In Vitro Studies of Plasmid-mediated Penicillinase from *Streptococcus faecalis* Suggest a Staphylococcal Origin

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Abstract

A strain of *Streptococcus faecalis* with plasmid-mediated penicillinase production was studied further. Partially purified penicillinase from the *S. faecalis* strain hydrolyzed penicillin, ampicillin, and ureido-penicillins but not penicillinase-resistant semisynthetic penicillins, cephalosporins, or imipenem; hydrolysis was inhibited by clavulanic acid. Hydrolysis of a given antibiotic correlated with a marked increase in the minimal inhibitory concentration (MIC) of that drug when a high inoculum was used. As with most enterococci, the MICs of cephalosporins and penicillinase-resistant semisynthetic penicillins were too high for clinical usefulness, although these agents did not show an inoculum effect. Based upon hybridization under stringent conditions of plasmid DNA from the *S. faecalis* strain to cloned penicillinase genes from *Staphylococcus aureus*, it appears that these resistance determinants are highly homologous and suggests that this enzyme was introduced into streptococci from staphylococci.

Introduction

Despite the widespread use of penicillin since the 1940's, β-lactamase–mediated penicillin resistance was unknown among streptococci until 1983 when we reported a β-lactamase–producing clinical isolate of *Streptococcus faecalis* in this journal (1). We also demonstrated that the ability of this strain to hydrolyze penicillin was plasmid-mediated and transferrable. This observation is of importance not only for enterococci but for all streptococci since many R-plasmids found in *S. faecalis* are able to transfer to other streptococcal species (2). The current study was undertaken in order to (*a*) investigate the substrate profile of the enterococcal β-lactamase, (*b*) determine the effect of β-lactamase inhibitors, and (*c*) seek genetic evidence that might indicate the origin of this resistance determinant.

Methods

Bacterial strains. S. faecalis strain HH22 was isolated from a urine specimen at Hermann Hospital, Houston, TX in 1981 and has been previously described (1). This strain is highly resistant (minimal inhibitory concentration [MIC][1] > 2,000 μg/ml) to gentamicin, is penicillin-resistant (PEN^R) at high inocula, and produces β-lactamase (Bla^+). HH22-PEN^S is a novobiocin-treated derivative of HH22 which no longer produces penicillinase but is still GM^R. Other major bacterial strains and plasmids used in this study are listed in Table I with their relevant properties. *Staphylococcus aureus* strain RN1753 containing pI524 was obtained from Richard Novick, The Public Health Research Institute of the City of NY (3), and the recombinant plasmid pJM13 was a gift from Jesse C. Rabinowitz (4), University of California, Berkeley, CA. Plasmids from gram-negative bacteria which were used in hybridizations include R6K(TEM-1), RP4(TEM-2), R57b(OXA-3), R16(OXA-2) (5) and pMG217(PSE-1) (supplied by G. A. Jacoby [6], Massachusetts General Hospital, Boston, MA).

Susceptibility testing. MICs were determined in brain-heart infusion broth by tube dilution as previously described (1); a final inoculum of 10^3 colony forming units (CFU)/ml is referred to as a "low inoculum" and 10^7 CFU/ml as a "high inoculum." Antibiotics used are shown in Tables II–IV; they and the β-lactamase inhibitors were obtained from their respective manufacturers or from Sigma Chemical Co., St. Louis, MO. The chromogenic cephalosporin nitrocefin was a gift from Glaxo Laboratories, Middlesex, England.

Enzyme preparation and hydrolysis studies. In order to obtain enzyme preparations for the hydrolysis assays, cultures of HH22 in brain-heart infusion broth were harvested by centrifugation. The supernatant was filter sterilized and then concentrated with a Diaflo Ultrafilter PM10 (Amicon Corp., Danvers, MA). The cellular pellet was resuspended in phosphate buffer (PB) (0.1 M $NaPO_4$ buffer, pH 7.0) with 0.02% sodium azide. This cellular suspension was sonicated extensively, centrifuged at 10,000 *g* for 20 min to remove cells, and then centrifuged sequentially at 30,000 *g* for 30 min and 110,000 *g* for 90 min. The pellets (resuspended in PB) and supernatants were tested for enzyme activity with nitrocefin. The supernatant obtained after centrifugation at 110,000 *g* was applied to a BioGel A 0.5 column (Bio-Rad Laboratories, Richmond, CA) equilibrated with PB with 0.02% sodium azide and eluted with the same buffer. The active fractions were pooled and used for the hydrolysis assays.

