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Effect of Disruption of a Gene Encoding an Autolysin of *Enterococcus faecalis* OG1RF

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A mutant (TX5127) of *Enterococcus faecalis* OG1RF was generated by disruption mutagenesis of a previously described autolysin gene. TX5127 formed longer chains (2 to 10 cells per chain) than wild-type OG1RF (mainly single cells) during growth in broth even though it had a growth rate similar to that of the parental strain as measured by turbidity and cell count. Autolysin activity, as defined by the ability to lyse heat-killed *Micrococcus lysodeikticus* cells, was absent in TX5127, while this activity was easily detectable in OG1RF. However, disruption of this autolysin gene did not block the ability of TX5127 to hydrolyze *E. faecalis* cell walls compared to that of OG1RF. The autolysis rate of cells of TX5127 in 10 mM sodium phosphate buffer (pH 6.8) was slower than that of wild-type OG1RF. TX5127 also showed a decreased rate of lysis in the presence of penicillin, as measured by changes in the turbidity of the culture during 24 h of incubation at 37°C and a slightly decreased effect of penicillin as measured by time-kill curves. The virulence of TX5127 was similar to that of OG1RF in the mouse peritonitis model, indicating that the autolysin of *E. faecalis* is not important for infection in this model.

Enterococci are among the more common causes of hospital-acquired infections and, among all enterococcal infections, *Enterococcus faecalis* is the most commonly recovered species (22). In recent years, the treatment of enterococcal infections has become more and more difficult because of the increasing antibiotic resistance of these organisms. One of the problems with enterococci is their relative resistance to penicillin and other β-lactams. Low-level resistance to β-lactams is intrinsic and appears to be due to the low affinity of enterococcal penicillin-binding proteins to penicillin (14, 34). In addition to this resistance, enterococci are often tolerant to β-lactams; that is, β-lactams have low bactericidal effects. The mechanism of tolerance of enterococci to β-lactams is still unclear. However, it has been demonstrated that resistance to penicillin and tolerance to penicillin are two distinguishable features of *E. faecalis*, because they could be elicited independently by in vitro exposure to penicillin (14). The tolerance of *E. faecalis* to β-lactams has been suggested as being associated with the autolysis system (29). Storch et al. (29) have shown that an increase in autolytic activity in clinical isolates was correlated with increases in penicillin-induced lysis and killing. In addition, Fontana et al. reported that *E. faecalis* strains which lacked or had diminished autolysin activity were less susceptible to the bactericidal activity of penicillin (13).

Autolysins of enterococci have been characterized primarily from *Enterococcus hirae* ATCC 9790. Two forms of autolysins have been reported in *E. hirae*, namely, muramidase-1, defined by the ability to lyse *E. hirae* cell walls, and muramidase-2, defined by the ability to lyse lyophilized *Micrococcus lysodeikticus* cells (9, 10, 17, 27). Muramidase-1 is a β-1,4-*N*-acetylmuramidase with an 87-kDa active form and a latent form that can be activated by trypsin (10), while muramidase-2 exists in a 125-kDa active form and a 75-kDa active form (9). Autolysin activities in *E. faecalis* have been reported, including one which could lyse heat-killed *M. lysodeikticus* cells and another which could lyse heat-killed *E. faecalis* cells; the proteins with autolytic activities were shown to have molecular masses and substrate specificities similar to those of *E. hirae* (13). An *E. faecalis* gene encoding an autolysin of unknown specificity has been cloned and sequenced by Béliveau et al. (1). These authors reported that two *E. coli* clones containing the gene had multiple active forms of autolytic activity which could lyse lyophilized *M. lysodeikticus* cells and *E. faecalis* cell walls that were contained in a renatured sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. This autolysin has a predicted size of 74 kDa. However, the physiological functions of the autolysin of *E. faecalis* are still unknown.

In our initial immunoscreening of a genomic library of *E. faecalis* OG1RF, a predominant antigen detected by human patient sera and immune rabbit serum (36) was found to be an autolysin encoded by the gene reported by Béliveau et al. (1). In this study, we generated an autolysin mutant and studied both the mutant and parental strains for autolytic activities, autolysis, penicillin-induced lysis, penicillin resistance, and virulence to determine whether this dominant antigen is a virulence factor. To our knowledge, this is the first report of a targeted disruption of an enterococcal autolysin.

