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Cloning and Expression of *Staphylococcus aureus* Plasmid-Mediated Quaternary Ammonium Resistance in *Escherichia coli*

JAN M. TENNENT, BRUCE R. LYON, MATTHEW T. GILLESPIE, JOHN W. MAY, AND RONALD A. SKURRAY*

Microbiology Department, Monash University, Clayton, Victoria, 3168, Australia

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The *Staphylococcus aureus* plasmid pSK1 carries Tn*4001*, a 4.7-kilobase (kb) transposon which specifies resistance to gentamicin, tobramycin, and kanamycin. In addition, pSK1 mediates resistance to trimethoprim and linked resistance to ethidium bromide (Eb^r) and to quaternary ammonium compounds (Qa^r). Restriction endonuclease analysis of pSK1 and a deleted derivative of pSK1 revealed that the gene(s) responsible for Eb^r Qa^r lies within a 5.2-kb *Hin*dIII fragment. This fragment has been cloned into the *Escherichia coli* plasmid vector pBR322, and transformants of an *E. coli* K-12 strain exhibited Eb^r Qa^r. Subcloning of the 5.2-kb insert, combined with data from electron microscopic analysis of deleted derivatives of pSK1, located the Eb^r Qa^r determinant(s) on a 2.3-kb segment of pSK1 DNA.

Recent studies of epidemic multiresistant strains of *Staphylococcus aureus* isolated from Australian hospitals have compared the patterns of antibiotic resistance with the plasmid DNA profiles of these strains (10, 11, 17). The results suggest that a common, plasmid-bearing strain of *S. aureus*, or its derivatives, has been responsible for the nationwide outbreak of nosocomial infections.

Plasmid-mediated resistance to the aminoglycoside antibiotics gentamicin, tobramycin, and kanamycin has been clearly established in these strains (10, 17), and we have identified an incompatibility group IncI plasmid, pSK1, as the prototype aminoglycoside resistance plasmid (10; B. Lyon, J. May, and R. Skurray, unpublished data). Plasmid pSK1 has been shown to carry a transposable element, Tn*4001*, which mediates resistance to gentamicin, tobramycin, and kanamycin. Furthermore, transfer in mixed cultures has revealed that pSK1 also encodes resistance to ethidium bromide (Eb^r) and quaternary ammonium compounds (Qa^r) (12).

We have recently isolated the plasmid pSK78, a derivative of pSK1, which, although still mediating aminoglycoside resistance, does not encode Eb^r Qa^r. In this paper, we describe the comparative restriction endonuclease digests of pSK1 and pSK78, which revealed a region of plasmid DNA on pSK1 responsible for Eb^r Qa^r. This region has been cloned and shown to express Eb^r Qa^r in an *Escherichia coli* vector-host system.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. aureus* SK430, which carries the Eb^r Qa^r plasmid pSK18 (12), was resistant to penicillin, methicillin, streptomycin, clindamycin, tetracycline, trimethoprim, ethidium bromide, benzalkonium chloride, and cetyltrimethylammonium bromide; we are grateful to P. Carson (Repatriation and General Hospital, Melbourne) for supplying this isolate.

Strain SK983, a chromosomal mutant of *S. aureus* SA113 (7) resistant to streptomycin (100 μg/ml) and fusidic acid (10 μg/ml), has been selected in this laboratory for use as a recipient in mixed culture transfer.

* Corresponding author.

S. aureus RN2425, carrying the plasmid pC194 (6), was kindly provided by R. P. Novick (Public Health Research Institute of the City of New York).

The *E. coli* K-12 strain employed was BHB2600 (F^- 803 *supE supF hsdR met*) (5), and the *E. coli* vector was pBR322 (2).

General methods. The media used and the method for the determination of antibiotic susceptibility have been previously described (11). Susceptibility to benzalkonium chloride, ethidium bromide, trimethoprim (Sigma Chemical Co., St. Louis, Mo.), and cetyltrimethylammonium bromide (BDH Chemicals Ltd., Poole, England) was tested at concentrations described below. MICs of antimicrobial agents were determined by dilution in agar with an inoculum of approximately 10^4 CFU. Mixed culture transfer was performed by the method of McDonnell et al. (13).

