Transduction and Plasmid Deoxyribonucleic Acid Analysis in a Multiply Antibiotic-Resistant Strain of *Staphylococcus epidermidis*

LINDA L. ROSENDOFF AND FRITZ H. KAYSER

Institute of Medical Microbiology, University of Zurich, Zurich, Switzerland

Received for publication 27 August 1974

A genetic analysis of a multiply antibiotic-resistant strain of *Staphylococcus epidermidis* was performed. Experiments designed to show reversion of organisms to antibiotic susceptibility, as well as studies of the influence of ultraviolet irradiation of phage on the transduction frequencies of the resistance markers, indicated that determinants of chloramphenicol (cml), tetracycline (tet), and neomycin (neo) resistance are present on separate plasmids, but the streptomycin marker is chromosomal. In 2 to 6% of tetracycline-resistant transductants, co-transduction of cml was also observed. By using CsCl-dye density gradients followed by neutral sucrose gradients, the plasmids carrying cml, tet, and neo could be isolated and their molecular weights could be determined. The tetracycline plasmid is shown to be incompatible with one of the cryptic plasmids of a recipient strain.

In recent years it has become apparent that under some circumstances *Staphylococcus epidermidis* can be considered as pathogenic as *Staphylococcus aureus*. Certain infections, such as endocarditis, urinary tract infections, and colonization of ventriculo-atrial shunts, are typically caused by *S. epidermidis* (9, 10, 11, 15). In addition, this organism can be isolated in pure culture from pyogenic material (26), although formerly such infections were attributed primarily to *S. aureus*. The isolated bacteria often prove to be quite antibiotic resistant (1, 4, 19); resistance to practically all chemotherapeutic agents has been demonstrated (25). Although there have been numerous studies of the mechanisms of antibiotic resistance in *S. aureus*, there have been few reports concerned with *S. epidermidis*. To gain some insight into the genetics of drug resistance in this organism, a study was undertaken to observe the stability of antibiotic resistance and the intraspecific transduction of determinants of these resistances, as well as to characterize the plasmids carrying these determinants.

**MATERIALS AND METHODS**

**Organisms.** All *S. epidermidis* strains were isolated from clinical material obtained in Zurich. Drug resistance was determined as previously described (14). Strain 109 is resistant to penicillin (Pen\(^{R}\)), chloramphenicol (Cml\(^{R}\)), tetracycline (Tet\(^{R}\)), neomycin (Neo\(^{R}\)), streptomycin (Str\(^{R}\)), and sulfamethoxazole (Sul\(^{R}\)); it is moderately resistant to methicillin (Mth\(^{R}\)). This strain is also resistant to cadmium acetate (Cd\(^{2+}\)) levels of \(6 \times 10^{-8}\) M. Strains 62, 108, 115, 247, and 294 are sensitive to all of the above antibiotics; 108, 115, and 294 are also sensitive to Cd\(^{2+}\). All strains are Baird-Parker subgroup II (2); however, 108 and 115 are lactose-negative variants and 247 is maltose negative, but all are phosphatase positive.

Reference strain *S. aureus* RN 1304 (containing the tetracycline plasmid T\(_{149}\)) was kindly provided by R. P. Novick, The Public Health Research Institute of the City of New York.

Lytic phages were a gift of J. Verhoef, Utrecht, Holland. Phage 448/407 (29) was propagated on *S. epidermidis* 109 as previously described (14); the resulting transducing lysate is identified as \(\phi 448/407\).\(^{109}\)

Elimination procedures. Experiments to determine spontaneous loss of antibiotic resistance and loss under various growth conditions and chemical treatment were performed according to Kaysers et al. (14). Colonies grown on drug-free brain heart infusion (BHI) agar were replicated onto BHI agar containing 25 \(\mu\)g of chloramphenicol per ml, 12.5 \(\mu\)g of tetracycline per ml, or 12.5 \(\mu\)g of neomycin per ml, or BTB agar (without the dye indicator); 17 containing 50 \(\mu\)g of streptomycin per ml. Drug susceptibilities of all strains which appeared to have lost antibiotic resistance were restested according to the disk method of Bauer et al. (3). Elimination of penicillin resistance was performed according to Schaefer (28) using LB broth (pH 8.0) and incubation at 43.5 C, followed by storage at 20 C for 48 h or 4 weeks. Penicillinase-producing organisms were selected for by using the
modified N-phenyl-1-naphthylamine-azo-o-carboxybenzene test of Novick and Richmond (22) or the iodide method of Perret (24), modified as described by Kayser and Wiedmer (13).

