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Effects of Multiple Deletions of Murein Hydrolases on Viability, Septum Cleavage, and Sensitivity to Large Toxic Molecules in *Escherichia coli*

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The multiplicity of murein hydrolases found in most bacteria presents an obstacle to demonstrating the necessity of these potentially autolytic enzymes. Therefore, *Escherichia coli* mutants with deletions in multiple murein hydrolases, including lytic transglycosylases, amidases, and DD-endopeptidases, were constructed. Even a mutant from which seven different hydrolases were deleted was viable and grew at a normal rate. However, penicillin-induced lysis was retarded. Most of the mutants were affected in septum cleavage, which resulted in the formation of chains of cells. All three enzymes were shown to be capable of splitting the septum. Failure to cleave the septum resulted in an increase in outer membrane permeability, and thus the murein hydrolase mutants did not grow on MacConkey agar plates. In addition, the hydrolase mutants not only could be lysed by lysozyme in the absence of EDTA but also were sensitive to high-molecular-weight antibiotics, such as vancomycin and bacitracin, which are normally ineffective against *E. coli*.

Because of the presence of a covalently closed bag-shaped murein (peptidoglycan) sacculus, it is generally believed that murein hydrolases are essential pacemaker enzymes for growth (10, 18, 20). Indeed, enzymes capable of cleaving bonds in the murein sacculus are ubiquitous among bacteria (22). In *Escherichia coli* 18 different murein hydrolases belonging to six different families have been described so far (9). Most (13 of 18) of these murein hydrolases can act as fatal autolysins. This fact is considered additional evidence that murein hydrolases have an essential, growth-supporting function in bacteria.

Unfortunately, until now no conclusive experimental proof has been presented that demonstrates the necessity of the potentially autolytic murein hydrolases. In fact, a triple mutant lacking three lytic transglycosylases (MltA, MltB, and Slt70) does not exhibit a significantly different phenotype (14). This finding may be due to the presence of such a large number of different murein hydrolases, which apparently can compensate for the loss of individual murein hydrolases. Therefore, we began to construct mutants with multiple deletions in murein hydrolases with the aim of obtaining mutants that express no or only marginal residual murein hydrolytic activity. Although we failed to obtain final proof that murein hydrolases have an essential function in general growth, we found that amidases, lytic transglycosylases, and even endopeptidases are involved in cell separation following cell division. Interestingly, the impairment of the cleavage of the murein septum observed for most of the mutants dramatically affected the integrity of the cell envelope.

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MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. Deletion mutants were constructed from *E. coli* MC1061 (1) and CS203 (16). The general cloning vector was pBluescript II SK+ (Stratagene, La Jolla, Calif.). Either the Kohara miniset λ library (17) was used for transfer of the plasmid-borne gene deletions into the chromosome or vector pMS7, a *sacB*-containing modification of pMAK700 (7), was employed for generation of chromosomal gene knockouts (13). The kanamycin resistance determinant was taken from pUC4K (Pharmacia, Uppsala, Sweden), the chloramphenicol resistance marker was obtained from pBCSK+ (Stratagene), and the tetracycline resistance gene was obtained from pBR322 (New England Biolabs, Beverly, Mass.).

Growth conditions. Bacteria were cultivated aerobically at 37°C in Luria-Bertani (LB) medium (15). Agar plates contained 1.5% agar (Gibco BRL, Paisley, Scotland), and MacConkey medium was prepared as described by Miller (15). Growth was monitored by determining optical density at 578 nm (OD_{578}) with an Eppendorf photometer (Eppendorf, Hamburg, Germany). Antibiotics were used at the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 20 μg/ml; and tetracycline, 12.5 μg/ml.

DNA manipulation and PCR. Standard techniques were used to manipulate DNA. *E. coli* was transformed by using the modified calcium chloride procedure (21). Restriction endonucleases were purchased from Roche Diagnostics (Mannheim, Germany), and oligonucleotides were obtained from MWG-Biotech (Ebersberg, Germany).

PCR was performed with an MJ Research PTC-200 (Biozym, Oldendorf, Germany) by using 0.5 U of Powerscript polymerase (PAN Systems GmbH, Nürnberg, Germany) per 25 μl to create products with deleted open reading frames or by using 0.7 U of *Taq* polymerase (MBI Fermentas, Vilnius, Lithuania) per 25 μl to screen for successful chromosomal gene deletions. Each primer was added to a final concentration of 0.5 μM. After initial denaturation for 3 min at 92°C, touchdown PCR (3) was performed at 72°C with 1 min of annealing, 1 to 3 min of extension (depending on the distance of the primer binding sites), and 0.5 min of denaturation at 92°C. The annealing temperature was initially 52°C and then was decreased in 12 cycles by 0.5°C each cycle. The final annealing temperature was 46°C, which was used for another 12 cycles.

