AmpC and AmpH, Proteins Related to the Class C β -Lactamases, Bind Penicillin and Contribute to the Normal Morphology of *Escherichia coli*

THOMAS A. HENDERSON, KEVIN D. YOUNG,* SYLVIA A. DENOME,† AND PAMELA K. ELF

Department of Microbiology and Immunology, School of Medicine, University of
North Dakota, Grand Forks, North Dakota 58202-9037

Received 23 May 1997/Accepted 15 July 1997

Two proteins that bind penicillin were observed in *Escherichia coli* infected with λ phages 141, 142, 650, and 651 from the Kohara genomic library. These phages carry chromosomal DNA fragments that do not contain any known penicillin binding protein (PBP) genes, indicating that unrecognized gene products were exhibiting penicillin binding activity. The genes encoding these proteins were subcloned, sequenced, and identified. One gene was *ampC*, which encodes a chromosomal class C β -lactamase. The second gene was located at about 8.5 min on the *E. coli* genomic map and is a previously uncharacterized open reading frame, here named *ampH*, that encodes a protein closely related to the class C β -lactamases. The predicted AmpH protein is similar in length to AmpC, but there are extensive alterations in the amino acid sequence between the SXXK and YXN motifs of the two proteins. AmpH bound strongly to penicillin G, cefoxitin, and cephalosporin C; was temperature sensitive; and disappeared from cells after overnight incubation in stationary phase. Although closely related to AmpC and other class C β -lactamases, AmpH showed no β -lactamase activity toward the substrate nitrocefin. Mutation of the *ampC* and/or *ampH* genes in *E. coli* lacking PBPs 1a and 5 produced morphologically aberrant cells, particularly in cell filaments induced by aztreonam. Thus, these two members of the β -lactamase family exhibit characteristics similar to those of the classical PBPs, and their absence affects cell morphology. These traits suggest that AmpC and AmpH may play roles in the normal course of peptidoglycan synthesis, remodeling, or recycling.

Penicillin binding proteins (PBPs) synthesize and modify peptidoglycan, the structural component of the bacterial cell wall (28, 35). Nine PBPs have been identified in *Escherichia coli*: PBPs 1a, 1b, 2, 3, 4, 5, 6, and 7, and DacD (3, 18, 28, 41). A tenth protein, PBP 1c, has recently been purified and cloned (39), and a protein previously designated PBP 8 is now known to be a proteolytic artifact of PBP 7 (17). The PBP family is related to the β -lactamases, which degrade a variety of β -lactam antibiotics (15, 16, 21, 27). The active sites of the two enzyme groups are similar, but the PBPs are inactivated by covalent binding of the antibiotic to the protein, while the β -lactamases hydrolyze their β -lactam substrates (15, 16). Thus, PBPs are the classic targets of β -lactams and β -lactamases are classic antagonists of these antimicrobial agents (14). Recent work addressing the question of how cells detect and respond to the presence of β -lactams has uncovered a relationship between peptidoglycan recycling and the induction of particular class C β -lactamases (33, 34). Thus, there appears to be a connection between β -lactamases and cell wall metabolism, at least at the level of gene regulation.

We report here that two proteins related to the class C β -lactamases bind covalently to particular β -lactams and that the absence of these proteins adversely affects the morphology of *E. coli* under certain circumstances. One protein is encoded by a known gene, *ampC*, and belongs to the family of class C β -lactamases. The predicted amino acid sequence of the protein encoded by a newly described gene, *ampH*, is similar to that of AmpC and identifies it as a close relative of this same family of enzymes. The penicillin binding characteristics of

AmpC and AmpH and the phenotypes of mutants suggest that there may exist additional, unrecognized contributions of the β -lactamases to the synthesis or metabolism of peptidoglycan.

MATERIALS AND METHODS

Bacterial strains, plasmids, phage, and PBP overexpression. The bacterial strains and plasmids used in this work are listed in Table 1. All bacteria were grown in Luria-Bertani medium (29) with appropriate antibiotics: ampicillin (100 μ g/ml), kanamycin (50 μ g/ml), and chloramphenicol (50 μ g/ml). Ampicillin was never used in growth media when penicillin binding proteins were to be assayed. Yeast extract and tryptone were from Difco (Detroit, Mich.). Nitrocefin was from D. Payne and G. Clarke (SmithKline Beecham, Brockham Park, United Kingdom). All other chemicals and antibiotics were from Sigma Chemical Co. (St. Louis, Mo.). Bacteriophages λ 141, λ 142, λ 650, and λ 651 were from the Kohara *E. coli* genomic library (22). Overexpression of PBPs in *E. coli* after infection by λ phage was performed exactly as described previously (18), except that Triton X-100 was omitted from all buffers. PBP labeling and visualization by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described previously (17).

Subcloning and insertional mutagenesis of the *ampH* and *ampC* genes. Plasmid preparations, restriction enzyme digestions, cloning, and other genetic manipulations were performed as described previously (37). Cloned genes were inactivated by removing restriction fragments internal to the open reading frame and replacing the wild-type sequence with an oligonucleotide linker sequence that contained a *Hind*III site. An internal DNA fragment was removed from the *ampH* gene by cleavage with *Bst*EII, the ends were blunted, a *Hind*III linker was added to each end, and a gene cassette was ligated into the new site. The inserted gene cassette consisted of a 2-kb *Hind*III DNA fragment containing the sequence *res-npt-res*, where *res* is the site at which resolvase acts and *npt* is the gene encoding kanamycin resistance (24). An internal DNA fragment was removed from the *ampC* gene by cleavage with *Hind*III and *Xho*I, *Hind*III linkers were added, and the *res-npt-res* cassette was inserted. The inactivated *ampH* and *ampC* genes were transferred from their respective plasmids to the chromosome of CS109 by the λ transduction method of Kulakauskas et al. (25), and the kanamycin resistance gene was removed from the truncated *ampH* and *ampC* genes by the resolvase-mediated method of Kristensen et al. (24). The complete procedure and gene diagrams will be described elsewhere (12).

β -Lactamase assay. β -Lactamase activity was assayed by using nitrocefin as the substrate and monitoring its destruction by the spectrophotometric method of O'Callaghan et al. (32). Nitrocefin stock solution (200 μ g/ml) was made in 50

* Corresponding author. Phone: (701) 777-2624. Fax: (701) 777-2054. E-mail: kyoung@mail.med.und.nodak.edu.

† Present address: AlphaGene, Inc., Woburn, MA 01801.

