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Evidence of Incorporation of the Chromosomal β-Lactamase Gene of *Enterococcus faecalis* CH19 into a Transposon Derived from Staphylococci

LOUIS B. RICE[1,2]* AND STEVEN H. MARSHALL[1]

Division of Infectious Disease, Department of Veteran's Affairs Medical Center,[1] and Department of Medicine, Case Western Reserve University School of Medicine,[2] Cleveland, Ohio 44106

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We recently reported the chromosomal location of the staphylococcal β-lactamase gene in four strains of *Enterococcus faecalis*. Transfer of this gene from strain CH19 to an enterococcal recipient was accompanied by transfer of numerous other antimicrobial resistance determinants in the absence of detectable plasmid DNA. A restriction map developed by comparing digestions of the regions surrounding the β-lactamase gene in donor and recipient chromosomes resembles published maps of previously described staphylococcal β-lactamase transposons, particularly in the area of the structural gene and its downstream region. In addition, DNA sequence analysis of the region immediately downstream of the β-lactamase gene from both CH19 and its transcipient, CX19, revealed the presence of a 121-bp inverted repeat region found in Tn*552* and Tn*4002*, two previously described staphylococcal β-lactamase transposons. These results suggest that the chromosomal β-lactamase gene of *E. faecalis* CH19 is incorporated into a transposonlike element derived from staphylococci.

Enterococcal β-lactamase production was first described in 1983 (5). Since then, β-lactamase-producing enterococcal isolates have been described from a wide range of geographic areas (6). In most instances, β-lactamase production has been plasmid mediated and associated with high-level resistance to gentamicin. The staphylococcal origin of this resistance mechanism was confirmed when the nucleotide sequence of the β-lactamase gene from *Enterococcus faecalis* HH22 (the first reported in the genus *Enterococcus*) was found to be identical to published sequences of three of the four staphylococcal type A β-lactamase genes (21). Although a number of β-lactamase-encoding staphylococcal transposons have been described, analysis of the regions surrounding the enterococcal plasmid-encoded β-lactamase genes has failed to reveal any significant similarities to known staphylococcal β-lactamase transposons (12, 16).

We recently reported the identification of chromosomal β-lactamase genes in strains of *E. faecalis* isolated during an outbreak of colonization with these strains at The Children's Hospital in Boston, Mass. (8). This determinant was transferable from one of the strains (CH19) to an enterococcal recipient at low frequency, where it was again found to be chromosomally integrated (8). Transcipients into which the β-lactamase gene transferred also acquired resistance to erythromycin, gentamicin, streptomycin, and tetracycline. In this report, we present evidence that the region surrounding the β-lactamase gene in these strains is identical in some respects to previously described staphylococcal β-lactamase transposons.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. faecalis* CH19 (Bla$^+$ Em^r Gm^r Sm^r Tc^r) is a clinical isolate in which all of the resistance determinants, including the β-lactamase gene, are located within the chromosome (8). *E. faecalis* JH2-7 (Fus^r Rif^r Thy$^-$) was used as a recipient in mating experiments (13). CX19 (Bla$^+$ Em^r Gm^r Sm^r Tc^r Fus^r Rif^r Thy$^-$) is the transcipient derived by mating CH19 and JH2-7 (8). *Escherichia coli* DH5α (3) and XL-1 Blue (Strategene, La Jolla, Calif.) were used as recipients in transformation experiments. pACYC184 (Cm^r Tc^r) (10) and pBCSK+ (Cm^r) (Stratagene) were used as vectors for cloning experiments.

Antimicrobial agents. All antimicrobial agents used in these experiments were purchased from Sigma Chemical Co., St. Louis, Mo.

DNA techniques. Genomic DNA was isolated from *E. faecalis* as previously described (8). Plasmid DNA was isolated from *E. coli* by using an alkaline lysis technique (Qiagen, Inc., Studio City, Calif.). Genomic DNA was digested with 5 to 10 U of restriction enzymes for 1 to 2 h as recommended by the manufacturer (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Partial digestions with restriction enzyme *Hae*III were performed for 5, 10, and 15 min at 37°C. Double digestions were performed on genomic DNA by using enzymes sequentially. Digested fragments were separated on 0.7 to 1% agarose gels and transferred to nylon filters by using a Vacugene negative-pressure transfer apparatus as recommended by the manufacturer (Pharmacia LKB Biotechnologies, Uppsala, Sweden). Filters were prehybridized and hybridized with digoxigenin-labelled probes under stringent conditions (68°C) as recommended by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The 840-bp *Hin*dIII-*Xba*I fragment of pJM13 (4), which contains the leader sequence and 80% of the structural gene that encodes the staphylococcal β-lactamase, was used as a probe for the enterococcal β-lactamase gene. Probes were labelled with digoxigenin by a random primer method as specified by the manufacturer. Hybridized fragments were detected by using an antidigoxigenin-alkaline phosphatase conjugate with a chromogenic enzyme substrate.

