Plasmid for Tetracycline Resistance in *Staphylococcus epidermidis*

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Received for publication 5 February 1973

A plasmid determining tetracycline resistance was demonstrated for *Staphylococcus epidermidis*. Tetracycline resistance was spontaneously lost from a *S. epidermidis* strain at a frequency of <0.1 to 1.3%. Frequency of loss was not appreciably enhanced by growth at 42 C or in ethyl violet. The rate of transduction of tetracycline resistance in three *S. epidermidis* crosses was decreased as a function of ultraviolet irradiation of the transducing phage. Plasmid deoxyribonucleic acid (DNA) associated with tetracycline resistance was isolated by CsCl-ethidium bromide dye-buoyant density gradients. The tetracycline plasmids from *S. aureus* RN 1304 (molecular weight 2.66 Mdals) and *S. epidermidis* 367(ϕ 367) (T_{367}) cosedimented in 5 to 20% neutral and alkaline sucrose gradients and are therefore of similar molecular weights. An extrachromosomal element in the recipient strain, isolated as covalently closed circular DNA, did not retain its integrity in an alkaline sucrose gradient.

The extrachromosomal nature of the deoxyribonucleic acid (DNA) determining several antibiotic resistances—particularly penicillin, tetracycline, and chloramphenicol—has been well established in certain strains of *Staphylococcus aureus* (18). Antibiotic resistances generally occur singly on separate plasmids in *S. aureus*, unlike the R factors of the *Enterobacteriaceae* (17). The reliability of the various criteria for establishing extrachromosomal elements (plasmids) as locations for genetic determinants has been extensively reviewed by Novick (17). In this study we report a plasmid which determines resistance to tetracycline in *S. epidermidis*. The extrachromosomal location of the genetic determinants for this antibiotic resistance was demonstrated by a relatively high spontaneous loss of resistance, by the kinetics of transduction of antibiotic resistance after ultraviolet (UV) irradiation of the transducing phage, and by isolation of plasmid-specific DNA. These techniques have been used by a number of workers to indicate plasmid-borne genes in *S. aureus*. Although evidence for antibiotic resistance plasmids in *Staphylococcus epidermidis* has been presented (3, 4, 21), these reports have not included the important criterion of UV-irradiation effects on transduction, nor has anyone previously identified the physical determinant for resistance carried by

this staphylococcal species. Characterization of the tetracycline resistance plasmid allows a comparison to be made to the plasmid for tetracycline resistance in *S. aureus*.

MATERIALS AND METHODS

Organisms. The *S. epidermidis* strains and the transducing phage, ϕ 367, have been previously described (15). The pertinent antibiotic sensitivity patterns and strain derivations are listed in Table 1. The location of chloramphenicol resistance on a separate plasmid in strain 20144 is inferred from other data.

Cultures were maintained on tryptone agar (TA; Oxoid) slants. Broth cultures were grown in Trypticase soy broth (TSB; BBL), tryptone, yeast extract broth (LB) (5), or CY (16).

Phage was propagated and assayed by the soft agar overlay technique (22) as previously described (15). Phage was induced from strain 367(ϕ 367) (T_{367}) by mitomycin C. An overnight TSB culture was diluted 1:10 into fresh TSB containing 1 μ g of the drug per ml. The culture was incubated for 3.5 to 4.0 h at 37 C, filtered, and stored at 4 C.

Plasmid elimination. Cultures were grown at an elevated temperature (42 C) in LB or in the presence of ethyl violet (EV) (Matheson, Coleman and Bell) (Schaeffer, see Discussion) to enhance spontaneous loss. Control cultures were incubated at 37 C for various time periods. The initial inoculum in most experiments was approximately 10^6 colony-forming units (CFU)/ml. Tetracycline-sensitive colonies were detected by replica plating to Trypticase soy agar

TABLE 1. Strain characteristics

Designation ^a	Antibiogram ^b			Strain derivation
	Pen	CM	Te	
367(ϕ 367)(T ₃₆₇)	R	S	R	Wild type, lysogenic
324	R	S	S	Wild type, indicator strain for ϕ 367
324(T ₃₆₇)	R	S	R	Tetracycline-resistant transductant of 324
21252(T ₂₁₂₅₂)	R	S	R	Wild type
21252(N _T)	R	S	S	Tetracycline-sensitive variant of 21252(T ₂₁₂₅₂)
20144(C ₂₀₁₄₄)	R	R	R	Wild type
(T ₂₀₁₄₄)				
20144(N _C)(N _T)	R	S	S	Tetracycline- and chloramphenicol-sensitive variant of 20144(C ₂₀₁₄₄) (T ₂₀₁₄₄)
RN1304	S	S	R	<i>S. aureus</i> , a transductant carrying T ₁₆₉ . Obtained from R. N. Novick