MATERIALS AND METHODS

Strains and media. The *E. faecalis* strain used in this study, OG1RF, has been described previously (23). pBluescript SK(−) was used for routine cloning (Stratagene, La Jolla, Calif.). Brain heart infusion (BHI) medium (Difco Laboratories, Detroit, Mich.) was used for growth of *E. faecalis* unless otherwise stated. SR medium (8) was used for electroporation of *E. faecalis*. The concentrations of antibiotics used for selection were as follows: for *Escherichia coli*, tetracycline, 12.5 μg/ml; kanamycin, 25 μg/ml; for *E. faecalis*, kanamycin, 2,000 μg/ml; fusidic acid, 25 μg/ml. Penicillin G was purchased from Sigma Chemical Co. (St. Louis, Mo.).

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DNA techniques. Plasmid DNA from *E. coli* was isolated by the alkaline SDS method as previously described (4). Transformation of *E. coli* was performed according to the method described by Calvin and Hanawalt (5). Transformation of *E. faecalis* by electroporation was carried out as described previously (18). Chromosomal DNA of *E. faecalis* and Southern blots were prepared as described previously (26, 35).

Mutagenesis. In order to generate a targeted autolysin mutant of *E. faecalis* OG1RF, an internal fragment (from 564 to 1,617 bp) of the previously described autolysin gene (1) was released from plasmid YX7 (36) and cloned into plasmid pTEX4577 (29), which contains a kanamycin resistance gene of gram-positive origin (12), by using *Sca*I and *Kpn*I sites. The resulting construct, pTEX4581, which carries the internal fragment of the autolysin gene, was electroporated into *E. faecalis* OG1RF followed by selection on SR agar plates with 2,000 μg of kanamycin per ml. Since pBluescript SK(−) lacks the origin of replication of a gram-positive plasmid, kanamycin-resistant colonies should represent mutants which integrated the recombinant plasmid into the autolysin gene by homologous recombination. The correct mutation was confirmed by Southern blotting analysis, and the autolysin mutant was designated TX5127.

Assay for autolysin (muramidase-2-like) activity. Determination of the ability of *E. faecalis* OG1RF and TX5127 to produce autolysin, as defined by the ability to lyse heat-killed *M. lysodeikticus* cells (muramidase-2-like lytic activity) (1, 16), was carried out as described previously (13). In brief, 10 μl of the bacteria to be assayed was spotted onto the surface of Todd-Hewitt (TH) agar containing heat-killed cells of *M. lysodeikticus* (*Micrococcus luteus*) (Sigma Chemical Co.) adjusted to an optical density at 600 nm (OD_{600}) of 0.5, and the plates were incubated at 37°C for 48 h. Bacteria that showed a clear lysis zone on heat-killed *M. lysodeikticus* cell plates were considered autolysin (muramidase-2-like lytic enzyme) producing (9, 17).

The ability to produce autolysin was further studied by SDS-PAGE (1). Briefly, 10 ml of overnight cultures of *E. faecalis* OG1RF and TX5127 was harvested by centrifugation and resuspended in 1 ml of denaturing buffer (2% dithiothreitol, 15% sucrose, 3.8% SDS). The culture supernatants were mixed with an equal volume of 2× denaturing buffer. Samples were then placed in a boiling water bath for 3 min. Twenty-five-microliter aliquots from cell or supernatant preparations were applied to an SDS-PAGE (10% polyacrylamide) gel containing 0.2% lyophilized *M. lysodeikticus* cells. After electrophoresis, the gel was renatured by incubation for 48 h in 25 mM Tris (pH 8) buffer containing 1% Triton at room temperature. Lytic activity could be visualized as clear bands on the opaque SDS-PAGE gel.

Assay for muramidase-1-like lytic activity. In order to determine whether the disruption of the gene coding for the cloned autolysin would alter the expression of other lytic enzymes, we also measured the ability of the wild type and the mutant strain to lyse *E. faecalis* cell walls (analogous to muramidase-1 activity of *E. hirae*, which was defined by the ability to lyse *E. hirae* cell walls) (10, 13). The preparation of enzyme and *E. faecalis* OG1RF cell walls and the assay of enzymatic activity were conducted as previously described (16).