Hydrolysis of penicillins was determined by a microiodometric assay (7) at 25°C using a substrate concentration of 0.2 mM. Hydrolysis of cephalosporins and imipenem was determined spectrophotometrically at 25°C with 0.1 mM substrate by recording changes in absorbance at a wavelength that corresponded to the peak absorbance for each compound (8, 9). These peaks were determined before the addition of enzyme; all were in the 250–300 nm range. The effects of sulbactam and clavulanic acid were studied in two ways. In order to determine the effect on the MIC of penicillin, 10^7 CFU/ml of HH22 were preincubated for 30 min with varying dilutions of the β-lactamase inhibitors before the addition of penicillin. The effect of inhibitors on the hydrolysis of nitrocefin was determined by preincubating 10^6 CFU of HH22 with various concentrations of inhibitor, then adding nitrocefin (250 μg/ml) in a final volume of 200 μl, and incubating at 25°C (8–10).

Plasmid isolation, DNA blotting, and hybridizations. Streptococcal

This work was presented in part at the 24th Interscience Conference on Antimicrobiol Agents and Chemotherapy, Washington, DC, October 1984.

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Received for publication 18 February 1985 and in revised form 4 September 1985.

J. Clin. Invest.

0021-9738/86/01/0289/05 $1.00
Volume 77, January 1986, 289–293

1. *Abbreviations used in this paper:* AMIK, amikacin; Bla, β-lactamase; bp, base pair; CA, clavulanic acid; CFU, colony-forming unit; EM, erythromycin; GM, gentamicin; KM, kanamycin; MIC, minimal inhibitory concentration; PB, phosphate buffer; PEN, penicillin; SB, sulbactam; SM, streptomycin; TC, tetracycline; TM, tobramycin.

Table I. Strains and Plasmids Used in Hybridization and Cloning Experiments in This Study

	Phenotype*	Reference/derivation
S. faecalis strains		
HH22	(GM, TM, KM, AMIK, SM)^R PEN^R(Bla$^+$) EM^R TC^R	1
HH22-PEN^S	(GM, TM, KM, AMIK, SM)^R PEN^S(Bla$^-$) EM^R TC^R	novobiocin-treated derivative of HH22
XH22	(GM, TM, KM, AMIK,)^R PEN^R(Bla$^+$)	HH22 × JH2-7 transconjugant (1)
XH22-(16)	(GM, TM, KM, AMIK, SM)^R EM^R	HH22 × JH2-7 transconjugant
XH22-(24)	(KM, AMIK, SM)^R EM^R	HH22 × JH2-7 transconjugant
Plasmids		
pI524	(Bla$^+$, Pb, Bi, Cd, Asa, Asi, Ant, Hg, Om)	a staphylococcal plasmid which encodes PEN^R(Bla$^+$) (22)
pJM13	PEN^R(Bla$^+$)TC^R	4.6 MD Eco RI *bla*-specifying fragment of staphylococcal plasmid pI258 cloned into pMB9 (4)
pMB9	TC^R	an *Escherichia coli* cloning vector which does not encode PEN^R or Bla (10)

Pb, Bi, Cd, Asa, Asi, Ant, Hg, and Om indicate resistance to lead, bismuth, cadmium, arsenate, arsenite, antimony, mercury, and organomercurials respectively. For aminoglycosides, resistance is defined as an MIC > 2,000 μg/ml.

and gram-negative plasmids were prepared by a modification of the method of LeBlanc and Lee (11) or as previously described (1). Staphylococci DNA was prepared in a similar fashion substituting lysostaphin (20 μg/ml) for lysozyme. Restriction endonuclease digestions were carried out according to the manufacturer's recommendations. Following electrophoresis in agarose gels, DNA was transferred to nitrocellulose (Schleicher and Schuell, Inc., Keene, NH) or Zeta-Probe filters (Bio-Rad Laboratories) by an adaptation of the method of Southern (12, 13).

