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The Non-Penicillin-Binding Module of the Tripartite Penicillin-Binding Protein 3 of *Escherichia coli* Is Required for Folding and/or Stability of the Penicillin-Binding Module and the Membrane-Anchoring Module Confers Cell Septation Activity on the Folded Structure

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Abstract

The *flsI*-encoded multimodular class B penicillin-binding protein 3 (PBP3) is a key element of the cell septation machinery of *Escherichia coli*. Altered *flsI* genes were overexpressed, and the gene products were analyzed with respect to the level of production, stability, penicillin affinity, and cell septation activity. In contrast to the serine β-lactamases and low-molecular-mass PBPs which are autonomous folding entities, the S-259-to-V-577 penicillin-binding module of M-1-to-V-577 PBP3 lacks the amino acid sequence information for correct folding. The missing piece of information is provided by the associated G-57-to-E-258 non-penicillin-binding module which functions as a noncleaved, pseudointramolecular chaperone. Key elements of the folding information reside within the motif 1-containing R-60-to-W-110 polypeptide segment and within G-188-to-D-197 motif 3 of the n-PB module. The intermodule interaction is discussed in the light of the known three-dimensional structure (at 3.5-Å [0.35-nm] resolution) of the analogous class B PBP2x of *Streptococcus pneumoniae* (S. Pares, N. Mouz, Y. Pétillot, R. Hakenbeck, and O. Dideberg, Nature Struct. Biol. 3:284-289, 1996). Correct folding and adoption of a stable penicillin-binding conformation are necessary but not sufficient to confer cell septation activity to PBP3 in exponentially growing cells. The in vivo activity of PBP3 also depends on the M-1-to-E-56 amino-terminal module which encompasses the cytosol, the membrane, and the periplasm and which functions as a noncleaved pseudo-signal peptide.

Building proteins according to modular patterns is an important driving force of evolution. Often, the properties of the multimodular proteins are more than the sum of the properties of the constitutive modules.

The penicilloyl serine transferases are attractive models for the study of protein modular design. They fall into two major groups. The first group includes the monofunctional low-molecular-mass penicillin-binding proteins (PBPs) and the β-lactamases. They each perform one single catalytic function and are autonomous folding entities. Experimental data strongly suggest that the TEM-1 β-lactamase folds via an intermediate that has the properties of a molten globule (28). This molecular species possesses extensive secondary structures but still lacks the persistent tertiary structures of the native state. The second group includes the high-molecular-mass PBPs which are multimodular proteins (7, 11). Essentially, the penicilloyl serine transferase moiety occurs as a penicillin-binding (PB) module that is fused to the carboxy end of a non-penicillin-binding (n-PB) module, forming a single polypeptide chain that folds on the outer side of the plasma membrane and is anchored to the membrane by an amino-terminal, noncleavable, pseudo-signal peptide.

As a polypeptide chain increases in length, finding the right fold may become a problem simply because the possibilities of misfolding increase. The in vivo folding of the *flsI*-encoded multimodular class B PBP3 of *Escherichia coli* (21) is the topic of this paper. PBP3 is synthesized as a 588-amino-acid-residue precursor. It is processed in *prc*$^+$ strains into mature M-1-to-V-577 PBP3 (20). Figure 1 (top) shows the modular design of PBP3 and identifies motifs 1 to 4 of the n-PB module and the three markers of the penicilloyl serine transferase-PB module (7, 11).

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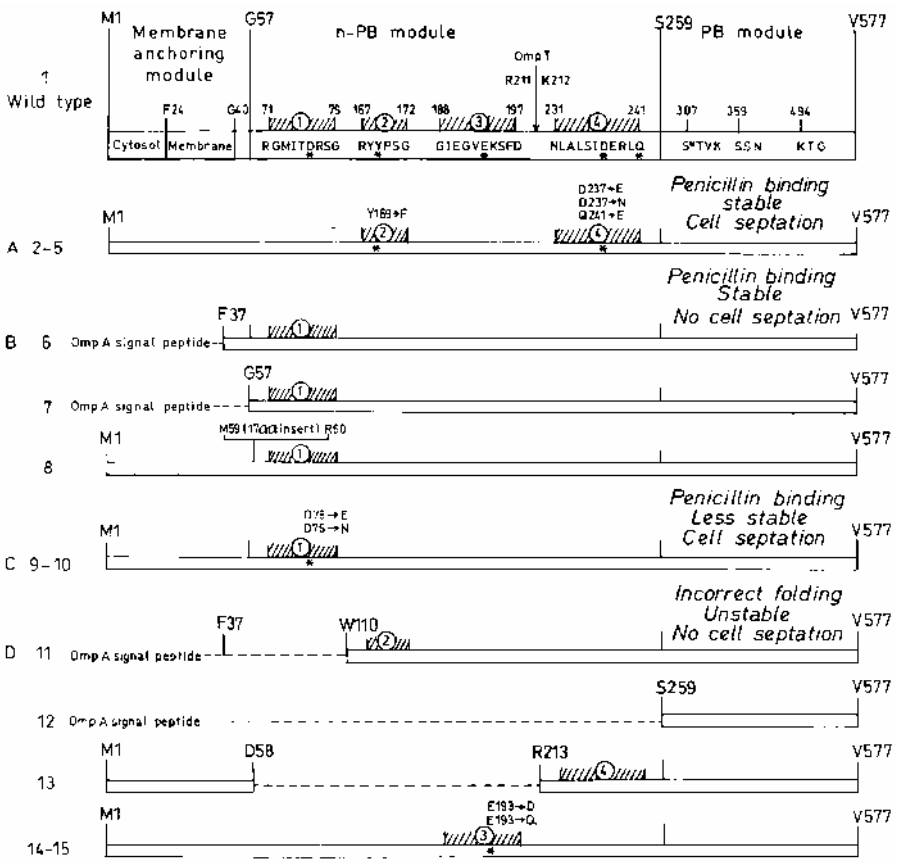
PBP3 was selected for this study for the following reasons. Its design is rather simple in comparison with those of other multimodular PBPs which contain inserts and extensions large enough to form additional modules. PBP3 is an essential component of a tightly regulated cell septation network, but the functioning of this network is far from being understood. The notion put forward by Matsuhashi (18) that the four multimodular PBPs of *E. coli* are bifunctional with transglycosylase (the n-PB modules) and transpeptidase (the PB modules) activities has stood the test of time for class A PBP1A and PBP1B. The PB module of the class B PBP3 is a (penicillin-sensitive) acyl serine transferase involved in wall peptidoglycan cross-linking. The question of what function is associated with the n-PB module is a matter of controversy (11).

