

Hybrid Proteins of the Transglycosylase and the Transpeptidase Domains of PBP1B and PBP3 of *Escherichia coli*

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The construction of hybrid proteins of PBP1B and PBP3 has been described. One hybrid protein (PBP1B/3) contained the transglycosylase domain of PBP1B and the transpeptidase domain of PBP3. In the other hybrid protein, the putative transglycosylase domain of PBP3 was coupled to the transpeptidase domain of PBP1B (PBP3/1B). The hybrid proteins were localized in the cell envelope in a similar way as the wild-type PBP1B. In vitro isolates of the strains containing the hybrid proteins had a transglycosylase activity intermediate between that of wild-type PBP1B-producing strain and that of a PBP1B overproducer. Analysis with specific antibiotics against PBP1A/1B and PBP3 and mutant analysis in strains containing PBP3/1B revealed no detectable effects in vivo compared with wild-type strains. The same was shown for PBP1B/3 when the experiments were performed in a *recA* background. The data indicate that the hybrid proteins cannot replace native penicillin-binding proteins. This finding suggests that functional high-molecular-weight penicillin-binding protein specificity is at least in part determined by the unique combination of the two functional domains.

The high-molecular-weight penicillin-binding proteins (HMW-PBPs) of *Escherichia coli* are responsible for the final stages of the synthesis of the peptidoglycan layer. *E. coli* contains four HMW-PBPs, called PBP1A, -1B, -2, and -3 (15). In vitro they have similar enzymatic activities, namely, a transglycosylase activity, situated in the amino-terminal part of the protein, and a transpeptidase activity, situated in its carboxy-terminal part. If the HMW-PBPs are truly bifunctional, with the same biosynthetic activities, what is the basis for their functional differences in vivo? To explore which part, or combination of parts, of a specific HMW-PBP is responsible for its unique functional task, hybrid proteins might be helpful. For instance if the transpeptidase domain of one PBP is combined with the transglycosylase domain of another, does this new combination function, and if so, does it more resemble the PBP from which the transpeptidase part has been derived, or more the PBP from which the transglycosylase domain was derived?

Construction of the fusion plasmids. To create hybrid proteins containing the transglycosylase domain of PBP1B and the transpeptidase domain of PBP3, and vice versa, the borders of the domains have to be localized. The parts that are important for the transpeptidase activity of the HMW-PBPs are all situated downstream of the active-site serine residue box of the respective HMW-PBPs (9). Little is known about the transglycosylase domain, its active center, and the importance of surrounding sequences in the HMW-PBPs.

For PBP1B, the active-site serine of the transpeptidase domain is amino acid 510 (14). den Blaauwen (3) suggested that the transglycosylase domain of PBP1B resembles the active center of lysozyme and contains amino acid 304 (Asp). For the construction of the hybrid proteins, it is therefore assumed that a good border for PBP1B is about halfway between the proposed transglycosylase center (amino acid 304) and the transpeptidase center (amino acid 510) (Fig. 1).

For PBP3, the active-site serine of the transpeptidase domain is amino acid 307 (13). It is known that certain mutants of PBP3 (all point mutations in the carboxy-terminal part of the protein) are more resistant to cephalixin (7). This is probably caused by a change in tertiary structure of the transpeptidase domain, and so cephalixin is not able to bind as well to PBP3 as to the wild-type protein. The most amino terminal of these high-resistance mutations is a point mutation at amino acid 258. On the other hand, an extremely cephalixin-sensitive mutation is known; this is a point mutation at amino acid 219 (19a). A plausible explanation for the increased sensitivity could be that it is caused by a change in the tertiary structure of the transglycosylase domain; binding of cephalixin to the transpeptidase domain therefore will handicap the protein in a dual way. If so, the border between these two domains could be located between the most downstream sensitive and the most upstream resistant mutant. Thus, for PBP3, a good border between the transglycosylase domain and the transpeptidase domain might be between amino acids 219 and 258 (Fig. 1).

The hybrid genes were constructed by the use of restriction enzyme *Pst*I. The *ponB* gene was cut by *Pst*I between the sequences coding for amino acids 423 and 424. The *pbpB* gene was cut by *Pst*I between the sequences coding for amino acids 241 and 242.

