

# Daughter Cell Separation by Penicillin-Binding Proteins and Peptidoglycan Amidases in *Escherichia coli*

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**As one of the final steps in the bacterial growth cycle, daughter cells must be released from one another by cutting the shared peptidoglycan wall that separates them. In *Escherichia coli*, this delicate operation is performed by several peptidoglycan hydrolases, consisting of multiple amidases, lytic transglycosylases, and endopeptidases. The interactions among these enzymes and the molecular mechanics of how separation occurs without lysis are unknown. We show here that deleting the endopeptidase PBP 4 from strains lacking AmiC produces long chains of unseparated cells, indicating that PBP 4 collaborates with the major peptidoglycan amidases during cell separation. Another endopeptidase, PBP 7, fulfills a secondary role. These functions may be responsible for the contributions of PBPs 4 and 7 to the generation of regular cell shape and the production of normal biofilms. In addition, we find that the *E. coli* peptidoglycan amidases may have different substrate preferences. When the DD-carboxypeptidase PBP 5 was deleted, thereby producing cells with higher levels of pentapeptides, mutants carrying only AmiC produced a higher percentage of cells in chains, while mutants with active AmiA or AmiB were unaffected. The results suggest that AmiC prefers to remove tetrapeptides from peptidoglycan and that AmiA and AmiB either have no preference or prefer pentapeptides. Mucopeptide compositions of the mutants corroborated this latter conclusion. Unexpectedly, amidase mutants lacking PBP 5 grew in long twisted chains instead of straight filaments, indicating that overall septal morphology was also defective in these strains.**

Most eubacteria owe their shape and mechanical stability to the peptidoglycan sacculus, a semirigid structure composed of multiple carbohydrate chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid that are interconnected by covalent cross-links between short peptides (14). Far from static, this structure exists in a dynamic state throughout the life of a cell. In *Escherichia coli*, the peptidoglycan is in constant turnover (34, 35), and the cutting of specific peptide and carbohydrate bonds is important for cell growth, division, separation of daughter cells, and lysis (10, 11, 13–15, 39).

Although enzymes capable of cleaving bonds in the murein sacculus are ubiquitous among the bacteria (39), surprisingly few of these proteins fit into a well-defined role in normal peptidoglycan biochemistry. One reason for this ambiguity is an apparent redundancy, so that the loss of any single enzyme may be masked by the presence of others with equivalent or compensatory activities (13, 39). The situation is exemplified by the widely distributed family of *N*-acetylmuramyl-L-alanine amidases, represented in *E. coli* by three closely related members: AmiA, AmiB, and AmiC (10, 11, 14). These periplasmic amidases remove side chains from peptidoglycan by cleaving the bond between the peptide and *N*-acetylmuramic acid (Fig. 1).

The peptidoglycan-specific amidases play an important role in cleaving the septum to release daughter cells after cell division in *E. coli* (10). Mutants lacking the AmiC protein separate poorly, in that ~30% of the population exists as chains of

three to six unseparated cells (10). If *amiA* alone is deleted, only 5 to 10% of the population is in chains of three to four cells, and deleting *amiB* by itself produces no chaining effect at all. Thus, AmiC appears to be the principle septum-cleaving enzyme, and because the enzyme localizes specifically to the septal ring, it is ideally positioned for cleaving the divisional septum (1). But even given the preeminent role of AmiC, cell chaining is exacerbated in mutants lacking all three amidases; in these strains, more than 90% of the cells exist as unseparated chains from 6 to 24 cells long (10). Thus, the three amidases have overlapping or redundant functions with regard to cell separation. Yet the continued existence of some short chains indicates that residual septum cleavage occurs, suggesting that at least one other enzyme can separate daughter cells, albeit with reduced efficiency.

The additional septum-cleaving enzymes belong to two other classes of peptidoglycan hydrolases: lytic transglycosylases, which cut the glycan chain, and endopeptidases, which cut peptide cross-links at a different site than that cleaved by amidases (Fig. 1). When the *sltY* gene, encoding lytic transglycosylase Slt70, was deleted from a triple amidase mutant, the chain length increased to 8 to 40 cells, indicating that this enzyme also plays a role in cell separation (10). Still, the existence of eight-cell chains suggests that cell separation occurs via another route, possibly due to the action of endopeptidases. An *E. coli* mutant lacking all three murein endopeptidases (PBP 4, PBP 7, and MepA) continues to separate normally and grows as single cells (11), indicating that these enzymes are not primary septum-cleaving factors. However, a mutant lacking the three endopeptidases and all three amidases grows as chains from 6 to 80 cells long (11), indicating that the chaining phenotype of the amidase mutant is exacerbated by removing the

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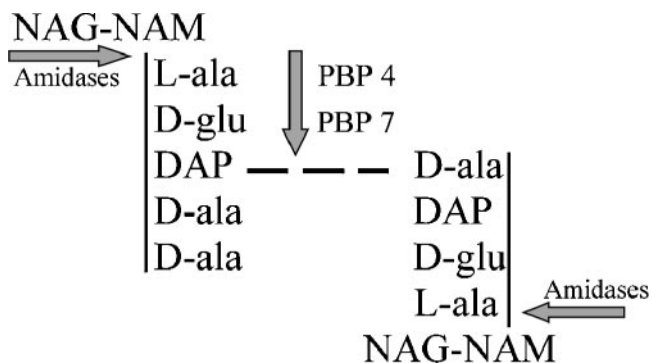


FIG. 1. Activities of endopeptidases and amidases on peptidoglycan subunits. Endopeptidases PBP 4 and PBP 7 cleave cross-linked peptide side chains, while amidases cut the bond connecting the peptide to the NAM component of the polymerized glycan backbone. The figure shows a pentapeptide monomer (upper left) cross-linked (dashed line) to a tetrapeptide monomer (lower right). NAG, *N*-acetylglucosamine; NAM, *N*-acetylmuramic acid; L-ala, L-alanine; D-glu, D-glutamic acid; DAP, diaminopimelic acid; D-ala, D-alanine.

endopeptidases. The cumulative effect of deleting all amidases, all endopeptidases, and Slt70 is even more severe (11). Therefore, although AmiC appears to be the major cell separation factor in *E. coli*, it is aided or can be replaced by other peptidoglycan hydrolases.

*E. coli* contains two low-molecular-weight (LMW) penicillin-binding protein (PBP) endopeptidases, PBP 4 and 7, which could play a role in septum cleavage either by directly cutting peptide side chains (Fig. 1) or by modifying the peptidoglycan to provide alternate substrates for the amidases. Because the individual endopeptidases were not examined for their contributions to cell separation (11), we deleted individual PBPs and amidases to see which enzymes facilitated cell separation. Amidase mutants lacking PBP 4 grew as longer chains, identifying a specific biological role for this endopeptidase in cell separation. PBP 7 appeared to play a secondary role. In addition, we found that amidases apparently prefer different peptidoglycan substrates: AmiC is more likely to remove tetrapeptides from the glycan chains, while AmiA and AmiB act on either tetrapeptides or pentapeptides. Finally, particular combinations of PBPs and amidases affected the overall septum geometry in that certain mutants formed highly abnormal cell chains.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, and phage.** Bacterial strains and plasmids are listed in Table 1. All bacteria were grown in Luria-Bertani medium with appropriate antibiotics where required: ampicillin (50  $\mu$ g/ml), kanamycin (50  $\mu$ g/ml), chloramphenicol (50  $\mu$ g/ml), or spectinomycin (50  $\mu$ g/ml). Yeast extract and tryptone were from Difco (Detroit, Mich.). All other chemicals and antibiotics were from Sigma Chemical Co. (St. Louis, Mo.) or Fisher Scientific (Pittsburgh, Pa.). Transduction via bacteriophage P1 was performed as previously described (27).

**Molecular techniques.** Plasmids were isolated using QIAprep spin miniprep kits (QIAGEN Corp., Valencia, Calif.) according to the manufacturer's instructions. Competent cells were prepared and transformed by electroporation, using a Gene Pulser apparatus from Bio-Rad (Hercules, Calif.). Chromosomal DNA for PCR amplification was prepared by using a DNeasy tissue kit (QIAGEN Corp.). DNA agarose gel electrophoresis was performed as previously described (38). DNA purifications from agarose gels were performed with a QIAquick gel extraction kit (QIAGEN Corp.). Restriction digests and ligations were performed with enzymes from New England Biolabs (Beverly, Mass.). Amplification

by PCR was performed in a 2400 GeneAmp Thermal cycler (Perkin Elmer, Boston, Mass.), and oligonucleotide primers for PCR were purchased from MWG Biotech (Highpoint, NC). Deoxynucleotide triphosphates were from Promega (Madison, Wis.), and Deep Vent DNA polymerase was from New England Biolabs (Beverly, Mass.). PCR was carried out as described previously (25, 26). The *res-npt-res* kanamycin resistance cassette was amplified from plasmid pBMM1 by using a MasterAmp Extra-long PCR kit from Epicenter (Madison, Wis.).

