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Cloning and Expression in *Escherichia coli* of Genes Encoding a Multiprotein Complex Involved in Secretion of Proteins from *Staphylococcus aureus*

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The genes encoding the multiprotein membrane-bound ribosomal protein (MBRP) complex (*mrp* genes), associated with membrane-bound ribosomes in *Staphylococcus aureus*, were cloned in *Escherichia coli*. All four components (molecular sizes 71, 60, 46, and 41 kilodaltons) of the MBRP complex were expressed from an 8.5-kilobase DNA fragment as judged by Western blot (immunoblot) analysis. The order of the individual genes within the cloned DNA fragment was determined by deletion mutagenesis and subcloning of various restriction fragments. Three RNAs, transcribed from the same DNA strand, were identified within the MBRP-coding region: one large RNA of approximately 5.9 kilobases, presumably coding for all four MBRP components, and two minor RNAs, coding for MBRP-71 and MBRP-60. The two minor RNAs seemed to be transcribed from promoters within the large transcription unit. Attempts to make insertional inactivations of the *mrp* genes with an internal 600-base-pair DNA fragment of the MBRP-coding region as a target were unsuccessful, presumably because such insertions are lethal.

Earlier work in this laboratory (1), aiming at the identification of the components of the apparatus for protein secretion in *Staphylococcus aureus*, has shown that membrane-bound but not cytoplasmic ribosomes are associated with a multiprotein complex (the membrane-bound ribosomal protein complex [MBRP complex]) of four proteins (molecular sizes 71, 60, 46, and 41 kilodaltons [kDa]). The membrane-bound fraction of the MBRP complex is bound to the 50S subunit of the ribosome (1) and is located between the inner surface of the cytoplasmic membrane and the membrane-bound ribosome (3). A free pool of the complex was also found in the cytoplasm (3). The distribution of the complex between the cytoplasm and the membrane was found to vary depending on the rate of exoprotein production (3), strongly suggesting a role of the MBRP complex in protein secretion. A similar complex, immunologically related to the MBRP complex, has also been identified in *Bacillus subtilis* (2, 5, 6). As there is no in vitro translation-secretion system in *S. aureus* or *B. subtilis* available by which the role of the MBRP complex in secretion can be studied, we decided to use a genetic approach instead. In the present paper we describe the cloning and expression of the MBRP-coding genes (*mrp* genes) in *Escherichia coli*. The genetic organization is analyzed by deletion mutagenesis and determination of transcripts. We also present evidence that the *mrp* genes are essential.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. *S aureus* V8 (ATCC 27733), from which the MBRP complex was originally isolated, was used as the source of the MBRP-coding genes. Restriction-weak *S. aureus* RN4220 (12) was the primary staphylococcal recipient of *E. coli*-propagated plasmids. Strain RN4220 has a decreased production of several extracellular proteins (unpublished results) similar to the decreased production in *exp* mutants (10). *E. coli* MC1061 (4) was the host in the cloning experiments and in the construction of inactivation plasmids. Propagation of bacteriophage M13 was done in *E. coli* JM103 (16). *S. aureus* cells were grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) or on nutrient agar (Difco). *E. coli* cells were grown in LB or on LB-agar (16).

Cloning vectors. The vector used for cloning and characterization of MBRP-coding DNA was pUN121 (21). This plasmid carries a tetracycline resistance gene which is suppressed by the bacteriophage lambda *c*I repressor (24). Insertion of a DNA fragment into the restriction sites within the *c*I gene confers tetracycline resistance. pUN121 was kindly provided by Mathias Uhlén (Royal Institute of Technology, Stockholm, Sweden).

For preparation of single-stranded DNA probes, appropriate DNA fragments were cloned in phage M13. The modified M13mp18 and M13mp19 (22) phages were used according to the protocol supplied by Pharmacia Fine Chemicals, Uppsala, Sweden.

For construction of hybrid plasmids for insertion inactivation experiments, the *E. coli* plasmid pSP64 (18) and the *S. aureus* plasmid pRN8103 (23) were used. pRN8103 is a derivative of the tetracycline resistance plasmid pT181 (11), with temperature-sensitive replication and with a new *Eco*RI site introduced at position 880.

