Identification of a *Staphylococcus aureus* Transposon (Tn4291) That Carries the Methicillin Resistance Gene(s)†

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We isolated a transposon (Tn4291) that carries the resistance gene(s) for methicillin in a secondary insertion site on the penicillinase plasmid pI524. Transposition of Tn4291 into pI524 occurred during the transduction of the tetracycline resistance plasmid pSN1 from a methicillin-resistant donor into a recipient that carried the mec allele in the primary site on the chromosome. Insertion of Tn4291 caused extensive rearrangement of pI524 and resulted in the formation of a 27.9-kilobase-pair plasmid (pIT103) which coded for resistance to methicillin and cadmium, but not penicillin. Although resistance to methicillin and cadmium were always linked, Tn4291 was stably maintained only in the presence of a chromosomal mec allele, while in its absence the plasmid was unstable and transposition to the primary site occurred. Subsequently, a 20.1-kilobase-pair plasmid, pIT203, was formed which retained cadmium resistance and regained the ability to express β-lactamase activity.

The characteristics of the genetic determinant for methicillin resistance (Mec+) in *Staphylococcus aureus* have been the focus of much speculation and controversy. Dornbach and co-workers (5, 6), by using strain DU4916, have suggested that Mec+ is a plasmid-borne determinant. Supporting genetic evidence for this view has been presented by Anear and Grubb (1) and Lacey (13), although subsequently, Lacey and Grinstead (14) were unable to associate the Mec+ determinant with plasmid DNA. The controversy seemed resolved when Sjostrom et al. (29) and Kuhl et al. (12) used transformational analysis to place the Mec+ determinant on the *S. aureus* chromosome. In fact, Kuhl et al. (12) used three-factor genetic crosses to map the Mec+ determinant specifically to the pyr-his-nov-pur region of linkage group II of the staphylococcal chromosome.

The methicillin resistance determinant has also been found to possess characteristics of a mobile genetic element. It has been shown by us (25) and by Sjostrom et al. (29) that methicillin resistance can be transduced into a recombination-deficient recipient at a frequency which closely approximates the transfer frequency into a wild-type recipient. In addition, an unusual association between a small tetracycline resistance plasmid, pSN1 (25), and the methicillin resistance element resulted in their cotransfer at a level of about 105 times greater than that predicted from the random cotransduction of unlinked genes (i.e., >25% of Tc transductants were also Mec+). Surprisingly, in the transductants Mec+ and Tc are not linked, and each element segregates independently. Consequently, this element may be likened to the site-specific staphylococcal transposon Tn534 (19-21, 23).

This study of methicillin resistance was undertaken to clarify the status of this determinant and to determine whether the putative transposon could be captured on a plasmid that acts as a target for a second-site insertion.

**MATERIALS AND METHODS**

**Bacterial strains.** The bacterial strains used in this study are described in Table 1.

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**MEDIA.** Brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) was used for the routine culturing of bacteria and for bacteriophage propagation (26). When agar plates were required, 1.5% agar (Difco Laboratories, Detroit, Mich.) was added.

**Marker analysis and elimination.** Antibiotic sensitivities were determined on BHI agar plates containing combinations of methicillin (5 μg/ml), and tetracycline (5 μg/ml) or cadmium nitrate (10 μg/ml). These plates were then replica plated onto methicillin-containing media, and the colonies were scored for the loss of Cd, the plasmid marker, β-lactamase assays were carried out with beta-lactam reagent disks (Marion Scientific, Div. Marion Laboratories, Inc., Kansas City, Mo.). Growth in 5 μg of ethidium bromide per ml overnight at 37°C, as described previously (25), was employed to eliminate plasmid-borne antibiotic resistance markers.

