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Additional DNA in Methicillin-Resistant *Staphylococcus aureus* and Molecular Cloning of *mec*-Specific DNA

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Additional DNA was shown to be present in methicillin-resistant *Staphylococcus aureus* by one- and two-dimensional restriction endonuclease analyses of the chromosomal DNA. A 3.5-kilobase *BglII* fragment, which was present in methicillin-resistant strains but not in the isogenic methicillin-sensitive parental strain, was cloned into newly constructed plasmid pWDB1 in *Escherichia coli*. Hybridization of this 3.5-kilobase *BglII* fragment with different methicillin-sensitive and methicillin-resistant *S. aureus* clinical isolates indicated that the fragment represents part of the methicillin resistance determinant (*mec*). In addition, the fragment carries a sequence that is present in some large staphylococcal plasmids, as well as in penicillinase plasmid pI524.

Methicillin is the first β-lactamase-resistant semisynthetic penicillin. Methicillin resistance in *Staphylococcus aureus* is due to intrinsic resistance to semisynthetic penicillins and cephalosporins (7). Methicillin resistance is not due to drug inactivation (8). The exact resistance mechanism is still not known. The methicillin resistance determinant (*mec*) is chromosomally located and is linked to the *purA-nov-his* cluster (10).

Although methicillin sensitivity and *purA*$^+$ are cotransducible at a frequency of 15%, *mec* cannot be cotransduced with *purA*$^+$. This behavior supports the hypothesis that *mec* resides on an inserted DNA sequence in *S. aureus* and that there is no allelic equivalent in methicillin-sensitive strains (17). The methicillin resistance determinant might be integrated into a transposable element (4).

Efforts to clone the methicillin resistance determinant by using molecular cloning methods have so far been unsuccessful, perhaps due to the rather large size or the unusual sequence of the methicillin resistance determinant. We attempted to identify and clone a methicillin-specific DNA fragment in order to obtain a hybridization probe for further investigations and for cloning the total methicillin resistance determinant.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation conditions. The bacteria and plasmids used in this study are listed in Table 1. The strains were grown in Luria-Bertani broth (12) at 37°C. Solid medium contained 1.5% agar (Difco Laboratories). Ampicillin (Bayer Leverkusen) was used at a concentration of 50 μg/ml for selection after transformation.

Isolation and cloning of DNA. Cellular DNA was prepared by phenol extracting a staphylococcal cell lysate and spooling out the DNA on a glass rod as described previously (3), except that the preparation was precipitated with spermine (6) before restriction nuclease analysis. Small-scale plasmid preparations of *Escherichia coli* were made by heat and alkali denaturation of a lysate and neutralization by using acidic phenol, as described by Kieser (9). For large-scale plasmid preparations the plasmids were amplified with chloramphenicol (12) before lysis and were purified by CsCl density gradient centrifugation. Ligation of DNA with T4 DNA ligase (Anglian Biotechnology Ltd.) was carried out as recommended by the supplier. Competent *E. coli* DH1 was transformed by using the method of Maniatis et al. (12).

Restriction enzyme analysis and DNA hybridization. All of the restriction enzymes used were obtained from Anglian Biotechnology Ltd. and were used as recommended by the supplier. Analytical and preparative DNA agarose gel electrophoresis and extraction of DNA fragments from agarose gels by using KI were done as described previously (1). For two-dimensional DNA electrophoresis the procedure of Yee and Inouye (18) was used. DNA was transferred from gels to nitrocellulose membranes (Millipore Corp.) by using the method of Southern (16). Probe DNA was labeled by nick translation (14) with [α-^{32}P]dATP (Amersham Corp.). DNA-DNA filter hybridizations were performed at 67°C overnight by using the conditions described previously (3). The filters were dried, and radioautographs were produced by using Curix RP1 PE (Agfa) film.

RESULTS

Restriction enzyme analysis of chromosomal DNA. Because of the genetic behavior of the methicillin resistance determinant (*mec*) in cotransductional crosses (17), it is generally believed that the DNA coding for *mec* is present only in Mec^r strains and not in isogenic Mec^s strains. Differences between the restriction nuclease patterns of the chromosomal DNAs of isogenic Mec^r and Mec^s strains were indeed observed in this study.