DNA manipulation procedures. The isolation of purified plasmid DNA from cultures of *S. aureus*, digestion with the restriction endonucleases *Bgl*II, *Eco*RI, *Hin*dIII, *Hpa*II, and *Pvu*II (New England Biolabs, Inc., Beverly, Mass.), agarose gel electrophoresis, and the estimation of DNA fragment sizes were performed as previously described (11). *Hin*dIII-*Eco*RI double digests of λ viral DNA (Miles Laboratories, Inc., Elkhart, Ind.) were employed as standards; fragment sizes in kilobases (kb) were taken from Daniels et al. (3). Procedures for alkaline phosphatase treatment of the *E. coli* plasmid vector and for the ligation of DNA, together with the techniques for *E. coli* transformation and plasmid DNA isolation (purified and rapid), have previously been described (15). DNA manipulations were performed with C1 physical containment as laid down by the Recombinant DNA Monitoring Committee, Australia.

Electron microscopy. Heteroduplex experiments were performed essentially as described by Davis et al. (4). In short, purified plasmid DNA was cleaved with the appropriate restriction endonuclease, alkali denatured, renatured, and mounted for electron microscopy. Molecules were visualized and photographed with a Phillips EM300 electron microscope at ×20,000. Contour lengths (expressed in kb) were determined relative to pC194 (2.91 kb) (6) from the average of at least 10 measurements with a Carl Zeiss MOP-1 digital analyzer.

