JOURNAL OF BACTERIOLOGY, Sept. 2003, p. 5342–5348 Vol. 185, No. 18
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.18.5342–5348.2003
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# Overproduction of Inactive Variants of the Murein Synthase PBP1B Causes Lysis in *Escherichia coli*

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Received 20 March 2003/Accepted 26 June 2003

**Penicillin-binding protein 1B (PBP1B) of *Escherichia coli* is a bifunctional murein synthase containing both a transpeptidase domain and a transglycosylase domain. The protein is present in three forms (α, β, and γ) which differ in the length of their N-terminal cytoplasmic region. Expression plasmids allowing the production of native PBP1B or of PBP1B variants with an inactive transpeptidase or transglycosylase domain or both were constructed. The inactive domains contained a single amino acid exchange in an essential active-site residue. Overproduction of the inactive PBP1B variants, but not of the active proteins, caused lysis of wild-type cells. The cells became tolerant to lysis by inactive PBP1B at a pH of 5.0, which is similar to the known tolerance for penicillin-induced lysis under acid pH conditions. Lysis was also reduced in mutant strains lacking several murein hydrolases. In particular, a strain devoid of activity of all known lytic transglycosylases was virtually tolerant, indicating that mainly the lytic transglycosylases are responsible for the observed lysis effect. A possible structural interaction between PBP1B and murein hydrolases in vivo by the formation of a multienzyme complex is discussed.**

The high-molecular-weight penicillin-binding proteins (PBPs) are responsible for the enlargement of the essential bacterial murein (peptidoglycan) sacculus by transpeptidation and transglycosylation of the murein precursors (17). In *E. coli* there are three bifunctional enzymes catalyzing both reactions, PBP1A, PBP1B, and PBP1C, and two monofunctional transpeptidases, PBP2 and PBP3 (12). PBP1A and -1B are the major bifunctional enzymes (13, 24), and a deletion of both is lethal for the cell (22). Encoded by a single gene (*ponB* or *mrcB*), PBP1B was shown to exist in three forms (α, β, and γ) which differ in the length of the short cytoplasmic part of the protein. Whereas PBP1Bβ is the result of a proteolytic cleavage of the first 24 N-terminal amino acids, PBP1Bγ arises from an alternative translational start at $M^{46}$ of the PBP1Bα form (14, 16, 18). Experimental data indicate that the α and γ form may have different functions during the cell cycle (3).

The hypothetical "three-for-one" model proposes a mechanism for the coordinated action of murein-synthesizing and -cleaving (-hydrolyzing) enzymes (11). It was suggested that hydrolases and synthases are synchronized in their activity by the formation of murein-synthesizing multienzyme complexes (10). Indeed, there is evidence that murein synthases interact with each other and with murein hydrolases (5, 19, 20, 25, 27). Until now, several protein-protein interactions involving PBP1B have been demonstrated by different methods: (i) PBP1B was shown to form dimers (27), which does not depend on the formation of disulfide bridges (4); (ii) purified PBP1B interacts with the cell division-specific transpeptidase PBP3 (T. Kast and J.-V. Höltje, unpublished data); and (iii) PBP1B interacts directly with the structural protein MipA, which itself interacts with the membrane-bound lytic transglycosylase A (MltA), a murein hydrolase, and a trimeric complex formed by these proteins could be reconstituted (25).

Because PBP1B is one of the major murein synthases and is involved in various protein-protein interactions, we tested the effect of overproduction of inactive PBP1B variants on cell viability. These variants contained single amino acid exchanges in the catalytic domains which prevent the catalytic reaction. If PBP1B is indeed part of murein synthesis multienzyme complexes, overproduced inactive variants could replace the active enzyme in the complexes. Because the murein hydrolases are still active in the complexes, one would expect a lytic effect. Indeed, we report in this communication that the overproduction of inactive PBP1B variants leads to lysis of the cells.

## MATERIALS AND METHODS

**Strains, growth media, and growth conditions.** *Escherichia coli* MC1061 (2) was grown in Luria broth (LB) medium at 37°C in a water bath with shaking. From the set of *E. coli* murein hydrolase mutants (9), strains MHD61 (Δ*amiA*::Cm Δ*amiB* Δ*mepA* Δ*dacB* Δ*pbpG*) and MHD82 (Δ*mltA*::Cm Δ*mltB*::Tc Δ*mltC* Δ*mltD* Δ*emtA*) were used. For testing tolerance at different pH values, complete medium (Bacto antibiotic medium 3; Difco, Detroit, Mich.) was used (7). The pH was adjusted to 5.0 with 1 M citric acid or to 7.2 with 1 M sodium hydroxide. The plasmids used in this study are described in Table 1. Kanamycin (Serva, Heidelberg, Germany) was added to strains harboring expression plasmids at a concentration of 50 μg/ml. Overnight cultures contained 1% glucose. Isopropylthiogalactoside (IPTG) was purchased from Gerbu, Gaiberg, Germany.

