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Susceptibility to Antibiotics and β-Lactamase Induction in Murein Hydrolase Mutants of *Escherichia coli*

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The antibiotic susceptibilities and capabilities to induce β-lactamases were studied in multiple *Escherichia coli* murein (peptidoglycan) hydrolase mutants. *E. coli* mutants lacking either three amidases, three amidases and one lytic transglycosylase, or six lytic transglycosylases showed higher levels of susceptibility to bacitracin, erythromycin, gallidermin, and vancomycin than the wild type. Mutant cells without three amidases lost viability in the presence of vancomycin and gallidermin, whereas the wild type was resistant to both antibiotics. β-Lactamase induction was studied after introduction of a plasmid carrying the *ampC* and *ampR* genes. Upon addition of cefoxitin to the growth medium, the wild type as well as a mutant lacking all known amidases and DD-endopeptidases induced β-lactamase, whereas a mutant lacking all known lytic transglycosylases was unable to induce β-lactamase, showing that lytic transglycosylase activity is essential for β-lactamase induction. Consequently, cells lacking lytic transglycosylase activity lysed in the presence of penicillin, despite the presence of the inducible β-lactamase system. We discuss the potential of murein hydrolase inhibitors for antibiotic therapy.

Murein (peptidoglycan) represents the stress-bearing structure (sacculus) that most bacteria require to withstand the internal turgor pressure and that surrounds the cytoplasmic membrane of most bacteria (11, 19). Murein hydrolases are ubiquitous among bacteria (22, 23). Autolysins are murein hydrolases capable of digesting the murein of the bacteria that produce them. Several functions have been associated with autolysins: they act as a pacemaker for cell wall growth (10, 11), they split the septum during cell division to separate the daughter cells (2, 7, 24, 25), they assist with murein turnover (5, 21), and they cleave murein for the assembly of large periplasm-spanning structures like the flagellum (9). Because they are capable of degrading the essential murein sacculus, the activities of the autolysins must be strictly controlled. Apparently, the mechanisms used to control autolysin activities fail upon the inhibition of murein synthesis, e.g., by penicillin, resulting in antibiotic-induced lysis (10, 27).

Escherichia coli has a large number ($n = 12$) of different autolysins that fall into three classes: three amidases (AmiA, AmiB, and AmiC) that cleave the amide bond between MurNAc and L-Ala; three DD-endopeptidases (PBP 4, PBP 7, and MepA) that hydrolyze the peptide cross-links; and six lytic transglycosylases (Slt70, MltA, MltB, MltC, MltD, and EmtA), a special class of muramidases that degrade the GlcNAc-MurNAc glycan strands resulting in the formation of a 1,6-anhydro-MurNAc residue at the released product (13). This high degree of redundancy makes it difficult, if not impossible, to define a distinct role for each of the enzymes. The construction and characterization of a set of multiple hydrolase mutants lacking up to seven different hydrolases have shed some light on their different functions (7, 8). In particular, the amidases and the lytic transglycosylases were found to be involved in the cleavage of the septum during cell division, because mutants lacking several amidases and/or lytic transglycosylases grow in chains of nonseparated cells (7, 8). One peculiar phenotype of these chaining murein hydrolase mutants is the higher degree of permeability of their outer membrane that results in their higher degrees of susceptibility to toxic compounds with high molecular weights, such as deoxycholate, vancomycin, and ramoplanin (8). The reason for the higher degrees of outer membrane permeability of chaining cells is unknown.

During normal growth of *E. coli* and other species, there is a massive release of murein fragments from the sacculus by the action of autolysins (5, 6), a process named murein turnover. The turnover products generated in the periplasm are recycled with a high degree of efficiency after transport to the cytoplasm and further processing (3, 4, 20). Upon inhibition of murein synthesis with β-lactams, the level of turnover products increases due to the uncontrolled actions of autolysins. Interestingly, in many gram-negative species the 1,6-anhydro-MurNAc-containing turnover products that are released from the sacculus by lytic transglycosylases serve as cytoplasmic signaling molecules for the induction of β-lactamase, an enzyme that is capable of destroying the β-lactam antibiotic (12, 14, 15, 18). Consequently, it was shown that *E. coli* mutants lacking one to three different lytic transglycosylases (16), as well as mutants lacking the AmpG transporter, which is mainly responsible for the uptake of the turnover products into the cytoplasm (17), showed reduced levels of β-lactamase induction.

