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Genetic Studies on Plasmid-Linked Cadmium Resistance in *Staphylococcus aureus*

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The genetic basis of cadmium resistance conferred by three penicillinase plasmids, PI_{524}, PI_{258}, and PII_{147}, of *Staphylococcus aureus* was examined by mutation, recombination, and deletion analysis. Three separate loci were identified: *cadA*, responsible for high-level resistance; *cadB*, giving a low-level resistance, nonadditive to *cadA;* and *mad*, a locus marginally *decreasing* the cadmium resistance of plasmid-positive staphylococci. The loci *cadA* and *mad* were present on all three plasmids, but *cadB* was only found on PII_{147}. Spontaneous deletions of *mad* involved up to three-fourths of the plasmid genome, which allowed derivation of a partial deletion map of PII_{147}, a plasmid with a contour length of 10.9 μm, corresponding to a molecular weight of 20.4×10^6.

In the enteric bacteria and in staphylococci, extrachromosomal genes are responsible for a considerable proportion of clinically significant antibiotic resistance and consequently have been subjected to detailed genetic and biochemical analysis. Doubtless of equal importance to the organisms—although perhaps less so to man—are extrachromosomal determinants of resistance to inorganic ions. Chief among these, in *Staphylococcus aureus* at least, is cadmium resistance, which is probably the most common and certainly the most effective of those so far discovered. Its study is therefore a key aspect of our attempts to achieve a comprehensive understanding of the biochemistry and genetics of plasmid-linked resistance in this organism. This paper describes a series of experiments showing that plasmid-linked cadmium resistance involves at least three distinct loci and that there are at least two different genetic patterns of these loci among three plasmids examined; these patterns seem to be correlated with plasmid incompatibility specificity.

MATERIALS AND METHODS

Media. CY broth, GL agar, phage agar, phage buffer, and lysing broth were used as previously described (9, 11). $Cd(NO_3)_2$, Na_2AsO_4, and erythromycin (a gift of Eli Lilly & Co.) were added sterilely to the various media directly before use.

Strains. *S. aureus* was used exclusively in these experiments. Strains are listed in Table 1. Cultures were grown in CY broth at 37 C with shaking. Culture densities were measured in a Klett-Summerson photoelectric colorimeter with a green (540 nm) filter; a reading of 100 in a standard Klett tube corresponded to 0.12 mg (dry weight)/ml or about 2×10^8 colony-forming units (CFU)/ml in an exponential culture at 37 C. Stock cultures in CY broth were quick-frozen with dry ice-alcohol and stored at −70 C.

Nomenclature of plasmids and markers is as described previously (18). An additional convention that we introduce here is the use of square brackets to denote deletions, the deleted markers being indicated within the brackets (see Table 6).

Bacteriophages 80α and ϕ11 (11) were used for generalized transduction. (Phage ϕ11 has previously been labeled P11. The P has been replaced with ϕ to eliminate confusion with penicillinase plasmids, which are also designated P.) Phage lysates were prepared in lysing broth, sterilized by membrane filtration, and stored at 4 C. Procedures for transduction were as previously described (9, 11).

Isolation of plasmid-linked mutations to cadmium sensitivity. A plasmid-positive strain was grown in CY broth to about 2×10^8 CFU/ml, diluted with an equal volume of phage buffer, infected with a transducing phage at an input multiplicity of about 2, and incubated for 15 min at 37 C. Ethyl methane sulfonate (Eastman Organic Chemicals) was then added to give a final concentration of 1 M. The culture was shaken at 37 C for an additional 30 min, and was then centrifuged, resuspended in lysing broth, and incubated at 37 C until lysis was complete—usually 1 to 1.5 hr. The resulting lysate was used to transduce a plasmid-negative strain (usually RN26 or RN27) with selection for resistance

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TABLE 1. *Staphylococcal strains*