**DNA purification.** The preparation of chromosomal DNA for use with DNA-modifying enzymes was described in an earlier report from our laboratory (9). Procedures for the preparation of cleared lysates and the purification of plasmid DNA by cesium chloride-ethidium bromide density gradient centrifugation have also been described previously (25). The DNA concentration was determined by measuring the A260 (1 unit of A260 = 50 μg/ml). Individual plasmids were obtained from dye-CsCl-purified plasmid DNA by separation in 0.8% agarose gels (26). Individual plasmid bands were excised with a clean razor blade and electroeluted (18) at 100 V for 1 h into TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA [pH 8.0]) containing 4.0 M NaCl and 20% glycerol. Plasmid DNA was precipitated by the addition of two volumes of cold 95% ethanol, and the precipitate was redissolved in TES buffer (30 mM Tris, 50 mM NaCl, and 2.5 mM EDTA [pH 7.8]).

**Transduction.** Transduction experiments were performed with bacteriophage 29 of the International Typing Series (2). Bacteriophage lysates were propagated and sterilized as described previously (7, 26). Transduction was carried out at a multiplicity of infection of 0.1, and both phage and uninfected recipient cells were plated as controls. All resulting clones were screened for the correct phenotype by replica plating on BHI agar containing the appropriate antibiotic.

**Transformation of *S. aureus* protoplasts with plasmid DNA.**
TABLE 1. Designation, phenotype, and plasmid profile of the *S. aureus* strains used in this studya

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype and derivation</th>
<th>Relevant phenotype</th>
<th>Plasmids present</th>
</tr>
</thead>
<tbody>
<tr>
<td>RN450</td>
<td>8325-4</td>
<td>Mec' Cd' Tc'</td>
<td>None</td>
</tr>
<tr>
<td>RN1753</td>
<td>8325-4(d11) (pI524)</td>
<td>Mec' Cd' Tc'</td>
<td>pI524</td>
</tr>
<tr>
<td>80CR3</td>
<td>PS80C r' m&quot;</td>
<td>Mec' Cd'</td>
<td>None</td>
</tr>
<tr>
<td>RN4220</td>
<td>8325-4 r&quot;</td>
<td>Mec' Cd'</td>
<td>None</td>
</tr>
<tr>
<td>DU4916</td>
<td></td>
<td>Mec' Cd' Tc'</td>
<td>pSN1, pSN2, pSN3</td>
</tr>
<tr>
<td>KS1320</td>
<td>RN1753 TD with DU4916</td>
<td>Mec' Cd' Tc'</td>
<td>pI524</td>
</tr>
<tr>
<td>KS1342</td>
<td>KS1320 TD with DU4916</td>
<td>Mec' Cd' Tc'</td>
<td>pIT101, pIT102, pIT103, pSN1</td>
</tr>
<tr>
<td>KS1352</td>
<td>80CR3 TF with KS1342 plasmids</td>
<td>Mec' Cd' Tc'</td>
<td>pIT203</td>
</tr>
<tr>
<td>KS1362</td>
<td>RN4220 TF with pIT103</td>
<td>Mec' Cd'</td>
<td>pIT203</td>
</tr>
<tr>
<td>KS1365</td>
<td>KS1362 cured of pIT203</td>
<td>Mec' Cd'</td>
<td>None</td>
</tr>
<tr>
<td>KS1370</td>
<td>KS1365 TF with pIT103</td>
<td>Mec' Cd'</td>
<td>pIT103</td>
</tr>
</tbody>
</table>

a Abbreviations: Mec, Methicillin; Cd, cadmium; Tc, tetracycline; r', restriction negative; r, resistant; s, sensitive; TD, transduced; TF, transformed.

Plasmid DNA was transformed into protoplasts of *S. aureus* 80CR3 and RN4220 by the method described by Chang and Cohen (3). Transformation mixtures were then spread onto each of several regeneration medium plates (31) and incubated at 37°C for 24 to 48 h. The cells were suspended in 0.5 ml of BH1, replated onto BH1 agar plates containing the appropriate antibiotic, and incubated at 37°C. The suspension method precluded accurate determination of the frequency of transformation.

