

Differential Susceptibilities of Enterococcal Species to Efamycin Antibiotics

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Received 29 December 1993/Returned for modification 17 February 1994/Accepted 10 May 1994

The efamycins are a class of naturally occurring antibiotics not currently used in the therapy of human disease. *Enterococcus faecium* and closely related species (*Enterococcus durans* and *Enterococcus hirae*) are susceptible to these antibiotics, while isolates of *Enterococcus faecalis* and other enterococcal species are highly resistant. Among enterococci, susceptibility or resistance to efamycins appears to be determined by the bacterial protein synthesis elongation factor EF-Tu. Efamycin susceptibility may be a useful adjunct for rapidly distinguishing *E. faecalis* and *E. faecium* in the clinical microbiology laboratory and/or as a supplementary test for use in determining the species of enterococci.

In the past decade, *Enterococcus* species have become an increasingly significant cause of nosocomial infections (8, 12). Of the infections attributable to enterococci, *E. faecalis* is currently responsible for approximately 75 to 80% of clinical infections and *E. faecium* is responsible for the majority of the remainder (12, 13). Identification of enterococci to the species level is important because it relates to the choice of empiric treatment. Historically, ampicillin resistance has often been used to differentiate *E. faecium* from *E. faecalis*, but because of current resistance patterns, this marker is no longer reliable. Consequently, a reproducible and economical alternative to the determination of enterococcal species is desirable.

Efamycins are naturally occurring antibiotics which inhibit bacterial growth by binding to the protein synthesis elongation factor EF-Tu (22). These antibiotics are unable to penetrate into many species of gram-negative bacteria, but they are able to inhibit the EF-Tu's of these species in cell-free protein synthesis systems (10, 22, 23). Among gram-positive species, susceptibility or resistance to efamycins appears to be determined, instead, by the properties of the EF-Tu's of the organisms. Staphylococci and some species of lactobacilli and *Bacillus* are resistant to efamycins (7, 9, 16, 20, 21), and resistance has been reported, but not extensively studied, among enterococci (14). We report on the susceptibility patterns of enterococci to efamycins and on the potential use for these antibiotics as an aid to identification to the species level.

We examined the susceptibilities of enterococcal isolates to three efamycin antibiotics. Kirromycin and aurodox were obtained by fermentation of *Streptomyces ramocissimus* ATCC 27529 and *Streptomyces goldiniensis* ATCC 21386, respectively (1, 19). Efratomylin was kindly provided by Merck Sharpe & Dohme. Solutions of the antibiotics were prepared in dimethyl sulfoxide or dimethylformamide and were diluted with the various assay media so that the final solvent concentration was not greater than 1.3%.

Enterococci were identified to the species level by using Rapid ID32 Strep galleries (Bio Mérieux) or according to

Guiney and Urwin (6). The following type strains were included in the study: *E. faecalis* NCTC 775, *E. faecium* NCTC 7171, *E. casseliflavus* NCIMB 11449, *E. avium* NCTC 9938, *E. durans* NCTC 8174, *E. hirae* NCIMB 6459, *E. raffinosus* NCTC 12192, and *E. gallinarum* NCTC 11428. The remaining isolates were either from the Royal London Hospital collection or from the Lepetit Research Center collection. The latter includes isolates from hospitals in several European countries and the United States; several of these isolates are vancomycin resistant.

MICs were determined by either of two methods: microdilution in Iso-Sensitest broth (Oxoid) or agar dilution in Iso-Sensitest agar. Incubation was for 18 to 24 h at 35 to 37°C. In addition, screening tests were carried out by using either an agar disk diffusion or a breakpoint agar dilution approach. Filter paper disks containing 100 µg of kirromycin were prepared and were dried at 35°C for 3 h in the dark. Disk susceptibility tests were performed by using inocula standardized to produce semiconfluent growth after overnight incubation at 35°C. The agar breakpoint test was performed by replicating the enterococci (10⁴ CFU) onto Iso-Sensitest agar without antibiotic and onto agar containing 0.1, 1, 10, and 100 µg of kirromycin per ml.

