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Paradoxical Response of *Enterococcus faecalis* to the Bactericidal Activity of Penicillin Is Associated with Reduced Activity of One Autolysin

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Ten clinical isolates of *Enterococcus faecalis* were examined for susceptibility to the bactericidal activity of penicillin. Four of these had MBCs of penicillin equal to 2 to 4× the MIC, and six exhibited a paradoxical response to penicillin, i.e., the bactericidal activity of the antibiotic had a concentration optimum at 2 to 4× the MIC and decreased significantly at concentrations above this. We found that the paradoxical response to penicillin was an intrinsic and stable property of a strain, but that its phenotypic expression was not homogeneous; only a fraction of the cell population that died at low concentrations was able to survive at high penicillin concentrations. The size of this fraction increased with increasing antibiotic concentration and reached a maximum in the late-log phase of growth. All 10 strains produced a lytic enzyme that was active on *Micrococcus luteus* heat-killed cells, whereas only some strains lysed *E. faecalis* heat-killed cells. Strains producing large amounts of the latter enzyme did not show the paradoxical response to penicillin, whereas mutants of these strains that lacked this enzymatic activity paradoxically responded to the antibiotic activity. In addition, from strains that showed paradoxical response to penicillin and produced only the enzyme that was active on *M. luteus*, it was possible to isolate mutants that were also capable of lysing *E. faecalis* cells and that were killed with similar efficiency by all concentrations above the MBC. On the basis of these findings, the paradoxical response to penicillin is explained as a property of certain strains of *E. faecalis*; this property is genetically characterized by alterations in synthesis or activity of one autolysin but phenotypically expressed only by a few cells that are in a particular physiological condition when exposed to high concentrations of antibiotics.

The so-called paradoxical effect of antibiotics was described in 1948 by Eagle and Musselman (3) as a particular response of some bacteria to beta-lactams, in which the bactericidal effect of these antibiotics has a concentration optimum. The phenomenon is very frequently observed in clinical isolates of staphylococci, group A and D streptococci, and *Streptococcus viridans* treated with beta-lactams (7) and poses certain problems in establishing whether a strain has to be considered prone to the killing activity of beta-lactams (20). This is important, since non-beta-lactam antibiotics that are potentially bactericidal against such strains are generally more toxic.

Several hypotheses have been suggested to explain the paradoxical effect. Blumberg and Strominger (1) proposed both the need for incorporation of un-cross-linked peptidoglycan into the cell wall for lethal action at appropriate penicillin concentrations and differential binding of penicillin to different protein targets. Mychajlonka et al. (13) suggested that the secondary inhibition of cellular RNA and protein synthesis is involved in the dose-dependent killing by penicillin. However, the biochemical mechanism responsible for the paradoxical effect has not been clarified.

In this study, we describe the properties of 10 clinical isolates of *Enterococcus faecalis*; some of these exhibited the paradoxical response to the bactericidal activity of penicillin. Evidence is presented that such a response to the antibiotic is shown by strains that do not produce or that produce low amounts of one autolysin which is particularly active on *E. faecalis* cells.

We suggest that this enzyme is apparently necessary for the killing of slowly growing cells, which have a particular physiological status when exposed to high penicillin concentrations.

MATERIALS AND METHODS

Strains. The *E. faecalis* strains used in these studies were clinical isolates obtained from different body areas. These isolates were initially identified as enterococci by growth in 6.5% salt broth and by hydrolysis of esculin in the presence of 40% bile (4). Subsequent identification was performed with the API-Strep system (API-System S.A.; Analytab Products). All experiments were carried out by growing bacteria in Mueller-Hinton broth (MHB) (Difco Laboratories) containing 1.5% agar when used as solid medium (MHA) to better correlate the response to bactericidal activity of penicillin with physiological and biochemical properties of the strains.

Antibiotics and reagents. Penicillin was purchased from E. R. Squibb & Sons. [^{14}C]penicillin (54 mCi/mmol) was purchased from Amersham Corp. All other chemicals were reagent-grade commercially available products.

