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Isolation and Characterization of a Kanamycin Resistance Plasmid from *Staphylococcus aureus*

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A circular covalently closed duplex deoxyribonucleic acid plasmid carrying genes for resistance to kanamycin/neomycin has been identified in *Staphylococcus aureus* E419. The plasmid has a molecular size of 9.2×10^6 daltons and can be transduced into or can transform competent susceptible strains of *S. aureus* to kanamycin/neomycin resistance.

The determinant for kanamycin/neomycin (Km/Nm) resistance in *Staphylococcus aureus* has been found to be situated on extrachromosomal elements or plasmids that were irreversibly lost from the host cell by growth with acridine dyes (9, 16) or at elevated temperature (2). This determinant mediates resistance to these antibiotics (and to paromomycin) through the action of a phosphoryltransferase that catalyzes the phosphorylation of these antibiotics. The molecular weight and contour length of one staphylococcal Km/Nm resistance plasmid have been reported by Chopra et al. (10). This paper describes the genetic and biophysical characterization of another covalently closed circular (CCC) deoxyribonucleic acid (DNA) molecule that mediates Km/Nm resistance.

MATERIALS AND METHODS

Bacterial strains and media. *S. aureus* E419, a clinical isolate with the antibiotic susceptibility pattern shown in Table 1, was chosen to study the genetic and biophysical properties of the Km/Nm determinant. The source and properties of the staphylococci and their plasmids are listed in Table 2. CY (1%) a yeast extract-casein hydrolysate medium, was prepared according to Novick (20). Other media were commercial products.

Transduction. Transduction of Km from E419 was performed by published methods (14), by using phage 53 or a host range mutant of phage 80 that was propagated on E419 for two lytic cycles by the soft agar method (1). Transduction from strain 8325 was accomplished by phage ϕ11 that was induced by ultraviolet irradiation (14) from a Km-resistant transductant of 8325(pI524). The recipients, 8325, 8325-4, or 8325(pI524), were mixed with phage at multiplicity of 0.1 to 1.0. The cultures were plated, in 0.1-ml portions, on brain heart infusion (BHI)-citrate agar (14) containing 100 μg of Km per ml.

Plasmid elimination. For elimination of plasmids, two to three colonies of a culture grown on a BHI (Difco) agar plate containing 100 μg of kanamycin per ml was inoculated into 15 ml of 1% CY broth. The culture was grown at 43 C for 24 h with vigorous orbital shaking. Dilutions were plated on BHI agar, incubated overnight at 37 C, and replica-plated to BHI-Km agar. The replica plates were read after 24 h at 37 C.

Preparation of cell-free lysate. Cultures of the various strains were grown in 1% CY broth at 37 C to logarithmic phage (3-h culture) and then harvested by centrifugation at 10,000 rpm for 10 min at 4 C. The cells were suspended in buffer at pH 7.0 (10 mM $MgCl_2$, 60 mM KCl, and 100 mM tris(hydroxymethyl)-aminomethane-chloride; 15) to which was added 50 μg of lysostaphin per ml (Schwarz-Mann). The suspension was incubated at 37 C until it turned translucent (30 to 60 min). The lysate was then centrifuged at 16,000 rpm for 45 min at 4 C, and the supernatant fluid was saved.

Inactivation of Km. A portion of the supernatant of a cell-free lysate of 8325-4, 8325-4(pSH2), or 8325(pSH2) was mixed with adenosine triphosphate to give a final concentration of 5 mM. Portions of this mixture were incubated overnight at 37 C with varying concentrations of Km. A 0.02-ml sample from each reaction mixture was placed on a sterile paper disk, 6 mm in diameter, on a heart infusion agar plate freshly spread with a culture of *Escherichia coli* ϕ (provided by R. Brubaker), a Km-susceptible indicator strain. The plates were incubated overnight at 37 C and the diameters of the zones of inhibited growth around each disk were measured.

Density gradients. The *S. aureus* strains were labeled with [*methyl*-^{3}H]thymidine (50 Ci/mmol; New England Nuclear) for at least two generations in 1% CY broth. The labeled cultures were lysed by the procedure of Novick and Bouanchaud (22) with minor modifications to be published elsewhere (P. W. Stiffler, H. M. Sweeney, and S. Cohen, J. Bacteriol., in press). Centrifugation of the cesium chloride-ethidium bromide (CsCl-EtBr)-DNA solution was in a Spinco type 65 rotor at 43,000 rpm for 24 h at 20 C. Thirty 0.2-ml fractions (18 drops) were collected. Samples of 5 μliters were spotted on filter paper squares, washed, and counted for radioactivity (24).

