{cat}/K_m was obtained from initial rates of hydrolysis at substrate concentrations $[S] < K_m$. For the R39 enzyme, kinetic parameters for AcKAThl were derived from the complete hydrolysis time course. Unless otherwise stated, all experiments were performed at 30°C in 0.1 M HEPES pH 7.5.

The interaction between the enzyme (E) and the various β-lactam compounds (C) was analyzed on the basis of the following model:

$$\text{E} + \text{C} \underset{}{\overset{K}{\rightleftharpoons}} \text{E·C} \overset{k_2}{\rightarrow} \text{E} - \text{C}_* \overset{k_3}{\rightarrow} \text{E} + \text{P}$$

where E · C is the Henri-Michaelis complex, E-C* is the inactive acyl-enzyme, P is the degraded β-lactam, and k_3 is a first-order rate constant characterizing the deacylation reaction. Hydrolysis of E-C* to restore the active enzyme (k_3) was slow and was thus neglected. The apparent first-order acylation rate constant (k_a) was measured at different β-lactam concentrations by recording the progressive decrease in the rate of hydrolysis of 100 μM AcKAThl. In all cases, the β-lactam concentration was at least 10 times greater than that of the enzyme. In the presence of the substrate, k_a is given by

$$k_a = k_2[C]/\{[C] + K(1 + [S]/K_m)\} \quad (1)$$

where [C] is the β-lactam concentration. In equation 1, K_m refers to the substrate, and under our experimental conditions [S] was well below K_m. In addition, the direct proportionality between k_a and [C] showed that [C] was negligible compared to $K(1 + [S]/K_m)$. Therefore equation 1 could be simplified to give $k_a = k_2[C]/K$. Values for k_2/K were obtained from the slope of the linear plots of k_a versus [C].

The intrinsic fluorescence of the R39 DD-peptidase decreases as a result of its interaction with various cephalosporins. This property was used to determine k_2/K for cephaloridine. Fluorescence quenching caused by cephaloridine was monitored at 20°C in 0.1 M Tris-HCl buffer, pH 7.8, containing 5 mM MgCl$_2$ with the help of a Bio-Logic SFM-3 stopped-flow apparatus (excitation wavelength = 280 nm; emission wavelength > 320 nm), and k_2/K was calculated from the slope of the linear plot of the apparent rate constant for acylation versus the β-lactam concentration, as previously described (13).

pH dependence of the activity. Hydrolysis of AcKAA was measured at 37°C at different pHs ranging from 3 to 12. The universal buffer system described by Teorell and Stenhagen (23) was used at all pHs.

Thermal unfolding curves. Thermal-denaturation melting curves were obtained as described by Vanhove et al. (26). The sample was heated at 1°C/min using a thermostatically regulated circulating water bath. Excitation wavelength was 284 nm and emission wavelengths were 335 and 330 nm for the R39 enzyme and PBP4a, respectively. Experiments were performed in 0.1 M Tris buffer, pH 7.8, using an SLM-AMINCO 8100 spectrofluorometer (Spectronic Instruments).

N-terminal sequencing. The N-terminal sequence was determined with 160 pmol of protein with the help of an Applied Biosystems Procise sequencer (PE Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands).

RESULTS

Expression of *dacC* from the *B. subtilis-E. coli* shuttle plasmid pMK4 and purification of PBP4a. The portion of the gene encoding the mature PBP4a was cloned in phase with the signal sequence of the *Bacillus licheniformis* 749i β-lactamase under the control of the *B. licheniformis blaP* promoter (15, 18). The resulting vector was first cloned in *E. coli* TG1, isolated, and used to transform *B. subtilis* 1A751, which was grown in LB medium for 16 h at 37°C. Under these conditions, the expected secretion of PBP4a into the culture medium was not observed. An SDS-PAGE analysis of the culture supernatant also did not reveal the presence of the target protein after staining with Coomassie brilliant blue, and no hydrolysis of AcKAA was detected. The reasons of the latter failure to express PBP4a remain unclear, although protease degradation of the PBP4a and inefficient processing by the signal peptidase are possible explanations. This was not further investigated since the same recombinant plasmid led to periplasmic production of PBP4a in *E. coli* TG1. Among the other transformed *E. coli* strains, DH5α, JM109, and JM110, the last yielded the best production of PBP4a. Although not highly overproduced, PBP4a was highly toxic when secreted into the periplasm of *E. coli*, and the significant cell lysis observed resulted in the detection of 50% of the total PBP4a in the culture supernatant. Pedersen et al. (19) also mentioned the toxicity of this protein for *E. coli*, at least when produced in a soluble processed form.