Strains BB262 and BB270 were derived from Mec^s strain BB255 by phage-mediated transduction. The donors of *mec* were two Mec^r *S. aureus* clinical isolates. Mec^s strain WB12 was derived from strain BB270 by phage-mediated transduction. The transducing phage originated from a Mec^s *purA102 nov-142* donor strain. Selection was for novobiocin resistance. Methicillin sensitivity cotransduced with *purA102* and *nov-142* at a frequency of 10%.

In *BglII* digests of chromosomal DNA, three additional bands at 3.5, 3, and 2.5 kilobases (kb) were observed in both Mec^r strains but not in the Mec^s strains (Fig. 1A). The 3- and 2.5-kb bands were recognized by their stronger staining intensities, as they comigrated with bands present in the Mec^s parent. *Eco*RI digests of the Mec^r strains contained a 21.7-kb fragment which was not found in the Mec^s digests (Fig. 1C).

Additional differences between Mec^r and Mec^s strains were apparent in two-dimensional agarose gels of restriction

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype	Relevant phenotype	Origin (reference)
S. aureus			
BB255	Same as NCTC 8325	Mec^s	This laboratory (3)
BB262	Same as NCTC 8325, *mec*	Mec^r	This laboratory; transduction of strain BB255 with phage 83A grown on strain E691 *mec* (originally isolated from a patient in 1976) and selection for Mec^r
BB270	Same as NCTC 8325, *mec*	Mec^r	This laboratory; transduction of strain BB255 with phage 80 α grown on strain E142 *mec* (originally isolated from a patient in 1967) and selection for Mec^r
WB12	Same as NCTC 8325, *nov-142 purA102*	Mec^s Nov^r	This laboratory; transduction of strain BB270 with phage 80 α grown on an isogenic Mec^s Nov^r host and selection for Nov^r
MS1C		Amp^s	MS1 cured of its penicillinase plasmid by ethidiumbromide treatment
MS1	*bla*	Mec^s Amp^r	Clinical isolate, Zürich, 1985
MS2		Mec^s	Clinical isolate, Zürich, 1985
MS3		Mec^s	Clinical isolate, Zürich, 1985
MS4		Mec^s	Clinical isolate, Zürich, 1985
MS5		Mec^s	Spontaneous Mec^s derivative of strain MR5
MR1	*mec*	Mec^r	Clinical isolate, Zürich, 1985
MR5	*mec*	Mec^r	Clinical isolate, Zürich, 1984
FK507	*mec*	Mec^r	Clinical isolate, Warsaw
FK276	*mec*	Mec^r	Clinical isolate, Hungary, 1969
13136	*mec bla*	Mec^r Amp^r	R. W. Lacey, United Kingdom (11)
13136 (mtc$^-$)		Mec^s Amp^s	R. W. Lacey, United Kingdom (11)
E. coli DH1	F$^-$ *recA1 endA1 gyrA96 thi-1 hsdR17* (*hsdR$^-$ hsdM$^+$*) *supe44*		D. Hanahan, obtained from B. Hohn (12)
Plasmids			
pHC79	*bla tet*		B. Hohn (5)
pWDB1	*bla tet*		This study; isolation of the pBR322 part of pHC79 after *Bgl*II digestion
pWDB2	*bla tet*		This study; recombinant plasmid (insertion of a 3.5-kb *Bgl*II fragment of strain BB270 into the *Bgl*II site of pWDB1)
pI524	*cad bis lea blaI blaZ asa asi incI merA merB*		E. Murphy (13)