In the work described here, we have further characterized the antibiotic susceptibilities and β-lactamase induction of multiple murein hydrolase mutants. The antibiotics chosen fall into different chemical classes and have different cellular tar-

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gets, but they have in common the fact that they are not active against or are only poorly active against gram-negative bacteria due to their high molecular weights: bacitracin is a cyclic peptide that prevents dephosphorylation of undecaprenyl pyrophosphate, erythromycin is a macrolide that targets protein biosynthesis, gallidermin is a membrane-active lantibiotic, and vancomycin is a glycopeptide that targets murein synthesis reactions. We show here that chaining murein hydrolase mutants are more susceptible to bacitracin, erythromycin, gallidermin, and vancomycin than wild-type cells. Furthermore, we describe the killing effects of vancomycin and gallidermin against a chaining hydrolase mutant but their lack of an effect on the viabilities of wild-type cells. In addition, we have investigated β-lactamase induction in various mutants in which the inducible β-lactamase system was introduced. Cells lacking the activities of all known lytic transglycosylases were unable to induce β-lactamase, whereas cells lacking all known amidases and DD-endopeptidases were able to do so. The inability to induce β-lactamase rendered cells without lytic transglycosylase activity susceptible to penicillin.

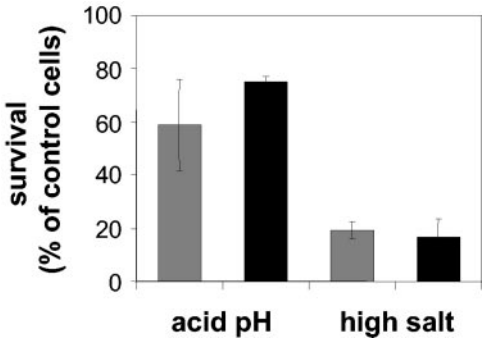

FIG. 1. Susceptibilities of murein hydrolase mutants to acid pH and to high salt concentrations. Cells of wild-type strain MC1061 (gray bars) or MHD52 (black bars) were exposed to pH 4.9 and 2.5 M NaCl. The rate of survival was compared to that of untreated control cells. The percent survival was determined in three independent experiments. Mutant and wild-type cells did not differ in their susceptibilities to low-pH or high-salt stress.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains used in this study were wild-type strain MC1061 (1) and the following murein hydrolase mutants (7, 8): MHD8 (MC1061 Δ*amiA*::Cm), MHD52 (MC1061 Δ*amiA*::Cm Δ*amiB* Δ*amiC*::Kan), MHD61 (CS203b Δ*amiA*::Cm Δ*amiB* Δ*mepA* Δ*dacB* Δ*pbpG*), MHD62 (CS203b Δ*amiA*::Cm Δ*amiB* Δ*amiC*::Kan Δ*mepA* Δ*dacB* Δ*pbpG*), MHD63 (MC1061 Δ*amiA*::Cm Δ*amiB* Δ*amiC*::Kan Δ*slt*), MHD79 (MC1061 Δ*mltA*::Cm Δ*mltB*::Tet Δ*mltC* Δ*mltD* Δ*mltE*, Δ*slt*::Kan), and MHD82 (MC1061 Δ*mltA*::Cm Δ*mltB*::Tet Δ*mltC* Δ*mltD* Δ*mltE*). Plasmid pJP1, which carries the *ampC* and *ampR* genes and a kanamycin resistance marker, was described before (16). Precultures of the cells were grown in Luria broth (LB) medium containing the appropriate antibiotics (tetracycline, 12.5 μg/ml; chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; ampicillin, 50 μg/ml) at 37°C in a shaking water bath. Bulgecin (40 μg/ml) was added to the medium in several experiments to inhibit the soluble lytic transglycosylase Slt70 (26).