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Genotype	Source or reference
<i>E. coli</i> strains		
CS109	W1485, λ^- <i>F^- thi glnV (supE) rph-1 rpoS</i>	C. Schnaitman
CS211-2	CS109 <i>dacA::res dacC::res</i>	12
CS336-3	CS211-2 <i>ampH::res</i>	12
CS337-1	CS211-2 <i>ampC::res</i>	12
CS214-1	CS109 <i>mrcA::res dacC::res</i>	12
CS357-3	CS109 <i>mrcA::res dacC::res ampH::res</i>	12
CS362-1	CS109 <i>mrcA::res dacC::res ampC::res</i>	12
CS420-2	CS109 <i>mrcA::res dacC::res ampC::res ampH::res</i>	12
XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI^qZAM15 Tn10</i>]	Stratagene (La Jolla, Calif.)
SP1070	<i>his supF dacA::Km dacC^a</i>	D. Edwards 11
Plasmids		
pBCSK ⁺	Cloning vector; Cam ^r	Stratagene
pBCKS ⁻	Cloning vector; Cam ^r	Stratagene
pTAH142HI	pBCSK ⁺ plus an 8-kb <i>HindIII</i> DNA fragment from λ 142 carrying <i>ampH</i> ⁺	This work
pTAH112	3.5-kb <i>BglII-EcoRI</i> DNA fragment from pTAH142HI cloned into the <i>BamHI-EcoRI</i> site of pBCSK ⁺ (<i>ampH</i> ⁺ <i>sbmA</i> ⁺)	This work
pTAH114	1.74-kb <i>NdeI-EagI</i> fragment removed from pTAH112, blunt ended, <i>BamHI</i> linkers inserted, cut, and religated (<i>ampH</i> ⁺ only)	This work
pSAD410-1	pBCKS ⁻ plus a 6-kb <i>EcoRI</i> DNA fragment from λ 650 carrying <i>ampC</i> ⁺	This work
pMOB45	Temperature-sensitive runaway replication vector based on plasmid R1; Tet ^r Cam ^r	C. Georgopoulos 7
pTAH116	pMOB45 plus <i>NdeI</i> (<i>BamHI</i> linker)- <i>HindIII</i> fragment (<i>ampH</i> ⁺) from pTAH112; Cam ^r Kan ^r	This work
pTAH106	pMOB45 plus 1.1-kb <i>HindIII-BamHI</i> fragment (<i>pbpG</i> ⁺)	This work

^a Same as strain JBS1002 (11).

mM NaHPO₄, pH 7.0. *E. coli* cells were grown to an A_{600} of 0.7 to 0.74, and 1.5 ml of cells was pelleted at $10,000 \times g$ and resuspended in 1.5 ml of assay buffer (10 mM Tris-HCl [pH 8.0], 1 mM MgCl₂, 0.1 mM dithiothreitol). The resuspended cells were broken by two passages through a French pressure cell (Aminco, Inc., Urbana, Ill.) at 16,000 lb/in². Half of the sample (whole-cell lysate) was assayed for β -lactamase activity without further treatment. The remaining half was centrifuged at $100,000 \times g$ in a Beckman TL100 centrifuge for 10 min at 4°C to remove cell debris and membrane vesicles, after which the supernatant was removed and assayed for β -lactamase. Whole-cell lysate and supernatant samples were diluted 1:2 to 1:100 in assay buffer, and 75 μ l of each sample was added to 25 μ l of nitrocefin stock. The change in absorbance over time at 482 nm was monitored at 30°C with a Beckman DU640 spectrophotometer. The protein concentration of each sample was determined by the enhanced microBCA assay (Pierce Chemical Co., Rockford, Ill.).

Competitive β -lactam binding assays. A sample of partially purified AmpH was prepared from a Toyopearl HW65F-HEGN dye column by eluting the protein with 1 M NaCl, as described previously for the purification of soluble PBP 8 (18). AmpH protein (14 μ g of protein/15 μ l) was added to 3 μ l of 100 mM β -mercaptoethanol and 3 μ l of an unlabeled β -lactam (35- or 140- μ g/ml stock solution, to give a 5- or 20- μ g/ml final concentration, respectively). The samples were incubated for 10 min at 37°C, after which 3 μ l of ¹²⁵I-labeled penicillin-X (100 μ g/ml) (17) was added and the incubation was continued for 15 min. SDS sample buffer (8.5 μ l) was added, and each sample was boiled for 4 min. Proteins in the samples were separated by SDS-PAGE and detected by autoradiography as described previously (17, 18).

DNA sequencing and analysis. DNA sequencing was performed by the dideoxynucleotide method of Sanger et al. (38), by using Sequenase enzyme (Amersham, Arlington Heights, Ill.) and double-stranded plasmid DNA as the substrate. Dideoxynucleotides and dideoxynucleotides were from Pharmacia Biotech (Piscataway, N.J.). Sequence analyses were performed with DNA Inspector IIe (Textco, West Lebanon, N.H.) and DNASIS (Hitachi Software Engineering America, Ltd., San Bruno, Calif.), and with sequence analysis utilities provided at the Baylor College of Medicine World Wide Web site (40).

RESULTS

Identification and subcloning of new PBPs from the Kohara λ library of the *E. coli* genome. The Kohara miniset chromosomal library of *E. coli* is composed of 476 λ phage clones (22). We assayed for the presence of PBPs expressed by these phages during infection of *E. coli* and found that Kohara

phages λ 141 and λ 142 (representing DNA from 8.43 to 8.64 min on the *E. coli* chromosome) each overexpressed a single new PBP that migrated to a position just lower than that of PBP 6 (Fig. 1, lanes 2 and 3). Phages λ 650 and λ 651 (representing DNA from 94.26 to 94.52 min) overexpressed a PBP that migrated at approximately the same position as PBP 6 (data not shown).

No known PBPs are located in the regions of the *E. coli*

FIG. 1. Expression of AmpH protein (H) by Kohara λ phage. *E. coli* SP1070 (Δ PBP 5, Δ PBP 6) was infected with Kohara phage λ 141 or λ 142, and the cells were harvested and labeled for PBP expression as described in Materials and Methods. Lane 1, uninfected *E. coli* SP1070; lanes 2 and 3, *E. coli* SP1070 after infection with λ 141 and λ 142, respectively.

FIG. 2. Penicillin binding by AmpH and AmpC in mutant strains and plasmid clones. PBPs were labeled in *E. coli* strains from which the *ampH* and/or *ampC* genes had been deleted, or in strains carrying plasmids containing the wild-type *ampH* and/or *ampC* genes. Lane 1, *E. coli* CS109 (wild type); lanes 2 and 8, CS211-2 (*dacA dacC*); lane 3, CS336-1 (*ampH dacA dacC*); lane 4, CS337-1 (*ampC dacA dacC*); lane 5, CS211-2/pTAH142HI (cloned *ampH*⁺); lane 6, CS211-2/pSAD410-1 (cloned *ampC*⁺); lane 7, CS211-2/pBCKS⁻ (vector control).

chromosome carried by these phages, implying that unrecognized proteins were binding penicillin; so we subcloned DNA fragments from these phages to identify the genes giving rise to these proteins. An 8-kb *Hind*III DNA fragment from phage λ 142 was cloned into the vector pBCKS⁺, creating pTAH142HI, and a 6-kb *Eco*RI DNA fragment from λ 650 was cloned into the vector pBCKS⁻, creating pSAD410-1 (Table 1). These plasmids were transformed into an *E. coli* mutant (CS211-2) from which PBPs 5 and 6 (*dacA* and *dacC*) had been deleted so that the new PBPs of similar molecular mass could be observed. The two plasmids overproduced two different proteins that bound ¹²⁵I-labeled penicillin-X (Fig. 2, lanes 5 and 6). By sequencing and deletion studies (described below), the lower-molecular-mass protein was identified as the product of a new gene, designated *ampH* (Fig. 2, lane 5), and the slightly larger protein was produced by a known gene, *ampC* (Fig. 2, lane 6).

The *ampH* product binds penicillin and is related to the class C β -lactamases. The gene from λ 142 and pTAH142HI was isolated on a 3.5-kb *Bgl*I-*Eco*RI fragment and was subcloned into the *Bam*HI-*Eco*RI-digested vector pBCKS⁺ to produce plasmid pTAH112 (Fig. 3). In this DNA segment is a known gene, *sbmA* (Fig. 3). pTAH112 was digested with *Nde*I and *Eag*I to remove the entire *sbmA* gene, from its predicted start codon (overlapping the *Nde*I site) through the end of the cloned fragment (the *Eag*I site is located in the multiple cloning site of the vector). The two ends of the cleaved plasmid were blunt ended, a *Bam*HI linker oligonucleotide was ligated to each end and cleaved with *Bam*HI, and the plasmid was religated to give plasmid pTAH114 (Fig. 3). This plasmid, containing 1,761 bp of cloned DNA, also overproduced the protein that bound penicillin (data not shown), indicating that the *sbmA* gene did not encode the new protein.

