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Molecular Cloning and Mapping of 16S-23S rRNA Gene Complexes of *Staphylococcus aureus*

AKIHITO WADA,†* HISAKO OHTA, KANOKVALAI KULTHANAN,‡ AND KEIICHI HIRAMATSU

Department of Bacteriology, Juntendo University, Hongo 2-1-1, Bunkyo-ku, Tokyo 113, Japan

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***Staphylococcus aureus* BB255, a derivative of NCTC8325, had six rRNA operons, and each operon contained two *Sma*I sites about 3 kb apart. By molecular cloning and pulsed-field gel electrophoresis, all operons were mapped at the junctions of *Sma*I fragments in the published map of NCTC8325 except one, which was connected to a previously unidentified 23-kb *Sma*I fragment.**

Staphylococcus aureus is one of the most medically important pathogens. Much information about mechanisms of pathogenicity and resistance to antibiotics has been accumulated as a result of recent progress in the molecular biology of this organism (3, 14, 17), and the chromosome map of *S. aureus* NCTC8325 has been established by P. A. Pattee and collaborators (16). Although most information about *S. aureus* has been derived from this strain (3, 17), studies of *S. aureus* clinical isolates sometimes identify a novel locus which is not present in NCTC8325 (1). To map a novel locus on the chromosome of NCTC8325, it is first necessary to identify another locus which is present both in the isolate and in NCTC8325 to estimate the distance between the two loci. For this purpose, information about a gene conserved on the chromosome is particularly useful. The rRNA gene is one candidate because this gene is well conserved among staphylococcal species (4, 20) and multiple copies of the gene are present on the chromosome in *S. aureus*. In this study, we cloned and mapped rRNA operons on the chromosome of BB225, a derivative strain of NCTC8325, by mapping previously unidentified small *Sma*I fragments between established large *Sma*I fragments of NCTC8325.

The *S. aureus* strains used in this study were MR108, a methicillin-resistant *S. aureus* strain described previously (7, 21), and BB255, a derivative of NCTC8325 cured of its penicillinase plasmid (a kind gift of B. Berger-Bächi) (2). *Escherichia coli* JM109, MC1061, K802 (*recA*), and DH5 were used as cloning hosts of genomic DNA fragments from the *S. aureus* strains.

DNA was extracted from *S. aureus* as described previously (5). pOH18, pOH4, and pOH3 were obtained from a *Hin*dIII genomic library of MR108 with pUC119 and JM109 by using a ^{32}P-labelled synthetic oligonucleotide probe (5′-GAGCGGAT CCTGAGTACGGCGG-3′) which corresponds to the sequence of the A domain of 23S ribosomal DNA from *Bacillus stearothermophilus* (9). Other probes were labelled with digoxigenin and detected with a DIG luminescent detection kit (Boehringer GmbH, Mannheim, Germany). AR1 was obtained from a *Pst*I library of BB255, constructed with pUC119 and MC1061, with probe A (see Fig. 1A). AR2, AR3, AR4, and AR5 were obtained with probe B (see Fig. 3A) from the same library. AR6 was obtained with charomid 10-36, a derivative of charomid 9-36 (18) (Nippon Gene, Tokyo, Japan), having a unique *Pst*I site at the polylinker site, and with K802 (*recA*). AR7 was obtained from a *Pst*I library containing partially digested DNA of BB255 cloned in charomid 10-36 and with DH5. Probe B was used to obtain these two clones.

Pulsed-field gel electrophoresis (PFGE) was performed to separate *Sma*I-digested DNA fragments of BB255. Detailed methods of DNA preparation and conditions of PFGE were described previously (23). Switching intervals were 20 s and 2 s for Fig. 4B and C, respectively. Nucleotide sequencing was carried out on both strands as described previously (19).

