The *Staphylococcus aureus* mec Determinant Comprises an Unusual Cluster of Direct Repeats and Codes for a Gene Product Similar to the *Escherichia coli* sn-Glycerophosphoryl Diester Phosphodiesterase

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The DNA sequence located between mecA, the gene that codes for penicillin-binding protein PBP2', and insertion sequence-like element IS431mec has been termed hypervariable because of its length polymorphism among different staphylococcal isolates. We sequenced and characterized the hypervariable region of the methicillin resistance determinant (mec) isolated from *Staphylococcus aureus* BB270. Within the 2,040-bp hypervariable region, we identified an unusual accumulation of long direct repeats. Analysis of the DNA sequence revealed a minimal direct repeat unit (dra) of 40 bp which was repeated 10 times within 500 bp. The dra sequences are responsible for the length polymorphism of mec. Moreover, we identified an open reading frame that codes for 145 amino acids (ORF145), whose deduced amino acid sequence showed 57% amino acid sequence similarity to the N terminus of the glycerophosphoryl diester phosphodiesterase (UgpQ) of *Escherichia coli*.

The methicillin resistance (Mc') determinant (mec) is responsible for the intrinsic resistance of staphylococci to penicillinase-resistant β-lactam antibiotics (21, 41, 45). The mec DNA is over 30 kb long and integrates into a specific site of the *Sma*I fragment of the *Staphylococcus aureus* NCTC 8325 chromosome (30). Methicillin-susceptible (Mc') strains lack an allelic site for mec. The mec determinant carries the mecA gene, which codes for the low-affinity penicillin-binding protein PBP2' (or PBP2a) (18, 47, 48), the component required for expression of Mc'. The mecA gene has been cloned and sequenced by different researchers (5, 27, 34, 38, 40, 41). Transformation of Mc' staphylococci with a plasmid bearing mecA renders the recipient strain resistant to methicillin (21, 32, 41, 45).

On the mec determinant, Barberis-Maino et al. (3) have identified an insertion sequence-like element, termed IS431 (also reported as IS257), which is associated with mecA (3, 25, 36). The intervening DNA sequence between IS431mec and mecA of various mec determinants has been claimed to be hypervariable, because the IS431mec-mecA region shows DNA restriction length polymorphism (11, 14, 22, 45). Virtually no other sequences on mec, except mecA and IS431mec, have been determined.

In staphylococci, many resistance determinants, located either on plasmids or on the chromosome, are flanked by single or multiple copies of insertion sequence-like elements, and insertion sequences have also been found to flank the *tra* genes of conjugative plasmids (5, 10, 13, 15). Insertion sequences or transposon attachment sites on the chromosome may function as target sites for recombination of different genetic elements such as, e.g., resistance genes, plasmids, bacteriophages, and transposons. Therefore, the possible involvement of IS431mec in gene transfer is a tempting hypothesis. The region comprising mecA and IS431mec seems indeed to be active in promoting rearrangements of mec-associated DNA segments. Trees and Landolo (44) have reported the transient mobilization of mec from the chromosome onto penicillinase plasmid pIL24 (44), while Matthews and Stewart (28) have shown that specific mec-associated sequences have the potential to amplify when the cells are grown under increasing concentrations of methicillin. The amplified DNA region involved in these events comprised IS431 and its flanking region. Deletions occurring in the mec region involving mec and associated flanking sequences have been observed repeatedly (17, 27, 31, 49). The elimination of different resistance determinants related to mec were found to be bound to loss of Mc'. The site of excision was often located within or near IS431 (22, 27, 31, 49). All of these phenomena account for the instability or fluctuation of mec-associated sequences and appear to share a common site, namely, the DNA comprising IS431 and its flanking DNA regions. To investigate the nature of this interesting DNA region and its associated length polymorphism, we subcloned and sequenced this DNA region of the mec determinant isolated from Mc' *S. aureus* BB270.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** Mc' *S. aureus* BB270 (7) was the DNA source for all of the experiments in this study. Mc' *S. aureus* BB565 was BB270 carrying penicillinase plasmid pIL24 (33), and Mc' *S. aureus* BB308 is a femA mutant of BB270 (6). The *Escherichia coli* strains used were JM109 (50) for the M13-based sequencing strategy and DH5α (Gibco BRL) for pTZ18R and pGEM11 cloning. Plasmid-encoded proteins were expressed in the maxicell system of *E. coli* CSR603 (35). *E. coli* BrzA95 (ugpQ pIsB pIsX), which does not grow on glycerophosphorylcholine as the phospholipid source except when complemented by ugpQ (9), was used for complementation analysis with plasmid pBB56. Plasmid pBB56 carries a 3.5-kb *Bgl*II insert which starts at