The DNA sequence of the cloned fragment in pTAH114 was determined (Fig. 4) and was identical to the sequence of an open reading frame located directly upstream of the *sbmA* gene (8). After we obtained these results, the entire sequence of the *E. coli* chromosome from 4 to 25 map minutes was deposited in GenBank (by Duncan and colleagues; accession no. U73857), and the sequence of the entire genome of *E. coli* was completed (by F. R. Blattner and colleagues; GenBank accession no. U00096). The sequence of our cloned DNA was identical to that of the open reading frame *yaiH*, corresponding to the open reading frame located at nucleotides (nt) 394354 to 395511 in the completed genomic sequence (U00096). The *ampH* gene is located immediately upstream of the *sbmA* gene and is transcribed in the opposite orientation; downstream are two unidentified open reading frames that are also transcribed in the direction opposite that of *ampH* (Fig. 3). Thus, *ampH* appears to be the only gene expressed from its transcript, and consistent with this interpretation, there are potential promoter (Fig. 4, nt 25 to 56) and terminator (Fig. 4, nt 1306 to 1342) sequences that bracket the *ampH* reading frame.

The derived amino acid sequence of the *ampH* gene, beginning at the GTG codon (Fig. 4, nt 166), predicts the production of a protein with a molecular weight of 40,814 and a pI of 9.37. This molecular mass is compatible with the position of the protein band just below PBP 6 on polyacrylamide gels (Fig. 2, lane 5). The highly basic pI was confirmed by two-dimensional nonequilibrium pH gel electrophoresis. The pI was greater

FIG. 3. Schematic of the *E. coli* chromosome and selected restriction enzyme sites from ~8.3 to 8.8 min. Long arrows, DNA contained in Kohara phages λ 141 and λ 142; thick lines, DNA fragments cloned into the three plasmids pTAH142HI, pTAH112, and pTAH114. Short arrows indicating the *ampH* gene and other genes and open reading frames also show the direction of transcription.

```

-35          -10          TTTGGTCAGCAGGAAAAAGATCGTG 24
TTTACGCCCGCTTACGTTTTTAACACATATTATTCACATAATTCGCCCTTTGCACATCATCAATGAG 90

TAGTATAAATACGCTCAGTTACCTTCATTCATCTATGCACACACCGTTGAACAGCTAGCTCTGCTT 156
* y k y a q l p s f n l w t p p l k r s l l

TTTTCGCGCGTGTGTGCGCGCTCATTGACCTCCGCTCCATGCGCGCGCAGCGGATCACTGAACCG 222
f s a v l c a a s l t s v h a a q p i t e f

GAGTTTGCCTCATATTTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 288
E F A S D I V D R Y A D H I F Y G S G A T G

ATGGCTCTGGTTGTTATCGACGCTAACGACGCGCTCTTTCGCGATTATGGCAAGCGGACCTGGT 354
M A L V V I D G N Q R V F R S Y G E T R P G

AATAACGTTTCGCGCGCTGATTCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 420
N N V R P Q L D S V V R I A S L T K L M T S

GAGATGCTGGTGAATTCGCTGACGCGCGGACCGTGAAGTAAACGATCCGCTAAGTAAATATGCT 486
E M L V K L L D Q G T V K L N D P L S K Y A

CCGCGAGCGCGACGCGCGGACCGCGCGGACCGCGCGGACCGCGCGGACCGCGCGGACCGCGCGGAC 552
P P G A R V P T Y N G T P I T L V N L A T H

ACCAGCGCGCTGCGCGCTGAACGCGCGGCTGCGCGCGGACCGCGCGGACCGCGCGGACCGCGCGGAC 618
T F A L P R E Q P G A A H R P F V F V W P T

CGCGAGCAACGCTGGAATACCTTTTACGCGCGGACCGCGCGGACCGCGCGGACCGCGCGGACCGCG 684
R E Q R W K Y L S T A K K L K A A P G S Q A A

TACTCTAACCTTGCCTTGCACCTGCTGCGAGTACGCGCGGACCGCGGACCGCGCGGACCGCGCGGAC 750
Y S N L A F D L A D A L A N A S G K P Y T

CAGTGTGTTGAAGAGCAGATTACCGCTCCATTTGGGATGAAGACACCACTACACCGCTTCCACCG 816
Q L F E E Q I T R P G M K D T T Y T S F

GATCAGCGCGCGCTTGAATGCTGCTGAGCGCGGACCGCGGACCGCGGACCGCGGACCGCGGACCG 882
D Q C R R L M V A R G A S P C N N T L A A

ATTGGCAGCGCGCGCTTATTCACGCGCTGCGGATGATGCGCTGATGCGGATGCGGATGCGGATGCGGAT 948
I G S G G V Y S T P G D M M R W M Q Q Y L S

TCTGATTTTATCAGCGGATTAACGAGCAGATCGATGCGGATGCGGATGCGGATGCGGATGCGGATGCG 1014
S D F Y Q R S N Q A D R M Q T L I Y Q R A Q

TTTACGAAGTATGCGGATGATGCGGCGCGGACCGCGGATGCGGATGCGGATGCGGATGCGGATGCGGAT 1080
F T K V I G M D V P G K A D A L G L G W V Y

ATGGCCCCGAAGAGGCTGCTCGCGGATTTATCAGAAGACGCGGCTGCGGCGCTTTCATTACC 1146
M A P K E G R P G I I Q K T G G G G G P I T

TATATGGCGATGATCCCGCAGAAGATTTGCGCGCTTGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGACG 1212
Y M A M I P Q K N I G A F V V V T R S P L T

CGCTTTAAATATGAGCGATGATCAATGACCTGGTACCGGAGCTAAGCGGGAATAAAGCGTTG 1278
R F K N M S D G I N D L V T E L S G N K F L

GTTATCCCGCGCTGCTGATTAATCTCTGAAGGCAACCGTTGATAGCGACCGTTGCTGCTTAT 1344
V I P A S *

CATTTGATATGCGCGCTTACCGAAGCAGCGAGAACTTCTGCGAGCAGACGCGGATATGCG 1410
TGATCGTTGATGATATATTCATCACCAATACGTCGCGCAATCTTCATTCAGTCATCAT 1476
TGATATTAATGTTGCGCGGATTTGCTGATGAAGATTATGCCAATGAGCTGCGCGTAAATTC 1542
CAGAATTC 1550
EcoRI

```

FIG. 4. Nucleotide and derived amino acid sequences for the *ampH* gene. Numbers to the right refer to the nucleotide sequence. Numbers to the left refer to the derived amino acid sequence, beginning at the GTG initiation codon located at nt 166 to 168 (underlined). This GTG and three other potential initiation codons are in boldface and are marked by an asterisk placed above each codon. Potential amino acids before GTG in the open reading frame are in lowercase letters. The amino acids of three penicillin binding active-site motifs are in boldface and underlined. The position of a potential promoter is indicated, beginning at nt 25, by underlining and labeling of the -35 and -10 regions. The stop codon of the *ampH* gene is denoted by an asterisk beneath the nucleotide sequence. A pair of inverted repeats that form a potential Rho-independent terminator is located between nt 1306 and 1342 and is identified by a pair of dashed lines.