The counts were normalized to the same total incorporation of radioactive material into cold trichloroacetic acid-precipitated material per optical density units at 540 nm to make them comparable. DNA from fractions 17 to 22 of the CsCl-EtBr gradient (see Fig. 1A) were pooled, extracted with isopropanol, and dialyzed against buffer containing 0.0015 M sodium citrate, 0.015 M NaCl, and 0.01 M Na_2ethylenediaminetetraacetic acid, pH 7.0. A 0.2-ml sample containing 0.1 ml of dialyzed ^{3}H-labeled pSH2 DNA and 0.1 ml of ^{14}C-labeled ColE1 DNA was layered on a 5-ml linear 20 to 31% neutral sucrose gradient made in 1 M NaCl, 0.05 M Na_2ethylenediaminetetraacetic acid, pH 7.5. The gradient was centrifuged for 180 min at 37,000 rpm in an SW39 rotor at 20 C. Fractions (30 to 32) of 0.15 ml (10 drops) were dropped on filter paper squares and then dried, washed, and counted for radioactivity (24).

TABLE 1. *Antibiotic disk susceptibility tests*

Antibiotic	Diameter (mm) of zone of inhibition of staphylococcal strain[a]:			
	8325	8325 (pSH2)	E419	E419 (cured of Km resistance)[b]
Kanamycin	20	0	0	22
Neomycin	18	0	0	20
Paromomycin	18	0	0	20
Amikacin (BB-K8)	18	16	14	20
Gentamicin	24	24	18	24
Sisomicin	22	22	20	24
Tobramycin	22	20	16	22
Lincomycin	22	20	0	0
Erythromycin	24	24	0	0
Penicillin	36	36	0	0
Methicillin	20	20	0	0

[a] Susceptibility tests were performed by a standard agar diffusion method (4).

[b] Km-susceptible clone of E419 detected after overnight growth at 43 C.

Electron microscopy. A sample of pSH2 DNA prepared for sucrose gradient analysis as described above was dialyzed against 0.15 M ammonium acetate, pH 6.0, and X-ray-irradiated with 9,000 rads to convert CCC molecules to open circular (OC) molecules. The irradiated DNA was prepared for examination in the electron microscope by the micro-drop technique of Lang and Mitani (18) and rotary shadowed with platinum-palladium (80 to 20%). The contour length of the OC DNA molecules were measured with a map measurer from photographic enlargements. A Fullam grating grid (54,864 lines/inch [about 2.54 cm]) was used as a measurement standard.

Transformation. Transformation was performed by a procedure based on those described for plasmid transformation in *E. coli* (13) and *S. aureus* (19) to be published elsewhere (Stiffler et al. J. Bacteriol., in press).

RESULTS AND DISCUSSION

The transduction of Km resistance is recorded in Table 3. Phage ϕ11, induced by ultraviolet irradiation (14) from a Km-resistant transductant of 8325(pI524), transduced Km/Nm resistance at a higher frequency than when phage 53 or 80 was used (Table 3). The observed decrease in transduction frequency of Km/Nm resistance after a 2-min exposure of the transducing phage to ultraviolet irradiation was consistent with the transduction of a plasmid-mediated determinant (3). Also, since 0.26% of cells in a culture of E419 irreversibly lost Km/Nm resistance after overnight growth at 43 C (Table 1), we inferred that the resistance determinant was a plasmid. It was designated pSH2.

To obtain physical evidence for the existence of CCC pSH2, the DNA content of 8325(pSH2) was analyzed by pycnographic centrifugation in CsCl-EtBr density gradients (Fig. 1A). Strain 8325 contained no detectable CCC DNA (Fig. 1A). The more dense peak of radioactivity in

TABLE 2. *Staphylococcal strains and plasmids*

Designation		Description[a]	Source
Staphylococcus	Plasmid		
8325		Propagating strain for typing phage 47. Harbors three prophages, ϕ11, ϕ12, ϕ13.	Received from Center for Disease Control, Atlanta, Ga.
8325-4		Strain 8325 cured of its three prophages.	Received from R. P. Novick (21).
	pI524	Staphylococcal penicillinase plasmid.	R. P. Novick (23).
E419		A clinical isolate of *S. aureus*. Not typed by phage at RTD. Phage type at 1,000 RTD was 7, 29, 47, 53, 54, 75, 77, 79.	Received from F. H. Kayser, Zurich, Switzerland.
	pSH2	The plasmid mediating Km/Nm resistance, transducible from *S. aureus* strain E419.	

