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Dissemination among Staphylococci of DNA Sequences Associated with Methicillin Resistance

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DNA probes consisting of pUC19 containing cloned *Staphylococcus aureus* chromosomal fragments were constructed from two methicillin-resistant *S. aureus* strains with different DNA sequences 5′ to *mecA*, the gene that mediates methicillin resistance. The probe from one strain, BMS1, contained a portion of the regulatory sequences (the terminal 641 bp of *mecR1* and all of *mecI*) associated with the induction and repression of *mecA* transcription (pGO195). The second probe, from strain COL (pGO198), contained DNA not found in strain BMS1. This DNA was within the sequences added at the site of a *mecR1* deletion. Genomic digests of 14 *S. aureus* isolates recovered between 1961 and 1969 all hybridized with pGO198. In contrast, 78% (36 of 46) of the *S. aureus* organisms isolated since 1988 hybridized with pGO195 but not with pGO198; the remainder hybridized with pGO198. No *S. aureus* isolates hybridized with both probes. *Staphylococcus epidermidis* digests hybridized with pGO198 (46%), pGO195 (14%), or both probes (35%); all 20 *Staphylococcus haemolyticus* isolates hybridized with pGO198. The restriction fragment length polymorphism patterns of all pGO198-hybridizing regions in *S. aureus* were identical to those in strain COL. In addition, the *mecR1* deletion junction nucleotide sequences of eight *S. aureus* and six *S. epidermidis* isolates were identical. However, 21 of 23 *S. epidermidis* and all 20 *S. haemolyticus* isolates had from 5 to more than 20 additional chromosomal bands that hybridized with pGO198; none of 21 *S. aureus* isolates had additional hybridizing bands. These data suggest that the additional DNA responsible for the *mecR1* deletion was part of a repetitive, and possibly mobile, element resident in coagulase-negative staphylococci but not in *S. aureus*. These data also support a hypothesis that the deletion event occurred in a coagulase-negative staphylococcus with subsequent acquisition of the interrupted sequences by *S. aureus*.

Resistance of staphylococci to all classes of beta-lactam antibiotics (methicillin resistance) is mediated by the production of an insensitive target enzyme. The insensitive target is a penicillin-binding protein (PBP), called PBP 2A, that is an addition to the normal, beta-lactam-susceptible complement of PBPs; it is not a mutant form of an existing PBP (8, 25). The gene encoding PBP 2A, *mecA*, is therefore not present in methicillin-susceptible staphylococci but is acquired by resistant cells (2, 25). It is a chromosomal gene and, in those *Staphylococcus aureus* strains that have been analyzed, always maps to the same location, between the genes encoding protein A and DNA gyrase (17). In addition to *mecA*, more than 30 kb of flanking sequences is also acquired, and the entire region is called *mec* or the *mec* locus (4). Both *mecA* and *mec* locus DNAs hybridize among all species of staphylococci that display the methicillin-resistant phenotype, suggesting the horizontal transfer of *mec* DNA (1, 2). There is considerable speculation about the origin and evolution of the *mec* locus. On the one hand, Kreiswirth et al. (13) recently provided evidence that all methicillin-resistant *S. aureus* evolved vertically from a single progenitor clone. On the basis of Southern blotting with DNA probes from *mecA* and Tn*554*, they suggested that *mecA* entered *S. aureus* on only one or possibly two separate occasions within the last 35 years, with little if any horizontal transfer within the species since the entry of *mecA*. On the other hand, Musser and Kapur (16) used multilocus enzyme electrophoresis to establish an evolutionary hierarchy for methicillin-resistant *S. aureus* and found that, although a group of isolates appeared to be closely related, most isolates exhibited a multilocus enzyme electrophoresis diversity more typical of the species as a whole than of a single clone. These latter data favor the continuous horizontal acquisition of *mec* sequences by *S. aureus*.

However, neither of the two studies cited above looked at coagulase-negative staphylococci as a source for *mec* DNA. Since both *mecA* and *mec* locus DNAs from coagulase-negative staphylococci have been shown to hybridize with comparable sequences from *S. aureus* (1, 2), it is reasonable to examine the role of coagulase-negative staphylococci in any possible horizontal acquisition of *mec* by *S. aureus*. In the current study, we looked at specific restriction fragment length polymorphism (RFLP) patterns and DNA sequence signatures in the region immediately 5′ to *mecA* in *S. aureus*, *Staphylococcus epidermidis*, and *Staphylococcus haemolyticus* isolates representing extensive geographic and chronologic diversities. This region contains two genes, *mecR1* and *mecI*, that are divergently transcribed from *mecA* (10, 21). On the basis of the amino acid similarities of their gene products to molecules regulating β-lactamase production in *S. aureus* and *Bacillus licheniformis* (9, 12, 18), it has been proposed that these genes encode a sensor-transducer and repressor, respectively. When these genes are intact, their products are capable of inducing and repressing *mecA* transcription and altering the phenotypic expression of methicillin resistance. In the present study, we found that early isolates of *S. aureus* contained a specific deletion of *mecR1* accompanied by the insertion of new DNA

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TABLE 1. Source of staphylococcal isolates used in the study