Previous studies led to the conclusion that PBP3 folds in vivo and adopts a stable, PB conformation independently of the membrane anchor-containing M-1-to-G-57 sequence (8). They also suggested that the folding of the PB module was dependent on the n-PB module (14).

Membrane anchor-free R-41-to-V-577 PBP3 (bearing a 10-amino-acid-residue amino-terminal extension) was produced in the cytoplasm of *E. coli*/pDML211 mainly in the form of granules that bind penicillin. Treatment of the granules with guanidinium chloride and refolding yielded a water-soluble PBP3 (1). Membrane anchor-free F-37-to-V-577 PBP3 and G-57-to-V-577 PBP3 were transported into the periplasm of *E. coli*/pDML219 and *E. coli*/pDML232, respectively, with the assistance of the OmpA signal peptide. Part of the truncated PBPs remained trapped in the membrane, but export was facilitated by increasing the SecB content of the transformants (8). The water-soluble (in 0.5 M NaCl) PBP3 derivatives each had the same thermostability and penicillin affinity as membrane-bound M-1-to-V-577 PBP3. In contrast, the L-240-to-V-577 PB module of PBP3 (bearing a 12-amino-acid-residue amino-terminal extension) that was produced in the cytoplasm of *E. coli*/pPH153 bound penicillin, but it was very unstable, having a half-life of 1 min or less at 37°C (14).

FIG. 1. *Schematic representation of the modular design of E. coli PBP3 and the truncated and mutated PBP3 derivatives.* Number 1 is the wild-type PBP3. The truncated and mutated PBPs fall into four groups, as indicated. Motifs 1 to 4 are markers of the multimodular class B PBPs. Motifs S*TVK (with S* being the active-site serine), SSN, and KTG are markers of the penicilloyl serine transferases. The vertical arrow in number 1 indicates the cleavage site of the OmpT protease. *, mutated amino acid residues.



In the experiments reported here, the role of the n-PB module in the in vivo folding of PBP3 was investigated. *E. coli* transformants which carried information for the overproduction of membrane-bound and water-soluble PBP3 derivatives from which the n-PB module had been eliminated and replaced by glutathione *S*-transferase and which had been truncated and altered at specific sites in motifs 1 to 4 were constructed. The effects of the modifications were probed by measuring the level of production of the derivatives and their thermostability and penicillin affinity compared with those of membrane-bound M-1-to-V-577 PBP3 and water-soluble F-37-to-V-577 PBP3 or G-57-to-V-577 PBP3 overproduced under identical conditions. The activity of the PBP3 derivatives in cell division was also probed by transforming *E. coli* RP41, which produces a chromosomal thermosensitive PBP3, with plasmids harboring the wild-type and modified *ftsI* genes and then testing the ability of the transformants to divide at 42°C.

MATERIALS AND METHODS

Expression vectors and PBP3 derivatives. Table 1 lists the vectors used in this study and the encoded wild-type, truncated, and mutated PBP3 forms. The arrows indicate cleavage sites. The numbers are the same as those in Fig. 1. In Fig. 1, the 14 PBP derivatives (derivatives 2 to 15) are classified into four groups: A, B, C, and D.

pDML214 (a pBR322 derivative) and pDML219 (a derivative of the runaway replication pIMIIA3) encoded OmpA signal peptide F-37-to-V-577 PBP3 (8).

pDML232 (a derivative of pIMIIA3) encoded OmpA signal peptide G-57-to-V-577 PBP3 (8).

(i) pDML239. Unidirectional truncation of the gene encoding OmpA signal peptide F-37-to-V-577 PBP3 was carried out with the double-stranded nested deletion kit from Pharmacia Biotech Benelux (Roosendaal, The Netherlands). A DNA duplex (Table 1) containing the *Sac*I and *Not*I sites was inserted into pDML219 at *Hin*dIII downstream from the OmpA signal peptide-encoding sequence, yielding pDML238. pDML238 was digested with *Sac*I (generating an exonuclease IΠ-resistant end) and *Not*I (generating an exonuclease Ill-sensitive end). Samples were treated with the exonuclease for various time intervals from 1 to 16 min and for 30 min and then with SI nuclease, and the DNA was recircularized. Of 100 *E. coli* RR1 transformants tested, one produced a 52-kDa protein that reacted with the anti-PBP3 antibodies. The gene had the expected sequence for the OmpA signal peptide-assisted transport of W-110-to-V-577 PBP3 bearing an additional phenylalanine residue (i.e., F-37 of PBP3) at the amino end. The gene was under the control of the *Ipp* promoter and the *lac* promoter-operator.

(ii) pDML235. The 1.3-kb *Hin*dII-*Eco*RI DNA fragment of pDML214 encoding the N-268-to-S-588 sequence of the PBP3 precursor was inserted into pIN OmpA *Hin*d (8) previously digested with *Hin*dIII and *Eco*RI. The insertion was made via a DNA duplex encoding the S-259-to-V-267 sequence of PBP3 and possessing protruding *Hin*dIII and *Hinc*II sites. The gene was under the control of the *Ipp* promoter and the *lac* promoter-operator.

(iii) pDML234. The 1.3-kb *Hinc*II-*Eco*RI DNA fragment of pDML214 was inserted into pGEX2T (Pharmacia) previously digested with *Bam*HI and *Eco*RI. The insertion was made via a DNA duplex encoding the S-259-to-V-267 sequence of PBP3 and possessing protruding *Bam*HI and *Hinc*II sites. The gene was under the control of the *tac* promoter-operator.

(iv) M13mp19-*ftsI* recombinants. The 2.7-kb *ftsI*-containing *Pvu*II fragment was inserted into the *Sma*I sites of the replicative form of M13mp19 under the control of the *lac* promoter-operator. Mutagenesis was carried out with mismatch repair-deficient *E. coli* BMH7118 *mutL* and the appropriate oligonucle-otides (Table 1). Clones were screened by hybridization with ^{32}P oligonucleo-tides. Among the clones constructed to produce the D-76→N PBP3 mutant, one underwent gene reorganization. Encoded M-1-to-M-59-(insert)-R-60-to-V-577 PBP3 contained a 17-amino-acid-residue repeat, the sequence of which was that of the wild-type R-60-to-D-76 sequence, except that D-76 was changed into N. The repeat occurred between M-59 and R-60 of wild-type PBP3 (Fig. 2).