Hybridization analysis. Agarose gel electrophoresis and the procedures for Southern blot hybridization and hybridization have been described previously (30). Nick-translated plasmid DNA was prepared by the method described by Rigby et al. (24). Restriction endonuclease digestions were performed as suggested by the manufacturer (New England BioLabs, Inc., Beverly, Mass.).

RESULTS

The strategy for the isolation of the methicillin resistance element was developed from the following observations. High cotransductional association was observed in wild-type and recombination-deficient cells (25) between the two unlinked genes, chromosomally expressed methicillin resistance and plasmid-borne tetracycline resistance. This suggested that the tetracycline resistance plasmid pSN1 provided vector functions for the transfer of a mobile element that encoded methicillin resistance. Furthermore, the single map site for mec on the chromosome (12) presumably also identified the insertion site of the mobile element and indicated a site specificity similar to that of **Tn554** (23). Therefore, to isolate the transposon, we blocked the presumed site of insertion with a chromosomal mec allele. During transduction for tetracycline resistance, we hypothesized two possible genetic arrangements. (i) If a repressor of transposition was produced by the resident Mec element located in the primary or preferred site, the incoming second copy should be trapped in the supposed plasmid vector pSN1; and (ii) in the absence of such a repressor, or even in its presence, as with **Tn554** (23), movement might occur into a second-site target provided by the plasmid pI524. To test this hypothesis, we constructed a recipient strain of *S. aureus* 8325-4 that was Mec' to block the insertion site on the chromosome. It also contained the plasmid pI524 to provide a potential secondary target site for transposition.

A bacteriophage 29 transducing lysate was prepared on *S. aureus* DU4916 as the donor of methicillin resistance and pSN1. Transductions were carried out, and tetracycline-resistant clones were isolated at a frequency of 9.5 × 10⁻⁷.

To determine which of the predicted results occurred, the plasmid content of 19 randomly chosen clones was examined for a plasmid (either pSN1 or pI524) that was increased in size due to the insertion of the methicillin resistance element. However, all contained the four plasmids shown in Fig. 1 (lane 3), with sizes of 2.4 (pIT101), 2.5 (pIT102), 4.2 (pSN1), and 27.9 (pIT103) kilobase pairs (kbp). Other than pSN1, none of these plasmids was analogous to the plasmids contained in the donor strain *S. aureus* DU4916 (Fig. 1, lane 1). One of the Tc' transductants (KS1342) was analyzed further.

Location of the methicillin resistance gene. The presence of pSN1 suggests that the methicillin resistance determinant is located elsewhere. Evidence that it had inserted into a plasmid was provided by its increased transducibility from strain KS1342 at frequencies (6.7 × 10⁻⁷) that were 600- to 3,300-fold greater than those obtained with chromosomal mec genes (KS1362, 1.1 × 10⁻⁶; DU4916, 2 × 10⁻⁸). Therefore, bulk plasmid DNA from KS1342 was purified by dye-buoyant density gradient centrifugation and was used to transform protoplasts of the restriction- and modification-deficient strain *S. aureus* 80CR3 (33) to methicillin resis-

FIG. 1. Agarose gel electrophoresis of CsCl-ethidium bromide density gradient-purified plasmid DNA from *S. aureus* DU4916 (lane 1), RN1753 (lane 2), KS1342 (lane 3), KS1352 (lane 4), KS1362 (lane 5), KS1370 (lane 6), and KS1365 (lane 7). Size designations along the right are in kilobase pairs.
tance. The plasmid content of several transformant clones was examined and found to consist only of a 20.1-kbp plasmid, pIT103 (Fig. 1, lane 4) that was approximately 7.8 kbp smaller than the largest plasmid contained in the transforming DNA preparation (i.e., Fig. 1, lane 3). Furthermore, all the methicillin-resistant clones were also found to be cadmium resistant (encoded by pIT24; Fig. 1, lane 2); this suggests that the methicillin resistance element indeed inserted into pIT24. pIT203 was too small to account for such an insertion, however, unless extensive deletion of pIT24 occurred in the process.