**Construction of expression plasmids.** The oligonucleotides (MWG Biotech, Gaiberg, Germany) used for cloning and site-directed mutagenesis are shown in Table 1. First, the *ponB* gene was amplified by PCR from chromosomal DNA of strain MC1061 with primers PBP1Baup and PBP1Bdown and Powerscript DNA polymerase (PAN Systems, Nürnberg, Germany). The PCR product was purified (Promega Wizard PCR Prep) and digested with *Eco*RI and *Bam*HI. After purification (Promega Wizard DNA Clean Up) the fragment was ligated (Roche Rapid DNA ligation kit) into plasmid pJFK118EH which had been digested with the same enzymes. The resulting plasmid, pUM1Bα, was transformed into MC1061. Plasmid pUM1bγ was constructed similarly by using the oligonucleotides PBP1Bcup and PBP1Bdown and plasmid pUM1Bα as the template for

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TABLE 1. Plasmids, oligonucleotides, and PBP1B variants

| Plasmid | Insert[a] | Oligonucleotides for cloning or site-directed mutagenesis | | Deduced protein[c] | Inactive domain[d] | Reference |
|---|---|---|---|---|---|---|
| | | Name | Sequence (5′→3′)[b] | | | |
| pJFK118EH | | | | | | 1 |
| pUM1Bα | *mrcB* gene ($A^1$TG-$TAA^{2535}$) | PBP1Baup<br>PBP1Bdown | TAT GAA TTC AAG CAT GGC CGG GAA TGA CC<br>CCA GGA TCC CGC TTA GAT GTT AAT TAC TAC C | PBP1Bα ($M^1$-$N^{844}$) | | This work |
| pUM1Bα* | *mrcB* gene ($A^1$TG-[$T^{1528}$→G]-$TAA^{2535}$) | PBP1Bmutup<br>PBP1Bmutdown | GCG TCG TTC GAT TGG TGC CCT TGC AAA ACC AGC G<br>CGC TGG TTT TGC AAG GGC ACC AAT CGA ACG ACG C | PBP1Bα* ($M^1$-[$S^{510}$→A]-$N^{844}$ | TP | This work |
| pUM1BTG*α | *mrcB* gene ($A^1$TG-[$G^{697}$→C]-$TAA^{2535}$) | 1bMutTGup<br>1bMutTGdown | GGA TAC TTT GCT GGC GAC ACA AGA CCG TCA TTT TTA CG<br>CGT AAA AAT GAC GGT CTT GTG TCG CCA GCA AAG TAT CC | PBP1Bα(TG*) ($M^1$-[$E^{233}$→Q]-$N^{844}$) | TG | This work |
| pUM1BTG*α*[e] | *mrcB* gene ($A^1$TG-[$G^{697}$→C; $T^{1528}$→G]-$TAA^{2535}$) | 1bMutTGup<br>1bMutTGdown | | PBP1Bα*(TG*) ($M^1$-[$E^{233}$→Q: $S^{510}$→A]-$N^{844}$) | TP + TG | This work |
| pUM1Bγ | *mrcB* gene, short form[f] ($A^{136}$TG-$TAA^{2535}$) | PBP1Bcup<br>PBP1Bdown | TAT GAA TTC ACC GAT GCC GCG CAA AGG | PBP1Bγ ($M^{46}$-$N^{844}$) | | This work |
| pUM1Bγ* | *mrcB* gene, short form[g] ($A^{136}$TG-[$T^{1528}$→C]-$TAA^{2535}$) | PBP1Bcup<br>PBP1Bdown | | PBP1Bγ* ($M^{46}$-[$S^{510}$→A]-$N^{844}$) | TP | This work |

[a] The numbers indicate the positions of the nucleotides in the *mrcB* gene.
[b] The underlined nucleotides are mutated for site-directed mutagenesis.
[c] The numbers indicate the positions of the amino acids in the PBP1Bα protein.
[d] TP, transpeptidase; TG, transglycosylase.
[e] Plasmid pUM1BTG*α* was generated by site-directed mutagenesis of pUM1Bα* with oligonucleotides 1bMutTGup and 1bMutTGdown.
[f] The insert of plasmid pUM1Bγ was amplified from pUM1Bα.
[g] The insert of plasmid pUM1Bγ* was amplified from pUM1Bα*.

PCR. Site-directed mutagenesis (Stratagene QuickChange site-directed mutagenesis kit) was performed according to the protocol of the manufacturer. The inserts of all plasmids were sequenced at the sequencing facility of the Max-Planck-Institut, Tübingen, Germany, and the correct sequences could be confirmed.