Species and contributor (no. of isolates)[a]	Dates of isolation (yr)	Place of isolation (no. of isolates)
S. aureus		
Public Health Research Institute (17)	1961–1971	England (6), Denmark (3), Uganda (1), Switzerland (1), Egypt (1), Kenya (1), Wash. (1), Oreg. (1), Md. (1), Tex. (1)
Public Health Research Institute (10)	1988–1992	N.Y. (6), Pa. (1), Canada (1), Ireland (1), Australia (1)
Bristol-Myers Squibb (10)	1971–1979	Calif. (2), La. (2), Mass. (1), Conn. (1), Wis. (1), Minn. (1), England (2)
Bristol-Myers Squibb (15)	1988–1992	Md. (2), N.C. (3), Pa. (2), Fla. (3), Tenn. (3), Conn. (1), United States[b] (1)
Centers for Disease Control (11)	1969–1979	N.Y. (3), Okla. (1), Wash. (1), Mo. (1), Conn. (1), Va. (1), Ky. (1), Fla. (1), United States[b] (1)
Centers for Disease Control (9)	1982–1985	N.J. (3), Calif. (2), N.Y. (1), Md. (1), S.C. (1), United States[b] (1)
Centers for Disease Control (10)	1989–1992	Iowa (3), Ga. (3), N.Y. (1), Calif. (1), United States[b] (2)
Medical College of Virginia Collection (9)	1980–1982	Va. (6), Fla. (3)
Medical College of Virginia Collection (3)	1990	Va. (3)
Memorial Hospital of Rhode Island (9)	1979–1984	S.C. (4), Mich. (1), Ga. (1), Ky. (2), Mass. (1)
M. C. de Freire Bastos (9)	1990–1992	Brazil (9)
Total *S. aureus* (112)		
S. epidermidis		
Medical College of Virginia Collection (58)	1970–1982	Va. (16), Mass. (17), Ala. (11), Oreg. (1), Canada (7)
Medical College of Virginia Collection (14)	1989–1991	Va. (8), Mich. (1), N.J. (1), Minn. (1), N.C. (2), Md. (1)
Bristol-Myers Squibb (1)	1969	United States[b] (1)
Total *S. epidermidis* (73)		
S. haemolyticus		
Bristol-Myers Squibb (10)	1990–1992	N.Y. (2), Pa. (1), Fla. (1), Ky. (1), Tex. (1), N.J. (1), Mo. (1), Ohio (1), Tenn. (1)
University of Toronto (10)	1984–1990	Canada (10)
Medical College of Virginia Collection (1)	1989	Va. (1)
Total *S. haemolyticus* (21)		

[a] Isolates from the Public Health Research Institute, New York, N.Y., were provided by B. Kreiswirth, isolates from Bristol-Myers Squibb were provided by M. Pucci, isolates from the Centers for Disease Control were provided by F. Tenover and R. Cooksey, isolates from the Memorial Hospital of Rhode Island were provided by S. Opal, isolates from the University of Toronto were provided by D. Low, and isolates from two hospitals in Rio De Janeiro, Brazil, were provided by M. C. de Freire Bastos.

[b] Isolates from unknown sites in the United States.

at the point of deletion; identical deletion junctions and added DNA were found in *S. epidermidis*. In addition, we provide evidence not only that the transfer of *mec* sequences is horizontal but also that the direction of transfer is from coagulase-negative staphylococci to *S. aureus*.

MATERIALS AND METHODS

Bacterial isolates. The sources of the staphylococci examined in the present study are given in Table 1. The type of specimen from which isolates were recovered was not known for most of the *S. aureus* and *S. haemolyticus* isolates. All *S. epidermidis* isolates were recovered from blood or intravenous catheters. We identified isolates as staphylococci by their appearance on Gram staining and catalase production; we determined species by coagulase production, API Staph-Ident strips (Analytab Products, Plainview, N.Y.), and specific carbohydrate utilization, all as described previously (2, 7). Phenotypic methicillin resistance was confirmed by growing the isolates on plates containing 10 μg of methicillin per ml (2). Heterotypic and homotypic expressions of resistance were determined by efficiency of plating, as described by Tomasz et al. (23).

DNA manipulation. Genomic DNA libraries were prepared from *S. aureus* isolates COL (1965; London, England) and BMS1 (1989; Long Branch, N.J.) and *S. haemolyticus* isolate Y176 (1988; Richmond, Va.). Cells were lysed, and genomic DNA was prepared by a variation of the Marmur technique (7). Partial *Sau*3A digests were made, and fragments were separated by sucrose density gradient centrifugation. Fractions with DNA fragments from 9 to 23 kb in size were collected, and the DNA was ligated into phage vector lambda DASH (Stratagene, La Jolla, Calif.) and packaged, all as described by the manufacturer. Phages were propagated to produce plaques on *Escherichia coli* LE392 and were lifted onto nitrocellulose; plaques were probed with ^{32}P-labelled pGO164. The probe, pG0164, is a 1.1-kb internal fragment from the *mecA* gene cloned into pUC18 and has been described previously (2). No target sequences hybridized with the vector alone. Lambda phage plaques were purified, and DNA was isolated and mapped with restriction endonucleases by standard techniques (19). The DNA fragments from lambda clones were subcloned into pUC19 or pBluescript (Stratagene) for further mapping and for use as probes against genomic staphylococcal DNA targets. All DNA cloning and Southern hybridization experiments were performed as described previously (22).

DNA sequences were determined from double-stranded DNA templates by using the Sanger dideoxynucleotide chain termination method and *Taq* polymerase (15). Sequence progression was accomplished by synthesizing successive primers.