^a Strains are designated by strain number followed by the number of any carried phage (ϕ) or plasmid in parenthesis. T and C indicate plasmids for tetracycline and chloramphenicol resistances, respectively; N indicates that a carried plasmid has been eliminated. The numerical subscript to T or C identifies the number of the original strain carrying the plasmid.

^b Antibiotic sensitivity determined on TSA with disks (BBL): penicillin (Pen), 2 units; tetracycline (Te), 30 μ g; chloramphenicol (CM), 30 μ g.

(TSA) containing 10 μ g of tetracycline per ml.

Transduction. Cells of recipient strains were washed from overnight cultures on TA slants with 2 ml of TSB. Transduction mixtures contained approximately 10⁸ CFU of bacteria and 10⁸ plaque-forming units (PFU) of bacteriophage in TSB. Multiplicities of infection (MOI) are noted in specific experiments. After incubation of the transduction mixture for 20 min at 37 C, an equal volume of 1% sodium citrate was added, and the mixture was centrifuged at 3,000 $\times$ g and resuspended to the original volume of the transduction mixture with 0.5% sodium citrate in TSB. One-tenth milliliter was then spread on TSA plates containing 0.5% sodium citrate as well as 10 μ g of tetracycline per ml. The transducing phage requires low levels of calcium at some point in its growth cycle and the sodium citrate, by binding the available calcium, prevented lysis of transductants by superinfecting phage. In all transduction experiments, controls were cells incubated in TSB and treated as the transduction mixtures. Colonies were counted after 24 h of incubation at 37 C.

Transducing phage lysates were irradiated in glass petri dishes by a General Electric 15-W germicidal lamp placed 53 cm above the dish. Two to 2.5 ml of phage lysate was placed in a dish and rotated during irradiation. Times of exposure were from 0 to 4 min.

Isolation of plasmid DNA. Exponentially growing cultures of *S. epidermidis* in CY broth were labeled for 40 min with 5 μ Ci of ³H-thymidine per ml (specific activity, 22 Ci/mmol). *S. aureus* RN 1304 was grown in 2 μ Ci of ¹⁴C-thymidine per ml (specific activity, 62 mCi/mmol) for 35 min.

Cells were harvested and washed by filtration, resuspended to 10¹⁰ CFU/ml, protoplasted with lyso-staphin (Schwarz/Mann; 15 μ g/ml for *S. aureus*, 30 μ g/ml for *S. epidermidis*), lysed with 1.5 volumes of a mixture of 1% Brij, 0.04% deoxycholate, and 0.3 M ethylenediaminetetraacetic acid (EDTA) (pH 8.0). The lysate was centrifuged at 49,000 $\times$ g for 30 min at 0 C. This method was employed by Novick and Bouanchaud (18) for the isolation of *S. aureus* plasmid DNA.

Covalently closed circular (CCC) DNA was isolated from nicked circular DNA and linear fragments in the lysate supernatant fluid above by centrifugation for 40 h at 43,000 rpm in a Spinco 50 or 65 fixed-angle rotor in cesium chloride-ethidium bromide (EB) dye-buoyant density gradients. The final concentration of EB was 250 μ g/ml. The resulting gradient was collected in 20-drop fractions from a hole punched in the bottom of the tube. Portions (50 μ liters) of each fraction were spotted onto 1-inch filter paper squares which were washed by the method of Bollum (8) and counted in a Nuclear-Chicago Unilux II scintillation counter.

Sucrose gradients. Fractions containing the CCC DNA isolated by CsCl-EB dye-buoyant density gradients were pooled, extracted with isopropanol to remove the EB (11), and dialyzed with dilute saline sodium citrate (0.015 M NaCl, 0.0015 M sodium citrate) in 0.05 M EDTA (pH 7.0). Sucrose solutions were also prepared in this buffer for neutral gradients. A 0.2-ml amount of *S. epidermidis* CCC DNA and 0.2 ml of the ¹⁴C-thymidine-labeled reference CCC DNA from *S. aureus* RN 1304 were layered onto 5 to 20% sucrose gradients with a 0.2-ml 50% sucrose cushion. The gradients were centrifuged at 45,000 rpm for 135 min at 5 C in a Spinco SW 50.1 rotor. Five-drop fractions were collected directly onto 1-inch filter paper squares, which were subsequently washed once each in 5% trichloroacetic acid, 95% ethanol, and diethyl ether, dried and counted.