Susceptibility testing. The MIC was determined by a macrodilution technique consisting of preparation of glass tubes containing serial twofold dilutions of antibiotic in 5 ml of MHB (range, 1 to 128 μg/ml) and inoculation with bacteria to obtain a final density of 10^7 CFU/ml. After 14 h of incubation, all tubes were vortexed; after 18 h, tubes were examined for visual turbidity, and the MIC was recorded. After

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TABLE 1. Response to penicillin of *E. faecalis* clinical isolates

Strain	Penicillin MIC (μg/ml)	% CFU surviving after 18 h of incubation at a penicillin concn (μg/ml) of:					% of PRP-positive cells[a]
		8	32	128	512	1,024	
E6	4	0.4	1.4	2.4	4.9	5.6	5.2
S/361	4	0.5	0.6	1.5	5.0	5.0	4.5
S/395	4	0.3	0.4	0.4	0.6	0.3	0.3
3626	4	1.1	5.6	3.7	6.4	6.4	5.3
3678	4	0.2	0.1	0.2	0.1	0.1	0.0
3741	2	1.5	1.7	1.9	5.7	10.0	8.5
4117	2	0.3	0.4	0.3	0.3	0.5	0.2
4119	4	0.1	0.2	0.2	0.1	0.1	0.0
4126	4	0.4	0.7	4.2	6.5	8.9	8.5
4133	2	0.3	0.3	0.9	2.0	5.0	4.7

[a] The percentage of the initial cell population that responded paradoxically to penicillin (PRP-positive cells) was evaluated by subtracting the percentage of CFUs surviving at 8 μg of penicillin per ml from the highest percentage of CFUs surviving at concentrations of 512 or 1,024 μg of penicillin per ml.

vortexing again, a 0.1-ml sample was taken from each tube without visual turbidity and serially diluted, and 0.1 ml of each dilution was spread onto two MHA plates. Taylor et al. (20) found that this procedure for MBC determination improved the killing rate of the antibiotic. CFUs were counted after 24 h of incubation. The MBC was considered the lowest concentration of penicillin that caused the lowest recovery of survivors. The standard definition of MBC (19) could not be used in this study, because in some strains no antibiotic concentration caused the stated 99.9% reduction in the initial cell population. For the purposes of this study, a paradoxical bactericidal effect of penicillin was considered to be represented by a significant increase in counts of survivors from at least three consecutive tubes containing antibiotic concentrations above the MIC, and strains exhibiting such behavior were regarded as positive in terms of paradoxical response to penicillin (PRP-positive strains).

Penicillin-induced lysis. A culture in the late-exponential phase of growth was diluted in fresh medium to obtain an A_{675} of 0.100 to 0.150 optical unit per ml by using a Beckman DU spectrophotometer. A penicillin solution was prepared at 20 times the desired final concentrations and suitably diluted in these cultures. After the addition of the antibiotic, cultures were incubated at 37°C and the A_{675} was measured.

Assay for lytic activity. The ability of *E. faecalis* strains to produce lytic enzymes was performed with a modification of the solid medium assay of Pooley et al. (14).

Heat-killed cells of *Micrococcus luteus* or *E. faecalis* to be used as substrate were added to melted MHA to obtain a final cell optical density (OD) of 0.5 optical unit per ml. After solidification, 10 μl of a stationary culture of the strains to be assayed was spotted on the surface of the medium, and plates were incubated for 24 to 48 h at 37°C.

The production of enzymes that lysed the cells used as substrate was shown by the appearance of a distinct clear zone around the spot. Since no differences in activity were observed that depended on the *E. faecalis* strain used as a substrate, further studies were carried out with *E. faecalis* E6 heat-killed cells.