To construct a hybrid protein (PBP1B/3) containing the transglycosylase part of PBP1B and the transpeptidase part of PBP3, the *ponB* gene-containing plasmid pBS99 and the *pbpB* gene-containing plasmid pPH115 were both cut with the restriction enzymes *Pst*I and *Bam*HI. Then the fragments were isolated and ligated (Fig. 1). The gene product (PBP1B/3) would thus contain amino acids 1 to 423 of PBP1B and amino acids 242 to 588 of PBP3. With the constructed plasmid (pCM12), wild-type strain MC4100 was transformed. A Western blot (immunoblot) of isolated cell envelopes of MC4100-(pCM12) showed the presence of the hybrid protein PBP1B/3 (Fig. 2) at 76 to 80 kDa in a sodium dodecyl sulfate-polyacrylamide gel (10). Because it contains the amino-terminal part of PBP1B, the hybrid occurs in an α form of 770 amino acids as well as a γ form of 728 amino acids.

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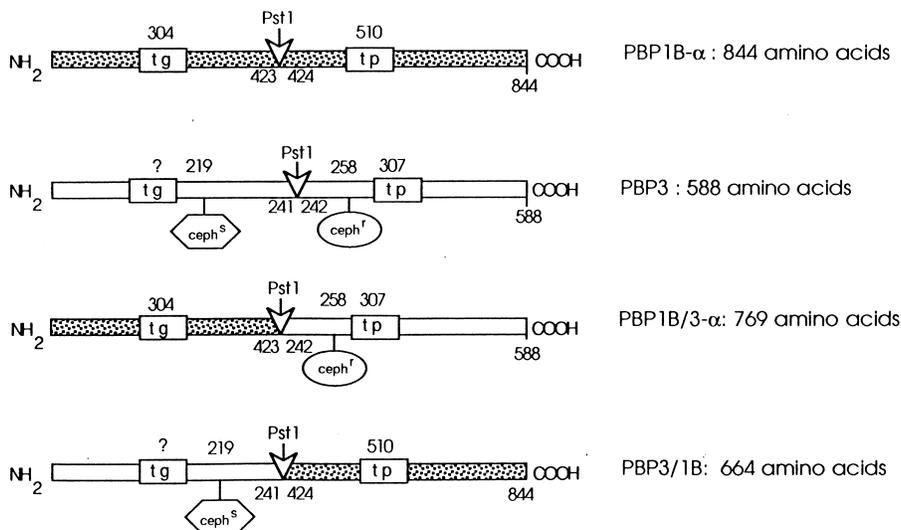


FIG. 1. Schematic depiction of PBP1B, PBP3, and the two hybrid proteins. NH₂, amino-terminal end of the protein; COOH, carboxy-terminal end of the protein; tg, proposed center for transglycosylase activity; tp, proposed center for transpeptidase activity; ceph^S, point mutation causing increased cephalaxin sensitivity; ceph^R, point mutation causing increased cephalaxin resistance.

To construct a hybrid protein (PBP3/31B) containing the transglycosylase part of PBP3 and the transpeptidase half of PBP1B, a similar procedure was followed. Plasmid pPH115 was cut with the restriction enzyme *Bam*HI; sticky ends were made blunt by DNA polymerase and then digested with *Pst*I. Plasmid pBS99 was digested with *Eco*RV and *Pst*I. The relevant fragments were isolated and ligated (Fig. 1). Thus, the hybrid protein (PBP3/1B) contains amino acids 1 to 241 of PBP3 and amino acids 424 to 844 of PBP1B (Fig. 1). With the constructed plasmid (pCM13), wild-type strain MC4100 was transformed. A Western blot of isolated cell envelopes of MC4100(pCM13) showed the presence of the hybrid protein PBP3/1B of 663 amino acids at the expected molecular mass of 62 kDa (Fig. 3). DNA techniques were performed as described by Maniatis et al. (12).

Cellular localization of the hybrid proteins. Both PBP1B and PBP3 are anchored to the inner membrane (1). It therefore seems likely that the hybrid proteins will also be translocated to the inner membrane. However, it is also possible that by the creation of the hybrids, sorting signals became mixed up. Therefore, the localization of the hybrid proteins was checked.