The following primers were used to create plasmid-encoded gene deletions: for deletion of Slt70, pSlt1/*Pst*I (5′-ATGAACCTGCAGGAGGAGTAGTTACCTTCCC-3′), pSlt2/*Sma*I (5′-GATCAGTAACGACGTCCCGGGAACTTGTTTGGCTTTTTCCATAA-3′), pSlt3/*Sma*I (5′-AAAAGCCAAACAAGTTCCCGGGACGTCGTTACTGATCCGCAC-3′), pSlt4/*Xba*I (5′-AGTCATCTAGATCTTCGGCGAAGGATCCTG-3′), and pSlt5(5′-TAGAATTCTAGACTGGCCCTGAATACGTCG-3′); for deletion of EmtA (MltE), pEmtA1/*Sal*I (5′-CTTATGTCGACTCCAGGTTGTAACCTGCGTC-3′), pEmtA3/*Sma*I (5′-ATGACTATACGAACCCGGGCTTCTCGCTCGAGAGAG-3′), pEmtA2/*Sma*I (5′-ATG

TABLE 1. Morphology of selected murein hydrolase mutants

Mutant	Deletions	Resistance marker(s)	% of cells in chains	No. of cells per chain
MHD52	*amiA*, *amiB*, *amiC*	Cm, Kan	90–100	6–24
MHD33	*mepA*, *dacB* (PBP4), *pbpG* (PBP7)	Kan		
MHD75	*mltC*, *mltD*, *mltE*		10–30	3–6
MHD79	*sltY*, *mltA*, *mltB*, *mltC*, *mltD*, *emtA*	Cm, Kan, Tet	30–50	3–8
MHD62	*amiA*, *amiB*, *amiC*, *mepA*, *dacB* (PBP4), *pbpG* (PBP7)	Cm, Kan	90–100	6–80
MHD64	*sltY*, *amiA*, *amiB*, *amiC*, *mepA*, *dacB* (PBP4), *pbpG* (PBP7)	Cm, Kan	100	20–100
MHD90	*amiB*, *amiC*, *mltC*, *mltD*, *emtA*	Kan	30–50	3–16
MHD91	*amiA*, *amiB*, *amiC*, *mltC*, *mltD*, *emtA*	Cm, Kan	90–100	6–24

ACTATACGAACCCGGGCTTCTCGCTCGAGAGAG-3′), pEmtA4/*Xba*I (5′-ACTAGATCTAGAAATGCTTACGTCAACGCTC-3′), and pEmtA5 (5′-TTG ACTAGATCTCCGCCTTATGAAAATCTTATTC-3′); for deletion of MltC, pMltC1/*Sal*I (5′-ATCATTGTCGACCGCGTTTCGGCATACCTT-3′), pMltC2/*Sma*I (5′-GGATAACATTTTTGCCCCGGGCACCATGTTAATGGTGACG C-3′), pMltC3/*Sma*I (5′-CCATTAACATGGTGCCCGGGCAAAAATGTTAT CCACATC-3′), pMltC4/*Xba*I (5′-TAATCATCTAGAAGATAAAGAAGATT GCCATACG-3′), and pMltC5 (5′-TAGATATCTAGAACGGGATGGTCAG AATGAAC-3′); for deletion of MltD, pMltD1/*Sal*I (5′-ATCATTGTCGACAA TTACATCTGGGTTTTGAA-3′), pMltD2/*Sma*I (5′-GGGAATCGGTGCCCG GGCGAGTAATATCGCTTTTGC-3′), pMltD3/*Sma*I (5′-GCGATATTACTC GCCCGGGCACCGATTCCCCCGGTG-3′), pMltD4/*Xba*I (5′-TAATCATCT AGAAGCTTCTGTACTGGTTACGC-3′), and pMltD5 (5′-TAATCATCTAG ATAGCTCACCACAGCGGCCC-3′); and for deletion of MepA, pMepA1/*Eco*RI (5′-CACCTGAATTCTCTCCGGTGTTTTTG-3′), pMepA2/*Sma*I (5′-G TAAAAAATGAATAAAACCCGGGAACAACAAAGCCTGAG-3′), pMepA3/*Sma*I (5′-GGCTTTGTTGTTCCCGGGTTTTATTCATTTTTTACCAGCG-3′), pMepA4 (5′-GTCATGAATCCCCTTCCAGATTGTCGC-3′), and pMepA5 (5′-GGTGAATTCATCAACAATAAATTTGCCGGAC-3′) (underlining indicates restriction sites).

Primers whose designations include the numbers 1 and 2 were used in pairs and resulted in PCR products upstream of the selected open reading frame, and primers whose designations include the numbers 3 and 4 were used in pairs and revealed PCR products downstream. The probes were mixed to obtain the final PCR product in a third PCR performed with the primers whose designations include the numbers 1 and 4. The DNA fragment that was finally obtained was ligated with pBluescript II SK+ and used to transform *E. coli* MC1061. The restriction sites introduced by primers whose designations include the numbers 2 and 3 were used for insertion of the resistance marker.