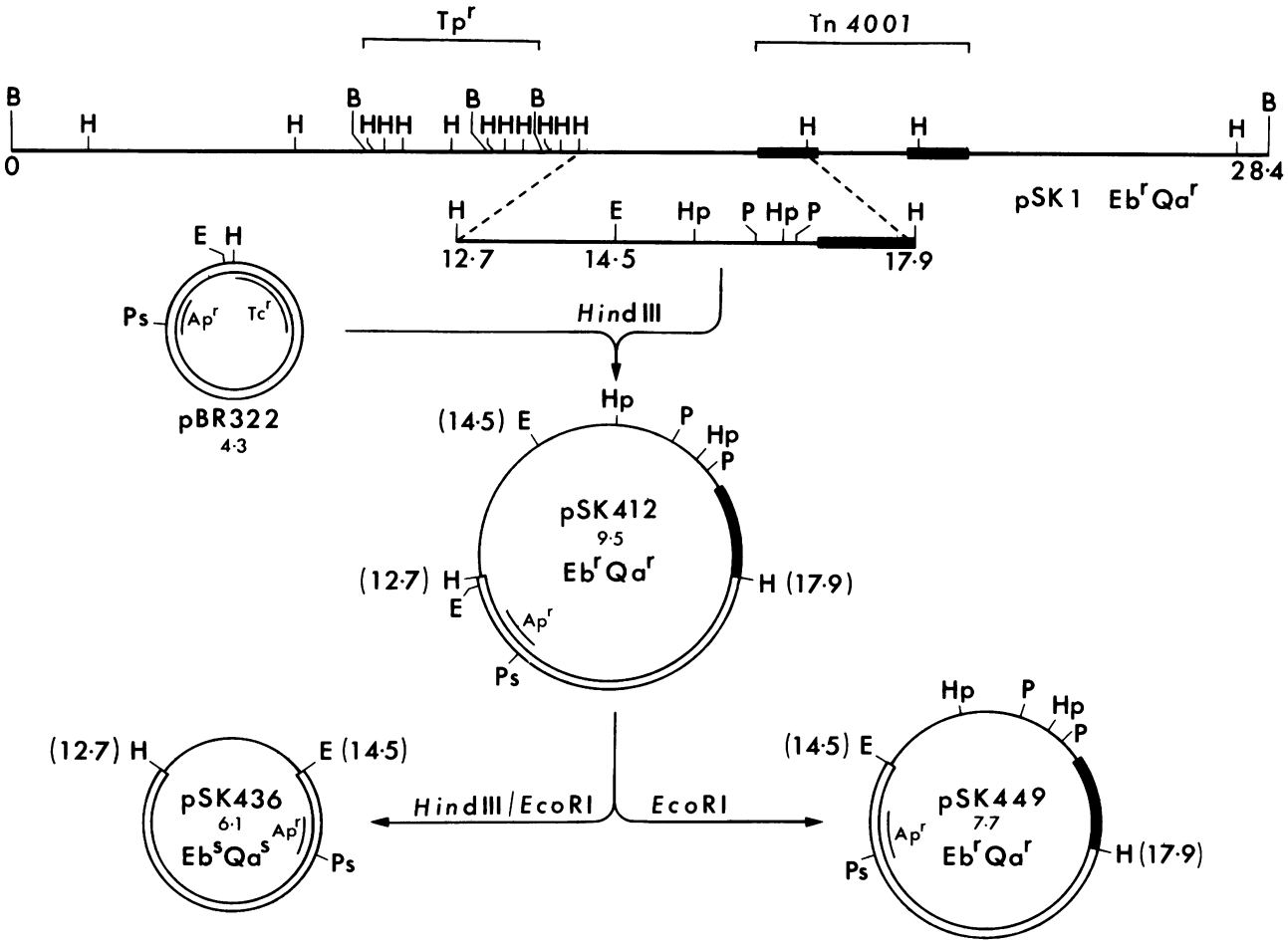


FIG. 1. Simplified restriction map of pSK1 showing the inverted repeats of the 4.7-kb aminoglycoside resistance transposon Tn*4001* (thick lines) and incorporating the strategy used to clone staphylococcal resistance to ethidium bromide (Eb^r) and quaternary ammonium compounds (Qa^r). The construction of plasmids pSK412, pSK436, and pSK449, which consist of fragments of pSK1 DNA cloned into pBR322 (double open lines), is described in the text; plasmid sizes and map coordinates are expressed in kb. Ap^r, Tc^r, and Tp^r designate plasmid DNA sequences which encode resistance to ampicillin, tetracycline, and trimethoprim, respectively. Restriction endonuclease sites are indicated by B (*Bgl*II), E (*Eco*RI), H (*Hin*dIII), Hp (*Hpa*II), P (*Pvu*II), and Ps (*Pst*I); only those *Eco*RI, *Hpa*II, and *Pvu*II sites of pSK1 lying within the 5.2-kb *Hin*dIII fragment (expanded) are shown.

RESULTS

In a previous communication (12), we reported that pSK1 encodes resistance not only to gentamicin, tobramycin, and kanamycin, but also to ethidium bromide and quaternary ammonium compounds; MICs of ethidium bromide, benzalkonium chloride, and cetyltrimethylammonium bromide were 120, 6, and 4 μg/ml, respectively, compared with 3, <1, and <1 μg/ml, respectively, for the plasmid-free laboratory strain SK983. Mixed culture transfer of pSK1 has demonstrated that it also encodes trimethoprim resistance (MIC > 800 μg/ml). The linkage of Eb^r and Qa^r on pSK1 was suggested by our observations that neither phenotype was lost or gained independently of the other in curing studies or in mixed culture transfer. In one such mixed culture transfer experiment with a donor carrying pSK1 and SK983 as the recipient, transcipients were obtained which were resistant to gentamicin, tobramycin, kanamycin, and trimethoprim but were Eb^s Qa^s. Plasmid DNA analysis revealed that such transcipients carried a 25.9-kb deleted derivative of pSK1 (data not shown). This plasmid has been designated pSK78.

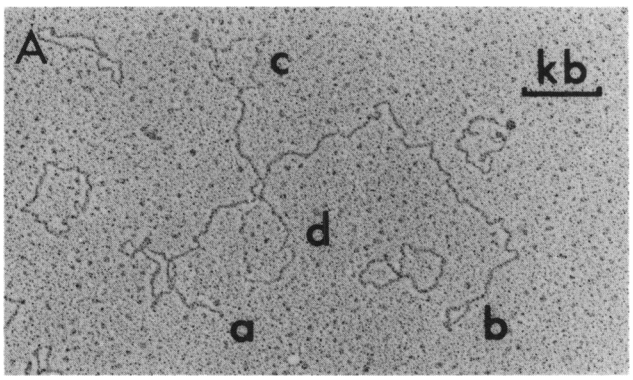
As a result of more detailed restriction mapping of both pSK1 and cloned fragments of pSK1 by polyacrylamide gel electrophoresis, the size of pSK1 has been recalculated to be 28.4 kb. The physical map of pSK1, including restriction sites for *Bgl*II and *Hin*dIII, is shown in Fig. 1. Plasmid pSK1 was cleaved by *Bgl*II into four fragments, the largest of which was replaced by a smaller fragment in pSK78 (data not shown). Based on this result, we determined that in the formation of pSK78 a deletion of approximately 2.5 kb had occurred within the largest *Bgl*II fragment of pSK1.