Ligations were performed by mixing the vector and insert DNAs in various ratios with T4 DNA ligase and ATP as recommended by the manufacturer (Boehringer Mannheim) and ligating them overnight at 17°C. Following ligation,

* Corresponding author.

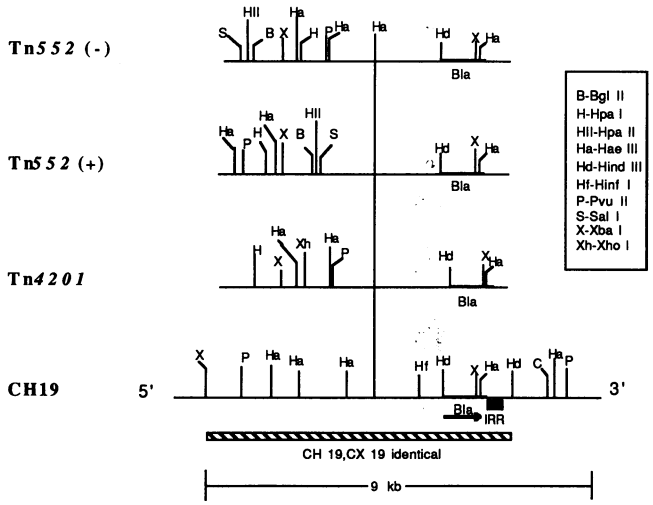

FIG. 1. Comparison of the restriction maps of staphylococcal β-lactamase transposons Tn*552* (shown with the invertible segments in the plus (+) and minus (−) orientations) (derived from reference 11) and Tn*4201* (from reference 17) with the chromosomal region surrounding the β-lactamase gene in CH19. The location of the right-hand inverted repeat (IRR) adjacent to the CH19 β-lactamase gene is indicated by the bold bar. The location of the β-lactamase gene is indicated, with the direction of transcription shown by the arrow. The hatched bar delineates the region of the restriction map similarity between Bla$^+$ donor (CH19) and recipient (CX19) strains. The alignment of the *Hae*III sites was used to facilitate comparison and is not meant to imply an exact correlation of these sites.

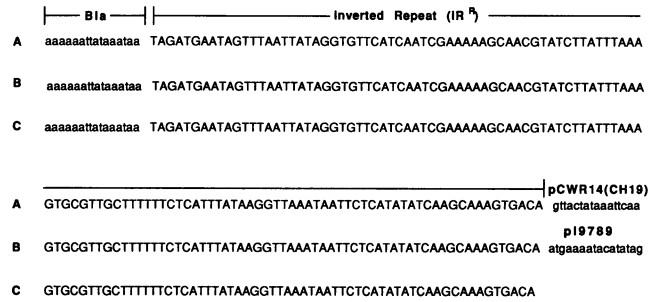

FIG. 2. Comparison of the nucleotide sequences from the ends of the β-lactamase genes and inverted repeat sequences from pCWR14 (A), Tn*552* (from reference 11) (B), and Tn*4002* (from reference 2) (C). Plasmid (pI9789) and chromosomal (CH19) junction sequences for Tn*552* and pCWR14 are denoted by the lowercase nucleotides at the end of the inverted repeat sequence and are included for comparison. The first nucleotide listed represents nucleotide 1163 of the β-lactamase sequence (2).

DNA was introduced into *E. coli* by electroporation as recommended by the manufacturer (Gene Pulser, Bio-Rad Laboratories, Richmond, Calif.). Transformed cells were selected on LB agar plates containing ampicillin (50 μg/ml) (for the β-lactamase gene-containing inserts) and chloramphenicol (30 μg/ml) or on plates containing chloramphenicol alone.

DNA sequencing was performed by using double-stranded DNA templates by the dideoxy-chain termination method with a commercially available sequencing kit (Sequenase; United States Biochemicals, Cleveland, Ohio). Fragments were labelled by incorporation of [^{35}S]dATP and separated on 6% polyacrylamide–urea gels (Base runner; International Biotechnologies Inc., New Haven, Conn.). T3 and T7 primers used in sequencing inserts in pBCSK+ were purchased from Stratagene.