The 5 to 20% alkaline sucrose gradients were prepared according to Blair et al. (6). These gradients contained 1.0 M NaCl, 0.001 M EDTA, and 0.3 M NaOH. They were centrifuged at 45,000 rpm for 90 min at 5 C.

RESULTS

Elimination of the tetracycline plasmid. Treatment of *S. epidermidis* 367(ϕ 367) (T₃₆₇) with EV or growth at elevated temperature did not significantly enhance the rate of loss of this

plasmid over the spontaneous frequency at 37 C (Table 2). Although curing conditions tended to increase the frequency of tetracycline-sensitive colonies, the results were variable. Similar results were observed with strains 21252(T_{21252}) and 20144(N_C) (T_{20144}). The spontaneous loss and the loss after enhancing treatments ranged from <0.1 to 3.6% in these three strains.

Effect of UV irradiation on the transduction of tetracycline resistance. A decrease in the transduction of tetracycline resistance as a function of UV dose was observed with UV irradiation of the transducing phage. This is in agreement with the expected behavior for plasmid-determined characteristics (1, 16). Three genetic crosses are illustrated: donor 367($\phi 367$) (T_{367}) with the recipient 324 (Fig. 1), a heterologous system; donor 21252(T_{21252}) with recipient 21252(N_T) (Fig. 2), and donor 20144(C_{20144}) (T_{20144}) with recipient 20144 (N_C) (N_T), both homologous systems with cured derivatives of the donors as recipients. The transducing phage was induced from the lysogenic donor by mitomycin C in the experiment illustrated in Fig. 1, and $\phi 367$ was propagated in the donors for the other experiments (Fig. 2 and 3). In all cases, the PFU of the transducing phage were also reduced with increased exposure to UV irradiation.

Isolation of plasmid-specific DNA. A denser band sedimenting as CCC DNA was isolated from all strains of *S. epidermidis* examined, including one strain (370-1) sensitive to all antibiotics which were tested. Therefore, to demonstrate plasmid DNA specific for the resistance marker, CCC DNA isolated by CsCl-EB dye-buoyant density gradients was sedimented through 5 to 20% sucrose gradients to separate the molecular species. The *S. aureus* tetracycline plasmid T_{100} , molecular weight

2.66 Mdals (18), was used as a reference DNA to estimate the molecular weight of the *S. epidermidis* tetracycline plasmid. A unique plasmid-determining tetracycline resistance was transduced from 367($\phi 367$) (T_{367}) to strain 324. This plasmid isolated from the donor and the transductant cosedimented with the *S. aureus* tetracycline plasmid T_{100} (Fig. 4A and 4D).

FIG. 1. Effect of UV irradiation on the transduction of tetracycline resistance. Transducing phage $\phi 367$ was induced by mitomycin C from the donor strain 367 ($\phi 367$) (T_{367}). Strain 324 was the recipient and phage indicator. MOI in the cross was 0.04. ■, PFU of bacteriophage per ml; ●, transductants per 10^6 input phage.

TABLE 2. Elimination of tetracycline resistance by elevated temperature or ethyl violet; strain: *S. epidermidis* 367($\phi 367$)(T_{367})

Growth conditions ^a	Expt no.	Colonies examined/no. sensitive					
		6 h	% ^b	24 h	% ^b	48 h	% ^b
37 C	(1)	1,336/0	<0.1	956/1	0.1	2,808/2	0.1
	(2)	1,446/19	1.3			802/3	0.36
	(3)						
42 C	(1)	1,089/0	<0.1	959/2	0.2		
37 C + ethyl violet, 0.25 μ g/ml	(1)					5,175/51	0.98
	(2)					584/21	3.6
	(3)					604/2	0.31

^a Growth medium was tryptone, yeast extract broth, pH 6.8.

^b Percent sensitive.

FIG. 2. Effect of UV irradiation on the transduction of tetracycline resistance. Transducing phage $\phi 367$ was propagated and assayed on the donor strain 21252(T_{21252}). The recipient strain 21252(N_T) was a tetracycline-sensitive variant of the donor. MOI in the cross was 0.03. ■, PFU of bacteriophage per ml; ●, transductants per 10^7 input phage.