Acid survival and high-osmolarity assays. Acid survival and high-osmolarity assays were performed essentially as described before (28). For the acid survival assay, the bacteria were grown in LB medium and received acetic acid to adjust the pH to 4.9, followed by incubation for 20 min at 37°C. For challenge with high osmolarity, the bacteria received 2.5 M NaCl and were incubated for 2 h at room temperature. The treated cultures as well as the nontreated control cultures were plated on LB agar, and the number of viable cells was counted after incubation for 18 h at 37°C. The percentage of surviving cells was determined as the mean value of three independent experiments.

Antibiotic susceptibility assay. Overnight precultures of different strains were diluted 1:50 and plated on LB agar without antibiotics. Sterile paper disks (diameter, 0.6 cm) received an antibiotic (bacitracin, gallidermin, erythromycin, or vancomycin) and were placed on the surfaces of the plates. After incubation at 37°C for 24 h, the diameters of the growth inhibition zones were measured. All experiments were performed in triplicate.

Killing assay with vancomycin or gallidermin. Bacteria were grown in LB medium (without antibiotics) at 37°C in a shaking flask. The culture was divided when the absorbance (578 nm) reached 0.2 to 0.3. One of the two parts received antibiotic, and one part served as a control. The growth was monitored by measuring the absorbance at 578 nm. The number of viable bacteria was determined in parallel. For this, samples were diluted and plated on LB agar. The numbers of colonies were counted after incubation for 18 h at 37°C.

β-Lactamase induction. For β-lactamase induction we followed a published procedure (16), with the following modifications. Cells (5 ml) from the exponential growth phase were diluted with 5 ml of medium containing 8 μg of cefoxitin per ml and were further incubated at 37°C for 30 min. Control samples received medium without cefoxitin. The cells were spun down, washed once with 50 mM sodium phosphate buffer (pH 7.0), resuspended in 1 ml of the same buffer, and stored at −20°C. To prepare cell lysates, 20 μl of resuspended cells was treated with 20 μl of lysis buffer (400 mM Tris-HCl, 8 mM EDTA, 200 μg of lysozyme per ml [pH 8.0]) and 40 μl of water, followed by a 5-min incubation at room temperature. The protein concentration of the lysate was determined by the Lowry method (Bio-Rad).

β-Lactamase enzyme assay. For quantification of β-lactamase activity, an enzyme assay with the chromogenic substrate nitrocefin was performed exactly as described before (16).

RESULTS

Different murein hydrolase mutants whose chaining phenotypes have been characterized (7, 8) were used in this study. Strain MHD52 (which lacks three amidases) forms chains of 6 to 24 cells and was tested for its susceptibility to low pH, high salt concentrations, and various antibiotics. MHD52 was also used in killing assays with vancomycin and gallidermin. The antibiotic susceptibilities of strain MHD52 and wild-type strain MC1061 were compared to those of strain MHD63, which has an additional deletion of a lytic transglycosylase (Slt70) and which produces longer chains of 8 to 40 cells; mutant MHD8, which lacks a single amidase and which shows only a weak chaining phenotype (20 to 30% of cells in chains of 3 to 6 cells); and strain MHD79, which lacks all six known lytic transglycoslyases and which forms chains of 3 to 8 cells.

Susceptibility of MHD52 to low-pH and high-salt stress. We have determined the susceptibilities of chain-forming MHD52 cells (which lack three amidases) to low-pH or high-salt stress. As shown in Fig. 1, there was no significant difference in the survival rates of MHD52 cells that were subjected to these challenges and cells of wild-type strain MC1061.

Susceptibilities of murein hydrolase mutants to different antibiotics. Wild-type strain MC1061 was not inhibited by bacitracin but had small and larger growth inhibition zones when it was incubated with gallidermin and erythromycin, respectively (Fig. 2). The MHD8 mutant, which lacked one amidase (AmiC), showed growth inhibition zones similar to those of wild-type strain MC1061; but multiple murein hydrolase mutants, such as MHD52 (which lacks three amidases), MHD63 (which lacks three amidases and one lytic transglycosylase), and MHD79 (which lacks six lytic transglycosylases), showed larger growth inhibition zones than strain MC1061 when they were incubated in the presence of bacitracin, erythromycin, and gallidermin. In some instances, we have observed a second