nuclease-digested DNAs. The *Eco*RI-digested DNAs were separated in the first dimension on an agarose gel; this was followed by digestion in situ with *Bgl*II and separation in the second dimension (Fig. 2). In two vertical lanes Mec^r strains BB270 and BB262 produced additional spots that were absent in Mec^s strain BB255; spots 1, 2, and 3 in the first lane were at 18, 3.5, and 2.9 kb, and spots 4 and 5 in the second lane were at 7.9 and 4.5 kb (Fig. 2A). On the other hand, the Mec^s strain produced DNA fragments that were not found in either Mec^r strain; spots 6 and 9 (8.9 and 3.8 kb) were present in Mec^s strain BB255 but not in the Mec^r strains (Fig. 2B). Other differences were found in pairs of spots (spots 7a and b and spots 8a and b). As judged by their staining intensities, Mec^s spots 7a and b together appeared to represent the same amount of DNA as the single spot (spot 7b) found in the Mec^r strains; the same was true of spots 8a and b compared with single spot 8b found in the same vertical lanes. This situation could be explained if in the Mec^s strains this part of the genome existed in two different configurations within a population, whereas in the Mec^r strains only one of these configurations was selected.

The one- and two-dimensional gels of Mec^r and Mec^s strains were originally prepared to identify putative *mec*-specific fragments that could then be isolated in preparative agarose gels. The 3.5-kb *Bgl*II fragment of Mec^r strain BB270, which was clearly separated from the surrounding bands in the one-dimensional gels (Fig. 1A), seemed to be the most suitable fragment for isolation and cloning.

Cloning of the 3.5-kb *Bgl*II fragment. Cloning vector pWDB1, which had a single *Bgl*II site, was derived from cosmid pHC79 (5) by removing the small *Bgl*II fragment containing the *cos* site and using the large *Bgl*II fragment with Amp^r and Tet^r, the replication functions, and some phage λ DNA. The 3.5-kb *Bgl*II fragment of *S. aureus* DNA was ligated into the *Bgl*II site of pWDB1. *E. coli* DH1 was transformed with the ligated mixture, and Amp^r colonies were selected. Since no change in the phenotype was expected due to the inserted staphylococcal DNA, the plasmid sizes of the clones had to be screened. One of nine Amp^r transformants carried a plasmid (pWDB2) of the desired size with a 3.5-kb insertion.

Analysis of the cloned 3.5-kb *Bgl*II fragment from *S. aureus*. The cloned 3.5-kb *Bgl*II fragment from *S. aureus* was isolated and used as a probe to hybridize *Bgl*II and *Eco*RI digests of the chromosomal DNAs of the isogenic Mec^s and Mec^r strains. In both Mec^r strains, which had obtained *mec* from different sources, the probe hybridized with a 3.5-kb *Bgl*II fragment (Fig. 1B and Fig. 3, lanes g and h) and with a 21.7-kb *Eco*RI fragment (Fig. 1D). The probe did not hybridize with DNAs isolated from the Mec^s strains (Fig. 1 and Fig. 3, lane f). This means that the cloned fragment consisted of additional DNA present only in strains carrying *mec*; it had to be located in the same chromosomal region as *mec*, since it was not present in Mec^s strains WB12, which was derived from Mec^r strain BB270 by selecting for *nov-142*.

Clinical Mec^r *S. aureus* isolates were stored both in stab cultures at room temperature and as lyophilized cultures. In some strains we observed a spontaneous loss of the Mec^r phenotype in stab cultures after 1 year, whereas this phenotype was retained in the lyophilized cultures. One pair of such isolates (from the original Mec^r culture and the spontaneous Mec^s culture) was analyzed (Fig. 3, lanes n and o).

