Cloning and Heterospecific Expression of the Resistance Determinant vanA Encoding High-Level Resistance to Glycopeptides in Enterococcus faecium BM4147

ANNE BRISSON-NOËL, SYLVIE DUTKA-MALEN, CATHERINE MOLINAS, ROLAND LECLERCQ, AND PATRICE COURVALIN

Unité des Agents Antibactériens, Unité Associée Centre National de la Recherche Scientifique 271, Institut Pasteur, 75724 Paris Cedex 15, and Service de Bactériologie-Virologie-Hygienne, Hôpital Henri Mondor, Université Paris XII, 94010 Créteil, France

Received 31 July 1989/Accepted 21 January 1990

Fragments of plasmid pIP816, which confers high-level glycopeptide resistance in Enterococcus faecium BM4147, were cloned into a conjugative gram-negative-gram-positive shuttle vector. The resulting hybrids were transferred by conjugation from Escherichia coli to Enterococcus faecalis and Bacillus thuringiensis. A 4-kilobase EcoRI fragment from pIP816 was found to confer vancomycin resistance in these hosts but not in E. coli or Bacillus subtilis.

Glycopeptides constitute an important class of antibiotics for the treatment of severe infections due to gram-positive microorganisms, especially in the case of intolerance or resistance to the beta-lactams (5, 6, 9). Certain gram-positive bacteria considered opportunistic pathogens, such as Leuconostoc spp. (2), Pediococcus spp. (3), and strains of Lactobacillus spp. (C. Thornsberry and R. Facklam, Antimicrob. Newsl. 1:8, 1984), are intrinsically resistant to glycopeptides. Staphylococci, streptococci, and enterococci were previously considered uniformly susceptible to these antibiotics (23). However, glycopeptide resistance in certain strains of coagulase-negative staphylococci (1, 15, 24) and of enterococci (8, 10, 11, 16, 17, 22) has been recently reported.

We previously established that resistance to high levels of vancomycin and teicoplanin in four clinical isolates of Enterococcus faecium was plasmid mediated and inducible by subinhibitory concentrations of glycopeptide antibiotics (10, 11). Plasmids conferring glycopeptide resistance in these strains were either nonconjugative (pIP816 in E. faecium BM4147) or self-transferable to E. faecium.

In this report, we describe the cloning of the resistance determinant vanA encoding inducible high-level resistance to glycopeptides in E. faecium BM4147 (10). A particular cloning strategy was developed, which enabled us to study the expression of this gene in several bacterial species.

Cloning strategy. Cloning of the vanA resistance determinant in Escherichia coli on the basis of vancomycin selection was not possible, since E. coli, like most gram-negative organisms, displays a natural resistance to glycopeptides which cannot cross the outer membrane. Although E. coli mutants susceptible to these drugs have been obtained under laboratory conditions (18), these strains exhibit a high reversion rate (A. Brisson-Noël, unpublished data) which excludes their use for cloning. It was therefore necessary to use a gram-positive organism as a host for cloning experiments. Direct cloning in gram-positive bacteria implies tedious techniques such as protoplast transformation or electroporation, which are often of poor efficacy. We therefore developed a two-stage procedure based on the use of the gram-negative-gram-positive shuttle vector pAT187 (20). This plasmid can replicate in both gram-positive and gram-negative organisms, where it confers kanamycin resistance, and can be mobilized from E. coli to various gram-positive bacteria. In order to allow cloning of EcoRI-generated DNA fragments, a pAT187 derivative with a unique EcoRI site was constructed as follows. Plasmid pAT187, which contains two EcoRI sites, was linearized by partial digestion with EcoRI, the cohesive ends were filled to generate blunt ends, and the plasmid was recircularized by ligation, giving plasmid pAT187-1.

Determinant vanA, conferring high-level resistance to glycopeptides in BM4147, is borne by plasmid pIP816 (34 kilobases [kb]) (10). An in vivo deletant of pIP816 conferring inducible resistance to glycopeptides, pIP816-1 (22 kb), was obtained after transformation into Streptococcus sanguis Challis (data not shown).

The strategy of cloning is schematically represented in Fig. 1. In the first step, EcoRI restriction fragments of pIP816-1 were cloned into pAT187-1 and introduced by transformation into E. coli JM83 (13). Multiple enzyme restriction profiles of recombinant plasmids from transformants resistant to kanamycin were analyzed by agarose gel electrophoresis and were compared with those of pIP816-1. Plasmid pAT211 contained a 10-kb insert that could result from a partial EcoRI digestion of pIP816-1. Plasmids pAT212 and pAT213 contained the 6- and 4-kb EcoRI portions of the 10-kb insert, respectively (Fig. 2).