RESULTS

Restriction mapping studies. Single, double, and partial digestions of genomic DNAs from both donor and transcipient strains and hybridization with the probe derived from the staphylococcal β-lactamase gene resulted in construction of the restriction map shown in Fig. 1. Restriction sites for enzymes shown on the map were found to be the same for both donor and transcipient DNAs for the region from the *Xba*I site approximately 5 kb upstream of the β-lactamase gene until the *Hin*dIII site approximately 600 bp downstream from the gene. Downstream of the *Hin*dIII site, restriction sites in CH19 and CX19 genomic DNAs for *Bgl*I, *Cla*I, *Hpa*I, and *Pvu*II were different. These results suggest that the element within which the β-lactamase gene transfers is at least 6 kb long and that one end lies 1 to 2 kb downstream of the β-lactamase gene. When this map is compared with previously published maps of Tn*552* and Tn*4201* (Fig. 1), similarities can be detected in the internal map of the structural β-lactamase gene and in the general location of the downstream end of the element. Some similarities are also evident in the upstream region. For example, the predicted size of the β-lactamase-containing *Hae*III fragment, given the known sequences of the β-lactamase gene and the upstream regions of previously described staphylococcal β-lactamase transposons (15), is 2,595 bp. The size of the measured *Hae*III fragment within the chromosomes of both CH19 and CX19 is 2.4 to 2.5 kb. Similarly, the predicted size of the *Hin*fI fragment in this region is 813 bp. The measured size of this fragment in the enterococcal chromosome is 900 to 1,000 bp. Marked differences between the restriction maps of the staphylococcal elements and the enterococcal chromosomal element are noted upstream of the *Hae*III site. Specifically, there are no *Bgl*II, *Hpa*II, or *Xho*I sites within this region of the enterococcal element.

Cloning and sequencing of the enterococcal β-lactamase region. Sequence analysis of staphylococcal β-lactamase transposons Tn*552* and Tn*4002* has shown that they share a 121-bp inverted repeat just downstream of the structural β-lactamase gene (2, 11). We cloned the 1.6-kb *Hin*dIII fragment of CH19, which contains the leader sequence, the structural β-lactamase gene, and approximately 600 bp downstream, into *E. coli* vector pACYC184, resulting in chimeric plasmid pCWR7. We then subcloned the 498-bp *Hin*cII-*Hin*dIII fragment (representing the region that extends from position 1065 of the β-lactamase sequence [2] to the *Hin*dIII site at the end of the insert) into *E. coli* phagemid pBCSK+ for sequencing. The resulting chimeric phagemid was designated pCWR14. A similar strategy was used to subclone the corresponding region of the 1.6-kb β-lactamase-encoding *Hin*dIII fragment from CX19, resulting in chimeric phagemid pCWR19.

Double-stranded DNA sequencing of the pCWR14 insert revealed a nucleotide sequence 234 bp long which is identical to published sequences for the region between the *Hin*cII site at position 1065 of the β-lactamase sequence and the end of staphylococcal β-lactamase transposons Tn*552* (11) and Tn*4002* (2), including the 121-bp inverted repeat region found in both of these transposons (Fig. 2). As expected, the β-lactamase sequence and the inverted repeat region were also found in pCWR19. In addition, the 264-bp region extending from the end of the inverted repeat to the down-

stream *Hin*dIII site were identical in pCWR14 and pCWR19, suggesting that the element within which the β-lactamase gene transferred from CH19 to JH2-7 extends beyond the inverted repeat region which defines the ends of staphylococcal β-lactamase transposons.

DISCUSSION

Enterococcal β-lactamase production is being recognized with increasing frequency, having been reported from numerous sites within this continent, as well as from South America and the Middle East (6). Two outbreaks of colonization or infections with these organisms have been reported, including the one in which CH19 was isolated (7, 18). While most reported β-lactamase production has been plasmid mediated (5, 12, 20), *E. faecalis* CH19 and the other strains isolated during this outbreak are unique in that the β-lactamase gene is located within the bacterial chromosome (8). A number of β-lactamase-encoding enterococcal plasmids have been analyzed, some in great detail (5, 12, 20). To date, there is no compelling evidence that any of these plasmid-mediated β-lactamase genes are incorporated into transposable elements resembling those found in staphylococci. The chromosomal location of the β-lactamase gene in our strains led us to postulate that it is incorporated into a transposable element. The results presented in this report provide further support for this hypothesis.