The strategy used for deletion of AmiA, AmiB, and AmiC has been described by Heidrich et al. (8), the strategy used for deletion of MltA and MltB has been described by Lommatzsch et al. (14), and the strategy used for deletion of PBP4 and PBP7 has been described by Nelson and Young (16).

Construction of deletion mutants. Based on a modification of the gene exchange method of Kulakauskas et al. (8, 13), deletions of the coding regions of the murein hydrolase genes were created. The principal procedure used was to generate a PCR product with a deletion of the gene of interest by using the primers mentioned above. For ligation to pBluescript II SK+ or directly to the modified vector pMS7 the introduced restriction sites were used (see above). A pBluescript II SK+ construct was necessary to introduce a selection marker at the position of the missing gene.

Formation of the recombinant pMS7 cointegrates, achieved by growth in liquid culture at 28°C for at least 6 h, was followed by plating on LB medium plates containing ampicillin and incubation at 42°C. Successful cointegrates were screened by PCR performed with primer T7 (New England Biolabs) and primer pSlt5, pEmtA5, pMltC5, pMltD5, or pMepA5. Selected clones with cointegrates were incubated at 28°C in liquid culture for at least 6 h and at 37°C on LB medium plates supplemented with 4% sucrose. Sucrose was harmful for the pMS7-carrying strains because of the gene locus *sacB* and thus selected for replacement of the constructed gene deletion with the chromosomal wild-type copy. Control for successful gene replacement was done by PCR performed with primers whose designations include the numbers 1 and 5 (see above). An existing chromosomal deletion was transferred into different mutant strains with bacteriophage P1 as described by Miller (15).

Multiple deletions were obtained either by P1 transduction (15) of an existing chromosomal gene deletion to a dedicated host if a selection marker was introduced into the site of gene deletion or by stepwise deletion of different genes by using recombinant vector pMS7 repeatedly.

Analysis of muropeptide composition. Murein sacculi were isolated as described previously (6). After digestion with α-amylase and pronase, the murein was hydrolyzed with cellosyl (kindly provided by Aventis [formerly Hoechst-Marion-Roussel AG], Frankfurt, Germany). The resulting muropeptides were reduced with sodium borohydride and fractionated by reverse-phase high-performance liquid chromatography as described by Glauner et al. (6).

Electron microscopy techniques. Bacterial cultures were harvested at an OD_{578} of about 1.0. Plasmolysis of cells was performed by incubation in an equal volume of 60% sucrose in LB medium for 10 min at room temperature. Bacteria were resuspended and fixed in 2.5% glutaraldehyde in phosphate-buffered saline The samples were then postfixed with 1% osmium tetroxide for 1 h on ice, and after they were rinsed with double-distilled water, they were treated with 1% aqueous uranyl acetate for 1 h at 4°C. Samples were dehydrated with a graded ethanol series and embedded in Epon. Ultrathin sections were viewed with a Philips CM10 electron microscope.

For scanning electron microscopy the fixed cells were dehydrated in ethanol and critical point dried from CO_2. The samples were sputter coated with 8-nm Au/Pd and examined at an accelerating voltage of 20 kV with a Hitachi S-800 field emission scanning electron microscope.

Sensitivity to different antibiotics. To study sensitivity to different antibiotics, the bacteria were grown in LB medium at 37°C to an OD_{578} of 0.1 to 0.2 and incubated with 1 mg of crystal violet per liter, 1 g of deoxycholate per liter, 0.1% Triton X-100, 20 μg of vancomycin per ml, 30 μg of lysozyme per ml, 20 μg of aztreonam per ml, 100 μg of mersacidin (kindly provided by H.-G. Sahl, Institut für Medizinische Mikrobiologie und Immunologie, Bonn, Germany) per ml, 25 μg of gallidermin (kindly provided by F. Götz, Institut für Genetik, Tübingen, Germany) per ml, 25 μg of ramoplanin per ml, or 5 μg of ampicillin per ml.

RESULTS

Phenotypes of mutants with multiple murein hydrolase deletions. Since constructing mutants with all possible combinations of deletions for all known murein hydrolases would have been a real Sisyphean task, we decided to construct mutants with deletions of whole families of potentially autolytic murein hydrolases first. We succeeded in obtaining mutants with deletions in all six lytic transglycosylases (MHD79), in all three periplasmic amidases (MHD52), and in all three endopeptidases (MHD33) (Table 1). We also combined various deletions with different enzyme specificities. One mutant, MHD64, lacked seven different murein hydrolases. Nevertheless, analysis of the growth rates and growth yields of all 82 mutants that were created did not reveal any major changes, except that mutants MHD62 [Δ(AmiA AmiB AmiC MepA PBP4 PBP7)], and MHD64 (MHD62 ΔSlt70) showed a dramatic decrease in CFU after incubation for more than 3 days in liquid culture and storage at 4°C for more than 2 weeks on agar plates (data not shown).