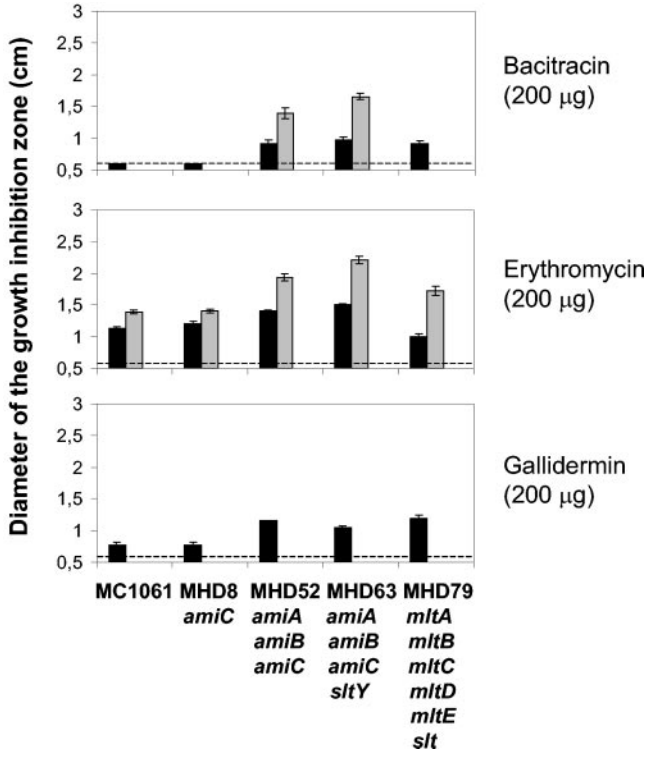

FIG. 2. Susceptibilities of murein hydrolase mutants to different antibiotics. Cultures of wild-type strain MC1061 or the different murein hydrolase mutants were tested in a diffusion assay (disk diameter, 0.6 cm; dashed line) with 200 μg of bacitracin, erythromycin, or gallidermin. Black bars, diameters of clear zones; gray bars, diameters of zones with reduced growth. The average values of three independent experiments are given. Mutants lacking multiple murein hydrolases were more susceptible to bacitracin, erythromycin, and gallidermin.

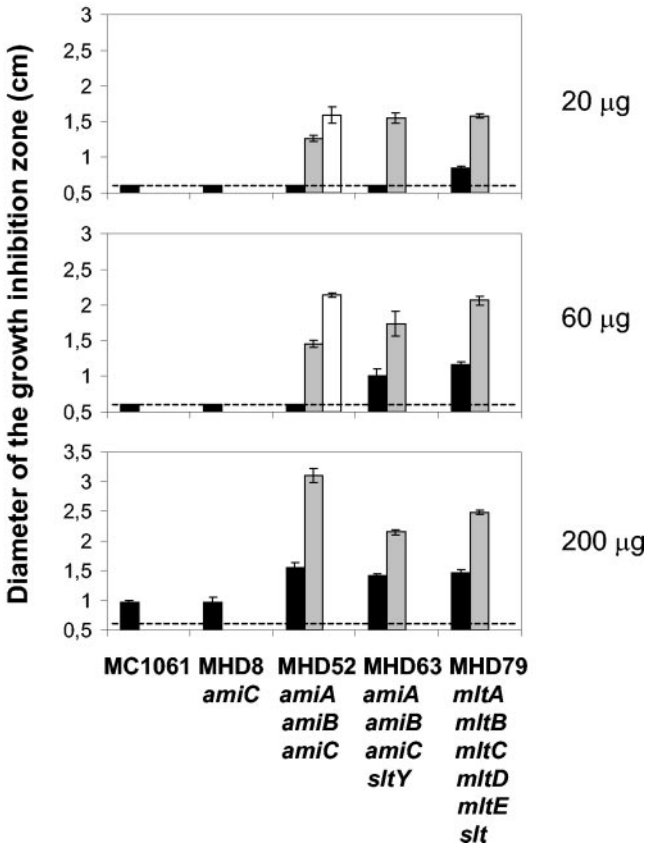

FIG. 3. Susceptibilities of murein hydrolase mutants to vancomycin. Cultures of wild-type strain MC1061 or the different murein hydrolase mutants were tested in a diffusion assay (disk diameter, 0.6 cm; dashed line) with 20, 60, or 200 μg of vancomycin. Black bars, diameters of clear zones; gray and white bars, diameters of two zones of reduced growth. The average values of three independent experiments are given. Mutants lacking multiple murein hydrolases were more susceptible to vancomycin.

circular zone around the disk with fewer colonies (gray bars in Fig. 2) than in the sites of confluent growth far from the disk.