DNA hybridization. All isolates were initially screened by dot blot hybridization (2). Probes consisted of insert plus

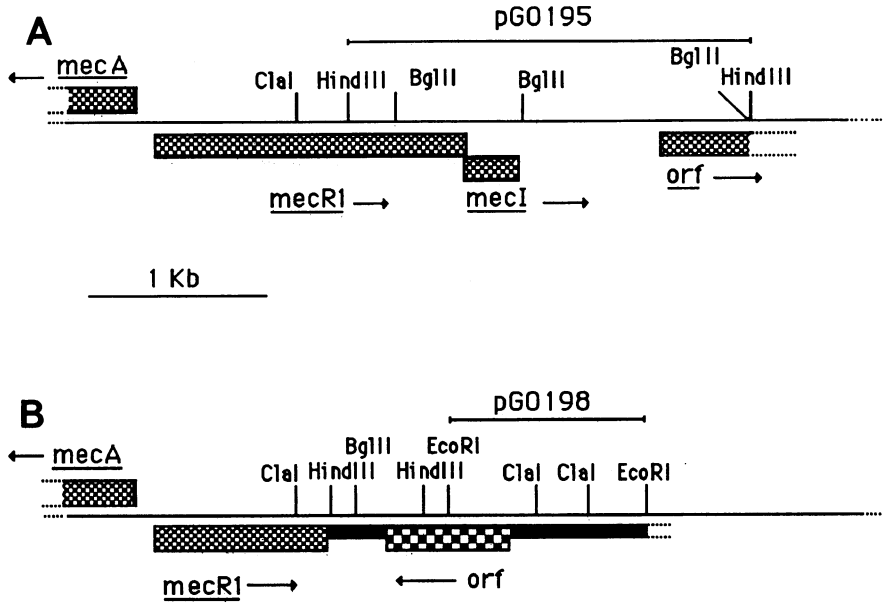

FIG. 1. Schematic representation of DNA sequences and probes used in the study. The BMS1 (A) and COL (B) *S. aureus* strains are shown. *mecA*, *mecR1*, and *mecI* are genotypic designations for the genes encoding PBP 2A and two putative regulatory genes, respectively. orf indicates ORFs of unknown function. Arrows designate the direction of transcription. Vertical lines denote restriction endonuclease cleavage sites. pGO195 and pGO198 are the designations for fragments that were cloned on pUC19 and that served as probes.

vector DNAs and were radiolabelled with [α-^{32}P]dCTP by nick translation. Vector sequences have previously been shown not to hybridize with staphylococcal DNA (2). Genomic DNA was extracted from selected isolates by a variation of the cetyltrimethylammonium bromide (CTAB; Sigma) minilysis method described previously for plasmid isolation (22, 24). For this procedure, staphylococci were grown overnight in brain heart infusion broth, and 3 ml of the growth was pelletted in a microcentrifuge tube. The pellet was resuspended in 500 μl of 10 mM Tris–1 mM EDTA plus 10 μl of a 1.5-mg/ml solution of lysostaphin (Applied Microbiology Inc., New York, N.Y.), and the cells were lysed by the addition of 30 μl of 10% sodium dodecyl sulfate; 3 μl of a 20-mg/ml solution of proteinase K was then added. Following the addition of 100 μl of 5 M NaCl, 80 μl of a 10% solution of CTAB in 0.7 M NaCl, and 650 μl of sevage (isoamyl alcohol [1 part] plus chloroform [24 parts]), the lysate was centrifuged and the supernatant was removed. The supernatant was treated with phenol, chloroform, and isoamyl alcohol, and the DNA was precipitated with ethyl alcohol. This total cellular DNA was then cleaved with restriction endonucleases and was electrophoresed through agarose. Genomic digests were then transferred to nitrocellulose by the Southern technique, probed, exposed to X-ray film, and analyzed for homology to probe DNA (dot blots) or RFLPs (genomic digests). All hybridization techniques and conditions have been described previously (2, 7, 22).

PCR amplification. PCR was used to analyze the deletion junction in selected staphylococcal clinical isolates by using genomic DNA that was purified by CTAB precipitation as described above. Primers were synthesized on a Biosearch 8700 DNA synthesizer (New Brunswick Scientific Co., New Brunswick, N.J.). The sequences of the two primers were 5′-GATGGACAATGACTGTG-3′ and 5′-GATGTCTGTC GAGGACTC-3′ (5′ representing the N terminus and 3′ representing the C terminus). PCR was performed on staphylococcal genomic DNA by using Vent polymerase (New England Biolabs, Beverly, Mass.) in a Microcyder (Eppendorf, Inc., Freemont, Calif.). The PCR products obtained were analyzed by agarose gel electrophoresis, purified, and directly sequenced by the dideoxynucleotide chain termination method as described above.

Nucleotide sequence accession numbers. The GenBank accession numbers for the nucleotide sequences presented here are L14017 for COL (3101 bp) and BMS1 L14020 for (3481 bp.).

RESULTS

Phenotypes of BMS1 and COL. The phenotypic expression classes of strains BMS1 and COL were determined by efficiency of plating. BMS1 was heterotypic in its resistance expression, similar to the isolates of expression class I, as defined by Tomasz et al. (23), while COL was completely homotypic (class IV). Neither isolate produced β-lactamase; BMS1 contained no plasmids, while COL had a single cryptic plasmid.

Cloning and sequencing of DNA 5′ to *mecA* in BMS1 and COL. Overlapping lambda clones of BMS1 and COL that hybridized to the *mecA* probe were mapped, and sequences 5′ to *mecA* were subcloned. These are shown in Fig. 1. Figure 2 shows the relevant nucleotide sequences of the 3,471 bases of DNA 5′ to *mecA* in BMS1 and the 2,969 bases in COL. For BMS1 and COL, the predicted translational start sites and promoter sequences for both *mecA* and *mecR1* were identical to each other and to published sequences (10). The sequence of BMS1 revealed two open reading frames (ORFs) on the opposite DNA strand and divergent from *mecA*. These sequences were identical to those reported by Hiramatsu et al. (10) for a Japanese isolate, N315, also reported to be extremely heterotypic in its resistance expression. These ORFs have been called *mecR1* (1,758 bp) and *mecI* (372 bp) because of their similarities to the *S. aureus* and *B. licheniformis* β-lactamase