However, one phenotype was dominant in most of the mutants: cleavage of the murein septum, which is essential for separation of the daughter cells during cell division, was often severely impaired. This resulted in formation of chains of cells

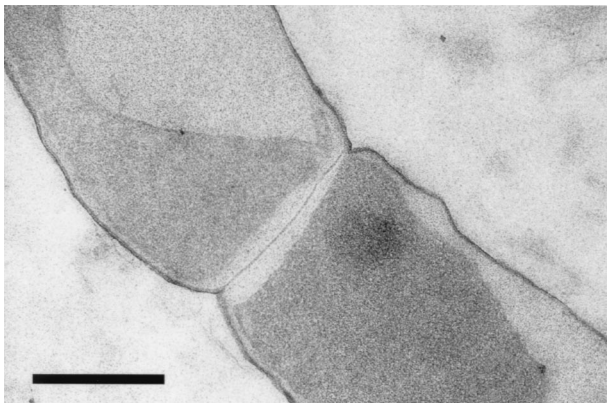

FIG. 1. Electron micrograph of a thin section of plasmolyzed triple amidase mutant MHD52. Plasmolysis was accomplished by incubating the cells in the presence of 30% sucrose in LB medium for 10 min at room temperature. Bar = 0.5 μm.

stuck together by uncleaved murein septa (Fig. 1 and Table 1). Whereas deletion of the lytic transglycosylases Slt70, MltA, and MltB did not affect the morphology, as shown previously (14), mutant MHD75, with *mltC*, *mltD*, and *mltE* deleted, grew with up to 30% of the cells in short chains containing three to six cells. Deletion of all known lytic transglycosylases (MHD79) resulted in growth of up to 50% of the cells in chains containing three to eight cells. Remarkably, the cells were slightly coccoid and were 0.7 to 1.3 μm long, compared to 2 μm for wild-type cells. This morphological alteration may indicate that cell elongation was impaired in this mutant. As described previously, predominantly *N*-acetylmuramyl-L-alanine amidases seem to be involved in splitting the septum (8). Deletion of a single amidase, AmiA, results in the formation of short chains (with three or four cells per chain), and the length of the chains increases as more members of the amidase family are deleted. By contrast, deletion of all three endopeptidases (MHD33) did not cause chain formation (Table 1). However, when this deletion was combined with deletions in lytic transglycosylases and/or amidases, the average number of cells in the chains increased further significantly. MHD62, which in addition to the amidases lacks the endopeptidases MepA, PBP4, and PBP7, forms chains containing 6 to 80 cells. Mutant MHD64, in which all three amidases, all three endopeptidases, and the major soluble lytic transglycosylase Slt70 were deleted, formed the longest chains, which consisted of 20 to 100 cells. We concluded that all three enzyme specificities (lytic transglycosylases, endopeptidases, and amidases) are capable of splitting the murein septum, although amidases seem to play a predominant role (8).

Murein structure. The structures of the mureins of the different mutants were changed, but surprisingly the changes were only minor (Table 2). In particular, the finding that the mutant lacking all known lytic transglycosylases (MHD79) exhibited no significant decrease in the amount of 1,6-anhydromuropeptides, which are the products of lytic transglycosylases, was unexpected. This may indicate that not only lytic transglycosylases but also other enzyme specificities can result in the formation of 1,6-anhydromuramyl chain ends. The possibility that these ends are artifacts generated during sample preparation is highly unlikely because of the careful control experiments that were done when the method used was developed (6). Indeed, it has been proposed that such ends might be formed during a specific termination step of the glycan strand polymerization reaction (12). The most dramatic changes were due to deletion of the endopeptidases in mutant MHD33. In accordance with the function of these enzymes, the overall cross-linkage was increased by about 20%. The level of trimers was increased by about 34%, and the level of tetramers was increased by roughly 170%. The changes that occur in amidase mutants, such as MHD52, have been described previously (8).

Loss of integrity of the outer membrane. To our surprise, all chain-forming mutants were unable to grow on MacConkey agar plates. Addition of the different ingredients that make up MacConkey medium individually to normal LB plates revealed that this phenomenon was due to the presence of deoxycholate, whereas crystal violet did not affect the viability of the mutants (Fig. 2A). Thus, it seems that somehow the barrier function of the outer membrane in the mutants is disturbed. To further characterize the change in permeability, several toxic compounds that normally do not harm wild-type *E. coli* cells were tested to determine their effects on the chain-forming mutants. The chain formers were sensitive not only to detergents such as 0.1% (wt/vol) Triton X-100 (data not shown) but also to high-molecular-weight antibiotics, including vancomycin (Fig. 2B), mersacidin, gallidermin, and even ramoplanin, which has a molecular mass of 2,550. An important finding is that the increase in permeability is directly correlated with the formation of unclipped septa. Specific inhibition of septum formation by the PBP3-specific monobactam aztreonam eliminated vancomycin and deoxycholate sensitivity (Fig. 2B).