**Preparation and analysis of membrane proteins.** Membranes of *E. coli* were prepared according to a published procedure (25) 1 h after induction and before the onset of lysis. Membranes were washed twice with 10 mM potassium phosphate–10 mM magnesium chloride (pH 6.8) and resuspended in 50 mM potassium phosphate–10 mM magnesium chloride (pH 7.0). Membrane proteins were analyzed by boiling of resuspended membrane preparations in sample buffer followed by discontinuous sodium dodecyl sulfate–8% polyacrylamide electrophoresis (SDS–8% PAGE) (15). The proteins in SDS gels were detected by staining with Coomassie blue. For the detection of PBP1B, the proteins in an SDS gel were transferred to nitrocellulose (Western blotting). The blot membrane was blocked by incubation over night at 4°C with TTBS (0.1 M Tris-HCl [pH 7.5], 0.9% NaCl, 0.2% Tween 20) containing 5% dehydrated skim milk. After four washes with TTBS, the blot membrane was incubated with a 1/1,000 dilution of a polyclonal rabbit anti-PBP1B antiserum (kindly provided by M. Nguyen-Disteche, Liege, Belgium) in TTBS containing 0.5% dehydrated skim milk. After washing (four times, 10 min each), a 1:2,000 dilution of donkey anti-rabbit–horseradish peroxidase conjugate (Amersham Life Science) was added and left for 1.5 h. The membrane was then washed as described above, and the PBP1B bands were visualized by peroxidase reaction with chloronaphthol as a substrate. For this, the blot membrane was developed with fresh chloronaphthol reagent (0.5 mg of chloronaphthol per ml in 0.1 M Tris-HCl [pH 7.4] plus 0.5 μl of 30% hydrogen peroxide per ml) until the bands appeared.

**Detection of PBPs.** The PBPs present in the membranes were labeled with biotinylated ampicillin and detected as described previously (6). Following SDS-PAGE and transfer to nitrocellulose, the PBPs were detected with streptavidin-horseradish peroxidase by using chloronaphthol as the substrate (see above).

**Preparation of lysing cells for light microscopy.** MC1061(pUM1Bα*) was grown at 37°C in LB medium containing 50 μg of kanamycin per ml. At an optical density (OD) of 0.1 the culture was divided into two parts, and 1 mM IPTG was added to one part. For comparison, wild-type MC1061 was grown in LB medium (without kanamycin). At an OD of 0.1 the culture was divided, and 100 μg of penicillin G was added to one part. At the onset of lysis, 70 min after the addition of IPTG or of penicillin G, the cells were fixed by treatment with 2% glutaraldehyde–0.1% formaldehyde for 30 min. After being washed with phosphate-buffered saline, the cells were examined by light microscopy.

## RESULTS

**Overproduction of PBP1B variants.** It was shown previously that the $Ser^{510}$→Ala exchange inactivates the transpeptidase domain and that the $Glu^{233}$→Gln exchange inactivates the transglycosylase domain of PBP1B, because these residues are essential for the catalytic reactions (24). Different variants of PBP1B were overproduced by the use of the multicopy plasmid pJFK118EH (1) containing the *ponB* gene behind the IPTG-inducible Ptac promoter. This plasmid (pUM1Bα) was changed by site-directed mutagenesis, resulting in the production of PBP1B variants in which residues in the active site of the transpeptidase or transglycosylase domain (or both) were replaced with similar amino acid residues (see above; Table 1).

Overproduction of the PBP1B variants in MC1061 upon induction with IPTG was confirmed by analysis of the membrane proteins (Fig. 1A). Analogous to the situation in wild-type cells, the overexpression of the *ponB* gene from the plasmid led to the appearance of three forms of the protein (α, β, and γ) with the expected molecular weights, with the amounts of α and γ form being largest (Fig. 1A). The level of overproduction, after induction with 1 mM IPTG for 1 h, was estimated to be about 50 to 100 times higher than that in wild-type cells. The produced proteins were recognized by an antiserum against PBP1B (Fig. 1B), and the activity was analyzed by a