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the end of the mecA gene and spans the hypervariable region (see Fig. 1A) and ends within IS431mec. Plasmid pBBB30 carries a 3.6-kb XbaI fragment; this insert starts within the mecA gene and spans the hypervariable region up to IS431mec. The cloning vector for pBBBS6 and pBBB30 was pTZ18R (USB, Cleveland, Ohio). Plasmid pBBB83 was pGEM11 (Promega, Madison, Wis.) carrying the MaelII-HindIII fragment spanning nucleotides (nt) 1000 to 1477 (see Fig. 2A).

Growth conditions. All bacterial strains were grown in LB broth (26) at 37°C. Ampicillin (50 μg/ml) was added where needed. For complementation analysis in the E. coli uppQ mutant, minimal medium A (29) containing 0.2% glucose and 1 mM glycerophosphorylcholine was used (9).

DNA manipulations. All DNA standard manipulations were done as described by Maniatis et al. (26) or Ausubel et al. (1). Nucleotide sequences were determined by dieodeoxy-chain termination (36) by using the Sequenase sequencing kit (USB). Transformation of S. aureus with recombinant plasmid DNA was done by electroporation by following the protocol described previously (8).

RNA manipulations. For extraction of staphyloccocal total RNA, 8-ml aliquots of exponentially growing cell cultures were pelleted at 10^6 rpm for 3 min in a Sorvall SS34 rotor at 4°C. All following manipulations were done rapidly and at 0°C to prevent RNA degradation. The cell pellet was suspended by vortexing in 700 μl of RNA extraction buffer (7.2 M urea, 1.2% sodium dodecyl sulfate, 35 mM NaCl, 15 mM EDTA, 10 mM Tris-HCl [pH 7.5] per ml), transferred to a microcentrifuge tube containing 300 μl of 25:24:1 phenol-chloroform-isooamyl alcohol (1), and quickly frozen in a dry ice-ethanol bath. The aliquots were then thawed at 65°C for 5 min, vortexed, and centrifuged at 4°C for 5 min in an SS34 rotor at 18,000 rpm. DNase I digestion and further purification steps were done as described by Ausubel et al. (1). Gel electrophoresis, transfer, and hybridization were done as described by Gilmann and Chamberlin (16), Stachel et al. (39), and Church and Gilbert (12), respectively.

Computer analysis. The DNA sequences were assembled with the DNA Inspector IIe program (Textco, Inc., West Lebanon, N.H.). All further analyses were performed with the GCG program (University of Wisconsin Genetics Computer Group, Madison). The NBRF, Swissprot, EMBL, and MIPX protein data banks were searched for amino acid homologies with ORF145. For the first search, we used the Wordsearch program.

Nucleotide sequence accession number. The nucleotide sequence of the hypervariable region of S. aureus BB270 has been submitted to the EMBL/GenBank data base under accession no. X52594.

RESULTS

Genetic organization of the hypervariable region of S. aureus. The genetic organization and a restriction map of the mec-associated DNA containing the hypervariable region of Mc' S. aureus BB270 is presented in Fig. 1A. To illustrate the variable section of the DNA, we show a Southern blot hybridization of different selected DNA's extracted from Mc' S. aureus clinical isolates collected at the University Hospital of Zurich during a period of over 20 years (Fig. 1B). To target the sequence of interest, the DNA was digested with TaqI and hybridized with the 1.473-bp XbaI-HindIII fragment containing the dru probe (Fig. 1A), which covers the entire putative hypervariable region (Fig. 1A). Restriction enzyme TaqI cuts six times within the XbaI-HindIII fragment of Mc' S. aureus BB270, giving rise to internal TaqI fragments of 52, 107, 135, 386, and 617 bp. Two additional fragments of 762 bp and 1.6 kb, located outside the dru probe, are designated as flanking fragments (Fig. 1A). The flanking 762-bp fragment overlapping the dru probe by only 19 bp and the internal 52-bp fragment escaped detection because of experimental limitations. From previous hybridization experiments, we knew that the flanking 1.6-kb fragment covering the 3' end of mecA and the flanking sequences starting within the XbaI site reaching into the IS431mec sequence (Fig. 1A) were not responsible for the observed length polymorphism of this DNA region. The Southern blot analysis shown in Fig. 1B narrowed down the variability to a DNA fragment ranging between 0.8 and 0.54 kb. The flanking fragments of 386, 135, and 107 bp were the same sizes in all of the strains analyzed (data not shown). One Mc' strain apparently had no variable DNA fragment and a band smaller than 386 bp (Fig. 1B, lane 7).