Construction of plasmids for insertion mutagenesis. As a tool for insertion inactivation of the MBRP-coding region by homologous recombination, the following plasmid was constructed. A 0.6-kilobase (kb) *Pst*I-*Hin*dIII fragment from the MBRP-coding region (a subfragment of fragment i in Fig. 3B) was inserted in the cloning cassette of pSP64 and transformed into *E. coli* MC1061. The resulting plasmid was opened with *Hin*dIII and ligated to a 3.8-kb *Hin*dIII fragment of pRN8103. This fragment, which contains the origin of replication and the tetracycline resistance genes, was obtained by partial *Hin*dIII digestion of the plasmid. The ligation mixture was transformed into *E. coli* MC1061. Tetracycline resistant transformants were checked by restriction endonuclease cleavage of plasmid minipreparations, the desired plasmid, pMBR600, was transferred to *S.*

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aureus RN4220 by protoplast transformation, and tetracycline-resistant clones were selected at 30°C.

In a similar way, pEXB383 was constructed. A 383-base-pair *Cla*I-*Sau*3AI fragment from the exoprotein-regulating *exp* locus of *S. aureus* (19) was ligated to *Bam*HI- and *Acc*I-digested pSP64 and then combined with the above-mentioned 3.8-kb *Hin*dIII fragment of pRN8103. The resulting plasmid was used as a positive control in insertion inactivation studies, since mutations in the *exp* locus are not lethal (10).

Construction of staphylococcal genomic library. A genomic library of *S. aureus* V8 chromosomal DNA was prepared as described by Löfdahl et al. (14). Fractionation of partially *Sau*3A-digested chromosomal DNA was made by sucrose gradient (10 to 30%) centrifugation, and 8- to 10-kb restriction fragments were collected and ligated into the *Bcl*I site of pUN121 and transformed into *E. coli* MC1061. A total of 3,000 clones able to grow on 5 μg of tetracycline per ml were isolated and grown in 200 μl of LB with 5 μg of tetracycline per ml overnight in 96-well microdilution plates. Glycerol was added to a final concentration of 15%, and the plates were stored at −70°C.

Antisera. An antiserum containing antibodies against MBRP-71 and MBRP-60 was obtained by immunization of rabbits with immunoprecipitates cut out from crossed-immunoelectrophoresis gels as previously described (1). An antiserum against each of the four MBRP components was prepared by immunizing with the individual proteins cut out from sodium dodecyl sulfate-polyacrylamide gels (3). The antiserum used for colony probing was freed from antibodies against *E. coli* antigens by passing it through a column of CNBr-Sepharose 4B (Pharmacia) to which had been coupled a whole-cell lysate of *E. coli* MC1061. Lysate was prepared by suspending the bacterial pellet in 100 mM $NaHCO_3$–0.5 M NaCl, pH 8.3, and freezing at −20°C. *E. coli* protein was added per swollen gel (10 mg/ml) and coupled according to the directions of the manufacturer.

Detection of clones producing MBRP antigens. To identify the clones of the staphylococcal genomic library that produce MBRP antigens, the antiserum against MBRP-71 and MBRP-60 was used. *E. coli* clones to be tested for the production of the MBRP antigens were transferred from the microdilution plates to LB plates with 5 μg of tetracycline per ml. After growth overnight at 37°C, the bacteria were lysed by incubation of the agar plates in saturated chloroform vapor for 30 min. Nitrocellulose filters (BA 85, 0.45-μm pore size; Schleicher & Schuell, Inc., Dassel, Federal Republic of Germany) were applied to the agar surface, and the plates were incubated at 4°C overnight. Filters were washed thoroughly several times in 20 mM Tris hydrochloride, pH 7.5–0.5 M NaCl–0.05% Tween 20 (TTBS) and incubated overnight in the same buffer plus antiserum. Further treatment of the filter and incubation with the second horseradish peroxidase-conjugated antibodies against rabbit immunoglobulins were the same as for Western blots (immunoblots) (see below).