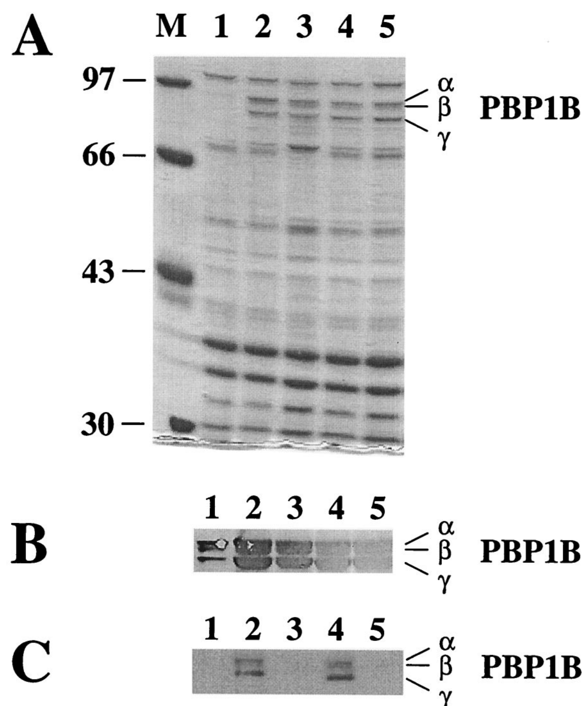


FIG. 1. Overproduction of PBP1B variants in MC1061. Different variants of PBP1B were overproduced by the induction with 1 mM IPTG for 1 h at 37°C. (A and B) Membranes were prepared, and either the proteins were separated by SDS–8% PAGE and stained with Coomassie blue (A) or the proteins were transferred to nitrocellulose and PBP1B was detected with a polyclonal antiserum (B). In order to detect active penicillin-binding protein, the samples were incubated with biotinylated ampicillin prior to SDS–8% PAGE and transfer to nitrocellulose. (C) The biotin label was recognized by streptavidin-horseradish peroxidase and visualized with chloronaphthol. Lane M, low-molecular-weight protein marker. Lanes 1, MC1061(pJFK118EH) (control). Lanes 2, MC1061(pUM1Bα) (overproduction of native PBP1B forms). Lanes 3, MC1061(pUM1Bα*) (overproduction of PBP1B forms with inactive transpeptidase domain). Lanes 4, MC1061(pUM1BTG*α) (overproduction of PBP1B forms with inactive transglycosylase domain). Lanes 5, MC1061(pUM1BTG*α*) (overproduction of PBP1B forms with inactive transpeptidase and transglycosylase domains). Numbers on the left are molecular weights in thousands.

PBP assay (Fig. 1C). As expected, only the variants with an intact transpeptidase domain showed a strong signal.

**Effect of overproduction of PBP1B variants on growth.** The different variants of PBP1B were overproduced in MC1061 growing in LB medium at 37°C. Cells overproducing native PBP1B did not show differences in growth compared to control cells harboring the empty plasmid (Fig. 2). However, the overproduction of PBP1B with inactive catalytic domains (either transpeptidase or transglycosylase or both) led to lysis of the cells (Fig. 2). Lysis started about 1 h after induction, and the lysis rates were identical for all three cases. The concentration of the inducer molecule IPTG was varied from 0.01 to 1 mM. The addition of 0.01 mM IPTG did not cause lysis, but lysis was observed upon induction with 0.03 mM IPTG, although lysis started later and was not as pronounced as that in the presence of 1 mM IPTG (Fig. 3A). Thus, the lysis effect correlates with the amount of overproduced inactive PBP1B. Lysis was not observed when the inactive short form PBP1Bγ was overproduced by the addition of 1 mM IPTG to MC1061(pUM1Bγ*).

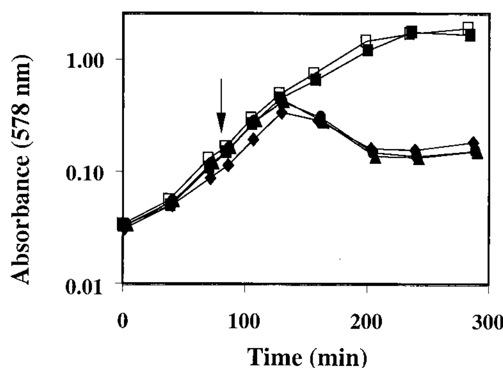


FIG. 2. Growth of MC1061 with overproduction of inactive PBP1B variants. MC1061 harboring either empty expression plasmid pJFK118EH (open squares) or the PBP1B expression plasmid pUM1Bα (closed squares), pUM1Bα* (diamonds), pUM1BTG*α (triangles), or pUM1BTG*α* (circles) was grown at 37°C in LB medium containing 50 μg of kanamycin per ml. At an OD of 0.1, the production of PBP1B variants was induced by the addition of 1 mM IPTG to the cultures.

However, with similar concentrations of IPTG, the amount of overproduced PBP1Bγ was smaller than the amount of PBP1Bαβγ (Fig. 3B). The reason for the smaller amount of inactive PBP1Bγ is unknown, since we could confirm by se-