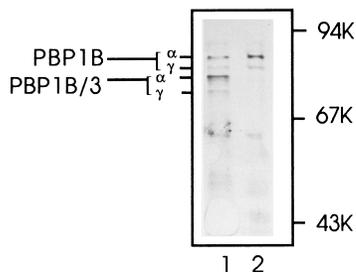


FIG. 2. Western blot containing isolated cell envelopes of MC4100(pCM12) containing the α and γ forms of the hybrid protein PBP1B/3 (lane 1) and wild-type strain MC4100 (lane 2). The blot was incubated with polyclonal antibodies against PBP1B. Blotting was performed as described by Towbin et al. (18), with the modifications described previously (21). The protein in isolated cell envelopes was determined as described by Lowry et al. (11). Polyclonal antibodies against PBP1B have been described by den Blaauwen et al. (5), and polyclonal antibodies against PBP3 were from B. Glauner.

Cells of strains containing the hybrid plasmids were sonicated, and after centrifugation, a cytoplasmic fraction and a cell envelope fraction were collected (21). The isolated cell envelopes were subfractionated by 0.5% (wt/vol) Sarkosyl (6) in a soluble inner membrane and a nonsoluble outer and peptidoglycan fraction. In Fig. 4 and 5, it can be seen that both hybrid proteins fractionated in a similar way as PBP1B. It was therefore assumed that both hybrids were translocated correctly.

In vivo phenotypic effects of the hybrids. To investigate whether the hybrid proteins were able to take over (parts of) the function of the HMW-PBPs from which they were created, they were analyzed in strains in which the respective HMW-PBPs were impaired or deleted. Attempts to transform pCM12 (PBP1B/3) to the PBP1B deletion strain SP1026 failed. It is therefore likely that this combination is lethal.

On the other hand, transformation of pCM13 (PBP3/1B) to SP1026 succeeded. Strain SP1026(pCM13) did not show differences compared with SP1026, with respect to growth rate and cell shape (data not shown). It is known that deletion of PBP1A plus PBP1B is lethal to the cell (20). We therefore checked whether pCM13 was able to compensate for their absence. However, deletion of the PBP1A gene (*ponA*) in SP1026(pCM13) by transduction was lethal. Thus, PBP3/1B is

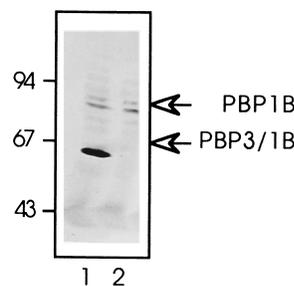


FIG. 3. Western blot containing isolated cell envelopes of MC4100(pCM13) containing the hybrid protein PBP3/1B (lane 1) and the wild-type strain MC4100 (lane 2). The blot was incubated with polyclonal antibodies against PBP1B (see the legend to Fig. 2). Sizes are indicated in kilodaltons.

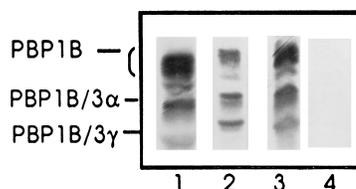


FIG. 4. Western blot of fractionated cells of MC4100(pCM12). Lanes: 1, soluble fraction after Sarkosyl treatment; 2, insoluble fraction after Sarkosyl treatment; 3, unfractionated cell envelopes; 4, cytoplasmic fraction.

not able to compensate for the absence of both PBP1B and PBP1A.

To investigate whether one of the hybrids could (partially) compensate for a mutated PBP3, the temperature-sensitive mutant LMC560 was transformed with each of the plasmids. It was found that, compared with the untransformed strain, there were no morphological changes, neither at the restrictive nor at the permissive temperature (data not shown). It was therefore concluded that the hybrid proteins were not able to take over the functions of their ancestors in the studied mutant strains.

Effects of antibiotics. To further assess the effects of the hybrid proteins, wild-type strains containing the hybrids were inhibited by antibiotics specific for PBP1B or PBP3. First, the strains were inhibited by the antibiotic cephalixin (2 μ g/ml), which has a high affinity for the PBP3 transpeptidase domain. It might be expected that PBP1B/3, containing the PBP3 transpeptidase domain, would also be able to bind cephalixin, whereas PBP3/1B, containing the PBP1B transpeptidase domain, would not. If PBP3/1B is able to take over (parts of) PBP3's task, it is presumed that this can be seen after inhibition with cephalixin. A growth experiment and morphological observations revealed that there is no difference in reaction of the hybrid-containing strains compared with the control strains (data not shown). Both strains, like the control strains, start filamentation by inhibition with cephalixin. Therefore, PBP3/1B (containing the PBP1B transpeptidase domain) is not able to take over (parts of) PBP3's function to prevent the strains from making cephalixin-induced filaments.