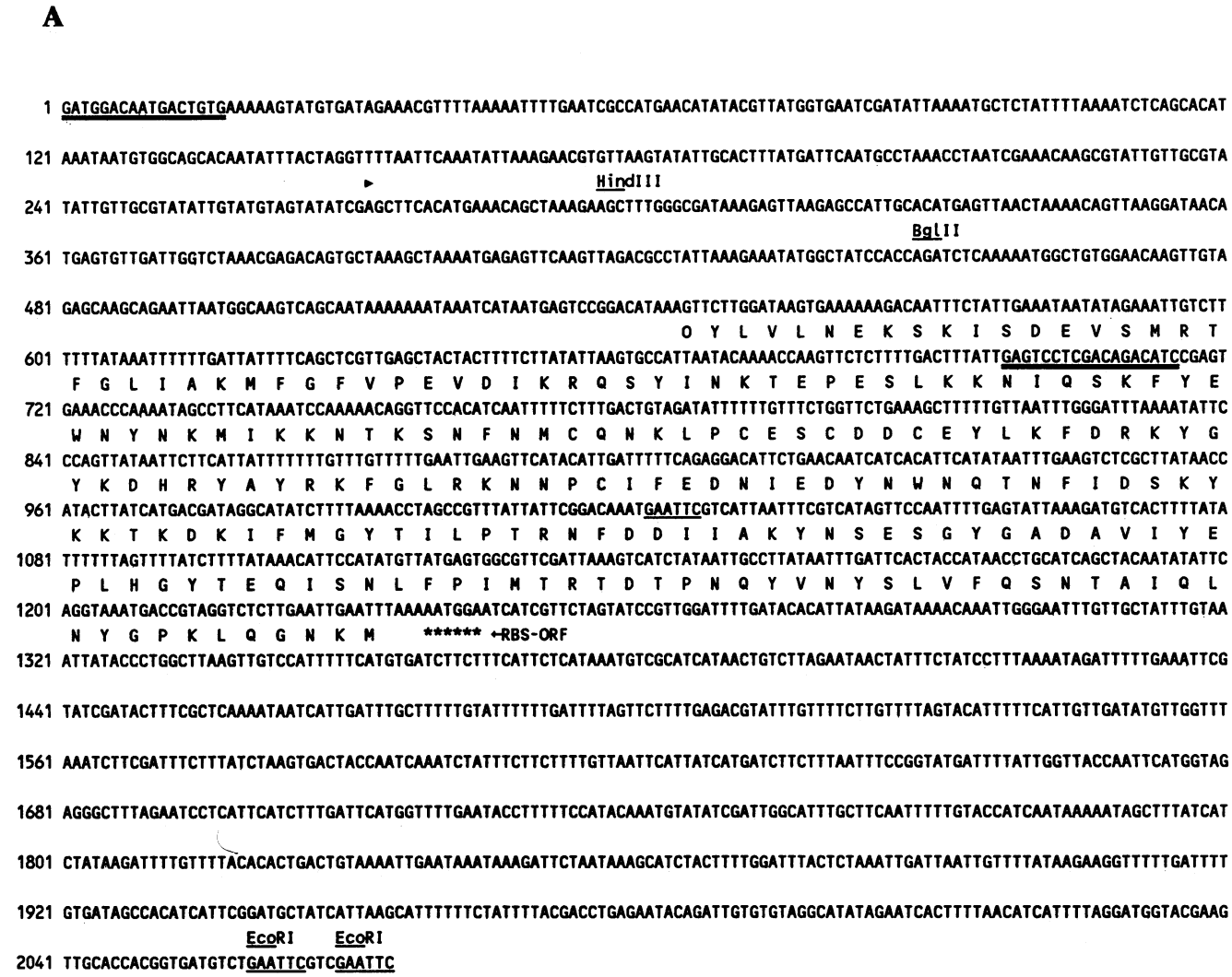

FIG. 2. Nucleotide sequences of probes and PCR primers used in the study. (A) Sequence of the 1,045-base *Eco*RI *mec* fragment from COL (bases 1021 to 2065) cloned in pUC19 to produce pGO198 (*Eco*RI restriction sites are underlined). Also included in panel A are 271 bases of *mecR1* before the divergence (arrowhead) marking the deletion of *mecR1-mecI* and the addition of new DNA. The positions of the PCR primers used to amplify fragments that included the deletion-addition junction are double underlined. The second PCR primer sequence (bases 698 to 715) is the opposite strand of what is shown here. An ORF (5′ to 3′ on the opposite strand) within the added DNA is also shown, with amino acids indicated above the nucleotide sequence; the putative ribosome-binding site (RBS) is indicated by asterisks, and the translational stop codon is indicated by O. (B) Sequence of the 2,355-base *Hin*dIII *mec* fragment (bases 147 to 2401) from strain BMS1 cloned on pUC19 to produce pGO195. The junction for divergence of the sequences in panels A and B is between bases 4 and 5, (marked by the arrow in panel B). The 5′ 971 bases of *mecR1* (not shown) are identical to those previously published (10), and a partial ORF contained within the cloned sequence is also shown. Putative ribosome-binding sites are indicated by asterisks. Sequences submitted to GenBank include the entire *mecR1* ORF for both strains COL and BMS1.

coinducer (*blaR1*) and repressor (*blaI* [9, 12, 18]). However, in COL the *mecR1* sequence was the same as that in BMS1 for 975 bases and then diverged completely, with termination of the *mecR1* ORF 987 bases from its start. The COL sequences following the point of divergence were completely different from the BMS1 sequences over the 1,006 bases of COL and the 2,438 bases of BMS1 that we determined. The DNA 3′ to this junction in COL contained one 688-bp ORF with a near-consensus ribosomal binding sequence on the opposite strand and oriented in the opposite direction from *mecR1* and a second ORF preceding the first; the second ORF consisted of 363 bases preceding a translational stop sequence with no identified translational start site (not shown in Fig. 2). In BMS1, a 515-bp partial ORF was also found 3′ to *mecI*, beginning 638 bp from the *mecI* translation stop. The sequences are illustrated schematically in Fig. 1.