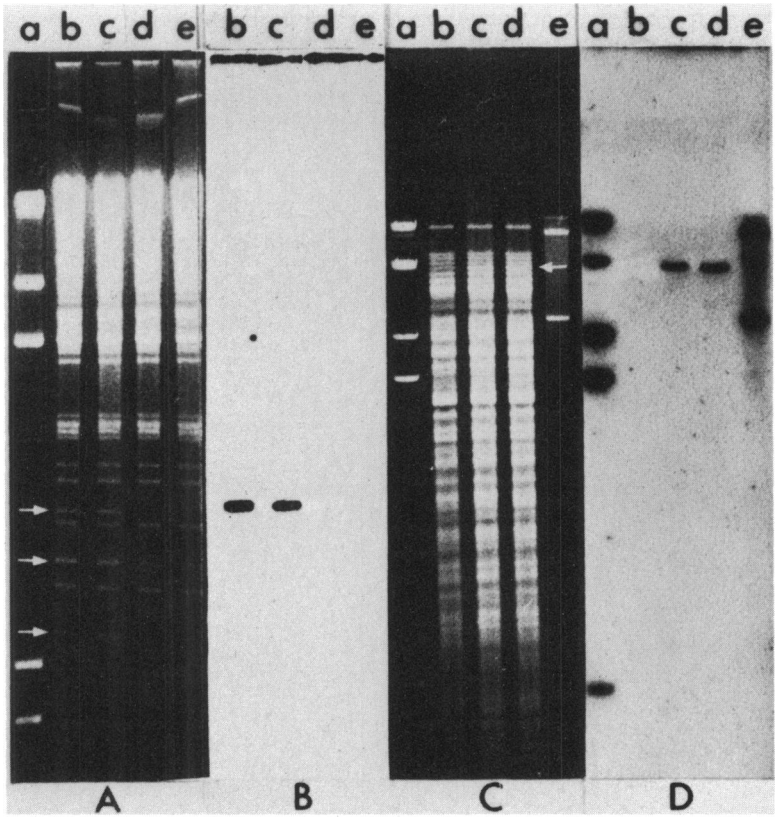


FIG. 1. Hybridization of the cloned 3.5-kb staphylococcal *Bgl*II fragment with *Bgl*II and *Eco*RI restriction nuclease digests of isogenic Mec^s and Mec^r strains. The *Bgl*II digests of chromosomal DNAs were separated on a 1.2% agarose gel (A), whereas the *Eco*RI digests were separated on a 0.5% agarose gel (C). Southern blots were made, and the cloned 3.5-kb staphylococcal *Bgl*II fragment was used as a hybridization probe. (B and D) Radioautographs of panels A and C, respectively. Some ^{32}P-labeled phage λ DNA was included in the hybridization mixture to label the molecular weight markers; it did not interfere with the staphylococcal DNA, as determined previously. (A and B) Lane a, Phage λ *Hin*dIII digest markers; lane b, Mec^r strain BB262; lane c, Mec^r strain BB270; lane d, Mec^s strain BB255; lane e, Mec^s strain WB12. (C and D) Lane a, Phage λ *Bgl*II digest markers; lane b, Mec^s strain BB255; lane c, Mec^r strain BB270; lane d, Mec^r strain BB262; lane e, phage λ *Xho*I digest markers. The additional bands found only in the Mec^r strains are indicated by arrows.

Hybridization of the 3.5-kb *Bgl*II fragment with *Bgl*II-digested DNA produced a strong band at 3.5 kb in only the Mec^r strain. Loss of the Mec^r phenotype seemed to be linked to excision of *mec* DNA.

The total DNAs of randomly chosen clinical isolates of *S. aureus* were purified; four strains were Mec^r, and four were Mec^s. The probe did not hybridize with *Bgl*II-digested DNAs of three Mec^s clinical strains (Fig. 3, lanes c through e). However, one Mec^s strain (Fig. 3, lane a) produced two strong hybridizing bands at about 5.5 and 0.5 kb. These bands disappeared after the strain was cured of its penicillinase plasmid (Fig. 3, lane b). This means that the bands were of plasmid origin.