The growth inhibition of the mutants was also determined with 20, 60, and 200 μg of vancomycin per disk. The multiple hydrolase mutants MHD52, MHD63, and MHD79 were more susceptible to vancomycin than MC1061 and MHD8 (Fig. 3). Of the strains tested, strain MHD79, which lacks all known lytic transglycosylases, was the most susceptible to vancomycin.

Killing of MHD52 by vancomycin and gallidermin. Mutant MHD52 was chosen to determine whether vancomycin and gallidermin exhibit bactericidal effects. For this study, cells of mutant MHD52 and its parental wild-type strain, strain MC1061, were grown in liquid culture to an absorbance (578 nm) of 0.2 to 0.3. The cultures were divided and antibiotic was added to one part. As shown in Fig. 4, the growth of wild-type strain MC1061 was not affected by the addition of 50 μg of vancomycin per ml. In contrast, the same concentration of vancomycin resulted in a moderate decrease in absorbance and an approximately 100-fold decrease in viable cell counts (numbers of CFU per milliliter) within 300 min for MHD52. Similarly, the growth of MC1061 was not affected by the addition of 50 μg of gallidermin per ml (Fig. 5). With 10 μg of gallidermin per ml, the increase in the absorbance (578 nm) of mutant MHD52 was lower. Upon addition of 50 μg of gallidermin per ml, the absorbance (578 nm) of MHD52 decreased and the cells rapidly lost viability, as indicated by the reduction in viable counts by a factor of about 1,000 within 100 min. Thus, both antibiotics, vancomycin and gallidermin, had killing effects against murein hydrolase mutant MHD52.

β-Lactamase induction in murein hydrolase mutants. The capacity to induce β-lactamase was tested in mutant strains carrying plasmid pJP1. The aim was to compare the results for a strain without lytic transglycosylase activity to those for a strain without all amidases and DD-endopeptidases (MHD61). Mutant strains carrying the same kanamycin resistance marker as pJP1, e.g., MHD79, which lacks all known lytic transglycosylases, could not be transformed with this plasmid. Nevertheless, kanamycin-susceptible mutant MHD82 (which lacks five lytic transglycosylases) grown in the presence of an inhibitor of Slt70, bulgecin, is devoid of the activities of all six lytic transglycosylases. Cefoxitin was used at a concentration of 4 μg/ml to induce β-lactamase expression. The addition of cefoxitin led to a 6.7-fold increase in β-lactamase activity in wild-type strain MC1061 and a 3.1-fold increase in mutant MHD61 (Fig. 6). The lower level of induction for MHD61 was mainly due to an already twofold increased basal level of β-lactamase produc-