Probe DNA included pI524 (31.8 kb), a 4.5-kb *Sal*I fragment from pI524 encoding β-lactamase production and cloned into pMB9 (this study), and an 840-base pair (bp) *Xba*I/*Hin*dIII fragment of pJM13 previously shown to code for the leader peptide and 80% of the mature staphylococcal β-lactamase (4). Plasmid DNA from XH22 was also used to probe the gram-negative β-lactamase–producing strains. Nick translation was performed with ^{32}P-labeled deoxycytidine triphosphate and cold nucleotides according to standard methods (12, 13). ^{32}P-labeled probe DNA was hybridized to blotted DNA and to colony lysates under conditions of high stringency (68°C in 6×SSC or 42°C with 50% formamide [12°C below the melting temperature of duplex DNA, T_m-12°C]) followed by high stringency washes (12).

Results

Enzyme and hydrolysis studies. No detectable hydrolysis of nitrocefin was produced by supernatant material from cultures at various phases of growth until this material was concentrated over 1,000-fold. Following sonication and removal of most intact cells, ~50% of the β-lactamase activity sedimented in the 30,000 *g* spin; ~60% of the remaining supernatant activity was sedimented by centrifugation at 110,000 *g*. Less extensive sonication resulted in more activity being sedimented in the first spin. Passage of the 110,000 *g* supernatant material over the BioGel A 0.5 column resulted in elution of β-lactamase activity in the void volume, indicating an apparent molecular weight of >500,000. Activity of pre- and postchromatographed material was also retained by Diaflo ultrafilter XM100A (Amicon Corp.) confirming a molecular weight of at least 100,000.

The chromatographed material was used to determine the rates of hydrolysis of various substrates (Table II). Penicillin, ampicillin, and the ureido penicillins were most rapidly hydrolyzed, followed by ticarcillin and carbenicillin; there was little or no hydrolysis of methicillin, nafcillin, the cephalosporin-like compounds, or imipenen. This pattern is typical of a penicillinase, such as that of *S. aureus*. The agents which were most rapidly hydrolyzed were also those which had the least efficacy against intact organisms. Table III shows the effect of high and low inocula on the susceptibility of HH22 to the various β-lactam agents. As previously reported, this organism is highly resistant to penicillin with an inoculum of 10^7 CFU/ml but is inhibited by 2 U/ml with 10^3 CFU/ml. The same marked effect of the high inoculum was seen with ampicillin, piperacillin, and mezlocillin, but little or no change was observed with the penicillinase-resistant penicillins, the cephalosporin-like compounds, or imipenem. The susceptibility to ticarcillin was moderately affected (eightfold) by the high inoculum.

The effects of β-lactamase inhibitors on the enterococcal β-lactamase are shown in Table IV. When clavulanic acid (CA) was tested with penicillin, the MIC of penicillin was 2 U/ml (with 50 μg/ml of CA), 4 U/ml (with 25, 10, 4, 2, and 1 μg/ml of CA), 256 U/ml (with 0.5 μg/ml of CA), and >1,000 U/ml

Table II. Relative Hydrolysis of β-Lactams by Partially Purified Enterococcal β-Lactamase

Antibiotics	Percent hydrolysis*
Penicillin	100
Ampicillin	157
Methicillin	3
Nafcillin	<1
Ticarcillin	29
Carbenicillin	44
Piperacillin	175
Mezlocillin	200
Cephalothin	<1
Cefazolin	<1
Cefoperazone	<1
Cephaloridine	<1
Cefotaxime	<1
Moxalactam	<1
Imipenem	<1

* Relative to the rate of hydrolysis of penicillin G.

Table III. Effect of Inoculum on the Susceptibility of Enterococcal Strain HH22 to β-Lactam Antibiotics

MIC (μg/ml)		
	Inoculum (*CFU/ml*)	
Antibiotic	10^3	10^7
Penicillin G	2*	1000
Ampicillin	2	>1000
Methicillin	32	32
Nafcillin	8	8
Ticarcillin	64	500
Piperacillin	2	1000
Mezlocillin	1	>1000
Cephalothin	32	64
Cefoperazone	16	16
Moxalactam	500	500
Imipenem	≤1	≤1

* MIC of penicillin is expressed in units per milliliters.