than 8.6 (glyceraldehyde-3-phosphate dehydrogenase) and less than 10.4 (PBP 7) (data not shown). The predicted AmpH protein contains the canonical SXXK, YXN, and KTG active-site motifs of the PBPs and β -lactamases (Fig. 4) (15, 16). A similarity search using the BEAUTY program (44) indicated that the encoded protein is related most closely to the family of class C β -lactamases—15 of the 16 most similar proteins belonged to this group (data not shown). Thus, the new gene was named *ampH* to indicate its close relationship to other *amp* genes of the class C family. The amino terminus (139 amino acids [aa]) of the predicted AmpH protein is 25% identical to the amino terminus (157 aa) of AmpC, the *E. coli* member of this family, and is 64% similar when conserved substitutions are included (Fig. 5). The carboxyl terminus (211 aa) of AmpH is 27% identical and 60% similar to the carboxyl domain (210 aa) of AmpC (Fig. 5).

```

V----LCAASL-TSVHAAQPIETEPEFASDIVDRYADHIFYSGSGATGMAIV 45
||| ||| . . . . . ||| |||
MFKTTLCA-LLITA--SCSTFAAPQINDIVHRTITPLIEQQKTPGMAVA 47

VI-DGNQVRFR-SYG--E-TRPGNNVRQLDSVVRIASLTK-----LMTSE 86
.|.| . . . . . ||| ||| . . . . . ||| |||
VIYQG--KPYFTWGYADIKK-QPV-TQ-QTLFELGSVSKTFTGVLGGD 92

MLVKLLDQGTVKLNPLSKY-A--PP----G-A--RVPTYNGTPTITVLN 126
. . . . . ||| ||| . . . . . ||| |||
AIARG-E---IKLSDPTTKYWPFLTAKWNGITLLHLATYTAGGLPL-QV 137

AT--HTSA-LPR-EQ---PGGAHRPVPVWPTREQRKYLSTAKLKAAFG 169
. . . . . ||| |||
PDEVKSSDDLRFYQNWQPA-----WAPG 161

SQAAYSNLAFLDADALANA-SGKPYTQLFEEQITRPL--GMK-DTTYT- 261
.|.| . . . . . ||| ||| . . . . . ||| |||
TQRLYANSSIGLFG-ALAVKPSGLSFEQAM--Q-TR-VFQPLKINHTWIN 255

-PSPDQCRRLMVA-ERGAFCNNTLAAIGSGGVYSTPGDMRWMMQ-QYLS 261
| . . . . . ||| ||| . . . . . ||| |||
VPEAEKNYAWGYREGKAVHVSFGALDAEAYGVKSLIEDMARVWQSN-LK 255

S-DFYQRS-NQA-DRM-QTLIYQRA-Q-F-TK--VI--GMDVP-GKADA- 298
. . . . . ||| ||| . . . . . ||| |||
PLDINEKTLQQGIQ-LAQSRVWGTGDMYQGLGWEMLDWPNV-PDSIINGS 303

---LGLGW--VY-MAPKEG--RPGIIQKTGGGGGFTYAMIPQKNIGAF 340
. . . . . ||| ||| . . . . . ||| |||
DNKIALAARFVKAITPPTPAVRASVWHTGTATGGGGSYVAFIPEKELGI- 352

VVVT-R--S-PLTRFKNMSDGINLVLTELSGNKPLVIPAS AmpH 375
| . . . . . ||| ||| . . . . . ||| |||
VMLANKNYPNP-ARV-D-A-AWQ-IL-N-ALQ AmpC 377

```

FIG. 5. Comparison of the amino acid sequences of AmpH and AmpC. Identical amino acids are identified by a line between the two sequences, similar amino acids are identified by a dot, and gaps in each sequence are indicated by dashes. The three penicillin binding active-site motifs in each sequence are in boldface and underlined. Top sequence, AmpH; bottom sequence, AmpC.

The strongest evidence that GTG is the start codon of *ampH* (Fig. 4, nt 166) comes from sequence comparisons with other members of the class C β -lactamases. If this is the true start site, then the length of the AmpH protein is almost equal to that of the AmpC protein (375 versus 377 aa). The canonical SXXK and YXN motifs of the two proteins can be aligned by assuming that significant changes have occurred in the gene sequence between these two motifs (Fig. 5, AmpH, aa 79 to 177). It appears that 26 or 27 aa may have been inserted between the SXXK and YXN sequences of the AmpH protein (Fig. 5, AmpH, aa 141 to 167). Such an insertion is similar to, though smaller than, the insertion between the analogous active-site motifs of PBP 4 from *E. coli* (31). Large segments of that putative insertion could be deleted from PBP 4 without significantly altering its ability to bind penicillin (30). A similar series of insertion or deletion events may have reconstructed the amino acid sequence of AmpH. In the case of AmpH and AmpC, compensatory additions and deletions in the region between the SXXK and YXN motifs appear to have occurred so that the distances between the two motifs are similar in the two proteins (92 aa in AmpH and 82 aa in AmpC) (Fig. 5).

Binding of β -lactams to AmpH. The specificity of β -lactam binding to the AmpH protein was determined by a competitive binding assay. The following β -lactams bound strongly to the AmpH protein so that it remained unlabeled when challenged with 125 I-penicillin-X: penicillin G, penicillin-X, cefoxitin, and cephalosporin C (Table 2 and Fig. 6, lanes 2, 3, 8, and 10, respectively). Of these, cefoxitin and cephalosporin C competed poorly if added at a concentration of 5 μ g/ml (data not shown). Cefmenoxime and cefotaxime competed slightly or moderately for binding to AmpH at a concentration of 20 μ g/ml (Fig. 6, lanes 6 and 7, respectively) but not at all when added at 5 μ g/ml (data not shown). Several other β -lactams, precursors, and derivatives did not compete for covalent binding to AmpH (Table 2). These results indicate that AmpH behaves like a classic PBP in the sense that it is covalently inactivated by a subset of β -lactams.

TABLE 2. AmpH interactions with β -lactams and derivatives^a

Binding to AmpH	Antibiotic
Yes	Penicillin G, penicillin-X, cefoxitin, cephalosporin C, cefmenoxime, ^b cefotaxime ^b
No	Ampicillin, 6-aminopenicillanic acid, <i>p</i> -hydroxyphenylacetic acid, 7-aminocephalosporanic acid, amoxicillin, aztreonam, bacampicillin, carbenicillin, cefaclor, cefadroxil, cefsulodin, ceftriazone, cefuroxime, cephalexin, cephaloglycin, cephadrine

^a Binding to AmpH was measured by incubation with unlabeled antibiotics at 5 or 20 μ g/ml prior to addition of ¹²⁵I-labeled penicillin-X.

^b These antibiotics competed moderately for binding to AmpH when they were added at a final concentration of 20 μ g/ml but did not bind when added at 5 μ g/ml.

β -Lactamase activity of AmpH. Because AmpH was so closely related to the class C β -lactamases (cephalosporinases), we tested for β -lactamase activity in *E. coli* cells that overexpressed this protein. The following strains were tested: *E. coli* XL1-Blue (negative control), XL1-Blue pBluescript SK⁺ (positive control; plasmid carrying a β -lactamase marker), XL1-Blue pSAD410-1 (positive control; plasmid carrying the cloned *ampC* gene), and XL1-Blue pTAH112 (plasmid carrying the cloned *ampH* gene). The AmpC protein exhibited a moderate level of β -lactamase activity toward nitrocefin compared with the β -lactamase encoded by the pBluescript plasmid (Table 3). However, the AmpH protein exhibited no demonstrable β -lactamase activity (Table 3), even though the protein was highly expressed in these cells, as measured by ¹²⁵I-penicillin-X-labeling (data not shown) (see the equivalent example of AmpH overexpression in a different *E. coli* strain in Fig. 2, lane 5). Thus, although AmpH is closely related by sequence to the class C β -lactamases, the protein exhibits no measurable β -lactamase activity against a common substrate. In this respect, AmpH again exhibits a characteristic common among the class C PBP.