With this expression system, a low periplasmic production of the TEM β-lactamase was also obtained, even in the absence of ampicillin in the culture medium. The proteins exhibit similar isoelectric pH values (pIs of 5.14 for PBP4a and 5.9 for the TEM enzyme), giving rise to overlapping elution profiles during the DEAE-cellulose purification step (see Materials and Methods). Other ion exchanger (DEAE- and Q-Sepharose) and hydrophobic-interaction (RESSOURCE ISO and RESSOURCE PHE; Pharmacia Biotech) columns did not allow the recovery of active PBP4a. The Ni^{2+}-nitrilotriacetic acid agarose, on which the TEM enzyme is naturally retained, was also tested, but PBP4a could not be eluted in an active form.

Homogeneous, β-lactamase-free PBP4a was eventually obtained by loading the sample obtained after the DEAE-cellulose purification step onto a molecular-sieve Sephacryl S100 column. This step had to be repeated in order to obtain the desired purity. Active enzyme could only be recovered when 10% ethyleneglycol was added to the buffer, suggesting that the protein interacts with the matrix in the absence of ethyleneglycol.

PBP4a exhibits an apparent molecular mass of 59 kDa on SDS-PAGE; the theoretical mass deduced from the protein sequence was 49.7 kDa.

Cloning of the *dacC* gene into pBAD/Myc-HisAK^r. The *dacC* gene without its signal sequence was amplified from genomic DNA by PCR using the following primers: 5′ CATGCCATGGCTGAAAAACAAGATGCACTTTCCGG3′ (the underlined sequence corresponds to an *Nco*I restriction site and contains a translation initiation codon in phase with the triplet coding for the first residue of the mature PBP4a) and 5′CGGAATTCGGTTCGACAAAGCGTTATTACAG3′ (the underlined sequence corresponds to an *Eco*RI site, and the last 23

TABLE 1. Hydrolysis parameters (k_{cat}/K_m and K_m) of the thiolesters for the R39 DD-peptidase and for *B. subtilis* PBP4a[a]

Thiolester	R39 DD-peptidase			*B. subtilis* PBP4a		
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
AcKAA	18[c]	280[c]	64,000[c]	N.D.[e]	N.D.	6,700
AcKAThl	5.4	42	130,000	N.D.	>400[b]	35,000
Bz-Gly-Thg	0.3[d]	30[d]	10,000[d]	2	470	4,300
Bz-D-Ala-Thg	5.6[d]	15[d]	336,000[d]	23	380	61,000
Bz-D-Ala-Thg + 10 mM D-Ala	N.D.	N.D.	N.D.	28	430	65,000
Bz-D-Ala-Thg + 50 mM D-Ala	N.D.	N.D.	N.D.	42	540	78,000

[a] Standard deviations did not exceed 10%.
[b] Initial rates of hydrolysis were proportional to substrate concentration up to 400 μM.
[c] From reference 27.
[d] From reference 5.
[e] N.D., not determined.

bases are complementary to nucleotides 1683 to 1705 in the sequence with accession no. Z34883 in the EMBL database). Therefore, the amplified fragment contains the sequence encoding the mature PBP4a with an additional N-terminal methionine as well as the endogenous stop codons and putative terminator sequence (25).

After verification of the sequence, the amplified fragment was inserted into expression plasmid pBAD/Myc-HisAK^r between the *Nco*I and *Eco*RI restriction sites without fusion to the polyhistidine coding region since preliminary assays of purification by affinity chromatography on Ni^{2+}-nitrilotriacetic acid agarose were unsuccessful.