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TABLE 1. *Vectors used for the overproduction of wild-type, truncated, and mutated PBP3 forms*

Vector(s)	PBP3 form[a]	No. in Fig. 1	Duplex and oligonucleotides
M13mp19-*ftsI* and pUCBM20-*ftsI*	M-1-to-V-577 (wild type)	1	
pDML214	OmpA signal peptide↓-F-37-to-V-577	6	
pDML219	OmpA signal peptide↓-F-37-to-V-577	6	
pDML232	OmpA signal peptide↓-G-57-to-V-577	7	
pDML239	OmpA signal peptide↓-F-37-(Δ38-to-109)-W-110-to-V-577[b]	11	5′AGCTTTTGGAGCTCATCTATGCGGCCGCA3′ AAACCTCGAGTAGATACGCCGGCGTTCGA′ *Hin*dIII *Sac*I *Not*I *Hin*dIII
pDML235	OmpA signal peptide↓-S-259-to-V-577	12	
pDML234	Glutathione *S*-transferase-LVPRG-S-259-to-V-577 ↑ (thrombin site)		
M13mp19-*ftsI* and pUCBM20-*ftsI*	M-1-to-D-58-(Δ59-to-212)-R-213-to-V-577	13	**5'AGAAGAAATATCTTCAATTACGCGACCATAGCCCTCT TT CACCAGCATATCCGGGGAGAT3 '**
	M-1-to-M-59-(insert)-R-60-to-V-577 (Fig. 2)	8	
	M-1-to-V-577 (D-76→E) (altered motif 1)	9	**5'ACCAGAACGTTCAGTAAT3'**
	M-1-to-V-577 (D-76→N) (altered motif 1)	10	**5'AGAACGGTTAGTAATCAT3'**
	M-1-to-V-577 (Y-169→F) (altered motif 2)	2	**5 ' GGACGGAAAGTAACG GCG3 '**
	M-1-to-V-577 (E-193→D) (altered motif 3)	14	**5 ' GAAACTCTTGTCAACGCC3 '**
	M-1-to-V-577 (E-193→Q) (altered motif 3)	15	**5'ACTCTTCTGAACGCCCTC3'**
	M-1-to-V-577 (D-237→E) (altered motif 4)	3	**5 ' CAGGCGTTCTTCAATACT3 '**
	M-1-to-V-577 (D-237→N) (altered motif 4)	4	**5 ' GCGTTCATTAATACTCAG3 '**
	M-1-to-V-577 (Q-241→E) (altered motif 4)	5	**5 ' CAGCGCCTÇCAGGCGTTC3 '**

[a] Arrows indicate cleavage sites.
[b] In short, W-110-to-V-577 PBP3.

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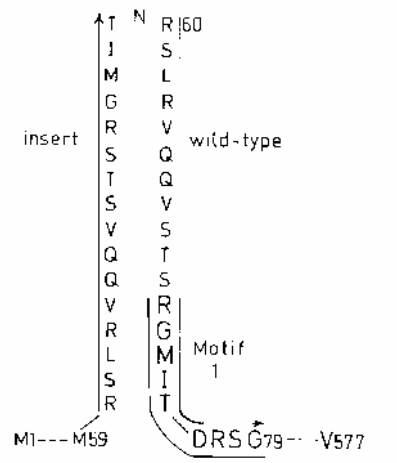
FIG. 2. *Position of the 17-amino-acid-residue repeat in M-1-to-M-59-(insert)-R-60-to-V-577 PBP3. Motif 1 of the n-PB module is indicated.*



(v) pUCBM20-*ftsI* recombinants. The *Eco*RI-*Bam*HI DNA fragments carrying the wild-type and mutated *ftsI* genes were excised from the M13mp19-*ftsI* recombinants and inserted into the corresponding sites of high-copy-number pUCBM20. The *ftsI* genes were under the control of their own promoter. The pUCBM20-*ftsI* recombinants were used for complementation assays.

Overproduction of the PBP3 derivatives, (i) OmpA signal peptide-transported F-37-to-V-577 PBP3 (pDML219), G-57-to-V-577 PBP3 (pDML232), W-110-to-V-577 PBP3 (pDML239), and S-259-to-V-577 (pDML235). *E. coli* RR1 transformants harboring pIMIIA3 (negative control) and the recombinant plasmids were grown at 37°C in Luria-Bertani medium containing 25 μg of kanamycin per ml to an A_{600} of 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) was added, and growth was continued for 3 h. The periplasmic, membrane, and cytoplasmic fractions were prepared via cell spheroplasting as described previously (8).

(ii) Glutathione *S*-transferase-(LVPRG)-S-259-to-V-577 PBP3 (pDML234). Cultures of *E. coli* HB101/pDML234 grown in Luria-Bertani medium at 37°C to an absorbance of 0.6 were induced with 2 mM IPTG and maintained for 2 additional h at 37°C. Granules accumulated in the cytoplasm. The cells were disrupted by sonication in 50 mM Tris-HCl (pH 8.0)-5 mM $MgCl_2$ containing 0.3 mg of lysozyme and 20 ng of DNase per ml, and the suspension was incubated for 15 min at 4°C. After the addition of 0.5 M NaCl, the granules (from 3.3 ml of culture) were pelleted at 10,000 × *g* and resuspended in 120 μl of 50 mM Lris-HCl (pH 8.0)-0.5 M NaCl-2.5 mM $CaCl_2$. Lhe suspension was incubated with thrombin (20 ng for 1 μg of total protein) for 1 h at 30°C and centrifuged at 10,000 × *g*, and the pellet was resuspended in 100 μl of the same buffer.