Nucleotide sequence. We determined the nucleotide sequence of the hypervariable DNA region isolated from Mc' S. aureus BB270 (Fig. 2A). The 2,040-bp DNA fragment spanned from the XbaI restriction site covering the 3' end of the published nucleotide sequence of IS431mec in S. aureus (34) up to the published 3' end of the mecA gene (38).

A self-homology matrix of the S. aureus hypervariable region (obtained with DNA Inspector IIe; data not shown) revealed several partially overlapping long direct repeats in the DNA sequence ranging from nt 330 to 820 (Fig. 2A). Beside this particular cluster, we found an open reading frame on the DNA sequence which could code for a protein of 145 amino acids (ORF145).

Analysis of dru in Mc' S. aureus BB270. The DNA fragment containing the long direct repeat cluster was restricted within 400 bp, ranging from nt 401 to 800 (Fig. 2). The identification of the minimal best repeated sequence was made by the correlation between the 401- to 800-bp segment and the complete 2,040-bp long DNA sequence. Significant values could be seen by shifting a multiple of 40 nt (data not shown). We concluded that the minimal repeated sequence was 40 bp long and was directly repeated at least nine times within the hypervariable region. We termed the 40-bp sequences the direct repeat unit (dru) and the DNA fragment carrying these sequences the dru element. A plot of the cross-correlation for each dru within the 401- to 800-bp segment was made to identify the best possible dru consensus sequence. By considering homology values greater than 60%, we propose for dru the following consensus sequence: CTAAATTACGTAGATAGGTATAAAG CAGTT, whereby we did not define (either theoretically or experimentally) the precise 5' and 3' ends of the dru consensus sequence, and it should therefore be considered as a circular string of probable positions for the given nucleotide within dru (Fig. 3). The nucleotides of the consensus sequence marked in boldface were conserved among all of the dru's studied in this work. Furthermore, we scanned the EMBL data bank for nucleotide sequence similarities to the consensus sequence of either dru or the dru element itself. However, no similarity to mobile genetic element structures or to other DNA sequences entries were found.

Analysis of ORF145. From nt 1040 to 1477, we found an open reading frame that codes for a protein of 145 amino acids (ORF145). ORF145 is located on the opposite DNA strand with respect to the transcription direction of the mecA gene and of the putative IS431mec-encoded transposase (Fig. 1A). A potential −10 region and a potential ribosomal
binding site were identified in the S' region of ORF145 (Fig. 2A), supporting our assumption that ORF145 is expressed in S. aureus.

Total RNAs of different Mc' S. aureus strains were analyzed by Northern (RNA) blotting for expression of the ORF145 gene. Here we show the result of the analysis of two Mc' S. aureus strains. The Northern blots were probed by using a 477-bp MaelIII-HindIII DNA fragment, the ORF145 probe (Fig. 1A), which covered ORF145. This experiment showed that ORF145 codes for a small mRNA of approximately 600 nt (Fig. 4), which would span the length of the open reading frame. With the ORF145 probe, we also detected a second transcript of 1,000 nt. This second band was not constantly apparent in the Mc' S. aureus analyzed.

Expression of ORF145 in E. coli maxicells. Recombinant plasmid pBBB30, carrying the entire hypervariable region, was used to transform E. coli CSR603 cells to analyze the plasmid-encoded proteins (Fig. 5). We detected a protein with an apparent molecular mass of 16.5 kDa that corresponded well to the predicted size of 16.3 kDa for the ORF145 protein.