**Construction of amidase deletion mutants.** Strain MHD41 was obtained from Heidrich et al. (10) and carries a deletion within *amiB* that begins 33 nucleotides downstream of the start codon and ends at nucleotide 1307 of the *amiB* gene. Because this strain did not contain a selectable marker within the *amiB* gene, a kanamycin resistance marker was inserted nearby so that the mutation could be moved by P1 transduction into new strains. For this purpose, a kanamycin gene cassette flanked by two *res* sites was inserted 13,838 bp upstream of the chromosomal position of *amiB*, into the noncoding region between *frdA* and *yjeA*, 25 bp downstream from the stop codon of the *frdA* gene. The *res-npt-res* cassette from plasmid pBMM1 (25, 26) was amplified by PCR using the following primers: 5'-CCCACAGCCACGCTACTTCAGGGTAAGTACCTGAAAAGTTACGG AATTCGAGCTCTGCAGTCC-3' and 5'-GTCAAATTTTCAGACTTATCCAT CAGACTATACTGTTGTACGATAAGCTTGCATGCCTGCAG-3'.

The primer pairs amplified the *res-npt-res* cassette and added sequences to the 5' and 3' ends that were identical to the chromosomal insertion site upstream of *amiB*.

The amplified PCR products were transferred by electroporation into *E. coli* KM-32, where they were incorporated into the chromosome via homologous recombination mediated by the  $\lambda$  phage recombination system, as previously described (26, 28, 40). Kanamycin-resistant colonies were selected, and colonies having correct insertions were identified by diagnostic PCR of chromosomal DNA by using the following primers: 5'-GGCAGCAATTGCAGCAGCGTA-3' and 5'-TAGCCCTCTTGCGCACTAAA-3'. This insertion mutation was named *yje25::res-npt-res*. The resulting strain was named KM32-1K, and a phage P1 lysate was prepared on this strain and used to transduce strain MHD41. Transductants were screened by PCR for the presence of the kanamycin cassette by using the above primers and were also screened for the presence of the *amiB* deletion by using the following primers: 5'-GTTTATGGGGATCCGCGATT-3' and 5'-TCGTCCTTAGTTTGGCAGCG-3'. One strain containing both the kanamycin cassette and the *amiB* deletion was selected and named MHD41-12K. A P1 lysate from this organism was used to transfer the *res-npt-res*-linked *amiB* deletion to other strains.

An *amiC* deletion mutant carrying *res-npt-res* within the *amiC* gene was also created. The *res-npt-res* cassette from plasmid pBMM1 was amplified by PCR, using the following primers: 5'-TCATCCCCTTCTCGCCAGCGTCCGCCAT CGGAAAATACGCGGAATTCGAGCTCTGCAGTCC-3' and 5'-GATAAG CTTGCATGCCGTCAGGATGACAGATTATGCGTCTTTTCGCTAAAAGTTT CCGGTCAA-3'. The primer pairs amplified the *res-npt-res* cassette and added sequences to its 5' and 3' ends that were identical to chromosomal sites 41 bases upstream of the *amiC* stop codon and 40 bases downstream of the *amiC* start codon. Kanamycin-resistant colonies were selected, and colonies having correct insertions were identified by diagnostic PCR of chromosomal DNA by using the following primers: 5'-CCATAAAAAAGCGCCATTCA-3' and 5'-GCGACTTT TTTATCAGTAATGCCGTG-3'. One strain carrying *res-npt-res* within *amiC* was selected and named RPKM179-1K.

**Microscopy.** Cultures were grown in LB medium at 37°C to an optical density (OD) of approximately 0.6. Cell samples (5  $\mu$ l) were placed on agarose-coated microscope slides and incubated at room temperature for 5 to 10 min to immobilize the cells. Bright-field microscopy was performed by using a  $\times$ 100 oil immersion objective on a Nikon EFD-3 microscope. Photographs were obtained with an attached Sensys charged-coupled-device camera and capture software (Photometrics Ltd., Tucson, Ariz.). Cells were counted and grouped into three categories: single cells, two cells attached as double cells, and three or more cells attached in chains. Each category was expressed as the percentage of the total number of cells present, whether in chains or not, to give the equivalent of total mass or units in each group. Data were plotted in Sigma Plot 8.0 and analyzed by a paired Student's *t* test.

**Peptidoglycan preparation.** Peptidoglycan was prepared as previously described (22). Bacteria from a fresh overnight culture were diluted 1:200 into 400 ml of LB medium and grown at 37°C to an OD at 600 nm (OD<sub>600</sub>) of 0.5 to 0.7. Cells were cooled rapidly to 4°C, harvested, and resuspended to 0.2 g/ml in distilled water. Resuspended cells were added dropwise, with vigorous stirring, to an equal volume of boiling 8% sodium dodecyl sulfate (SDS) solution. The solution was boiled for 30 min, after which the lysate was allowed to cool overnight to room temperature. Insoluble peptidoglycan was pelleted by ultra-

