Vancomycin Resistance Is Encoded on a Pheromone Response Plasmid in Enterococcus faecium 228

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In Enterococcus faecium 228, vancomycin resistance is encoded on a 55-kilobase conjugative plasmid, pHKK100. This plasmid was transferred with high frequency into susceptible strains of Enterococcus faecalis and conferred responses to pheromones produced by E. faecalis and Streptococcus sanguis. pHKK100 is the first plasmid described that mediates both vancomycin resistance and pheromone response.

Antibiotic resistance among gram-positive bacteria has been increasing over the last few decades (5). Within the past 2 years, clinical isolates of vancomycin-resistant enterococci have been described (10, 11, 17, 19, 20). In the present study, we describe an isolate of Enterococcus faecium in which vancomycin resistance is mediated by a conjugative 55-kilobase (kb) plasmid that also confers beta-hemolysin production and pheromone responsiveness.


E. faecium 228 was isolated from a patient in Spain in 1987 and was the generous gift of L. McDougal, Centers for Disease Control, Atlanta, Ga. Enterococcus faecalis 236R is a hemolysin-negative, rifampin-resistant derivative of ATCC 23655. JH2-2 (gift of D. Clewell, University of Michigan, Ann Arbor), OG1RF, and FA373 are E. faecalis strains which produce multiple pheromones (4). FA373 harbors the pheromone response plasmid pAM373 (2). Streptococcus sanguis Challis V288 was the gift of G. Pozzi, Istituto di Microbiologia, Siena, Italy.

E. faecium 228 was resistant to vancomycin (MIC, >256 µg/ml), teicoplanin (MIC, 32 µg/ml), erythromycin (MIC, >64 µg/ml), chloramphenicol (MIC, 40 µg/ml), and gentamicin (MIC, 64 µg/ml), as determined by twofold dilutions of antibiotic in brain heart infusion broth with an inoculum of 10⁶ CFU. When overnight cultures were diluted 1:10 into brain heart infusion broth containing 50 µg of vancomycin per ml, the lag phase for cultures grown overnight in medium containing 1 µg of vancomycin per ml was 2 h less than that for cultures grown overnight without antibiotic, suggesting that resistance was inducible in strain 228, as in previously characterized isolates of resistant enterococci (11, 15, 17, 20).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate cell membrane preparations as previously described (6). Preparations from strain 228 grown in 10 µg of vancomycin per ml demonstrated a 38,000-dalton protein similar to the inducible membrane protein associated with vancomycin resistance in other isolates of E. faecalis and E. faecium (15, 17, 20). This protein was not visualized when 228 was grown without vancomycin.

Vancomycin-susceptible progeny of strain 228 were obtained by incubation with 1/2 the MIC of ciprofloxacin or at 42°C for 18 h. Plasmid DNA was isolated by the procedure of Portnoy and White (3) and identified by agarose gel electrophoresis. Strain 228 demonstrated four plasmids ranging from 4 to 55 kb in size. The 55-kb plasmid was present in all resistant derivatives tested and absent in susceptible derivatives. Revertants were susceptible to teicoplanin but retained resistance to other antibiotics. When preparations of purified plasmid DNA (13) from strain 228 were used to transform protoplasts of E. faecalis 236R (21), vancomycin-resistant transformants were obtained at a frequency of 3.5 × 10⁻⁴ µg of DNA. All transformants demonstrated the 55-kb plasmid. Taken together, these findings suggest that in 228, vancomycin resistance is mediated by the 55-kb plasmid, pHKK100. Leclercq and colleagues described four related plasmids that mediate vancomycin resistance in E. faecium (11, 12). pHKK100 appears to differ from these plasmids in its larger size (55 versus 30 to 40 kb) and its lack of other antibiotic resistance determinants.

Vancomycin resistance also could be transferred from strain 228 to susceptible strains of E. faecalis by conjugation. In filter mating experiments (18) with 236R as recipient, mean conjugation frequencies of 2.5 × 10⁻² per donor were obtained. One transconjugant, 23030, was selected for further study. Plasmid DNA from 23030 was used for restriction digests (SalI, AvaI, BamHI, and Sphi) as previously described (16), yielding an estimated size of pHKK100 of 55 kb based on summation of fragments.