(iii) M-1-to-M-59-(insert)-R-60-to-V-577 PBP3, M-1-to-D-58-(Δ59-to-212)-R-213-to-V-577 PBP3, and PBP3 mutants. Cultures (4 ml) of *E. coli* LG1 or *E. coli* XL1Blue were grown in 2XYL medium at 37°C to an absorbance of 0.3 and infected with the M13mp19-*ftsI* recombinants. The cultures were induced with 1 mM IPLG, and growth was continued for 1 h at 37°C. Lotal cell extracts were prepared by suspending the cells in 120 μl of 10 mM Lris-HCl (pH 8.0)-1 mM EDLA and freezing and thawing. Alternatively, the cells were suspended in 120 μl of 30 mM Tris-HCl (pH 8.0)-20% (wt/vol) sucrose-5 mM EDLA and converted to spheroplasts by incubating the suspension with 100 μg of lysozyme for 15 min at 4°C. The preparation was then supplemented with 10 mM $MgCl_2$, 15 mM $CaCl_2$, and 20 μg of DNase. Samples (10 μl) were frozen at -20°C.

Penicillin binding. Lhe level of production of the PBP3 derivatives by the *E. coli* transformants was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of samples labelled with 10^{-4} M [^{3}H]benzylpenicillin (5 Ci/mmol; Radiochemical Centre, Amersham, United Kingdom) and then by Coomassie blue staining and fluorography of the gels (8). Lhe values of the second-order rate constant of acylation (k_{+2}/K [in per molar per second]) by the radioactive penicillin (direct binding) and nonradioactive cephalexin (competition with radioactive penicillin) were determined under conditions in which a 25-fold increase in the β-lactam concentration caused an increase in the extent of enzyme acylation from 10 to 90%. With *D* being the antibiotic concentration (in molar) required to achieve 50% saturation and *t* being the time (in seconds) of incubation, the equation $k_{+2}/K = 0.69/D \cdot t$ was used (12).

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Immunoblot assays. Immunological detection was carried out with monoclonal or polyclonal anti-PBP3 antibodies according to the Bio-Rad protocol. The antigen-antibodies complexes were detected with alkaline phosphatase coupled to anti-mouse or anti-rabbit immunoglobulin G.

RESULTS

ftsI-encoded, membrane-bound M-1-to-V-577 PBP3 occurs in a few tens of molecules per cell in the wild-type *E. coli* strains. It binds benzylpenicillin and cephalexin with high and low affinities, respectively. It is denatured completely and loses its penicillin affinity after a 10-min incubation at 52°C and pH 7.8 to 8.0 (8). Overproduction of PBP3 to a few thousands of copies per cell does not alter the cell division activity of the PBP (3).

Depending on the effects caused by the changes introduced in encoding *ftsI*, the altered PBP3 derivatives fell into four groups, A to D. They are shown in Fig. 1. Like the wild-type PBP3, the PBP3 derivatives were assumed to be processed so that the last 11 amino acid residues were eliminated and to terminate at the valine residue which is at position 577 of the wild-type PBP. The location of the insert in M-1-to-M-59-(insert)-R-60-to-V-577 PBP3 is shown in Fig. 2. The expression vectors used to produce the wild-type and modified PBPs are listed in Table 1.

The decreased amounts of PBP3 derivatives (in comparison with those of wild-type PBP3 or membrane anchor-free PBP3 used as controls) that were detected in the experiments described below were attributed for the following reasons to a decreased stability of the proteins and not to variations in the level of gene expression. For each group of experiments (except for the production of the glutathione *S*-transferase-PB module hybrid), the promoter and the vector copy number were the same and, therefore, could not influence the comparison of the levels of production of the PBP3 derivatives. With constructs containing deletions, changes in RNA structure and translation alterations might result in reduced levels of synthesis. This possibility seems unlikely, because single-amino-acid changes in PBP3 derivatives produced from constructs containing point mutations were sufficient to alter the thermostability of the PBP.

The hybrid glutathione *S*-transferase-PB module. Production of a protein (or part of a protein) in the wrong cellular compartment often results in polypeptide misfolding. However, membrane anchor-free PBP3 was overproduced in the cytoplasm of *E. coli* in the form of PB granules, and the granules were converted in an active water-soluble PBP3 form (see the introduction).

Attempts to produce the S-259-to-V-577 PB module of PBP3 in the cytoplasm and to stabilize it by fusing the amino end of the module to the carboxy end of glutathione *S*-transferase via a pentapeptide linker containing the thrombine cleavage site were made. Glutathione *S*-transferase is a cytoplasmic protein whose size (240 amino acid residues) is similar to that of the n-PB module. *E. coli* HB 101/pDML234 had the information for the IPTG-induced synthesis of the hybrid.

Gene overexpression resulted in the production of large quantities of a protein which reacted with the anti-PBP3 antibodies and had the molecular weight of 60,000 expected for the chimeric protein. However, the protein occurred almost exclusively in the form of inclusion bodies, the purified granules gave only a faint signal upon reaction with [^{3}H]benzylpenicillin, and the granules were very resistant to thrombin action. Increasing the GroEL and -ES content of the cells by cotransformation with pGroESL (13) increased the quantity of the granules produced and not the synthesis of a soluble chimeric protein from which the PB module could be released with thrombin.

The n-PB module is essential for proper folding in vivo. *E.*

coli RR1/pDML219, -232, -239, and -235 had the information for IPTG-inducible overproduction and OmpA signal peptide-assisted transport of membrane anchor-free F-37-to-V-577 PBP3 derivative 6 (Fig. 1), G-57-to-V-577 PBP3 derivative 7, W-110-to-V-577 PBP3 derivative 11, and S-259-to-V-577 PBP3 derivative 12, respectively. W-110-to-V-577 PBP3 lacked motif 1 of the n-PB module. S-259-to-V-577 PBP3 consisted of the PB module alone. The transformants were grown at 37°C and were IPTG induced under identical conditions without increasing the SecB content of the cells (see the introduction). The cytoplasmic, membrane, and periplasmic fractions were isolated and analyzed.

F-37-to-V-577 PBP3 (Fig. 1 [derivative 6]) and G-57-to-V-577 PBP3 (Fig. 1 [derivative 7]) were overproduced in large amounts in a stable, PB form, and more than 60% of the synthesized PBPs were transported in the

periplasm (Fig. 3). In contrast, W-110-to-V-577 PBP3 (Fig. 1 [derivative 11]) and the S-259-to-V-577 PB module (Fig. 1 [derivative 12]) could be detected only in negligible amounts. The small quantities of W-110-to-V-577 PBP3 that were detected essentially remained trapped in the membrane (Fig. 3). Immunoblotting of the gels with anti-PBP3 antibodies failed to detect any protein bands other than the faint ones identified by PB, suggesting that the synthesized polypeptides were short-lived and underwent complete degradation.