Mutagenesis and selection procedure of autolysin-altered mutants. Stationary-phase cells were treated with ethyl methanesulfonate as described by Pooley et al. (14). Mutagenized cells were diluted and plated into MHA containing heat-killed *E. faecalis* E6 cells to obtain 100 to 300 colonies per plate. Clones showing the desired phenotype were selected for further analysis.

Assay for PBPs. Penicillin-binding proteins (PBPs) were assayed with growing cells as described by Fontana et al. (5, 6), with the sole exception that *E. faecalis* cells were lysed by treatment with lysozyme (100 μg/ml, final concentration) and mutanolysin (10 μg/ml, final concentration in 0.1 M phosphate buffer [pH 7.2]) for 30 min at 37°C, followed by sonication.

RESULTS

Response of *E. faecalis* strains to penicillin activity. Ten clinical isolates of *E. faecalis* were examined for susceptibility to growth inhibition and killing by penicillin (Table 1). In six strains (E6, S/361, 3626, 3741, 4126, and 4133), the bactericidal activity of penicillin had a concentration optimum of 2 to 4× the MIC, but at concentrations above this it decreased significantly. In these strains only a fraction of the cell population used as the inoculum exhibited the paradoxical response to penicillin (Table 1). In fact, the great majority of cells (90 to 95%) were killed even by the highest penicillin concentration used, and a small percentage (0.3 to 1.5%) survived also at the lowest concentration. On the basis of the definition stated in Materials and Methods, these strains were considered to be PRP positive.

In the remaining four strains (S/395, 3678, 4117, and 4119), all penicillin concentrations above 2 to 4× the MIC had similar killing activity; the fraction of the cell population which survived at the highest concentrations was equal to or only a little higher than that which survived at low concentrations. These strains were considered to be PRP negative. MIC determinations and evaluations of the number of cells surviving after 18 h at various penicillin concentrations were repeated three times for all strains without finding any significant difference in results.

We then studied the effect of cell culture age and inoculum size on the percentage of bacteria paradoxically responding to penicillin in *E. faecalis* E6 (PRP-positive strain) and *E. faecalis* 3678 (PRP-negative strain). Table 2 shows the effect of cell culture age on the paradoxical response to penicillin. The percentage of PRP-positive cells of *E. faecalis* E6 reached a maximum (8.9%) in the late-log phase of growth and decreased after overnight incubation (1.7%). A small increase in PRP-positive cells was also observed in the late-log-phase cultures of *E. faecalis* 3678 (from 0 to 0.2%).

In contrast, the number of bacteria used as the inoculum did not significantly influence either the MIC or the percentage of cells surviving at various penicillin concentrations in either *E. faecalis* E6 or *E. faecalis* 3678 (Table 3).

We then evaluated whether the PRP-positive phenotype was due to heterogeneity of the microbial population caused by the appearance of peculiar mutants resistant to killing by high but not low penicillin concentrations or was an intrinsic

TABLE 2. Effect of growth phase on response of PRP-positive and -negative *E. faecalis* strains to bactericidal activity of penicillin

Strain	Growth phase[a]	% CFU surviving after 18 h of incubation at a penicillin concn (μg/ml) of:			% of PRP-positive cells[b]
		8	32	128	
E6 (PRP positive)	Early log	0.1	1	1	0.9
	Mid log	0.2	0.5	0.7	0.5
	Late log	0.1	2.1	9	8.9
	Late stationary	0.1	0.2	1.8	1.7
3678 (PRP negative)	Early log	0.1	0.1	0.1	0
	Mid log	0.1	0.1	0.1	0
	Late log	0.1	0.1	0.3	0.2
	Late stationary	0.2	0.1	0.2	0.1

[a] Overnight cultures were diluted 1:20 in fresh medium and incubated at 37°C. After two doublings of the OD (early log), 8 doublings (mid log), 16 doublings (late log), and overnight incubation (late stationary), a 1-ml sample was taken from the culture and suitably diluted in a series of tubes containing various penicillin concentrations to obtain a density of 10^7 CFU/ml. The MIC of penicillin was 4 μg/ml for both strains at all phases.