The penicillin binding activity of AmpH is sensitive to temperature and growth phase. We cloned the *ampH* gene into the temperature-sensitive runaway replication vector pMOB45 (7). pTAH112 was digested with *Nde*I, the ends were blunted, and *Bam*HI oligonucleotide linkers were attached. From this plasmid, the *Bam*HI-*Hind*III fragment containing only the *ampH* gene was subcloned into the *Bam*HI-*Hind*III sites of pMOB45, creating pTAH116. *E. coli* containing pTAH116 was grown at 30°C and at an *A*₅₅₀ of ~0.2 was shifted to 45°C for 70 to 80 min. Although AmpH was overexpressed and strongly labeled at 30°C (Fig. 7, lanes 5 and 6), the protein labeled very poorly

at 45°C (Fig. 7, lanes 7 and 8). The reduction in labeling was not an artifact of the overproduction system because PBP 7, when cloned and expressed in the same manner, was affected much less at the higher temperature (Fig. 7, lanes 1 to 4). Normal amounts of overexpressed AmpH were visible in cells shifted to 37°C, and a reduced amount was visible at 42°C, though the reduction was not as great as that observed at 45°C (data not shown).

The previous results were obtained in cells expressing a cloned *ampH* gene, in which the AmpH protein was produced at levels 20 to 100 times greater than normal. At the higher temperatures a low level of residual labeling continued to be observed, perhaps because of the increased amount of protein. In cells containing a single chromosomal copy of *ampH*, the level of AmpH decreased by approximately half when the cells had been shifted to 37°C and was below detection at 42°C (data not shown). When RNA was prepared from these cells and hybridized with an *ampH*-specific DNA probe, *ampH*-derived mRNA was present in equal amounts after growth of cells at 30, 37, and 42°C (data not shown). Thus, the reduction in AmpH protein at the higher temperatures was not the result of decreased transcription. It appears that the AmpH protein is temperature sensitive, binding penicillin less well as the temperature approaches and exceeds 42°C.

In addition, in wild-type *E. coli* the AmpH protein disappeared from cells in stationary phase at 30 or 37°C, but not from cells in which AmpH had been overproduced from a runaway replication vector (data not shown).

AmpH is nonessential. The *ampH* gene was deleted from *E. coli* by insertional mutagenesis of the cloned gene, followed by transfer of the inactive gene to the chromosome by λ transduction (see Materials and Methods). *E. coli* CS211-2 is a strain from which PBPs 5 and 6 have been deleted (Fig. 2, lane 2) (12). When *ampH* was deleted from CS211-2 to create strain CS336-1, a PBP disappeared that was the same size as the protein expressed by the cloned AmpH gene (Fig. 2, lane 3). Thus, the *ampH* gene is expressed at a low level in wild-type *E. coli*. *ampH* mutants were viable and exhibited no overt growth defects (data not shown), indicating that the gene was not essential under normal laboratory conditions.

AmpC binds penicillin. Phage λ 650 overexpressed a PBP that migrated at approximately the same position as PBP 6 (data not shown). This λ phage contains DNA from ~94.2 to 94.5 min on the *E. coli* genomic map, which includes the *ampC* gene (36). Because the AmpH protein is related to the AmpC protein, we cloned the *ampC* gene to determine if AmpC represented this "new" PBP. pSAD410-1 contains the *ampC* gene on a 6-kb *Eco*RI DNA fragment from λ 650 (Table 1), and *E. coli* containing this plasmid overproduced a protein to which ¹²⁵I-penicillin-X bound strongly (Fig. 2, lane 6).

A question that remained was whether wild-type levels of AmpC would bind penicillin. The location of AmpC on polyacrylamide gels is the same as that of PBP 6, which is one of the major penicillin binding proteins (see, e.g., Fig. 2, lane 1). Under normal circumstances, binding of penicillin by AmpC would be obscured by PBP 6. Therefore, we deleted the *ampC* gene from a strain of *E. coli* from which PBPs 5 and 6 had both been deleted, so that any AmpC-specific penicillin binding activity could be visualized. In such cells, a PBP band disappeared (Fig. 2, lane 4), and the location of this band was the same as the location of overproduced AmpC protein (Fig. 2, lane 6). Inspection of Fig. 2, lanes 3 and 4, shows that the AmpC and AmpH proteins are encoded by separate genes and that each can be deleted from *E. coli* without affecting cell viability.

FIG. 6. Competitive binding of β -lactams to AmpH. Aliquots of purified AmpH protein were incubated with individual unlabeled β -lactams, derivatives, or precursors before AmpH was labeled with ¹²⁵I-penicillin-X, and the samples were separated by SDS-PAGE and exposed to Kodak X-Omat X-ray film. Binding of a particular β -lactam to AmpH prevents subsequent labeling with the radiolabeled compound, thereby reducing the intensity of the AmpH band. Unlabeled β -lactams were added individually to AmpH as follows: lane 1, no β -lactam (control); lane 2, penicillin G; lane 3, penicillin-X; lane 4, ampicillin; lane 5, 6-aminopenicillanic acid; lane 6, cefmenoxime; lane 7, cefotaxime; lane 8, cefoxitin; lane 9, 7-aminocephalosporanic acid; lane 10, cephalosporin C.

TABLE 3. β -Lactamase activity encoded by cloned *ampC* and *ampH* genes

Plasmid ^a	Gene	Whole-cell lysate			Supernatant		
		Protein (mg/ml)	Destruction of nitrocefin (μ mol/min) ^b	Sp act ^c	Protein (mg/ml)	Destruction of nitrocefin (μ mol/min) ^b	Sp act ^c
None	None	7.6	1.8 $\pm$ 0.13	240	5.0	1.45 $\pm$ 0.02	290
pBluescript	<i>bla</i>	8.0	75.0 $\pm$ 3.2 ^d	9,400	ND ^e	ND	ND
pSAD410-1	<i>ampC</i>	7.7	7.10 $\pm$ 1.5	920	5.1	4.9 $\pm$ 0.6	960
pTAH112	<i>ampH</i>	7.6	0.78 $\pm$ 0.18	100	5.0	0.68 $\pm$ 0.09	140

^a *E. coli* XL1-Blue was the host strain.^b Values are from 5 to 10 individual measurements (of appropriately diluted samples) from two independent experiments. Destruction of 10⁻⁴ mol of nitrocefin increases the A₄₈₂ value by ~1.0.^c Nanomoles of nitrocefin destroyed per minute per microgram of protein. A one-way analysis of variance indicated that every combination of results was significantly different at a *P* value of <0.05, except that there was no significant difference between the activities in whole cell-lysates and supernatants.^d Values are from three individual measurements.^e ND, not done.