All Mec^r strains produced a strong band in their *Bgl*II digests between 3.4 and 3.5 kb that hybridized with the cloned fragment (Fig. 3, lanes g through k, m, and o). Strain 13136 had (in addition to the 3.5-kb *mec*-determined band) a 3.6-kb band and a 0.5-kb band, as well as a weak 2.2-kb band (Fig. 3, lane m). These bands were also of plasmid origin, since they all disappeared in derived Mec^s strain 13136 (Mtc$^-$) (Fig. 3, lane 1), which had lost its penicillinase plasmid concomitantly with *mec*. These additional hybridizing bands comigrated with *Bgl*II bands produced from another penicillinase plasmid, pI524, which also hybridized with the 3.5-kb *Bgl*II fragment (Fig. 3, lane p). An analysis of *Eco*RI and *Bgl*II restriction nuclease digests of pI524 revealed that there were three plasmid regions which hybridized with the 3.5-kb *Bgl*II fragment (Fig. 4). One of these regions was localized to the left of *Bgl*II fragment B of pI524, and one was localized to the right of this fragment, which is supposed to carry the mercury resistance determinant. These regions were about 0.5 kb long. A third region, which had a lower level of homology, was also found on pI524, as shown by the weaker hybridization of *Eco*RI fragment D and *Bgl*II fragment F (Fig. 4). The 0.5-kb fragments are of interest since they were found on penicillinase plasmids, as well as on the chromosome of Mec^r strains linked to *mec* DNA.

In some Mec^r and Mec^s clinical isolates bands showing weak hybridization with the probe were also observed (Fig. 3, lanes i through l and o). The relationship of these bands to *mec* has not been analyzed yet.

DISCUSSION

In this study we showed that Mec^r strains contain additional DNA sequences compared with isogenic sensitive strains. The cloned 3.5-kb *Bgl*II fragment which was assumed to be associated with *mec* did not hybridize with the chromosomal DNAs of Mec^s strains. Hybridization of the 3.5-kb *Bgl*II fragment with the two isogenic Mec^r strains (Fig. 1B) and the Mec^r clinical isolates (Fig. 3), as well as the lack of any hybridization with Mec^s chromosomal DNA,

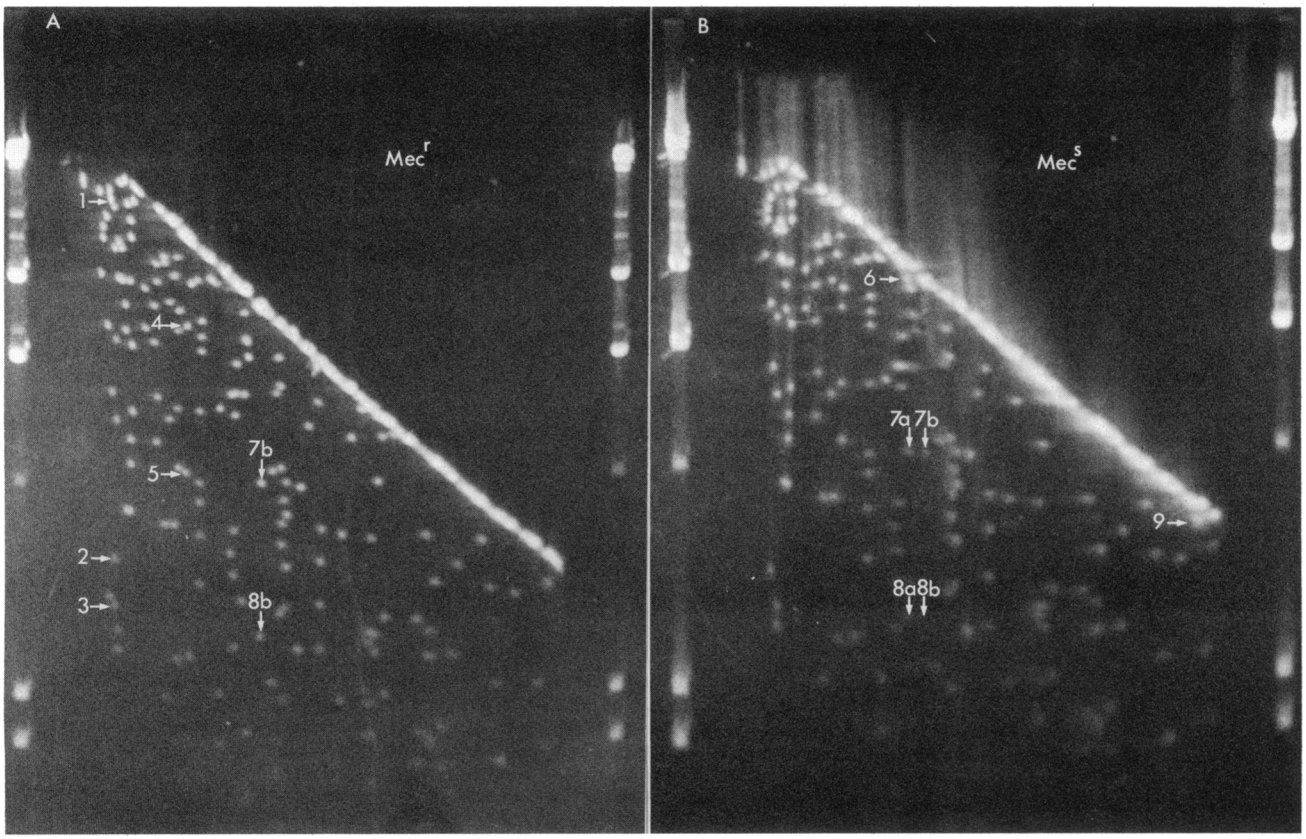
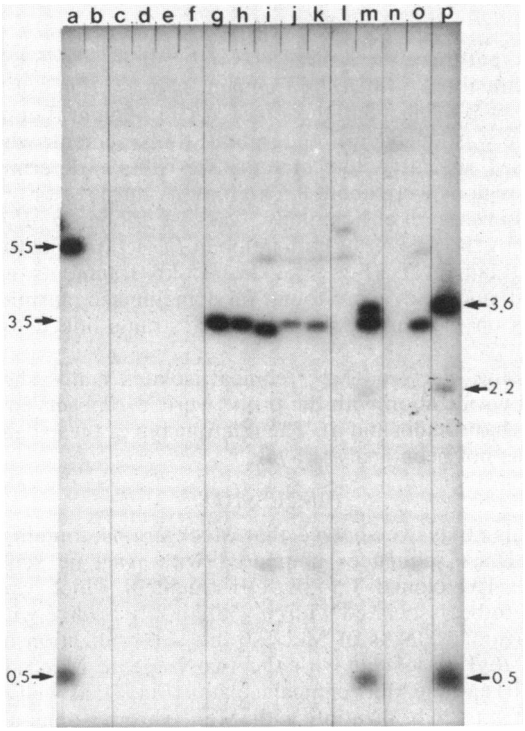