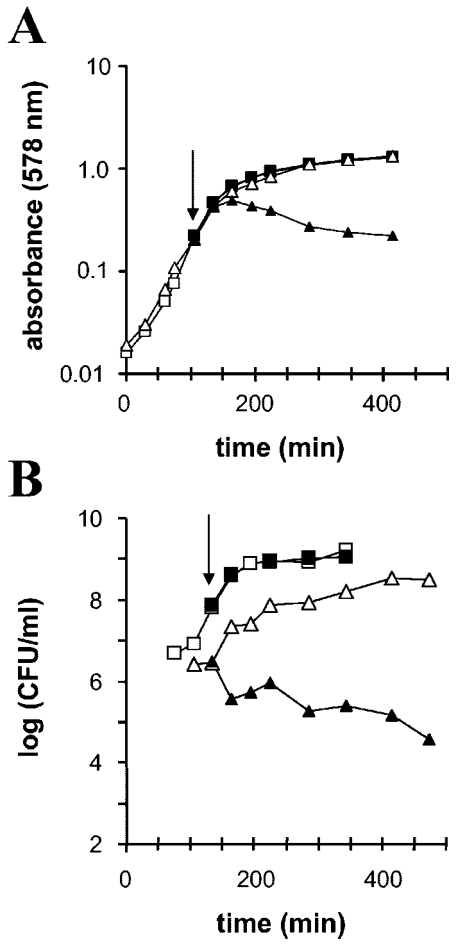

FIG. 4. Killing of MHD52 by vancomycin. Cells of MC1061 (squares) or MHD52 (triangles) were grown at 37°C in LB medium. When the cells reached an absorbance (578 nm) of about 0.2 to 0.3 (arrows), the cultures were divided and one part received 50 μg of vancomycin per ml (closed symbols). Control cultures did not receive antibiotic (open symbols). The absorbance (578 nm) of the cultures was monitored (A), and the viable counts were determined after serial dilution and plating on LB agar (B). Whereas wild-type strain MC1061 was resistant to vancomycin, mutant MHD52, which lacked three amidases, was killed by vancomycin.

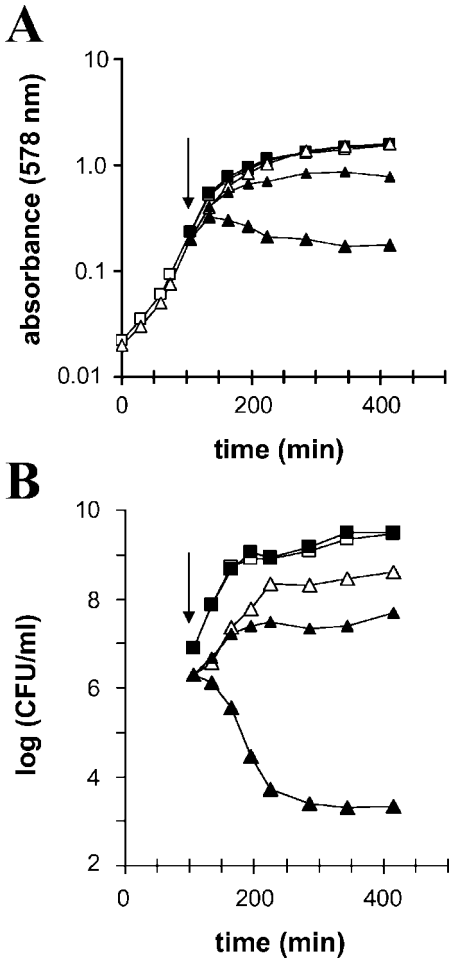

FIG. 5. Killing of MHD52 by gallidermin. Cells of MC1061 (squares) or MHD52 (triangles) were grown at 37°C in LB medium. When the absorbance (578 nm) reached about 0.2 to 0.3 (arrows), the cultures were divided and one part received 10 μg of gallidermin per ml (small closed symbols) and a second part received 50 μg of gallidermin per ml (large closed symbols); control cultures did not receive antibiotic (open symbols). The absorbance (578 nm) of the cultures was monitored (A), and the viable counts were determined after serial dilution and plating on LB agar (B). Wild-type strain MC1061 was resistant to gallidermin, but mutant MHD52, which lacked three amidases, was killed by gallidermin.

tion without cefoxitin, whereas the β-lactamase levels in MC1061 and MHD61 were similar by induction with cefoxitin. MHD82 cells grown in the presence of bulgecin had a low basal level of β-lactamase activity and cefoxitin failed to induce further β-lactamase activity.

Susceptibilities of murein hydrolase mutants to penicillin. Wild-type strain MC1061, as well as strains MHD62 (which lacks three amidases and three DD-endopeptidases) and MHD79 (which lacks six lytic transglycosylases), lysed upon the addition of 100 μg of penicillin G per ml (Fig. 7). Penicillin-induced lysis was slightly delayed in MHD62 and occurred at a slightly slower rate in MHD79 than in the wild type. The presence of plasmid pJP1, which encodes the inducible β-lactamase, prevented lysis upon addition of 20 μg of penicillin G per ml in MC1061 and MHD61 but not in MHD82 (grown in the presence of bulgecin). Thus, the activities of the lytic transglycosylases are required to induce β-lactamase and to prevent lysis by β-lactams.