(with 0.25 μg/ml or less). In the microtiter assay with nitrocefin, CA inhibited hydrolysis at 1.0 μg/ml but not 0.5 μg/ml following 4 h of incubation; at 18 h, there was also hydrolysis with 1.0, 2.0, and 4.0 μg/ml of CA but not with higher concentrations. With sulbactam (SB), the MIC of penicillin was 4 U/ml with 50 μg/ml SB, 16 U/ml with 25 μg/ml SB, 32 with 8 μg/ml SB, and 1,000 U/ml with 2 μg/ml or less (Table IV). In the hydrolysis inhibition assay, 50 μg/ml of SB inhibited visible evidence of hydrolysis at 4 h, with very slight hydrolysis with 25 μg/ml; at 18 h, hydrolysis had occurred even in 100 μg/ml of SB.

Hybridizations. Initial studies showed hybridization of the staphylococcal plasmid pI524 to an 82 kb (54 megadalton [MD]) plasmid from HH22 and its transconjugant XH-22, and to 21.8-kb (14.4 MD) and 5.1-kb (3.4 MD) *Eco*RI digestion fragments of plasmid DNA from the same strains (not shown). The 4.5-kb *Sal*I fragment of pI524 which carries the β-lactamase gene was cloned into pMB9 (which has no β-lactamase gene) and then used as a crude probe for β-lactamase sequences in the enterococcal plasmids. Fig. 1 shows that labeled probe hybridized with the 54-MD (82 kb) plasmid in HH-22 and its β-lactamase-producing (Bla^+) transconjugant XH22. There was no hybridization to plasmids from Bla^- derivatives (XH22-(24) [shown in Fig. 1], XH22-(16), or HH22-PEN^S (Bla^-) [not shown]). This smaller probe hybridized only to a 5.1-kb (3.4 MD) fragment in *Eco*RI digests of plasmid DNA isolated from Bla^+ strains; again, there was no hybridization demonstrated with digested DNA isolated from Bla^- strains (data not shown).

To ensure that the above results represented hybridization due to β-lactamase gene sequences, hybridization was performed using the 840-bp *Xba*I/*Hin*dIII fragment of pJM13 known to be located within the β-lactamase gene of the staphylococcal plasmid pI258 (4). Fig. 2 shows that this probe hybridized only to a 5.1-kb *Eco*RI fragment of HH22 while there was no hybridization to HH22-PEN^S (Bla^-). This fragment appears to be part of a doublet or triplet of similarly sized fragments (better seen in other gels). Neither this probe nor labeled XH22 hybridized to DNA from the gram-negative strains that produced TEM, OXA, or PSE type β-lactamases.

Discussion

Although enterococci are intrinsically more resistant to penicillins than other streptococci (14), β-lactamase production is a new mechanism of resistance for these organisms and in fact is new for the genus *Streptococcus.* In our initial report, we observed that enterococcal strain HH22 was resistant to high levels of penicillin using a heavy inoculum and that penicillin activity was rapidly destroyed in cultures in which this organism was growing (1). We have now shown that this β-lactamase acts like a typical penicillinase, such as produced by staphylococci (15, 16), since it hydrolyzes penicillin, ampicillin, ureido penicillins, and to a lesser extent, carboxy penicillins, but does not hydrolyze penicillinase-resistant penicillins such as methicillin, the cephalosporins, or imipenem (17–19). The hydrolysis results correlate with marked increases in resistance to these compounds when heavy inocula are used. Unfortunately, the β-lactams that resist hydrolysis are in general those, such as the cephalosporins and methicillin, which have little intrinsic activity or clinical usefulness against enterococci (14). They should be useful if this plasmid-mediated resistance determinant moves into other