Stock no.	Host	Plasmid	Derivation (reference)
A. Primary strains			
RN1	8325	None known	Naturally occurring
RN4	8325	PI_{524}[a]	Transductant (9)
RN8	8325	PII_{147}	Transductant (15)
RN24	8325	PI_{258}*penI443*[b]	Transductant (15)
RN25	8325-3	None known	RN1 cured of prophages ϕ11 and ϕ12
RN26	8325-3(ϕ11)	None known	RN25 lysogenized with ϕ11
RN27	8325-3(80α)	None known	RN25 lysogenized with 80α (9)
RN31		PI_{258}	Transductant (15)
RN92	147(N)	None known	Original host for PII_{147} (15)
RN223	8325-3	PII_{147}*penI300mer*$^+$	Transductant (15)
RN240	8325(80α)*str*	PII_{147}*pen-401cadA4*	Transductant (16)
RN241	8325(80α)*str*	PII_{147}*pen-401cadA7*	Transductant (16)
RN242	8325(80α)*str*	PII_{147}*pen-401cadA11*	Transductant (16)
RN243	8325(80α)*str*	PII_{147}*pen-401cadA17*	Transductant (16)
RN244	8325(80α)*str*	PII_{147}*pen-401cadA22*	Transductant (16)
RN245	8325(80α)*str*	PII_{147}*pen-401cadA29*	Transductant (16)
RN246	8325(80α)*str*	PII_{147}*pen-401cadA37*	Transductant (16)
RN450	8325-4	None known	RN25 cured of prophage ϕ13
RN654	8325-4	PI_{258}*pen-401asa-53cadA52ero*$^+$	Transductant
RN900	147(N)*seg-9*	PI_{258}*penI443*	Transductant
RN923	147(N)*seg-9*	PII_{147}*penI220*	Transductant
RN1008	8325	PI_{258}*pen-401cadA52mer-14ero*$^+$	Transductant
RN1014	8325-3(ϕ11)	PII_{147}*penI300mer-30asa*$^+$	Transductant
RN1030	8325-4(ϕ11)*his-7recA1*	None known	Mutation of RN450 (L. Wyman, Ph.D. Thesis, 1971)
RN1337	147(N)*seg-9*	None known	Mutation of RN92
RN1465	8325-4	PII_{147}	Transductant RN8 $\xrightarrow[\text{cad}]{80\alpha}$ RN450[c]
RN1467	8325-4	PII_{147}*penI300mer*$^+$	Transductant RN223 $\xrightarrow[\text{cad}]{80\alpha}$ RN450[c]
B. Derivative strains (new mutants)			
RN966	8325-3(ϕ11)	PII_{147}*penI300cadA114*	RN223 $\xrightarrow[\text{asa}]{\phi 11\ \text{EMS}^b}$ RN26
RN1422	8325-4(ϕ11)*his-7recA1*	PII_{147}*penI300cadA114*	RN966 $\xrightarrow[\text{asa}]{80\alpha}$ RN1030
RN1424	8325-3(ϕ11)	PII_{147}*penI300cadA114cadB1*	RN966 $\xrightarrow[\text{asa}]{\phi 11\ \text{EMS}}$ RN26
RN1425	8325-3(ϕ11)	PII_{147}*penI300cadA114cadB2*	
RN1426	8325-3(ϕ11)	PII_{147}*penI300cadA114cadB3*	
RN1427	8325-3(ϕ11)	PII_{147}*penI300cadA114cadB4*	
RN1467	8325-4	PII_{147}*penI300cadA114*	RN966 $\xrightarrow[\text{asa}]{80\alpha}$ RN450
RN1468	8325-4	PII_{147}*mad-2[cadA pen asa]*	Spontaneous *mad* mutants of PII_{147}*penI300cadA114* (RN966) transduced into RN450
RN1471	8325-4	PII_{147}*mad-1 cadA114[pen]*	
RN1472	8325-4	PII_{147}*mad-3[mer cadA pen]*	
RN1501	8325-4	PII_{147}*mad-4[mer cadA pen asa]*	
RN1582	8325-3	PII_{147}*penI300mad-6*	Spontaneous *mad* mutants of PII_{147}*penI300* (RN223) transduced into RN25
RN1583	8325-3	PII_{147}*penI300mad-7*	
RN1584	8325-3	PII_{147}*penI300mad-8*	
RN1585	8325-3	PII_{147}*penI300mad-9*	
RN1487	8325-3	PII_{147}*penI300mad-11*	
RN1588	8325-3	PII_{147}*penI300mad-12*	
RN1589	8325-3	PII_{147}*penI300mad-13*	—