By using the 23S rRNA gene probe from *B. stearothermophilus*, three recombinant clones, pOH18, pOH4, and pOH3, were obtained from MR108 and analyzed for restriction maps (Fig. 1A). Interestingly, the *Sma*I site in each recombinant was conserved. *Sma*I is a rare cutter for the chromosome of *S. aureus*, for which a low GC content (32 to 36%) is characteristic. By using this enzyme, P. A. Pattee constructed a physical chromosome map of *S. aureus* NCTC8325 (16). Sequencing experiments of the *Hin*dIII-*Eco*RI fragment of pOH4 corresponding to probe A (Fig. 1A) showed that these clones actually contained a part of rRNA sequences. Figure 1B illustrates a sequence comparison between probe A and an rRNA operon of *B. stearothermophilus* (9). Probe A shared 82.2% of the nucleotides with the 23S rRNA gene of *B. stearothermophilus*.

For further cloning and a mapping study of RNA operons, we chose BB255 because this strain showed the same PFGE pattern as NCTC8325 when digested by *Sma*I (data not shown) and has no plasmid which may interfere with PFGE analysis. Southern hybridization analysis of BB255 after conventional gel electrophoresis with probe A revealed two bands of approximately 3 kb by *Sma*I digestion, five bands by *Pst*I, six bands by *Hin*dIII, and two bands of approximately 1 kb by *Eco*RI (Fig. 2A). The data could be interpreted as showing six copies of rRNA operons present in BB255, with *Sma*I and *Eco*RI sites well conserved in each rRNA operon. The 3-kb *Sma*I fragments have not been identified on the published chromosome map of NCTC8325 (16). To map these 3-kb *Sma*I fragments on the chromosome and to identify the chromosomal location of rRNA operons, we tried to clone the 3-kb *Sma*I fragments together with upstream and downstream sequences of them. Figure 2B shows a hybridization study with probe A after single or double digestion by *Sma*I, *Sma*I plus *Pst*I, *Sma*I plus *Hin*dIII, and *Sma*I plus *Eco*RI. Only in the case of additional *Pst*I digestion did the mobilities of the *Sma*I-digested bands remain unaltered (Fig. 2B, lanes 1 and 2). This indicated that *Pst*I sites should be present outside of the two

* Corresponding author.
† Present address: Department of Bacteriology, The National Institute of Health, Toyama 1-23-1, Shinjuku-ku, Tokyo 162, Japan.
‡ Present address: Board of Dermatology, Immunology Section, Institute of Dermatology, Bangkok 10400, Thailand.

