ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1997, p. 1409 Vol. 41, No. 6
0066-4804/97/$04.00+0

Vancomycin Dependence in a VanA-Producing *Enterococcus avium* Strain with a Nonsense Mutation in the Natural D-Ala-D-Ala Ligase Gene

Vancomycin-dependent enterococci (VDE) selected either in vivo or in vitro have been previously described (3, 4, 6). A hypothesis to explain the phenomenon of vancomycin dependence is that VDE acquired the *van* operon, encoding the glycopeptide-inducible mechanism of resistance, but lost the capacity to produce the natural chromosome-encoded D-Ala-D-Ala ligase (3, 6). The absence of the latter would result in the lack of synthesis of the natural pentapeptide precursor (1) and therefore the absence of growth, unless the Van system was turned on to synthesize the new UDP-MurNAc-tetrapeptide-D-lactate precursor.

In our previous study (6) of the clinical *Enterococcus avium* isolate Ea1 (MICs: vancomycin, 16 μg/ml; teicoplanin, 32 μg/ml) which expresses the VanA type of resistance, we selected in vitro (on vancomycin, 64 μg/ml) the vancomycin- and teicoplanin-dependent mutant Ea3. We also selected Ea31, a derivative of Ea3 (on vancomycin, 128 μg/ml), which grew in the absence of glycopeptides and therefore expressed glycopeptide resistance constitutively. Without induction, Ea1 synthesized only the natural precursor UDP-MurNac-pentapeptide, which, after induction by vancomycin, was associated with large amounts of UDP-MurNAc-tetrapeptide-D-lactate (6). The latter was the only precursor found in the vancomycin-dependent strain Ea3 and in the constitutive strain Ea31, even after precursor accumulation. This suggested the inability of strains Ea3 and Ea31 to synthesize the pentapeptide precursor, and therefore a nonfunctional or absent chromosomal ligase.

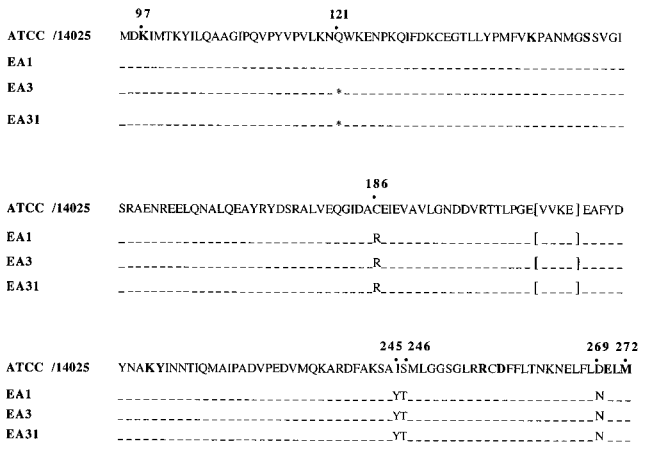

FIG. 1. Amino acid sequences deduced from the amplified fragments of strains Ea1, Ea3, and Ea31. The amino acid sequence of the reference strain *E. avium* ATTC 14025 is shown. Only the stretches which have been determined in common (reference 2 and the present work) are shown. The numbering system was taken from that for the *Escherichia coli* DdlB ligase as described previously (2); the sequence VVKE, present in all *E. avium* strains but not in the *E. coli* ligase, was therefore not counted and is shown in brackets. Bold letters represent conserved amino acids. Positions 121, 186, 245, 246, and 269 are those where amino acid differences were found. Asterisks represent the position of the stop codon.

We have now exploited the recently published sequence data for chromosome-encoded ligases of enterococci, including *E. avium* (2), and amplified a 652-bp fragment of the chromosomal ligase genes of Ea1 and it two mutants using the following primers: Eav1, 5′-TTGGATATGCCTTACGTTG-3′, covering positions 13 to 31 of the sequenced fragment of the ligase gene of *E. avium* (ATCC 14025), and the degenerate primer Eav2, 5′-TCAAGCCCATYTTTTCCCA-3′, covering the 3′-terminal positions 1023 to 1005 of the *E. faecalis* ligase gene. The reverse primer was chosen on the basis of the high degree of sequence conservation between the ligases of enterococci and because the fragment thus amplified covered almost the total gene. Reactions were performed in a DNA thermal cycler (PHC-2; Techne, Cambridge, United Kingdom) 40 cycles of 1 min of denaturation at 92°C, 1 min of annealing at 52°C, and 1 min of extension at 72°C.

The amplified fragments were purified and directly sequenced as previously described (5) by using the primers described above. The deduced amino acid sequences are shown in Fig. 1. Compared with the reference ligase from *E. avium* ATCC 14025 (2), some differences were observed. For Ea1, four amino acid substitutions were found. More importantly, in Ea3 and its derivative Ea31, a C-to-T transition resulted in the replacement of the glutamine codon at position 121 by a stop codon. It is then very likely that the vancomycin-dependent strain Ea3 and the constitutive strain Ea31 synthesize a truncated ligase lacking more than half of the C-terminal sequence.

This observation reinforces the above-mentioned hypothesis and supports our previous result (6), which showed no trace of UDP-MurNac-pentapeptide in strains Ea3 and Ea31, even after accumulation, but does not explain the constitutive expression of glycopeptide resistance in strain Ea31.

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Farid Sifaoui
Laurent Gutmann
L.R.M.A.
Université Paris VI
15, rue de l'Ecole de Médecine
75270 Paris Cedex 06, France