[a] PI_{524}, PII_{147}, and PI_{258} have been previously referred to as α, β, and γ, respectively (15, 18).

[b] Point mutational markers have been introduced by ethyl methane sulfonate mutagenesis followed by transduction to non-mutagen-treated host strains. Detailed pedigrees are reported elsewhere (16, 18; Novick, *unpublished data*). See Materials and Methods.

[c] Strains newly constructed for this study. Our convention for transductional crosses is: donor strain $\xrightarrow[\text{marker}]{\text{phage}}$ recipient strain.

to erythromycin or arsenate. Transductant plasmids carrying mutations to cadmium sensitivity were identified by replication to GL agar supplemented with $Cd(NO_3)_2$. For first-step mutants, the $Cd(NO_3)_2$ concentration was 50 μM; for second-step mutants, 10 μM. Mutants thus identified were cloned, tested for other plasmid markers (16), quick-frozen, and stored at −70 C. Reversions to cadmium resistance were selected by plating dilutions of CY broth cultures on GL agar supplemented with $Cd(NO_3)_2$ at various concentrations as specified.

Resistance tests. Cadmium resistance was quantitated by standard twofold serial broth dilution tests with the use of organisms from overnight GL plates at 10^4 CFU/tube. A volume of 1 ml in 13-mm tubes was used, and the tubes were shaken for 24 hr at 37 C. The $Cd(NO_3)_2$ concentration in the first tube not showing turbidity was taken as the end point. As variations in end point occurred with different batches of media, results are expressed as the ratio of test strain end point to a control strain end point. The control strain, RN1, was included in each set of tests. Small fluctuations in resistance ratios were also observed. These reflect the limits of accuracy of the test and are probably due to factors such as inoculum variations. Other resistance markers were scored by disc susceptibility tests or by plating on inhibitor-containing agar, as previously described (16).

Incompatibility specificity. Plasmid incompatibility specificity was scored with a host strain thermosensitive for plasmid maintenance. The mutational defect of this strain, RN1337, is manifested as an inability to maintain type I plasmids above 32 C; type II plasmids are also unstable in this host but only above 37 C (Novick and Brodsky, *unpublished data*). Plasmids to be scored were transduced into RN1337, and their stability was assessed by staining colonies grown at 32, 37, and 42 C for penicillinase activity (12). The results were evaluated by comparison with control strains RN900 and RN923, which are derivatives of RN1337 harboring plasmids of known incompatibility type.

Penicillinase. Penicillinase activity in colonies was assessed by the *N*-phenyl-naphthylamine-azo-*o*-carboxybenzene stain technique (15). Quantitative penicillinase activities were determined by the iodometric method of Perret (17). Penicillin G was a gift from Chas. Pfizer & Co.

Plasmid recombination. Crosses between suitably marked compatible plasmids were carried out by the construction of plasmid merodiploids (15) which were used as donors of plasmid markers to a plasmid-negative strain. Recombinant plasmids were obtained by joint selection for a marker from each of the component plasmids; recombinants were screened for cadmium resistance by replica plating. Other unselected markers were scored as described above.