AmpC binds aztreonam. The β -lactam antibiotic aztreonam binds PBP 3 and causes *E. coli* to filament due to the inactivation of this essential PBP, which is intimately involved in the formation of septal peptidoglycan (2, 9). For some time, aztreonam has been used as a PBP 3-specific antibiotic in studies of septation, because in competitive binding assays aztreonam binds most strongly to PBP 3 (42). We reconfirmed previous observations (13) that AmpC binds aztreonam just as strongly as does PBP 3. *E. coli* 336-3 (in which PBPs 5 and 6 and AmpH are deleted) was incubated with 0.1 to 10 μ g of unlabeled aztreonam/ml, after which the remaining active PBPs were labeled with ¹²⁵I-penicillin-X. PBP 3 disappears in such a competitive binding assay at the lowest concentration of aztreonam (0.1 μ g/ml) (Fig. 8, lane 2). However, the AmpC protein also disappears at this same concentration (Fig. 8, lane 2). No other PBP binds aztreonam so avidly (although PBP 1a is inhibited by 50 to 90% when the aztreonam concentration reaches 10 μ g/ml [data not shown]).

AmpC and AmpH participate in determination of normal cell shape. We deleted *ampC*, *ampH*, or both genes from *E. coli* CS214-1 (from which PBPs 1a and 5 had previously been deleted [12]) and observed the growth and morphological characteristics of the strains. The mutants grew at the same rate as the parent, CS214-1, as measured by increase in optical density (data not shown), but over half the mutant cells from mid-logarithmic-phase cultures had diameters noticeably larger than that for the parental strain (Fig. 9). In addition, cell poles of many of the mutants were not rounded but were squared, flattened or "flared," sometimes appearing as though two cell

poles had been partially formed at the end of a cell (Fig. 9). Cells devoid of both *ampC* and *ampH* looked even worse; individual cells had uneven contours, and the constrictions between dividing cells were aberrant and asymmetric (Fig. 9).

The variations in cellular morphology were accentuated when *ampC* and/or *ampH* mutants were exposed to aztreonam, which causes *E. coli* to filament due to inhibition of PBP 3-dependent septation. After 100 min of exposure to aztreonam, the CS214-1 parent formed long filaments that were uniform in length and diameter and had a smooth outer contour (Fig. 10A). However, 40 to 50% of the filaments of *ampC* or *ampH* mutants had increased diameters, which usually varied significantly along the length of a single filament (Fig. 10A). About 30 to 40% of the filaments exhibited bifurcations at one or both poles, some of which were long enough to be considered "branches." Other bifurcations appeared in the midsection of a filament, and a large number of cells took on even more bizarre shapes (Fig. 10A). After 280 min of filamentation, the CS214-1 parental strain formed extremely long filaments, approximately 2 to 4% of which exhibited a small bifurcation at one pole (Fig. 10B). In both the *ampC* and *ampH* mutants, these bifurcations were 10 to 20 times more

FIG. 7. Effect of temperature on the labeling of AmpH and PBP 7. Cells were grown at 30°C, and half of each culture was shifted to growth at 45°C for 70 to 80 min. Cells were labeled with ¹²⁵I-penicillin-X, separated by SDS-PAGE, and visualized by exposure to X-ray film. Lanes 1 and 2 (30°C) and lanes 3 and 4 (45°C), *E. coli* CS109 containing plasmid pTAH106 (cloned PBP 7 gene [*pbpG*] in temperature-sensitive runaway replication vector). Lanes 5 and 6 (30°C) and lanes 7 and 8 (45°C), *E. coli* CS109 containing plasmid pTAH116 (cloned *ampH* gene in temperature-sensitive runaway replication vector). Whole cells were labeled in lanes 1, 3, 5, and 7, and cell lysates were labeled in lanes 2, 4, 6, and 8.

FIG. 8. Competitive binding of aztreonam to PBP 3 and AmpC. Prior to labeling with ¹²⁵I-penicillin-X, *E. coli* cells were exposed to aztreonam at the following concentrations: lane 1, no aztreonam (control); lane 2, 0.1 μ g/ml; lane 3, 1.0 μ g/ml; lane 4, 2.0 μ g/ml. The question mark denotes an unidentified penicillin binding protein.

FIG. 9. Morphology of *ampH*, *ampC*, and *ampC ampH* mutants of *E. coli*. Derivatives of the parent strain, *E. coli* CS214-1 (*mrcA::res* [Δ PBP 1a] *dacA::res* [Δ PBP 5]), were created by deleting the following genes: *ampH* (strain CS357-3), *ampC* (CS362-1), or *ampH* and *ampC* (CS420-2). Cells were harvested during logarithmic growth, visualized by phase-contrast microscopy with a 100 $\times$ oil objective, and photographed by cold charge-coupled device camera capture. The magnification and enlargement of each photo in this figure and in Fig. 10 are the same, so that direct comparisons of length and width can be made between individual cells. The parental strain in this figure is approximately 1.6 to 1.8 μ m in length in the absence of antibiotic. Three examples of each of the derivatives are shown.

numerous and frequently appeared at both ends of a single filament (Fig. 10B). A few poles exhibited three “knobs” instead of two (Fig. 10B).

When both *ampC* and *ampH* were deleted from CS214-1, the morphological aberrations were more obvious than in either single mutant, even as soon as 45 min after the addition of aztreonam (Fig. 10A). After 45 min of filamentation, double mutants displayed uneven contours, many were curved or bent at acute angles, and the cell diameter often increased significantly (Fig. 10A). After 100 min of filamentation, dramatic aberrations appeared in the double mutants, with filaments exhibiting wildly variable diameters and shapes (Fig. 10A).

In the cases described in Fig. 9 and 10, many sacculi were present that had apparently lysed and thus contained no cytoplasm, as indicated by their transparency in phase microscopy and the absence of 4',6-diamidino-2-phenylindole (DAPI)-stainable DNA (data not shown). The shapes of these empty sacculi were as aberrant as those of living cells (Fig. 10A), indicating that overall cell shape was determined by the structure of the peptidoglycan, as is true for wild-type cells.

When *ampC* or *ampH* was deleted from the original parent, *E. coli* CS109, none of the morphological alterations described above occurred (data not shown). Thus, loss of either protein individually was not sufficient to produce the observed effects. When *ampC* or *ampH* was deleted from a strain lacking PBP 5 or when *ampH* was deleted from a strain lacking PBP 1a, a small percentage (<2 to 5%) of cells exhibited slightly increased diameters or irregular wall contours but there were no bifurcations at the ends of aztreonam-induced filaments (data not shown). Only when *ampC* was deleted from a mutant also deficient in PBP 1a was there a measurable, but still very low (5 to 10%), increase in the number of cells exhibiting significant increases in diameter or having terminal bifurcations at the ends of cell filaments (data not shown). Thus, the phenotypes associated with loss of AmpC and/or AmpH became

most apparent in an *E. coli* strain that was also deficient in at least two of the classical PBPs.

DISCUSSION

An arbitrary definition divides the PBPs from the β -lactamases: PBPs form long-lived covalent bonds with penicillin and its derivatives, while β -lactamases hydrolyze these compounds. Unfortunately, this distinction is not absolute—many proteins bind some β -lactams but hydrolyze others, and closely related proteins may exhibit great diversity in the substrates they bind or hydrolyze and in the efficiency with which they do so. Thus, classifying a protein as a PBP or β -lactamase is an exercise in discriminating between degrees of activity, and the resulting categories, though they may identify proteins with common properties, may not represent protein classes having common physiological functions.