To determine whether pIT103 formed by rearrangement after a second-site insertion of the methicillin resistance element into pIT24, pIT103 was nick-translated and used to probe the plasmids pIT103, pSN1, and pIT24. pIT103 did not hybridize with pSN1 (Fig. 2, lane 2), but it did hybridize with pIT24 (Fig. 2, lane 3). pIT101 and pIT102 were also isolated and used as probes of various restriction endonuclease digests of pIT24. These two plasmids were found to hybridize to regions of pIT24 that were deleted during the formation of pIT103. pIT101 is homologous to the region of pIT24 that contains one of the inverted repeat sequences preceding the penicillinase genes (22, 28), and pIT102 is homologous to a region between the mercury resistance genes and the mcr functions (22, 28) (data not shown). The 4.2-kbp plasmid in KSI342 was shown to hybridize with pSN1 (data not shown). Furthermore, HindIII restriction endonuclease digests of the 4.2-kbp plasmid and pSN1 (27) were examined and found to be identical (data not shown). The 27.9-kbp plasmid from KSI342 (pIT103) was then purified and used to transform protoplasts of the restrictionless host RN4220 for resistance to methicillin and cadmium. The transformants that were obtained were screened for plasmid content and were again found to contain the smaller 20.1-kbp plasmid pIT203 (Fig. 1, lane 5).

These data imply that the methicillin resistance element of pIT103 is unstable, and in the absence of a chromosomal copy of the mec gene, transposition occurs by a zygotic induction-like mechanism. To determine whether this was the case, pIT103 was transformed into S. aureus RN4220 that contained a copy of the methicillin resistance element on the chromosome. Selection was for resistance to cadmium, and transformants were examined for plasmid content. All the clones selected were found to contain the larger plasmid pIT103 (Fig. 1, lane 6), indicating that if a copy of the element occupies the primary site on the chromosome, the second copy on pIT103 is unable to transpose. The presence of the methicillin resistance element on this plasmid was verified by subsequent transformation of the plasmid from this strain into S. aureus RN4220 (data not shown) with selection for methicillin resistance. All the methicillin-resistant transformants examined were also resistant to cadmium and underwent a transposition event with regeneration of the 20.1-kbp plasmid pIT203.

One of these transformants, KSI62, was cured of pIT203 by growth in the presence of 5 μg of ethidium bromide per ml. The culture was screened for the loss of resistance to cadmium nitrate and the plasmid, which occurred at a rate of 7% (Fig. 1, lane 7), yet 100% of the culture retained the methicillin resistance phenotype.

Characterization of pIT103. A restriction endonuclease map of pIT103 is shown in Fig. 3. As a result of the original insertion event, the target plasmid pIT24 sustained extensive deletion. Approximately 11.7 kbp of the plasmid, which corresponded to one of the inverted repeats (28) that preceded the bla genes and the DNA that conformed to the plasmids pIT101 and pIT102, were deleted. The deleted DNA was replaced with Tn4291. The filled portion corresponded to the region of the plasmid which contained non-pIT24 DNA and represented the probable location of the transposon. This fragment was 7.8 kbp and was bounded by HindIII restriction sites. The remaining regions of pIT103 showed homology to both pIT24 and pIT203. Also shown in Fig. 3 is the location of the cadmium resistance determinant and the inactivated β-lactamase determinant (see below), which were detected and localized by using specific probes for these genes.

Transposition resulted in a precise excision of Tn4291 from pIT103 and the subsequent formation of pIT203. To determine those regions of pIT103 that were deleted following transposition, both pIT103 and pIT203 were used to probe the same blot of restriction endonuclease digestions of pIT103 (Fig. 4). From these data, it can be seen that a 7.8-kbp HindIII fragment (Fig. 4, compare lanes 6 and 9) and the corresponding 6.8- and 1.0-kbp fragments in the HindIII-EcoRI double digestion (Fig. 4, compare lanes 5 and 8) were lost.