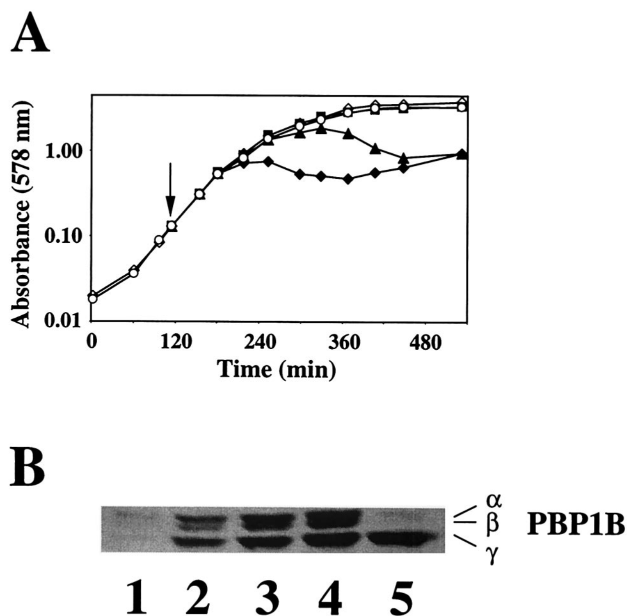


FIG. 3. Overproduction of variants of PBP1Bαβγ and PBP1Bγ with different inducer concentrations. (A) Cultures of MC1061(pJFK118EH) (open diamonds) (control), MC1061(pUM1Bα*) (squares, triangles, and closed diamonds), or MC1061(pUM1Bγ*) (circles) were grown in LB medium with 50 μg of kanamycin per ml. At an OD of 0.1, the MC1061(pJFK118EH) and MC1061(pUM1Bγ*) cells received 1 mM IPTG, whereas the culture of MC1061(pUM1Bα*) was divided into three aliquots which received 0.01 (squares), 0.03 (triangles) or 0.05 mM (closed diamonds) IPTG. The growth was monitored by OD measurement. (B) One hour after the addition of IPTG, one aliquot was removed from each culture and membranes were isolated as described in Materials and Methods. The membrane proteins were separated by SDS–8% PAGE, and after blotting on nitrocellulose, PBP1B was detected with an antiserum. Lane 1, MC1061(pJFK118EH); lane 2, MC1061(pUM1Bα*) with 0.01 mM IPTG; lane 3, MC1061(pUM1Bα*) with 0.03 mM IPTG; lane 4, MC1061(pUM1Bα*) with 0.05 mM IPTG; lane 5, MC1061(pUM1Bγ*) with 1 mM IPTG.

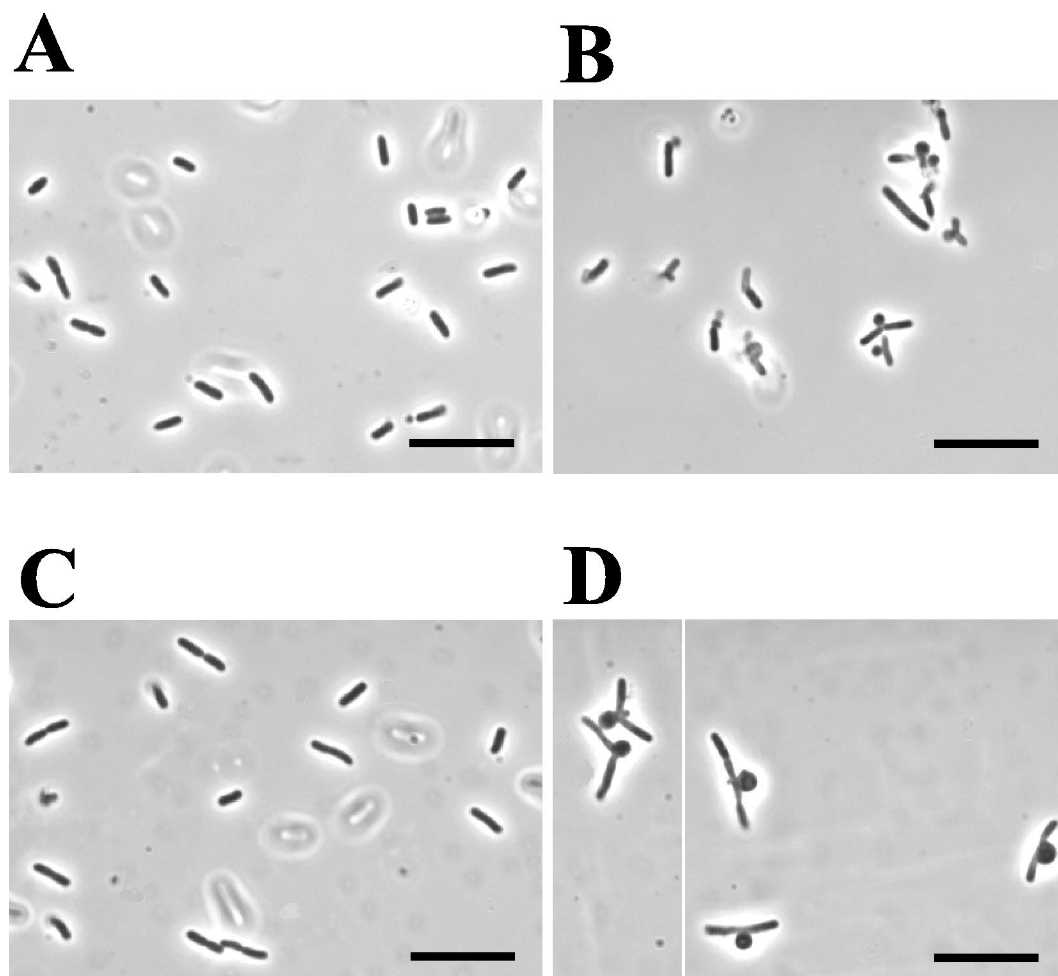


FIG. 4. Light microscopy of cells at the onset of lysis. MC1061(pUM1Bα*) was grown in LB medium with 50 μg of kanamycin per ml. Wild-type MC1061 was grown in LB medium. At an OD of 0.1, the cultures were divided. In MC1061(pUM1Bα*) the overproduction of inactive PBP1B was induced by the addition of 1 mM IPTG (B), whereas MC1061 received 100 μg of penicillin G per ml (D). After 70 min, the cells were fixed for light microscopy as described in Materials and Methods. Control cultures of MC1061(pUM1Bα*) (A) and MC1061 (C) received neither IPTG nor penicillin G. Bars, 10 μm.

quencing the correctness of the promoter region on the plasmid. There was no difference in lytic response when inactive PBP1B was overproduced upon addition of 1 mM IPTG in mutants lacking either PBP1A, PBP1B, or MipA. With 0.05 mM IPTG, the lytic response was similar in wild-type cells and mutants without PBP1B or MipA but was faster in a mutant lacking PBP1A (data not shown).