DNA methods. Restriction enzymes and T4 DNA ligase were purchased from Boehringer GmbH, Mannheim, Federal Republic of Germany, and used according to the instructions of the manufacturer. *Sty*I was obtained from New England BioLabs, Inc., Beverly, Mass. Transformation of *E. coli* was done by standard procedures (16), and transformants were grown on LB-agar plates with 5 μg of tetracycline per ml or 50 μg of ampicillin per ml. Protoplast transformation of plasmids into *S. aureus* was performed by the method of Murphy et al. (20), and cells were regenerated on DM3 (20) agar with 5 μg of tetracycline per ml. Staphylococcal chromosomal DNA was prepared by the method of Löfdahl et al. (14). Plasmids from *E. coli* were prepared by an alkaline plasmid preparation method (8) and used directly for restriction analysis, deletion mutagenesis, or preparation of specific DNA fragments. To prepare plasmids from *S. aureus*, the cell walls were broken with lysostaphin (Sigma Chemical Co., St. Louis, Mo.), and thereafter the *E. coli* protocol was followed (8). Agarose gel electrophoresis (0.8%, wt/vol) was run according to Maniatis et al. (16) by using phage lambda DNA cleaved with *Hin*dIII as a size marker. Southern blots were performed with Biodyne A nylon membranes (Pall Ultrafine Filtration Corp., Glen Cove, N.Y.) according to the instructions of the manufacturer. DNA fragments, separated by agarose gel electrophoresis, were recovered by the centrifugal filtration (30). DNA probes were radiolabeled with an oligomer-primed DNA-labeling kit (Boehringer GmbH) by using [α-^{32}P]dATP (3,000 Ci/mmol; Amersham Corp., Arlington, Heights, Ill.). Probed filters were exposed to Fuji RX film with intensifying screens (Cronex Lightning-Plus; Du Pont Co., Wilmington, Del.) at −70°C.

Northern blot (RNA blot) hybridization. Total RNA from *S. aureus* V8 was prepared by lysostaphin treatment on ice, followed by a hot phenol extraction procedure as previously described (10). Total RNA from *E. coli* was prepared according to von Gabain et al. (28). All RNA preparations were DNase treated with RQ 1 DNase I (Promega Biotech, Madison, Wis.). Denaturing RNA gels containing formaldehyde were run by the method of Maniatis et al. (16). An RNA ladder of 9.5 to 0.24 kb (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used as a size marker, which was detected either by ethidium bromide staining or by hybridization with radiolabeled phage lambda DNA. Separated RNA was transferred by capillary blotting to Biodyne A filters, which were baked at 80°C for 1 h. Hybridization to the probe in 50% formamide at 42°C and subsequent washings were performed as described by Thomas (26). When the single-stranded DNA of the phage M13 clones was used as a probe, 50 ng of the single-stranded DNA was mixed with 50 ng of radiolabeled M13mp18 replicative-form DNA and the mixture was heated to 95°C for 10 min and added to the hybridization buffer.

Protein electrophoresis and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in 12% acrylamide gels by the method of Laemmli (13). Acrylamide and prestained standards (catalog no. 161-0305) were purchased from Bio-Rad Laboratories, Richmond, Calif. Protein concentrations were assayed as described by Lowry et al. (15). Extracts of *E. coli* clones to be analyzed were prepared as follows. Bacterial pellets (0.5 A_{550} units of cells) were suspended in 250 μl of sample preparation buffer (13) and boiled for 10 min. After sonication for 20 s, insoluble material was removed by centrifugation at 10,000 × *g* for 15 min. A 5-μl sample of the supernatant was subjected to electrophoresis. Extracts of *S. aureus* were prepared by dissolving protoplast pellets (1) in sample preparation buffer. Total protein (1 μg) was loaded onto the gel. Electrotransfer to nitrocellulose filters (BA 85, 0.45-μm pore size; Schleicher & Schuell) was done in a Bio-Rad Transblot cell in 25 mM Tris–192 mM glycine–20% (vol/vol) methanol, pH 8.3, at 6 V/cm overnight (27). Residual binding sites of the filter were blocked by incubation in TTBS for 30 min. The MBRP antibodies were then added in TTBS, and the filter was incubated at 4°C for 3 h. After the filter was washed three times for 10 min each time in TTBS,

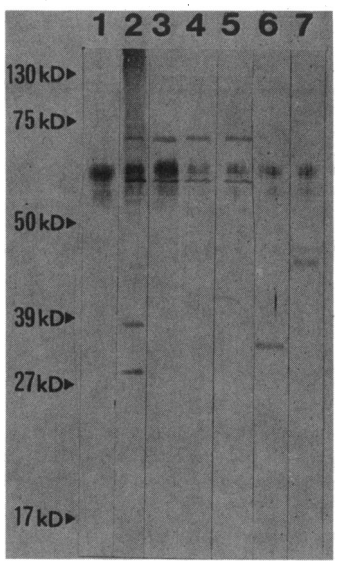

FIG. 1. Western blot analysis of clones from the staphylococcal genomic library. The extracts were from *E. coli* MC1061 carrying cloning vector without insert (lane 1), *S. aureus* V8 (lane 2), clone 8A2 (lane 3), clone 3G9 (lane 4), clone 10A11 (lane 5), clone 9F12 (lane 6), and clone 15C1 (lane 7). The absorbed MBRP-71–MBRP-60 antiserum was used for probing. Names of clones are given as coordinates of microdilution plates of the genomic library. Positions of prestained standards are indicated (kD, kilodaltons).