Table IV. Effect of β-Lactamase Inhibitors on Penicillin Susceptibility of HH22

	Concentration of inhibitor									
	0	0.25	0.5	1	2	4	10	25	50	100
CA (*μg/ml*)										
MIC of penicillin (*U/ml*)*	1,000	1,000	256	4	4	4	4	4	2	2
Hydrolysis‡										
4 h	+	+	+	−	−	−	−	−	−	−
18 h	+	+	+	+	+	±	−	−	−	−
SB (*μg/ml*)										
MIC of penicillin (*U/ml*)*	1,000	1,000	1,000	1,000	1,000	32	32	16	4	4
Hydrolysis‡										
4 h	+	+	+	+	+	+	+	±	−	−
18 h	+	+	+	+	+	+	+	+	+	+

* With an inoculum of 10^7 CFU/ml of HH22. ‡ Hydrolysis of nitrocefin by HH22 (see text).

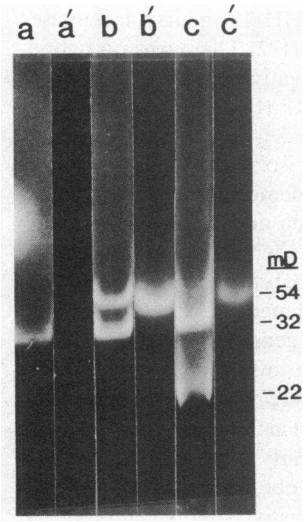

Figure 1. Agarose gel electrophoresis of plasmid DNA from (*a*) a PEN^S (Bla$^-$) transconjugant, XH22-(24) (KM, AMIK, SM)^{R}EM^R (Table I), (*b*) a PEN^R transconjugant, XH22 (GM, TM, KM, AMIK)^R PEN^R (Bla$^+$), (*c*) the original clinical isolate HH22 (GM, TM, KM, AMIK, SM)^{R}PEN^R (Bla$^+$) EM^{R}TC^R, and the corresponding autoradiograph (a′, b′, c′) following hybridization to a ^{32}P-labeled Sal I Bla$^+$ fragment of the staphylococcal plasmid pI524. Approximate sizes of the plasmids of HH22 are given as megadaltons (MD).

streptococci, but they are not likely to have a therapeutic role for enterococcal infections. Imipenem, which shows promise as an effective anti-enterococcal agent in other studies (17, 20), was the only β-lactam intrinsically active as well as resistant to hydrolysis by this enzyme. Vancomycin inhibited this strain (data not shown) as did the combination of CA with penicillin; SB was not as potent as CA, which is again similar to its effect on staphylococcal β-lactamase (21).

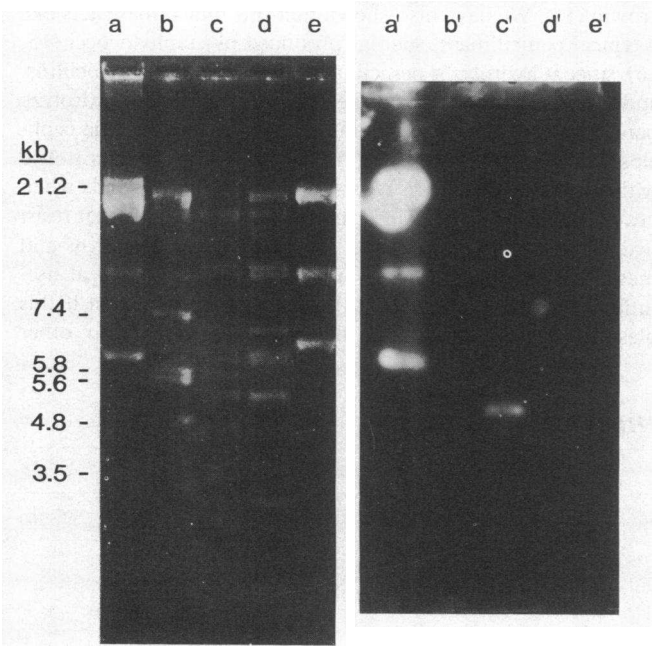

Figure 2. On the left, agarose gel electrophoresis of DNA from (*a*) pJM13 (partially digested), (*b*) lambda digested with *Eco*RI, (*c*) HH22, digested with *Eco*RI, (*d*) the novobiocin-generated derivative, HH22-PEN^S (Bla$^-$), digested with *Eco*RI, and (*e*) lambda digested with *Hin*dIII. On the right is the autoradiograph of this DNA following hybridization to an 840-bp ^{32}P-labeled *Xba*I/*Hin*dIII fragment of pJM13 which encodes most of the staphylococcal *bla* gene (4). The sizes on the left are derived from lambda digested with *Eco*RI (lane b) in kilobases (kb).