Dividing these enzymes into two families colors our ideas of their functions: PBPs synthesize and remodel peptidoglycan, and β -lactamases protect bacteria by inactivating β -lactams. Such a conclusion correctly describes the two possible extremes, but many of these enzymes occupy what may be a middle ground. For example, PBPs 5 and 6 have long been regarded as “weak β -lactamases” (10) that hydrolyze penicillin G with half-lives of $\sim$ 10 to 12 and $\sim$ 32 min, respectively (1). These rates are much higher than those of other PBPs but are about 1,000-fold less than the rates of the class A β -lactamases, with class C β -lactamases generally exhibiting intermediate rates. Moreover, the boundaries between the enzymatic characteristics of the PBPs and β -lactamases are not inviolable. Directed mutagenesis can decrease the rate of penicillin hydrolysis by PBP 5 to almost zero, leave the hydrolysis rate unchanged while destroying penicillin binding, or increase the hydrolysis rate two- or threefold over that of the wild-type enzyme (43). Thus, PBP 5 can be manipulated to become more like a nonhydrolyzing PBP or more like a β -lactamase. Similarly, a mutated class A β -lactamase may take on enzymatic characteristics normally associated with PBP 5 and other DD-carboxypeptidases (26). In addition, some wild-type β -lactamases catalyze transpeptidation reactions similar to those carried out by PBPs (14), and individual class C β -lactamases can form long-lived β -lactam-enzyme complexes (14). Sequence comparisons suggest that the source of these similarities between β -lactamases and PBPs may be explained by their evolutionary origin; the class A and class C β -lactamases apparently arose from a common PBP ancestor, an actinomycete DD-carboxypeptidase (20, 21). In short, PBPs and β -lactamases can be viewed as a continuum of related enzymes with overlapping activities.

The results reported in this paper highlight the close functional relationships between proteins described as PBPs and those characterized as β -lactamases. Two proteins in *E. coli* behave as do the classical PBPs, as defined by the ability to form a stable covalent complex with 125 I-penicillin-X and other β -lactams: one is a known protein, AmpC, and the other is a newly recognized protein, AmpH. Sequence similarities place AmpH among the class C β -lactamases, of which AmpC is also a member, but although AmpC displays marked β -lactamase activity as measured by hydrolysis of nitrocefin, AmpH displays no such activity toward this substrate. The observation that AmpC binds β -lactams has been made previously: an AmpC-aztreonam complex is extremely stable, with a deacylation rate of less than 0.0001 sec $^{-1}$, and an AmpC-moxalactam complex has a deacylation rate of less than 0.001 sec $^{-1}$ (13). (That AmpC binds aztreonam as well as does PBP 3 suggests cautious interpretation of experiments conducted under the pre-

FIG. 10. Morphology of *ampH*, *ampC*, and *ampC ampH* mutants of *E. coli* in the presence of the β -lactam aztreonam. The parent and derivative strains of *E. coli* (described in the legend to Fig. 9) were exposed to aztreonam (10 μ g/ml) for the times indicated, and individual cells were photographed. The magnification and enlargement of each photo in this figure and in Fig. 9 are the same, so that direct comparisons of length and width can be made. (A) The black arrow indicates a photograph of an empty cell sacculus which contains no cytoplasm. (B) The white arrow indicates a cell pole with multiple "knobs."

sumption that inactivation of septation via PBP 3 inhibition is the sole effect of aztreonam.) The stability of AmpC- β -lactam complexes and the inability of AmpC to hydrolyze aztreonam or moxalactam are exactly the characteristics expected of classic PBPs vis-à-vis β -lactams. This indicates that the differences between AmpC (or AmpH) and the classic PBPs are differences of degree and definition—the two groups differ in the spectrum of β -lactams with which they form long-lived covalent complexes.

A logical implication of their behavior as PBP-like proteins is that AmpC and/or AmpH might also play normal physiological roles in *E. coli*, perhaps in concert with the classic PBPs. In other species of the *Enterobacteriaceae*, AmpC can protect cells against exogenous β -lactams (4), and in these bacteria the *ampC* gene is inducible and is regulated by the *ampR* and *ampDG* gene products (19, 33). However, this ability of AmpC does not address its role, if any, in normal cellular physiology. In *E. coli*, in contrast to these other enteric bacteria, AmpC production is not induced in response to β -lactam exposure and the transcriptional regulation of the gene is different (4). The only reported phenotypes associated with AmpC include the following: the growth rate of an *E. coli ampC* mutant decreases suddenly, but not dramatically, about midway through the exponential phase; overexpression of AmpC slows the growth rate of *E. coli* during exponential phase; and AmpC severely inhibits cell growth if expression is induced during the lag phase (6). Still, in none of these cases is there an indication of a normal physiological function of the AmpC protein. It has been proposed, on the basis of structural similarities between substrates, that AmpC might function as a peptidoglycan LD-endopeptidase in addition to its known β -lactamase activity (5), but this supposition remains unproved.

We present evidence (Fig. 9 and 10) which illustrates that loss of AmpC and AmpH disturbs the production of correctly shaped *E. coli* cells in at least one genetic background. In particular, *E. coli* loses the ability to maintain a normal diameter, contour, or overall shape; individual filaments exhibit high rates of branching; and the extent and frequency of morphological aberrations increase when both AmpC and AmpH are absent. Thus, these proteins appear to participate in the normal processes of cell wall growth and/or maturation. We cannot explain why these morphological defects should appear more severe in a bacterial strain that also lacks PBPs 1a and 5, although the most commonly invoked explanation for similar situations among the PBPs is duplication of function.

The physiological characteristics of AmpH provoke the speculation that it could serve as a target for β -lactamase induction in members of the *Enterobacteriaceae* other than *E. coli*. AmpH binds cefoxitin (a strong inducer of β -lactamase expression in these other bacteria) but does not bind aztreonam or ampicillin (weak inducers). In addition, AmpH protein disappears in stationary phase and at high temperatures, conditions in which β -lactamase induction is decreased or absent (23). Such a system of induction would make biological sense if AmpC was being called on to compensate for inactive AmpH.

The synthesis and metabolism of peptidoglycan have been the subjects of intense research for several decades. Nevertheless, the discovery that the AmpC and AmpH proteins may play roles in this pathway reinforces two themes. First, there is still a great deal to learn about how bacteria form and maintain their cell walls, and second, some members of the β -lactamase family apparently contribute more to normal bacterial physiology than has been appreciated.

ACKNOWLEDGMENTS

This work was supported by a grant from SmithKline Beecham Pharmaceuticals.

We especially thank David Knowles, Graham Clarke, and David Payne of SKB for their support; Joachim-Volker Hölftje for providing results prior to publication; and Karla Glick for preparing graphics.