Overproduction of PBP4a is detrimental to the viability of *E. coli* when the enzyme is secreted into the periplasm (our first expression system and reference 19). Therefore, a maximal repression of arabinose promoter pBAD was obtained by growing the transformed LMG194 cells in minimal RM medium containing 0.2% glucose. Different inducer concentrations were tested, and the best production was obtained with 0.2% arabinose and 4 h of culture at 37°C.

The cells from 25 ml of culture were treated as described previously (9), and the different cellular contents were analyzed by SDS-PAGE; as expected from the genetic construction, the cytoplasm contained a significantly overproduced soluble protein (data not shown). Moreover, the cytoplasmic extract exhibited a clear hydrolytic activity on AcKAA, which indicated a correct folding of PBP4a. The enzyme was purified as described above from 1 liter of culture after breaking the cells in cell disruption equipment (Constant Systems Ltd., Warwick, United Kingdom). The final yield was 8 mg of pure enzyme per liter of culture. The N-terminal sequence, AEKQDALS, confirmed the identity of the purified enzyme as PBP4a, with the loss of the first theoretical methionine residue. The enzyme produced with the help of the second expression system (pBAD/Myc-HisAK^r) was used for the biochemical characterization.

Kinetic characterization of PBP4a. (i) DD-peptidase activity and interaction with thiolester substrates. PBP4a clearly exhibits a DD-carboxypeptidase activity on AcKAA. Surprisingly, however, the enzyme is inhibited by an excess of substrate (data not shown) and the K_m value could not be determined. By contrast, k_{cat}/K_m could be determined by measuring the initial rate of hydrolysis at low concentrations of AcKAA (Table 1). Thiolesters are also efficiently hydrolyzed by PBP4a

(Table 1). K_m values for AcKAThl, Bz-Gly-Thg, and Bz-D-Ala-Thg are, however, much higher that those reported for the R39 DD-peptidase.

(ii) Transpeptidase activity. The kinetic parameters for the hydrolysis of Bz-D-Ala-Thg in the presence of 10 and 50 mM D-alanine are included in Table 1. If D-alanine can be used as an acceptor in a transpeptidation reaction, and assuming that the deacylation step is rate limiting, increases in both k_{cat} and K_m are expected. This was indeed observed, although the increases in k_{cat} and K_m are relatively modest. In addition, similar to what was reported for the R39 enzyme (27), a slight but significant increase in k_{cat}/K_m was also measured.

(iii) Inactivation by β-lactam antibiotics. Table 2 shows values of k_2/K determined with various β-lactam compounds. Despite a very high similarity to the R39 DD-peptidase, which is extremely sensitive to β-lactams, *B. subtilis* PBP4a is significantly more resistant to these compounds with the exception of cephaloridine, by which it is efficiently inactivated.

pH dependence of the activity of PBP4a. The rate of hydrolysis of AcKAA was measured at 37°C between pH 3 and 12. The activity of PBP4a was undetectable between pH 3 and 5.5. From pH 6 to 12, however, the activity increased linearly (not shown). Despite a maximum of activity at pH 12, all the kinetic measurements were performed at physiological pH values.

Thermostability of PBP4a. Thermal unfolding curves for PBP4a and the R39 DD-peptidase are shown in Fig. 1. Activity measurements at the end of the transition indicated that unfolding was not reversible, and therefore only apparent melting

TABLE 2. Comparison of the k_2/K values for the *B. subtilis* PBP4a and R39 DD-peptidase with various penicillins and cephalosporins

β-lactam	k_2/K (M^{-1} s^{-1})[a] for:	
	B. subtilis PBP4a	R39 DD-peptidase
Benzylpenicillin	1,400	300,000[b]
Ampicillin	4,800	70,000[b]
Cephaloridine	46,000	270,000
Cefuroxime	740	3,900[b]
Ceftiofur	860	45,000[c]
β-Iodopenicillanate	160	7,600[b]

[a] Standard deviations did not exceed 10%.
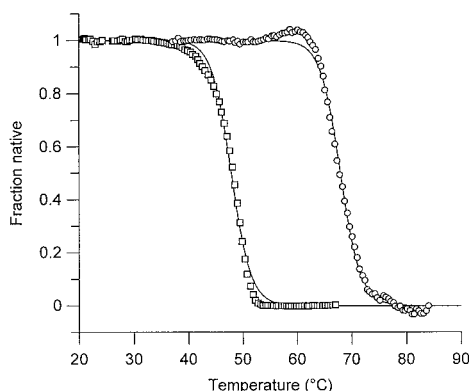[b] From reference 10.
[c] Sophie Lepage, personal communication.