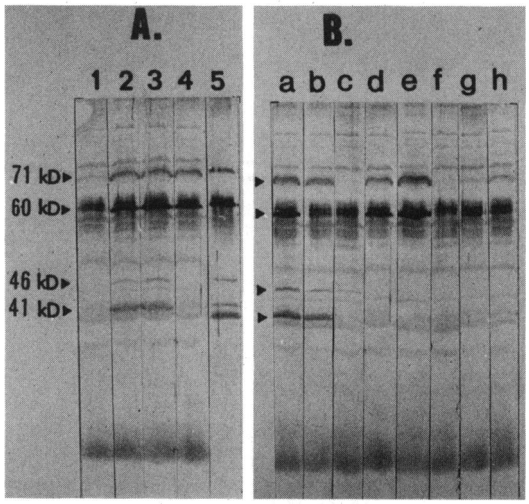

FIG. 2. Western immunoblot analysis of MBRP-positive clones from the staphylococcal genomic library and deletion mutants, using a mixture of the four monospecific antisera directed against the 71-, 60-, 46-, and 41-kDa components of the MBRP complex. Arrowheads indicate positions of the MBRP antigens. (A) MBRP production of the originally isolated *E. coli* clones. The extracts are from *E. coli* MC1061 carrying vector without insert (negative control; lane 1), 8A2 (pMBR1) (lane 2), 3G9 (pMBR2) (lane 3), 10A11 (pMBR3) (lane 4), and *S. aureus* V8 (control; lane 5). (B) Production of MBRP antigens in *E. coli* from various subcloned DNA fragments. The fragments are designated by the lowercase letters used in Fig. 3B.

horseradish peroxidase-conjugated swine antiserum directed against rabbit immunoglobulin (catalog no. 170-6515; Bio-Rad) was added in a 1:500 dilution in TTBS, and the filter was incubated for 1 h at room temperature. The filter was washed twice for 10 min each time in TTBS and then washed for 10 min in the same buffer without Tween 20. After this treatment, the antibodies were detected by the method of Haid and Suissa (7) by using 4-chloro-1-naphthol (Sigma).

RESULTS

Detection of MBRP-producing clones. All 3,000 clones of the staphylococcal genomic library were transferred to nitrocellulose filters and probed with the absorbed antiserum against MBRP-71 and MBRP-60. Nine colonies gave a stronger positive signal and were further analyzed by Western blotting by using the antiserum described above for antigen detection. Three of these clones contained antigens with the same mobility as MBRP-71 and MBRP-60 (Fig. 1, lanes 3 to 5). Note that in the staphylococcal lysate, at least three bands were seen in addition to MBRP-71 and MBRP-60 (Fig. 1, lane 2), indicating that the antiserum was not completely specific for MBRP-71 and MBRP-60. Three clones produced a protein with an apparent molecular size of 30 kDa (one is shown in Fig. 1, lane 6) which was not seen in the staphylococcal control. The possibility that this band represented a truncated form of MBRP-71 or MBRP-60 was excluded by the fact that it was not detected with the more specific antiserum against MBRP-71 or MBRP-60 and because the restriction map of the cloned DNA was completely different from that of the clones producing full-length MBRP-71 or MBRP-60 (data not shown). Three clones gave bands that probably represented protein A (one is shown in Fig. 1, lane 7) since they were detected with the conjugated antibodies alone (data not shown). The apparent molecular sizes, 45 and 48 kDa, are consistent with data reported by Löfdahl et al. (14) on the expression of the staphylococcal protein A gene by *E. coli*. Also, 30 clones giving weakly positive signals were analyzed by Western blotting, and none of them produced MBRP antigens (data not shown).

When the three MBRP-producing clones were analyzed by Western blotting with a mixture of the four monospecific MBRP antisera, it was found that clones 8A2 and 3G9 produced all four MBRP proteins and that clone 10A11 only produced the 71- and 60-kDa proteins (Fig. 2A, lanes 2 to 4). The plasmids of these three clones were designated pMBR1, pMBR2, and pMBR3, respectively.