FIG. 2. Two-dimensional agarose gel of *Eco*RI and *Bgl*II restriction nuclease-digested chromosomal DNAs from isogenic Mec^r and Mec^s strains. (A) Mec^r strain BB262. (B) Mec^s strain BB255. An *Eco*RI restriction nuclease digest of chromosomal DNA was separated on a 0.8% agarose gel in the first dimension, the lane was cut out, and the DNA fragments were digested in situ with *Bgl*II and separated on a 0.8% gel in the second dimension. Phage λ *Hin*dIII digest markers were run at the ends of the gels. A second Mec^r strain, strain BB270, produced the same pattern as strain BB262 (data not shown). Additional spots are indicated by arrows and are numbered.



strongly suggests that the cloned fragment is a part of *mec* DNA. Its chromosomal location in the *mec-purA-nov-his* cluster was confirmed by the absence of hybridization in Mec^s derivative WB12. Interestingly enough, the size of the 3.5-kb *Bgl*II fragment seemed to be conserved in the various clinical isolates which we analyzed. The probe originated from a clinical Mec^r strain isolated in 1967, whereas the other Mec^r strains analyzed were isolated between 1969 and 1985 and originated from different parts of the world.

The unexpected hybridization of the 3.5-kb *Bgl*II fragment with certain penicillinase plasmid fragments from Mec^s and

FIG. 3. Hybridization of the cloned 3.5-kb staphylococcal *Bgl*II fragment with *Bgl*II restriction nuclease-digested DNAs from various Mec^s and Mec^r *S. aureus* strains. Total DNAs were isolated from randomly chosen clinical isolates of *S. aureus*, restricted with *Bgl*II, and separated on a 1.2% gel. A Southern blot was made, and the cloned 3.5-kb *Bgl*II fragment was used as a hybridization probe. Lanes a through f, Mec^s strains (lane a, strain MS1; lane b, strain MS1C; lane c, strain MS2; lane d, strain MS3; lane e, strain MS4; lane f, strain BB255); lanes g through k, Mec^r strains (lane g, strain BB262; lane h, strain BB270; lane i, strain MR1; lane j, strain FK507; lane k, strain FK267). Lanes l and m contained a strain pair [lane l, Mec^s strain 13136 (mtc$^-$); lane m, Mec^r strain 13136], as did lanes n and o (lane n, Mec^s strain MS5; lane o, Mec^r strain MR5). Lane p contained plasmid pI524. The numbers indicate fragment sizes (in kilobase pairs).

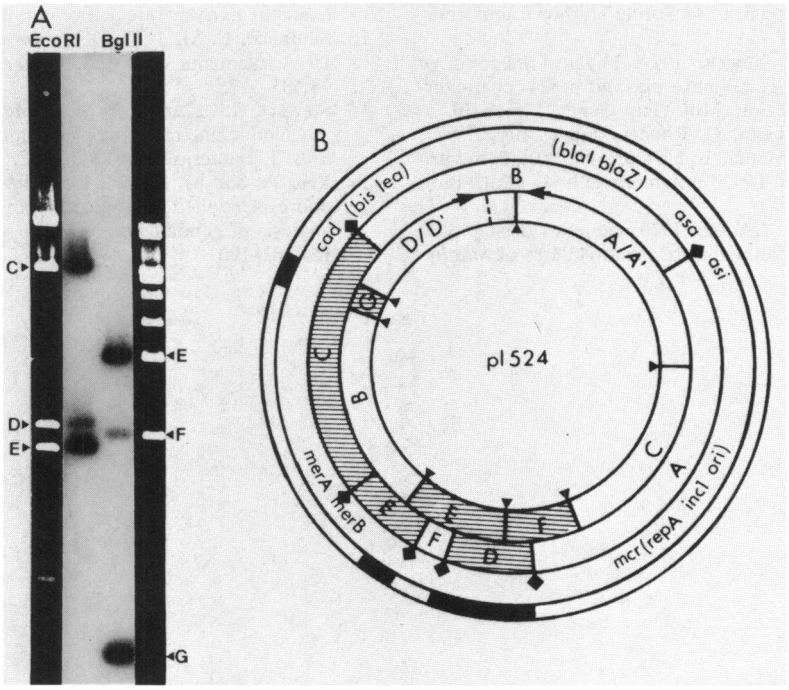


FIG. 4. Localization of regions of homology of plasmid pI524 with the cloned 3.5-kb *Bgl*II fragment. *Eco*RI and *Bgl*II restriction nuclease digests of pI524 were separated on a 1% agarose gel. A Southern blot was made by using the ^{32}P-labeled 3.5-kb *Bgl*II fragment as a probe. (A) The outer lanes are ethidium bromide-stained gel lanes, and the inner lanes are the corresponding radioautographs. (B) Physical genetic map of pI524 (13), with the *Bgl*II cleavage sites (▼) indicated on the inner circle and the *Eco*RI cleavage sites (◆) indicated on the outer circle. The restriction fragments which hybridized with the 3.5-kb probe are cross-hatched. The solid bars on the outermost circle indicate regions of homology between pI524 and the 3.5-kb probe.

Mec^r strains (Fig. 3), as well as with two distinct 500-base pair fragments of penicillinase plasmid pI524 flanking the mercury resistance determinant (Fig. 4), suggests that an IS-like sequence may be located on the 3.5-kb fragment. This hypothesis is supported by various observations. Shafer and Iandolo (15) showed that *mec* may sometimes be transiently associated with nonlinked markers, such as *entB* and small Tet^r plasmids, during elimination or transduction. There is an increase in the frequency of *mec* transduction in the presence of penicillinase plasmids in recipients (15), as well as an increase in the transduction efficiency of Tn*551* in Mec^r recipients (2). This indicates that there may be some kind of interaction between *mec* and transposable elements, probably by a sharing of common or similar recombination enzymes. However, our data do not support any hypothesis concerning a specific function of the 500-base pair fragment.