E. coli TGI was infected with M13mpl9-*ftsI* recombinants having the information for the IPTG-inducible overproduction of membrane-bound M-1-to-V-577 PBP3 derivative 1 (Fig. 1) and M-1-to-D-58-(Δ59-to-212)-R-213-to-V-577 PBP3 derivative 13 (Fig. 1). The missing M-59-to-D-212 segment contained motifs 1, 2, and 3 of the n-PB module. The transformants were grown at 37°C and induced under identical conditions. Analysis of total cell extracts in 10 mM Tris-HCl (pH 8.0)-1 mM EDTA showed that M-1-to-V-577 PBP3 was overproduced in a stable and active form. In contrast, M-1-to-D-58-(Δ59-to-212)-R-213-to-V-577 PBP3 was detected only in very small amounts (Fig. 4).

If misfolded, a protein is often unstable, incorrectly translocated, and rapidly degraded. The above observations showed that the G-57-to-E-258 n-PB module, but not the membrane anchor-containing M-1-to-G-57 polypeptide segment, was essential for mediating proper folding of PBP3 in vivo and that a key element of the folding information resided in the G-57-to-W-110 polypeptide segment that contained conserved motif 1.

D-76 of motif 1 and E-193 of motif 3 of the n-PB module contribute to the folding amino acid sequence information.

The above studies raised the possibility that not only motif 1 but also motifs 2 to 4 of the G-57-to-S-259 n-PB module played a role in the folding process.

E. coli TG1 or XL1Blue was infected with M13mp19-*ftsI* recombinants having the information for the IPTG-inducible overproduction of membrane-bound M-1-to-V-577 PBP3 derivative 1 (Fig. 1), M-1-to-M-59-(insert)-R-60-to-V-577 PBP3 derivative 8, and M-1-to-V-577 PBP3 mutants 2 to 5, 9 and 10, and 14 and 15.

The levels of production of the PBP3 mutants were estimated by incubating the samples with [^{3}H]benzylpenicillin at 37°C. Their thermostabilities were estimated by incubating the samples for 10 min at 37, 42, 47, and 52°C and then measuring the amounts of PBP left in an active form by the subsequent binding with [^{3}H]benzylpenicillin for 10 min at 30°C (unless otherwise stated). Their affinities for benzylpenicillin and cephalexin in terms of the second-order rate constant of acylation were also determined at 30°C. A temperature of 30°C was used in these experiments because the level of labelling of M-1-to-M-59-(insert)-R-60-to-V-577 PBP3 by [^{3}H]benzylpenicillin was higher at 30 than at 42°C (see below). This difference was attributed to a reversible denaturation of a certain proportion of the PBP at 42°C. Indeed, with all the other conditions being identical, samples of M-1-to-M-59-(insert)-R-60-to-V-577 PBP3 first maintained 10 min at 42°C and then treated with [^{3}H]benzylpenicillin for 10 min at 30°C showed the same extent of labelling as the samples treated directly with penicillin at 30°C without preincubation at 42°C.

Y-169 → F (motif 2) PBP3 mutant 2 (Fig. 1), D-237 → E, D-237 → N, and Q-241 → E (motif 4) PBP3 mutants 3 to 5, and M-1-to-M-59-(insert)-R-60-to-V-577 PBP3 mutant 8 were overproduced in amounts comparable to that of M-1-to-V-577 PBP3 (Fig. 4). They were somewhat more stable than the wild-type PBP (Fig. 5). The values for the second-order rate constant of acylation with benzylpenicillin ranged from ≈700 M^{-1} s^{-1} to ≈1,900 M^{-1} s^{-1} (Table 2). These values were just outside the limits of experimental error. Cephalexin was a very weak acylating agent (≈20 M^{-1} s^{-1}).

M-1-to-M-59-(insert)-R-60-to-V-577 PBP3 mutant 8 (Fig. 1) was susceptible to proteolytic cleavage within the insert. The product was similar to G-57-to-V-577 PBP3 with respect to molecular mass and solubilization in 0.5 M NaCl. At 30°C, the decrease of the larger PBP3 form and the increase of the smaller one as a function of time were commensurate, with a 50% conversion being observed after a 30-min incubation (Fig. 6). M-1-to-M-59-(insert)-R-60-to-V-577 PBP3 was also incubated with [^{3}H]benzylpenicillin at 42°C. The fluorograms were more difficult to interpret, because the two PBP forms each underwent denaturation at this temperature. Clearly, however, an appreciable amount of the noncleaved PBP was still present after a 60-min incubation (Fig. 6).

D-76 → E and D-76 → N (motif 1) PBP3 mutants 9 and 10 (Fig. 1) bound penicillin at 30°C with the same affinity as M-1-to-V-577 PBP3 (Table 2), but they were detected in lesser amounts (Fig. 4), and they were more thermolabile (Fig. 5). The D-76 → N mutation was especially damaging. Denaturation, however, was not complete after a 10-min incubation at 42°C.

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E-193→D (motif 3) PBP3 mutant 14 (Fig. 1) was detected in very small quantities. The amount of E-193→Q (motif 3) PBP3 mutant 15 that could be detected by fluorography or immunoblotting of the gels did not exceed that attributable to the chromosome-encoded PBP3 of the host (Fig. 4). At 42°C, the E-193→D and E-193→Q PBP3 mutants could not be detected.

From the foregoing, it followed that motifs 1 and 3 of the n-PB module were parts of the folding amino acid sequence information. D-76 of motif 1 contributed to the stability of the native state structure. E-193 of motif 3 was essential for proper folding.

FIG. 3. *Production of OmpA signal peptide-transported W-110-to-G-577 PBP3 by IPTG-induced E. coli RR1/pDML239.* Low-resolution (7-cm) SDS-PAGE of membrane (M) and periplasmic (P) fractions isolated from *E. coli* RR1/pIMIIA3, showing the level of production of the chromosome-encoded PBP3 of the host (lane 1), *E. coli* RR1/pDML219, showing the level of production of F-37-to-G-577 PBP3 (lane 2), and *E. coli* RR1/pDML239, showing the level of production of W-110-to-G-577 PBP3 (lane 3), is shown. The membrane fractions (M) and periplasmic fractions (P) corresponded to 400 and 133 μl, respectively, of cell cultures at an absorbance of 1.2. Polyclonal anti-PBP3 antibodies were used for the immunoblots. K-211-to-V-577 PBP3 resulting from OmpT cleavage of the R-210-K-211 peptide bond is also identified.