Autolysis assay. Cell autolysis was determined by a modification of the method of Massidda et al. (20). Six-milliliter cultures of OG1RF or TX5127 grown in THGB (TH broth [Difco Laboratories] supplemented with 2% glucose) were removed at different growth phases (exponential phase, late exponential phase, and stationary phase), chilled on ice, and filtered (0.45-μm pore size; Millipore Corp., Bedford, Mass.), washed three times with distilled water at 4°C, and resuspended in 6 ml of 10 mM sodium phosphate buffer (pH 6.8) with or without trypsin (0.5 μg/ml). Trypsin was used because it has been reported that in *E. hirae*, muramidase-1 is present in two forms, the active form and the latent form, which could be activated by trypsin (25, 27). The suspension was then incubated at 37°C, and the OD_{675} was measured at 15-min intervals for up to 6 h.

Penicillin effects. Penicillin-induced lysis was measured by the methods previously described (13). In brief, bacterial cells from a late-exponential-phase culture were diluted 1:20 in fresh BHI medium to an OD_{660} of 0.08 to 0.10. Penicillin (stock solution, 1,024 μg/ml) was added to obtain the desired concentrations. Cultures were incubated at 37°C, and 1-ml aliquots were removed at different time points to measure the OD_{660}.

MICs were determined by agar dilution as described previously (24) with increments of 0.25 μg/ml, and *E. faecalis* ATCC 29212 was employed as a control. Counts for time-kill assays ($n = 6$) were performed according to the method previously described (21) to compare the bactericidal activities of penicillin against *E. faecalis* OG1RF and TX5127. Statistical analysis was performed with Student's two-tailed *t* test.

Morphological examination and growth rate. To determine whether mutation of the gene encoding autolysin caused any change in *E. faecalis* OG1RF morphologically, we examined the cells of OG1RF and TX5127 by light microscopy. Cells were stained with Gram stain (Difco Laboratories) according to the protocol supplied and were examined with an American Optical microscope.

To determine the growth rate, overnight cultures of *E. faecalis* OG1RF and TX5127 were diluted 1:20 in BHI and grown at 37°C with shaking. The turbidity was measured at different time points with a Manostat turbidometer (Manostat, New York, N.Y.). CFU were determined by serial dilution of the cultures in saline and plating them onto BHI agar plates in duplicate.

Mouse peritonitis model. *E. faecalis* OG1RF and TX5127 were grown overnight in BHI broth. The cells were harvested by centrifugation, washed once with 0.9% saline, and then resuspended in saline. Serial dilutions were made in saline and were mixed (1:10) with 50% sterile rat fecal extract (SRFE). Groups of six outbred (ICR) female mice 4 to 6 weeks old (weighing 22 to 25 g) were challenged intraperitoneally with different inocula (15). A control group of mice was injected with 50% SRFE only. Survival was monitored every 12 h. Determination of Kaplan-Meier survival curves and log rank analysis were performed as described previously (28).

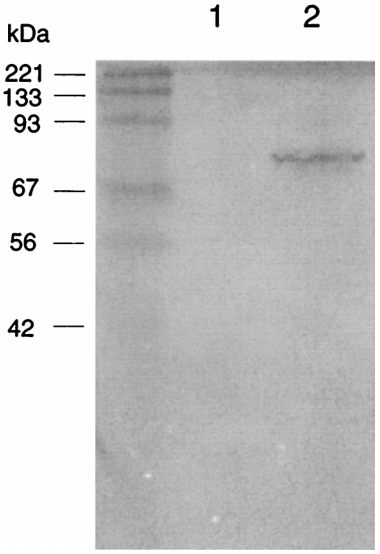

FIG. 1. Cell wall lytic activity of OG1RF and TX5127 against *M. lysodeikticus* cells in renatured SDS-PAGE. Lane 1, whole cells of TX5127; lane 2, whole cells of OG1RF.

RESULTS AND DISCUSSION

Mutagenesis. After transformation of 100 μl of *E. faecalis* cells with 5 μl of pTEX4581, the construct carrying the internal fragment of the autolysin gene, 28 Kan^r colonies were recovered on SR medium-kanamycin (2,000 μg/ml) plates. Hybridization of Southern blots of the chromosomal DNA from one of these colonies by using the internal fragment of the autolysin gene as a probe showed the correct chromosomal insertion in the autolysin gene (data not shown); this insertion mutant derivative was designated TX5127.