Two subclones prepared from strains BMS1 and COL were used in probing wild-type staphylococcal isolates (Fig. 1). pGO198 consisted of a 1,045-bp *Eco*RI fragment from COL, cloned into pUC19, that contained DNA beyond the *mecR1* deletion junction and that had no homology, by nucleotide sequence analysis or hybridization, to the DNA from BMS1. pGO195, a 2,255-bp *Hin*dIII fragment from BMS1, also cloned into pUC19, contained the terminal 641 bp of *mecR1*, all of *mecI*, and 1,243 bp of downstream DNA including the initial 515 bp of the unidentified ORF.

Probing clinical isolates for *mec* sequences. All of the clinical isolates listed in Table 1 were initially screened by dot

B

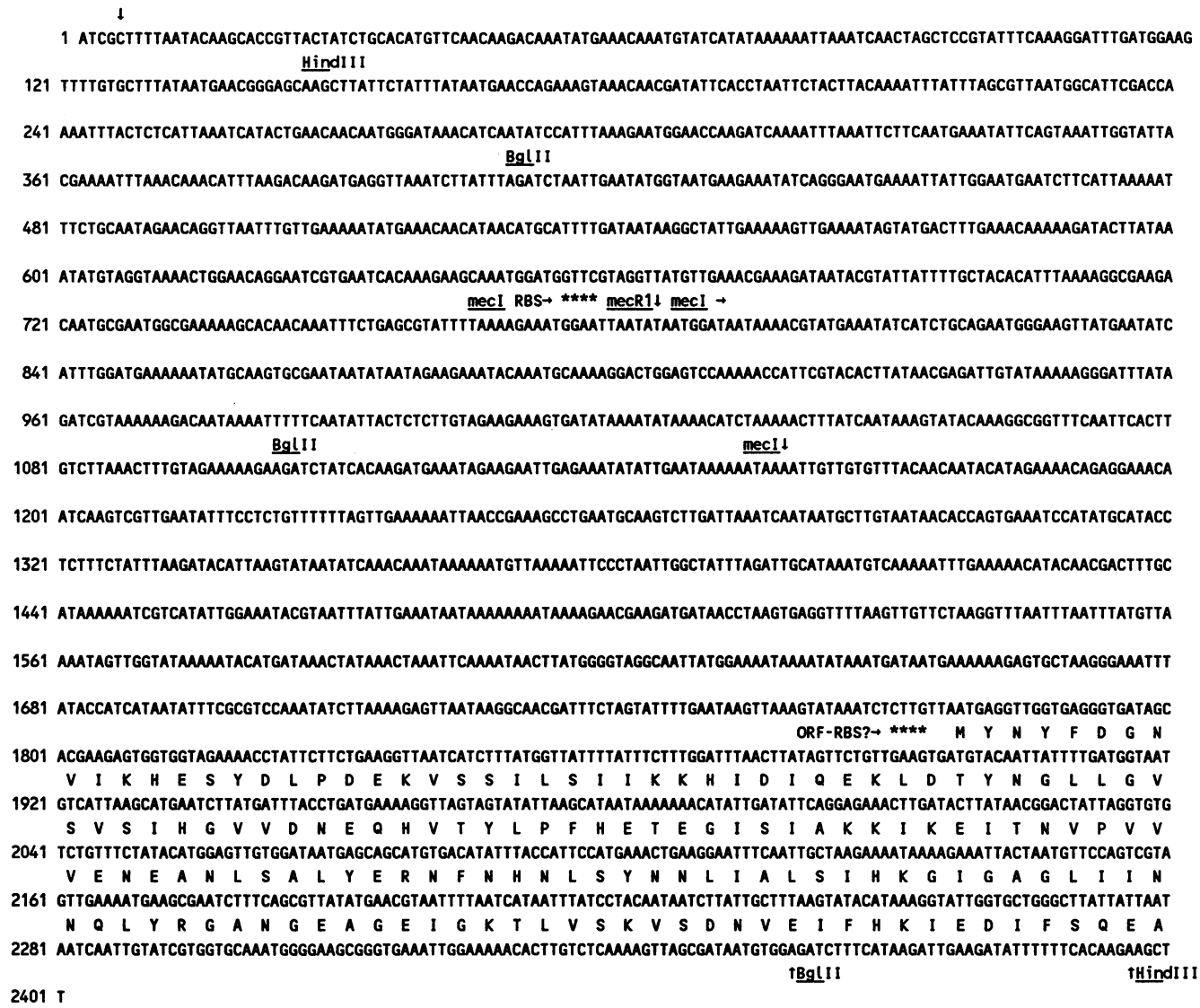

FIG. 2—*Continued.*

blot hybridization with pGO164 to confirm the presence of *mecA*. Only *mecA*-positive isolates were analyzed further. All isolates were then screened with pGO198 to identify homologs of COL DNA added at the deletion junction or pGO195 to identify homologs of BMS1 terminal *mecR1*, *mecI*, and downstream sequences. The results are presented in Table 2. Of the 112 *S. aureus* isolates tested, 110 hybridized only with pGO198 or pGO195; no isolate hybridized with both probes, but 2 isolates failed to hybridize with either probe. These latter two isolates were reconfirmed as *mecA* positive by Southern blot analysis of genomic DNA. *S. aureus* isolates were then investigated for hybridization to pGO198 or pGO195 according to the organism's year of recovery (Table 3). All of the 15 methicillin-resistant *S. aureus* isolates recovered in Europe and Africa between 1960 and 1968 hybridized only with pGO198; 2 isolates recovered in 1970 and 1971 hybridized only with pGO195. Thus, the first methicillin-resistant *S. aureus* isolates recovered from clinical specimens had interrupted regulatory sequences. Intact *mecR1* and *mecI* sequences were not found in *S. aureus* until the 1970s, but these sequences predominated among isolates from the United States, Australia, Brazil, and Europe recovered in the late 1980s and 1990s.