An efamycin-resistant derivative of *E. hirae* ATCC 8043 was selected by placing a total of approximately 2 × 10⁹ CFU on Iso-Sensitest agar plates containing 100 µg of kirromycin per ml. One of the three colonies appearing after 24 h of incubation at 37°C was kept for further study, after confirmation (by using a Rapid ID32 Strep gallery) that it was *E. hirae*. To assay cell-free protein synthesis, *E. hirae* ATCC 8043 and its kirromycin-resistant derivative (G1679) were grown to the mid-logarithmic phase (ca. 2 × 10⁸ CFU/ml) in Iso-Sensitest broth and were centrifuged at 13,000 × g for 10 min at 4°C. The bacteria were washed twice by resuspension in 20 ml of saline (0.85% NaCl) and centrifugation and were then suspended in 5 ml of buffer (10 mM Tris-HCl [pH 7.8], 5 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride, 10 µg of leupeptin per ml, 10 µg of trasylyol per ml, 100 µg of lysozyme per ml, 100 µg of lysostaphin per ml, 10 µg of mutanolysin per ml) and incubated for 45 min at 37°C. (All reagents were from Sigma Chemical Co., St. Louis, Mo.) Concentrated supernatants (S-100) and NH₄Cl-washed ribosomes were prepared as described previously (10). Cell-free polyuridylylate-directed polyphenylalanine

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TABLE 1. Susceptibility of enterococci to kirromycin

Species (total no. of isolates)	No. of isolates tested in:		MIC Range ($\mu\text{g/ml}$)	Inhibition zone diam (mm) ^c
	Broth ^a	Agar ^b		
<i>E. faecalis</i> (75)	35	40	>128 >128	6-8
<i>E. avium</i> (6)	2	4	>128 >128	6
<i>E. casseliflavus</i> (7)	3	4	>128 >128	6
<i>E. gallinarum</i> (8)	7	1	>128 >128	6
<i>E. liquefaciens</i> (4)	4		>128	
<i>E. zymogenes</i> (2)	2		>128	
<i>E. raffinosus</i> (4)		4	>128	6
<i>E. faecium</i> (79)	39	40	0.5-2 4	27-33
<i>E. durans</i> (9)	7	2	0.5-2 4	27
<i>E. hirae</i> (5)	4	1	0.13-2 4	27

^a Iso-Sensitest broth microdilution method with inocula of 10^6 CFU/ml.

^b Iso-Sensitest agar dilution method with inocula of 10^5 CFU.

^c Disk diameter, 6 mm.

synthesis was performed as described previously (9). After hot trichloroacetic acid treatment, the samples were filtered through glass fiber filters (Whatman GF/C). The filters were immersed in 10 ml of Ultima Gold (Packard) scintillation fluid and were counted in a Packard model 1600TR liquid scintillation spectrometer.

The MICs of kirromycin for several species of enterococci are given in Table 1. The same pattern of results was obtained with efrotomycin and aurodox and when kirromycin was tested on Iso-Sensitest agar containing lysed horse blood or on MacConkey agar (data not shown). Broth microdilution MICs for susceptible isolates were slightly lower when lower inocula (10^4 CFU/ml) were used, but the susceptibility pattern remained unchanged (data not shown). The disk susceptibility test clearly distinguished resistant and susceptible species (Table 1). When 67 selected isolates were tested in an agar breakpoint screening test by using a lower inoculum (10^4 CFU) than that used for agar dilution MICs (10^5 CFU), all of the susceptible isolates failed to grow on agar containing 1 μg of kirromycin per ml, whereas all of the resistant isolates grew on agar containing 100 μg of the antibiotic per ml. Because the agar dilution MICs were 4 $\mu\text{g/ml}$ for all of the susceptible isolates tested, it would seem reasonable to use 10 μg of kirromycin per ml for routine breakpoint testing.