[a] RTD, Routine test dilution.

strain 8325(pSH2) was characteristic of CCC DNA (Fig. 1A). The less dense peak contained chromosomal fragments and any linear or OC DNA molecules that were present.

The fractions containing CCC DNA molecules isolated from 8325(pSH2) were pooled, extracted with isopropanol, and dialyzed. A sample was cosedimented through a linear 20 to 31% neutral sucrose gradient with differentially labeled CCC ColE1 DNA which has a sedimentation coefficient of 23*S* (6). The calculated sedimentation coefficients of 32*S* and 23*S* (8) correspond to the CCC and OC plasmid molecules, respectively, with a molecular size of approximately 9×10^6 daltons (5). When mixtures of differentially labeled pSH2 DNA and ColE1 DNA were sedimented in alkaline sucrose gradients, there was the expected shift in sedimentation coefficients for CCC and OC DNA molecules (data not shown; 25). The CCC DNA was about 0.6% of the total cell DNA. Using a value of 5.5×10^{-15} g as the normal DNA content of a staphylococcus (22), these results are equivalent to a minimum of two copies of pSH2 per cell. However, since approximately half of the counts in Fig. 1B are in the OC form, we infer that pSH2 is relatively fragile and this may account for the low recovery of CCC DNA in the CsCl gradient (Fig. 1A).

Figure 2 is an electron micrograph of the OC form of pSH2. The average contour length of the OC molecules was 4.46 μm $\pm$ 0.06 (standard error). This corresponds to a molecular weight of 9.2×10^6, assuming a mass of 2.07×10^6 daltons per μm (12). This agreed very closely with the estimated molecular weight of 9×10^6 from the sedimentation coefficient (5).

A portion of the isopropanol-extracted, dialyzed pSH2 DNA was used as transforming DNA. pSH2 transformed Km/Nm resistance to susceptible strains (Table 4). The biophysical properties of the CCC DNA molecules isolated from transformants, by the methods described above, were identical to those of pSH2. Subsequent serial transformations of pSH2 were possible (Table 4).

The inactivation of Km by the cell-free extracts of the resistant transductant and transformant strains is shown in Table 5. Since 8325(pSH2) and 8325(pI524)(pSH2) were resistant to paromomycin, Km, and Nm, and susceptible to gentamicin, tobramycin, sisomicin, and BB-K8 (Table 1), we assume that the strains

TABLE 3. *Transduction of Km resistance to S. aureus 8325(pI524)*

Phage	Phage titer	Exposure time of phage to ultraviolet (min)	No. of transductants
53/E419[a]	4.5×10^8	0	135[b]
		2	100
80/E419[c]	1.25×10^9	0	331[b]
		2	285
8325(pI524)(pSH2)[d]	1.4×10^9	0	937
		2	392

[a] Typing phage 53 propagated on strain E419.
[b] Phage lysis of some transductants was apparent on the selective plates, evidently reducing the recovery of transductant clones.
[c] Typing phage 80 propagated on strain E419.
[d] ϕ11 was induced by ultraviolet irradiation of its host strain.

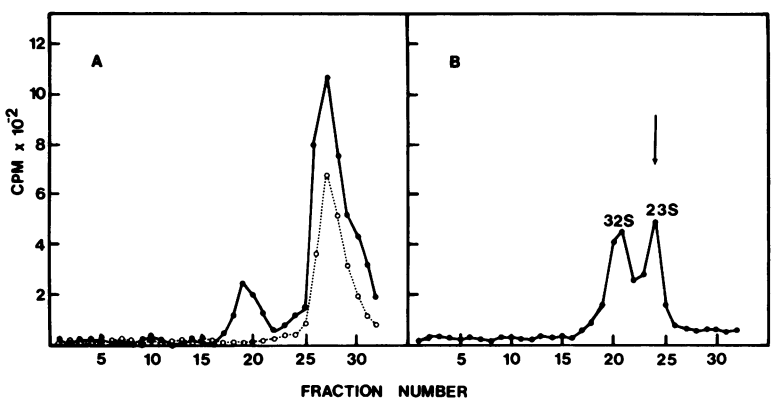

FIG. 1. *Centrifugal analyses of [^{3}H]thymidine-labeled DNA from Km-resistant and -susceptible strains of S. aureus 8325. (A) Equilibrium centrifugation of separate, cleared lysates of 8325 (○) and 8325(pSH2)(●) in CsCl-EtBr solutions. The separately centrifuged gradients were superimposed in the plot. (B) Neutral sucrose gradient analysis of CCC pSH2 (●). The arrow indicates the position of CCC ^{14}C-labeled ColE1 DNA (23S) in this gradient.*