[b] The percentage of PRP-positive cells was calculated as described in footnote *a* of Table 1.

property phenotypically expressed only by a fraction of the population. To this end, 50 clones each of two PRP-positive strains, *E. faecalis* E6 and 3626, and of one PRP-negative strain, *E. faecalis* 3678, isolated from among the cells surviving after 18 h of incubation in the presence of 128 μg/ml, were tested for their response to the bactericidal activity of penicillin. All of the cultures from these clones demonstrated the same MICs as the parent and contained a similar percentage of PRP-positive cells (data not shown).

Penicillin-induced lysis in *E. faecalis* strains. Several authors have suggested that resistance to bactericidal activity of penicillin in enterococci results from a low activity of the endogeneous autolytic enzyme system (7, 12, 16, 21). To test this hypothesis, exponentially growing cells of three PRP-positive and three PRP-negative strains were incubated with penicillin concentrations ranging from 2 to 32 times the MIC, and culture turbidity was monitored at intervals.

Figure 1 shows that growth of PRP-positive strains was inhibited to a similar extent by all penicillin concentrations above the MIC. OD values, after an initial increase lasting 30 to 60 min, remained constant or decreased slowly and after 24 h dropped to values near those of the starting cultures (Fig. 1A, B, and C). Growth of PRP-negative strains was apparently less inhibited by 2× the MIC than growth of PRP-positive strains; in the presence of 2× the MIC of penicillin, the OD of one strain showed an increase similar to that of the control for at least 60 min, and the OD of two strains showed a similar increase for at least 120 min. Higher concentrations caused a significant inhibition of cell growth similar to that caused in PRP-positive strains. After 24 h of incubation, all penicillin-treated cultures showed a substantial drop in turbidity values, which ranged from 30 to 90% of the OD of starting cultures (Fig. 1D, E, and F).

Lytic activity pattern of *E. faecalis* strains. The difference in the rate of lysis induced by penicillin in PRP-positive and PRP-negative strains prompted us to examine all 10 isolates of *E. faecalis* for lytic enzyme production. For this purpose, we evaluated the ability of all 10 strains of *E. faecalis* to form clear halos on MHA containing heat-killed cells of either *M. luteus* or *E. faecalis* strains. All strains produced similar halos of lysis on *M. luteus* cells by action of an enzyme which hereafter, for reasons of simplicity, we will call the "ML enzyme," whereas only five strains also lysed *E. faecalis* cells (Fig. 2). This lytic activity will be called hereafter the "EF enzyme." Of the five strains that lysed *E. faecalis* cells, four (S/395, 3678, 4117, and 4119) produced large amounts of enzyme and were PRP negative (Table 1), whereas one (S/361) produced a relatively low amount of enzyme and was PRP positive.

These results clearly indicated an association between the ability to respond paradoxically to the bactericidal activity of penicillin and lack of, or poor, EF enzyme activity.

Isolation and characterization of mutants with altered autolytic phenotype. To evaluate the possible relationship between absent or diminished EF enzyme activity and the paradoxical response of *E. faecalis* to penicillin, we attempted the isolation both of mutants of *E. faecalis* E6 (a strain which did not apparently produce EF enzyme and was PRP positive) eventually capable of lysing *E. faecalis* cells and of mutants of *E. faecalis* 3678, a PRP-negative strain

TABLE 3. Effect of inoculum size on paradoxical response to penicillin[a]

Strain	Inoculum size (CFU)	% CFU surviving after 18 h of incubation at a penicillin concn (μg/ml) of:			% of PRP-positive cells[b]
		8	32	128	
E6 (PRP positive)	7.2×10^6	0.3	1.3	2.5	2.2
	7.2×10^4	0.6	1.3	2.9	2.3
	7.2×10^2	0.1	1.7	2.1	2.1
3678 (PRP negative)	9×10^6	0.1	0.2	0.2	0.1
	9×10^4	0.1	0.1	0.2	0.1
	9×10^2	<0.1	<0.1	<0.1	0

[a] The MIC of penicillin was 4 μg/ml for both strains at all inoculum sizes.

