JOURNAL OF BACTERIOLOGY, June 1989, p. 3025–3030
0021-9193/89/063025-06$02.00/0

Vol. 171, No. 6

Penicillin-Binding Protein 2 Is Essential in Wild-Type *Escherichia coli* But Not in *lov* or *cya* Mutants

TERU OGURA,[1] PHILIPPE BOULOC,[2] HIRONORI NIKI,[1] RICHARD D'ARI,[2] SOTA HIRAGA,[1] AND ALINE JAFFÉ[2]*

Department of Molecular Genetics, Institute for Medical Genetics, Kumamoto University Medical School, Kumamoto 862, Japan,[1] and Institut Jacques Monod, Centre National de la Recherche Scientifique, Université Paris 7, 2 Place Jussieu, 75251 Paris Cedex 05, France[2]

Received 18 November 1988/Accepted 3 March 1989

Penicillin-binding protein 2 (PBP2), target of the β-lactam mecillinam, is required for rod morphology and cell wall elongation in *Escherichia coli*. A new temperature-sensitive PBP2 allele and an in vitro-constructed insertion deletion allele were shown to be lethal in wild-type strains, establishing that the activity of this protein is essential. Mutations in the *lov* or *cya* genes, conferring mecillinam resistance, compensated for the deleterious effect of the absence of PBP2. The resulting double mutants grew as spheres. In a *cya* mutant lacking PBP2, the restoration of a Cya$^+$ phenotype by addition of cyclic AMP caused lethality and a block in cell division. These results show that in wild-type cells, PBP2 is essential for growth and division.

The rod shape of *Escherichia coli* and other bacilli is maintained by the rigid, highly cross-linked peptidoglycan layer. The growth of rod-shaped *E. coli* cells involves the insertion of new cell wall material to elongate the cylindrical cell surface and cross-wall synthesis or septation. Mutants defective in the elongation process grow as spherical cells, which suggests that elongation is not essential for bacterial division. They carry mutations in the *rodA-pbpA* region at 15 min on the genetic map (2, 17, 31, 35) or in the *mre* region at 71 min (37, 40). The *pbpA* gene codes for penicillin-binding protein 2 (PBP2), which is the specific target of the β-lactam antibiotic mecillinam (22, 30, 32). Rod-shaped bacteria become round in the presence of this antibiotic, and after several divisions they lyse (19, 24, 28, 39). Although mecillinam kills wild-type cells, it is possible to select viable resistant mutants that are spherical at 42°C; these include *pbpA*(Ts) and *rodA*(Ts) mutants. In a *pbpA*(Ts) mutant cultivated at 42°C, no PBP2 can be detected by penicillin binding and fluorography, whereas a *rodA*(Ts) mutant shows normal β-lactam binding to PBP2 (30, 34). The *pbpA* and *rodA* genes are contiguous (31) and lie in the same operon (25). All of the PBPs are involved in cell wall metabolism. PBP2 has peptidoglycan synthesis activity, catalyzing the transpeptidase (cross-linking) and transglycosylase reactions; the latter activity requires the presence of RodA (16). Both of these proteins are involved in determining bacterial cell shape, and they seem to act specifically in cell wall elongation.

A special class of mecillinam-resistant mutants exists: *cya* and *crp* mutants (1, 9, 18) and the recently described *lov* mutant (4, 5). These strains, in contrast to *pbpA* and *rodA* strains, have short, rod-shaped morphology, associated with a slow growth rate; in the presence of mecillinam, they become spherical but continue to grow. These mutants are probably affected in the regulation of cell wall synthesis. It has been speculated that the *lov* gene product may be involved in evaluation of the growth rate and coordination of the elongation and septation processes with growth rate (5).

During the cell cycle, peptidoglycan synthesis is required for lateral elongation of the cell wall, septum formation, and determination of bacterial cell shape. PBP2 and the RodA protein, as discussed above, seem to be specific to the elongation process. PBP3, on the other hand, is involved in septum formation (15); *pbpB*(Ts) mutants, coding for a temperature-sensitive PBP3, stop dividing at high temperature and form long filaments (38). Interactions between gene products involved in these activities have been suggested. (i) An interaction between PBP2 and the RodA protein is suggested by the finding that the transglycosylation reaction associated with PBP2 is detected only in the simultaneous presence of RodA (16). (ii) An interaction between the RodA protein and PBP3 was suggested by Begg et al. (3), who selected a mutation in the *rodA* gene (*sui*) specifically able to suppress a particular mutant allele of *pbpB*. (iii) An interaction between PBP3 and the FtsA protein was demonstrated by Tormo et al. (36); FtsA, whose role has not yet been determined, is involved in a late step of cell division (10, 11). The lethal action of mecillinam, which is in striking contrast to the viability of *pbpA*(Ts) mutants at high temperature, may reflect interactions of this kind, whereby mecillinam, by binding to PBP2, can indirectly affect another protein (e.g., RodA, PBP3, or FtsA) involved in cell wall elongation, septation, or shape determination.