Even the sensitivity of the chain formers to lysozyme (14,000 Da) was dramatically increased, as demonstrated by the occurrence of lysozyme-induced lysis. Normally, the presence of EDTA is essential for permeation of lysozyme across the outer membrane. A quite peculiar finding was the formation of barbell-like ends on the chains treated with lysozyme (Fig. 3a), especially during treatment of mutant MHD64 with lysozyme. Thin sections of these structures indicated that they were formed by fusion of several spheroplasts that were enclosed by a huge outer membrane envelope (Fig. 3b). The electron micrographs suggest that the action of lysozyme may begin at the ends of the chains and then proceed toward the middle.

The integrity of the murein of the murein hydrolase mutants

TABLE 2. Muropeptide compositions of murein hydrolase mutants

Muropeptides	Relative amt (%) in *E. coli* wild type[a]	% Changes in mutants[b]			
		MHD33	MHD52	MHD62	MHD79
Monomeric	55.4	−14.4	−8.6	−6.1	4.0
Dimeric	37.8	16.0	6.1	13.7	−5.0
Trimeric	4.3	34.5	53.5	14.8	−16.0
Tetrameric	0.2	171.0	155.0	54.6	−8.0
Anhydro	3.1	20.0	93.5	16.9	−10.0
Cross-linkage	22.5	19.5	12.9	9.7	−6.0
Average chain length (disaccharide units)[a]	27.5	−16.3	−30.5	−11.0	8.0

[a] The relative amounts of the muropeptides and the degrees of cross-linkage were calculated as described by Glauner et al. (6).

[b] Percent increase or decrease compared with the fraction in the wild type. For example, the value for monomeric muropeptides in strain MHD33 was determined as follows: 55.4 × (100 − 14.4)/100 = 47.4%.

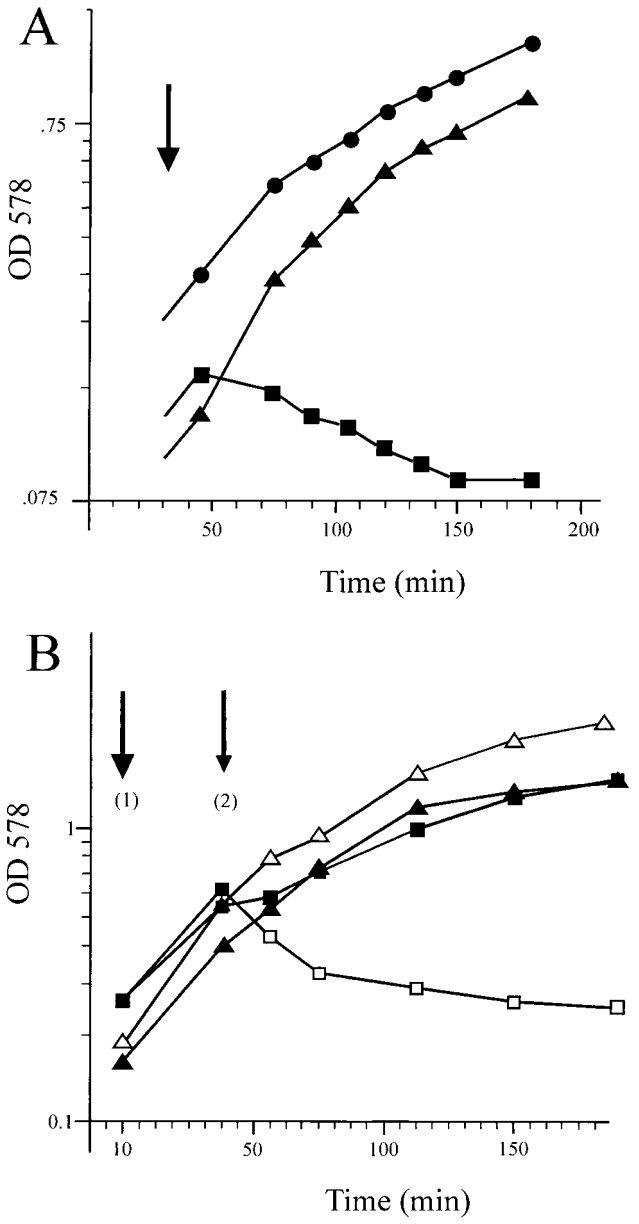

FIG. 2. Effects of deoxycholate and crystal violet on *E. coli* MHD52 (Δ*amiABC*). *E. coli* MHD52 and wild-type *E. coli* MC1061 were grown aerobically at 37°C in LB medium. (A) At the time indicated by the arrow deoxycholate (1 g/liter) or crystal violet (1 mg/liter) was added. Symbols: ●, MC1061 plus deoxycholate; ▲, MHD52 plus crystal violet; ■, MHD52 plus deoxycholate. (B) At the times indicated by the arrows vancomycin (20 μg/ml) (arrow 1) and/or aztreonam (20 μg/ml) (arrow 2) were added. Symbols: △, MC1061 plus vancomycin; □, MHD52 plus vancomycin; ▲, MC1061 plus aztreonam; ■, MHD52 plus vancomycin and aztreonam.