TABLE 1. *E. coli* strains

Strain (reference) <sup>a</sup>	Genotype <sup>b</sup>	PBP(s) deleted <sup>c</sup>	Amidase(s) deleted
CS109	W1485 <i>rph rpoS</i>		
APCS-1 (25)	CS109 <i>yhbE-3650::res-npt-res</i> (Kan <sup>r</sup> cassette insertion near <i>dacB</i> <sup>+</sup> )		
APCS-3 (25)	CS109 <i>yeh-7310::res-npt-res</i> (Kan <sup>r</sup> cassette insertion near <i>pbpG</i> <sup>+</sup> )		
BMCS04-1K (26)	CS109 <i>mrcA::res-npt-res</i>	1a	
BMCS16-1K (25)	CS109 <i>ybe-9207::res-npt-res</i> (Kan <sup>r</sup> cassette insertion near <i>dacA</i> <sup>+</sup> )		
BUDDY (16)	MC4100 <i>tatC::spc</i>		
KM-32 (28)	<i>argE3 his-4 leuB6 proA2 thr-1 ara-14 galK2 lacY1 mlt-1 xyl-5 thi-1 rpsL31 tsx-33 supE44 Δ(recC ptr recB recD)::Plac-bet exo cmr</i>		
KMRP-1K	KM-32 <i>yje25::res-npt-res</i> (Kan <sup>r</sup> cassette insertion near <i>amiB</i> <sup>+</sup> )		
MC1061 (3)	<i>araD139 Δ(ara- leu)7697 ΔlacX74 galU galK hsdR2 rpsL</i>		
MHD8 (10)	MC1061 <i>amiC::Kan</i>		AmiC
MHD9 (10)	MC1061 <i>amiA::Cam</i>		AmiA
MHD41 (10)	MC1061 <i>amiB</i>		AmiB
MHD41-12K	MC1061 <i>amiB yje25::res-npt-res</i>		AmiB
RPKM179-1K	KM-32 <i>amiC::res-npt-res</i>		AmiC
RP1	CS109 <i>amiC::Kan</i>		AmiC
RP3	CS203-1B <i>amiC::Kan</i>	4, 7	AmiC
RP4	CS204-1 <i>amiC::Kan</i>	5, 7	AmiC
RP8	CS219 <i>amiC::Kan</i>	4, 5	AmiC
RP9	CS220-1 <i>amiC::Kan</i>	4, 6	AmiC
RP12	CS322-1 <i>amiC::Kan</i>	4, 5, 6	AmiC
RP14	CS446-1 <i>amiC::Kan</i>	4, 5, 6, 7	AmiC
RP19	CS703-1 <i>amiC::Kan</i>	1a, 4, 5, 6, 7, C, H	AmiC
RP20	CS702-1 <i>amiC::Kan</i>	1b, 4, 5, 6, 7, C, H	AmiC
RP21	CS219 <i>amiA::Cam</i>	4, 5	AmiA
RP22-1K	CS219 <i>amiB yje25::res-npt-res</i>	4, 5	AmiB
RP24-1K	CS204 <i>amiB yje25::res-npt-res</i>	5, 7	AmiB
RP25-1K	CS11-2 <i>amiB yje25::res-npt-res</i>	4	AmiB
RP26	CS11-2 <i>amiA::Cam</i>	4	AmiA
RP27-1K	CS9-19 <i>amiB yje25::res-npt-res</i>	7	AmiB
RP28	CS9-19 <i>amiA::Cam</i>	7	AmiA
RP29-1K	CS12-7 <i>amiB yje25::res-npt-res</i>	5	AmiB
RP30	CS12-7 <i>amiA::Cam</i>	5	AmiA
RP31	CS12-7 <i>amiC::Kan</i>	5	AmiC
RP32	CS11-2 <i>amiC::Kan</i>	4	AmiC
RP33	CS9-19 <i>amiC::Kan</i>	7	AmiC
RP37	CS109 <i>amiA::Cam</i>		AmiA
RP40	RP24-1 <i>amiA::Cam</i>	5, 7	AmiA, AmiB
RP41	RP22-1 <i>amiA::Cam</i>	4, 5	AmiA, AmiB
RP42	RP29-1 <i>amiA::Cam</i>	5	AmiA, AmiB
RP43	RP25-1 <i>amiA::Cam</i>	4	AmiA, AmiB
RP44	RP27-1 <i>amiA::Cam</i>	7	AmiA, AmiB
RP45	CS14-2 <i>amiC::Kan</i>	C	AmiC
RP46	CS15-3 <i>amiC::Kan</i>	H	AmiC
RP48	CS17-1 <i>amiC::Kan</i>	6	AmiC
RP49	CS109 <i>amiB yje25::res</i>		AmiB
RP50	RP30 <i>ybe-9207::res-npt-res</i>		AmiA
RP52	RP21 <i>ybe-9207::res-npt-res</i>	4	AmiA
RP53	RP21 <i>yhbE-3650::res-npt-res</i>	5	AmiA
RP60-1K	CS16-1 <i>amiB yje25::res-npt-res</i>	1b	AmiB
RP61-1K	CS17-1 <i>amiB yje25::res-npt-res</i>	6	AmiB
RP64-1K	CS703-1 <i>amiB yje25::res-npt-res</i>	1a, 4, 5, 6, 7, C, H	AmiB
RP65-1K	BMCS04 <i>amiB yje25::res-npt-res</i>	1a	AmiB
RP70	CS331-1 <i>amiC::Kan</i>	5, 6, 7	AmiC
RP75	RP49 <i>amiA::Cam</i>		AmiA, AmiB
RP77	RP75 <i>amiC::Kan</i>		AmiA, AmiB, AmiC
RP79	CS17-1 <i>amiA::Cam</i>	6	AmiA
RP80	CS18-3K <i>amiA::Cam</i>	DacD	AmiA
RP102	RP19 <i>amiA::Cam</i>	1a, 4, 5, 6, 7, C, H	AmiA, AmiC
RP103	RP30 <i>amiC::Kan</i>	5	AmiA, AmiC
RP104	RP32 <i>amiA::Cam</i>	4	AmiA, AmiC
RP106	RP43 <i>amiC::Kan</i>	4	AmiA, AmiB, AmiC
RP107	RP44 <i>amiC::Kan</i>	7	AmiA, AmiB, AmiC
RP108	RP42 <i>amiC::Kan</i>	5	AmiA, AmiB, AmiC
RP109	RP40 <i>amiC::Kan</i>	5, 7	AmiA, AmiB, AmiC
RP110	RP41 <i>amiC::Kan</i>	4, 5	AmiA, AmiB, AmiC
RP113	RP4 <i>amiA::Cam</i>	5, 7	AmiA, AmiC

Continued on following page

TABLE 1—Continued

Strain (reference) <sup>a</sup>	Genotype <sup>b</sup>	PBP(s) deleted <sup>c</sup>	Amidase(s) deleted
RP114	RP8 <i>amiA</i> ::Cam	4, 5	AmiA, AmiC
RP117	CS315-1 <i>amiA</i> ::Cam	4, 5, 7	AmiA
RP118-1K	CS315-1 <i>amiB yje25::res-npt-res</i>	4, 5, 7	AmiB
RP120	RP117 <i>amiC</i> ::Kan	4, 5, 7	AmiA, AmiC
RP130	RP3 <i>amiA</i> ::Cam	4, 7	AmiA, AmiC
RP139	RP14 <i>amiA</i> ::Cam	4, 5, 6, 7	AmiA, AmiC
RP144	RP118-1 <i>amiC</i> ::Kan	4, 5, 7	AmiB, AmiC
RP145	RP144 <i>amiA</i> ::Cam	4, 5, 7	AmiA, AmiB, AmiC
RP149	CS609-1 <i>amiC</i> ::Kan	1b, 4, 5, 7, C, H	AmiC
RP152	RP149 <i>amiA</i> ::Cam	1b, 4, 5, 7, C, H	AmiA, AmiC
RP154	CS610-1 <i>amiA</i> ::Cam	1b, 4, 6, 7, C, H	AmiA
RP156	CS203-1B <i>amiB yje25::res</i>	4, 7	AmiB
RP157	RP156 <i>amiA</i> ::Cam	4, 7	AmiA, AmiB
RP158	RP157 <i>amiC</i> ::Kan	4, 7	AmiA, AmiB, AmiC
RP160	CS447-1 <i>amiC</i> ::Kan	4, 6, 7, C	AmiC
RP164	RP160 <i>amiA</i> ::Cam	4, 6, 7, C	AmiA, AmiC
RP167	CS316-1 <i>amiC</i> ::Kan	4, 6, 7	AmiC
RP168-1K	CS316-1 <i>amiB yje25::res-npt-res</i>	4, 6, 7	AmiB
RP169	CS703-1 <i>amiA</i> ::Cam	1a, 4, 5, 6, 7, C, H	AmiA
RP172	RP168-1 <i>amiA</i> ::Cam	4, 6, 7	AmiA, AmiB
RP173	RP172 <i>amiC</i> ::Kan	4, 6, 7	AmiA, AmiB, AmiC
RP175	CS703-1 <i>tatC</i> ::Spc	1a, 4, 5, 6, 7, C, H	
RP178	RP154 <i>amiC</i> ::Kan	1b, 4, 6, 7, C, H	AmiA, AmiC
RP184-1K	CS446-1 <i>amiC::res-npt-res</i>	4, 5, 6, 7	AmiC
RP185-1K	CS331-1 <i>amiC::res-npt-res</i>	5, 6, 7	AmiC
RP188	RP184-1 <i>yhbE-3650::res-npt-res</i>	5, 6, 7	AmiC
RP189	RP184-1 <i>ybe-9207::res-npt-res</i>	4, 6, 7	AmiC
RP191	RP184-1 <i>yeh-7310::res-npt-res</i>	4, 5, 6	AmiC

<sup>a</sup> Strains having prefixes CS or RP were derived from *E. coli* CS109. CS strains have been described previously (4, 25, 26), and RP strains were created for this work.

<sup>b</sup> Strains were created by moving genes by P1 transduction from the following source strains: *amiC*::Kan (from MHD8), *amiA*::Cam (from MHD9), *amiB yje25::res-npt-res* (from MHD41-12K), *amiC::res-npt-res* (from RPKM179-1K), and *tatC::spc* (from BUDDY). Strains carrying the *res-npt-res* allele have a suffix K. After removal of the kanamycin gene from these strains by using the ParA resolvase (21, 28), the suffix K was removed from the strain name.

<sup>c</sup> Numbers refer to the respective PBPs: 1a, PBP 1a; 1b, PBP 1b; 4, PBP 4; 5, PBP 5; 6, PBP 6; 7, PBP 7; C, AmpC; H, AmpH.

centrifugation at 100,000 × *g* for 60 min at 20°C, and the pellet was washed and resuspended in distilled water. This step was repeated six times until the SDS concentration fell below 1 µg/ml, as determined by the methylene blue assay (9). Pelleted peptidoglycan was resuspended in 1 ml of distilled water and disaggregated by sonication (1.5 min; 90% duty cycle; 4.5 output), using a Branson sonifier 450. Glycogen contamination was removed by adding α-amylase (final concentration, 100 µg/ml) and imidazole (final concentration, 0.32 M) and incubating at 37°C for 2 h. Afterwards, pronase was added to 200 µg/ml, and the sample was incubated at 60°C for 1.5 h to remove lipoprotein. After pronase treatment, the solution was added to an equal volume of boiling SDS with vigorous stirring and boiled for 15 min. Insoluble peptidoglycan was pelleted, washed free of SDS as described above, and resuspended in 200 to 300 µl of distilled water containing 0.02% NaNO<sub>3</sub>. Samples were stored at -20°C.