In the second step, pAT211, pAT212, and pAT213 were transformed into E. coli JM83 containing the conjugative plasmid pRK24 (4), which can mobilize the shuttle vector pAT187-1 and its derivatives. The resulting transformants were then used as donors in mating experiments (20) with Enterococcus faecalis JH2-2 (7) and a Bacillus thuringiensis HD1 cry mutant (12). Kanamycin-resistant transconjugants were tested for resistance to glycopeptides. In parallel experiments, the same plasmids were introduced by transformation into Bacillus subtilis BS168 (19) and E. coli 16 (18), a mutant susceptible to glycopeptides, and transfor-
mants resistant to kanamycin were tested for glycopeptide resistance. The plasmid content of the transconjugants and of the transformants was studied by agarose gel electrophoresis of crude bacterial lysates after digestion with EcoRI endonuclease.

Expression of glycopeptide resistance in \(E.\ faecalis\) JH2-2. \(E.\ faecalis\) JH2-2 containing pAT211 expressed glycopeptide resistance at a high level, similar to that of \(E.\ faecium\) BM4147 (Table 1) (10). In contrast, pAT213 conferred low-level resistance and pAT212 did not confer glycopeptide resistance. These results indicate that a determinant conferring glycopeptide resistance is present on the 4-kb fragment. However, additional sequences in the 6-kb EcoRI fragment appear to be required for full expression of the glycopeptide resistance phenotype mediated by pIP816, pIP816-1, and pAT211. Study of the induction of glycopeptide resistance revealed that \(E.\ faecalis\) JH2-2 containing plasmids pAT211 and pAT213 displayed the same pattern of inducibility as did the original \(E.\ faecium\) clinical isolate BM4147 (10; Fig. 3).

**Heterospecific expression of the \(vanA\) determinant.** The results listed in Table 1 show that the glycopeptide resistance encoded by \(vanA\) is expressed in \(E.\ faecalis\) JH2-2 and a \(B.\ thuringiensis\) HD1 cry mutant. However, whereas \(vanA\) confers in \(E.\ faecalis\) the same level of resistance as it does in the original \(E.\ faecium\) host (10), the expression appeared to be lower in \(B.\ thuringiensis\). No expression of resistance was found in \(B.\ subtilis\) BS168 or in \(E.\ coli\) 16. To rule out the possibility that lack of expression was due to deletions, plasmids pAT211 and pAT213 were purified from the \(B.\ subtilis\) and \(E.\ coli\) transformants, transferred into \(E.\ coli\) JM83 containing pRK24, and transferred by conjugation into \(E.\ faecalis\) JH2-2. In all cases, vancomycin resistance was fully expressed in \(E.\ faecalis\) JH2-2. Since most enterococcal promoters function in \(E.\ coli\) (21) and in \(B.\ subtilis\) (14),

**FIG. 1.** Strategy used for the cloning of gene \(vanA\). Fragments of pIP816-1 inserted into pAT187-1 are depicted by a thick line. Km, Kanamycin; Vm, vancomycin; S, susceptible.

**TABLE 1.** Heterospecific expression of the \(vanA\) resistance determinant

| Host                        | MIC (µg/ml) of glycopeptide against strain harboring\(^a\): |   |   |   |   |   |   |   |   |
|-----------------------------|----------------------------------------------------------|---|---|---|---|---|---|---|---|
|                             | pIP816 | no plasmid | pAT211 | pAT212 | pAT213 | Vm | Tei | Vm | Tei | Vm | Tei | Vm | Tei |
| \(E.\ faecium\) BM4147      | 1,024   | 512        | 4      | 1      | —\(^b\) |     |     |     |     |     |     |     |     |
| \(E.\ faecalis\) JH2-2      | —       | —          | 2      | 0.5    | 4,000  | 128 | 2   | 0.5 | 128 | 32  |     |     |     |
| \(B.\ thuringiensis\) HD1 cry mutant | —       | —          | 0.5    | 0.5    | 64     | 16  | 0.5 | 0.5 | 8   | 4   |     |     |     |
| \(B.\ subtilis\) BS168      | —       | —          | 0.5    | 0.5    | 0.5    | 0.5 |     |     |     |     |     |     |     |
| \(E.\ coli\) 16             | —       | —          | 8      | 32     | 8      | 32  | —   | —   | —   | —   |     |     |     |

\(^a\) Vm, Vancomycin; Tei, teicoplanin.

\(^b\) —, Not determined.
Levels of glycopeptide resistance could therefore result from features affecting the transport or the proper positioning of the resistance protein into the membrane or both.

We thank P. Trieu-Cuot for helpful advice and discussions and M. Arthur for critical reading of the manuscript. A. Brisson-Noël was a recipient of a fellowship from Laboratoires Léderlé, Rungis, France.