In this work, we describe a new class of *pbpA* mutants that are characterized by the fact that they are mecillinam sensitive yet grow as spherical cells at high temperature. Among them, one mutant is thermosensitive for growth and lyses at 42°C, mimicking the lethal action of mecillinam on wild-type strains. This finding, together with results for a previously reported *pbpA* lethal mutant, SP6 (29), suggested that PBP2 could be an essential protein. To test this hypothesis, an internal fragment of the *pbpA* gene was replaced in vitro with a kanamycin cassette to inactivate PBP2. The *pbpA*::*kan* gene could not be introduced into a wild-type strain in the absence of a mini-F *pbpA*$^+$ plasmid. We demonstrate that the mutated gene is lethal, leading to a cell division block and loss of viability. The lethality can be suppressed by *lov* and *cya* mutations, which are known to suppress the lethality due to mecillinam (1, 4). The *lov pbpA*::*kan* and *cya pbpA*::*kan* strains grow as spheres. These results suggest that PBP2 plays a double role in cell growth: a nonessential activity required for cell wall elonga-

* Corresponding author.

TABLE 1. Bacterial strains

Strain	Genotype
W3110	Prototroph
SH2	W3110 *trpE5 his*
SH3208	SH2 (λ)
C600	*thr leu thi lac*
SP45	*trp tyr ilv supD126 pbpA45*(Ts)
KJB21	*thr leu pro his thy ftsI lip zbf*::Tn*5*
SK2255	*thy zbf*::Tn*10*
PP7860	*argH his* Δ*cya*
FS1576	C600 *recD1009*
B1617	SP45 *recA441*(Tif)
GC3344	C600 *eda*::Tn*10 lov-1*
GC3388	*lov-1 rpsL282*
SH3234	SH3208 *pbpA12*
SH3235	SH3208 *pbpA13*
SH3236	SH3208 *pbpA18*
SH3237	SH3208 *pbpA51*
SH3238	SH3208 *pbpA63*
SH3249	SH3238 Rif^r
GC7309	SH3249 *ilv*::Tn*5* Δ*cya*
GC7315	SH3249 *eda*::Tn*10 lov-1*
GC7353	W3110 *pbpA51 zbf*::Tn*5*
GC7364	FS1576 *pbpA*::*kan*
GC7368	GC7364 *pbpA*$^+$ *zbf*::Tn*10*
GC7370	GC3344 R4
GC7378	PP7860 *pbpA*::*kan*

tion and an essential activity that can be bypassed in *lov* and *cya* mutants.

MATERIALS AND METHODS

Bacterial strains and plasmids. All strains used were *E. coli* K-12 derivatives (Table 1). Strains W3110 (21), SH3208 (13), SP45 (31), and FS1576 (33) have been described. KJB21 and SK2255 were kindly provided by Ken Begg, and PP7860 was given to us by Agnes Ullmann.

The cosmid vector pHSG262 (6) was obtained from the Japanese Cancer Research Resources Bank and used to prepare a library of chromosomal DNA segments of strain W3110. The plasmid vector pACYC184 (8) was used for subcloning chromosomal DNA fragments. Plasmids pBS47 and pLG346 (34) were kindly provided by Brian Spratt. The temperature-sensitive runaway replication plasmid pOU82 (12) was received from S. Molin. Plasmid pXX747 was constructed by joining the *Bam*HI DNA segment containing the *lacZ* gene of *E. coli* and a *Bam*HI DNA segment of pOU82 (13). Mini-F plasmid pXX326, which had the partition segment carrying the *sopA*, *sopB*, and *sopC* genes but not the *ccd* segment, was described previously (27). Plasmid pACYC177 (8) was the source of the kanamycin fragment used to construct a plasmid containing a disrupted *pbpA* gene.

Media. Cells were grown in LB broth (26) or P medium (1% polypeptone, 0.5% NaCl, pH 7.4) or in appropriately supplemented ME or M63 salts (26). Agar P plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml were used for β-galactosidase expression tests. Antibiotics were used at the following concentrations (micrograms per milliliter): chloramphenicol, 20; tetracycline, 10; kanamycin, 20 or 40; ampicillin, 25; mecillinam, 1, 15, or 25.

Isolation of *pbpA* mutants. Mutants were isolated in the course of developing a method for detection of anucleate cell-producing mutants. The strategy used has been extensively described elsewhere (13). It consists of detecting blue colonies at 42°C on X-Gal plates after ethyl methanesulfonate (EMS) mutagenesis of strain SH3208 containing the thermosensitive runaway replication plasmid pXX747.

Fluorescent DNA staining. The DNA staining technique developed by Hiraga et al. consists of observing 4,6-diamidino-2-phenylindole (DAPI)-stained bacterial DNA in a Zeiss microscope, using simultaneous phase contrast and fluorescence (13).

Genetic techniques and DNA manipulations. Genetic techniques and procedures used for DNA manipulations have been described previously (23, 26).

RESULTS

Characterization and mapping of morphological mutants. *E. coli* mutants showing a rod shape at 30°C and a spherical shape at 42°C were detected as described in Materials and Methods after EMS mutagenesis of strain SH3208 harboring the plasmid pXX747. The selection was set up to detect bacterial mutants spontaneously producing a high level of anucleate cells. Three classes of mutants were found. (i) One mutant produced minicells; it carried a mutation at 25 min, in or near the *min* locus. (ii) Several mutants produced normal-size anucleate rod-shaped cells; one of them, called *mukA*, was shown to carry a *tolC* mutation at 65 min (13). (iii) Five mutants had a spherical shape at 42°C and a rod shape at 30°C.