**HPLC analysis.** Mucopeptides were produced by digesting peptidoglycan with *N*-acetylmuramidase SG (USB Specialty Biochemicals, Amersham Life Sciences, Cleveland, Ohio). The reaction mixture consisted of the following: 50 µl peptidoglycan (*A*<sub>208</sub> = 0.3), 7.5 µl of 0.2 M phosphate buffer, pH 5.5, 2.5 µl of 0.2% NaNO<sub>3</sub>, 5.0 µl of H<sub>2</sub>O, and 10 µl of *N*-acetylmuramidase SG (1 mg/ml stock in 20 mM NaPO<sub>4</sub> buffer, pH 5.5). Samples were digested by incubation at 37°C overnight, after which the sample was dried under centrifugal vacuum. The resulting mucopeptides were reduced with sodium borohydride and fractionated by reverse-phase high-pressure liquid chromatography (HPLC), as previously described (8, 36). The peaks were collected, purified by HPLC using a trifluoroacetic acid-acetonitrile gradient system, identified by amino acid-amino sugar analysis (24), and quantified as previously described (8).

**Scanning electron microscopy (SEM).** Cells were grown to mid-log phase in LB medium, collected, resuspended in phosphate-buffered saline, spotted onto glass coverslips coated with poly-L-lysine, fixed in Karnovsky's fixative overnight, postfixed in 1% OsO<sub>4</sub>, and prepared as described previously (2, 18). Cells were observed and photographed with a Hitachi 4700 field emission scanning electron microscope.

## RESULTS

### LMW PBPs contribute to cell separation in *amiC* mutants.

To test whether PBPs play a role in cell separation, we deleted *amiC* from strains lacking various PBPs. We chose this route because deleting *amiC* produces a higher percentage of cells in chains than the deletion of *amiA* or *amiB* alone (10). In *amiC* mutants, the numbers of cells in chains increased when certain sets of multiple PBPs were deleted (Fig. 2). At the extremes, 37% of the cell population grew in chains in the control strain RP1 (Δ*AmiC*), whereas the numbers of cells in chains reached 78% in strain RP14, with deletions of PBPs 4, 5, 6, and 7 and *AmiC* (ΔPBP4,5,6,7 Δ*AmiC*), and 92% in strain RP19 (ΔPBP1a,4,5,6,7 ΔAmpC ΔAmpH Δ*AmiC*) (Fig. 2). Since deleting the high-molecular-weight PBPs 1a or 1b had no differential effects (Fig. 2, strains RP19 and RP20), the results indicated that one or more LMW PBPs played a role in cell separation in *amiC* mutants. Note that all these results depended on mutating one or more of the *ami* genes, because no chaining was observed in any PBP mutant that expressed all three wild-type amidases.

Because strains showing increased chaining were missing four or more LMW PBPs, including the endopeptidases PBP 4 and PBP 7, we analyzed *amiC* mutants lacking PBPs alone and in combination to determine which were most important for cell separation. In strain RP8 (ΔPBP4,5 Δ*AmiC*) 57% of cells grew in chains (*P* = 0.002 compared to either RP1 or RP4), but



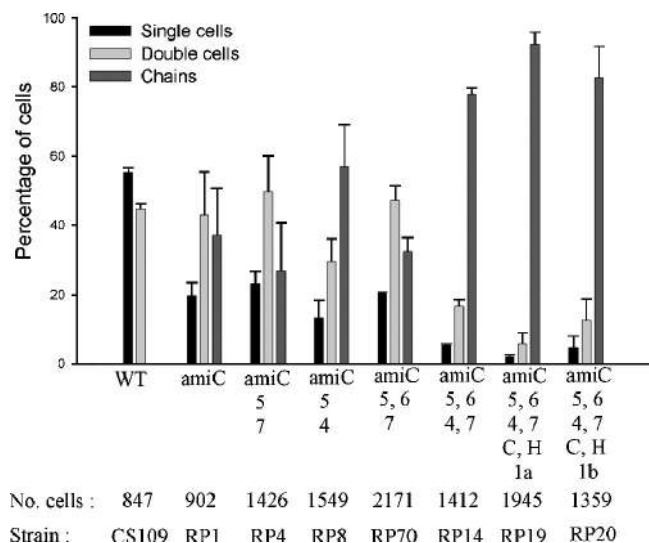


FIG. 2. Multiple PBP deletions increase chaining in *amiC* mutants. Strains were grown in LB medium at 37°C to an OD<sub>600</sub> of 0.6. Cells were counted as described in Materials and Methods. Data are from three independent experiments, and the error bars represent the standard deviation. Deleted genes are indicated on the x axis, and numbers refer to the respective PBPs (e.g., 1a is PBP 1a). C, AmpC; H, AmpH; WT, wild-type parent, CS109; No. cells, the total number of cells counted for each strain. The respective strain names are given at the bottom of the figure. The relevant paired *t* test results were as follows: RP1 versus RP4, *P* > 0.05 (not significant); RP1 versus RP8, *P* = 0.002 (significant); RP4 versus RP8, *P* = 0.002 (significant).

in RP4 ( $\Delta$ PBP5,7  $\Delta$ AmiC) only 27% of the cells were in chains (*P* > 0.05 compared to RP1) (Fig. 2). Likewise, for strain RP14 ( $\Delta$ PBP4,5,6,7  $\Delta$ AmiC) 78% of the cells grew in chains, but only 32% were in chains for strain RP70 ( $\Delta$ PBP5,6,7  $\Delta$ AmiC) (Fig. 2). Two strains equivalent to RP14 and RP70 (RP184-1K and RP185-1K, respectively) were generated independently by inserting a different cassette into the *amiC* gene. Although the absolute values of cells in chains (66% and 42%) (Table 2) were slightly different from those of RP14 and RP70 (Fig. 2), the trend was the same as in all other cases. In each pairwise comparison, deletion of PBP 4 was associated with an increase in chaining, whereas deletion of PBP 7 had little or no effect under the conditions employed. However, when PBPs were

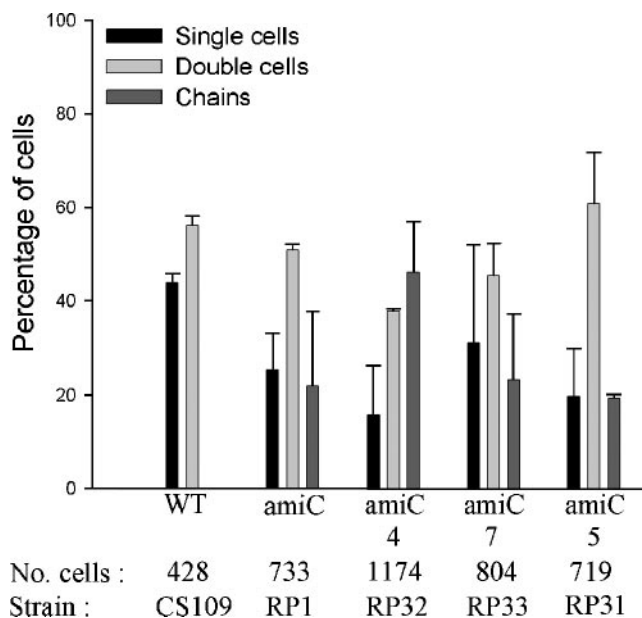


FIG. 3. Deleting PBP 5 does not increase chaining in *amiC* mutants. Growth conditions, graphical presentation, and abbreviations are described in the legend of Fig. 2. Data are from two independent sets of experiments. Paired *t* test results between RP1 and any of the strains lacking PBPs are not significant (*P* > 0.05).

removed individually from an *amiC* mutant, chaining was not increased to a significant degree (*P* > 0.05) in mutants lacking the endopeptidases PBP 4 (Fig. 3, strain RP32), PBP 7 (Fig. 3, strain RP33), or MepA (data not shown), nor was chaining increased in *amiC* mutants lacking the D<sub>D</sub>-carboxypeptidases PBP 5 (Fig. 3, strain RP31) or DacD (data not shown).