The product of ORF145 is related to E. coli UgpQ glycerolphosphoryl diester phosphodiesterase. The deduced amino acid sequence of ORF145 was used for a homology search of the protein data banks. The amino acid sequence alignment in Fig. 2B showed a significant similarity of 57% (total amino acid identity of 36%) to UgpQ, a glycerophosphoryl diester phosphodiesterase, the product of the fifth gene (ugpQ) of the inducible sn-glycerol phosphate uptake regulon in E. coli (9, 24, 43). The similarities were located in the N-terminal sequence and covered the putative catalytic domain of UgpQ (42).

Furthermore, plasmid pBBB56 was able to complement ugpQ E. coli mutant Brz495, allowing growth within 48 h on minimal medium when supplemented with glycerophosphorylcholine as the phospholipid source, whereas the vector pTZ18R alone did not, suggesting that ORF145 can act as a glycerophosphoryl diester phosphodiesterase in the gram-negative host.

**DISCUSSION**

In the present work, we characterized the mec-associated hypervariable region of Mc' S. aureus BB270. We showed...
that the DNA length polymorphism of the staphylococcal mec determinant is due to the presence of a very unusual cluster of 10 long direct repeats with a best minimal repeated sequence of 40 nt. We termed this novel structure the mec determinant of S. aureus dru and the DNA sequence in which the dru’s are located the dru element. We calculated a consensus sequence on the basis of the best probability that a certain nucleotide would occur at the same position within the different dru’s. We did not determine the 5’ and 3’ ends of dru, and it is to be handled as a circular sequence. The dru’s are contiguous, with exception of a 1-nt shift between dru481 to dru522 and dru722 to dru763. dru441, dru481, and dru562 are identical and, like dru682, have 95% homology to the calculated consensus sequence. As for other repeated sequences in prokaryotes (19), the function and perpetuation of these elements remain to be established. An additional intriguing observation of the hypervariable region was made by analyzing its G+C content. As shown in Fig. 3A, we found a DNA fragment with an unusual base composition 5’ of the dru element (from nt 280 to 380), namely, a high G+C content of 47%. This value is considerably higher than the mean for a fragment of staphylococcal DNA of the same length (approximately 31%). This G+C-rich fragment was preceded by a long perfect inverted repeat of 16 nt.

Whether the dru element is functionally connected to IS431 mec or is a residual of multiple imprecise insertion and excision events of other mobile DNA elements is not known. The search for similarities in the GenBank-EMBL DNA Sequence Data Library revealed that the dru’s and the dru element are not related to any known sequences. The dru element is an intriguing feature of mec DNA, and its biological function, its impact on Mc' regulation or virulence, and its role in the evolution of multiple drug resistance in Mc' staphylococci remain to be investigated. The hypervariable region seemed to have no observable function or to affect directly the expression of Mc', since the cloned 4-kb HindIII fragment, isolated from two different sources, which carried only mecA but not ORF145 or dru, was able to confer Mc' to susceptible S. aureus strains (32, 45). Interestingly, a region of length polymorphism associated with a cluster of direct repeats and an insertion sequence has been recently described for Mycobacterium tuberculosis complex strains.
The various ORF145-specific probes, coagulase-negative staphylococci were analyzed. We identified ORF145, which codes for a protein with an amino acid sequence comparable to that of the glycophosphoryl diester phosphodiesterase (UgpQ) of E. coli (9, 24, 43). We found that transcription of ORF145 in coagulase-negative +positive Mc' staphylococci was constitutive. Furthermore, by Northern blot analysis using the ORF145-specific probe, we detected two different mRNAs. One, 600 nt long, had approximately the size predicted for ORF145. The longer mRNA was not always detectable, and the hybridization signal was variable among the various RNA preparations and strains. The role and nature of this 1-kb transcript are currently being investigated. The contribution of ORF145 to Mc' expression in staphylococci remains to be elucidated. It would be interesting to know whether ORF145 has a biologically active function in Mc' staphylococci and whether it can interact with native staphylococcal phosphate metabolism. The nature of the staphylococcal mec determinant has yet to be determined. It may be that the mec determinant contains genes or gene fragments of chromosomal origin from other species. Moreover, the overall organization of gene clusters of different species seems to be conserved within smaller or larger sections of the chromosome. In E. coli, the ugp operon (min 76) maps close to genes for cell wall-metabolizing enzymes, a striking similarity to the organization of mec, where PBP2' is located next to ORF145, a ugpQ-related enzyme (2, 9). One could then speculate that a section(s) of the mec determinant might be of chromosomal origin and has been truncated and mobilized, presumably by the dru element or IS431.

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