Journal of Antimicrobial Chemotherapy (1995) **36**, 821–825

Characterization of vancomycin resistance in *Enterococcus durans*

E. Cercenado[a,*], S. Ünal[a], C. T. Eliopoulos[a], L. G. Rubin[b], H. D. Isenberg[b], R. C. Moellering[a], Jr and G. M. Eliopoulos[a]

[a]*Deaconess Hospital, Boston, Massachusetts 02215 and Harvard Medical School, Boston, Massachusetts 02115,* [b]*Long Island Jewish Medical Center, the Long Island Campus for Albert Einstein College of Medicine, New Hyde Park, New York 11042, USA*

During investigation of an outbreak of vancomycin resistant *Enterococcus faecium* in a paediatric hospital, an isolate of *Enterococcus durans* resistant to vancomycin, teicoplanin, ampicillin and highly resistant to gentamicin and streptomycin was found in the stools of a patient also colonized with a strain of *E. faecium* with the same resistance pattern. Minimal inhibitory concentrations of vancomycin and teicoplanin were 512 and 64 mg/mL, respectively. Resistance to vancomycin as well as high-level resistance to gentamicin was transferable to an *E. faecium* recipient strain. Both multiresistant *E. faecium* and *E. durans* isolates as well as the transconjugant presented only one plasmid. The *vanA* gene was detected and localized to the high molecular weight plasmid by DNA hybridization with a *vanA* gene probe. Growth in vancomycin resulted in induction of an approximately 40 kDa protein visible in membrane preparations from these cells. Genetic linkage between vancomycin and gentamicin resistance genes in the same plasmid is suggested.

Introduction

Resistance to vancomycin has now been described in several enterococcal species. The genetic basis of moderate to high levels of resistance to vancomycin in *Enterococcus faecium* and *Enterococcus faecalis*, and of lower level resistance to vancomycin in *Enterococcus gallinarum* and *Enterococcus casseliflavus*, have been extensively investigated. The *vanA* gene which mediates high-level resistance to vancomycin as well as resistance to teicoplanin, in *E. faecium* and *E. faecalis* has been sequenced and has been localized to a transposable genetic element designated *Tn*1546. DNA sequences of genes from *E. faecalis* and *E. faecium* with moderate to high levels of resistance to vancomycin but susceptibility to teicoplanin (*van*B and *van*B2) and from *E. gallinarum* and *E. casseliflavus* which demonstrate low-level vancomycin resistance (*van*C and *van*C2) have also been determined (Moellering, 1995).

Rare isolates of *Enterococcus avium* (Uttley *et al.*, 1989), *Enterococcus mundtii* (Green, Barbadora & Michaels, 1991) and *Enterococcus durans* (Green *et al.*, 1991, Hall, Chen & Williams, 1992) resistant to vancomycin have been described. Susceptibility testing of a previously reported vancomycin resistant strain of *E. durans* (MIC of

*Correspondence address: Dr Emilia Cercenado, Servicio de Microbiología, Hospital General Gregorio Marañón, Dr. Esquerdo 46, 28007 Madrid, Spain. Tel: +34-(91)-5868453 Fax: +34-(91)-5868018.

0305–7453/95/110821+05 $12.00/0

vancomycin > 256 mg/L, teicoplanin 16 mg/L) revealed a class A glycopeptide resistance pattern (Hall, *et al.*, 1992), but to our knowledge, mechanisms of resistance in this species have not been further explored. During investigation of an outbreak of colonization with vancomycin resistant *E. faecium* in a paediatric hospital (Rubin *et al.*, 1992), an isolate of *E. durans* resistant to vancomycin, teicoplanin, ampicillin and highly resistant to gentamicin and streptomycin was found in the stools of a patient also colonized with *E. faecium* resistant to vancomycin, teicoplanin, ampicillin and highly resistant to gentamicin and streptomycin. In the present study, we investigated the basis of glycopeptide resistance in this isolate of *E. durans* and suggest the acquisition of a vancomycin resistance plasmid from *E. faecium* as well as the genetic linkage of resistance to vancomycin and high-level resistance to gentamicin on the same plasmid.