Transduction procedures. The techniques used by Kayser et al. (14) for transduction with S. aureus were also used in these S. epidermidis transduction experiments. Lysates of phage 448/407 grown on strain 109 usually had titers of 10^8 to 10^9 plaque-forming units per ml. Recipient organisms were grown with shaking until the end of logarithmic growth. Amounts of 1 ml of the recipient culture and 1 ml of phage were mixed and incubated with gentle shaking at 37 C. Under these conditions, 80% of the phages will usually be absorbed after 10 min of incubation. In most experiments, the phage-to-bacteria ratio was from 0.05 to 0.3. After 30 min of incubation, further absorption was halted by adding 0.5% sodium citrate, followed by centrifuging at 4 C and two washings with BHI broth plus 0.5% sodium citrate (BHI-citrate). The pellet was resuspended in 0.5 ml of BHI-citrate, mixed undiluted or in appropriate dilutions with 2.5 ml of BHI soft agar, and plated onto BHI agar plates. The soft overlay agar as well as the base agar contained one of the following drugs: streptomycin, 100 µg/ml; chloramphenicol, 50 µg/ml; neomycin, 25 µg/ml; tetracycline, 12.5 µg/ml; methicillin, 6.25 or 3.1 µg/ml; penicillin, 0.5 or 0.1 µg/ml; or cadmium acetate, 6 x 10^-4 M. In the ultraviolet (UV) irradiation experiments, the same phage lysate was used in the transfer of resistance against chloramphenicol, neomycin, tetracycline, and streptomycin.

In selecting streptomycin-resistant transductants, the washed pellet was resuspended in 4 to 7 ml of BHI-citrate broth and reincubated at 37 C for 1 to 2 h. After centrifugation, selection was carried out as described above.

Dye-CsCl density gradient. The method of Novick and Bouanchaud (21) was used to determine the presence of plasmid deoxyribonucleic acid (DNA). The bacteria under study were labeled for 1 h with 1 µCi of [methyl-^3H]thymidine per ml (specific activity, 19 Ci/mmol), and control cultures were labeled with 0.1 µCi of[^14C]thymidine per ml (specific activity, 62 mCi/mmol). After 0.5 h refrigeration at 4 C, cultures were combined and lysed. The lysate-dye-CsCl mixture was centrifuged at 42,000 rpm in a Beckman SW50 rotor for 38 h at 20 C. Two-drop fractions were collected on Whatman GF/B glass filters and the amount of radioactivity was determined.

To isolate plasmid DNA in a larger amount, approximately 10 ml of the lysate-dye-CsCl mixture were centrifuged in a Beckman 50 Ti fixed angle rotor at 42,000 rpm for 40 h at 20 C. Three-drop fractions were collected in tubes and 10 µl of each fraction was spotted on Whatman filters and counted to determine the location of the plasmid peak. The appropriate fractions were pooled and dialyzed for 24 h at 4 C against two changes of 0.01 M ethylenediaminetetraacetic acid, pH 7.5.

Sucrose gradients. Neutral sucrose gradients (5 to 20%) were formed according to the method of Guerry et al. (8). One-tenth milliliter of plasmid-containing solution was then layered onto the gradients and centrifuged at 40,000 rpm in a Beckman SW50 rotor at 15 C for 2 to 2.5 h. Two-drop fractions were collected directly on Whatman filters and radioactivity was counted.