The absolute number of cells in chains sometimes varied from day to day for any set of strains being compared, but their relative behavior remained constant. For example, the control strain RP1 ( $\Delta$ AmiC) exhibited variation in chaining percentage on different days (e.g., compare RP1 in Fig. 2 with Fig. 3), but PBP mutations caused similar changes in chaining each time. The causes of this variation are unknown, but the most likely considerations are subtle differences in medium composition, growth conditions, age at harvest, rotatory flask speed,

TABLE 2. PBPs complement chaining phenotype of *amiC* mutants

Strain <sup>a</sup>	PBPs deleted <sup>b</sup>	PBP replaced <sup>c</sup>	Percentage of cells arranged as:			No. of cells counted
			Single cells	Double cells	Chains	
CS109			41.3 ± 0.2	58.6 ± 0.2	0.00 ± 0	416
RP1			24.1 ± 1.8	62.3 ± 4.3	13.4 ± 6.2	503
RP185-1K	5, 6, 7		19.0 ± 0.8	39.0 ± 3.0	41.8 ± 2.2	758
RP184-1K	4, 5, 6, 7		10.5 ± 2.9	23.7 ± 2.8	65.7 ± 5.7	638
RP188	5, 6, 7	4	15.9 ± 8.6	36.2 ± 7.1	47.7 ± 15.9	437
RP189	4, 6, 7	5	13.3 ± 5.9	30.8 ± 9.5	55.7 ± 15.7	786
RP191	4, 5, 6	7	19.4 ± 1.0	35.3 ± 2.8	45.3 ± 4.2	535
RP12	4, 5, 6		19.6 ± 0.8	38.5 ± 3.4	41.8 ± 4.3	1066
RP167	4, 6, 7		22.1 ± 3.7	44.0 ± 0.3	33.5 ± 3.3	828

<sup>a</sup> All strains contain an *amiC* deletion except for *E. coli* CS109. Strains were grown in LB medium at 37°C until the OD<sub>600</sub> = 0.6. Data was from two independent experiments, and the mean ± standard deviation is reported.

<sup>b</sup> Numbers refer to the respective PBPs deleted: 4, PBP 4; 5, PBP 5; 6, PBP 6; 7, PBP 7.

<sup>c</sup> Denotes that the wild type versions of the indicated PBPs replaced genes originally deleted from the chromosome of RP184-1K.

or variation in collection procedures. Though we standardized these parameters, the phenomenon of cell separation may be sensitive to unknown factors. In any case, the relative behavior of the mutants (increases or decreases in chaining) was consistent in any one experiment and was taken into account by using paired statistical tests.

To confirm the results obtained by gene deletion, we complemented the chaining phenotype by reinserting wild-type PBP genes into the chromosome of selected *amiC* mutants. For this purpose, multiply mutated strains were constructed by using an *amiC::res-npt-res* insertion mutant from which the kanamycin gene could be removed by site-specific recombination (4, 28, 29). The kanamycin cassette was removed from the *amiC* locus in these strains, and mutated PBP genes were replaced with their wild-type alleles by selecting for a kanamycin resistance marker inserted nearby on the chromosome.

When wild-type genes for PBPs 4, 5, or 7 were reinserted into *E. coli* RP184-1K ( $\Delta$ PBP4,5,6,7  $\Delta$ AmiC), each resulting strain exhibited a decrease in chaining (Table 2). When wild-type endopeptidases PBP 4 or PBP 7 were reinserted into RP184-1K, the number of cells in chains decreased by 27% and 32%, respectively, bringing the chain numbers to levels equivalent to those observed for mutants lacking only three PBPs (RP185-1K and RP12, respectively). Reinserting wild-type PBP 5 produced only a 15% decrease in chaining, and this number was 64% higher than the number of cells in chains for strain RP167, from which PBPs 4, 6, and 7 were deleted originally (Table 2).

Because the chaining phenotype was complemented completely or partially by any one of the three LMW PBPs, the results suggest that each protein affects cell separation in this genetic background. The effect of PBPs 4 and 7 indicates that the activities of these two endopeptidases are additive when multiple PBPs are missing from a single strain. Thus, even though deleting PBP 7 had no effect on cell separation when PBP 4 was present (Fig. 3), PBP 7 contributed to separation in the absence of PBP 4. The explanation for the incomplete complementation after reintroducing PBP 5 is not clear, but in light of the possibility that amidases exhibit different substrate preferences (see below), the mutants may have adapted their physiology to increase the rate of removal of pentapeptide side chains, which would be reduced when PBP 5 was reintroduced. In summary, of all the LMW PBPs, PBP 4 contributed most toward cell separation in *amiC* mutants, and PBP 7 played a role only if PBP 4 was inactive.

**Peptidoglycan amidases may prefer different peptide substrates.** Deleting PBP 5 by itself did not significantly increase chaining in *E. coli* RP31 ( $\Delta$ PBP5  $\Delta$ AmiC) compared to RP1 ( $\Delta$ AmiC) (Fig. 3). However, the AmiA and AmiB amidases remained active in these strains. The only known activity of PBP 5 is to remove the terminal D-alanine from pentapeptides, thereby creating tetrapeptide side chains; so in the absence of PBP 5, a surplus of pentapeptides accumulates (20, 22). It was possible that the loss of PBP 5 had no effect on cell separation because AmiA or AmiB were able to substitute for AmiC by removing pentapeptides from the glycan chains in strain RP31 (Fig. 3) as well as tetrapeptides from strain RP1 (Fig. 3). This led us to speculate that AmiC acted on a different spectrum of peptide side chains than did AmiA and AmiB.

To test the idea that the amidases process different sub-

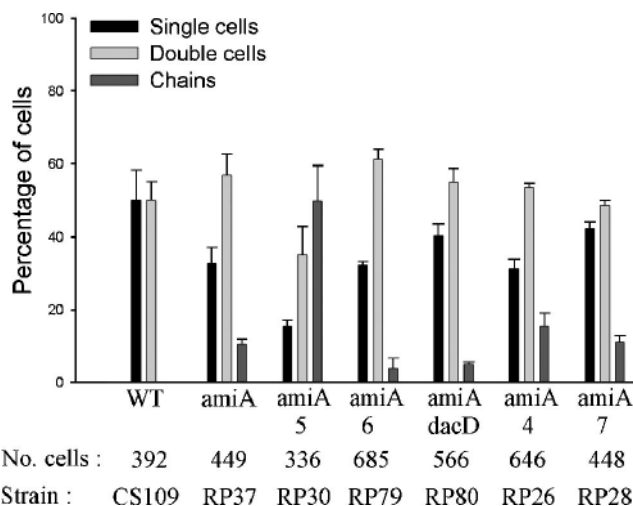


FIG. 4. Deleting PBP 5 increases chaining in *amiA* mutants. Growth conditions, graphical presentation, and abbreviations are described in the legend of Fig. 2. Data are from two independent sets of experiments.

strates, we deleted combinations of PBPs from *amiA* or *amiB* mutants. If AmiA and AmiB cleaved pentapeptide side chains almost as efficiently as tetrapeptides and if AmiC cleaved tetrapeptides more efficiently, then a mutant lacking PBP 5 and either AmiA or AmiB should exhibit increased chaining because pentapeptides would accumulate but would not be removed by AmiC. In fact, only ~10% of the population of *E. coli* RP37 ( $\Delta$ AmiA) was composed of chains, indicating that AmiC was actively removing tetrapeptides, but deletion of PBP 5 from strain RP30 ( $\Delta$ PBP5  $\Delta$ AmiA) resulted in ~50% (Fig. 4) and up to 70% (Fig. 5) of the population growing in chains, suggesting that AmiC was much less effective in removing pentapeptides than was AmiA. Deletion of no other single PBP increased chaining significantly in *amiA* mutants (Fig. 4).

To confirm that the increase in chaining was due to the activity of PBP 5, we reinserted the wild-type allele of the PBP 5 gene into the chromosome of *E. coli* RP30 ( $\Delta$ PBP5  $\Delta$ AmiA). This new strain, RP50 ( $\Delta$ AmiA) exhibited ~10% chaining as opposed to ~70% chaining for the parent strain RP30 ( $\Delta$ PBP5  $\Delta$ AmiA) (Fig. 5A). We also reinserted the wild-type PBP 4 or PBP 5 genes into the chromosome of *E. coli* RP21 ( $\Delta$ PBP4,5  $\Delta$ AmiA). Adding back PBP 5 reduced chaining to background levels in strain RP52 ( $\Delta$ PBP4  $\Delta$ AmiA), whereas adding back PBP 4 reduced chaining only slightly in strain RP53 ( $\Delta$ PBP5  $\Delta$ AmiA) (Fig. 5B), perhaps because PBP 4 exhibits a very low DD-carboxypeptidase activity. The results indicate that the chaining phenotype was controlled predominantly by the presence or absence of PBP 5.