Mutants known to be spherical at high temperature are affected either in the *pbpA* and *rodA* genes, at 15 min, or in the *mre* region, at 71 min on the *E. coli* chromosome; all of these mutants are affected in sensitivity to the β-lactam mecillinam. The plating efficiencies of the five new spherical mutants, the parental strain SH3208, and the *pbpA*(Ts) mutant SP45 were measured in the presence and absence of mecillinam at 30 and 42°C. Plating efficiency at 42°C in the presence of mecillinam (25 μg/ml) varied from 10^{-2} to 10^{-3} for the five mutants compared with the same cultures plated at 42°C in the absence of antibiotic. Plating efficiencies of the parental strain and strain SP45 were 10^{-4} and 0.8, respectively, at 42°C. At 30°C, all strains had a plating efficiency of about 10^{-4}. One of the five mutants was shown to be thermosensitive for growth, giving a ratio of 10^{-2} in colony-forming ability on LB plates at 42°C compared with 30°C.

The mutant strain showing thermosensitive growth and a spherical shape at 42°C (SH3238) was used for preliminary mapping. A chromosomal DNA library of strain W3110 was constructed in the cosmid vector pHSG262 (Km^r) and used to infect strain SH3238. Colonies resistant to kanamycin were isolated at 42°C. Among 100 colonies tested by microscopic observation, one was found to contain rod-shaped bacteria at 42°C and to have recovered thermoresistance. The cosmid DNA was extracted and analyzed by restriction enzyme cleavage, and the pattern was compared with the whole cleavage map of the *E. coli* chromosome (21). The complementing cosmid pAX224 was found to contain a segment located near 15 min and covering the *pbpA-rodA* genes. To confirm the location of the mutation, P1 phage transductions were carried out with P1 lysates prepared on strains carrying a Tn*5* or Tn*10* transposon close to the *pbpA-rodA* genes (KJB21 and SK2255). After infection of SH3238, 50 to 80% of the transductants recovered a wild-type phenotype. To determine whether the new mutations were in the *pbpA* or *rodA* gene, we introduced plasmids pBS47 and pLG346, carrying the *pbpA*$^+$ and *rodA*$^+$ genes, respectively (34). All five mutations were suppressed by

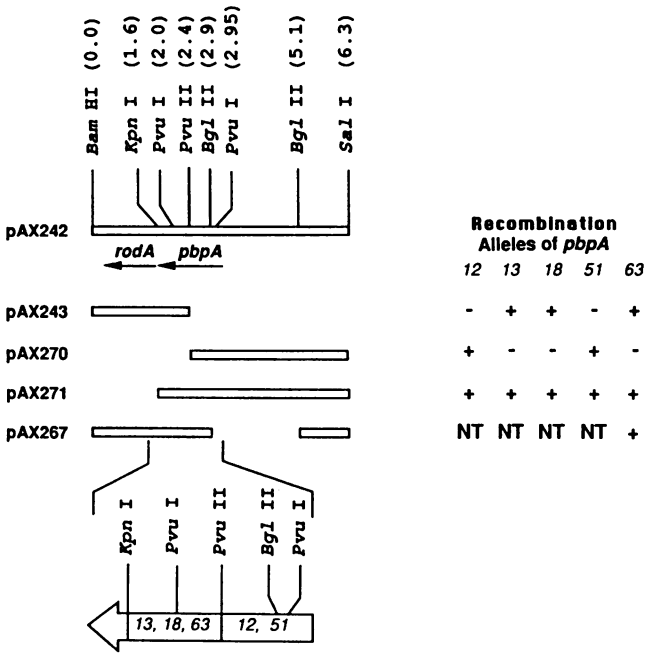

FIG. 1. *pbpA-rodA* region and new *pbpA* alleles. The ability (+) or inability (−) of DNA fragments to give rise to rod-shaped recombinants in the five new *pbpA* mutants is indicated; NT, not tested. The five *pbpA* alleles are located with respect to the *Pvu*II site. Numbers in parentheses represent the distance in kilobases from the *Bam*HI site.

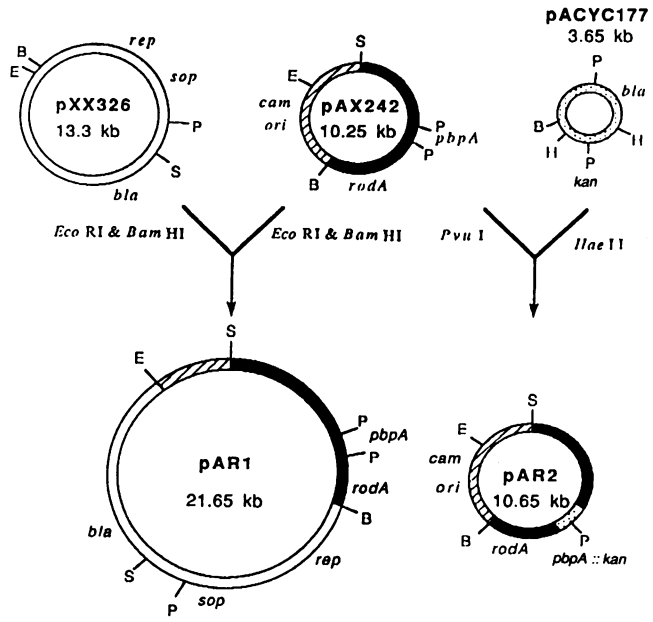

FIG. 2. Construction of plasmids pAR1 and pAR2. The *Hae*II sites are shown only in pACYC177. Abbreviations and symbols: *rep* and *sop*, Mini-F replication and partition regions, respectively; *ori*, replication origin of pACYC184; *kan*, *cam*, and *bla*, kanamycin, chloramphenicol, and ampicillin resistance genes, respectively; ▬, chromosomal DNA; ▭, mini-F pXX326 DNA; ▨, pACYC184 DNA; ▩, pACYC177 DNA. Abbreviations for restriction endonucleases: B, *Bam*HI; E, *Eco*RI; H, *Hae*II; P, *Pvu*I; S, *Sal*I.