DISCUSSION

The reason for the higher degrees of susceptibility of chaining murein hydrolase mutants of *E. coli* to different antibiotics is not known. It is likely that the permeability of the outer membrane is increased, and that could be caused, for example, by a loss of integrity of certain outer membrane proteins. Alternatively, more indirect effects could cause higher degrees of susceptibility to antibiotics, for example, reduced drug efflux pump activity. There is no obvious difference in the appearance of the outer membranes of thin sections of MHD52 cells by electron microscopy (W. Vollmer, unpublished results), and we did not observe a significant difference in the protein com-

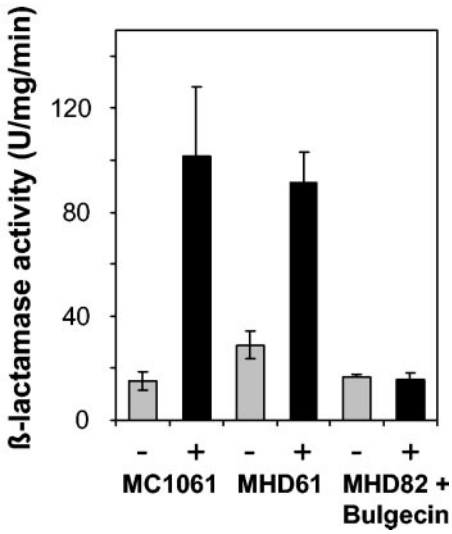

FIG. 6. β-Lactamase induction in murein hydrolase mutants. Cleared lysates of cells of wild-type strain MC1061 and mutants MHD61 and MHD82 (grown with bulgecin) that were treated with cefoxitin (+) or left untreated (−) were assayed for their β-lactamase activities by using the chromogenic substrate nitrocefin. β-lactamase was induced in MC1061 and MHD61 but not in MHD82, which was grown with bulgecin.

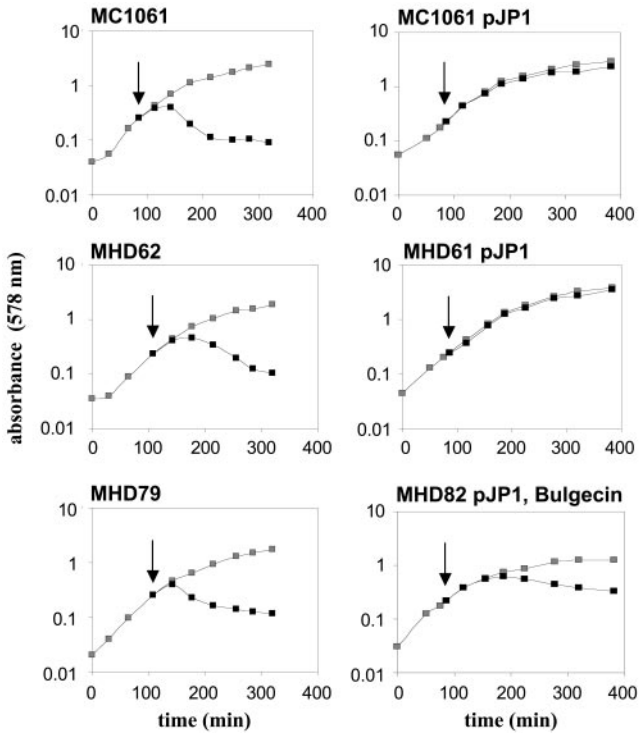

FIG. 7. Penicillin-induced lysis in different murein hydrolase mutants. The different strains (indicated above the growth curves) were grown in LB medium at 37°C. When the cultures reached an absorbance (578 nm) of 0.2 to 0.3 (arrows), they were divided into two parts. One part served as a nontreated control (gray symbols). The second part received 100 μg (MC1061, MHD62, and MHD79) or 20 μg (strains with pJP1) of penicillin G per ml. Lysis was observed in MHD62 (which lacked three amidases and three DD-endopeptidases) and MHD79 (which lacked six lytic transglycosylases), although lysis was slower in the latter mutant. The inducible β-lactamase expressed from pJP1 enabled MC1061 and MHD61 (which lacked two amidases and three DD-endopeptidases) to grow upon penicillin G treatment, whereas cells of MHD82(pJP1) (which lacked five membrane-bound lytic transglycosylases and in which Slt70 was inhibited with bulgecin) lysed because the β-lactamase gene was not induced.