The similarity of the substrate profile of the enterococcal β-lactamase to that reported for staphylococcal β-lactamases suggested that their gene sequences might be related and that the enterococcal *bla* gene may be derived from the staphylococcal gene pool. Initial hybridizations using pI524 and its 4.5-kb *Sal*I β-lactamase–specifying fragment suggested that the enterococcal gene was carried on the 82-kb plasmid and was highly homologous to the staphylococcal gene. However, there was concern that the homology demonstrated might be due to non-β-lactamase sequences carried on the pI524 fragment. The 840-bp *Hin*dIII/*Xba*I fragment of pJM13 carries only 75 bp upstream of the *bla* promoter in addition to the promoter, leader sequence, and 80% of the β-lactamase structural gene (4). Thus, it is an extremely specific probe and its hybridization, under stringent conditions, to a 5.1-kb *Eco*RI fragment in digests of plasmid DNA isolated from HH22 and β-lactamase–producing transconjugants but not to the novobiocin "cured" derivative HH22-PEN^S (Bla$^-$) suggests that the β-lactamase gene of HH22 is highly related to that found in *S. aureus*. Other hybridizations using the entire 31.8-kb plasmid pI524 as a probe showed homology with only one additional fragment, which suggests that, with the exception of the penicillinase determinants, the plasmids are largely unrelated. Furthermore, attempts to transfer the plasmid to *S. aureus* have failed (unpublished observations). A number of other antibiotic resistance determinants and transposons in the two genera have been found to be homologous (22); moreover, transfer of certain macrolide-lincosamide-streptogramin resistance plasmids from streptococci to staphylococci and vice versa has been demonstrated (23). With respect to the β-lactamase gene, Shalita et al. (24) have suggested on the basis of the genetic data reported by Ashehov (25) that the *bla* gene and a flanking invertible sequence may constitute a transposable unit. A similar organization of the *bla* gene in HH-22 would strengthen the suggestion that it was introduced from *S. aureus*.

It is interesting that the enterococcal β-lactamase appears to be completely cell bound with a molecular weight of over 500,000. Most β-lactamase producing *S. aureus* strains secrete their β-lactamase as an exoenzyme of ~29,000. A few strains, however, secrete <10% of the enzyme that they produce (26). Nielsen and Lampen (27) showed that *S. aureus* 8325-4 containing pI524 secreted two-thirds and retained one-third of the total β-lactamase as a membrane-bound form (27). The membrane-bound form differed from the exoenzyme in retaining part of the leader sequence which also carried a lipophilic modification (27, 28). The *bla* gene of the staphylococcal plasmid pI258 has also been shown to produce a completely membrane-bound product in *Bacillus subtilis* (28). Thus, the proportion of β-lactamase that is processed to form membrane-bound lipoprotein may be dependent on the host cells. Efforts to solubilize the streptococcal enzyme and determine the basis of its cell binding are in progress.

In summary, the activity of the streptococcal β-lactamase is similar to that of a typical penicillinase. Specific hybridization to staphylococcal β-lactamase gene probes suggests that the penicillinase determinant may have spread from *S. aureus* to streptococci. One could speculate that the difference in the cellular binding of these two enzymes is due to genetic divergence leading to closely related but different proteins or to different processing of identical genes by the different host strains. Further studies will be required to determine which if either of these hypotheses is correct.

Acknowledgments

We especially thank J. Rabinowitz for the important contribution of pJM13 and D. LeBlanc and L. Lee for the many conversations and helpful suggestions regarding plasmid purification.

This work was supported in part by the National Institutes of Health grant AI-19011 to Dr. Murray, by the Medical Research Council of Canada grant MA7288 to Dr. Brunton, and by a fellowship from the Ontario Ministry of Health to Barbara Mederski-Samoraj.

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