[b] The percentage of PRP-positive cells was evaluated by subtracting the percentage of CFUs surviving at a penicillin concentration of 8 μg/ml from that surviving at a penicillin concentration of 128 μg/ml.

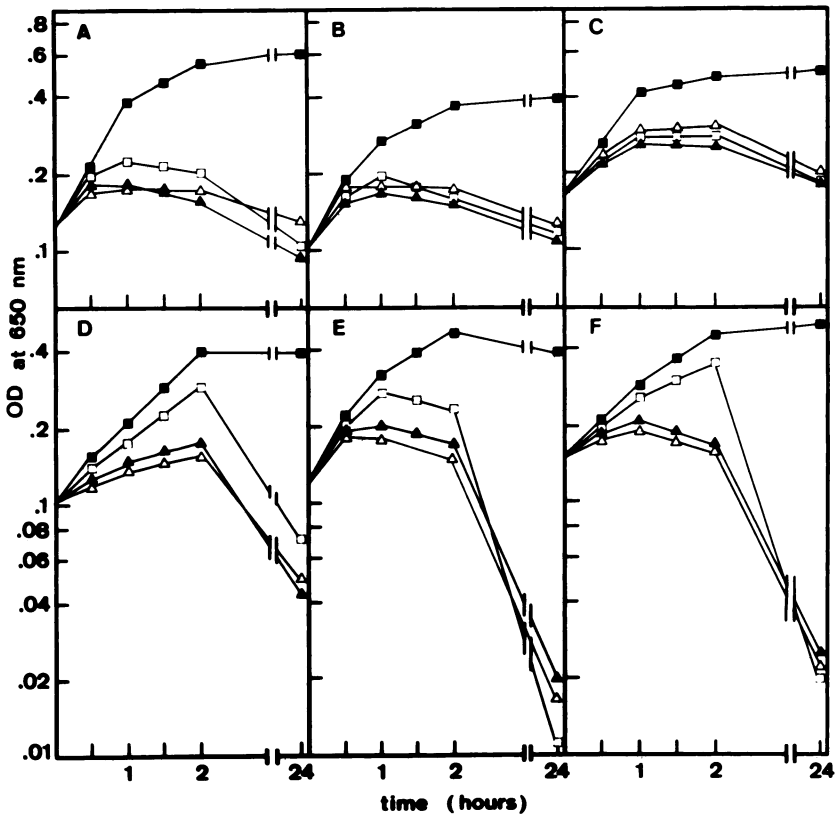

FIG. 1. Effect of penicillin on growth of three PRP-positive strains (A, E6; B, 3626; C, S/361) and three PRP-negative strains (D, S/395; E, 3678; F, 4117). Penicillin was added at 8 (□), 32 (▲), or 128 (△) μg/ml (final concentrations) at zero time. Untreated cultures (■) were used as controls.

with high-level EF enzyme activity but lacking the ability to lyse the above cells.

After mutagenesis with ethylmethane sulfonate, several clones of *E. faecalis* E6 capable of lysing *E. faecalis* cells were isolated. The mutant strains differed in the level of lytic activity; they produced lysis halos of different sizes. Two clones producing large halos and one producing a small halo were selected for analysis of response to bactericidal activity of penicillin.

From mutagenized cultures of *E. faecalis* 3678, several mutants lacking the ability to lyse *E. faecalis* cells were isolated. Two of these were selected for further characterization.

The two clones derived from *E. faecalis* E6 (*E. faecalis* E6/A and E6/B), which produced large halos of lysis on *E. faecalis* cells, failed to show a significant paradoxical response to penicillin (Table 4). On the contrary, the clone producing the smaller lysis halo (E6/1) still maintained the PRP-positive phenotype of the parent. This was not surprising, as a wild-type strain of *E. faecalis* (S/361) with low EF enzyme activity was also shown to be PRP positive (Fig. 3 and Table 1).