Isolation and measurement of plasmid DNA. Circular duplex plasmid deoxyribonucleic acid (DNA) was isolated from cleared lysates (2) of plasmid-positive derivatives of RN450 (14) by equilibrium dye-buoyant density centrifugation (19). The denser band was collected visually, the ethidium bromide was extracted with isopropanol (D. R. Helinski, *personal communication*), and the preparation was dialyzed exhaustively against SSC (0.15 M NaCl + 0.015 M sodium citrate). The preparations were stored at 4 C for 1 week to permit the accumulation of single-stranded scissions, and electron microscope measurements were carried out on DNA molecules spread by Lang's microdrop modification (8) of the Kleinschmidt and Zahn technique (7) and rotary-shadowed with uranium. Photomicrographs were taken on a Phillips electron microscope 300 at a magnification of 5,500 times. The photographic plates were projected with a photographic enlarger and were traced; contour lengths were measured with a map measurer. Magnification was calibrated with a diffraction grating (28,800 lines/inch [2.54 cm]). Phage PM2 (4), kindly provided by J. Dahlberg, served as a source of circular DNA for a contour length reference standard.

RESULTS

Cadmium-sensitive mutants. New mutations to cadmium sensitivity were isolated for three different plasmids, PI_{258}, PI_{524}, and PII_{147}, and their residual cadmium resistance was measured by broth dilution assays in CY broth. Several previously isolated mutants (16) were also included. The results of these assays (Fig. 1) showed that, whereas most of the PI_{258} and PI_{524} mutants were as sensitive to cadmium as the plasmidless host strain, none of 18 PII_{147} mutants was fully sensitive; all had residual cadmium resistance levels at least 8- to 10-fold greater than their plasmid-negative host. Because this result suggested that PII_{147} might have two cadmium loci, a PII_{147} derivative carrying one of the first-step mutations, *cadA114*, was used for the preparation of a mutagen-treated transducing lysate for the isolation of second-step cadmium-sensitive mutations. Among plasmid transductants of RN27, selected for arsenate resistance, eight were more sensitive to $Cd(NO_3)_2$ than the *cadA114* donor; all eight, in fact, were fully sensitive, as shown in Fig. 1.

Disc sensitivity tests revealed that all of the following parental plasmid markers were unaffected by either the first- or second-step cadmium mutations: *pen*, *mer*, *bis*, *asa*, *lea*. Incompatibility specificity (10) was also unaffected.

These results suggest that, whereas PI_{258} and PI_{524} have a single major determinant of cadmium resistance, PII_{147} has at least two independently mutable *cad* loci, hereinafter designated *cadA* and *cadB*. If this is true, then the five first-step mutants, 11, 107, 108, 111, and 113 (see Fig. 1), with only partially diminished levels, could be either *cadB* or leaky *cadA*