**Characterization of the lysis effect.** Cells lysing as a result of the production of inactive PBP1B variants were examined by light microscopy (Fig. 4). These cells were slightly smaller than wild-type cells, which could be due to the presence of kanamycin in the growth medium. Otherwise, lysis of cells upon production of inactive PBP1B was almost indistinguishable from that of wild-type cells lysing as a result of the action of penicillin G (Fig. 4); like in the case of penicillin-induced lysis, the disruption of the cells appears to occur most often at the site of cell division, indicating that the site of cell division is most prone to both types of lysis. This similarity in lysis phenotype is paralleled by the almost-identical growth response of cells that either overproduced inactive PBP1B or received 100 μg of penicillin G per ml (Fig. 5A). It was reported before that cells growing in medium with a low pH of 5.0 show a tolerance to penicillin-induced lysis (7). Under these conditions, a tolerance phenotype was also seen upon overproduction of inactive PBP1B (Fig. 5B). Thus, the overproduction of inactive PBP1B results in a lysis effect similar to the lysis following the inactivation of the PBPs by exogenous penicillin.

**Overproduction of inactive PBP1B variants in multiple murein hydrolase mutant strains.** To test whether the lytic effect is the result of uncontrolled activity of autolytic enzymes, the inactive PBP1B variants were overproduced in murein hydrolase mutant strains. Although a library of multiple murein hydrolase mutants exists, due to the antibiotic resistance markers, most of these strains are incompatible with the plasmids used in this study. Nevertheless, overproduction of PBP1B variants could be studied in strain MHD61, lacking two amidases (AmiA and AmiB) and three endopeptidases (MepA, PBP4, and PBP7), and in strain MHD82, lacking five membrane-bound lytic transglycosylases (MltA, MltB, MltC, MltD, and EmtA). In both strains, the overproduction of active

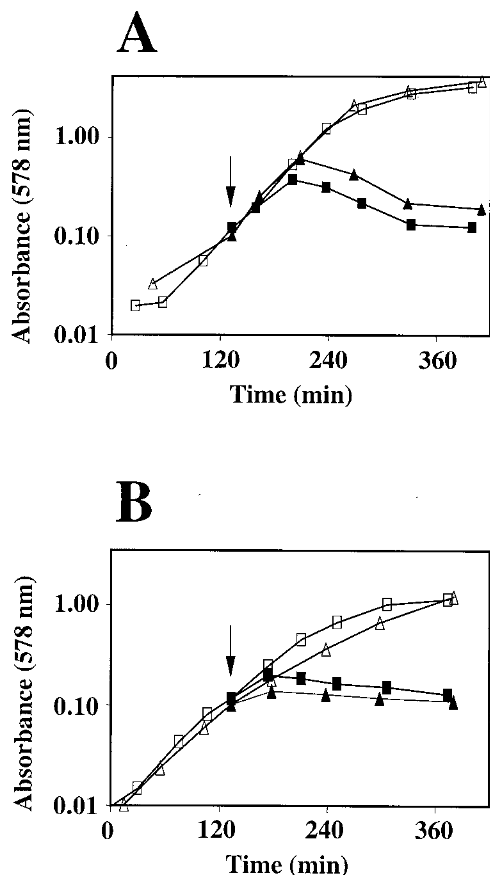


FIG. 5. Penicillin-induced lysis and lysis upon overproduction of inactive PBP1B at pH 7.2 and 5.0. MC1061(pUM1Bα*) (squares) or MC1061 (triangles) was grown at 37°C in Bacto antibiotic medium 3 at a pH of 7.2 (A) or 5.0 (B). At an OD of 0.1 (arrow), the cultures were divided and one part (closed symbols) received either 1 mM IPTG to induce the overproduction of inactive PBP1B [MC1061(pUM1Bα*)] or 100 μg penicillin G per ml (MC1061).

PBP1B had no effect on growth (Fig. 6A). Compared to that in the wild type, lysis upon overproduction of inactive PBP1B was slightly delayed in MHD61 and was significantly slower in MHD82 (Fig. 6B). Upon the addition of bulgecin, an inhibitor of the soluble lytic transglycosylase Slt70 (23), the MHD82 cells were devoid of activity of all known lytic transglycosylases. Under these conditions, these cells were virtually tolerant towards lysis as a result of overproduction of inactive PBP1B, and a microscopic examination revealed a dramatically lower frequency of lysing MHD82 cells compared to wild-type cells (data not shown). The amounts of overproduced inactive PBP1B were similar in MHD82 and in wild-type cells (Fig. 6C). Thus, the lysis observed in wild-type cells is caused mainly by the activity of the lytic transglycosylases.