Identification and restriction mapping of the MBRP-coding DNA. The plasmids of the three MBRP-producing clones were restriction mapped with *Eco*RI, *Pst*I, *Acc*I, *Sty*I, *Xba*I, and *Hin*dIII (Fig. 3A). To determine if all three MBRP-producing strains were derived from the same chromosomal locus, radiolabeled plasmids from each of the clones were used as probes in Southern blot hybridization experiments with chromosomal DNA from *S. aureus* V8 cleaved separately with *Eco*RI and *Pst*I. All three probes detected the same three *Pst*I fragments of 0.8, 8.0, and 8.5 kb and two *Eco*RI fragments of 10.5 and 7.0 kb (data not shown; compare restriction map, Fig. 3B), indicating that they were derived from the same chromosomal locus. This locus we named *mrp*.

Localization of the *mrp* genes. To determine the genetic organization of the MBRP complex, various fragments from pMBR1, pMBR2, and pMBR3 were deleted by cleaving plasmids with the appropriate restriction enzyme and religating the whole mixture. The restriction enzymes were chosen so that the antibiotic resistance genes or the origin of replication were not affected. Deletion mutations were confirmed by restriction analysis. Thus, from pMBR1, a 7.1-kb *Eco*RI fragment, a 5.2-kb *Pst*I fragment, and a 6.1-kb *Sty*I