pBS47 but not by pLG346, which showed that all lie in the *pbpA* gene; the allele in strain SH3238 was named *pbpA63*. The 11.95-kilobase *Bam*HI fragment of the complementing cosmid pAX224 containing the *pbpA-rodA* region was cloned in plasmid pACYC184. The resulting plasmid, pAX225, corrected the *pbpA63* mutation, as expected. A *Sal*I deletion derivative of pAX225, called pAX242 (Fig. 1), also corrected *pbpA63* and the other four *pbpA* mutations. A series of deletion derivatives of pAX242 were constructed (Fig. 1). None corrected the five *pbpA* mutations. However, some of the *pbpA* mutants carrying different plasmids showed a mixture of rod-shaped and spherical cells after 24 h of incubation at 42°C, which indicated that recombination had occurred between the plasmid and the chromosome to produce a *pbpA*$^+$ gene. By this means, the alleles *pbpA63*, *pbpA13*, and *pbpA18* were mapped between the *Pvu*II and *Kpn*I sites, and the alleles *pbpA12* and *pbpA51* were mapped on the other side of the *Pvu*II site (Fig. 1).

Division block and lysis of *pbpA63* at 42°C. The plating efficiency of mutant *pbpA63* (SH3249) was lowered by a factor of 100 at 42°C compared with its colony-forming ability at 30°C. On shift of a *pbpA63* culture from 30 to 42°C, lysed cells accumulated in the culture. Microscopic observation revealed that the change in morphology occurred progressively, starting with rods which increased in diameter, became ovoid, and finally became spherical at 150 min. Upon measurement of the average rod length at time zero and sphere diameter at 180 min, it was striking that despite the change in cell shape from rod to sphere, the length (or diameter) remained constant. The DNA mass at 180 min was much larger, as judged by microscopic fluorescence. The constant length and the volume and DNA mass increases, which are common features of mutants changing from rod to spherical shape, will be discussed elsewhere (W. Donachie and K. Begg, submitted for publication).

Suppression of the lethality of *pbpA63* by *lov* and *cya* mutations. The lethality of strain *pbpA63* mimics the lethal action of the antibiotic mecillinam, which is known to bind specifically to PBP2 (30, 32). On the other hand, the rod-shaped mutants *lov* and *cya* have been shown to be mecillinam resistant, although no defect in PBP2 binding activity could be detected (5, 18). The possibility that these mutations might counteract the lethality of *pbpA63* at 42°C was tested. The plating efficiencies of the double mutants *pbpA63 lov* (GC7315) and *pbpA63 cya* (GC7309) were shown to be identical at 30 and 42°C, and lysis did not occur, which showed that the lethal effect due to *pbpA63* mutation at 42°C could be suppressed by the presence of either the *lov* or the *cya* mutation. Furthermore, the double mutants remained spherical at 42°C, which showed that although the *lov* and *cya* mutations suppressed the lethality of *pbpA63*, the elongation process was still inhibited in the double mutants. These observations suggested that PBP2 may be an essential protein in wild-type cells.

Complete inactivation of PBP2 by disruption of the *pbpA* gene. To test the hypothesis that PBP2 is essential, we constructed an inactive *pbpA* gene in vitro. The *pbpA* gene in plasmid pAX242 was inactivated by deleting the internal *Pvu*I segment within the *pbpA* gene and replacing it with the *Hae*II fragment from plasmid pACYC177 containing a kanamycin resistance gene; one of the resulting plasmids was named pAR2 (Fig. 2). We then attempted to introduce this *pbpA*::*kan* deletion-insertion gene into the chromosome by transforming the *recD* strain FS1576 with the larger linear DNA fragment of pAR2 digested with *Bam*HI and *Sal*I, selecting for kanamycin resistance. As a control, the same strain harboring the mini-F *pbpA*$^+$ *rodA*$^+$ plasmid pAR1 was transformed in parallel. The plasmid constructions are de-

scribed in Fig. 2. There were about eight times more Km^r transformants in FS1576 harboring pAR1 than in FS1576. The Km^r transformants obtained from the haploid strain FS1576 were chloramphenicol resistant and rod shaped; they were shown to harbor an intact pAR2 plasmid, which contaminated the linearized DNA fragment. After 48 h of incubation, microcolonies appeared on the transformation plates. Only one was able to grow after purification. This clone, GC7364, contained a homogeneous population of spherical, mecillinam-resistant cells. Southern blot analysis of chromosomal DNA from this strain confirmed that the *pbpA* gene was disrupted (not shown).