To determine molecular weights of the plasmids, 247Tet^R, 247Cml^R, and 247Neo^R were labeled with [methyl-^3H]thymidine as before and reference strain RN 1304 was labeled with 0.2 µCi of[^14C]thymidine per ml. After lysis, plasmid DNA was isolated and subsequently dialyzed as described above. Fifty microliters of the reference plasmid plus 50 µl of a tritium-labeled plasmid were mixed together and layered onto sucrose gradients, and 0.1-ml fractions were collected on filters. Sedimentation constants and molecular weights were then calculated according to Clowes (5) and Hudson et al. (12). All resistant transductants, which were analysed for the presence of plasmid DNA, were obtained in an experiment in which the same phage lysate was used.

Electron microscopy. Plasmid-containing fractions were collected in tubes from sucrose gradients and stored for several weeks at 4 C to allow covalently closed circular molecules (CCC) of plasmid DNA to convert to open circular forms.

Copper grids were prepared according to the spreading procedure of Kleinschmidt (10) and examined with a Phillips E201 electron microscope. Contour lengths were calculated from the known magnification determined with a calibration grid. Molecular weights were estimated on the assumption that 1 µm corresponds to 1.96 x 10^4 of DNA (18).

RESULTS

Elimination of resistance markers. Neomycin was the most unstable of the antibiotic resistances in S. epidermidis 109 (Table 1). The neomycin (neo) marker had a 0.4% rate of spontaneous loss, which increased to 1.8% (χ^2 = 10.4; P = 0.001) when the organisms were grown at 43.5 C, and to 8.0% (χ^2 = 141.7; P < 0.001) when stored at 37 C. Chloramphenicol (cml) and tetracycline (tet) appeared to be relatively stable markers. Elimination of resistance did occur, but the frequency of loss was very low (0.2 to 0.4%) with all the procedures employed. In none of the almost 20,000 colonies tested was the streptomycin (str) marker eliminated. Attempts to eliminate penicillinase production were also unsuccessful in the approximately 12,000 colonies of strain 109 studied. At the same time, 10 other penicillinase-producing S. epidermidis strains were studied using a combination of different elimination methods. In 32 different experiments, 184,964 colonies were tested and all were found to be penicillinase producers. No attempts were made to eliminate either the methicillin (mth) or the sulfamethoxazole-(sul) markers in strain 109.
Transduction of resistance markers. *S. epidermidis* strains 62, 108, and 247 were used as recipients in most of the transduction experiments. The transduction frequency was similar with all three recipients. The phage-to-bacteria ratio (multiplicity of infection) varied between 0.05 to 0.3. Neomycin and *tet* markers were transduced at rates of $10^{-6}$ to $10^{-7}$ transductants per plaque-forming unit (Table 2), and *cml* was transduced at the high frequency of $10^{-3}$ to $10^{-4}$. Streptomycin-resistant transductants could be selected only when the phage lysate was previously irradiated for several minutes, and when the bacteria were incubated in 4 to 7 ml of broth for at least 0.5 h before selecting resistant organisms, the so-called phenotypic lag phenomenon. Chloramphenicol was consistently co-transduced along with the *tet* marker in 2 to 6% of the organisms; no other co-transduction was observed.

Attempts to transduce penicillin resistance were unsuccessful. Since markers affecting response of Cd²⁺ have been shown to be linked to the penicillinase (*pen*) marker in *S. aureus* (23), we tried to transduce resistance to this metal into three cadmium-sensitive *S. epidermidis* strains; this was unsuccessful as well. We were also unable to transduce the *mth* marker into strains 62, 108, or 247. Cohen and Sweeney (6) have observed that transduction of *mth* is more likely to occur in *S. aureus* if the recipient strains contain penicillinase plasmids. For this reason, we tried to transduce *mth* into an additional 12 *S. epidermidis* strains which were penicillinase producers, although it was not known if these resistances were due to chromosomal or plasmid genes. Transduction was not possible using these recipients either. Multiplicities of infection in all unsuccessful transduction experiments varied between 0.1 to 1. No experiments were performed to transduce the *sul* marker.