Methods

Identification and susceptibility testing of the bacterial strains

E. durans SF 170 and *E. faecium* SF 169 were stool isolates from a paediatric oncology patient hospitalized in Long Island, NY. The organisms were identified by the API Rapid Strep System (Analytab Products, Plainview, NY). Testing for β-lactamase was performed using a nitrocefin disk (BBL Microbiology Systems, Cockeysville, MD, USA). Antimicrobial susceptibility was determined by an agar dilution technique (National Committee for Clinical Laboratory Standards, 1990) on Mueller-Hinton II agar (MHA, BBL) with an inoculum of approximately 10^4 cfu per spot. Testing for high-level resistance to streptomycin and gentamicin utilized MHA plates containing 2000 and 500 mg/L of each aminoglycoside, respectively. Ampicillin was obtained from Bristol Laboratories, Evansville, IN; vancomycin was from Eli Lilly & Co., Indianapolis, IN.; teicoplanin was a gift of Marion Merrell Dow, Cincinnati, OH; gentamicin was from Elkins-Sinn, Inc., Cherry Hill, NJ; and streptomycin was purchased from Sigma Chemical Company, St. Louis, MO, USA.

Conjugation studies

Transfer of vancomycin resistance and of high-level resistance to gentamicin was assessed by a cross-streak mating technique utilizing *E. faecium* GE-1 (Eliopoulos *et al.*, 1988) as a recipient. Screening for transconjugants was performed on plates containing vancomycin 20 mg/L, rifampin 100 mg/L, and fusidic acid 25 mg/L.

DNA hybridization studies

Plasmid DNA from *E. durans* SF 170 and from *E. faecium* SF 169 were obtained using the alkaline extraction technique of Birnboim with modifications as described previously (Eliopoulos *et al.*, 1988). Plasmid DNA was separated by agarose gel electrophoresis and visualized under ultraviolet light after staining with ethidium bromide. DNA was transferred on to nylon membranes (Amersham Corp., Arlington Heights, IL) by blotting. Probe DNA was generated by PCR amplification of a *vanA* template from *E. faecium* 228 (Handwerger, Pucci & Kolokathis, 1990) using primers internal to the structural gene: primer 1, corresponding to nucleotides 407 to 425 of *vanA*, 5′-GGGGGTTGCTCAGAGGAGC-3′; primer 2, the reverse complement of nucleotides

1339 to 1360, 5′-TGCAATACCTGCAGCGGCCTAC-3′. Digoxigenin labelling of probe DNA and hybridization under stringent conditions were performed according to instructions of the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA).

Cell membrane protein analysis

Membranes were prepared from cells of *E. durans* SF 170 grown to late stationary phase in glucose phosphate broth medium (Scott Laboratories, West Warwick, RI, USA) with 0.1% citrate sodium dihydrate, with and without vancomycin 20 mg/L. Cells from 1 L overnight broth cultures were pelleted, washed, and resuspended in 10 mL of 10 mM sodium phosphate buffer, pH 7.0, with lysozyme 100 mg/L (Sigma) and 5 mM EDTA and incubated for 30 min at 35°C; cells were then disrupted by sonication. Unbroken cells were removed by low speed centrifugation and cell membranes collected by ultracentrifugation at 135,000*g*. Proteins were separated on 8% SDS-polyacrylamide gels and visualized after staining with Coomassie brilliant blue dye.