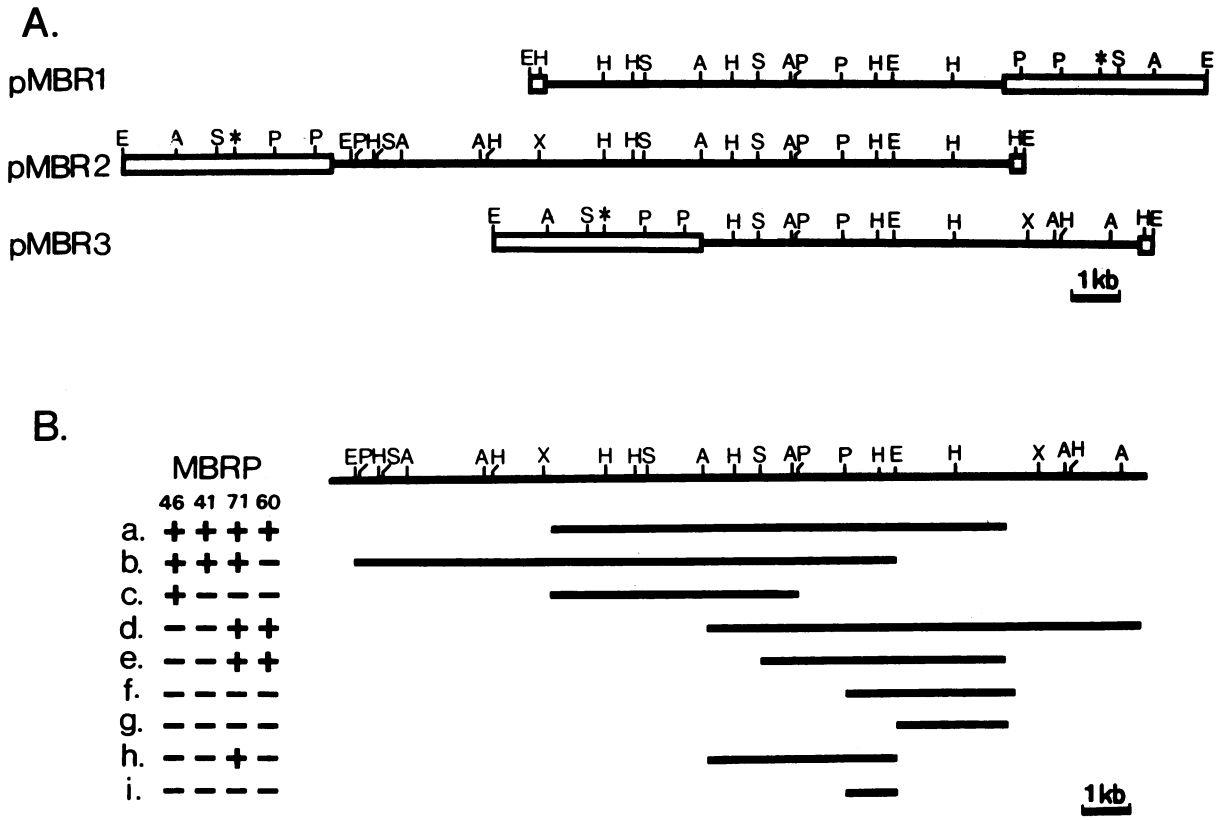

FIG. 3. Restriction maps of plasmids pMBR1, pMBR2, and pMBR3 from MBRP-producing *E. coli* clones (8A2, 3G9, and 10A11, respectively) and localization of the coding regions. Abbreviations: E, *Eco*RI; P, *Pst*I; H, *Hin*dIII; A, *Acc*I, S, *Sty*I; X, *Xba*I. Size markers of 1,000-base-pair region are shown at the bottom of each panel. (A) Restriction maps of original MBRP clones. ——, Cloned DNA; ▭, vector; *, origin of replication of the vector. (B) Production of MBRP components (at left) from subcloned DNA fragments as indicated by solid lines. The different fragments are referred to in the text by the letters at the left.

fragment were deleted, respectively. The extent of the *S. aureus* DNA in these constructs is shown in Fig. 3B (fragments g, c, and e, respectively). From pMBR2, an 11.0-kb *Pst*I fragment was deleted (Fig. 3B, fragment f), and from pMBR3, a 5.5-kb *Eco*RI fragment was deleted (Fig. 3B, fragment h). Furthermore, we cloned the 10.5-kb *Eco*RI fragment (Fig. 3B, fragment b) from pMBR2 in both orientations into the *Eco*RI site of pUN121. The 1.0-kb *Pst*I-*Eco*RI fragment (Fig. 3B, fragment i) of pMBR1 was also cloned in pUN121. *E. coli* clones containing these various plasmids were then analyzed for the production of MBRP components by Western blot analysis (Fig. 2B). These results are summarized in Fig. 3B.

Removal of nucleotides from the right side of the originally cloned DNA led to the progressive loss of first MBRP-60 and then MBRP-71 plus MBRP-41 (Fig. 3B, fragments b and c). Accordingly, deletion of DNA fragments from the left side of the insert resulted in the loss of the ability to code for synthesis of MBRP-46 and MBRP-41 (Fig. 3B, fragments d and e). These results suggest that the order of the genes from left to right is MBRP-46, MBRP-41, MBRP-71, and MBRP-60.

The subcloned 10.5-kb *Eco*RI fragment of pMBR2 produced MBRP-71, MBRP-46, and MBRP-41 but not MBRP-60, irrespective of the orientation of the insert in the vector (data not shown), indicating that the corresponding transcripts are promoted within the fragment.

Transcripts from MBRP-coding DNA. To determine the transcripts corresponding to the *mrp* genes, total RNA from *S. aureus* V8 was analyzed by Northern blot hybridization. One major transcript of 5.9 kb and two smaller transcripts of 2.8 and 1.6 kb were seen when the subcloned and purified 10.5-kb *Eco*RI fragment of pMBR2 (Fig. 3B, fragment b; Fig. 4A, lane 1) and the subcloned and purified 1.0-kb *Pst*I-*Eco*RI fragment of pMBR1 (Fig. 3B, fragment i; Fig. 4B, lane 1) were used as probes. These RNAs were not detected when the vector pUN121 was used alone as the probe (data not shown). To decide from which DNA strand the transcripts were synthesized, the 1.0-kb *Pst*I-*Eco*RI fragment was cloned into phages M13mp18 and M13mp19. The single-stranded phage DNAs were then used as probes in Northern blot hybridization experiments against staphylococcal total RNA. The M13mp18 clone hybridized with the same three RNAs as did the double-stranded probe (Fig. 4B, lanes 1 and 2), whereas no hybridization was seen with the M13mp19 probe. The probes were checked by Southern blot hybridization against *Eco*RI- and *Pst*I-cleaved chromosomal staphylococcal DNA. Both probes hybridized to the same 10.5-kb *Eco*RI and 8-kb *Pst*I fragments to the same extent (data not shown). The M13 clones were also confirmed by restriction cleavage of replicative-form DNA.

The MBRP-specific transcripts in *E. coli* containing pMBR1 were analyzed by using the purified 10.5-kb *Eco*RI fragment of pMBR2 as a probe. Three RNAs with the same mobilities as those seen in *S. aureus* appeared (Fig. 4A, lane 2).