Effect of phage irradiation on transduction frequency. Phage lysates were UV irradiated for varying periods of time and the transduction frequencies after irradiation were determined. As illustrated in Fig. 1, the number of phage plaque-forming units decreased logarithmically with progressive irradiation. Transduction of the *tet* and *neo* markers also decreased logarithmically during the first 8 min of irradiation, but then the decrease became less pronounced with further irradiation. In the case of the *cml* marker, there was little change in transduction

<table>
<thead>
<tr>
<th>TABLE 1. Elimination of antibiotic resistance in <em>S. epidermidis</em> 109</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type of elimination experiment</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Spontaneous loss</td>
</tr>
<tr>
<td>Growth at 43.5 C</td>
</tr>
<tr>
<td>Growth in ethidium bromide</td>
</tr>
<tr>
<td>Storage at room temperature (3 weeks)</td>
</tr>
<tr>
<td>Ultraviolet irradiation</td>
</tr>
<tr>
<td>Storage at 37 C (23 days)</td>
</tr>
</tbody>
</table>

* Number in parenthesis indicates percentage.

<table>
<thead>
<tr>
<th>TABLE 2. Transduction of antibiotic resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibiotic</strong></td>
</tr>
<tr>
<td>Tetracycline</td>
</tr>
<tr>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>Streptomycin</td>
</tr>
<tr>
<td>Neomycin</td>
</tr>
<tr>
<td>Penicillin</td>
</tr>
<tr>
<td>Cadmium acetate</td>
</tr>
<tr>
<td>Meticillin</td>
</tr>
<tr>
<td>Meticillin</td>
</tr>
</tbody>
</table>

* Number of transductants per nonirradiated plaque-forming unit. NO, Not observed. 
frequency during the first 15 min of irradiation, and even after 35 min of exposure to UV the

decrease was still minimal. In contrast to the \textit{tet}, \textit{neo}, and \textit{cml} markers, the frequency of transduction of the \textit{str} marker increased following irradiation. The frequency peaked at 4 min of irradiation and leveled off after about 10 min of UV exposure at a rate that was higher than when nonirradiated phage lysates were used.

\textbf{Isolation of plasmid DNA.} Plasmid CCC DNA was separated from chromosome DNA in CsCl-ethidium bromide density gradients. We hoped to show that no plasmid DNA was present in the recipient strains, but as seen in Fig. 2, the recipient strain 247 contained plasmid DNA. Similar studies also showed the presence of plasmid DNA in recipient strains 62 and 108. In addition, the donor strain 109, in which three antibiotic resistances (\textit{tet}, \textit{cml}, and \textit{neo}) had been eliminated (109\textit{TetsCmlsNeos}), still contained appreciable amounts of plasmid DNA.

Plasmid CCC DNA was then layered onto sucrose gradients to isolate individual plasmids. Recipient strain 247 contained two distinct plasmids (Fig. 3) with sedimentation constant values of 29\textit{S} and 16\textit{S} (CCC molecules); their functions are unknown (cryptic). After transduction of the \textit{str} marker, no additional peak could be seen. However, following transduction of the \textit{cml} or \textit{neo} markers, a third peak with