LITERATURE CITED

1. Arioli, V., and R. Pallanza. 1987. Teicoplanin-resistant coagulase-negative staphylococci. Lancet i:39.
2. Bus-Hoi, A., C. Branger, and J. F. Acar. 1985. Vancomycin-resistant streptococci or Leuconostoc spp. Antimicrob. Agents Chemother. 28:458–460.
3. Colman, G., and A. Efstradiou. 1987. Vancomycin-resistant leuconostocs, lactobacilli, and now pediococci. J. Hosp. Infect. 10:1–3.
4. Figurski, D. H., and D. Helsinki. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA 76:1646–1652.
5. Geraci, J. E., and W. R. Wilson. 1981. Vancomycin therapy for infective endocarditis. Rev. Infect. Dis. 3:S250–S258.
6. Gund, D. W. 1981. Vancomycin for treatment of bacterial meningitis. Rev. Infect. Dis. 3:S289–S292.
7. Jacob, A. F., and S. J. Hobbs. 1974. Conjugal transfer of plasmid-borne multiple antibiotic resistance in Streptococcus faecalis var. zymogenes. J. Bacteriol. 117:360–372.
8. Kaplan, A. H., P. H. Dillard, and R. R. Facklam. 1988. Recovery of resistant enterococci during vancomycin prophylaxis. J. Clin. Microbiol. 26:1216–1218.
9. Kirby, W. M. 1981. Vancomycin therapy in severe staphylococcal infections. Rev. Infect. Dis. 3:S236–S239.
10. Leclercq, R., E. Derlot, J. Duval, and P. Courvalin. 1988. Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium. N. Engl. J. Med. 319:157–161.
11. Leclercq, R., E. Derlot, E. Weber, J. Duval, and P. Courvalin. 1989. Transferable vancomycin and teicoplanin resistance in Enterococcus faecium. Antimicrob. Agents Chemother. 33:10–15.
12. Lereclus, D. H., G. Menou, and M. Lecadet. 1983. Isolation of a DNA sequence related to several plasmids from Bacillus thuringiensis after a mating involving the Streptococcus faecalis plasmid pAM1. Mol. Gen. Genet. 191:307–313.
13. Messing, J. 1979. A multipurpose cloning system based on the single-stranded bacteriophage M13. Recomb. DNA Tech. Bull. 1:43–44.
14. Moran, C. P., N. Lang, S. F. Le Grice, G. Lee, M. Stephens, A. L. Sonenshein, J. Pero, and R. Losick. 1982. Nucleotide sequences that signal the initiation of transcription and translation in Bacillus subtilis. Mol. Gen. Genet. 186:339–346.
15. Schwalbe, R. S., J. T. Stapleton, and P. H. Gilligan. 1987. Emergence of vancomycin resistance in coagulase-negative staphylococci. N. Engl. J. Med. 316:927–931.
16. Shaes, D. M., S. Al-Obeid, J. H. Shaes, A. Boisvin, and R. Williamson. 1989. Inducible, transferable resistance to vancomycin in Enterococcus faecium, D399. J. Antimicrob. Chemother. 23:503–508.
17. Shaes, D. M., A. Bouveret, C. Devine, J. H. Shaes, S. Al-Obeid, and R. Williamson. 1989. Inducible, transferable resistance to vancomycin in Enterococcus faecalis A256. Antimicrob. Agents Chemother. 33:198–203.
18. Shaes, D. M., J. H. Shaes, J. Davies, and R. Williamson. 1989. Escherichia coli susceptible to glycopeptide antibiotics. Antimicrob. Agents Chemother. 33:192–197.
19. Spizizen, J. 1958. Transformation of biochemically deficient strain of Bacillus subtilis by deoxyribonucleic acid. Proc. Natl. Acad. Sci. USA 44:1072–1078.
20. Trieu-Cuot, P., C. Carlier, P. Martin, and P. Courvalin. 1987. Plasmid transfer by conjugation from Escherichia coli to Gram-
positive bacteria. FEMS Microbiol. Lett. 48:289–294.

21. Trieu-Cuot, P., A. Klier, and P. Courvalin. 1985. DNA sequences specifying the transcription of the streptococcal kanamycin resistance gene in Escherichia coli and in Bacillus subtilis. Mol. Gen. Genet. 198:348–352.

22. Uttley, A. H., C. H. Collins, J. Naidoo, and R. C. George. 1988. Vancomycin-resistant enterococci. Lancet i:57–58.

23. Watanakunakorn, C. 1981. The antibacterial action of vancomycin. Rev. Infect. Dis. 3:S210–S215.

24. Wilson, A. P. R., M. D. O’Hare, D. Felmingham, and R. N. Gruneberg. 1986. Teicoplanin-resistant coagulase negative Staphylococcus. Lancet ii:973.