To study whether PBP1B/3 can take over (parts of) the task of PBP1B and/or PBP1A, wild-type strain MC4100 containing pCM12 was inhibited by cefsulodin. Cefsulodin is an antibiotic with a high affinity for the active center of the transpeptidase domain of PBP1A and PBP1B. If PBP3/1B, the hybrid containing the transpeptidase domain of PBP1B, is able to bind cefsulodin, cells containing pCM13 should react in a similar way to cefsulodin as wild-type cells. On the other hand, PBP1B/3, containing the transpeptidase part of PBP3, is expected not to be able to bind cefsulodin.

When cefsulodin (40 μ g/ml, 30°C) is used, MC4100(pBR328) and MC4100(pCM13) (containing PBP3/1B) start to lyse after

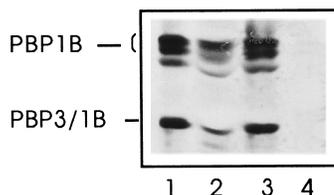


FIG. 5. Western blot of fractionated cells of MC4100(pCM13). Lanes: 1, soluble fraction after Sarkosyl treatment; 2, insoluble fraction after Sarkosyl treatment; 3, unfractionated cell envelopes; 4, cytoplasmic fraction.

TABLE 1. Transglycosylase activities of isolated cell envelopes

Strain	Activity (14 C cpm) ^a		
	Expt 1	Expt 2	Expt 3
SP1026	1,341 \pm 543	950 \pm 70	706 \pm 23
MC4100	15,336 \pm 1,624		
MC4100(<i>recA</i>)	15,188 \pm 1,591		
MC4100(pCM12)	22,194 \pm 1,921		
MC4100(pCM13)	20,424 \pm 2,330		
MC4100(pBS99)	23,558		
MC4100(<i>recA</i>)(pCM12)	19,543 \pm 1,143		
MC4100(<i>recA</i>)(pBS99)	22,052		
SP1026(pCM13)	4,159 \pm 770		
SP1026(pBS99)	5,621		

^a For each experiment, the activity of the pBS99-containing strain was taken as a standard, and the other values were related to that value.

50 min (data not shown). This was to be expected because it is known (20) that simultaneous inhibition of PBP1B and PBP1A causes cell lysis. MC4100(pCM12) (containing PBP1B/3) and the PBP1B-overproducing strain MC4100(pBS99) each showed a decreased growth rate, and filamentation could be seen (data not shown). Because the overproducing strain and the strain containing the hybrid PBP1B/3 reacted identically, we wondered whether, in the case of the hybrid protein, this was caused by recombination of the hybrid gene and whether filamentation was solely due to overproduction of PBP1B.

To investigate this possibility, pCM12 and pBS99 were transformed to the *recA* strain MC4100(*recA*). It can be seen (Fig. 6) that in this genetic background, the pCM12-containing strain behaves like the wild-type strain and not like the PBP1B-overproducing strain (see above). Therefore, it is likely that the effect found in MC4100 is due to recombination and that the filamentation is caused by the overproduction of PBP1B. If so, this finding also indicates that PBP1B/3 is not able to take over the function of either PBP1B or PBP1A.

In vitro transglycosylase activity. To investigate the activity of the hybrid proteins in vitro, isolated cell envelopes from strains containing the constructed plasmids were assayed for transglycosylase activity (4). The hybrids were first assayed in a wild-type background. It was found that both hybrids had a transglycosylase activity higher than that of the wild type (MC4100). In fact, their activities came closer to that of the PBP1B overproducer MC4100(pBS99) (Table 1). These results suggest that the hybrids contributed to the overall transglycosylase activity.

Because it is known that the transglycosylase activity of PBP1B overrules all other transglycosylase activities in vitro (16), the experiment was also performed in a PBP1B-less background (SP1026). Since this deletion strain could not be transformed with pCM12, the experiment was done only with pCM13. As can be seen (Table 1), pCM13 showed an activity somewhat lower than that of SP1026(pBS99). The activity of pCM12 was also measured in a *recA* strain because of the stability problems with this plasmid. Similar results were obtained as in the *recA* wild-type background; i.e., pCM12 showed an activity between that of the *recA* strain and that of the *recA* strain containing the PBP1B overproduction plasmid pBS99 (Table 1). From these results, it can be concluded that both pCM12 and pCM13 contributed to an increased transglycosylase activity in vitro.