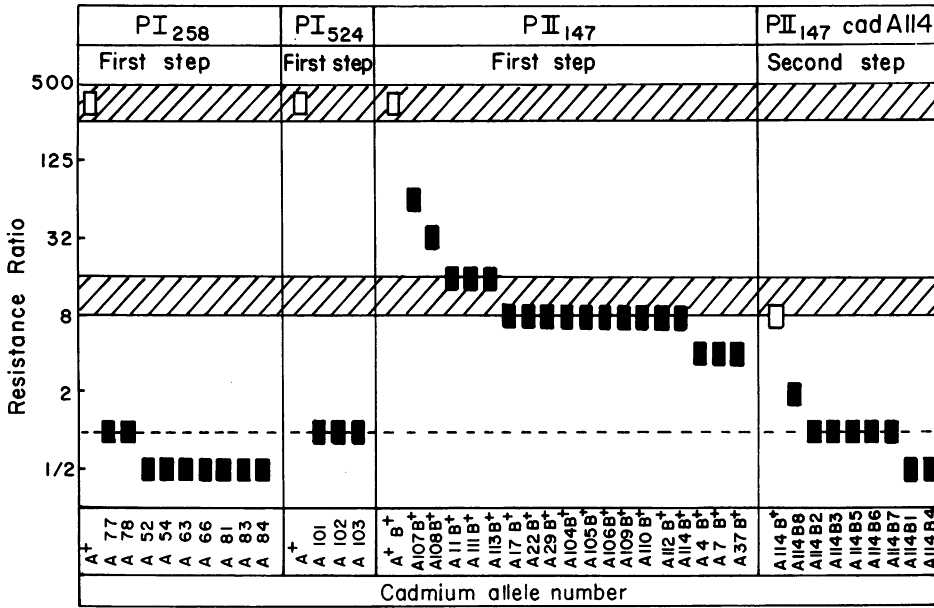

FIG. 1. *Resistance ratios for cadmium-sensitive mutants. Each solid block represents the residual resistance to cadmium nitrate due to a plasmid mutation, whose number appears at bottom. The open blocks represent the resistance levels due to the parental plasmids from which each set of mutants was derived. These values, the results of broth dilution tests, are shown as resistance ratios which were obtained by dividing the end point concentration for the mutant by that for the plasmidless control strain (dashed line). The upper and lower cross-hatched areas represent ranges for wild type and for the most usual first-step mutants.*

mutants. Evidence presented below favors the latter alternative.

To determine the levels of resistance conferred separately by the two *cad* loci and to reveal their phenotypic and genotypic relationships, we next performed a series of reversion and recombination tests with the various mutant plasmids. As the single *cad* locus carried by PI_{258} and PI_{524} corresponds phenotypically to the *cadA* locus of PII_{147}, it too will be designated *cadA*.

Separation of cadmium loci by recombination. If the *mcrII* plasmid, PII_{147}, has two distinct loci for cadmium resistance, they should be separable by recombination. This separation was realized in a pair of crosses between PII_{147}, wild type for cadmium, and PI_{258}, carrying the *cadA52* point mutation. The latter marker, which reverts at a frequency of 10^{-8} or less (16), was used because of its stability. In these crosses, two plasmid diploids were constructed by the transduction of two different PI_{258} derivatives into RN223 and RN1014, strains carrying $PII_{147}cad^{+}$ plasmids. The genotypes of these diploid transductants, selected for erythromycin resistance, are shown below. (Note: only relevant plasmid alleles are indicated. All others are wild type [see Fig. 4, and also reference 18].) Although *pen-401* maps in or near the structural locus, it has not been assigned to the Z cistron; strains carrying it produce about 10% of wild-type penicillinase activity, but the enzyme itself appears to be structurally unaltered (M. H. Richmond, *personal communication*).

(i) PI_{258}*pen-401 cadA52 asa-53 ero*$^{+}$/
PII_{147}*penI300 mer-30 asa*$^{+}$

(ii) PI_{258}*pen-401 cadA52 mer-14 ero*$^{+}$/
PII_{147}*penI300 mer*$^{+}$

Recombinant plasmids were isolated by transduction from these diploids into strain RN27 (a plasmid-negative) with simultaneous selection for one marker from each of the component plasmids. With the first diploid, selection was for AsaR EroR; with the second, MerR EroR. In both cases, the doubly resistant transductants were replica-plated for the scoring of cadmium resistance levels. With diploid i as donor, 14 of 900 AsaR EroR transductants had an intermediate level of cadmium resistance. Of the total, about 59% were *cadA*$^{-}$ and about 40% were *cad*$^{-}$. With diploid ii, 30 of 650 MerR EroR transductants had an intermediate level. About 84% of the total were