The Km^r transformants derived from FS1576 harboring the *pbpA*$^+$ plasmid pAR1 were analyzed and classified in four types: (i) 10% of the clones contained only rod-shaped cells and were found to harbor an intact pAR2 plasmid (Cm^r); (ii) 35% of the clones contained only rod-shaped cells and lost the Ap^r and Km^r markers concomitantly during incubation without selection, which indicated that these clones contained the *pbpA*$^+$ gene on the chromosome and the *pbpA*::*kan* gene on the mini-F plasmid (it has been shown that mini-F plasmids are unstable in *recD* mutants [26a]); (iii) 10% of the clones contained only rod-shaped cells but segregated neither Ap^s nor Km^s segregants during incubation without selection, which indicated that the mini-F plasmid was integrated in the chromosome and that one of the two *pbpA* genes had been replaced by the *pbpA*::*kan* gene; and (iv) 45% of the clones contained a mixed population consisting of a large majority of rod-shaped cells and few spherical cells; these were found to have the *pbpA*::*kan* gene on the chromosome and an extra-chromosomal mini-F *pbpA*$^+$ *rodA*$^+$ plasmid (not shown). The mini-F plasmid is unstable in *recD* strains (26a), and the spherical cells presumably arose from plasmid-free segregants. These results show that transformants carrying the disrupted *pbpA* gene on the chromosome are very rare in the absence of a *pbpA*$^+$ plasmid. A P1 lysate prepared on strain GC7364 was used to attempt to transduce the *pbpA*::*kan*-disrupted gene into C600. No Km^r transductants could be obtained on either rich medium or minimal glucose medium. The inability to transduce the disrupted gene suggests that the absence of PBP2 activity is deleterious to the bacteria but that compensatory mutations can arise to allow bacterial growth; this was the case for clone GC7364, as described below.

Compensation for lack of PBP2 by *lov* and *cya* mutations. In view of the fact that *lov* and *cya* mutations were able to suppress the lethality of the *pbpA63* mutant without affecting its spherical morphology at 42°C, we attempted to transduce the disrupted *pbpA*::*kan* gene of GC7364 into *lov* and *cya* mutants. In both cases, 100 to 300 Km^r transductants were obtained. The cells were spherical, which indicated the absence of PBP2 activity, and Southern blot analysis of the *cya pbpA*::*kan* strain confirmed that the *pbpA* gene was disrupted (not shown).

Two other derivatives of the *lov* mutant were also used. They were (i) GC7370, a pseudorevertant of *lov* isolated as a fast-growing *lov* derivative (*lov* R4) retaining the mecillinam resistance character and (ii) the *lov rpsL282* strain GC3388, in which the *rpsL282* allele suppresses both the slow growth rate and the mecillinam resistance of the *lov* mutant; these two strains have been described recently (4, 5). In LB broth at 37°C, the generation times of wild-type (C600), *lov* (GC3344), *lov* R4 (GC7370), and *lov rpsL282* (GC3388) strains were 22, 60, 30, and 40 min, respectively. In the fast-growing *lov* R4 derivative, which remained mecillinam resistant, 50 to 100 Km^r transductants were obtained; the cells were spherical and the *pbpA* gene was disrupted, which showed that this strain was able to grow in the absence of PBP2. In contrast, in the *lov rpsL282* strain, which is also a fast-growing *lov* derivative but mecillinam sensitive, no Km^r transductants were obtained. These results suggest that the capacity to survive in the absence of PBP2 is not related to the growth rate but rather to the mecillinam resistance associated with the *lov*, *lov* R4, and *cya* mutations.

In view of this finding, it was thought that the original *pbpA*::*kan* transformant, GC7364, might have acquired a mutation in a gene (different from *pbpA*) conferring mecillinam resistance. The *pbpA*::*kan* gene in strain GC7364 was transduced to *pbpA*$^+$ by using a P1 lysate prepared on SK2255, which contains a Tn*10* transposon 88% cotransducible with the *pbpA* gene. The Tc^r Km^s transductants obtained were rod shaped. One was tested for mecillinam sensitivity. The plating efficiency of the *pbpA*$^+$ transductant (GC7368) in the presence of mecillinam was 1,000-fold higher than that of the parental (*pbpA*$^+$) strain FS1576. The suppressor mutation conferring mecillinam resistance in strain GC7368 was not in the *cya* or *crp* gene, as tested by the ability to grow on maltose as carbon source. Preliminary results suggest that it may be located in or near the *lov* locus.

Lack of PBP2 affects cell division. The double mutant *cya pbpA*::*kan* (GC7378) allowed us to analyze the fate of PBP2-deficient cells by adding cyclic AMP (cAMP) to a culture. After 7 h of incubation, samples were observed by DAPI staining. Cell size in the untreated culture was essentially homogeneous, with a majority of spherical cells 2 to 4 μm^3 in volume and 5 to 10% large spheres about 25 μm^3 in volume, as calculated from diameter measurements on photomicrographs; a few lysed cells were present in the culture. In contrast, the cAMP-treated culture exhibited a mixture of distorted, sphere-shaped DNA-containing cells and a large number of ghosts heterogeneous in size. The volume of the nucleate cells varied from 25 to 150 μm^3, which represented an increase of 50-fold compared with the volume of the untreated bacteria. These results show that in a Cya$^+$ context, the lack of PBP2 causes cell division inhibition and lysis.

We compared the perturbed division pattern in the absence of PBP2 with that observed after induction of the SOS-dependent division inhibitor SfiA (14), known to block the essential division protein FtsZ (20). The *recA441*(Tif) mutation alters the RecA protein in such a way as to induce the SOS response spontaneously at 42°C in the presence of adenine without any perturbation of DNA replication (7). A culture of the double mutant *recA441 pbpA45* was shifted to 42°C in the presence of adenine, and DAPI-stained samples were analyzed in a fluorescent microscopy. After 3 h of incubation at 42°C, strain B1617 (*recA441 pbpA45*) showed a population of spherical DNA-containing cells whose volume varied from 25 to 200 μm^3, as calculated from diameter measurements on photomicrographs. This experiment demonstrates that combining inhibition of PBP2 transpeptidase activity with a well-defined cell division block results in the same phenotype as that observed in the total absence of PBP2.