Assay for autolysin (muramidase-2-like) activity. On plates containing heat-killed *M. lysodeikticus* cells, OG1RF showed a clear zone of lysis around the colonies, while the autolysin mutant failed to show clearing (data not shown), indicating that the gene product had been inactivated or was not readily secreted. Similarly, with renatured SDS-PAGE gels containing lyophilized *M. lysodeikticus* cells, no band of clearing was shown for whole cells of TX5127, while OG1RF showed clear bands similar to those previously reported (Fig. 1) (1). Similar results were shown for supernatants of TX5127 and OG1RF, respectively (data not shown). The fact that TX5127 lost the ability to hydrolyze the lyophilized cells of *M. lysodeikticus* would classify this gene product as a muramidase-2-like enzyme based on the definitions used for *E. hirae* (17).

Assay for muramidase-1-like lytic activity. To determine whether the inactivation of this autolysin would alter the effect of other autolytic enzymes, we measured muramidase-1-like activity in OG1RF and TX5127. Hydrolysis of *E. faecalis* cell walls by TX5127 from different growth phases was similar to that of wild-type OG1RF (data not shown).

Autolysis assay. In order to detect whether inactivation of this autolysin gene affected the autolysis of *E. faecalis* cells, cultures of OG1RF and TX5127 from different growth phases

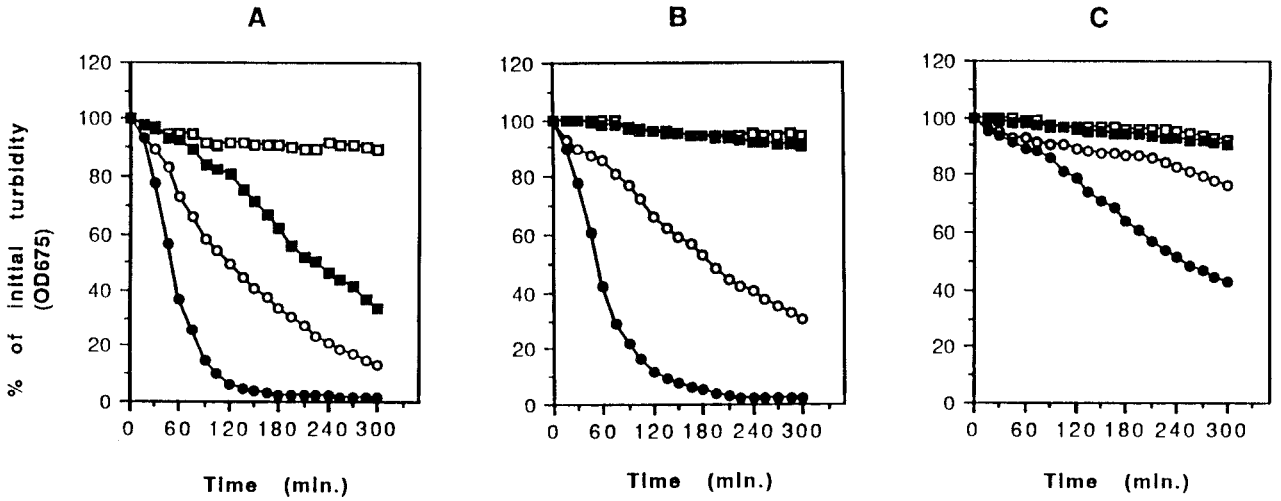

FIG. 2. Autolysis of OG1RF (circles) and TX5127 (squares). (A) Cells from early exponential phase. (B) Cells from late exponential phase. (C) Cells from early stationary phase. Cells were collected on filters and resuspended in 10 mM sodium phosphate (pH 6.8). The changes in turbidity were measured by a spectrophotometer at 675 nm. Open symbols, without trypsin; solid symbols, with trypsin. Standard errors were in a range of between 0 and 6% at OD_{675}.

(early exponential phase, late exponential phase, and stationary phase) were used in an autolysis assay. As shown in Fig. 2, there is a partial inhibitory effect of the mutation on exponential-phase cells' autolysis but a much more dramatic inhibitory effect on late-log or stationary-phase cells' autolysis. One interpretation is that other enzymes are involved in the early exponential phase but that the muramidase-2-like enzyme mainly works in the late log or stationary phase. Perhaps the expression of lytic enzymes is regulated in different growth phases. Another possible explanation is that the substrates (cell wall) of this autolysin and other lytic enzymes are different in different growth phases. The effect of trypsin on autolysis was also investigated. TX5127 showed a slower autolysis rate than OG1RF in the presence or absence of trypsin, although in the presence of trypsin, there was more rapid autolysis of both OG1RF and TX5127 (Fig. 2), similar to that shown by Cornett et al. with both a wild-type strain and a muramidase-2 mutant strain of *E. hirae* (7). This suggests that this autolysin and other lytic enzymes are involved in autolysis and that other lytic enzymes may be activated by trypsin.