The demonstration of classic transposition in a recombination-deficient environment has been rare with the previously described staphylococcal β-lactamase transposons. Only Tn*4201* has been conclusively shown to exhibit such movement (17). The transposability of staphylococcal β-lactamase transposons Tn*552*, Tn*4002*, and Tn*3852* (which may all be the same mobile element) has been inferred largely on the basis of circumstantial evidence and the fact that the genetic organization of Tn*552* has been found to resemble that of the Tn*21* subgroup of Tn*3* family transposons (11). The presence of a 121-bp nucleotide sequence immediately adjacent to the enterococcal β-lactamase gene which is identical to the right-hand inverted repeat of previously described staphylococcal β-lactamase transposons leaves little doubt that this gene was, at least at one point, integrated into a staphylococcus-derived transposon.

While our results demonstrate conclusively that the regions downstream of the β-lactamase genes in CH19 and CX19 are identical to staphylococcal β-lactamase transposons, the composition of the upstream region is open to question. We have shown that there are similarities between the *Hae*III and *Hin*fI sites upstream of the β-lactamase genes and the upstream regions of staphylococcal β-lactamase transposons (11, 15), suggesting that the upstream regions are identical as well. However, since both of these enzymes cut at relatively frequent intervals, it is possible that such similarities of fragment size are coincidental. Nucleotide sequence analysis is required before definitive statements can be made regarding the composition of the region between the upstream *Hae*III site and the *Hin*dIII site at the beginning of the β-lactamase gene in our enterococcal strains.

The upstream regions of Tn*552* have been shown to encode the β-lactamase inducer (*blaI*) and repressor (*blaRI*) genes, and the nucleotide sequences of these genes have been elucidated (11, 15). We have been unable to detect an increase in the penicillin MIC after exposure of CH19 to antibiotics known to induce the staphylococcal β-lactamase, suggesting that β-lactamase production in CH19 is not inducible (8). Formal induction studies examining relative β-lactamase production have not been done, however, so these results must be interpreted with caution. In any case, the extent to which the staphylococcal enzyme is inducible in an enterococcal background is not clear (21). Definitive knowledge of the composition of the upstream region in CH19 awaits DNA sequence analysis.

The presence of the inverted repeat region notwithstanding, we have been unable to document self-directed movement of the enterococcal chromosomal β-lactamase gene. The fact that it appears by restriction mapping to be integrated into a separate site within the enterococcal chromosome in recipient strain CX19 originally suggested to us that the element was capable of independent movement. However, the nucleotide sequences that extend beyond the inverted repeat in donor and recipient strains are also identical, raising the possibility that the β-lactamase transposon suggested by the presence of the inverted repeat is incorporated into an even larger mobile element. Large conjugative elements, in some cases representing the integration of a smaller conjugative transposon into a second element carrying a different resistance determinant, have been described in *Streptococcus agalactiae* (1).

Alternatively, the movement to the recipient chromosome may have been the result of a recombination event that occurred during conjugation. Torres et al. have demonstrated intercellular transfer of disparate chromosomal genes between enterococcal strains, apparently under the control of functions encoded by conjugative transposon Tn*925*, by a process postulated to resemble a cell fusion event (14). Tetracycline resistance is encoded in CH19 by a *tetM* gene incorporated into a 19-kb conjugative transposon (9). The presence of this conjugative transposon in CH19 raises the possibility of a conjugation event similar to that described with Tn*925*, resulting in the transfer of a range of chromosomal antimicrobial resistance determinants, adding yet another potential mechanism by which this troublesome genus is able to spread antibiotic resistance. Further work is required to shed light on the mechanism(s) by which the β-lactamase gene of CH19 transfers between organisms.

An intriguing possibility raised by our results is that the chromosomal integration of a staphylococcal β-lactamase transposon was the initial mechanism by which the β-lactamase gene was introduced into the enterococcus, with subsequent plasmid-encoded genes representing the result of an intracellular recombination event between chromosome and plasmid. Such an integration could have occurred during a conjugative transposon-facilitated cell fusion event between enterococcus and staphylococcus or via a bacteriophage-mediated transduction event. Interestingly, CH19 is infected with a bacteriophage capable of transducing gentamicin resistance into an enterococcal recipient (19).

It is also possible that the chromosomal integration of the β-lactamase gene in CH19 represents an isolated event. To our knowledge, the strains isolated in this outbreak continue to represent the only examples of chromosomally encoded β-lactamase production in enterococci. In addition, comparison of the genomic DNA of this strain with DNA from a number of other β-lactamase-producing isolates suggests no clonal relationship between them (6). Elucidation of the relative importance of transposition in the evolution of enterococcal β-lactamase production requires a more detailed understanding of the nature of the transferable chromosomal β-lactamase and its relationship to plasmid-encoded elements and close observation of future patterns of dissemination. The history of antimicrobial resistance in the

genus *Enterococcus* teaches us that today's isolated event is tomorrow's growing problem. We anticipate further reports of chromosomally encoded β-lactamase genes.

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