Results and discussion

SF 170 was identified as *E. durans* and SF 169 as *E. faecium* with characteristic API Rapid Strep profiles. MICs of vancomycin and teicoplanin for *E. durans* SF 170 were 512 and 64 mg/L, respectively. The isolate was also resistant to ampicillin (MIC 32 mg/L) but did not produce β-lactamase, and it exhibited high-level resistance to both streptomycin and gentamicin. MICs of vancomycin and teicoplanin for *E. faecium* SF 169 were 512 and 128 mg/L, respectively. The isolate was also resistant to ampicillin (MIC 128 mg/L) but did not produce β-lactamase, and showed high-level resistance to both streptomycin and gentamicin. Both *E. durans* SF 170 and *E. faecium* SF 169 possessed a single high molecular weight (approximately 70 kb) plasmid. Hybridization of the labelled *vanA* gene probe to both plasmids was unequivocal (not shown). Vancomycin resistance as well as high-level resistance to gentamicin was transferred from *E. durans* SF 170 into *E. faecium* GE-1 by cross-streak mating. Frequency of transfer was not formally determined, but numerous transconjugants appeared on selective plates. Control experiments verified that neither donor nor recipient organisms grew on these plates. Neither high-level resistance to streptomycin, nor resistance to ampicillin was transferred. As compared with those derived from cells grown in broth alone, cytoplasmic membranes prepared from *E. durans* SF 170 grown to stationary phase in the presence of vancomycin 20 mg/L demonstrated a substantial increase in the density of a *c.* 40 kDa protein band (Figure).

Hybridization of a *vanA* probe with DNA of *E. durans* Sf 170 confirms that the class A glycopeptide resistance phenotype demonstrated by this strain is due to the *vanA* gene, as in *E. faecium* SF 169. Localization of the resistance gene to plasmid DNA and transfer of resistance into suitable recipients by conjugation are other features of the class A phenotype associated with the *vanA* gene in *E. faecium* and *E. faecalis* (Dutka-Malen *et al.*, 1990*a*). Introduction of synthesis of an approximately 40 kDa cytoplasmic membrane protein upon growth in vancomycin seen with the *E. durans* isolate corresponds precisely with observations of *E. faecium* BM4147, the prototype class A (*vanA*) strain (Dutka-Malen *et al.*, 1990b).

Isolation of *E. durans* SF 170 and of *E. faecium* SF 169 both with the same resistance pattern from the same patient's sample suggests that the *E. durans* probably acquired

resistance from the vancomycin resistant *E. faecium*, an explanation confirmed by molecular evidence because the 70 kb plasmid was the only plasmid present in both strains and conferred resistance to vancomycin and high-level resistance to gentamicin.

Another point of interest is the presence of high-level resistance to both gentamicin and streptomycin in *E. durans*. These resistances have not frequently been reported in this species; moreover, it seems that resistance to vancomycin and gentamicin was present on the same plasmid of our isolates. We have not determined if an *aac6'aph2"* probe hybridizes with the 70 kb plasmid, but the presence in the transconjugant of this plasmid as well as its pattern of resistance to both vancomycin and gentamicin suggests genetic linkage of these two important resistances on the same plasmid.

E. durans is an uncommon cause of human infections (Moellering, 1995). Nevertheless, observations with the strain studied here raise three major concerns. First, glycopeptide resistance occurred in a strain which also demonstrated significant resistance to ampicillin and high-level resistance to both streptomycin and gentamicin. In this manner, *E. durans* SF 170 resembles multiple antibiotic resistant *E. faecium* which creates significant therapeutic difficulties and major infection control concerns. Secondly, demonstration of conjugal transfer of both vancomycin and gentamicin resistance from *E. durans* into a recipient strain of *E. faecium* indicates that isolates of *E. durans* could play an important role in dissemination of resistance genes irrespective of their own potential to cause invasive disease in humans. Thirdly, although gentamicin and vancomycin resistances