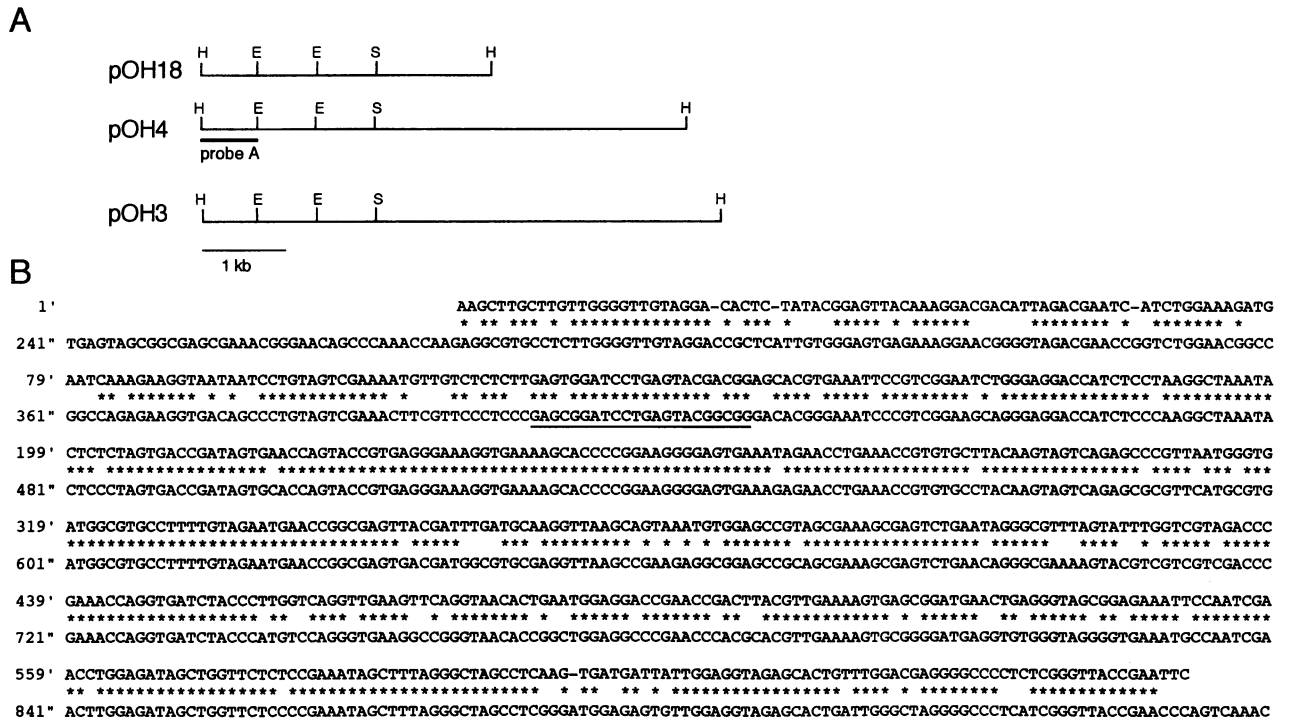

FIG. 1. (A) Mapping of three clones, pOH18, pOH4, and pOH3, from MR108. The *Hin*dIII-*Eco*RI fragment of pOH4 corresponding to probe A is shown by a thick line. Abbreviations of restriction enzymes: H, *Hin*dIII; E, *Eco*RI; S, *Sma*I. (B) Nucleotide sequence of probe A and comparison with the sequence of the 23S rRNA gene of *B. stearothermophilus* (EMBL entry name, BSRRNA23). The upper sequence is of probe A, and the lower is of BSRRNA23. A high homology rate of 82.8% was observed throughout the nucleotide sequence of probe A. The sequence of the synthetic oligonucleotide probe used for the cloning is indicated by underlining.

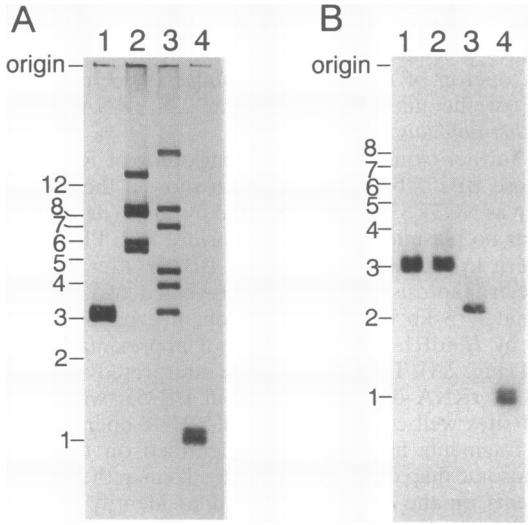

FIG. 2. Southern blot analysis of BB255 DNA with probe A containing 23S rRNA gene. (A) DNA of BB255 digested by enzymes *Sma*I (lane 1), *Pst*I (lane 2), *Hin*dIII (lane 3), and *Eco*RI (lane 4); (B) DNA of BB255 digested by enzymes *Sma*I (lane 1), *Sma*I plus *Pst*I (lane 2), *Sma*I plus *Hin*dIII (lane 3), and *Sma*I plus *Eco*RI (lane 4). The molecular weight marker is a 1-kb DNA ladder (Bethesda Research Laboratories, Gaithersburg, Md.). The sizes are indicated to the left of the blots in kilobases.