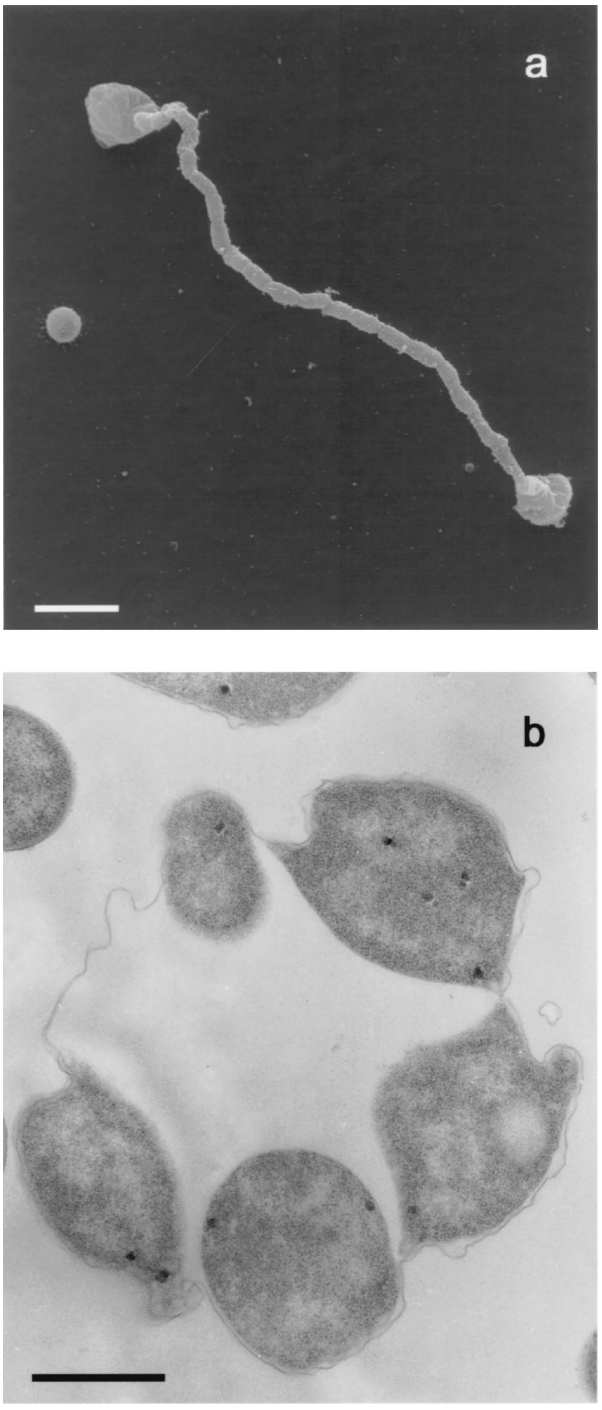

FIG. 3. Morphological changes of *E. coli* MHD64 treated with lysozyme. Lysozyme (10 μg/ml) was added to a growing culture (37°C) of *E. coli* MHD64 (*sltY amiABC mepA dacB pbpG*) in LB medium at an OD_{578} of about 0.7. Samples ware taken 20 min later and prepared for electron microscopy as described in Materials and Methods. (a) Scanning electron microscopy. Bar = 5 μm. (b) Ultrathin section of a ball-like structure like those shown in panel a. Bar = 1 μm.

seems not to be affected because osmotic shock experiments did not reveal differences between wild-type cells and chain formers. In addition, no significant change in the overall pattern of lipopolysaccharides, in lipoprotein content, or in outer membrane proteins was observed (data not shown).

Formation of aberrant membrane structures. Impairment of septum cleavage was accompanied in 1 to 5% of the population by the formation of wavy septa consisting of only cytoplasmic membrane (Fig. 4b). This phenotype was observed most clearly with mutant MHD64, which lacks seven different murein hydrolases. Thus, septation of the cytoplasm can take place without the formation of a murein septum. In addition to the