## DISCUSSION

Most bacteria contain autolytic enzymes which, under certain conditions, may degrade the murein sacculus, causing autolysis of the cell (21). Spontaneous lysis of growing cells occurs after inhibition of the formation of the murein precursor molecules in the cytoplasm and also by the inhibition of the two final periplasmic steps of murein synthesis, the transpeptidation by β-lactam antibiotics (such as penicillin) or the transglycosylation by moenomycin.

In this communication we report a novel bacteriolytic phenomenon: in contrast to lysis due to the inactivation of murein synthetic enzymes by exogenous antibiotics, lysis is induced by the overproduction of inactive variants of a bifunctional transpeptidase-transglycosylase, PBP1B, in the presence of wild-type PBP1B. Overproduction of the native (active) protein to the same extent did not result in lysis. Because the inactive variants contained a single exchange of an amino acid in the active site, it is likely that the three-dimensional structure of the protein was not disturbed and that lysis was the consequence of the nonfunctionality of the catalytic domains. It should be pointed out that lysis occurred in wild-type cells in which the whole set of murein-metabolizing enzymes, including active PBP1B, is present. Lysis occurs only if PBP1B is overproduced in an inactive (and not in the active) form, showing that the lytic response is not caused merely by the overproduction of the protein per se, as observed frequently for other overproduced proteins.

Several details of the described lysis phenomenon deserve comments. First, the amount of inactive PBP1B that led to lysis was rather large. Lysis is a complex phenomenon related to the structural integrity of the murein sacculus, and we do not know the minimal number and the size of defective sites (or holes) needed to cause a lytic effect. Also, we do not know if the cell has repair mechanisms for correcting defects in the sacculus.

Second, we could never observe lysis when an inactive variant of only the short form PBP1Bγ was overproduced in the presence of up to 5 mM IPTG. With similar concentrations of IPTG, inactive PBP1Bγ expressed from pUM1Bγ* was present in smaller amounts than all three forms of inactive PBP1B expressed from the full-length *ponB* gene from the plasmid pUM1Bα*. The reason for the lower production of PBP1Bγ is unknown. It remains to be elucidated whether the cells did not lyse because of the smaller amount of PBP1Bγ or whether PBP1Bα and PBP1Bγ participate in different processes that are different in their proneness towards lysis. Recently, different roles for PBP1Bα and PBP1Bγ in murein synthesis during cell division and cell elongation, respectively, were proposed (3).

Third, and strikingly, the growth curves as well as the microscopic appearance of cells lysing upon overproduction of inactive PBP1B were quite similar to those of cells lysing due to the activity of an exogenous β-lactam antibiotic. In both situations, lysis often starts during cell division at the midpoint of the cell, which is the site of focused murein synthesis. The tolerance to lysis at low pH was also comparable for both the lysis induced by penicillin and the lysis induced by overproduction of inactive PBP1B. In the case of β-lactam-induced lysis, this tolerance effect was speculated to be the consequence of a suppressed murein hydrolase (autolytic) activity (6). Thus, we concluded that the observed lysis upon overproduction of inactive PBP1B is due to the unbalanced activity of murein hydrolases as in the case of penicillin-induced lysis.

In *E. coli*, there is an astonishing number of known autolytic enzymes, i.e., six lytic transglycosylases, three amidases, and three endopeptidases (8, 12), making it difficult to define specific roles for each of these hydrolases. Nevertheless, we could