*Sma*I sites, 3 kb apart. We then screened the *Pst*I genomic library of BB255 by using probes A and B (Fig. 1A and 3A) to obtain six recombinant clones: AR1, AR2, AR3, AR4, AR5, and AR6. The lengths of these clones were as follows: AR1, 5.4 kb; AR2, 5.9 kb; AR3, 8.0 kb; AR4, 8.0 kb; AR5, 8.4 kb; and AR6, 14 kb. These lengths corresponded very well to the sizes estimated by Southern blot analysis of BB255 with probe A (Fig. 2A, lane 2). Figure 3A shows restriction maps of these clones. It is noticeable that the upstream portions containing two *Sma*I sites 3 kb apart were well conserved, whereas the downstream portions were all different. In addition, a part of the cloned fragments at the upstream portion contained restriction sites comparable to those of MR108 (Fig. 1A and 3A), suggesting good conservation of 23S rRNA genes between BB255 and MR108. The similar but distinct electrophoretic mobilities of the two bands generated by *Sma*I and *Eco*RI (Fig. 2A, lanes 1 and 4) could be explained by a small difference in the size of the *Eco*RI-*Hin*dIII fragments in the six clones (Fig. 3A, arrows). Besides this, the restriction enzyme maps in Fig. 3A accounted for all of the banding patterns of Fig. 2. A subsequent preliminary sequencing experiment of AR1 revealed the locations of 16S and 23S rRNA sequences (Fig. 3A). The two conserved *Sma*I sites were both located in the rRNA operon, the left one in the 16S rRNA gene and the right one in the 23S rRNA gene. Recently, sequences of 23S and 16S rRNA genes of *S. aureus* were published by other laboratories under EMBL accession numbers X68425 (12) and X70648, respectively. The published sequence data correspond well to the restriction sites shown in Fig. 3.

For mapping of rRNA operons on the chromosome of BB255, specific probes were prepared from the downstream