In the sucrose profile of the recipient 324 a small peak of ³H-thymidine-labeled material was observed in the same area of the gradient as the ¹⁴C-thymidine-labeled T_{189} reference DNA (Fig. 4B). This peak probably represents molecules of the faster sedimenting DNA which were damaged by the isopropanol extraction procedure. When the material was dialyzed to remove the EB but not extracted with isopropanol, this small peak was not observed (Fig. 4C).

Material isolated as CCC DNA in CsCl-EB dye-buoyant density gradients from strains 324 and 324(T_{367}) was sedimented through 5 to 20% alkaline sucrose gradients. In the transductant 324(T_{367}), a peak cosedimenting with the reference CCC DNA T_{189} was observed (Fig. 5B). However, the integrity of the fast sedimenting DNA molecules isolated from strain 324 appears to be lost under these conditions (Fig. 5A) and sediments more slowly than the reference DNA.

DISCUSSION

Previous studies have indicated that penicillin (3, 21), chloramphenicol, and tetracycline resistances (4) may be determined by plasmids in certain strains of *S. epidermidis*, based on frequencies of loss after physical (growth at elevated temperature) or chemical (acridine orange, ethidium bromide) treatments. Schaeffer (S. Schaeffer, 1970, Bacteriol. Proc., p. 61) reported pH-dependent preferential elimination of tetracycline resistance over penicillin resistance with EV in *S. aureus*. In this study of the tetracycline plasmid, we observed a fairly high spontaneous loss, <0.1 to 1.3%, which was not appreciably enhanced by growth at 42°C or in the presence of EV. These results may reflect differences in strains, the plasmids, or simply the variability from experiment to experiment which we observed with our strains. Schaeffer (21) also reported variations from experiment to

FIG. 3. Effect of UV irradiation on the transduction of tetracycline resistance. Transducing phage $\phi 367$ was propagated and assayed on the donor strain 20144(C_{20144}) (T_{20144}). The recipient strain 20144(N_C) (N_T) was a tetracycline- and chloramphenicol-sensitive variant of the donor. MOI in the cross was 0.13. ■, PFU of bacteriophage per ml; ●, transductants per 10^7 input phage.

FIG. 4. Sucrose gradient profiles of the denser DNA band isolated by CsCl-EB dye-buoyant density gradients. A, 0.2-ml amount of ^3H -thymidine-labeled material from the *S. epidermidis* strains (●) and 0.2 ml of ^{14}C -thymidine-labeled tetracycline plasmid DNA from *S. aureus* RN 1304 (○) were layered onto 5 to 20% neutral sucrose gradients, performed on a 50% sucrose cushion. The gradients were centrifuged 135 min at 45,000 rpm in an SW 50.1 rotor at 5 C. Five-drop fractions were collected directly onto 1-inch filter paper squares from a hole punched in the bottom of the tube. The papers were washed, dried, and counted. A, The profile of strain 367(ϕ 367) (T_{367}), the tetracycline-resistant donor. B, Profile of the recipient strain 324. C, Profile of strain 324 with DNA preparation dialyzed only to remove EB. A 0.1-ml amount of the reference DNA was used in this gradient. D, Profile of a tetracycline-resistant transductant 324(T_{367}). The reference plasmid, T_{189} , of strain RN 1304 has a reported molecular weight of 2.66 Mdals (Novick and Bouanchaud, 1971).

experiment in "curing" antibiotic resistances from strains designated *S. epidermidis* BV. The most efficient elimination of tetracycline resistance occurred with growth at 45 C in LB medium, pH 6.8.

Loss of antibiotic resistance at a greater frequency than expected by mutation is suggestive of extrachromosomal inheritance, but the kinetics of loss should be examined for this single criterion to be conclusive (17). Such a kinetic study by May et al. (14) established the extrachromosomal nature of tetracycline resistance in *S. aureus* strain E₁₈₉. However, the frequency of loss effected by treatment must be

very much higher than those which we observed with *S. epidermidis* for a study of the kinetics of loss to be practical.