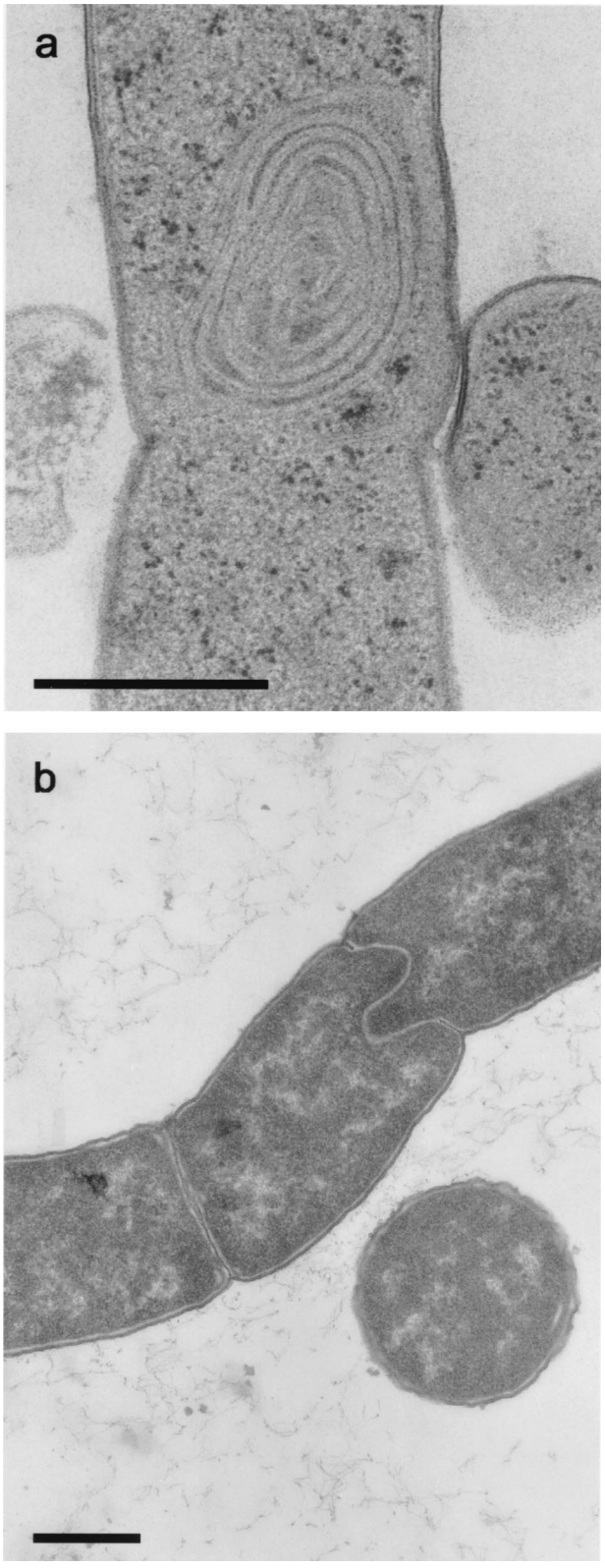

FIG. 4. Electron micrographs of thin sections of mutant MHD64 [Δ(*sltY mltA mltB amiA amiB amiC mepA dacB pbpG*)]. Bars = 0.5 μm.

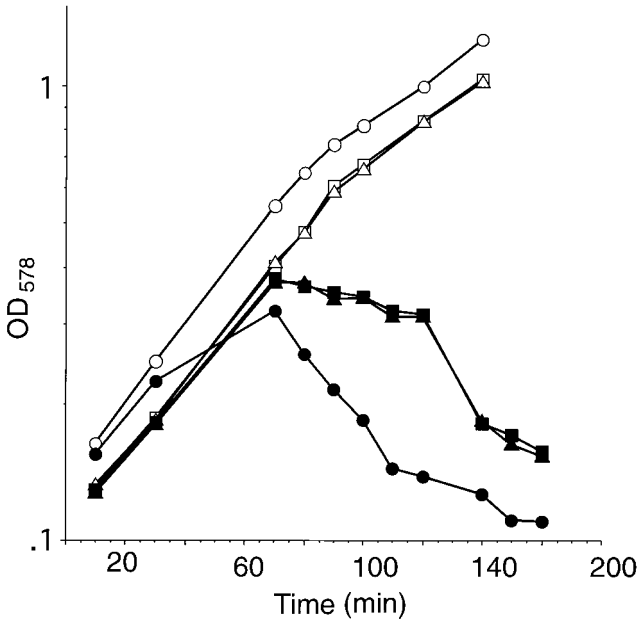

FIG. 5. Ampicillin-induced lysis of mutants with multiple murein hydrolase deletions. *E. coli* MC1061 (wild type), MHD90 [Δ(*mltC mltD mltE amiB amiC*)], and MHD91 [Δ(*mltC mltD mltE amiA amiB amiC*)] were grown aerobically at 37°C in LB medium. After 90 min some cultures (solid symbols) received 5 μg of ampicillin per ml. Circles, MC1061; squares, MHD90; triangles, MHD91.

presence of murein-free membrane septa, extramembrane material was occasionally deposited in spiral packages (Fig. 4a). However, even in plasmolyzed cells formation of blebs of membrane material at cell division sites, such as those that have been described to occur in mutants lacking lipoprotein (23), was not observed (Fig. 1).