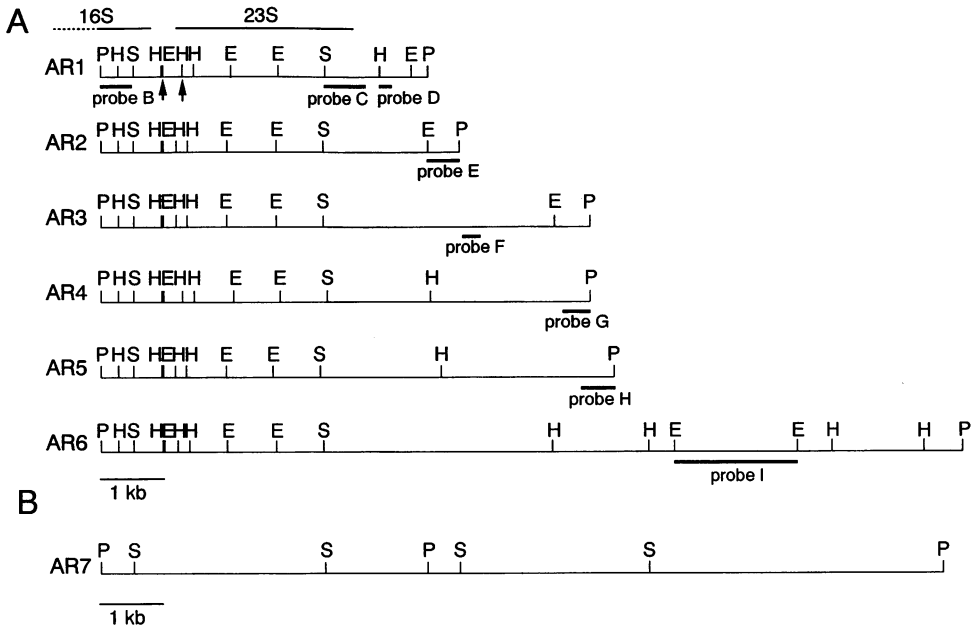

FIG. 3. (A) Restriction maps of the six clones containing rRNA operons of BB255. Restriction enzyme abbreviations are the same as those described in the legend to Fig. 1A, with the following addition: P, *Pst*I. The arrows show the *Eco*RI-*Hin*dIII fragment, which appears in slightly different sizes depending on the clones. The location of the probes for cloning and mapping studies are indicated by thick lines under the restriction maps. The positions of 16S and 23S rRNA operons are shown at the top of the restriction maps. (B) Restriction map of AR7 cloned from BB255. It consists of two *Pst*I fragments. The upstream part is identical to AR1, and the downstream part is identical to AR5.

portion of each clone (Fig. 3A). However, it was impossible to obtain specific probes from the upstream side of the left *Sma*I site because this portion was conserved in all clones. DNA of BB255 was digested by *Sma*I, separated by conventional gel electrophoresis or by PFGE, and hybridized with the various probes (Fig. 4). Probe B, located at the upstream end of AR1, was found to hybridize to a 2.1-kb fragment and to a 23-kb fragment in addition to fragments too big for size analysis by conventional gel electrophoresis (Fig. 4A, lane 1). Curiously, probe C, located downstream of the right *Sma*I site of AR1, also hybridized to the same 2.1-kb fragment but not to the 23-kb fragment (Fig. 4A, lane 2). Moreover, probes B and D, which were located at both ends of AR1 (Fig. 3A), were found to hybridize to exactly the same five large *Sma*I fragments separated by PFGE (Fig. 4B, lanes 3 and 5), whereas probe C reacted with different sets of large *Sma*I fragments (Fig. 4B,

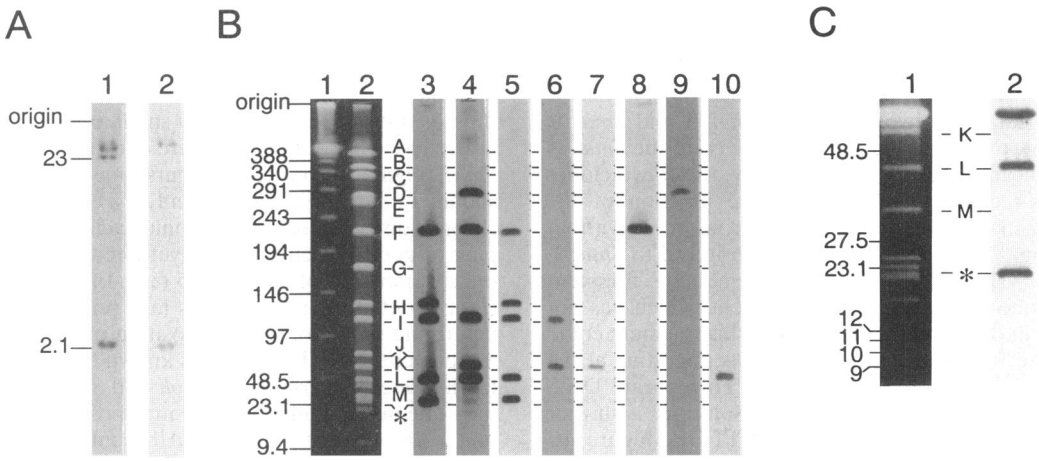

FIG. 4. (A) Southern hybridization analysis after conventional gel electrophoresis of *Sma*I-digested BB255 DNA with probes B and C for lanes 1 and 2, respectively. The size of each band is shown in kilobases. (B) Separation of large *Sma*I fragments of BB255 by PFGE with a switching interval of 20 s and its Southern hybridization analysis. Lanes: 1, concatemers of lambda phage DNA used as molecular size markers, with sizes indicated in kilobases; 2, *Sma*I-digested DNA of BB255 (the name of each *Sma*I fragment is based on the published map of NCTC8325 [16]; the 23-kb fragment which was not identified in the map is shown by an asterisk); 3 to 10, probes B, C, D, E, F, G, H, and I, respectively. (C) Separation of small *Sma*I fragments of BB255 by PFGE with a switching interval of 2 s (lane 1) and its Southern blot analysis with probe B (lane 2). The sizes of DNA markers are indicated in kilobases. The names of the fragments are the same as those described for panel B. For a description of each probe, see the legend to Fig. 3A.