As for AmiB, 6.5% of the cells were in chains in *E. coli* RP49 ( $\Delta$ AmiB), but deletion of PBP 5 increased this proportion to 23% in *E. coli* RP29-1K ( $\Delta$ PBP5  $\Delta$ AmiB) (data not shown). This increase in chaining was less than that observed in an *amiA* background but could be explained if AmiA cleaved pentapeptides more readily than did AmiB. In any case, the results support the hypothesis that AmiC was less effective in removing pentapeptides than were AmiA or AmiB.

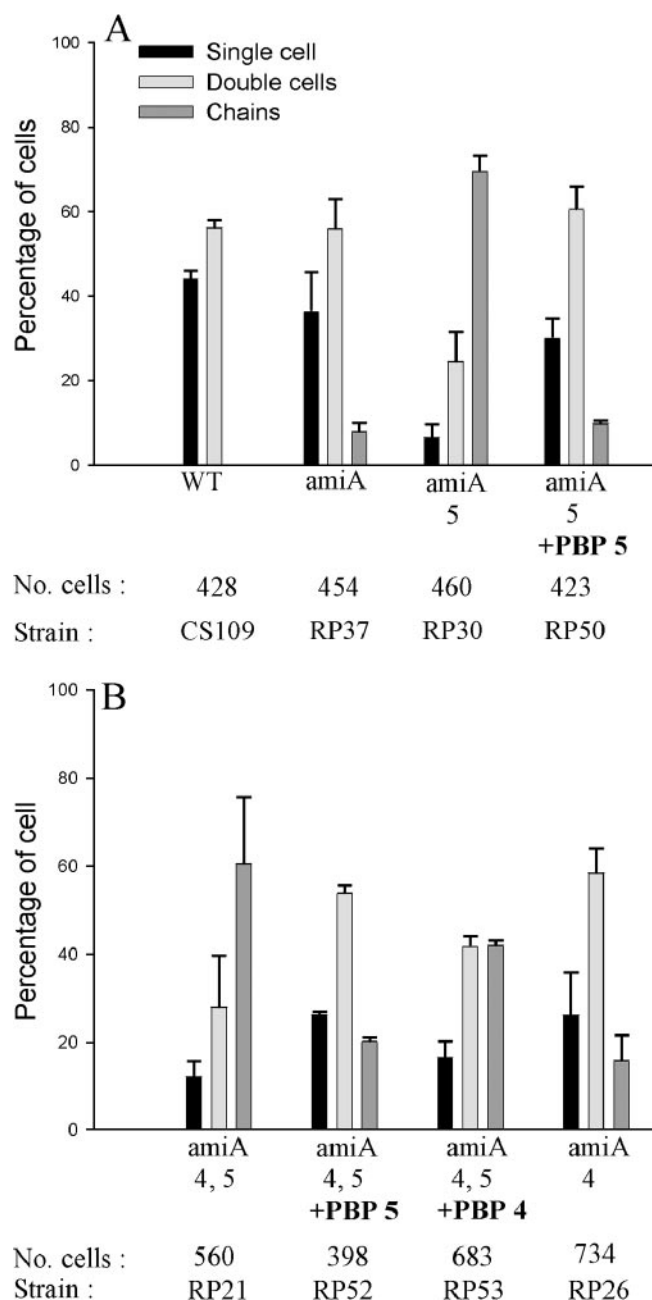


FIG. 5. PBPs complement chaining phenotype in *amiA* mutants. Growth conditions, graphical presentation, and abbreviations are described in the legend of Fig. 2. Data are from two independent sets of experiments. PBPs replaced by the wild-type allele are noted in bold. (A) Effect of replacing wild-type PBP 5 in *amiA* mutants lacking PBP 5. (B) Effect of replacing wild-type PBP 5 or PBP 4 in an *amiA* mutant lacking PBPs 4 and 5.

**Pentapeptide levels increase in *amiA* and *amiB* deletion mutants.** If the substrate preferences of the amidases differ, then overall peptidoglycan structure would be expected to vary in the presence or absence of different amidases. To test this, we compared the muropeptide composition of mutants by growing the cells to mid-log phase, isolating the sacculi from each strain, digesting with muramidase, and analyzing the

products by reverse-phase HPLC. In the wild-type strain CS109 where DD-carboxypeptidases were active, few pentapeptides were present in the cell wall (~0.4%) (Table 3). However, *E. coli* CS703-1, which lacks the major DD-carboxypeptidases, had a much higher pentapeptide content (~34%) (Table 3). The increase in pentapeptides was accompanied by a roughly equivalent decrease in the amounts of tetrapeptides and tripeptides (Table 3), because PBP 5 does not process the pentapeptide side chains.

To determine the effects of amidases on muropeptide composition, we deleted the *amiA*, *amiB*, or *amiC* genes from CS703-1. Compared to this strain, the proportions of pentapeptides in *E. coli* RP169 ( $\Delta$ AmiA) and RP64-1K ( $\Delta$ AmiB) rose from 34.3 to 39.7 (a 16.7% increase) and from 34.3 to 41.4 (a 20.6% increase), respectively, while the proportions of tetrapeptides declined by similar amounts (Table 3). Also, the proportion of pentapeptides rose from 34.3 to 37.9 (a 10.5% increase) in strain RP102 ( $\Delta$ AmiA  $\Delta$ AmiC) (Table 3). In each case, in the absence of AmiA or AmiB, the level of pentapeptides increased. These changes are consistent with the prediction that deleting either AmiA or AmiB reduces a strain's ability to remove pentapeptides from the glycan chains of peptidoglycan. Conversely, deleting AmiC produced a different effect: the proportion of pentapeptides was not altered in strain RP19 ( $\Delta$ AmiC) but instead remained equal to the levels observed in CS703-1 (Table 3). These results are consistent with the prediction that AmiC may remove pentapeptides poorly or not at all.

**Deleting PBP 5 produces twisted chains in amidase mutants.** While constructing double amidase deletion mutants, we observed that strain RP139 ( $\Delta$ PBP4,5,6,7  $\Delta$ AmiA  $\Delta$ AmiC) produced coiled chains but that strain RP75 ( $\Delta$ AmiABC) grew in long straight chains (Fig. 6). This suggested that the LMW PBPs are not only involved in cell separation but also contribute to the gross morphology of the chains. To determine which PBP contributed to the formation of this twisted phenotype, we constructed double and triple amidase mutants lacking combinations of PBPs. Twisted chains appeared only when the PBP 5 gene, *dacA*, was deleted in concert with mutations of *amiA* and *amiC*. The phenotype was never observed in strains carrying wild-type PBP 5 (Fig. 6, and data not shown). Also, twisted chains were not observed in mutants lacking a single amidase even when PBP 5 was deleted.

TABLE 3. Muropeptide profiles of amidase mutants

Strain <sup>a</sup>	Amidase(s) deleted	Percentage of muropeptide <sup>b</sup>		
		Tripeptide	Tetrapeptide	Pentapeptide
CS109		13.5 ± 0.3	86.1 ± 0.4	0.4 ± 0.1
CS703-1		5.7 ± 0.6	60.0 ± 0.5	34.3 ± 0.9
RP169	AmiA	<b>4.4 ± 0.3</b>	<b>55.9 ± 0.3</b>	<b>39.7 ± 0</b>
RP64-1K	AmiB	<b>4.6 ± 1.1</b>	<b>54.0 ± 2.2</b>	<b>41.4 ± 3.2</b>
RP19	AmiC	<b>4.7 ± 0.9</b>	60.1 ± 2.2	35.2 ± 3.1
RP102	AmiA AmiC	<b>4.1 ± 0.3</b>	58.1 ± 0.3	<b>37.9 ± 0</b>

<sup>a</sup> All strains are derived from CS703-1 (except for CS109) and carry deletions eliminating PBPs 1a, 4, 5, 6, 7, AmpC, and AmpH (4, 26). CS109 is the original parent strain.