The 3.5-kb *Bgl*II fragment is probably only part of the DNA brought into cells with *mec*. The size of *mec* cannot be determined yet, although this gene is likely to be more than 3.5 kb long. In *Eco*RI-*Bgl*II double digests of Mec^r chromosomal DNA, the additional DNA in spots 1 to 5 added up to 36.7 kb (Fig. 2A). Other differences which were observed in the double digests between Mec^s and Mec^r strains suggest that additional changes may have occurred in the chromosome concomitantly with the insertion of *mec*.

The cloned *Bgl*II fragment will be used in further studies to isolate larger DNA fragments from a cosmid library of staphylococcal DNA. We hope that these fragments will carry the complete methicillin resistance determinant.

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LITERATURE CITED

1. **Bächi, B.** 1980. Physical mapping of the *Bgl*I, *Bgl*II, *Pst*I and *Eco*RI restriction fragments of staphylococcal phage φ11 DNA. Mol. Gen. Genet. **180**:391–398.
2. **Berger-Bächi, B.** 1983. Increase in transduction efficiency of Tn*551* mediated by the methicillin resistance marker. J. Bacteriol. **154**:533–535.
3. **Berger-Bächi, B.** 1983. Insertional inactivation of staphylococcal methicillin resistance by Tn*551*. J. Bacteriol. **154**:479–487.
4. **Cohen, S., H. M. Sweeney, and S. K. Basu.** 1977. Mutations of prophage φ11 that impair the transducibility of their *Staphylococcus aureus* lysogens for methicillin resistance. J. Bacteriol. **129**:237–245.
5. **Hohn, B., and J. Collins.** 1980. A small cosmid for efficient cloning of large DNA fragments. Gene **11**:291–298.
6. **Hoopes, B. C., and W. R. McClure.** 1981. Studies on the selectivity of DNA precipitation by spermine. Nucleic Acids Res. **9**:5493–5504.
7. **Kayser, F. H.** 1980. Die Resistenz methicillin-resistenter Staphylokokken gegenüber neuen Cephalosporin-Antibiotika. Infection **8**:165–170.
8. **Kayser, F. H., E. J. Benner, R. Troy, and P. D. Hoeprich.** 1971. Mode of resistance against β-lactam antibiotics in staphylococci. Ann. N.Y. Acad. Sci. **182**:106–117.
9. **Kieser, T.** 1984. Factors affecting the isolation of ccc DNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid **12**:19–36.
10. **Kuhl, S. A., P. A. Pattee, and J. N. Baldwin.** 1978. Chromosomal map location of the methicillin resistance determinant in *Staphylococcus aureus*. J. Bacteriol. **135**:460–465.
11. **Lacey, R. W.** 1972. Genetic control in methicillin-resistant strains of *Staphylococcus aureus*. J. Med. Microbiol. **5**:497–508.
12. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular

cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Murphy, E., and R. P. Novick.** 1979. Physical mapping of *Staphylococcus aureus* penicillinase plasmid pI524: characterization of an invertible region. Mol. Gen. Genet. **175**:19–30.
14. **Rigby, P. W., J. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labelling deoxyribonucleic acid to high specific activity in vitro by nick-translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
15. **Shafer, W. M., and J. J. Iandolo.** 1979. Genetics of staphylococcal enterotoxin B in methicillin-resistant isolates of *Staphylococcus aureus*. Infect. Immun. **25**:902–911.
16. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
17. **Stewart, G. C., and E. D. Rosenblum.** 1980. Genetic behavior of the methicillin resistance determinant in *Staphylococcus aureus*. J. Bacteriol. **144**:1200–1202.
18. **Yee, T., and M. Inouye.** 1982. Two-dimensional DNA electrophoresis applied to the study of DNA methylation and the analysis of genome size in *Myxococcus xanthus*. J. Mol. Biol. **154**:181–196.