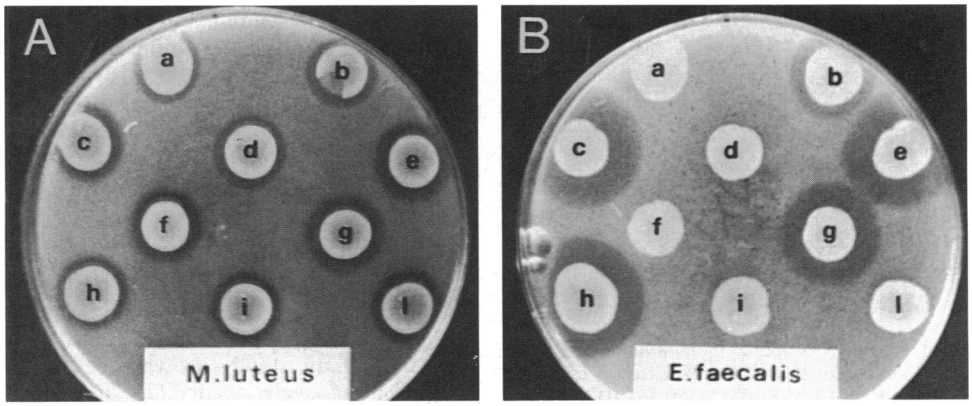

FIG. 2. Lysis of *M. luteus* (A) and *E. faecalis* (B) heat-killed cells by the following *E. faecalis* strains: E/6 (a), S/361 (b), S/395 (c), 3626 (d), 3678 (e), 3741 (f), 4117 (g), 4119 (h), 4126 (i), and 4133 (l).

TABLE 4. Properties of *E. faecalis* E6 and 3678 mutants with altered lytic enzyme pattern[a]

Strain	Lytic enzyme phenotype[b]		% CFU surviving after 18 h of incubation at a penicillin concn (μg/ml) of:			% of PRP-positive cells[c]
	ML	EF	8	32	128	
E6 (wild type)	+	−	0.4	1.4	2.4	2
E6/A	+	+	0.4	0.4	0.7	0.3
E6/B	+	+	0.3	0.7	0.8	0.5
E6/1	+	±	0.7	1.6	3.2	2.5
3678 (wild type)	+	++	0.2	0.1	0.2	0.1
3678/A	+	−	0.2	0.6	2.9	2.7
3678/B	+	−	0.4	0.6	1.8	1.4

[a] The MIC of penicillin was 4 μg/ml for all strains.
[b] Lytic activity was determined as described in the legend to Fig. 2: −, no lysis halo; +, 2-mm-diameter halo; ± and ++, halo diameters measuring less and more, respectively, than 2 mm.
[c] The percentage of PRP-positive cells was evaluated as described in footnote *a* to Table 1.

The two clones derived from *E. faecalis* 3678 (3678/A and 3678/B) and lacking the EF enzyme, as opposed to the parent, showed a PRP-positive phenotype.

All mutants derived from *E. faecalis* E6 and 3678 apparently did not show any alteration in ML enzyme activity.

Interaction of penicillin with PBPs in *E. faecalis* strains. *E. faecalis* E6 (PRP-positive strain) and 3678 (PRP-negative strain) in the late-exponential phase of growth were incubated for 60 min with 8, 32, and 128 μg of cold penicillin per ml. Unbound PBPs were then saturated with radioactive penicillin and visualized by fluorography.

The strains had similar PBP patterns; the only difference was the relative amount of PBP 4 (Fig. 3). Since this PBP appeared a little darker in both strains when membranes were incubated with radioactive penicillin for 60 min (Fig. 3, lanes a and b), it is possible that this protein belongs to the class of low-affinity PBPs already described for this and other enterococcal species (6). In E6 and 3678 strains, 8 μg of penicillin per ml bound or saturated PBPs 1, 2, and 3, whereas higher concentrations also saturated PBPs 4 and 5 (Fig. 3).