Concluding remarks. The results so far indicate that these hybrids cannot replace the native forms in vivo. It seems that the bifunctional activity of the HMW-PBPs in vivo is determined, at least in part, by the unique combination of the two

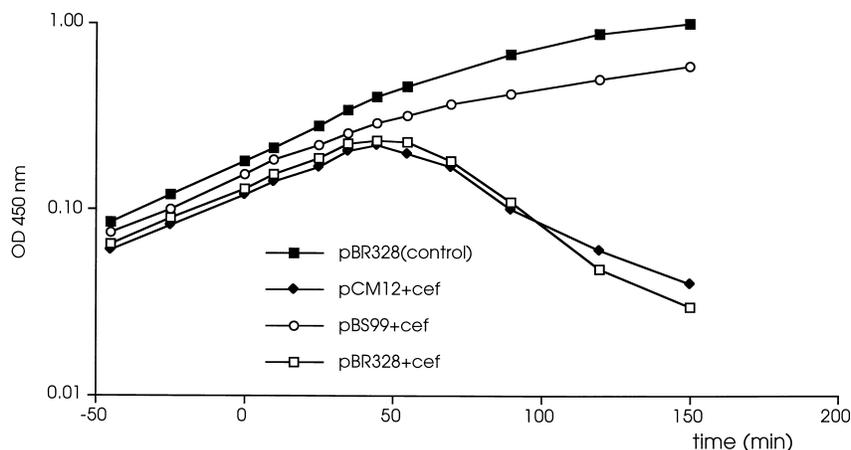


FIG. 6. Growth curve of the indicated strains after incubation with cefsulodin (cef) (40 $\mu\text{g/ml}$). The antibiotic was added at 0 min. All indicated plasmids were transformed in a MC4100(*recA*) background. Cells were grown in TY medium, which contained 1% tryptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco), and 0.5% NaCl. Liquid cultures were grown at 37°C in a water bath shaker if not stated otherwise. Plates contained TY medium with 2% Bacto-Agar (Difco). When necessary, ampicillin (100 $\mu\text{g/ml}$; Gist-Brocades, Delft, The Netherlands), chloramphenicol (35 $\mu\text{g/ml}$; Serva, Heidelberg, Germany), spectinomycin (50 $\mu\text{g/ml}$; Sigma, St. Louis, Mo.), or kanamycin (25 $\mu\text{g/ml}$; Sigma) was added. The cell concentration was determined by measuring the absorbance (optical density [OD]) at 450 nm in a T-1 spectrophotometer (Gilford Instrument Laboratories Inc., Halstead, Essex, England) of 2-ml samples taken from liquid cultures. To the sample, 0.5 ml of 0.5% formaldehyde was added. All bacterial strains are derivatives of *E. coli* K-12. The following strains were used: CSH26 [Δ (*lac-pro*) *ara* *thi*], MC4100 [F^- *araD139* Δ (*argF-lac*)*U169 rpsL150 flbB5301 ptsF25 deaC1 rbsR relA*] (2), MC4100(*recA*) [F^- *araD139* Δ (*argF-lac*)*U169 rpsL150 flbB5301 ptsF25 deaC1 rbsR relA recA*], SP1026 [*his supF* Δ (*ponB::Spe^r*)] (20), Sp1027 [*his supF* Δ (*ponA::Kan^r*)] (20), and LMC560 [F^- *araD139?* Δ (*araF-lac*)*U169 deoC1 FlbB5301 lysA1 pbpB(R1) ptsF25 rbsR relA rpsL150*] (17). pBS99 was used as the PBP1B-overproducing plasmid (1); pPH115 was used as a PBP3-overproducing plasmid (7).

enzymatic domains. For instance, inhibition of PBP1B by cefsulodin cannot be overcome in the presence of PBP1B/3. Likewise, inhibition of PBP3 by cephalixin cannot be overcome by PBP3/1B. On the other hand, in vitro transglycosylase activity in hybrid-containing strains appeared to be higher than in the wild-type strain. This finding suggests that the two hybrid PBPs contain potentially functional transglycosylase domains; i.e., the border between the two enzymatic domains in each hybrid was probably chosen correctly. It should, however, be noted that there is conflicting evidence with respect to transglycosylase activity of PBP3. Positive evidence was published by Ishino and Matsushashi (8); negative evidence has been published by van Heijenoort et al. (19). If PBP3 is not bifunctional, then perhaps the effect found with the PBP3/1B protein is an indirect one and PBP3 is able to promote a transglycosylase activity.

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