TABLE 2. Dot blot hybridization of clinical staphylococcal isolates with *mec* probes

Species (no. of isolates)	No. of isolates hybridizing with probe:			
	pG0198	pG0195	Both	Neither
S. aureus (112)	42	68	0	2
S. epidermidis (73)	34	10	25	4
S. haemolyticus (20)	20	0	0	0

Among coagulase-negative staphylococci, all 20 of the *S. haemolyticus* isolates investigated hybridized only with pGO198. In contrast, 46% of *S. epidermidis* isolates hybridized with pGO198 alone and 34% hybridized with both pGO198

TABLE 3. Hybridization of *S. aureus* isolates with *mec* probes by date of isolation

Probe	Percentage of isolates hybridizing by date of isolation (no. hybridizing/no. tested)			
	1961–1971	1971–1985[a]	1988–1992	Total
pGO198	88 (15/17)[b]	37 (17/46)	21 (10/47)	38 (42/110)
pGO195	12 (2/17)	63 (29/46)	79 (37/47)	62 (68/110)

[a] There was no difference in hybridization distribution between isolates recovered from 1971 to 1979 (19 isolates) and those recovered from 1979 to 1985 (27 isolates).

[b] All 15 of the isolates that hybridized with pGO198 were recovered between 1961 and 1968; the 2 isolates that hybridized with pGO195 were recovered in 1970 and 1971.

and pGO195 (Table 2). Only 10 isolates (14%) did not hybridize with the pGO198 probe. For *S. epidermidis*, there was no difference in hybridization with pGO198 or pGO195 between isolates collected in 1970 to 1980 in comparison with the hybridization of isolates collected from 1988 to 1991. We could find no methicillin-resistant *S. epidermidis* isolates recovered in the early 1960s. No other species of coagulase-negative staphylococci were examined because we could find no more than two or three isolates of any other species that were *mecA* positive.

RFLPs among clinical isolates. The *Cla*I fragment signatures of the isolates that hybridized with pGO198 are shown in Fig. 3. Two fragments, one of 1.35 kb that included the deletion junction and a second of 1.2 kb, had the characteristic RFLP pattern of *S. aureus* or *S. epidermidis* isolates that hybridized with pGO198 (Fig. 3, lanes 1, 2, and 4). Some isolates had three *Cla*I fragments that hybridized with pGO198. The third fragment represented a partial digest because of incomplete cleavage at one of the two internal *Cla*I sites (Fig. 1). *S. aureus* and *S. epidermidis* isolates that hybridized with pGO195 had a 4.8-kb *Cla*I fragment (Fig. 3, lanes 6 to 8) and a 2.25-kb *Hin*dIII fragment (data not shown). Thirty *S. aureus* isolates that hybridized with pGO198 were examined, and all had the characteristic *Cla*I RFLP signature; no additional hybridizing bands were seen.

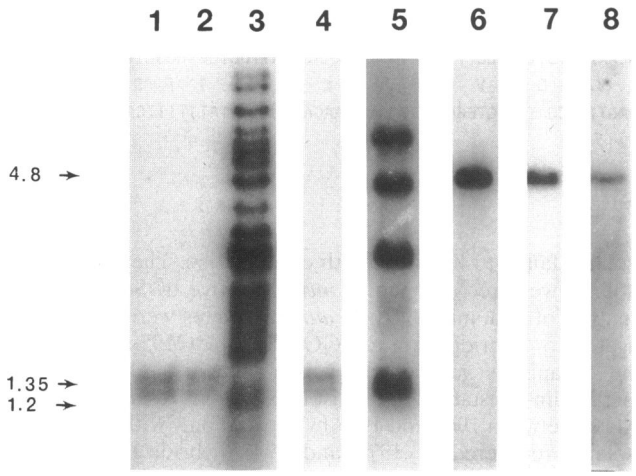

FIG. 3. Autoradiographs of staphylococcal target genomic DNA cut with *Cla*I and probed with ^{32}P-labelled pGO198 or pGO195. Lanes 1 through 5 were probed with pGO198; lanes 6 through 8 were probed with pGO195. Lane 1, *S. aureus* COL; lane 2, *S. epidermidis* SE5; lane 3, *S. haemolyticus* Y176; lane 4, *S. epidermidis* 43; lane 5, *S. epidermidis* SE64; lane 6, *S. epidermidis* SE43; lane 7, *S. aureus* BMS1; lane 8, *S. aureus* BK160. The numbers to the left indicate the size (in kilobases) of the two bands that hybridized with pGO198 in lanes 1, 2, and 4 (1.35 and 1.2) and the single bands that hybridized with pGO195 in lanes 6 through 8 (4.8).

Hybridization of *S. haemolyticus* isolates with pGO198 revealed the multiple banding pattern seen in lane 3 of Fig. 3. Each of the 20 isolates tested had multiple hybridizing bands. However, 15 epidemiologically distinct isolates all had different RFLP patterns, while 5 isolates from one hospital in Toronto, Ontario, Canada, which were previously shown to be epidemiologically related (14), all had nearly identical patterns.