The 7.8 kbp HindIII fragment was then used to probe chromosomal digests of a methicillin-sensitive strain,
RN4220, and a methicillin-resistant strain. The methicillin-resistant strain KS1370 was formed by transposition of Tn4291 from pIT103 to the chromosome of RN4220 with subsequent curing of pIT203. Chromosomal DNA was digested with HindIII, subjected to agarose gel electrophoresis, and probed. The probe hybridized to a 9.0-kbp fragment in the KS1370 chromosomal digest (Fig. 5, lane 2), identifying the insertion of the transposon into the chromosome, but did not hybridize to any DNA in the RN4220 chromosomal digest (Fig. 5, lane 1). pIT203 did not hybridize to this 9.0-kbp band (data not shown). This experiment also substantiates the fact that the HindIII restriction sites are external to the transposon, or a 7.8-kbp fragment would have been generated in the chromosomal DNA preparations.

A comparison among the plasmids pI524, pIT103, and pIT203 is given in Table 2. pIT103 is the only plasmid that confers methicillin resistance. The presence of the other pI524 resistance genes on pIT103 and pIT203 indicates that these regions are conserved subsequent to the insertion of Tn4291, verifying the restriction data. It is significant that only strains containing pIT103 lacked the ability to produce β-lactamase, but those containing pI524 or pIT203 were penicillin resistant and β-lactamase positive. Therefore, in the formation of pIT103, Tn4291 appeared to have insertionally inactivated a region of the penicillin genes of pI524 and on transposition, precise excision occurred which restored β-lactamase activity.

**DISCUSSION**

The early observations of Dornbusch and co-workers (5, 6) and Shafer and Iandolo (25) concerning the high level of cotransducibility of the genes for tetracycline resistance, methicillin resistance, and enterotoxin B production provided the first evidence which suggested that methicillin resistance resides on a mobile genetic element. Moreover, the lack of an allelic equivalent of mec in methicillin-sensitive cells further implies this resistance trait as an accessory gene. When the chromosomal site of methicillin resistance was mapped by Kuhl et al. (12) and was found to be stably located in linkage group II, speculation on the transposability of methicillin resistance lessened. However, the issue resurfaced when Shafer and Iandolo (25, 26) and Sjostrom et al. (29) showed that the methicillin resistance determinant is capable of chromosomal integration independently of the host recombination system. Therefore, if methicillin resistance is located on a transposable element, it is probably similar to the site-specific transposon Tn554 (23).

The results presented here demonstrate the existence of Tn4291, a new site-specific mobile element in S. aureus that encodes methicillin resistance. From results of our earlier work (25), we anticipated that the transposon would be associated with pSN1, but we were never able to isolate variants of pSN1 or other plasmids that contained the methicillin resistance genes. Therefore, while evidence for such a complex remains strong (25), it seems clear that this association, in contrast to the second-site insertion, is unstable in the presence of a chromosomal mec allele. Cotransductional association of chromosomal methicillin resistance and plasmid-encoded tetracycline resistance may occur by mechanisms that are related to the chromosomal integration of pTI181 identified by Gillespie et al. (10) and reported in a recent review by Lyon and Skurray (17, and references therein). pTI181 is integrated into the chromosome of methicillin-resistant strains and is flanked by sequences similar to IS257. Four copies of similar sequences were also found in cloned DNA associated with methicillin resistance. These sequences may provide focus for recombination events that generate mixed oligomers of pSN1 and mec alleles that are packaged during transducing particle production (8) but that are unstable and fully resolvable on insertion in the recipient.