The isolation of transducing phages from *S. epidermidis* (15, 23) has provided additional evidence of plasmid-linked antibiotic resistances in this species. The decline in the rate of transduction of extrachromosomally inherited traits as a function of UV dose was described by Arber (1) for *Escherichia coli*. Novick's work (16) with the penicillinase plasmid of *S. aureus* demonstrated that the effect of UV irradiation on the transduction of plasmid-linked genes was applicable in staphylococci as well. Asheshov

FIG. 5. Alkaline sucrose gradients of the denser DNA band isolated by CsCl-EB dye-buoyant density gradients. A 0.2-ml amount of ^3H -thymidine-labeled material from the *S. epidermidis* strains (●) and 0.2 ml of ^{14}C -thymidine-labeled tetracycline plasmid DNA from *S. aureus* RN 1304 (○) were layered onto 5 to 20% alkaline sucrose gradients. The gradients were centrifuged at 45,000 rpm in an SW 50.1 rotor for 90 min at 5 C. A, The profile of the recipient strain 324. B, Profile of a tetracycline-resistant transductant 324(T_{367}).

(2), Poston (19), and other workers (12, 13) have applied this criterion to tetracycline resistance in *S. aureus*. Their results indicated a plasmid-linked genetic marker.

The kinetics of transduction of tetracycline resistance in *S. epidermidis* strains with UV irradiation of the $\phi 367$ were those expected with plasmid-linked determinants. In no case was there stimulation of transduction rates when optimal conditions were employed. MOI values were low, and superinfecting phage was inactivated to prevent lysis of tetracycline-resistant transductants of the phage-sensitive recipients.

The demonstration of plasmid-specific DNA determining tetracycline resistance was complicated by material isolated as CCC DNA in CsCl-EB dye-buoyant density gradients from the recipient strain, 324. Also, what appeared to be multiple species of CCC DNA were isolated from strains 21252(T_{21252}) and 20144(C_{20144}) (T_{20144}). Strain 324 was also resistant to penicillin, but penicillinase production was not spontaneously lost from this strain nor was it eliminated by "curing" treatments such as growth at 42 C or in EB at a frequency of 0.1% (unpublished results). However, it was possible to identify one species of DNA unambiguously as

the tetracycline plasmid by its acquisition in a tetracycline-resistant transductant and its disappearance (unreported studies) from the donor strain upon loss of tetracycline resistance.

The molecular species in the sucrose profiles of 324 and 324(T_{367}) which sediments faster than the tetracycline plasmids T_{169} (*S. aureus*) and T_{367} (*S. epidermidis*) was presumed to be CCC DNA on the basis of its isolation as a denser band in CsCl-EB dye-buoyant density gradients. If this peak is representative of supercoiled DNA, its behavior in alkaline gradients (Fig. 5A) indicates that the DNA may be associated with some other material, perhaps RNA. Blair et al. (7) have recently presented evidence of an alkaline- and ribonuclease-sensitive portion in covalently closed Col E₁ DNA accumulating in *E. coli* when protein synthesis is inhibited by chloramphenicol.

Isopropanol extraction of EB also affected this faster-sedimenting material so that a second peak, which sedimented with T_{169} , was observed in the neutral sucrose gradient of the recipient strain 324 (Fig. 4B). Omission of the extraction step yielded only a single peak, indicating that the additional peak is a product of extraction with isopropanol. It is interesting

that, when the ratio of the distances migrated is calculated for the faster-sedimenting (?CCC DNA) and slower sedimenting (second peak) material, the ratio is in the range expected for a CCC DNA and a linear form of the CCC DNA molecule of the same molecular weight, but not the ratio expected for CCC DNA and open circular DNA (9). In alkaline gradients, linear DNA would be denatured and may account for the sucrose profile observed (Fig. 5A).

Since the physical sizes of the tetracycline resistance plasmids of *S. aureus* and *S. epidermidis* are similar, if not identical, the implication of genetic relatedness cannot be avoided. Baldwin et al. (3) have suggested that antibiotic-resistant *S. epidermidis* might be involved in the emergence of resistant strains of *S. aureus* by in vivo transduction of plasmid-linked determinants. Interspecific transduction has been reported (23), and the appearance of antibiotic resistance is often first observed in coagulase-negative staphylococci (10, 20). DNA hybridization may provide evidence of genetic homology between antibiotic resistance plasmids of the two species.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AI-03093 from the National Institute of Allergy and Infectious Diseases, by Public Health Service training grant 2E-122 from the National Institute of General Medical Sciences, and by General Research Support grant 5 S01 RR 05426-11.

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