Twenty *S. epidermidis* isolates that hybridized with both pGO195 and pGO198 were also examined. All had a 4.8-kb *Cla*I fragment identical in size to fragments seen in *S. aureus* and *S. epidermidis* isolates that hybridized only with pGO195 (Fig. 3, lanes 6 to 8). However, the pGO198 RFLP patterns were of three types. One type showed only the characteristic two- or three-band *Cla*I pattern (2 isolates; Fig. 3, lane 4), a second type showed multiple bands (5 to 10) of various sizes with no characteristic strain COL-like RFLP pattern (6 isolates; Fig. 3, lane 5), and a third type included both multiple bands and the characteristic signature pattern (12 isolates). The third type of *S. epidermidis* RFLP pattern was also seen in three isolates that hybridized only with pGO198. Plasmid DNAs were obtained from all 20 *S. haemolyticus* isolates and were subjected to Southern hybridization with pGO198. Only 4 of the 20 isolates had any plasmid DNA that hybridized with the probe, confirming the predominance of chromosomal sequences responsible for the multiple hybridization signals. We did not hybridize *S. epidermidis* plasmid DNA with the probe. While all *S. haemolyticus* isolates and 90% of the *S. epidermidis* isolates examined for RFLP patterns by using pGO198 as a probe had multiple chromosomal fragments that hybridized with pGO198, none of 21 *S. aureus* isolates examined had more than the signature COL-like RFLP pattern.

PCR and sequence analysis of deletion junctions. One PCR primer was generated to match a sequence just past the COL deletion junction, and a second primer was generated to match a sequence within the first 716 bases of the *mecR1*-coding sequence (Fig. 2). All eight *S. aureus* isolates (four isolated in the 1960s and four isolated in 1988 to 1992) and two *S. epidermidis* isolates examined that hybridized with pGO198 and that gave only the single COL-like signature RFLP patterns yielded PCR fragments of the same size. All had deletion junctions, as determined by DNA sequence analysis, identical to the junction found in COL. Of 11 *S. epidermidis* isolates examined that had both COL-like RFLP signatures and multiple additional bands that hybridized with pGO198, four yielded a single PCR fragment. Those fragments also contained a junction sequence identical to that in COL. The other seven isolates yielded multiple PCR fragments of various sizes or no PCR product. Likewise, multiple PCR fragments were generated from each of four *S. haemolyticus* isolates. However, a lambda clone that included the *mecA* gene was obtained from a genomic library of one *S. haemolyticus* isolate, Y176. The sequence of the DNA 5′ to *mecA* identified a deletion junction different from that seen for COL. The deletion site was 17 bp into the *mecR1* sequence, in comparison with the COL deletion, which was 975 bp into the *mecR1* sequence. Furthermore, the DNA added after the Y176 deletion junction was completely dissimilar by sequence analysis to that found in COL. This accounts for the absence of a 1.35-kb *Cla*I signature fragment in the hybridization pattern of Y176 shown in Fig. 3. The two *S. aureus* isolates that hybridized with neither pGO198 nor pGO195 yielded no PCR product. Thus,

eight *S. aureus* isolates obtained from patient specimens 30 years apart on three different continents and six *S. epidermidis* isolates collected in the 1970s and 1980s all had identical sites for the deletion event found in COL in 1965.

DISCUSSION

We analyzed 105 methicillin-resistant *S. aureus* isolates recovered from patient specimens collected over 30 years in Europe, Africa, South America, Australia, and the United States. We took care to ensure that each isolate was unique, as determined by the site and time of isolation or the phenotype. Thus, if methicillin-resistant *S. aureus* were clonal, as proposed by Kreiswirth et al. (13), there should be a hierarchy of rearrangements in the DNA immediately 5′ to *mecA*, all explained by evolution from a single progenitor. In contrast, we found that there were at least two classes of genetic organization of the *mec* gene that cannot be readily accounted for by the evolution of one from the other. The earliest methicillin-resistant *S. aureus* isolate examined in our study, which was obtained from the collection reported by Kreiswirth et al. (13), was isolated in Cairo, Egypt, in 1961. This isolate contained a deletion of *mecR1* and *mecI*, the regulatory genes commonly found in recently isolated methicillin-resistant *S. aureus* strains. In addition, the early isolate contained new DNA added at the point of deletion that was not found in recent isolates. All of the isolates that we examined from the 1960s contained the identical deletion junction; intact *mecR1* and *mecI* sequences did not appear in methicillin-resistant *S. aureus* isolates until the 1970s. Data suggesting a similar temporal sequence for the appearance of rearranged sequences 5′ to *mecA* were recently presented by Hurlimann-Dalel et al. (11) in a survey of methicillin-resistant *S. aureus* isolates recovered from patients in Switzerland since 1965 and by Suzuki et al. (20). Clearly, the late appearance of intact regulatory sequences could not have arisen by the restoration of DNA deleted from earlier isolates.

Two additional pieces of evidence argue against the evolution of all methicillin-resistant *S. aureus* isolates from a single clone. First, 26 of the *S. aureus* isolates that we examined were common to our study and the study of Kreiswirth et al. (13). Isolates were found within two of their six major classes that had both the deletion and the intact *mecR1* and *mecI* genotype. Thus, these two classes (classes II and IV), the classes containing the largest number of isolates, were not likely to have represented single clonal groups. Second, Musser and Kapur (16), using multilocus enzyme electrophoresis, found that while methicillin-resistant *S. aureus* isolates from the 1960s had a single multilocus genotype, the more recent isolates represented the breadth of the chromosomal diversity of *S. aureus*, suggesting multiclonality. All of these data can be interpreted by invoking the appearance of an early clone of methicillin-resistant *S. aureus* in the 1960s and the reappearance of a second, possibly multiclonal, group of isolates in the 1970s. Rather than evolution of *mec* DNA within the species *S. aureus*, horizontal transfer from a separate bacterial species or genus seems more likely.