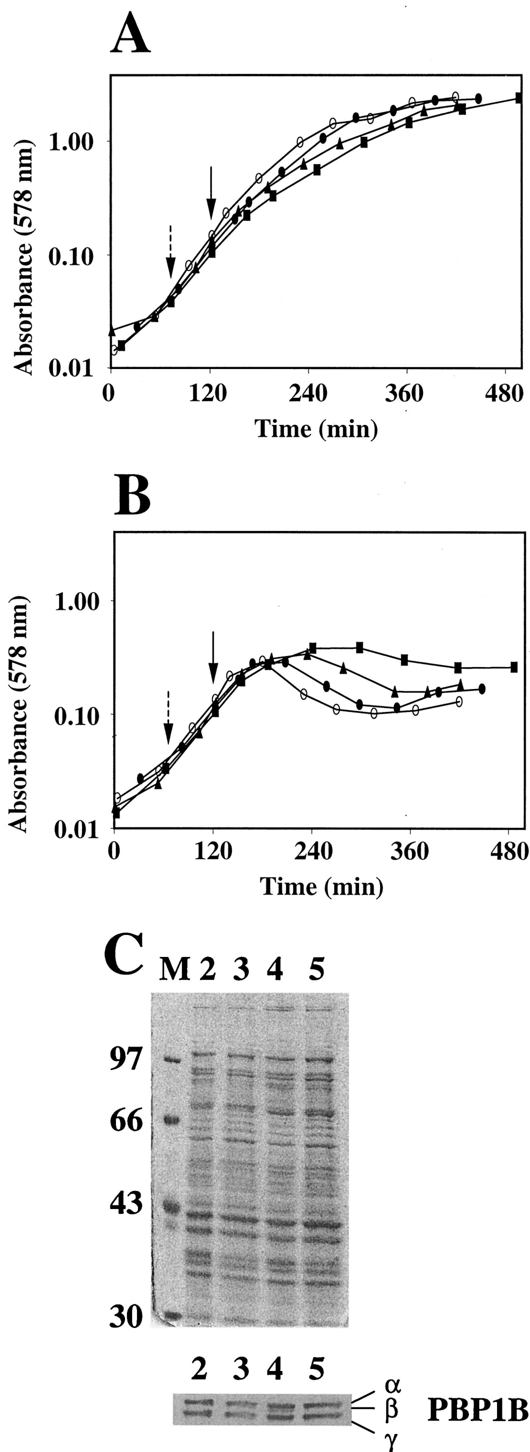


FIG. 6. Overproduction of active or inactive PBP1B in murein hydrolase mutants. (A and B) Wild-type MC1061 (open circles), MHD61 (closed circles), or MHD82 (triangles and squares) harboring either pUM1Bα (A) (control, overproduction of active PBP1B) or pUM1Bα* (B) (overproduction of inactive PBP1B) was grown at 37°C in LB medium. At an OD of 0.1 (arrow), the cultures received 1 mM IPTG. One culture of MHD82 (squares) also received 20 μg of bulgecin per ml at the indicated time (dashed arrow) to inhibit the soluble lytic transglycosylase. (C) To compare the amounts of overproduced PBP1B variants in MHD82 grown in the presence of bulgecin and in MC1061, cells were harvested 1 h after induction with 1 mM IPTG and boiled in SDS sample buffer. The total protein was analyzed by SDS–8% PAGE following Coomassie blue staining (upper panel). PBP1B variants were detected with antiserum after blotting on nitrocellulose (lower panel). Lane M, molecular weight marker; lane 2, MC1061(pUM1Bα) (overproduction of active PBP1B); lane 3, MC1061(pUM1Bα*) (overproduction of inactive PBP1B); lane 4, MHD82(pUM1Bα) (overproduction of active PBP1B); lane 5, MHD82(pUM1Bα*) (overproduction of inactive PBP1B). Numbers on the left are molecular weights in thousands.

show that lysis due to the overproduction of inactive PBP1B was caused mainly by the action of lytic transglycosylases. A mutant lacking five membrane-bound lytic transglycosylases and with the soluble lytic transglycosylase Slt70 inhibited with bulgecin was virtually tolerant towards overproduction of inactive PBP1B.

It was found previously that purified PBP1B interacts with MltA via the structural protein MipA, and a trimeric complex formed by these proteins could be reconstituted on the surface of a BIAcore sensor chip (25). Furthermore, we detected the binding of PBP1B present in a crude membrane extract to immobilized MltB by affinity chromatography (W. Vollmer and J.-V. Höltje, unpublished data). These results suggest that there may be structural interactions between PBP1B and different lytic transglycosylases in vivo.

The observation that overproduction of inactive PBP1B results in lysis due to the action of murein hydrolases raises the question of how the presence of an inactive enzyme can affect the function of another enzyme. Shortly after the recognition of the murein sacculus as a covalently closed, bag-shaped macromolecule, Weidel and Pelzer proposed the enzymatic balance model for the growth of the sacculus (26). Accordingly, two opposing enzyme systems, hydrolases and synthases, are function in a balanced way, with the former introducing nicks into the murein structure and the latter inserting new material at these sites. Therefore, bacteriolysis following the inhibition of a step of murein synthesis, either the precursor formation or the insertion of the precursors into the sacculus, has been explained as being the consequence of an imbalance between the synthetic and hydrolytic enzyme activities.

However, the precise mechanism of lysis after inhibition of murein synthesis remained elusive. The observed lysis effect upon overproduction of inactive PBP1B could be explained in different ways: (i) inactive PBP1B could still bind lipid II, leading to a depletion of precursor molecules, a situation that is known to cause lysis, or (ii) assuming a limited number of murein synthesis sites per sacculus, PBP1B could block such sites by preventing the binding of active synthases and thus inhibit murein synthesis, leading to lysis. However, the question of by which mechanism a possible depletion of precursor or a blocking of murein synthesis sites would cause lysis remains.

On the basis of a previous model of a multienzyme complex combining both synthetic and hydrolytic enzymes in a murein synthesis machine, we favor the following, alternative explanation for the lysis caused by the overproduction of inactive PBP1B. Overproduced inactive PBP1B is likely to replace the active enzyme in the complex. The resulting complexes are no longer capable of catalyzing some synthetic reactions (peptide cross-linking and/or glycan strand polymerization) but still con-

tain a fully active hydrolase subcomplex. The accumulation of these semifunctional or "unbalanced" complexes, which continue to insert cuts into the murein sacculus but fail to add proper new material, would then result in a lytic effect. As expected, the inactivation of the transpeptidase or the transglycosylase domains (or both domains) in the overproduced enzyme results in lysis. In our view, the lytic response upon overproduction of inactive PBP1B variants could be an indication of the existence of multienzyme complexes containing PBP1B and autolytic enzymes in vivo.

## ACKNOWLEDGMENTS

We thank Astrid Ursinus for excellent technical assistance and Uli Schwarz for his interest and support.

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