Penicillin effects. It has been suggested that autolysins of *E. faecalis* are associated with the bactericidal activity of penicillin (13)—that is, that suppression of the activity of a cell's autolytic activity could protect bacteria from the bacteriolytic antibiotics like penicillin. To test this possibility, penicillin at concentrations of 4, 16, and 64 μg/ml was added to *E. faecalis* OG1RF and TX5127 cultures, and the turbidities of the culture were monitored. With penicillin at 4 and 16 μg/ml, TX5127 showed less decrease in OD_{660} than OG1RF after 24 h of incubation (Fig. 3). When a higher concentration of penicillin was used, the difference in OD_{660} of these two strains was less pronounced, as was the decrease in OD_{660}, suggestive of an Eagle effect (11). Overall, a concentration of 4 μg/ml generated the greatest decrease in OD_{660} for both strains. These results suggest that inactivation of this autolysin may partially suppress penicillin-induced lysis. However, mutation of this autolysin gene did not change the MIC for *E. faecalis*. The MICs of penicillin for both OG1RF and TX5127 are the same (3.5 μg/ml with 0.25-μg/ml increments), indicating that disruption of the autolysin gene does not increase penicillin resistance.

Time-kill curves were used to test whether inactivation of this autolysin would protect bacteria from the killing effect of penicillin—that is, whether inactivation would increase the tolerance of TX5127 to penicillin compared to that of OG1RF. OG1RF was killed moderately well by 5 μg of penicillin per ml with a decrease of 2.8 ± 0.2 $\log_{10}$ CFU/ml at 24 h. Both OG1RF and TX5127 could still grow slowly in 2 μg of penicillin per ml, but OG1RF showed about 0.6 log less growth than TX5127 at 24 h ($P < 0.001$) (Table 1). In 5 and 10 μg of penicillin per ml, TX5127 showed about 0.6 less log reduction than OG1RF ($P = 0.03$ and 0.04, respectively) (Table 1), a small but statistically significant difference. These data indicate that inactivation of this autolysin may partially protect the bacterial cells from the lytic effect of penicillin, but does not convert the mutant into a strain as tolerant as those in vitro-selected tolerant strains reported by Hodges et al., which did not show a decrease in CFU per milliliter after 24 h of exposure to 10× the MICs of penicillin (14).

Morphological examination and growth rate. Examination of OG1RF and TX5127 by light microscopy showed that TX5127 formed chains compared with parental strain OG1RF. Figure 4 shows the distribution of the number of cells in the chains of OG1RF and TX5127. The majority of the chains of TX5127 had 2 to 10 cells per chain, while OG1RF mainly existed as single cells. Morphological change in *E. hirae* has been previously reported by Lleò et al. for thermosensitive mutants, which showed great reduction in the production of muramidase-1 and formed elongated cells at nonpermissive temperature (19). Llèo et al. postulated that muramidase-1 might be associated with the formation of septa, while muramidase-2 might be involved in separation of daughter cells. Our results in *E. faecalis* are consistent with their hypothesis for *E. hirae*. The formation of chains of TX5127 indicates that this autolysin (muramidase-2-like enzyme) is required for appropriate cell separation in *E. faecalis*.

Since it appeared that this autolysin affected cell separation, we also examined the growth rate of the TX5127 in comparison with that of OG1RF. The growth rate of TX5127 in BHI broth was similar to that of OG1RF measured in Klett units (data not shown). The number of CFU of TX5127 was somewhat lower than that of OG1RF (data not shown), probably due to the formation of chains. However, this autolysin of *E. faecalis* clearly was not essential for cell growth, and there were still