positions of the outer membranes of MHD52 and the wild type (Vollmer, unpublished). This is in line with the findings that multiple hydrolase mutants grow at similar rates as the wild type (8) and that the triple amidase mutant MHD52 survived high-salt and low-pH stress similarly to the wild type (this work). Thus, the lack of several hydrolases and, consequently, the chaining phenotype do not seem to reduce the overall fitness of the mutants under laboratory conditions. We found that different chain-forming multiple murein hydrolase mutants show increased susceptibilities to antibiotics like bacitracin, erythromycin, gallidermin, and vancomycin, which differ in their modes of action and which normally act exclusively against gram-positive bacteria (Fig. 2 and 3). An important finding is that vancomycin, which inhibits murein synthesis, as well as gallidermin, a membrane-active lantibiotic, showed bactericidal effects against the chain-forming triple amidase mutant.

In *E. coli*, a strong chaining phenotype is associated only with multiple deletions and not with single deletions in hydrolase genes, possibly because the enzymes can replace each other in cell separation function. We found that only multiple hydrolase mutants had reduced susceptibilities to the antibiotics tested. For example, triple amidase mutant MHD52 (which lacks *amiA*, *amiB*, and *amiC*) and MHD79 (which lacks six lytic transglycosylases) have stronger chaining phenotypes and are more susceptible to all antibiotics tested than single amidase mutant MHD8 (which lacks *amiC*) (Fig. 2 and 3). The percentage of cells present in chains and the number of cells per chain varies among the different mutants (7, 8). Also, exponentially growing mutant cells generally have a greater tendency to chain than stationary-phase cells. MHD52 cells lost viability 100-fold with vancomycin and 1,000-fold with gallidermin. It is likely that the culture contained some short chains of cells (with lower outer membrane permeabilities) that might have escaped killing by the antibiotic.

In several gram-negative bacterial species, a β-lactamase (AmpC) is induced in response to challenge with β-lactam antibiotics. The induction of β-lactamase is regulated by murein turnover products that contain a 1,6-anhydro-MurNAc residue; these are the products of lytic transglycosylases. We introduced the *ampC-ampG* system on a plasmid into *E. coli* mutants lacking several murein hydrolases. Wild-type cells and a mutant without two amidases and three DD-endopeptidases (MHD61) induced β-lactamase in the presence of cefoxitin to similar levels. A strain that lacked the activities of all known lytic transglycosylases (MHD82 grown with bulgecin) completely lost the capability to induce β-lactamase, presumably because of its inability to produce 1,6-anhydro-MurNAc-containing inductor molecules. Whereas all classes of autolysins (amidases, DD-endopeptidases, and lytic transglycosylases) are involved in penicillin-induced autolysis (Fig. 7, panels on the left), only the lytic transglycosylases are essential for the induction of β-lactamase. As a consequence, a strain without

lytic transglycosylase activity, strain MHD82(pJP1), which was incubated with bulgecin, could not induce β-lactamase even in the presence of the inducible β-lactamase system and is lysed in the presence of penicillin due to the actions of amidases and DD-endopeptidases (Fig. 7).

Murein hydrolases were not regarded as possible targets for antimicrobial therapy for several reasons. First, multiple hydrolases are present in most bacteria, although the total number is not usually as high as the numbers present in *E. coli*. Second, murein hydrolases are not essential for bacterial growth. However, the same arguments could be made for the murein synthases, the penicillin-binding proteins (PBPs), which are present in multiple forms in every bacterial species and which are, with a few exceptions, not essential. Nevertheless, as murein synthesis inhibitors, the β-lactams are among the most successful antibiotics because they do not inactivate a single enzyme but, rather, inactivate the whole set of PBPs. Our results indicate that general inhibitors of classes of murein hydrolases might be of value for combined antibiotic therapy for infections caused by gram-negative bacteria. First, inhibitors of amidases that cause chaining could be used to sensitize gram-negative bacteria to a number of antibiotics that are otherwise restricted to use as treatments for infections caused by gram-positive organisms. Second, the inhibition of lytic transglycosylases might be a way to prevent the induction of β-lactamases in many gram-negative bacteria, making them susceptible to β-lactam antibiotics.

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