Another type of experiment was carried out to observe the effect of inactivating PBP2. As mentioned above, our five new *pbpA* mutants are mecillinam sensitive at 42°C, although they grow as spheres at this temperature. By choosing one of the thermoresistant alleles, we were able to observe the effect of mecillinam on an actively growing population of spherical cells. Strain *pbpA51* (GC7353) was incubated at 42°C in rich medium for 2 h, which is the time

required to obtain a homogeneous spherical cell population. Mecillinam (1 μg/ml) was then added to the culture. After 3 h of incubation in the presence of mecillinam at 42°C, the culture contained enormous DNA-containing spherical cells and a large number of ghosts, just as in the culture of strain GC7378 after addition of cAMP. The diameter of the ghosts was heterogeneous, approximately corresponding to spheres of 20 to 100 μm^3; this finding shows that lysis is not caused by high osmotic pressure but occurs independently of cell volume. In the *pbpA51* mutant, PBP2 activity was severely restricted at 42°C, but cell division proceeded normally in the absence of mecillinam. The addition of a low concentration of mecillinam perturbed the division pattern exactly as in mecillinam-treated wild-type cells (19) or in the *cya pbpA*::*kan* strain growing in the presence of cAMP.

DISCUSSION

Previously described spherical mutants altered in PBP2 elongation activity were selected as derivatives resistant to mecillinam, a β-lactam specific to PBP2. In this paper, we describe five new spherical mutants affected in the *pbpA* gene, which codes for PBP2. These mutants differ from the earlier ones by their sensitivity to mecillinam, which shows that alterations in PBP2 can inactivate the transpeptidase activity of the protein without abolishing mecillinam sensitivity. Similarly, it has recently been shown that single amino acid replacements in PBP2 can result in mutant proteins that retain penicillin-binding capacity but have no transpeptidase activity (H. Adachi, T. Andoh, T. Ohta, and H. Matsuzawa, Bacterial Cell Surfaces in Bioscience, Ito, Japan, 1988, p. 30). Thus, PBP2 transpeptidase activity and penicillin-binding capacity (or mecillinam sensitivity) can clearly be separated.

One of the new mutant alleles, *pbpA63*, was lethal at 42°C. At the nonpermissive temperature the cells became spherical, division was blocked, and lysis occurred, mimicking the action of mecillinam on wild-type cells. This finding suggested that PBP2 might be an essential protein despite the existence of viable spherical *pbpA* mutants unable to carry out cell wall elongation. To test this idea, we constructed in vitro a *pbpA* gene in which an internal fragment was deleted and replaced with a kanamycin cassette. The *pbpA*::*kan* gene could not be readily recombined into the chromosome of a wild-type strain unless a second *pbpA*$^+$ gene was present on a plasmid, consistent with the hypothesis that PBP2 is essential.

The *lov* and *cya* mutations are known to confer mecillinam resistance. These mutants are rod shaped in the absence of mecillinam but grow as spheres in its presence; they thus suppress the lethal effect of mecillinam but not its inhibition of cell wall elongation. Both of these mutations suppressed the lethality of the *pbpA63* and *pbpA*::*kan* alleles. In the total absence of PBP2, as in the presence of mecillinam, the cells grew as spheres, with no lateral elongation. It is therefore clear that PBP2 is required for maintenance of the rod shape and that, even when shape is not maintained, PBP2 is essential for growth in *lov*$^+$ *cya*$^+$ strains. The essential role of PBP2 was studied in three different conditions: in the *pbpA*::*kan cya* strain cultivated in the presence of cAMP, in the *pbpA63* lethal mutant cultivated at 42°C, and in the *pbpA51* nonlethal mutant cultivated at 42°C in the presence of mecillinam. In all cases, the cells stopped dividing and formed large spheres, with volume increases of up to 50-fold. Similar spherical monsters were obtained after inhibiting the division of the *pbpA45* mutant by induction of the SOS-dependent division inhibitor SfiA.

The perturbed division pattern observed in the absence of PBP2 could reflect the reduction of total transpeptidase activity (a quantitative change) or the loss of another activity associated with PBP2 (a qualitative change). In the first case, the lethality and cell division block observed could occur through a reduction in peptidoglycan cross-linking due to the total absence of PBP2-associated transpeptidase activity. In this case, it must be assumed that viable spherical *pbpA*(Ts) mutants, although lacking sufficient transpeptidase activity to maintain a rod-shaped cell wall, have retained enough activity to divide and survive. This hypothesis is difficult to reconcile with the fact that the lethality associated with total absence of PBP2 is suppressed by *lov* and *cya* mutations, which confer mecillinam resistance but are not known to affect the PBPs (5, 18). The ability of *lov* and *cya* mutants to support the absence of PBP2 cannot be explained by growth rate differences, since one such strain (GC7370, *lov* R4) actually grew faster than a Mec^r *lov* derivative (GC3388, *lov rpsL282*), which could not accept the *pbpA*::*kan* gene.

In the hypothesis of a qualitative change, PBP2 is assumed to have two activities, a nonessential activity required for cell morphology and an essential activity involved in cell division. This second function of PBP2 is presumably indirect, since septation can take place in the complete absence of PBP2 (e.g., in *lov pbpA*::*kan* and *cya pbpA*::*kan* strains). It may be regulatory in nature, proceeding via interactions of PBP2 with other components directly involved in cell division, such as PBP3, which may interact with RodA (3). The role of the *lov* gene product in these interactions is currently under investigation; the role of the cAMP-catabolite gene activator protein complex may be to regulate *lov* expression, as previously suggested (4).