E. faecium, *E. durans*, and *E. hirae* were susceptible to the elfamycins, while isolates of *E. faecalis* and all other species tested were resistant. Resistance to penicillins, aminoglycosides, or glycopeptides had no apparent effect on the pattern of susceptibility to elfamycins (data not shown). These results suggest that susceptibility to elfamycin antibiotics could be used as a rapid screening test to distinguish between the two species most often encountered in clinical specimens (*E.*

faecalis and *E. faecium*). Furthermore, it appears that the test has the potential to distinguish *E. faecium* from *E. gallinarum* and *E. casseliflavus*. At present, this differentiation is based on motility and pigment production, tests which tend to be unreliable and may be inconvenient to perform in the clinical laboratory (18). An additional test may be of value, particularly since *E. gallinarum* and *E. casseliflavus* are intrinsically resistant to vancomycin (5, 15).

We have demonstrated previously that elfamycin resistance in *E. faecalis* ATCC 7080 was mediated by the intrinsic resistance of its EF-Tu (10). As expected from its susceptibility to elfamycins, a cell-free protein synthesis system from *E. hirae* ATCC 8043 was inhibited by 50% at concentrations of ≤ 1 μg of kirromycin or efrotomycin per ml, while protein synthesis in a system derived from the isogenic resistant strain was not inhibited by drug at 100 $\mu\text{g/ml}$ (data not shown).

On the basis of these studies with *E. hirae* and previous studies with other gram-positive bacteria (7, 9, 10, 16, 20, 21), it appears that resistance or susceptibility to elfamycins in enterococci is mediated by EF-Tu. Although this protein is considered to be highly conserved among eubacteria (3, 11), the elfamycin-binding site appears to be less conserved than other portions of the molecule (10). Studies with *Escherichia coli* have shown that a single amino acid change in EF-Tu can lead to elfamycin resistance (2, 17), and the relative ease of isolation in the laboratory of a resistant derivative of *E. hirae* suggests that this is generally true for bacterial EF-Tu's. However, the fact that in the wild different species within the genus *Enterococcus* have different susceptibilities to elfamycins, independently of their ecological niches, suggests that selective pressure is low at present, despite the use of these antibiotics in animal husbandry (4). One may hope, therefore, that clinical isolates of enterococci will maintain their species-related susceptibility pattern in the future and that an elfamycin may be incorporated into a rapid screening test to aid in the determination of species of enterococci.

REFERENCES

- Berger, J. H. H. Lehr, S. Teitel, H. Maehr, and E. Grunberg. 1973. A new antibiotic X-5108 of *Streptomyces* origin. I. Production, isolation and properties. *J. Antibiot.* **26**:15-22.
- Duisterwinkel, F. J., J. M. DeGraff, B. Kraal, and L. Bosch. 1981. A kirromycin resistant elongation factor EF-Tu from *Escherichia coli* contains a threonine instead of an alanine in position 375. *FEBS Lett.* **131**:89-93.
- Filer, D., and A. V. Furano. 1980. Portions of the gene encoding elongation factor Tu are highly conserved in prokaryotes. *J. Biol. Chem.* **255**:728-734.
- Foster, A. G., and D. L. Harris. 1976. Efrotomycin, a drug for swine dysentery control. *J. Anim. Sci.* **43**:252.
- Green, M., K. Barbadora, and M. Michaels. 1991. Recovery of vancomycin-resistant gram-positive cocci from pediatric liver transplant recipients. *J. Clin. Microbiol.* **29**:2503-2506.
- Guiney, M., and G. Urwin. 1993. Frequency and antimicrobial susceptibility of clinical isolates of enterococci. *Eur. J. Clin. Microbiol. Infect. Dis.* **12**:362-366.
- Hall, C. C., J. D. Watkins, and N. H. Georgopapadakou. 1989. Effects of elfamycins on elongation factor Tu from *Escherichia coli* and *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **35**:2366-2370.
- Jarvis, W. R., and W. J. Martone. 1992. Predominant pathogens in hospital infections. *J. Antimicrob. Chemother.* **29**(Suppl. A):19-24.
- Landini, P., M. Bandera, B. P. Goldstein, F. Ripamonti, A. Soffientini, K. Islam, and M. Denaro. 1992. Inhibition of bacterial protein synthesis by elongation factor Tu-binding antibiotics MDL 62879 and efrotomycin. *Biochem. J.* **283**:649-652.
- Landini, P., M. Bandera, A. Soffientini, and B. P. Goldstein. 1993. Sensitivity of elongation factor Tu (EF-Tu) from different bacte-