<sup>b</sup> Muropeptide percentages were determined by HPLC and are reported as the mean ± standard deviation of two independent measurements. Bolded results are significantly different from the respective measurements for CS703-1 ( $P < 0.05$ ).



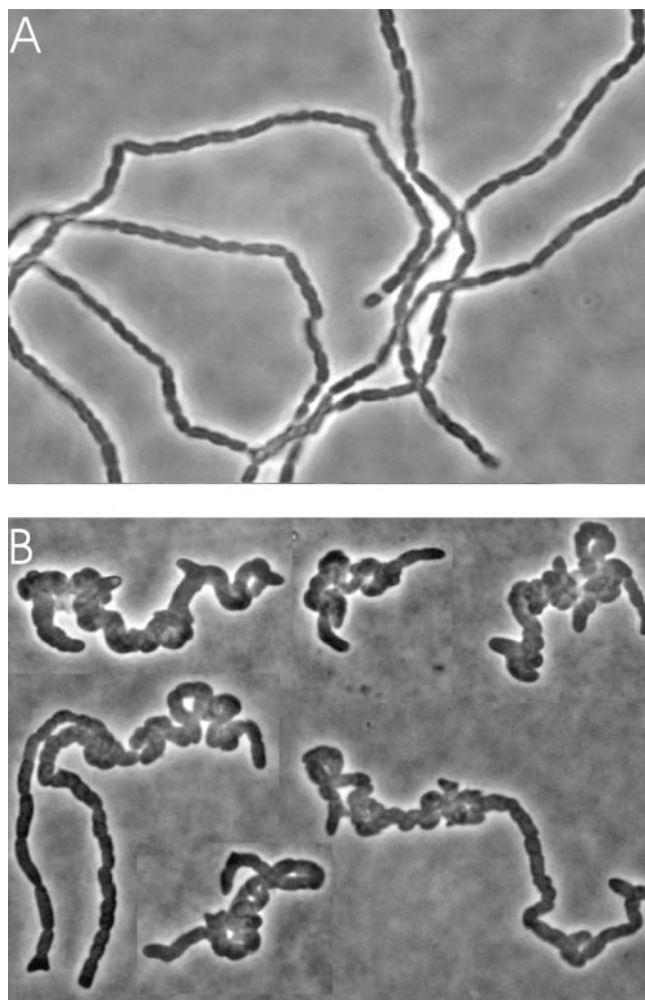


FIG. 6. Deletion of PBP 5 produces spirally coiled chains in amidase mutants. Strains were grown in LB medium at 37°C to mid-log phase. Cells were affixed to agarose-coated slides and observed by phase-contrast microscopy with a  $\times 100$  oil immersion objective. (A) RP164 (genes deleted: PBPs 4, 6 and 7; AmpC, AmiA, and AmiC). (B) RP139 (genes deleted: PBPs 4, 5, 6 and 7; AmiA and AmiB). Image B is a composite from different fields.

In *E. coli* RP108 ( $\Delta$ PBP5  $\Delta$ AmiABC) only 1% of the cell population grew in twisted chains, whereas in RP102 ( $\Delta$ PBP1a,4,5,6,7  $\Delta$ AmpC  $\Delta$ AmpH  $\Delta$ AmiA  $\Delta$ AmiC) and RP152 ( $\Delta$ PBP1b,4,5,7  $\Delta$ AmpH  $\Delta$ AmpC  $\Delta$ AmiA  $\Delta$ AmiC), which each lack multiple LMW PBPs, 20 to 25% of the chains were twisted dramatically and extensively (Fig. 7), and  $\sim 70\%$  of the chains were twisted to a lesser degree (data not shown). Furthermore, when AmiB was deleted so that all three amidases were absent, virtually 100% of the cells were present in extraordinarily long, convoluted and twisted chains (data not shown). This suggested that although the absence of PBP 5 was essential for forming the twisted coils, the absence of other LMW PBPs also contributed to the phenotype. SEM of strain RP164 ( $\Delta$ PBP4,6,7  $\Delta$ AmpC  $\Delta$ AmiA  $\Delta$ AmiC) revealed rod-shaped cells arrayed in chains, while strains RP102 and RP152 produced coiled chains (Fig. 7A and B and data not shown). These twisted chains did not have a specific pattern and were

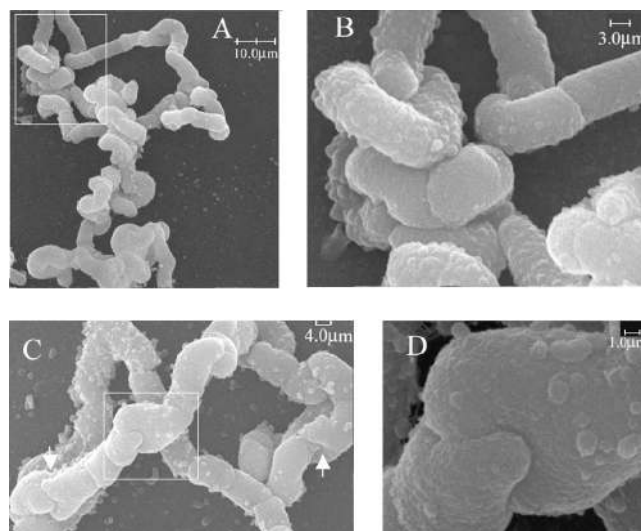


FIG. 7. SEM analysis of twisted chains. (A) RP102 (genes deleted: PBPs 1a, 4, 5, 6 and 7; AmpC, AmpH, AmiA, and AmiC). (B) Higher magnification of boxed area shown in panel A. (C) Examples of “saddle joint” cell junctures, one boxed and two others indicated by white arrows. (D) Higher magnification of saddle joint in boxed area of panel C.

coiled in random directions. Curiously, neighboring cells in some of the chains were connected to one another in a novel manner. Instead of being separated by a perpendicular septum, the cells were connected by a “saddle joint,” an interlocking pair of saddle-shaped surfaces at the poles of each cell in which finger-like projections were interdigitated (Fig. 7C and D). The implication is that a combination of amidase and PBP mutations impairs the proper shape of the septum, leading to asymmetric division planes, which upon further growth and division produce twisted chains of unseparated cells.

**Deleting *tatC* produces the twisted chain phenotype in PBP mutants.** AmiA and AmiC are transported to the periplasm via the twin arginine (Tat) pathway (1, 16). Therefore, because amidase mutants grow as chains of unseparated cells, cells defective in the Tat pathway exhibit the same phenotype (1). To confirm that the twisted chain phenotype depended on the absence of AmiA, AmiC, and PBPs, we deleted *tatC* from *E. coli* strains lacking multiple LMW PBPs. *E. coli* RP175 ( $\Delta$ PBP1a,4,5,6,7  $\Delta$ AmpC  $\Delta$ AmpH  $\Delta$ TatC) grew as twisted chains, which were not observed in a strain lacking *tatC* by itself (data not shown). Again, twisted chains only appeared when PBP 5 was deleted and most often when additional PBPs were absent (data not shown). These results are consistent with the interpretation that the phenotype depends on the absence of AmiA and AmiC activity in cells lacking PBP 5 and other LMW PBPs.

## DISCUSSION

Following bacterial division, daughter cell separation requires that the common peptidoglycan wall be cut carefully and precisely; to do otherwise risks breaking structurally essential bonds and triggering cell lysis. To accomplish this feat, the relevant hydrolytic enzymes must somehow choose and cleave



a select group of chemical bonds among what seem to be equivalent substrates. Somewhat surprisingly, this process includes the activities of several different classes of hydrolytic enzymes (10, 11), each with its own slightly different substrate preferences. The ballet-like balancing of these activities is critical, highlighted by the extreme sensitivity of dividing cells to lysis by antibiotics specific for these enzymes (5, 10, 19). The results presented here represent an extension of what we need to know about how these hydrolases interact with one another and choose their substrates.

**Endopeptidase functions.** Most gram-negative bacteria produce several LMW PBPs with well established biochemical capabilities but whose biological functions are ill-defined or completely unknown (14). Of the seven LMW PBPs in *E. coli*, only four have been associated with a physiological function. The DD-carboxypeptidase PBP 5 plays an important role in creating the uniform rod shape of each cell (30–32), and AmpC augments the resistance of *E. coli* to  $\beta$ -lactam antibiotics if its expression is increased by mutation (17, 23). PBPs 4 and 7 play unspecified auxiliary roles in maintaining regular cellular morphology (25, 31), enhance the ability of *E. coli* to form normal biofilms (7), and help separate daughter cells after cell division when the amidases or lytic transglycosylases cannot do so (10, 11). Our data support and extend this last conclusion by showing that PBP 4 facilitates cell separation in the absence of AmiC, the principle peptidoglycan amidase, with PBP 7 supplying secondary assistance.