In *E. coli* containing a deleted variety of pMBR1 (Fig. 3B, fragment e) which produced only MBRP-71 and MBRP-60,

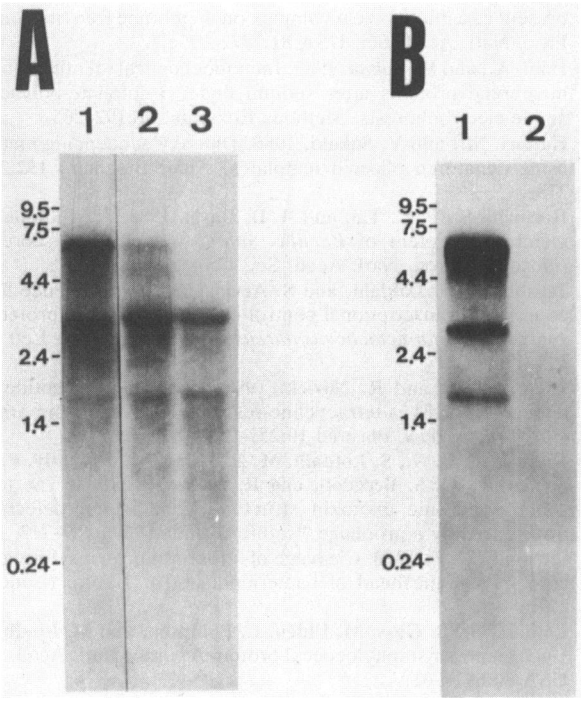

FIG. 4. Northern blot hybridization showing sizes of transcripts and direction of transcription of MBRP-coding DNA. The RNA standards (sizes in kilobases) are indicated at the left of each panel. (A) Total RNA from *S. aureus* V8 (lane 1), *E. coli* containing pMBR1 (lane 2), and *E. coli* containing a *Sty*I-deleted pMBR1 (Fig. 3B, fragment e) (lane 3) probed with the purified 10.5-kb *Eco*RI fragment of pMBR2 (Fig. 3B, fragment b). (B) Total RNA from *S. aureus* V8 probed with single-stranded DNA of the 1-kb *Pst*I-*Eco*RI fragment (Fig. 3B, fragment i) cloned in M13mp18 (lane 1) and M13mp19 (lane 2).

only the two small RNAs of 1.6 and 2.8 kb were seen (Fig. 4A, lane 3). The same results were obtained with three different RNA preparations.

Insertion inactivation of the *mrp* genes. Plasmid pMBR600, which carries a 0.6-kb internal fragment of the *mrp* operon, was used for insertion mutagenesis. This plasmid, which has temperature-sensitive replication and also carries a tetracycline resistance gene, was established in *S. aureus* V8 at 30°C. Clones containing the appropriate plasmids were grown overnight in tryptic soy broth at the nonpermissive temperature (42°C). After serial dilution, the bacteria were spread on nutrient agar plates with and without tetracycline and incubated at 42°C.

Tetracycline-resistant colonies were recovered at a frequency of approximately 10^{-6}. Chromosomal DNA from 10 of these clones was digested with *Eco*RI and analyzed by Southern blot hybridization by using as a probe the 0.6-kb *Pst*I-*Hin*dIII fragment. None of the clones had integrated the plasmid at the expected position in the *mrp* operon (data not shown). Integration of pMBR600 by homologous recombination (Campbell integration [29]) over the 0.6-kb *Pst*I-*Hin*dIII fragment would result in two new *Eco*RI fragments of 1.0 and 13.9 kb as compared with a 10.5-kb *Eco*RI fragment in the wild type. In 10 tetracycline-resistant clones examined, the 10.5-kb fragment was seen together with one or two other fragments (approximately 2.5, 3.0, and 9.5 kb), indicating that the plasmid had integrated outside the *mrp* region owing to some sequence homology between the vector and the staphylococcal genome. This was supported by the finding that in a parallel experiment, using only the hybrid vector, the frequency of tetracycline-resistant colonies was the same as with pMBR600.

As a positive control of our insertion inactivation strategy, we used pEXB383, which is identical to pMBR600 except that it contains a 383-base-pair fragment of a nonessential *S. aureus* gene instead of the *mrp* fragment. With this plasmid, the frequency of tetracycline-resistant colonies after the selection procedures at the nonpermissive temperature was approximately 1,000-fold higher than with pMBR600. Southern blot analysis of six clones revealed that all had integrated the plasmid at the expected position within the *expB* gene (data not shown). These results suggest that inactivation of the *mrp* operon is lethal.