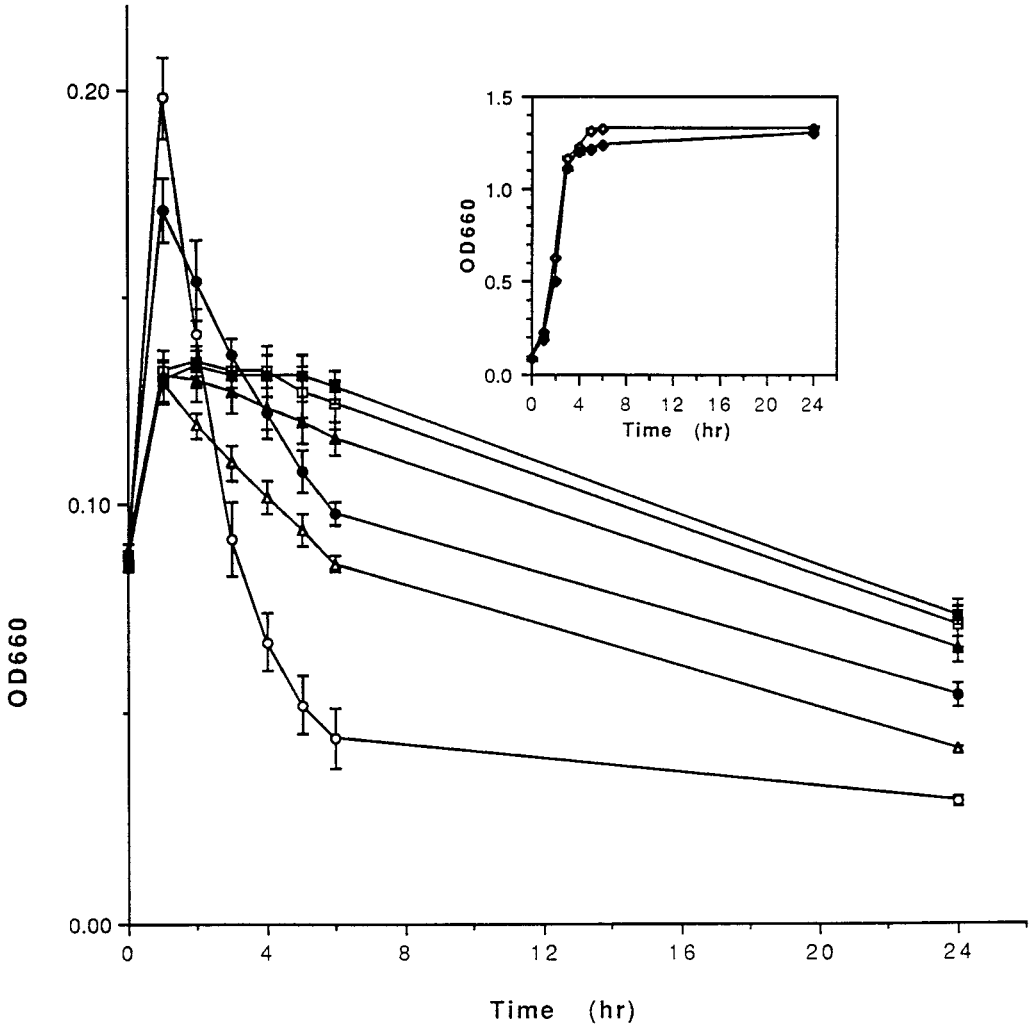

FIG. 3. Penicillin-induced lysis of OG1RF (open symbols) and TX5127 (solid symbols). Penicillin stock solution was added at time zero to obtain final concentrations of 4 (circles), 16 (triangles), and 64 (squares) μg/ml. Turbidity was monitored at OD_{660}. Insert, OG1RF (open diamonds) and TX5127 (solid diamonds) without penicillin. Standard errors are indicated by error bars.

some single cells in the culture of TX5127, suggesting that a muramidase-1-like autolysin of *E. faecalis* or other lytic enzymes may substitute for the function of this autolysin. Double mutation of the muramidase-1-like autolysin and this autolysin may be able to further address this question.

Mouse peritonitis model. Berry et al. have reported that following the interruption of the major autolysin gene, *Streptococcus pneumoniae* type 2 and type 3 strains were less virulent (2, 3). However, Tomasz et al. reported that interruption of the autolysin gene in type 3 pneumococci had no effect on virulence (31). This led to the hypothesis that the role of autolysin in pneumococcal infections might vary from serotype to serotype (3). It has been suggested that the possible role of autolysin in infections by pneumococci may be to facilitate the release of toxins and/or inflammatory cell wall breakdown products (6, 32). Using a mouse peritonitis model, we studied the virulence of TX5127 in comparison to that of OG1RF. The 50% lethal dose (LD_{50}) of TX5127 was 3.0×10^8 CFU, similar to the LD_{50} of OG1RF (3.2×10^8 CFU). The time course of death for TX5127 was also similar to that of OG1RF (e.g., $P = 0.2735$ by log rank test for survival after inoculation of 3.2×10^8 CFU of OG1RF versus 3.0×10^8 CFU of TX5127 [data not shown]), suggesting that this enterococcal autolysin does not play an important role in infection in the model that we used or that inactivation of autolysin could be compensated for by other factors.