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Transduction frequencies as a function of ultraviolet irradiation of transducing phage \textphi448/407-109. Symbols: \textDelta, survival of phage; \textbullet, transduction frequency of \textit{tet} (multiplicity of infection [MOI] = 0.2); \textcircled{a}, transduction frequency of \textit{cml} (MOI = 0.3); \textsquare, transduction frequency of \textit{neo} (MOI = 0.05); \textbullet, transduction frequency of \textit{str} (MOI = 0.05).}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Dye-CsCl density gradients of bacterial lysates. Density increases from right to left. The left peak represents the denser, faster sedimenting plasmid DNA peak. (A) \textcircled{a}, \textsuperscript{3}H-labeled \textit{S. epidermidis} 247; \textbullet, \textsuperscript{14}C-labeled \textit{S. epidermidis} 109. (B) \textcircled{a}, \textsuperscript{3}H-labeled \textit{S. epidermidis} 109\textit{TetsCmlsNeos}; \textbullet, \textsuperscript{14}C-labeled \textit{S. epidermidis} 109.}
\end{figure}
Fig. 3. Neutral sucrose gradients (5 to 20%; from right to left) of plasmid DNA. (A) O, \(^{3}H\)-labeled S. epidermidis 247. (B) O, \(^{3}H\)-labeled 247Str^r; O, \(^{14}C\)-labeled 247. (C) O, \(^{3}H\)-labeled 247Cm^R; O, \(^{14}C\)-labeled 247. (D) O, \(^{3}H\)-labeled 247Neo^r; O, \(^{14}C\)-labeled 247. (E) O, \(^{3}H\)-labeled 247Tet^r; O, \(^{14}C\)-labeled 247. (F) O, \(^{3}H\)-labeled 247Tet^R Cm^R; O, \(^{14}C\)-labeled 247.
sedimentation constants of 21S and 20S, respectively, was observed. CCC configuration of all three plasmids was confirmed by electron microscopy. Interestingly, after transduction of the tet marker, an additional peak of 20S was present, but the 16S plasmid of the recipient strain had disappeared. The same phenomenon could be seen with strain 247TetCmR, in which these two antibiotic-resistance markers were co-transduced. Strain 109TetCmlRNeo contained at least three plasmid peaks (Fig. 4). Because of this, rather difficult situation analysis of the neomycin, tetracycline, and chloramphenicol plasmids in segregants of strain 109 was not performed. Sedimentation values and molecular weights of the 3 to 4 cryptic plasmids of strain 109 were not determined.

The neomycin and tetracycline plasmids sedimented with the marker plasmid, T16, which has a molecular weight of 2.66 x 10⁶ (21). The chloramphenicol plasmid was calculated to have an approximate molecular weight of 2.8 x 10⁶, and the large and small cryptic plasmids were 6.4 x 10⁶ daltons and 1.3 x 10⁶ daltons, respectively.

**Electron micrography of plasmids.** Figure 5 illustrates the three different size plasmids that could be isolated. The largest and the smallest plasmids were the cryptic plasmids of the recipient strain 247. The medium-size plasmid was the transduced chloramphenicol plasmid. Contour length measurements of 18 open circular molecules of the chloramphenicol plasmid gave an average value of 1.5 μm per molecule. On the assumption that 1 μm corresponds to 1.96 x 10⁶ daltons of DNA (18), the molecular weight was calculated to be 3.0 x 10⁶. The difference (7.1%) between this value and the 2.8 x 10⁶ dalton value obtained in the sedimentation experiments can be explained by the intercalation of residual ethidium-bromide molecules between nucleotide pairs, resulting in an increase in length (7). This is also indicated by the smooth appearance of the circular molecules, which do not show sharp bends, and by the observation that twisted molecules are rarely seen. Molecular weights based on contour lengths of open circular molecules of the two cryptic plasmids were calculated to be 15% higher for the small and 9.3% higher for the large plasmid.