One of the possible sources of *mec* DNA was suggested by our analysis of the DNA sequence 5′ to *mecA* in the two coagulase-negative staphylococci displaying the greatest resistance to beta-lactams and other antimicrobial agents, *S. epidermidis* and *S. haemolyticus*. All 20 of the *S. haemolyticus* and 90% (20 of 23) of the *S. epidermidis* isolates examined had multiple chromosomal bands that hybridized with pGO198. In contrast, all *S. aureus* isolates examined had the hybridization pattern typical of the single insertion and subsequent deletion event 5′ to *mecA*. Furthermore, in addition to the multiple hybridizing chromosomal bands, *S. epidermidis* also contained nucleotide sequences at *mecR1* deletion junctions identical to those in *S. aureus*. These data suggest that DNA in the pGO198 probe, and therefore, DNA added at the deletion junction in *S. aureus* COL, may be part of a mobile genetic element, possibly an insertion sequence. Analysis of DNAs from four *S. haemolyticus* Y176 lambda clones that hybridized with pGO198 identified four different insertion sites for an element 1,943 bp long with features characteristic of an insertion sequence (3). However, the complete insertion sequence-like sequence was not present at the various *mecR1* insertion-deletion junctions, suggesting that further DNA rearrangements took place after the initial insertion event. Thus, it is possible that coagulase-negative staphylococci are the reservoir for an insertion sequence-like element responsible for an insertion-deletion event that involves *mecR1*. The presence of the identical insertion-deletion junction in both *S. epidermidis* and *S. aureus* raises the possibility that the initial insertion sequence-mediated *mecR1* insertion-deletion event occurred in coagulase-negative staphylococci, with the subsequent horizontal transfer of rearranged sequences to *S. aureus*.

Retention of the precise insertion-deletion junction sequence in two different staphylococcal species isolated over a period of 30 years suggests that the original insertion event and the subsequent horizontal transfer occurred in the past and that clones containing this rearrangement have been favored and have persisted. The persistence of isolates with this precise rearrangement of *mec* DNA may be explained as proposed by Suzuki and colleagues (20). Functional *mecR1-mecI* sequences serve to repress the production of *mecA*, and both methicillin and oxacillin are poor inducers of resistance. Thus, these strains appear susceptible when exposed to methicillin and would be at a disadvantage in an environment in which semisynthetic, penicillinase-resistant penicillins are used. Interruption of these sequences would lead to the constitutive expression of *mecA* and clinical methicillin resistance. Thus, in the 1960s, when methicillin was introduced, *S. aureus* isolates with the COL-type interruption of *mecR1* and *mecI* were favored and became the predominant methicillin-resistant clone. Later isolates that had an intact *mecR1-mecI* region by DNA hybridization also expressed phenotypic methicillin resistance, but in this case, the regulatory sequences may be dysfunctional because of mutations. Mutations in the *mecI* genes of clinical methicillin-resistant *S. aureus* isolates from Japan have been documented by Suzuki et al. (20). Mutations were of multiple types (missense, nonsense, and deletion mutations), most of which would be predicted to alter MecI function. Further methicillin- or oxacillin-inducible regulation of *mecA* in strains with mutant or altered *mecR1-mecI* genes can be provided by plasmid-encoded β-lactamase regulatory genes (25). It is teleologically unclear why *S. aureus* would originally acquire a noninducible repressor linked to a resistance gene that essentially renders the gene nonfunctional. One explanation is that the resistance gene and regulatory sequences were acquired en bloc from another organism in which the genes were inducible and functional in response to signals relevant to that organism's survival. Modification and selection were required in the new host in order to allow expression of resistance to semisynthetic penicillins.

Alternative explanations may also explain the appearance and evolution of rearranged sequences. Both the intact and mutant *mecR1-mecI* sequences and the COL-type insertion-deletion event may have existed in *S. aureus* and the sequences may have coevolved. The absence of *mecR1-mecI* sequences in clinical methicillin-resistant *S. aureus* isolates until the 1970s may have reflected selective pressures favoring one genotype

over the other. This explanation does not invoke the horizontal transfer of *mec* sequences from coagulase-negative staphylococci. It is impossible to exclude this possibility; early staphylococcal isolates representing geographic diversity are not available for phenotypic and genotypic analyses. However, two observations favor not only the horizontal transfer of *mec* sequences but also the directionality from coagulase-negative staphylococci to *S. aureus*. First, as stated above, is the apparent residence of a putative insertion sequence responsible for the *mecR1* interruption in coagulase-negative staphylococci but not *S. aureus*.

Second, is the observation that *S. epidermidis* isolates obtained in the 1970s contained not only RFLP signatures and DNA sequence evidence of typical *S. aureus* COL-type deletion junctions but also hybridization evidence of intact *mecR1-mecI* sequences. This suggests that *S. epidermidis* may have been an intermediate in the acquisition and transfer of *mecR1-mecI* sequences to *S. aureus*. The 1970s marked the decrease in the prevalence of the original *S. aureus* clone from the 1960s and the emergence of multiresistant *S. aureus* isolates, most of which hybridize with the pGO195 *mecR1-mecI* probe, that currently predominate and that are responsible for the worldwide methicillin-resistant *S. aureus* pandemic (5, 6).

Our hypothesis assumes that a single *mecR1* insertion-deletion event occurred, most likely in a coagulase-negative staphylococcus, with subsequent dissemination of those particular rearranged sequences. Alternatively, the *mecR1* site may be a hot spot for the insertion of this insertion sequence-like element. If this were true, then insertion sequence-mediated insertion events could have occurred on multiple occasions and could be ongoing. However, the presence of the same insertion junctions, deletions, and rearrangements of both *mec* and inserted DNAs in *S. aureus* and *S. epidermidis* argue strongly for the dissemination of sequences resulting from a single mutational event.

The ultimate sources in nature of *mecA*, *mecR1-mecI*, and the putative pGO198-hybridizing insertion sequences identified in this report are not known, but the evidence that we presented suggests that coagulase-negative staphylococci played important roles as intermediaries and reservoirs for the DNA sequences that eventually appeared in *S. aureus*.

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