REFERENCES

1. Amanuma, H., and J. L. Strominger. 1980. Purification and properties of penicillin-binding proteins 5 and 6 from *Escherichia coli* membranes. *J. Biol. Chem.* **255**:11173–11180.
2. Ayala, J. A., T. Garrido, M. A. de Pedro, and M. Vicente. 1994. Molecular biology of bacterial septation, p. 73–101. In J.-M. Ghuyssen and R. Hakenbeck (ed.), *Bacterial cell wall*. Elsevier Science B.V., Amsterdam, The Netherlands.
3. Baquero, M.-R., M. Bouzon, J. C. Quintela, J. A. Ayala, and F. Moreno. 1996. *dacD*, an *Escherichia coli* gene encoding a novel penicillin-binding protein (PBP6b) with DD-carboxypeptidase activity. *J. Bacteriol.* **178**:7106–7111.
4. Bennett, P. M., and I. Chopra. 1993. Molecular basis of β -lactamase induction in bacteria. *Antimicrob. Agents Chemother.* **37**:153–158.
5. Bishop, R. E., and J. H. Weiner. 1992. Coordinate regulation of murein peptidase activity and AmpC β -lactamase synthesis in *Escherichia coli*. *FEBS Lett.* **304**:103–108.
6. Bishop, R. E., and J. H. Weiner. 1993. Complementation of growth defect in an *ampC* deletion mutant of *Escherichia coli*. *FEMS Microbiol. Lett.* **114**:349–354.
7. Bittner, M., and D. Vapnek. 1981. Versatile cloning vectors derived from the runaway-replication plasmid pKN402. *Gene* **15**:319–329.
8. Borodovsky, M., J. D. McIninch, E. V. Koonin, K. E. Rudd, C. Medigue, and A. Danchin. 1995. Detection of new genes in a bacterial genome using Markov models for three gene classes. *Nucleic Acids Res.* **23**:3554–3562.
9. Botta, G. A., and J. T. Park. 1981. Evidence for involvement of penicillin-binding protein 3 in murein synthesis during septation but not during cell elongation. *J. Bacteriol.* **145**:333–340.
10. Broome-Smith, J., and B. G. Spratt. 1984. An amino acid substitution that blocks the deacylation step in the enzyme mechanism of penicillin-binding protein 5 of *Escherichia coli*. *FEBS Lett.* **165**:185–189.
11. Broome-Smith, J. K. 1985. Construction of a mutant of *Escherichia coli* that has deletions of both the penicillin-binding protein 5 and 6 genes. *J. Gen. Microbiol.* **131**:2115–2118.
12. Denome, S. A., P. K. Elf, T. A. Henderson, and K. D. Young. Unpublished data.
13. Dubus, A., S. Normark, M. Kania, and M. G. P. Page. 1994. The role of tyrosine 150 in catalysis of β -lactam hydrolysis by AmpC β -lactamase from *Escherichia coli* investigated by site-directed mutagenesis. *Biochemistry* **33**:8577–8586.
14. Frère, J.-M. 1995. Beta-lactamases and bacterial resistance to antibiotics. *Mol. Microbiol.* **16**:385–395.
15. Ghuyssen, J.-M. 1994. Molecular structures of penicillin-binding proteins and β -lactamases. *Trends Microbiol.* **2**:372–380.
16. Ghuyssen, J.-M., P. Charlier, J. Coyette, C. Duez, E. Fonze, C. Fraipont, C. Goffin, B. Joris, and M. Nguyen-Distèche. 1996. Penicillin and beyond: evolution, protein fold, multimodular polypeptides, and multiprotein complexes. *Microb. Drug Resist.* **2**:163–175.
17. Henderson, T. A., P. M. Dombrosky, and K. D. Young. 1994. Artifactual processing of penicillin-binding proteins 7 and 1b by the OmpT protease of *Escherichia coli*. *J. Bacteriol.* **176**:256–259.
18. Henderson, T. A., M. Templin, and E. S. Moland. 1995. Identification and cloning of the gene encoding penicillin-binding protein 7 of *Escherichia coli*. *J. Bacteriol.* **177**:2074–2079.
19. Jacobs, C., L.-J. Huang, E. Bartowsky, S. Normark, and J. T. Park. 1994. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for β -lactamase induction. *EMBO J.* **13**:4684–4694.
20. Kelly, J. A., O. Dideberg, P. Charlier, J. P. Wery, M. Libert, P. C. Moews, J. R. Knox, C. Duez, C. Fraipont, B. Joris, J. Dusart, J.-M. Frère, and J.-M. Ghuyssen. 1986. On the origin of bacterial resistance to penicillin: comparison of a beta-lactamase and a penicillin target. *Science* **231**:1429–1431.
21. Kirby, R. 1992. Evolutionary origin of the class A and class C β -lactamases. *J. Mol. Evol.* **34**:345–350.
22. Kohara, Y., K. Akiyama, and K. Isono. 1987. 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.
23. Korfmann, G., C. C. Sanders, and E. S. Moland. 1991. Altered phenotypes associated with *ampD* mutations in *Enterobacter cloacae*. *Antimicrob. Agents Chemother.* **35**:358–364.
24. Kristensen, C. S., L. Eberl, J. M. Sanchez-Romero, M. Givskov, S. Molin, and V. de Lorenzo. 1995. Site-specific deletions of chromosomally located DNA segments with the multimer resolution system of broad-host-range plasmid RP4. *J. Bacteriol.* **177**:52–58.
25. Kulakauskas, S., P. M. Wikström, and D. E. Berg. 1991. Efficient introduc-

- tion of cloned mutant alleles into the *Escherichia coli* chromosome. J. Bacteriol. **173**:2633–2638.
26. Lewis, E. R., K. M. Winterberg, and A. L. Fink. 1997. A point mutation leads to altered product specificity in β -lactamase catalysis. Proc. Natl. Acad. Sci. USA **94**:443–447.
 27. Lindberg, F., and S. Normark. 1986. Contribution of chromosomal β -lactamases to β -lactam resistance in enterobacteria. Rev. Infect. Dis. **3**(Suppl.): S292–S304.
 28. Matsubashi, M. 1994. Utilization of lipid-precursors and the formation of peptidoglycan in the process of cell growth and division: membrane enzymes involved in the final steps of peptidoglycan synthesis and the mechanism of their regulation, p. 55–71. In J.-M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier Science B.V., Amsterdam, The Netherlands.
 29. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 30. Mottl, H., P. Nieland, G. de Kort, J. J. Wierenga, and W. Keck. 1992. Deletion of an additional domain located between SXXX and SXN active-site fingerprints in penicillin-binding protein 4 from *Escherichia coli*. J. Bacteriol. **174**:3261–3269.
 31. Mottl, H., P. Terpstra, and W. Keck. 1991. Penicillin-binding protein 4 of *Escherichia coli* shows a novel type of primary structure among penicillin-interacting proteins. FEMS Microbiol. Lett. **78**:213–220.
 32. O'Callaghan, C. H., A. Morris, S. M. Kirby, and A. H. Shingler. 1972. Novel method for detection of β -lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents Chemother. **1**:283–288.
 33. Park, J. T. 1995. Why does *Escherichia coli* recycle its cell wall peptides? Mol. Microbiol. **17**:421–426.
 34. Park, J. T. 1996. The convergence of murein recycling research with β -lactamase research. Microb. Drug Resist. **2**:105–112.
 35. Pellon, G. 1990. Biosynthesis of peptidoglycans from eubacterial cell envelopes. Bull. Inst. Pasteur **88**:203–236.
 36. Rudd, K. E. 1992. Alignment of *E. coli* DNA sequences to a revised, integrated genomic restriction map, p. 2.3–2.43. In J. H. Miller (ed.), A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 37. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 38. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
 39. Schiffer, G., M. F. Templin, and J.-V. Höltje. Unpublished data.
 40. Smith, R. F., B. A. Wiese, M. K. Wojzynski, D. B. Davison, and K. C. Worley. 1996. BCM search launcher—an integrated interface to molecular biology data base search and analysis services available on the World Wide Web. Genome Res. **6**:454–462.
 41. Spratt, B. G., and A. B. Pardee. 1975. Penicillin-binding proteins and cell shape in *E. coli*. Nature **254**:516–517.
 42. Sykes, R. B., and D. P. Bonner. 1985. Discovery and development of the monobactams. Rev. Infect. Dis. **7**:S579–S593.
 43. van der Linden, M. P. G., L. de Haan, O. Dideberg, and W. Keck. 1994. Site-directed mutagenesis of proposed active-site residues of penicillin-binding protein 5 from *Escherichia coli*. Biochem. J. **303**:357–362.
 44. Worley, K. C., B. A. Wiese, and R. F. Smith. 1995. BEAUTY: an enhanced BLAST-based search tool that integrates multiple biological information resources into sequence similarity search results. Genome Res. **5**:173–184.