The only previously reported lethal *pbpA* mutant, SP6, selected by B. Spratt at 30°C as a mecillinam-resistant mutant (29), grew as round cells at 30°C in the absence of the drug; introduction of a complementing F′ *pbpA*$^+$ plasmid into SP6 resulted in the growth of cells as rods, but some resistance to mecillinam was still expressed (30). It is likely that this strain had a totally inactive PBP2 and had acquired a secondary mutation conferring mecillinam resistance to compensate. Mutants that are able to suppress the lethal effect of PBP2 deficiency are under investigation. Characterization of their products should help define the multicomponent functional complexes required for cell shape determination and bacterial division.

ACKNOWLEDGMENTS

We thank Ken Begg for encouragement and for correction of the manuscript. We are grateful to Brian Spratt for the gift of bacterial strains and plasmids and for critical reading of the manuscript. We thank Willie Donachie and Conrad Woldringh for stimulating discussions and our colleagues Bunichi Ezaki, Chiyome Ichinose, Danièle Joseleau-Petit, Jean-Claude Liébart, Hirotada Mori, and Aline Robin for their warm support.

This work was supported in part by the Association pour la Recherche sur le Cancer (grant 6696), the Fondation pour la Recherche Médicale, and by a Grant-in-Aid for Scientific Research on Priority Areas, a Grant-in-Aid for Scientific Research, and the Monbusho International Scientific Research Program for Joint Research from the Ministry of Education, Science and Culture, Japan.

LITERATURE CITED

1. **Aono, R., M, Yamasaki, and G. Tamura.** 1979. High and selective resistance to mecillinam in adenylate cyclase-deficient or cyclic adenosine 3′,5′-monophosphate receptor protein-deficient mutants of *Escherichia coli*. J. Bacteriol. **137**:839–845.
2. **Bachmann, B. J.** 1983. Linkage map of *Escherichia coli* K-12,

edition 7. Microbiol. Rev. **47**:180–230.
3. **Begg, K. J., B. G. Spratt, and W. D. Donachie.** 1986. Interaction between membrane proteins PBP3 and RodA is required for normal cell shape and division in *Escherichia coli*. J. Bacteriol. **163**:615–622.
4. **Bouloc, P., A. Jaffé, and R. D'Ari.** 1988. Preliminary physiologic characterization and genetic analysis of a new *Escherichia coli* mutant, *lov*, resistant to mecillinam. Rev. Infect. Dis. **10**: 905–910.
5. **Bouloc, P., A. Jaffé, and R. D'Ari.** 1979. The *Escherichia coli lov* gene product connects peptidoglycan synthesis, ribosomes and growth rate. EMBO J. **8**:317–323.
6. **Brady, G., H. M. Jantzen, H. U. Bernard, R. Brown, G. Schutz, and T. Hashimoto-Goto.** 1984. New cosmid vectors developed for eukaryotic DNA cloning. Gene **27**:223–232.
7. **Castellazzi, M., J. George, and G. Buttin.** 1972. Prophage induction and cell division in *E. coli*. I. Further characterization of the thermosensitive mutation *tif-1* whose expression mimics the effect of UV irradiation. Mol. Gen. Genet. **119**:139–152.
8. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134**: 1141–1156.
9. **D'Ari, R., A. Jaffé, P. Bouloc, and A. Robin.** 1988. Cyclic AMP and cell division in *Escherichia coli*. J. Bacteriol. **170**:65–70.
10. **Donachie, W. D., K. J. Begg, and N. F. Sullivan.** 1984. Morphogenes of *Escherichia coli*, p. 27–62. *In* R. Losick and L. Shapiro (ed)., Microbial development. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Donachie, W. D., and A. C. Robinson.** 1987. Cell division: parameter values and the process, p. 1578–1593. *In* F. C. Neidhardt, J. L. Ingraham, B. Magasanik, K. B. Low, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
12. **Gerdes, K., J. E. L. Larsen, and S. Molin.** 1985. Stable inheritance of plasmid R1 requires two different loci. J. Bacteriol. **161**:292–298.
13. **Hiraga, S., H. Niki, T. Ogura, C. Ichinose, H. Mori, B. Ezaki, and A. Jaffé.** 1989. Chromosome partitioning in *Escherichia coli*: novel mutants producing anucleate cells. J. Bacteriol. **171**:1496–1505.
14. **Huisman, O., R. D'Ari, and S. Gottesman.** 1984. Cell division control in *Escherichia coli*: specific induction of the SOS function SfiA protein is sufficient to block septation. Proc. Natl. Acad. Sci. USA **81**:4490–4494.
15. **Ishino, F., and M. Matsuhashi.** 1981. Peptidoglycan synthetic enzyme activities of highly purified penicillin-binding protein 3 in *Escherichia coli*: a septum-forming reaction. Biochem. Biophys. Res. Commun. **101**:905–911.
16. **Ishino, F., W. Park, S. Tomioka, S. Tamaki, I. Takase, K. Kunugita, H. Matsuzawa, S. Asoh, T. Ohta, B. G. Spratt, and M. Matsuhashi.** 1986. Peptidoglycan synthetic activities in membranes of *Escherichia coli* caused by overproduction of penicillin-binding protein 2 and RodA protein. J. Biol. Chem. **261**: 7024–7031.
17. **Iwaya, M., C. W. Jones, J. Khorana, and J. L. Strominger.** 1978. Mapping of the mecillinam-resistant, round morphological mutants of *Escherichia coli*. J. Bacteriol. **133**:196–202.
18. **Jaffé, A., Y. A. Chabbert, and E. Derlot.** 1983. Selection and characterization of β-lactam-resistant *Escherichia coli* K-12 mutants. Antimicrob. Agents Chemother. **23**:622–625.
19. **James, R., J. Y. Haga, and A. B. Pardee.** 1975. Inhibition of an early event in the cell division cycle of *Escherichia coli* by FL 1060, an amidino penicillanic acid. J. Bacteriol. **122**:1283–1292.
20. **Jones, C., and I. B. Holland.** 1985. Role of the SulB (FtsZ) protein in division inhibition during the SOS response in *Escherichia coli*: FtsZ stabilizes the inhibitor SulA in maxicells. Proc. Natl. Acad. Sci. USA **82**:6045–6049.
21. **Kohara, Y., K. Akiyama, and K. Isono.** 1988. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50**:495–508.
22. **Lund, F., and L. Tybring.** 1972. 6-β-Amidinopenicillanic acids, a new group of antibiotics. Nature (London) New Biol. **236**: 135–137.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Matsuhashi, S., T. Kamiryo, P. M. Blumberg, P. Linnett, E. Willoughby, and J. L. Strominger.** 1974. Mechanism of action and development of resistance to a new amidino penicillin. J. Bacteriol. **117**:578–587.
25. **Matsuzawa, H., S. Asoh, K. Kunai, K. Muraiso, A. Takasuga, and T. Ohta.** 1989. Nucleotide sequence of the *rodA* gene, responsible for the rod shape of *Escherichia coli*: *rodA* and the *pbpA* gene, encoding penicillin-binding protein 2, constitute an operon. J. Bacteriol. **171**:558–560.
26. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