How might the LMW PBPs contribute to cell separation? Amidases remove peptide side chains from *N*-acetylmuramic acid by cleaving the bond between the peptide and the glycan chain (Fig. 1). The reaction removes cross-links between glycan chains, which is evidently required to separate daughter cells after septation (10, 11). Because the endopeptidases PBP 4 and PBP 7 cleave a bond internal to the peptide linkage (Fig. 1), they, too, can sever previously connected glycan chains, which appears to have the same overall effect as far as cell separation is concerned. PBP 4 was more efficient in this regard because complementation by PBP 7 was observed only in PBP 4 mutants. However, the fact that PBPs 4 and 7 can effect cell separation only when AmiC is missing reinforces the idea that amidases are the principal septum-cleaving enzymes (10, 11). Thus, PBPs 4 and 7 may have a still unrecognized primary function other than their contribution to cell separation.

A potentially important inference from the current results is that PBPs 4 and 7 may act primarily at the septum. Since the activity of these two PBPs contributes to cell shape (25, 31) and since shape seems to depend on the formation and manipulation of inert peptidoglycan at cell poles (6, 33), PBPs 4 and 7 might regulate the composition or structure of septal peptidoglycan, which would be consistent with their ability to split the septum during cell separation. Also, PBP 7 cleaves intact sacculi but not isolated muropeptides (37), which would harmonize with the idea that PBP 7 requires a particular three-dimensional septal structure as its substrate. Either improper cell shape or a defect in septation may explain why the loss of PBPs 4 and 7 affects biofilm formation (7). Aside from these suppositions, what seems clear is that the data are at odds with the idea that either PBP 4 or PBP 7 is essential for insertion of new glycan chains into preexisting cell wall (12, 14). In particular, because all the endopeptidases can be deleted without

affecting cell growth or extension of the lateral wall (4, 11), the data are also inconsistent with the idea that these proteins are essential components of a multienzyme peptidoglycan synthesis complex.

**Amidase substrates and functions.** Our current results suggest that the amidases may prefer different substrates in vivo, with AmiC removing tetrapeptide chains more efficiently while AmiA and AmiB may remove both tetrapeptides and pentapeptides, with a possible preference for the latter. When Heidrich et al. assayed the activities of AmiA and AmiC, using peptidoglycan from a  $\Delta$ *amiABC*  $\Delta$ *slyY* mutant as substrate, AmiA released mostly tetrapeptides, and AmiC released roughly equal amounts of tri- and tetrapeptides (10). This is not at odds with the idea presented in this work that AmiA cleaves pentapeptides because the substrate in the experiments of Heidrich et al. was prepared from cells with functional PBP 5, meaning there was such a small amount of pentapeptide to begin with that any release would go unnoticed. Even so, their results show that the two enzymes do have different in vitro preferences: AmiA showed a preference for tetrapeptide substrates over tripeptides while AmiC acted equally on both.

Of the three amidases, AmiC is clearly the most important for separating cells after septation (10, 11), which makes sense considering the possible substrate preferences we report. Because tetrapeptides comprise the principle type of side chains in wild-type *E. coli*, AmiC would be expected to be more active during cell separation than AmiA or AmiB. These latter two enzymes would be more active on the less abundant pentapeptide side chains, with AmiA more active than AmiB in this regard. However, in the absence of AmiC, AmiA and AmiB clearly retain sufficient activity toward tetrapeptides to allow cell separation to proceed more slowly.

The results of Heidrich et al. (10) are intriguing in another way. In their in vitro assay, AmiA appeared to release ~10 times more material than did AmiC when acting on same substrate, suggesting either that AmiA is a more active amidase or that AmiC is more specific in its substrate requirements. In fact, AmiA is distributed uniformly in the periplasm, but AmiC localizes to the septum in a division-specific manner (1), and, when added to isolated murein sacculi, AmiC appears to degrade the thick septal rings more effectively than sidewall peptidoglycan (10). Thus, it is quite possible that AmiC recognizes and degrades muropeptides located mostly at the septum. This would account for the smaller total amount of muropeptides released by AmiC in vitro because the septum is a minor fraction of the sacculi. It would also account for our in vivo results, where the absence of AmiA produces an increase in wall-associated pentapeptides as opposed to little change in the absence of AmiC. In this scenario, AmiC would prefer to cut tri- or tetrapeptides at the septum, whereas AmiA would cleave pentapeptides located anywhere around the sacculus.

**Relationships among PBPs and peptidoglycan hydrolases.** Other than the preeminence of AmiC, we cannot assign specific roles or percentages of effort to other amidases or PBPs during septum cleavage. The possibility that the amidases have different substrate preferences suggests that LMW PBPs other than the endopeptidases might also affect cell separation by modifying the muropeptide substrates available to these hydrolytic enzymes. For example, deleting PBP 5 alters the ratio of pentapeptides to tetrapeptides in the sacculus, and mutating

multiple combinations of LMW PBPs results in the accumulation of different numbers of peptide side chains (22). If the amidases have different substrate preferences, the changes probably alter the relative roles of these enzymes during cell growth and separation.

One overt sign of this relationship is that deleting PBP 5 in concert with *amiA* and *amiC* altered the morphology of chains of unseparated cells. Deleting the amidases by themselves caused *E. coli* to grow as straight chains, but amidase mutants lacking PBP 5 grew as grossly twisted chains. The most likely explanation for the phenotype is that the increase in pentapeptide side chains affects septum formation or processing, resulting in abnormally shaped poles (6, 31, 32). The simplest explanation for the twisted chains is that daughter cells remain attached to one another at these asymmetric poles, which may themselves be the result of an imbalance of amidase actions during septum formation or cleavage. In effect, deleting the amidases to cause chaining creates a more sensitive visual assay for the cell shape changes caused by loss of the LMW PBPs.

As for the hydrolases themselves, there is a difference in the compounds left behind by the actions of PBPs 4 and 7 and those left by amidases. Cleavage by an endopeptidase leaves two peptide side chains attached to the glycan chains which are therefore available for future cross-linking reactions during the insertion of new peptidoglycan. Conversely, amidases remove the peptides entirely, leaving no side chains at all, meaning that these particular glycan subunits cannot participate as recipients in new cross-linking reactions. Thus, the location and extent of amidase activity may dictate the future course of peptidoglycan insertion in specific areas of the sacculus. This might have a bearing on the chemical nature of "inert peptidoglycan," which is a functional description without a structural explanation.

**Peptidoglycan structure.** Because of the large differences in chaining phenotype caused by deleting various hydrolases, it seems the cells should exhibit significant cell wall differences. Unexpectedly, the muropeptide compositions of hydrolase mutants are surprisingly constant (11; data not shown). In particular, the profiles do not change significantly among mutants of differing chain length, and where the profiles do change, they do so to approximately the same degree in mutants that chain (no amidases) as in those that do not chain (no endopeptidases) (11). Thus, muropeptide profiles do not correlate with the chaining phenotype, implying that cell separation defects are related to differences in peptidoglycan structure rather than to overall composition. The nature of these structural variations is unknown, and no current techniques address this important issue.

**Mysteries.** Although we are accumulating more information about what enzymes are active during septum formation and cell separation, many fundamental questions remain unanswered. How does the cell split septal peptidoglycan so that the daughter cells separate while not cutting the wall so that the cell lyses? How do the hydrolases distinguish which bonds to cut and which to leave? Why do some enzymes act only at the septum and not elsewhere, thus sparing the side wall? What is the chemical nature of the peptidoglycan that remains after cell separation? Do these areas have no peptide side chains on their outer surfaces, or is there a surplus of dangling cross-

linked peptides? Why are amidases the principle splitters rather than endopeptidases? Why are multiple hydrolases required for efficient separation, and why does a lytic transglycosylase contribute to separation at all? Why does a surfeit of peptidoglycans create abnormal septation and poles? It seems clear that answering these questions will describe fundamental relationships between the PBPs and hydrolases, as well as clarifying their roles in regulating bacterial growth and cell morphology.

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