To map more precisely the region of pSK1 DNA which was absent from pSK78, we formed heteroduplex molecules between pSK78 and the Eb^r Qa^r plasmid pSK18 (Fig. 2A). Plasmid pSK18 is structurally similar to pSK1, but lacks both Tn*4001* and a 4.0-kb segment of pSK1 DNA associated with trimethoprim resistance (12; B. Lyon, J. Tennent, J. May, and R. Skurray, manuscript in preparation). Hybridized *Bgl*II fragments of pSK18 and pSK78 were found to differ from each other in two respects; first, by a stem and loop structure (Fig. 2A, c), indicating the presence of Tn*4001* on pSK78, and second, by a single-stranded loop of pSK18 DNA (Fig. 2A, d) which was interpreted to represent the size and position of the DNA deleted from pSK78. Contour length measurements of the stem and loop structure of Tn*4001* present in this and other heteroduplexes have deter-

mined 4.7 kb to be a more accurate estimation of the size of this transposon, previously reported as 4.5 kb (12). Similar measurements established that in forming pSK78, a 2.5 ± 0.2-kb deletion of pSK1 had occurred at a site immediately adjacent to the inverted repeat of Tn*4001* and approximately 2.6 kb from the *Bgl*II fragment end (Fig. 2A, a). A representation of the heteroduplex molecule formed between pSK18 and pSK78 aligned against a restriction map of the large *Bgl*II fragments of the two plasmids is depicted in Fig. 2B. Compared with pSK1, the Eb^s Qa^s plasmid, pSK78, lacks an *Eco*RI site and two sites each for *Hpa*II and *Pvu*II (data not shown). All of these sites map on the segment of DNA shown to be missing from pSK78 (Fig. 2B, d) and are contained within the 5.2-kb *Hin*dIII fragment of pSK1 (Fig. 1).

When purified pSK1 DNA was digested with *Hin*dIII, only 10 of the known 15 *Hin*dIII fragments of this plasmid could be visualized with the agarose gel system employed (Fig. 3A, lane b). On comparison, the deleted plasmid, pSK78, was found to lack a 5.2-kb *Hin*dIII fragment, but instead produced a unique *Hin*dIII fragment, approximately 2.7 kb in size (Fig. 3A, lane c). To demonstrate that the region of pSK1 DNA encoding Eb^r Qa^r was situated within this 5.2-kb *Hin*dIII fragment, purified pSK1 DNA was digested with *Hin*dIII and cloned into the *Hin*dIII site of pBR322 (Fig. 1). The ligated DNA was then transformed into *E. coli* BHB2600, and the resultant ampicillin-resistant, tetracycline-susceptible transformants were screened for Eb^r. All Eb^r transformants obtained were also Qa^r, a result which further supports the linkage between Eb^r and Qa^r. The MICs of ethidium bromide, benzalkonium chloride, and cetyltrimethylammonium bromide for such clones were 380, 60, and 60 μg/ml, respectively, compared with 50, 12, and 20 μg/ml, respectively, for *E. coli* BHB2600 carrying pBR322 alone.

DNA analysis of all Eb^r Qa^r transformants showed them to contain a hybrid plasmid consisting of the 5.2-kb *Hin*dIII fragment of pSK1 ligated to pBR322 (Fig. 3A, lane d); one such plasmid has been designated pSK412 (Fig. 1). The orientation of insertion of the pSK1 DNA fragment into pBR322 was established by digesting pSK412 with *Eco*RI. Fragments of 1.8 and 7.7 kb resulted (data not shown), indicating that the *Eco*RI site of the 5.2-kb insert DNA mapped approximately 1.8 kb to the right of the *Eco*RI site present in pBR322 (Fig. 1). A hybrid plasmid in which the pSK1 fragment is inserted in the opposite orientation was also shown to express Eb^r Qa^r, suggesting that the gene(s) encoding this phenotype is contained entirely within the 5.2-kb *Hin*dIII fragment.