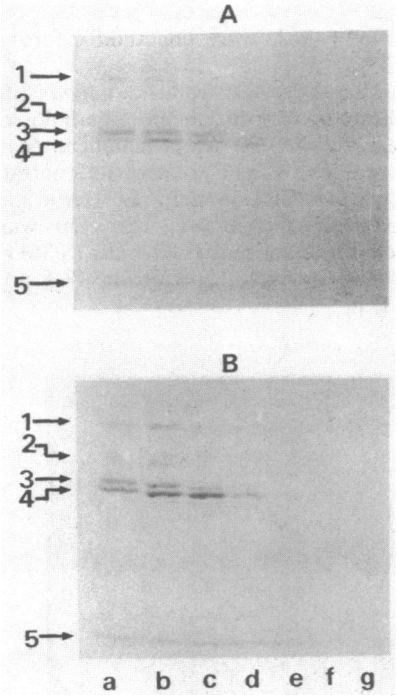

FIG. 3. Binding of 100 μM [^{14}C]benzylpenicillin to PBPs of *E. faecalis* E/6 (A) and *E. faecalis* 3678 (B) grown for 60 min in the presence of cold penicillin at 4 (c), 8 (d), 16 (e), 64 (f), or 128 (g) μg/ml (final concentrations). a and b were control samples exposed to radioactive penicillin for 15 and 60 min, respectively.

DISCUSSION

The data presented in this paper allow a more complete definition of the paradoxical response of enterococci to bactericidal activity of penicillin and a likely explanation of its mechanism. (i) Only a number of strains were capable of responding paradoxically to the antibiotic, and in these strains such behavior was genetically determined. (ii) In all of the PRP-positive strains only part of the cell population actually showed the paradoxical response to high penicillin concentrations, and this percentage strongly decreased in exponentially growing cells. (iii) PRP-negative strains were more prone to penicillin-induced lysis than were PRP-positive strains. (iv) PRP-positive strains differed from PRP-negative strains in the diminished activity or lack of one autolysin, the EF enzyme.

On the basis of these findings we suggest that killing of enterococci by penicillin involved the activity of two autolysins. One of these, the ML enzyme, was sufficient for penicillin to be bactericidal against exponentially growing cells (Table 2) and against a substantial proportion (ranging from 90 to 99.6%; Tables 1 and 2) of non-exponentially growing cells, whereas the activity of the other autolysin, the EF enzyme, or the synergistic activity of both enzymes was apparently required for killing a relatively small proportion (4.5 to 8.5%) of non-exponentially growing cells exposed to high penicillin concentrations.

The resistance of a number of non-exponentially growing cells of PRP-positive strains to the bactericidal activity of high penicillin concentrations might be explained by assuming (i) that the penicillin-triggered lethal event involving the activity of the ML enzyme occurred only in one phase of the cell division cycle and (ii) that slowly growing cells that were in this phase when exposed to penicillin survived only when peptidoglycan synthesis and growth were rapidly inhibited. This hypothesis is supported both by the evident dependence of the paradoxical response on the rate of growth and by the penicillin concentration used.

In fact, in slowly growing cells a single chromosome-replicating cycle occurs (2), and the cell cycle phase of resistance to bactericidal activity of penicillin may be temporally and physically separated from the susceptibility

phase. As a consequence, a single cell may eventually be able to respond paradoxically to penicillin as long as the phase of resistance lasts. On the contrary, in exponentially growing cells, where chromosome replication cycles initiate before completion of the previous cycle (2), the susceptibility phase of one division cycle may overlap and predominate over the resistance phase of the other division cycle, and a single cell may be susceptible to the bactericidal activity of penicillin for almost its entire life.