Delayed lysis response to β-lactams. Penicillin tolerance, which is defined as growth inhibition in the absence of bacteriolysis, has been observed in bacteria with reduced endogenous murein hydrolase activity (24). The obvious reduction in murein hydrolytic activity in the multiple-deletion mutants was therefore expected to cause a tolerant response when the cells were treated with cell wall synthesis inhibitors. Although there was not a complete absence of lysis, the mutants survived significantly longer in the presence of the antibiotics tested than the corresponding control cells survived (Fig. 5). Whereas the wild-type strain started to lyse about 30 to 40 min after the addition of ampicillin, significant lysis in mutants MHD90 [Δ(*amiA amiB amiC mltC mltD*)] and MHD91 [Δ(*amiA amiB amiC mltC mltD emtA*)] was not seen before 90 min after addition of the antibiotic following a period of growth arrest. Thus, there was a significant effect on the lag time between addition of the inhibitor and onset of bacteriolysis. However, the mutants did not exhibit complete tolerance. Also, the lysis response to endogenously produced plasmid-encoded lysis protein E of bacteriophage ϕX174 (25) was not affected in mutants MHD52, MHD31, and MHD79 (data not shown).

DISCUSSION

Although we did not create a mutant lacking all known murein hydrolases, it is quite surprising that even a mutant

with deletions in seven murein hydrolases of different specificities was still able to grow without any obvious change in its phenotype. The only growth event that absolutely depends on the action of murein hydrolases is the splitting of the murein septum that allows cell separation. Interestingly, cleavage of the murein septum can be performed by enzymes with different specificities. The phenotypes of the different deletion mutants indicate that amidases play the major role in this process, followed by lytic transglycosylases. Endopeptidases seem to play a minor role since as long as amidases and/or lytic transglycosylases were present, deletion of endopeptidases did not increase the length of the chains. However, in the absence of amidases and lytic transglycosylases, deletion of the endopeptidases significantly increased the length of the chains that were formed.

One may ask whether hydrolases are also essential for general growth (that is, enlargement of the cells), which in the case of rod-shaped bacteria is characterized by elongation of the cells. As discussed elsewhere (11), one could speculate that opening of meshes, which is an absolute requirement for insertion of new murein precursors in order to widen the murein net, can be accomplished by enzymes that do not behave as hydrolases but behave as transferases. Such enzymes catalyze a two-step reaction; first, a bond in a donor substrate is cleaved, and concomitantly a covalent enzyme-substrate intermediate is formed. In the second step the donor substrate moiety is covalently bound to the acceptor substrate and the enzyme is released. One example is the DD-transpeptidation reaction that is catalyzed by the high-molecular-weight molecules PBP 1a, PBP 1b, PBP 2, and PBP 3 of *E. coli* (5). Therefore, we speculate that insertion of murein precursors into the preexisting murein sacculus may be catalyzed by transferases that couple the opening of meshes (that is, cross bridges) in the sacculus with the covalent attachment of new subunits to the murein mesh. If such enzymes do not cleave cross bridges in the absence of acceptor molecules, they do not turn into fatal autolysins. By contrast, splitting of the septum that does not occur along with growth of the sacculus could be catalyzed by simple hydrolytic enzymes. Such murein hydrolases are, however, prone to act in an uncontrolled fashion as autolytic enzymes. As a matter of fact, antibiotic-induced bacteriolysis predominantly takes place at the site of cell division (2).

It is shown in this paper that accumulation of unclipped murein septa in the chain formers affects the barrier function of the outer membrane, allowing penetration of large molecules, such as vancomycin and lysozyme. This raises the question, why should inhibition of septum cleavage interfere with the integrity of the outer membrane? In context with the observation that in these mutants septa made solely of cytoplasmic membrane are formed (that is, there is no murein cross wall) and the observation that extramembrane material accumulates in form of spirals, one may argue that the increase in the membrane surface is greater than the increase in the murein surface when cleavage of the septum is blocked. It seems that under such conditions both cytoplasmic material and outer membrane material continue to be formed at normal rate despite the fact that the increase in the murein surface is greatly diminished because no new polar caps are formed. Polar caps have been calculated to represent almost two-thirds of the total murein surface (26). Although the rate of murein synthesis is strictly coupled to the rate of phospholipid synthesis, as shown by the stringent responses of both phospholipid synthesis and murein synthesis (4, 19), a control mechanism that couples surface increase in the murein sacculus to membrane synthesis does not seem to exist.

The increase in outer membrane permeability as a result of inhibition of the splitting of the septum, which is most dramatically shown by vancomycin sensitivity, indicates that inhibitors of the septum-cleaving hydrolases are promising candidates for molecules that render gram-negative bacteria sensitive to antibiotics that normally cannot pass through the outer membrane. In particular, amidase inhibitors might be useful for improving chemotherapeutic strategies for gram-negative infections.

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