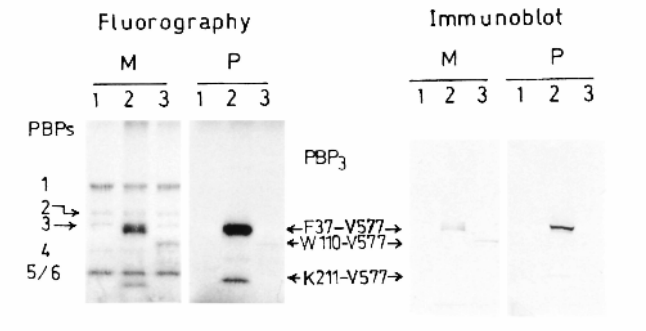

FIG. 4. *Production of the M-1-to-D-58-(Δ59-to-212)-R-213-to-V-577 PBP3 (Del.), M-1-to-M-59-(insert)-R-60-to-V-577 PBP3 (Ins.), and M-1-to-V-577 PBP3 mutants by IPTG-induced E. coli TG1/M13mp19-ftsI derivatives 2 to 4, 9 and 10, and 14 and 15, respectively (Table 1 and Fig. 1).* High-resolution (25-cm) SDS-PAGE of total cell extracts in 10 mM Tris-HCl (pH 8.0)-1 mM EDTA is shown for the following: *E. coli* TG1 (TG1), showing the level of production of chromosome-encoded PBP3 of the host; *E. coli* TG1/M13mp19-ftsI number 1 (WT), showing the level of production of M-1-to-V-577 PBP3; *E. coli* TG1/ M13mp19-*ftsI* mutant 13 (Del.), showing the level of production of M-1-to-D-58-(Δ59-to-212)-R-213-to-V-577 PBP3 (marked with two asterisks); E. coli TG1/ M13mp19-*ftsI* mutant 8 (Ins.), showing the level of production of M-1-to-M-59-(insert)-R-60-to-V-577 PBP3 (marked with an asterisk); and (as indicated) E. coli TG1/M13mp19-*ftsI* mutants 9 and 10 (motif 1), 2 (motif 2), 14 and 15 (motif 3), and 3 and 4 (motif 4), showing the levels of production of the corresponding PBP3 mutants. The total cell extracts corresponded to 0.5 ml of cell culture at an A_{600} of 0.8. Monoclonal anti-PBP3 antibodies were used for the immunoblots.

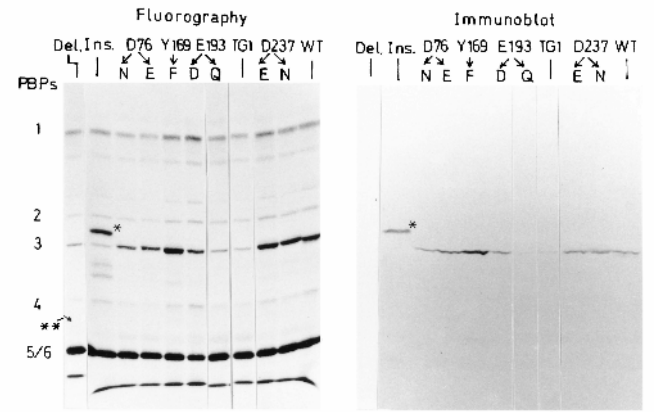

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FIG. 5. *Thermostability of the M-1-to-M-59-(insert)-R-60-to-V-577 PBP3 and PBP3 mutants.* Total cell extracts of IPTG-induced E. coli XL1Blue infected with M13mp19-ftsI recombinants were prepared in 30 mM Tris-HCl (pH 8.0)-sucrose-$MgCl_2$-$CaCl_2$ as described in Materials and Methods. Samples were maintained for 10 min at various temperatures as indicated. The amounts of PBP left in an active form were estimated by [^{3}H]benzylpenicillin binding at 30°C as measured by SDS-PAGE and fluorography. Before the labelling with radioactive penicillin, 0.3 μM cloxacillin was added to the reaction mixture to inactivate the AmpC β-lactamase of the host. The results are expressed as percentages of labelling obtained after the 10-min preincubation of the samples at 37°C. ●, M-1-to-V-577 PBP3; Δ, Y-169→F, D-237→E, and D-237→N PBP3 mutants; ○, D-76→E PBP3 mutant; □, D-76→N PBP3 mutant.

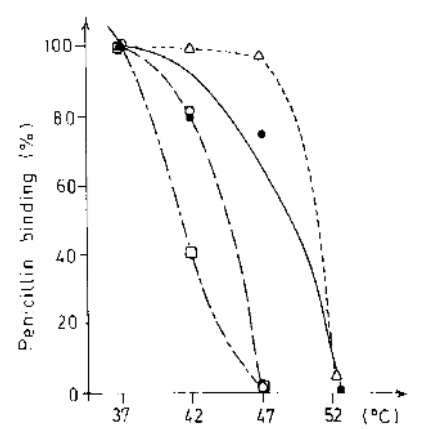
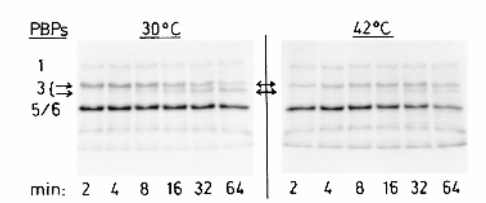


TABLE 2. *Second-order rate constants of acylation for wild-type PBP3 and PBP3 mutants with benzylpenicillin and cephalexin at 30°C*

PBP3	k_{+2}/K (M^{-1} s^{-1})[a]	
	Benzylpenicillin	Cephalexin
M-1-to-V-577 (wild type)	680	17
M-1-to-M-59-(insert)-R-60-to-V-577	1,240	7
M-1-to-V-577 (D-76→N)	820	10
M-1-to-V-577 (D-76→E)	720	ND
M-1-to-V-577 (Y-169→F)	1,280	23
M-1-to-V-577 (D-237→E)	1,920	23
M-1-to-V-577 (D-237→N)	1,900	23
M-1-to-V-577 (Q-241→E)	1,045	8

[a] ND, not determined.