DISCUSSION

The structural genes of the four polypeptides of the *S. aureus* MBRP complex were cloned in *E. coli* and were found within an 8.5-kb chromosomal DNA fragment. On the basis of the molecular sizes of the MBRP components (41, 46, 60, and 71 kDa), the theoretical minimal coding sequence should be approximately 6 kb. The very close coupling between the genes strongly supports our previous findings that the MBRP complex is a structural and functional unit (1, 3).

By analysis of the synthesis of different MBRP components from various deleted and subcloned DNA fragments, the order of the genes from left to right was found to be MBRP-46, MBRP-41, MBRP-71, and MBRP-60.

Three different RNA species (approximately 5.9, 2.8, and 1.6 kb), transcribed in the same direction (from left to right), were found within the MBRP-coding (*mrp*) region. They were all detected with the same 1-kb DNA probe from the right half of the *mrp* region, indicating that they are partly identical. Since the same RNAs were seen both in *S. aureus* and in *E. coli* clones producing all four MBRP components, attempts were made to analyze these RNAs in relation to the synthesis of the different MBRP components in *E. coli*. Considering its length (5.9 kb), the largest RNA could be a polycistronic mRNA coding for all four MBRP components. The two smaller RNAs would then be stable degradation products or specifically processed forms of 5.9-kb RNA. However, two different DNA fragments (Fig. 3B, fragments d and e) missing the left part of the *mrp* region, including the putative promoter of the 5.9-kb RNA, allowed the synthesis of the two small RNAs and the production of both MBRP-71 and MBRP-60. These results suggest that MBRP-71 and MBRP-60 are coded for by the 2.8- and 1.6-kb RNAs, respectively, since the 2.8-kb RNA is too small to code for both proteins.

To explain the finding that all three RNAs hybridized with the same 1-kb *Pst*I-*Eco*RI fragment, one must assume that both the 2.8- and the 1.6-kb RNAs are transcribed from promoters which are internal to the largest transcriptional unit. This would mean that the 2.8-kb RNA terminates within the 1-kb *Pst*I-*Eco*RI fragment and that the 1.6-kb RNA starts within this fragment. However, a DNA fragment extending approximately 3 kb to the right of the above-mentioned *Pst*I site (Fig. 3B, fragment f), which should contain the entire MBRP-60 gene, gave no detectable production of this protein. This cannot be explained on the basis of our present results but awaits more detailed studies, including nucleotide sequencing and determination of the transcriptional starts and terminations.

Our main reason for cloning the *mrp* genes was to be able to study the function of the MBRP complex. Our first

attempt to do so was made by insertion inactivation of the *mrp* region by using the internal 600-base-pair *PstI-Hin*dIII fragment as a target. According to our present model of the *mrp* operon, insertion of a foreign DNA in this fragment would abolish the synthesis of MBRP-71 and MBRP-60. To achieve this, the appropriate 600-base-pair DNA fragment was cloned into a tetracycline resistance plasmid with a temperature-sensitive replicon and transformed into *S. aureus*. A very low frequency of tetracycline-resistant colonies (10^{-6}) was found when the transformants were grown at the nonpermissive temperature. None of the survivors had integrated the plasmid at the desired position, indicating an inefficient homologous recombination or indicating that the recombinants did not survive. The possibility that inactivation of the *mrp* operon was lethal was supported by control experiments using the same temperature-sensitive plasmid containing a DNA fragment that is not essential. With this construct, the frequency of tetracycline-resistant recombinants was 1,000-fold higher and all examined survivors had integrated the plasmid at the desired position.

Although there is at present no conclusive evidence that the MBRP complex of *S. aureus* or its *B. subtilis* equivalent (S complex) is involved in the secretion of proteins, several indications for such a function have been presented. The most important are the association of the complex with membrane-bound ribosomes (1, 3, 5), its location between the membranes and the ribosomes (3, 9), and the correlation between the amounts of membrane-bound complex and exoprotein production (3, 17). Assuming a role for the MBRP complex in protein secretion, several reasons for a lethal effect of inactivation of the *mrp* operon can be suggested, such as accumulation of toxins and hydrolytic enzymes in the cytoplasm or disturbance of vital membrane functions due to stacking of secreted proteins in the membrane. In *E. coli* several genes coding for components of the secretion machinery seem to be essential (25). The exact reason for this is, however, not known.

On the basis of the present results, we hope to be able to elucidate the function of the MBRP complex by more specific and gentle in vitro mutagenesis and complementation experiments.

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