A number of investigators (4, 5, 11, 25, 32) have observed an increase in the transduction frequency of mec when the

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**TABLE 2. Comparison among plasmids pI524, pIT103, and pIT203**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mec</th>
<th>Pc</th>
<th>Cd</th>
<th>Hg</th>
<th>Asa</th>
<th>Asi</th>
<th>Inverted repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>pI524</td>
<td>s</td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>Present</td>
</tr>
<tr>
<td>pIT103</td>
<td>r</td>
<td>s</td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>Absent</td>
</tr>
<tr>
<td>pIT203</td>
<td>r</td>
<td>s</td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>r</td>
<td>Absent</td>
</tr>
</tbody>
</table>

a Abbreviations: Mec, Methicillin; Pc, penicillin; Cd, cadmium; Asa, arsenate; Asi, arsenite; r, resistant; s, sensitive.

b Penicillin resistance was determined by a penicillinase assay.

c The presence of inverted repeats was determined by the observation of half-molar bands in restriction endonuclease digests.
recipient cell contains a penicillinase plasmid such as pI524. Our results suggest that these penicillinase plasmids play a more active role in the transduction of methicillin resistance and perhaps other resistance determinants by providing secondary insertion sites for mobile elements that carry such genes. The formation of pIT103 is the result of the resolution of such a transduction intermediate which occurred in the absence of an available primary insertion site.

Transposition of Tn4291 into an alternative site occurred even in the presence of a chromosomal copy of the transposon, suggesting that movement does not appear to be under the control of a repressor. Tn554 also transposes to a second site in the presence of a chromosomal copy, but it has been shown to produce a repressor of transposition (19). However, the repressor only affects transposition into the primary site. A similar situation may also be found with Tn4291, but as yet this question remains open.

After transposition of Tn4291 from pIT103 to the chromosomal region of the residual plasmid pIT203, lacked DNA sequences that hybridized to Tn4291. However, a 9.0-kbp chromosomal HindIII fragment homologous to the 7.8-kbp HindIII fragment of pIT103 containing Tn4291 can be demonstrated. These data therefore demonstrate the sequential transposition of Tn4291 from its chromosomal location in strain DU4916 to a plasmid insertion site on pI524 and back to a chromosomal location.

The insertion of Tn4291 caused extensive deletion of about 11.7 kbp of pI524, leaving a plasmid of only 27.9 kbp (pIT103). The data indicate that Tn4291 originally inserted into pI524 between two HindIII sites that were either originally quite close together or juxtaposed by the extensive rearrangement that occurred. We conclude that the precise Tn4291 insertion site is within the bia region of pIT103 and interrupts its expression. Support for this conclusion rests not only on hybridization data but also on the observation that after transposition the resultant plasmid pIT203 regained β-lactamase activity. This finding also indicates that excision of the transposon is precise and similar to that exhibited by Tn554 (21) and temperate bacteriophage such as lambda (15) or staphylococcal phage L54a (16).

The regions of the original pI524 that remained appeared to be evolutionarily conserved sequences. These plasmids presumably evolved from one ancestral plasmid with an approximate molecular weight of 10,000 that included the cadmium resistance gene(s) and the necessary mcr functions (28). In fact, the determinants for cadmium, mercury, arsenate, and arsenite resistance and β-lactamase production remain with pIT103.

The exact size of the transposon is not clear. We have not been able to detect the DNA that remains in pIT203 after transposition that is homologous to the 7.8-kbp HindIII segment of DNA which would indicate that the transposon consists of essentially all of the fragment. The apparent size of the element indicates that it consists of several genes, at least one of which relates to antibiotic resistance (possibly an altered penicillin-binding protein [17]) and one or more genes of which are concerned with transposition (19, 20).

Two new cryptic plasmids (pIT101 and pIT102) also appeared whose origin could be traced to pI524. Their small size (2.4 and 2.5 kbp, respectively) raises the question of their inability to replicate autonomously. We were unable to isolate strains of S. aureus in which these plasmids existed singly. This may indicate that they are unable to replicate independently in the pI524 replicon or that they integrate into the chromosome as silent units. These possibilities are currently under investigation.

**ACKNOWLEDGMENTS**

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