FIG. 6. *Proteolytic cleavage and thermostability of M-1-to-M-59-(insert)-R-60-to-V-577 PBP3.* Total cell extracts of IPTG-induced *E. coli* XL1Blue infected with M13mp19-*ftsI* mutant 8 were prepared in 30 mM Tris-HCl (pH 8.0)-sucrose-$MgCl_2$-$CaCl_2$ as described in Materials and Methods. Samples (10 μl) were supplemented with 10^{-4} M [^{3}H]benzylpenicillin and incubated at 30 and 42°C for increasing periods of time up to 64 min. Before the labelling, the reaction mixtures were supplemented with 0.3 μM cloxacillin to inactivate the AmpC β-lactamase of the host. The samples were submitted to low-resolution (7-cm) SDS-PAGE, and the gels were analyzed by fluorography.



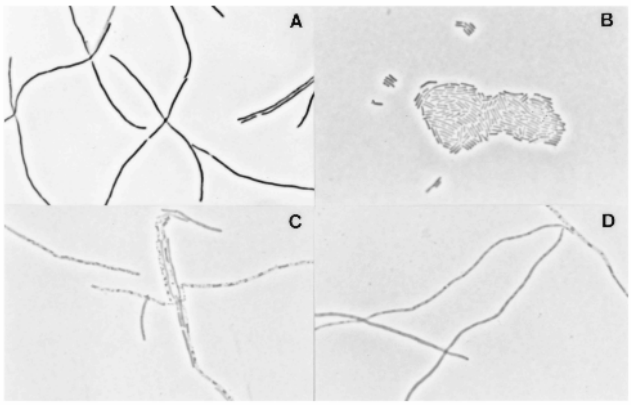
The membrane anchor-containing M-1-to-G-57 polypeptide segment, although not required for proper folding, is essential for the cell septation activity of PBP3. *E. coli* RP41 contains an *ftsI* allele that encodes a thermosensitive PBP3 (10). At 30°C, the strain grows and divides normally. At 42°C, it grows as filaments in liquid media (Fig. 7A) and fails to form colonies on agar plates. The thermosensitive PBP3 has two amino acid substitutions: G-191→D in motif 3 and D-266→N close to the junction between the n-PB and PB modules.

E. coli RP41 was transformed with pDML214 that encoded truncated F-37-to-V-577 PBP3 mutant 6 (Fig. 1) and with pUCBM20-*ftsI* recombinants that encoded membrane-bound M-1-to-V-577 PBP3 wild type 1, M-1-to-M-59-(insert)-R-60-to-V-577 PBP3 mutant 8, and PBP3 mutants 2 to 5, 9 and 10, and 14 and 15. The transformants were grown in liquid (2XYT and Luria-Bertani) media containing 50 μg of ampicillin per ml (to ensure maintenance of the plasmids) at 30 and 42°C without IPTG induction.

At 42°C and in exponentially growing cultures, *E. coli* RP41 transformants producing plasmid-encoded, membrane-bound M-l-to-V-577 PBP3 divided normally and were rod shaped (Fig. 7B). As low ampicillin concentrations cause cell filamentation, the observed complementation activity showed that the plasmid-encoded β-lactamase was produced in amounts sufficient to reduce the antibiotic concentration below the level at which cell filamentation is induced.

Under the same growth conditions, complementation was also observed with the membrane-bound D-76→E, D-76→N (motif 1), Y-169→F (motif 2), D-237→E, D-237→N, and Q-241→E (motif 4) PBP3 mutants. In contrast, membrane anchor-free F-37-to-V-577 PBP3 and the membrane-bound E-193→D and E-193→Q (motif 3) PBP3 mutants (Fig. 7C) and the membrane-bound M-1-to-M-59-(insert)-R-60-to-V-577 PBP3 mutant (Fig. 7D) lacked complementation activity. The lack of activity of the latter PBP3 derivative is most likely due to conformational changes in the polypeptide chain immediately upstream from R-60 and not to the proteolytic cleavage of the insert, since, as can be seen from Fig. 6, the rate of cleavage is low.

FIG. 7. *Complementation of E. coli RP41 at 42°C. Cells in the exponential phase of growth are shown. (A) E. coli RP41 harboring pUCBM20 (control). (B to D) E. coli RP41 harboring pUCBM20-ftsI recombinants encoding membrane-bound M-1-to-V-577 PBP3 (B), the E-193→D PBP3 mutant (C), and M-1-to-M-59-(insert)-R-60-to-V-577 PBP3 (D).*



DISCUSSION

In marked contrast with the monofunctional PBPs which are autonomous folding entities, the PB module of PBP3 does not fold properly by itself. Chaperones assist folding of a newly synthesized polypeptide chain into a correctly folded protein by blocking aggregation and other off-pathway reactions (5). Molecular chaperones such as GroEL and -ES provide an appropriate environment in which the polypeptide folds by itself (9, 15, 17). Prosequences broadly classified as intramolecular chaperones facilitate the folding of the polypeptide chain to which they are covalently linked. The concept of prosequence-assisted protein folding was first demonstrated for serine proteases (6, 25). As the PB module of PBP3 requires the presence of the associated n-PB module to acquire a stable, PB conformation, the question of what mechanisms and properties determine the folding assistance function of the n-PB module is discussed in light of the serine proteases.

The prosequence (166 residues) of the α-lytic protease of *Lysobacter enzymogenes* (26) and the n-PB module (258 residues) of *E. coli* PBP3 are more than two-thirds the length of the corresponding protease (198 residues) or PB module (318 residues). Folding of the protease in the absence of the prosequence results in no significant formation of an active protease. Similarly, folding of the PB module of PBP3 in the absence of the n-PB module or folding of a PBP3 in which the n-PB module is truncated or modified by single-amino-acid substitutions in

motif 3 results in the lack of significant formation of an active PBP derivative.