TABLE 1. Bactericidal killing by penicillin

Strain	Decrease in cell count (log_{10} CFU/ml) with penicillin concn of[a]:		
	2 μg/ml	5 μg/ml	10 μg/ml
OG1RF	0.75 ± 0.11	3.96 ± 0.16	3.62 ± 0.18
TX5127	0.18 ± 0.08[b]	3.36 ± 0.13[c]	3.03 ± 0.08[d]

[a] Initial inocula were about 10^7 CFU/ml. Data are expressed as mean (log_{10} CFU per ml at 24 h of growth without penicillin $-log_{10}$ CFU per milliliter at 24 h of growth with penicillin) ± standard deviation ($n = 6$).
[b] $P < 0.001$ versus OG1RF by Student's two-tailed t test.
[c] $P = 0.03$ versus OG1RF by Student's two-tailed t test.
[d] $P = 0.04$ versus OG1RF by Student's two-tailed t test.

Conclusions. It has been proposed that β-lactam-induced lysis of bacteria is the result of inhibition of biosynthesis of cell

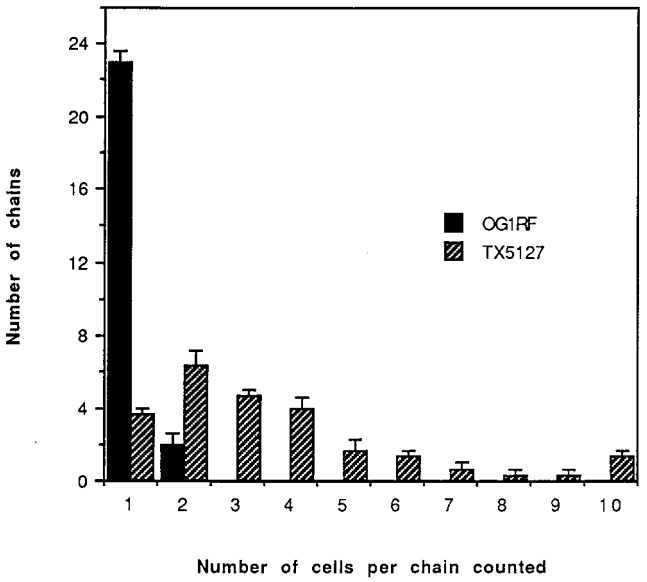

FIG. 4. Distribution of the number of cells in the chains of OG1RF and TX5127. The chains counted were randomly chosen. Standard errors are shown by error bars.

walls and the hydrolysis of cell walls by cellular autolytic enzymes (30, 33). Tomasz et al. showed that suppression of or a defect in autolytic enzyme(s) in pneumococci was associated with a simultaneous increase in viability of pneumococci cells in the presence of penicillin (30). Storch et al. have shown that increases in penicillin-induced lysis in clinical isolates of *E. faecalis* were correlated with an increase in autolytic activity (29). It was also shown by Fontana et al. that in *E. faecalis*, reduction of the ability to lyse *E. faecalis* cell walls was associated with decreased bactericidal activity of penicillin against *E. faecalis* clinical isolates (13). In our study, even though we found that interruption of the muramidase-2-like hydrolytic activity gene decreased the rates of autolysis and penicillin-induced lysis, we did not detect an increase in penicillin resistance (by MIC), and interruption produced, at most, only a small increase in tolerance by time-kill assay. These data suggest that alterations in the autolysin gene previously characterized by Béliveau et al. (1) or its expression are not responsible for the tolerance of some clinical or in vitro-derived isolates of *E. faecalis* to β-lactam killing effects. We have also shown here, by using intraperitoneal challenge of mice, that this autolysin gene had no significant effects on the virulence of *E. faecalis*. Further study would be needed to determine whether this autolysin is involved in other enterococcal infections or contributes to the virulence of other strains of *E. faecalis*.

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