**DISCUSSION**

By using the criteria of genetic stability and the effect of irradiated phage on the transduction frequency, Minshew and Rosenblum (20) have shown that tetracycline resistance is plasmid borne in their strains of *S. epidermidis*. In our multiply antibiotic-resistant strain, we have shown that chloramphenicol and neomycin resistance, as well as tetracycline resistance, are plasmid borne. Spontaneous loss of these antibiotic markers occurred, and prolonged storage at 37°C significantly increased elimination of markers, although other curing experiments gave less significant results. In addition, no increase in transduction frequency after UV irradiation of transducing lysates could be observed. Momentarily we do not have an explanation why the small neomycin plasmid at low UV doses exhibited greater UV sensitivity than transducing phage. This behavior, however, can be constantly demonstrated. On the other hand, streptomycin was seen to be genetically stable, and irradiating the transducing phages for several minutes increased the transduction frequency, therefore indicating its chromosomal location. Penicillinase production appeared to be a stable marker, both in this strain and in several other strains which we examined. Since we were unable to transduce this trait, it is not possible to ascertain whether it is chromosomally located or not. Strain 109TetCmlRNeo still contained numerous plasmid peaks; it is
Sucrose gradients of plasmid DNA also substantiate the chromosomal location of \textit{str} and the plasmid location of \textit{tet}, \textit{cml}, and \textit{neo}. The recipient strains primarily used in the transduction experiments were seen to contain two distinct plasmids. The function of these plasmids is unknown; the organism was sensitive to all antibiotics tested and was nonhemolytic on human and sheep blood agar. The 16S plasmid, at least, appeared to be unnecessary for the survival of the organism since it could be eliminated without observable change in the organism. Following transduction of the \textit{str} marker into this recipient strain, there was no additional plasmid peak. Although a streptomycin plasmid could have the same molecular weight as one of the cryptic plasmids, this is unlikely. After transduction of \textit{cml}, \textit{tet}, and \textit{neo}, an additional peak was seen. The three plasmids are approximately the same size, somewhat less than $3 \times 10^8$ daltons. The tetracycline and neomycin plasmids sedimented with the reference tetracycline plasmid \textit{T}$_{\text{16S}}$ of \textit{S. aureus}. The tetracycline plasmid that Minshew and Rosenblum (20) studied also sedimented with \textit{T}$_{\text{16S}}$. The tetracycline plasmid from our strain 109 appeared to be incompatible with the 16S plasmid of strain 247. The organisms in which this phenomenon were observed, 247Tet$^R$ and 247Tet$^R$Cml$^R$, were obtained from separate experiments; it seems improbable that by chance two organisms were studied in which the 16S plasmid has been spontaneously lost. The phenomenon of plasmid incompatibility has been observed in \textit{S. aureus} (27) and occurs, perhaps, as a result of competition for the same membrane attachment site.

According to Novick and Bouanchaud (21), there are two types of plasmids in \textit{S. aureus}: large plasmids (e.g., the penicillinase plasmid, approximately $20 \times 10^8$ daltons) which replicate with the chromosome and are present in two to three copies per cell, and small plasmids (e.g., tetracycline and chloramphenicol, approximately $2.6 \times 10^4$ daltons) which replicate independently are present in multiple copies per cell. Our tetracycline, chloramphenicol, and neomycin plasmids probably fall into the latter category. The low rate of marker elimination could be explained on the basis of multiple copies. We have not calculated the exact number of plasmids per cell; however, the chloramphenicol plasmid is probably present in more copies than the neomycin and tetracycline plasmids. Its radioactivity profile in sucrose gradients is consistently higher than neomycin and tetracycline. This would also give an answer for the co-transduction of \textit{cml} with \textit{tet}. One explanation for co-transduction could be that some transducing phage particles acquire both the tetracycline and chloramphenicol plasmids, since in strain 247Tet$^R$Cml$^R$, the 16S plasmid of the recipient disappeared (incompatibility with tetracycline) and the amount of radioactivity in

![Electron micrographs of open circle plasmid DNA. (A) 29S plasmid of \textit{S. epidermidis} 247. (B) 16S plasmid of \textit{S. epidermidis} 247. (C) 21S chloramphenicol plasmid of \textit{S. epidermidis} 109.](image-url)
the tetracycline-chloramphenicol peak was comparatively high (multiple copies of chloramphenicol).

Several preliminary experiments to transduce these plasmids into S. aureus were unsuccessful; however, similar sizes of our plasmids and those with Novick and Bouanchaud (21) describe would indicate that they are similar, if not the same. Perhaps antibiotic-resistant S. epidermidis serve as a reservoir of resistance plasmids which can be transferred to sensitive S. epidermidis and S. aureus strains.

ACKNOWLEDGMENTS

We wish to thank E. Huf for technical assistance and L. Ruegg for help in preparing this manuscript. We are grateful to H. Koblet for criticisms, and to A. Vogel and Th. Koller for help and advice in preparing the electron micrographs.

LITERATURE CITED