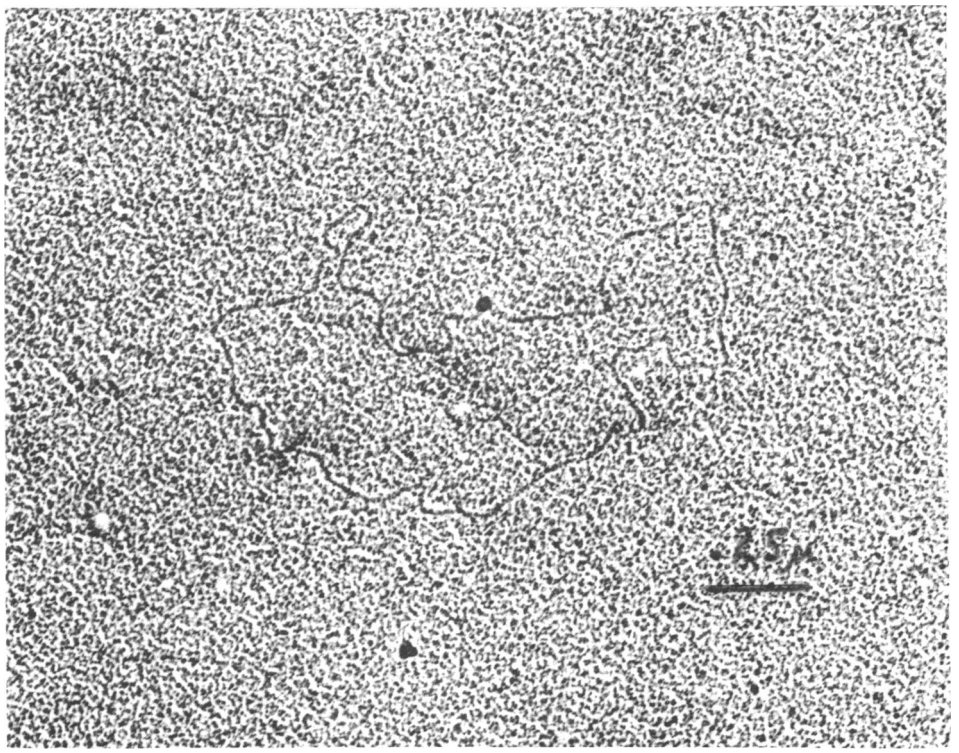

FIG. 2. *Electron micrograph of pSH2. Magnification, × 44,480. The bar in the micrograph represents 0.25 μm.*

TABLE 4. *Transformation of Km resistance to S. aureus 8325*

Donor DNA[a]	Frequency	No. of transformants isolated
8325(pSH2)[b]	8.2×10^{-8}	42
8325(pSH2)[c]	5.7×10^{-8}	38

[a] Each transformation mixture contained 1 μg of donor plasmid DNA and 5×10^8 recipient cells in a volume of 1 ml.

[b] Donor organism acquired pSH2 by transduction from E419.

[c] Donor organism acquired pSH2 by transformation from 8325(pSH2).

TABLE 5. *Inactivation of Km by cell-free extracts of strains bearing a Km/Nm resistance plasmid*

Cell-free extract	Diameter (mm) of zone of inhibition at given concn of Km solution treated with *S. aureus* lysate			
	400 (μg/ml)	200 (μg/ml)	100 (μg/ml)	0
8325-4	12	10	9	0
8325-4(pSH2)	0	0	0	0
8325(pSH2)	7	0	0	0

bearing pSH2 inactivated Km, Nm, and paromomycin by means of a Km/Nm phosphoryltransferase (7).

pSH2 is different in size from the Km/Nm resistance plasmid described by Chopra et al. (10). The plasmid that they described had a molecular size of 5.9×10^6 daltons by calculation from its contour length and a copy number per cell of 9 to 16. Evidently there exist diverse plasmids mediating Km/Nm resistance in staphylococci. It is impossible to say at this time whether there is formation de novo of Km/Nm plasmids in *S. aureus* or the extensive modification of a primordial Km/Nm plasmid by recombination, deletion, or other genetic reactions (17).

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