26a. **Niki, H., C. Ichinose, T. Ogura, H. Mori, M. Morita, M. Hasegawa, N. Kusukawa, and S. Hiraga.** 1989. Chromosomal genes essential for stable maintenance of the mini-F plasmid in *Escherichia coli*. J. Bacteriol. **170**:5272–5278.

27. **Ogura, T., and S. Hiraga.** 1983. Mini-F plasmid genes that couple host cell division to plasmid proliferation. Proc. Natl. Acad. Sci. USA **80**:4784–4788.
28. **Park, J. T., and L. Burman.** 1973. A new penicillin with a unique mode of action. Biochem. Biophys. Res. Commun. **51**:863–868.
29. **Spratt, B. G.** 1975. Distinct penicillin binding proteins involved in the division, elongation and shape of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **72**:2999–3003.
30. **Spratt, B. G.** 1977. The mechanism of action of mecillinam. J. Antimicrobiol. Chemother. **3**(Suppl. B):13–19.
31. **Spratt, B. G., A. Boyd, and N. Stoker.** 1980. Defective and plaque-forming lambda transducing bacteriophage carrying penicillin-binding protein-cell shape genes: genetic and physical mapping and identification of gene products from the *lip-dacA-rodA-pbpA-leuS* region of the *Escherichia coli* chromosome. J. Bacteriol. **143**:569–581.
32. **Spratt, B. G., and A. B. Pardee.** 1975. Penicillin-binding protein and cell shape in *E. coli*. Nature (London) **254**:515–517.
33. **Stahl, F. W., I. Kobayashi, D. Thaler, and M. M. Stahl.** 1986. Direction of travel of *Rec*BC recombinase through bacteriophage lambda DNA. Genetics **113**:215–227.
34. **Stoker, N. G., J. K. Broome-Smith, A. Edelman, and B. G. Spratt.** 1983. Organization and subcloning of the *dacA-rodA-pbpA* cluster of cell shape genes in *Escherichia coli*. J. Bacteriol. **155**:847–853.
35. **Tamaki, S., H. Matsuzawa, and M. Matsuhashi.** 1980. Cluster of *mrdA* and *mrdB* genes responsible for the rod shape and mecillinam sensitivity of *Escherichia coli*. J. Bacteriol. **141**: 52–57.
36. **Tormo, A., J. A. Ayala, M. A. de Pedro, M. Aldea, and M. Vicente.** 1986. Interaction of FtsA and PBP3 proteins in the *Escherichia coli* septum. J. Bacteriol. **166**:985–992.
37. **Wachi, M., M. Doi, S. Tamaki, W. Park, S. Nakajima-Iijima, and M. Matsuhashi.** 1987. Mutant isolation and molecular cloning of *mre* genes, which determine cell shape, sensitivity to mecillinam, and amount of penicillin-binding proteins in *Escherichia coli*. J. Bacteriol. **169**:4935–4940.
38. **Walker, J., A. Kovarik, J. Allan, and R. Gustafson.** 1975. Regulation of bacterial cell division: temperature-sensitive mutants of *Escherichia coli* that are defective in septum formation. J. Bacteriol. **123**:693–703.
39. **Waxman, D. J., and J. L. Strominger.** 1983. Penicillin-binding proteins and the mechanism of action of β-lactam antibiotic. Annu. Rev. Biochem. **52**:825–869.
40. **Westling-Häggström, B., and S. Normark.** 1975. Genetic and physiological analysis of an *envB* spherelike mutant of *Escherichia coli* K-12 and characterization of its transductants. J. Bacteriol. **123**:75–82.