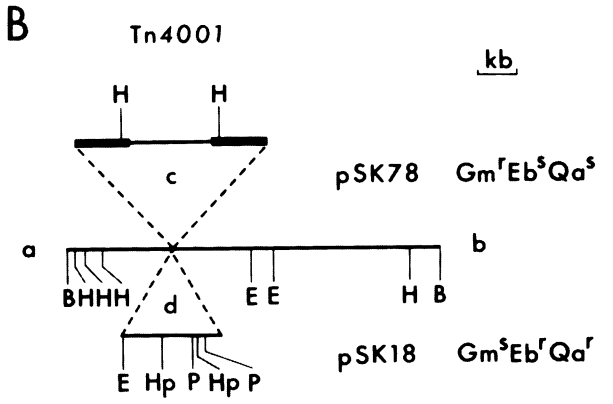


FIG. 2. (A) Heteroduplex molecule formed between *Bgl*II-digested pSK18 and pSK78; a 1-kb scale marker is shown. The ends of the heteroduplex molecule are denoted by a and b; the 4.7-kb double-stranded stem and loop structure of Tn*4001* is indicated by c; and d indicates a single-stranded loop of 2.5 kb. Plasmid pC194 (6) was included as a size standard. (B) Diagrammatic representation of the heteroduplex in (A) formed between pSK18 and pSK78. A restriction map of the largest *Bgl*II fragments of the two plasmids is included relative to a 1-kb scale marker; a, b, c, and d are as defined above. Restriction endonuclease sites are indicated by B (*Bgl*II), E (*Eco*RI), H (*Hin*dIII), Hp (*Hpa*II), and P (*Pvu*II); resistance or susceptibility to gentamicin (Gm), ethidium bromide (Eb), and quaternary ammonium compounds (Qa) is indicated.

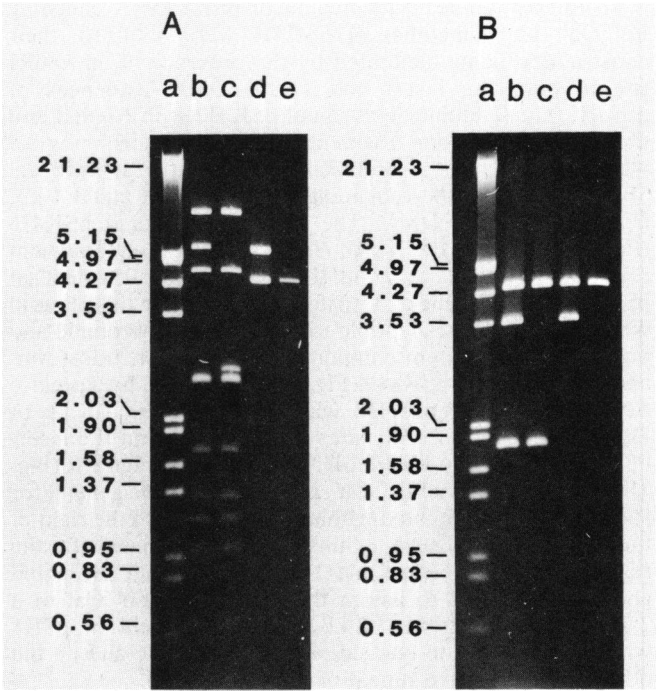


FIG. 3. (A) Agarose gel (1.2% [wt/vol]) electrophoresis of *Hin*dIII digests of purified plasmid DNA from *S. aureus* and *E. coli* strains. Lanes a, λ DNA cleaved with *Hin*dIII and *Eco*RI; fragment sizes (in kb) are shown at left; b, pSK1; c, pSK78; d, pSK412; e, pBR322. The fourth band from the well in lane b is a doublet consisting of the 2.45- and 2.5-kb *Hin*dIII fragments of pSK1 which have not been completely resolved in this gel. (B) Agarose gel (1.2% [wt/vol]) electrophoresis of *Hin*dIII-*Eco*RI digests of rapidly isolated plasmid DNA from *E. coli* strains. Lanes a, λ DNA cleaved with *Hin*dIII and *Eco*RI; fragment sizes (in kb) are shown at left; b, pSK412; c, pSK436; d, pSK449; e, pBR322. The <0.1-kb *Hin*dIII-*Eco*RI fragment of pBR322 (lanes b and e) could not be detected under these conditions.