- rial species to the antibiotics efrotomycin, pulvomycin and MDL 62879. *J. Gen. Microbiol.* **139**:769–774.
11. Ludwig, W., M. Weizenegger, D. Betzl, E. Leidel, T. Lenz, A. Ludvigsen, D. Möllenhoff, P. Wenzig, and K. H. Schleifer. 1990. Complete nucleotide sequences of seven eubacterial genes coding for the elongation factor Tu: functional, structural and phylogenetic evaluations. *Arch. Microbiol.* **153**:241–247.
 12. Murray, B. E. 1990. The life and times of the enterococcus. *Clin. Microbiol. Rev.* **3**:46–65.
 13. Ruoff, K. L., L. de la Maza, M. J. Murtagh, J. D. Spargo, and M. J. Ferraro. 1990. Species identities of enterococci isolated from clinical specimens. *J. Clin. Microbiol.* **28**:435–437.
 14. Selva, E., G. Beretta, N. Montanini, G. S. Saddler, L. Gastaldo, P. Ferrari, R. Lorenzetti, P. Landini, F. Ripamonti, B. P. Goldstein, M. Berti, L. Montanaro, and M. Denaro. 1991. Antibiotic GE 2270 A: a novel inhibitor of bacterial protein synthesis. *J. Antibiot.* **44**:693–701.
 15. Shlaes, D. M., L. Etter, and L. Guttman. 1991. Synergistic killing of vancomycin-resistant enterococci of classes A, B, and C by combinations of vancomycin, penicillin, and gentamicin. *Antimicrob. Agents Chemother.* **35**:776–779.
 16. Smith I., and P. Pares. 1978. Genetic and biochemical characterization of kirromycin resistance mutations in *Bacillus subtilis*. *J. Bacteriol.* **135**:1107–1117.
 17. Swart, G. W. M., A. Parmeggiani, B. Kraal, and L. Bosch. 1987. Effects of the mutation glycine 222 to aspartic acid on the functions of elongation factor Tu. *Biochemistry* **26**:2047–2054.
 18. Vincent, S., R. G. Knight, M. Green, D. F. Sahn, and D. M. Shlaes. 1991. Vancomycin susceptibility and identification of motile enterococci. *J. Clin. Microbiol.* **29**:2335–2337.
 19. Vos, C., and J. D. Admiral. December 1975. Antibiotic MYC 8003 and process for producing same. U.S. patent 3,927,211.
 20. Woerner, W., C. Gloeckner, M. Mierzowski, and H. Wolf. 1983. On the heterogeneity of elongation factor Tu among eubacteria. *FEMS Microbiol. Lett.* **18**:69–73.
 21. Woerner, W., and H. Wolf. 1982. Kirromycin-resistant elongation factor Tu from wild-type of *Lactobacillus brevis*. *FEBS Lett.* **146**:322–326.
 22. Wolf, H., G. Chinali, and A. Parmeggiani. 1974. Kirromycin, an inhibitor of protein biosynthesis that acts on elongation factor Tu. *Proc. Natl. Acad. Sci. USA* **71**:4910–4914.
 23. Wolf, H., G. Chinali, and A. Parmeggiani. 1977. Mechanism of inhibition of protein synthesis by kirromycin. *Eur. J. Biochem.* **75**:67–75.