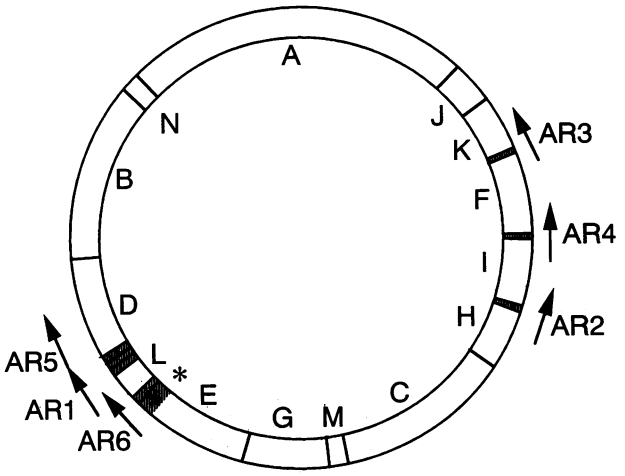

FIG. 5. Chromosome mapping of the six rRNA operons on the basis of the published map of NCTC8325 (16). The names of the fragments are the same as those described in the legend to Fig. 4B and C. Arrows indicate the direction of transcription of each rRNA operon determined by partial nucleotide sequencing of AR1. Novel, small *Sma*I fragments mapped in this study are indicated by hatched areas. AR1 and AR5 are connected as shown in Fig. 3B.

lane 4). Probe D also hybridized to the 2.1-kb *Sma*I fragment (data not shown). These data suggested that AR1 is juxtaposed with one of the other clones. This, in turn, suggests that two rRNA operons would repeat in tandem. This prediction was proven by direct cloning of genomic DNA fragment AR7, constructed of AR1 and AR5, connected directly in tandem (Fig. 3B). In AR7, the distance between the two *Sma*I sites across the central *Pst*I site was 2.1 kb, and this 2.1-kb *Sma*I fragment was flanked by two 3-kb *Sma*I fragments, one from AR1 and the other from AR5. This result was consistent with the Southern hybridization data of Fig. 2A and 4A. A subsequent mapping study of rRNA operons was based on the published chromosome map of NCTC8325 (16). Because probe H from AR5 hybridized to the *Sma*I-D fragment specifically (Fig. 4B, lane 9), and the *Sma*I-L fragment was a member of those fragments detected by probe B (lane 3), the AR1 and AR5 complex could be mapped at the junction of the *Sma*I-L and *Sma*I-D fragments (Fig. 5). Although probe E from AR2 hybridized to two fragments, *Sma*I-I and *Sma*I-K (Fig. 4B, lane 6), probe F from AR3 hybridized to the *Sma*I-K fragment specifically (lane 7). Consequently, these two clones as well as AR4 could be mapped at the junction of the established *Sma*I large fragments on the chromosome without redundancy (Fig. 5). Probe I from AR6 hybridized to *Sma*I-L fragment (Fig. 4B, lane 10), and the remaining fragment detected by probe B was the 23-kb fragment, which has not been identified in the published map (16). This fragment could not be separated from other small fragments migrating at the lower portion of the gel (Fig. 4B, lane 2). To separate DNA fragments of 10 to 50 kb with a good resolution, a shorter switching interval of 2 s was applied for PFGE. Figure 4C shows the separation of small *Sma*I fragments of BB255 of less than 50 kb. Probe B could detect the 23-kb fragment as well as other fragments, including those too large to be separated under these conditions (Fig. 4C, lane 2). These data showed that the *Sma*I-L fragment and the 23-kb fragment were connected by the 3-kb *Sma*I fragment of AR6 and that the rRNA operon in this clone was located at this junction (Fig. 5).