To further delineate the region of pSK1 DNA encoding Eb^r Qa^r, two subclones of pSK412 were obtained, their construction being facilitated by the presence of an *Eco*RI site in both the insert and vector DNA sequences of pSK412.Thus, double digestion of pSK412 with *Hin*dIII and *Eco*RI produced four fragments (Fig. 3B, lane b), two of which derived from the pBR322 portion of the hybrid (Fig. 3B, lane e), with the remaining fragments, 1.8 and 3.4 kb, from the inserted DNA. The derivative plasmid pSK436 (Fig. 1) consists of the 1.8-kb *Hin*dIII-*Eco*RI insert fragment religated to the large *Hin*dIII-*Eco*RI vector fragment of pSK412 (Fig. 3B, lane c); transformants carrying this plasmid were found to be susceptible to both ethidium bromide and quaternary ammonium compounds. In contrast, transformants which carried pSK449 (Fig. 1), constructed by a precise *Eco*RI deletion of pSK412 which removed the 1.8 kb of DNA between the *Eco*RI sites of the vector and the insert but retained the 3.4-kb *Hin*dIII-*Eco*RI insert fragment (Fig. 3B, lane d), displayed Eb^r Qa^r, indicating that the gene(s) for such resistance is located within this fragment to the right of the *Eco*RI site. These results, taken together with the restriction endonuclease and heteroduplex data presented above, allowed us to assign the gene(s) for Eb^r Qa^r to a 2.3-kb segment of the 5.2-kb *Hin*dIII fragment of pSK1 which is bounded on one side by an *Eco*RI site and on the other by the inverted repeat of Tn*4001* (Fig. 1).

DISCUSSION

Resistance to ethidium bromide and to quaternary ammonium compounds has been shown to be encoded by pSK1, an *S. aureus* plasmid of incompatibility group IncI which also encodes aminoglycoside resistance. In a previous analysis, we have shown that pSK1 belongs to a family of at least six structurally related plasmids which mediate resistance to gentamicin, kanamycin, and tobramycin in Australian isolates of multiresistant *S. aureus* (10). Like pSK1, these plasmids also encode resistance to ethidium bromide and quaternary ammonium compounds and, on the basis of restriction endonuclease digests, also carry Tn*4001* (B. Lyon, J. May, and R. Skurray, unpublished data). The association of Qa^r with aminoglycoside resistance plasmids recovered from Australian strains of *S. aureus* has also been noted by other workers (16), and the determinants for Eb^r have previously been located on aminoglycoside resistance plasmids (1, 8) and on a penicillinase plasmid (9) isolated elsewhere.

We believe that staphylococcal aminoglycoside resistance plasmids have evolved by the insertion or deletion of defined DNA segments which arise during transposition or recombination events (11, 12). Evidence supporting this hypothesis is provided by the isolation of two naturally occurring Eb^r Qa^r plasmids, pSK7 and pSK18, which do not encode aminoglycoside resistance. On the basis of restriction endonuclease and heteroduplex analyses, both plasmids share structural homology with pSK1 (10, 12; unpublished data) and may therefore represent plasmids either before the insertion of Tn*4001* or after its excision. The extensive application of quaternary ammonium compounds as disinfectants may well account for the emergence of Qa^r staphylococci. It is possible that the further selective pressure provided by aminoglycoside antibiotics in hospital environments has been responsible for the maintenance and dissemination of Tn*4001* on these Qa^r vectors.

When the gene(s) for Eb^r Qa^r from *S. aureus* was cloned into pBR322, *E. coli* transformants carrying the hybrid plasmids pSK412 or pSK449 demonstrated resistance levels for ethidium bromide and quaternary ammonium compounds up to 15 times higher than those of Eb^r Qa^r strains of *S. aureus*. This effect may have been due to the relocation of the *S. aureus* Eb^r Qa^r gene(s) onto a high-copy-number replicon which effectively increased the gene dosage. However, since resistance to both ethidium bromide and quaternary ammonium compounds is thought to arise by the same mechanism, that is, by a reduction in the uptake of these agents by a cell (9, 14), and as there is a clear distinction between the MICs for susceptible strains of *S. aureus* and *E. coli*, it is likely that the dissimilar resistance levels reflect differences in the innate permeability of these organisms toward such agents.

The cloning of Qa^r from *S. aureus* into *E. coli* has placed the resistance determinant(s) into a background where the effects of the gene products are more readily observed and which is accessible to DNA manipulative procedures that may assist in further elucidating the mechanism of staphylococcal quaternary ammonium resistance.

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