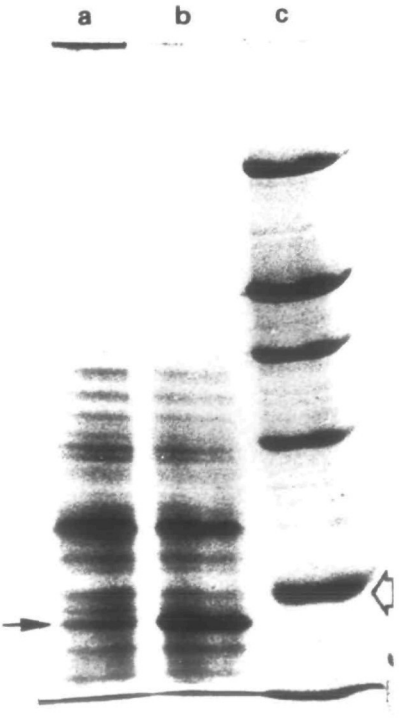

Figure 1. Cell membrane protein analysis of *Enterococcus durans* SF 170. Lane a, strain grown in the absence of vancomycin; lane b, strain grown in the presence of vancomycin 20 mg/L; lane c, molecular weight standard, bands correspond to 200, 116, 97.4, 66 and 45 kDa. Black arrow indicates the increase in the density of a *ca.* 40 kDa protein band. Open arrow indicates the situation of the 45 kDa standard protein.

have been shown to occur together in some strains, this is to our knowledge, the first demonstration of genetic linkage between vancomycin and gentamicin resistance genes in the same plasmid. If such multiresistance plasmids emerge, they would have serious implications.

Acknowledgements

E. Cercenado was supported by a grant from the Fondo de Investigación Sanitaria del Instituto Nacional de la Salud, Ministerio de Sanidad y Consumo, Spain.

References

Dutka-Malen, S., Leclercq, R., Coutant, V., Duval, J. & Courvalin, P. (1990*a*). Phenotypic and genotypic heterogeneity of glycopeptide resistance determinants in Gram-positive bacteria. *Antimicrobial Agents and Chemotherapy* **34,** 1875–9.

Dutka-Malen, S., Molinas, C., Arthur, M. & Courvalin, P. (1990*b*). The VANA glycopeptide resistance protein is related to D-analyl-D alanine ligase cell wall biosynthesis enzymes. *Molecular and General Genetic* **224,** 364–72.

Eliopoulos, G. M., Wennersten, C., Zighelboim-Daum, S., Reiszner, E., Goldmann, D. & Moellering, R. C. (1988). High-level resistance to gentamicin in clinical isolates of *Streptococcus (Enterococcus) faecium*. *Antimicrobial Agents and Chemotherapy* **32,** 1528–32.

Green, M., Barbadora, K. & Michaels, M. (1991). Recovery of vancomycin-resistant gram-positive cocci from pediatric liver transplant recipients. *Journal of Clinical Microbiology* **29,** 2503–6.

Hall, L. M. C., Chen, H. Y. & Williams, R. J. (1992). Vancomycin-resistant *Enterococcus durans*. *Lancet* **340,** 1105.

Handwerger, S., Pucci, M. J. & Kolokathis, A. (1990). Vancomycin resistance is encoded on a pheromone response plasmid in *Enterococcus faecium* **228.** *Antimicrobial Agents and Chemotherapy* **34,** 358–60.

Moellering, R. C. (1995). *Enterococcus* species, *Streptococcus bovis* and *Leuconostoc* species. In *Principles and Practice of Infectious Diseases* (Mandell, G. L., Bennett, J. E. & Dolin, R., Eds), pp. 1826–35. Churchill Livingstone, New York.

National Committee for Clinical Laboratory Standards. (1990). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically—Second Edition Approved Standard M7–A2*. NCCLS, Villanova, PA.

Rubin, L. G., Tucci, V., Cercenado, E., Eliopoulos, G. M. & Isenberg, H. D. (1992). Vancomycin-resistant *Enterococcus faecium* in hospitalized children. *Infection Control and Hospital Epidemiology* **13,** 700–5.

Uttley, A. H. C., George, R. C., Naidoo, J., Woodford, N., Johnson, A. P., Collins, C. H. *et al.* (1989). High-level vancomycin-resistant enterococci causing hospital infection. *Epidemiology and Infection* **103,** 173–81.

(*Received 3 February 1995; returned 17 February 1995; revised 10 May 1995; accepted 26 June 1995*)