Like the wild type, strain 23030 was able to transfer vancomycin resistance in filter matings. Conjugation frequencies with recipient E. faecalis strains JH2-2 and OG1RF were 3.7 × 10⁻¹ and 1.1 × 10⁻¹ per donor, respectively. The very high conjugation frequencies observed with pHKK100 compared with those of other plasmids that mediate vancomycin resistance (11, 12, 15) suggested that this plasmid confers a pheromone response.

Pheromone response plasmids described in E. faecalis are generally 45 to 60 kb in size. Strains harboring these plasmids aggregate in response to sex pheromones (clumping inducing agents) excreted by recipient cells. The clumping response allows conjugal transfer to occur with high frequency in both broth cultures and solid media (2, 4). Pheromone production has been demonstrated in several gram-
positive species, but pheromone response plasmids have been described only in \textit{E. faecalis} (2, 4).

To determine whether pHKK100 conferred a pheromone response, matings were performed in broth and the response to filtrates of pheromone-producing strains was tested. Both strain 228 and transconjugant 23030 were able to transfer vancomycin resistance in broth matings to \textit{E. faecalis} FA373, JH2-2, and OG1RF at frequencies ranging from $4 \times 10^{-3}$ to $1 \times 10^{-5}$ per donor, suggestive of a pheromone response.

Clumping inducing agent assays were performed as described by Dunny and colleagues (4), using 228 and 23030 as the responder strains. The results are expressed as the highest dilution demonstrating a clumping response. Both strains responded to filtrate of JH2-2 (1:16), which produces multiple pheromones (2). The addition of EDTA (50 mM) dispersed these clumps, again indicative of pheromone-mediated aggregation (22). Filtrates of \textit{S. sanguis} Challis also caused a clumping response (1:16). Thus, the pheromone to which pHKK100 responds differs from the previously described pheromones cAMY1, cAMY2, cAMY3, cPD1, and cAD1, since strains harboring plasmids responding to these pheromones do not clump upon exposure to filtrates of strain Challis (2). These observations suggested that pHKK100 responded to cAM373, which is produced by strain Challis (2). However, 228 showed a clumping response to filtrate of FA373 (1:4) and was able to mate with this strain in broth. Therefore, pHKK100 appears to respond to a pheromone different from cAM373, since production of a given pheromone is inhibited in cells harboring this pheromone to which pheromone responds; i.e., FA373, which harbors pAM373, does not produce cAM373 (8). pHKK100 may respond to a yet uncharacterized pheromone.

Pheromone responsiveness has been described for several plasmids that confer resistance to other classes of antibiotics in clinical isolates of \textit{E. faecalis}, including the $\beta$-lactamase-producing strain HH22 (1, 14). However, pheromone response has not been previously described in \textit{E. faecium} or in association with glycopeptide resistance.

Screening of vancomycin-resistant transconjugants on 4% horse blood agar revealed that transfer of pHKK100 also conferred beta-hemolysis, commonly associated with pheromone response plasmids (4). Recent evidence suggests that enterococcal hemolysins play a role in pathogenicity. Ike and colleagues demonstrated increased virulence in experimental murine infections with \textit{E. faecalis} harboring the hemolysin plasmid pAD1 compared with that of transposon insertion-generated hemolysin-defective mutants (9).

Thus, our findings suggest that pHKK100 encodes a possible virulence factor and has a high potential for conjugative transfer of resistance. Although the effect of pheromone responsiveness on the rate of conjugal transfer in vivo is unknown, clumping inducing agent response is more common among antibiotic-resistant clinical isolates of \textit{E. faecalis} than among susceptible isolates (4). Both hemolysin production and pheromone responsiveness are observed with greater frequency among clinical isolates of enterococci than among fecal colonizers (7). Although pheromones are produced by some \textit{S. sanguis} and \textit{Staphylococcus aureus} isolates, transfer of pheromone response plasmids to these organisms has not been demonstrated (2). The implications of pheromone responsiveness for the spread of resistance to other species remain unclear.

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LITERATURE CITED