According to the studies of *E. coli* (8) and *Bacillus subtilis* (24), all of the rRNA operons are present in approximately one-half of the circular chromosome and centered around the bidirectional origin of DNA replication (*oriC*). This could be applied to the case of *S. aureus* as follows. The locations of the six rRNA operons were confined to approximately one-half of the circular chromosome of *S. aureus*, and the direction of the transcription of each rRNA operon was divergent from the *Sma*I-G fragment (Fig. 5), on which the genes associated with the origin of replication, *dnaA-dnaN-recF-gyrB-gyrA*, have been mapped (13).

The upstream portion of the clones shared conserved restriction sites, except for a small difference in the size of the *Eco*RI-*Hin*dIII fragment (Fig. 3A). This fragment, based on the partial nucleotide sequencing, contained the spacer region lying between 16S and 23S rRNA genes. In *E. coli* (15) and *B. subtilis* (22), different numbers of tRNA genes are identified in this region. A similar variation of the spacer region in *S. aureus* may account for the observed slight difference in the *Eco*RI-*Hin*dIII fragments. Recently, a tRNA gene cluster located downstream of an rRNA operon was cloned from *S. aureus* (6). By comparison of the sequences of the cluster and the restriction sites of our clones (not shown in Fig. 3), the cluster seems to be located at the downstream portion of AR3. It was thought that other tRNA genes would be located near the rRNA operons (6). To determine the precise positions and the numbers of tRNA genes, further sequencing experiments are required.

In this study, we provided the direct evidence that some large *Sma*I fragments of the chromosome of NCTC8325 were connected by the 3-kb *Sma*I fragments and, at these junctions, rRNA operons were present. PFGE data were consistent with the published map of NCTC8325 (16), which enabled us to locate the rRNA operons on the chromosome. The clone AR6 was mapped at the junction of the *Sma*I-L fragment and the previously unidentified 23-kb fragment. Thus, it seems most likely that the 3-kb *Sma*I fragment from AR6 and the 23-kb fragment occupy the position between the *Sma*I-L and *Sma*I-E fragments (Fig. 5). As mentioned by P. A. Pattee (16), several other small *Sma*I fragments have not yet been mapped. However, only four *Sma*I fragments, which ranged from 9 to 23 kb in size, remain to be mapped as shown by the PFGE results of BB255 (Fig. 4B and C). The latest edition of the chromosome map of NCTC8325 (16) contains information about enzyme sites other than the *Sma*I site. It is expected that multiple-enzyme digestion and the PFGE technique will reveal the positions of the unmapped small *Sma*I fragments.

Because rRNA operons were located at the junctions of *Sma*I fragments, the clones containing rRNA operons corresponded to linking clones around *Sma*I sites of the NCTC8325 chromosome. These clones would facilitate the precise mapping of genes which have not yet been mapped physically on the chromosome of NCTC8325 (16). In addition, these linking clones containing rRNA genes may serve as reference loci to determine the chromosomal position of a novel gene which is not present in NCTC8325. It is known that, of members of the family *Enterobacteriaceae*, *E. coli* and *Salmonella* species have the same number and the same map position of rRNA operons on the chromosomes (10, 11). All examined clinical isolates of *S. aureus* to date had 3-kb *Sma*I fragments detected by probe A (data not shown). These observations suggest that rRNA operons would be located at conserved *Sma*I sites of the chromosomes in other *S. aureus* strains, thus supporting the use of the clones containing rRNA operons for physical mapping of other gene(s) on the chromosome of *S. aureus* strains.

Nucleotide sequence accession number. The sequence has

been deposited in the EMBL data library under accession number D12572.

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