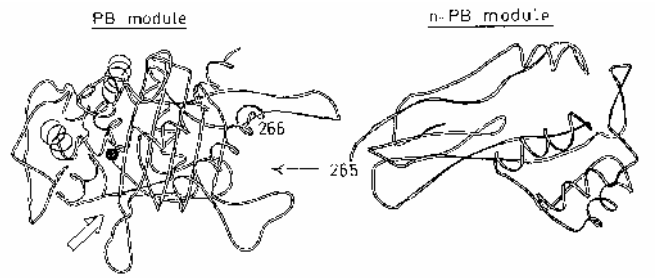
The prosequences of the subtilase family have conserved amino acid sequence motifs (25). Similarly, the motif 1-containing G-57-to-W-110 segment and the motif 3-containing G-188-to-D-197 segment of the n-PB module of PBP3 are key elements of the folding information. As was shown by the effects of single-amino-acid changes, D-76 of motif 1 contributes to the stability of the folded structure, and E-193 of motif 3 is essential for proper folding. Other studies highlight the major importance of motif 3. As was mentioned above, the G-191→D mutation confers thermosensitivity at 42°C to the *E. coli* RP41 PBP3 (perhaps independently of the accompanying D-266→N mutation). As was reported by Spratt and Cromie (27), plasmid-borne *ftsI* was mutagenized, and a collection of plasmids having information for the synthesis of thermosensitive PBP3 mutants that no longer functioned in cell division were obtained. Of the nine mutants that were analyzed, eight had amino acid substitutions in the PB module. One had a single-amino-acid substitution affecting E-190 in motif 3 of the n-PB module.

The prosequences of the serine proteases interact with the folded proteins (6, 25). As was shown previously (8), OmpT cleavage of the R-210-K-211 peptide bond in wild-type M-1-to-V-577 PBP3 and truncated F-37-to-V-577 PBP3 or G-57-to-V-577 PBP3 generates two fragments. These fragments remain associated with each other and behave like the uncleaved protein during chromatography under nondenaturing conditions. Labelling the cleaved PBP with radioactive benzylpenicillin and subjecting it to SDS-PAGE allowed a 38-kDa, PB fragment to be isolated. This fragment (shown in Fig. 3) was identified by amino acid sequencing as truncated K-211-to-V-577 PBP3.

The pre- and prosequences of the serine pre- and proproteases are cleaved off when transport and folding of the protease are attained. PBP3 behaves differently. The M-l-to-E-56 membrane anchor-containing module (or the pseudopresequence) and the n-PB module remain parts of the final structure in its native state. Knowledge of the three-dimensional structure of PBP3 would help an understanding the nature of the intermodule interactions.

The structure of PBP3 is not known. However, *Streptococcus pneumoniae* membrane anchor-free PBP2x has been crystallized (4), and its structure has been resolved at 3.5 Å (0.35 nm) (22). The PB module has the signature fold topology and active site of the penicilloyl serine transferases superfamily, and the associated n-PB module is shaped like a pair of sugar tongs (Fig. 8). The PB module has extensions that form an additional groove, the head of the pair of sugar tongs of the n-PB module fits into the noncatalytic groove of the PB module, and the hole (about 10-Å [0.1-nm] diameter) formed by the sugar tongs of the n-PB module is well exposed to the solvent, about 65 Å (0.65 nm) away from the active-site serine.

FIG. 8. *Schematic representation of the PB module and n-PB module of S. pneumoniae PBP2x (adapted from reference 22). The PB module and n-PB module are presented as disconnected entities. PBP2x is a single polypeptide chain. Residue 265 of the n-PB module is peptide linked to residue 266 of the PB module so that the head of the pair of sugar tongs of the n-PB module fits into the groove of the PB module (black arrow). Open arrow, catalytic site of the PB module; black ball, active-site serine.*



The four motifs characteristic of the n-PB modules of the multimodular class B PBPs were first identified by hydrophobic cluster analysis of the amino acid sequences (7). As PBP2x and PBP3 are members of the same class B, one may safely assume that these motifs are spatially equivalent in the two PBPs. The atomic coordinates of PBP2x are not available, but from a close inspection of the figures presented in reference 22, it appears that motifs 1, 2, 3, and 4 of PBP2x and, by extension, PBP3 are segments forming the head of the pair of sugar tongs of the n-PB module and, therefore, are important structural elements of the intermodule interaction.

The OmpT-susceptible R-210-to-K-211 peptide bond is on a lateral branch of the sugar tongs, a structural feature that well explains the behavior of the nicked PBP3 (see above). One may also note that motif 4 at the end of the n-PB module is part of helix al of the PB module of PBP2x. When it is defined as the S-259-to-V-577 polypeptide segment, the PB module of PBP3 presumably lacks al. It is unstable and does not bind penicillin (see Results). When it is defined as the L-240-to-V-577 polypeptide segment (14), the PB module of PBP3 presumably possesses al. It is also unstable, but it binds penicillin (see the introduction).

The n-PB module of PBP3 assists the folding of the PB module and thus functions as a prosequence. The n-PB module and the PB module of PBP3 are in close interaction and form an autonomous folding entity. Whether the PB module of PBP3, once folded, can be dissociated from the n-PB module while retaining its native state stability is not known. Whether the n-PB module can promote the folding of the PB module when expressed in *trans* is also not known. Putting these properties together, it is proposed to tentatively classify the n-PB module of PBP3 and by extension, the n-PB modules of the other class B PBPs as noncleaved, pseudointramolecular chaperones.

Another conclusion of this work is that the cell septation activity of PBP3 not only requires correct folding of the periplasmic part of the protein, it also depends on the M-1-to-E-56 amino-terminal module which encompasses the cytosol, the membrane, and the periplasm.

In the course of evolution, monofunctional penicilloyl serine transferases were fused to other polypeptides. As a result of these evolutionary events, the PB module of the *E. coli* PBP3, while it retained the same basic three-dimensional structure, lost its ability to fold autonomously, and traces of amino acid sequence similarity with the low-molecular-mass PBPs, other than with the active site-defining motifs, almost completely disappeared. At the same time, PBP3 became part of a multiprotein complex that synthesizes the septal peptidoglycan in a cell cycle-dependent fashion. It has been proposed (16, 23) that PBP3 interacts in the periplasm with bienzyme (transglycosylase-transpeptidase) multimodular class A PBP1b, endopeptidase monofunctional PBP7, and soluble lytic transglycosylase Slt70. It has also been proposed (2) that the intracellular M-1-to-R-23 segment of PBP3 interacts in the cytosol with FtsZ, a GTPase similar to tubulin (19), and FtsA, an ATPase similar to actin (24). The most plausible picture which emerges from these advances is that the n-PB module and the membrane anchor-containing module of PBP3 are important wiring elements of the network, providing recognition sites through which the acyl serine transferase (transpeptidase)-PB module is linked to other cell septation proteins.

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