The bactericidal activity of antibiotics at relatively low concentrations against slowly growing cells that are in the resistance phase may be because unbalanced growth and peptidoglycan synthesis (which may occur at relatively low antibiotic concentrations as these do not saturate all PBPs) are per se lethal or because such unbalanced growth allows the cells to continue their cycle and overcome the resistance phase. On the contrary, high penicillin concentrations may freeze these cells in the resistance phase through the rapid inhibition of peptidoglycan synthesis (which may occur as these concentrations saturate more PBPs) and cell growth, resulting in a reduced bactericidal effect.

Direct experimental evidence of a relationship between paradoxical effect and cell division cycle may emerge from studies on *E. faecalis* synchronized cultures, but at present these experiments cannot be performed, since (i) *E. faecalis* cannot be synchronized because cells form long chains, and (ii) media, such as a chemically defined medium, that reduce chain formation (8) inhibit bactericidal activity of penicillin and render the paradoxical effect less evident (unpublished observations).

However, we and other authors have already shown that susceptibility to growth inhibition by beta-lactams varies in different phases of the cell cycle (9, 15, 18) and in different growth conditions (5), and it is likely that this also occurs for susceptibility to bactericidal activity.

Several authors have suggested that resistance to bactericidal activity of penicillin in enterococci results from a low activity of the endogenous autolytic enzyme system (7, 12, 16, 17, 21), but a relationship between autolysins and the paradoxical response to bactericidal activity of penicillin has never been shown or postulated.

In our study, strains showing ML enzyme activity only but no or only poor EF enzyme activity lysed very slowly in the presence of penicillin and were PRP positive, whereas strains showing high-level EF enzyme activity lysed more extensively in the presence of penicillin and were PRP negative. The isolation of *E. faecalis* E6 (a strain that apparently did not produce the EF enzyme and was PRP positive) and of several mutants capable of lysing *E. faecalis* cells and the finding of a PRP-positive wild type producing a smaller amount of this enzyme suggest that the gene for the synthesis of EF enzymes is present in all *E. faecalis* strains but that the synthesis or activity of this enzyme is strongly repressed in some strains.

The role of the EF enzyme in the bactericidal activity of penicillin and in the suppression of the paradoxical effect was also strongly supported by the finding that *E. faecalis* E6 mutants with high levels of this enzyme activity were PRP negative and that *E. faecalis* 3678 mutants with no EF enzyme activity were PRP positive. However, the assignment of a precise function of the EF enzyme in the paradoxical response requires the development of the genetic of enterococcal autolysin genes.

The lytic enzymes produced by *E. faecalis* appear to be quite similar to the two peptidoglycan hydrolases produced by *Enterococcus hirae* (*Streptococcus faecium*) ATCC 9790 (10, 11). Kawamura and Shockman have demonstrated that both enzymes have *N*-acetylmuramoylhydrolase activity but different substrate specificity; one (muramidase 1) is active on homologous cell walls but not on *M. luteus* cell walls, and the other (muramidase 2) is poorly active on *E. hirae* cell walls but very active on *M. luteus* (10, 11). It is possible that all enterococcal species possess a similar lytic enzyme pattern and that a low activity of one of these enzymes is responsible for the paradoxical response to penicillin also in other enterococcal species. Further studies are in progress to establish whether two enzymes are responsible for the lytic activities in *E. faecalis*.

The results presented in this paper also have important clinical implications. They showed that, as opposed to what is generally believed, not all *E. faecalis* strains are tolerant to penicillin. In 4 of the 10 strains we studied, penicillin concentrations of 2 to 4× the MIC reduced the inoculum by 99.7 to 99.9%, values very close to that required to define the activity of an antibiotic as bactericidal (19).

The strains possessing both enzymatic activities might also be more susceptible to lysis and killing by penicillin in vivo, and infections due to these strains do not necessarily require synergistic treatment with penicillin plus aminoglycoside or more toxic drugs.

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