FIG. 1. Thermal unfolding curves for the R39 DD-peptidase (squares) and PBP4a from *B. subtilis* (circles). Apparent T_ms deduced from the curves are 47.9 and 67.9°C for the R39 and the PBP4a enzymes, respectively.

temperatures (T_m) could be computed. Apparent T_ms were 47.9 and 67.9°C for the R39 and PBP4a DD-peptidases, respectively, highlighting a very significantly higher stability for the latter enzyme.

DISCUSSION

PBP4a from *B. subtilis*, a new member of the class C low-molecular-weight PBP family, was overexpressed in a soluble and active form in *E. coli*. The first expression system using the *B. subtilis-E. coli* shuttle vector directed the PBP4a to the periplasm of *E. coli*. This resulted in significant lysis of the different strains tested, indicating a high toxicity of the protein. By contrast, the expression of PBP4a from the pBAD/Myc-HisAK[r] plasmid resulted in cytoplasmic production without any observable cell lysis in *E. coli* LMG194.

The purification of PBP4a was particularly difficult since the protein was rapidly inactivated on several ion exchangers and was not eluted from molecular-sieve columns in the absence of ethyleneglycol. However, PBP4a was successfully purified to homogeneity by chromatography on a weak ion exchanger (DEAE-cellulose) followed by filtration on Sephacryl S100 in a buffer supplemented with 10% ethyleneglycol. The final yield was 8 mg per liter of culture.

In vitro, the protein clearly exhibited DD-carboxypeptidase and thiolesterase activities on short compounds exhibiting D-Ala-D-Ala and D-Ala-thiolactate or D-Ala-thioglycolate C termini. Interestingly, the enzyme is inhibited by an excess of AcKAA, but inhibition occurs at relatively high substrate concentrations (>10 mM), and it is not clear whether this observation has any physiological relevance. Kinetic parameters for Bz-D-Ala-Thg measured in the presence of D-alanine indicate that the PBP4a can use the latter as an acceptor in a transpeptidation reaction. By contrast to the R39 DD-peptidase, which is very sensitive to inactivation by β-lactams, the *B. subtilis* PBP4a is only weakly inhibited by many of these compounds, although the two proteins exhibit a high degree of homology (61% similarity and 46% strict identity). This low affinity of PBP4a for penicillin explains the observation of Pedersen et al. (19), who failed to visualize the protein in membranes of *B. subtilis* incubated with fluorescein-hexanoic-6-aminopenicillanic acid.

We also show here that the *B. subtilis* PBP4a is much more thermostable than the related R39 enzyme ($\Delta T_m = 20$°C). Many analyses to relate the protein conformational characteristics to thermostability have been carried out (for a review, see reference 21); however, no unambiguous rules can be established to predict how substituted amino acids improve the thermostability of a protein, particularly in the absence of three-dimensional structures. The prolyl contents and the percentages of hydrophobic residues are quite similar for the two enzymes. The only significant difference is in the percentage of positively charged residues (mainly due to the number of lysine residues): 8.4% in PBP4a versus 1% in the R39 DD-peptidase. The percentage of negatively charged residues being almost similar in both proteins, there is an unfavorable balance of charged amino acids in the R39 enzyme (ratio of positive to negative charges: 0.31 versus 0.79 for PBP4a), resulting in a particularly low isoelectric pH value for the R39 enzyme (<3.6). As pointed out by Vanhove et al. (26), this unfavorable balance might contribute to the particularly low stability of the R39 DD-peptidase.

In *B. subtilis*, PBP4a expression is initiated at the end of the stationary phase. The data from Pedersen et al. (19) and Popham et al. (20) indicate that the PBP4a product has no significant role in spore peptidoglycan production. Data for insertional *dacC* mutants indicate also that this gene is dispensable under normal growth conditions.

Although a DD-carboxypeptidase activity was clearly assigned in vitro to the PBP4a product, the physiological role of the whole protein and the function of the additional domain remain to be determined.

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