cadA⁺, but only about 9% of the total were *cad*⁻. The complete scoring of these crosses will be detailed elsewhere. The intermediate-level transductants were purified, scored for unselected markers, and assayed by broth dilution for cadmium resistance levels. Figure 2 is a diagrammatic representation of these crosses which makes use of the marker order for PII_{147} obtained by deletion analysis (see Fig. 4) and that for PI_{258} obtained by recombination mapping (Novick and Brodsky, *unpublished data*). Note that in cross i the required genotype involves four crossovers, and in cross ii, two crossovers; this may account for the threefold greater frequency of recombinants in cross ii. The results are shown in Table 2, where it can be seen that 37 of 44 had resistance levels similar to those of the first-step (*cadA*) mutants described above (see Fig. 1). These evidently represent the expression of *cadB* and support the conclusion that PII_{147} has two *cad* loci that are separable by recombination as well as by mutation. These results suggest also that *cadB* segregates with the *mer* region but independently of *pen* (see Fig. 4). It is not known whether or not the *cadA52* marker of the PI_{258} parent is present in any of the intermediate-level recombinants. Seven of the 44 recombinants had higher intermediate cadmium resistance levels. The genotypes of these are uncertain; however, some were unstable in their cadmium resistance and therefore appeared to be heterozygous. These seven were not examined further.

Reversion of cad mutants. All of the cadmium-sensitive mutants gave rise to spontaneous phenotypic revertants on GL agar plus 50 μM $Cd(NO_3)_2$. The apparent reversion frequencies of the first-step mutants, but not of the second-step mutants, were strongly inoculum-dependent at this cadmium concentration, and so reversion frequencies were scored with the lowest practicable inocula, namely, 10^7 to 10^8 CFU/plate. When this was done, both first-step and second-step mutants had reversion frequencies in the range of 10^{-6} to 10^{-8}/CFU (Table 3).

Series of independent CadR derivatives of several strains were examined in some detail (Table 4). As the CadR colonies were selected at random for these tests, the numbers given in Table 4 under "No. of isolates found" are an indication of the approximate relative frequencies of the various types of revertants isolated in any particular experiment.

Considering first strain RN966, which carries a first-step mutation, *cadA114*, we found that some of the reversions were not plasmid-linked, as transduction into a new host with selection for plasmid-linked arsenate resistance resulted in the transfer of a plasmid indistinguishable from the *cadA114* parent element. These reversions were not studied further. Those that were plasmid-linked could be divided into two major phenotypic classes, i and ii, on the basis of cadmium resistance levels (Fig. 3). The class i revertants had relatively high levels of cadmium resistance. Four of these were tested by broth dilution and found to be slightly less resistant than the original CadR strain from which the mutant was derived. The possibility that their Cad⁺

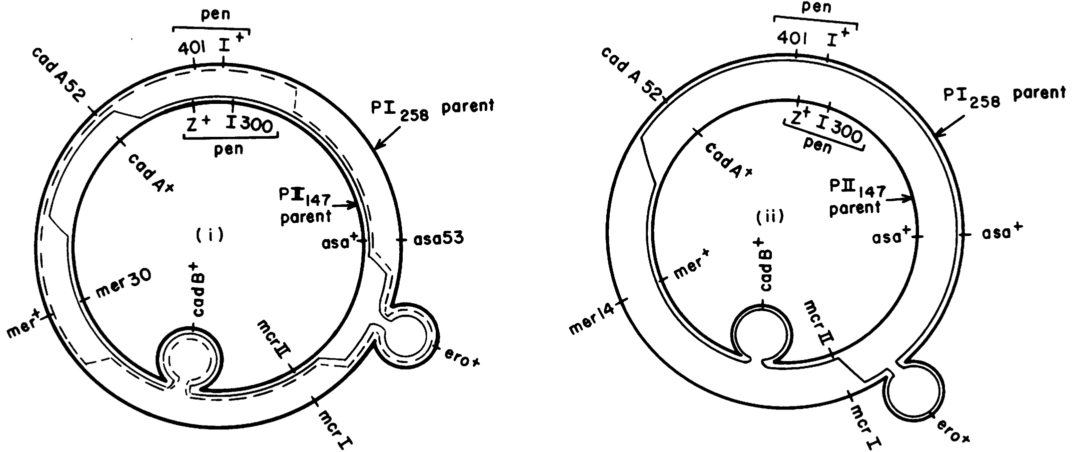

FIG. 2. *Plasmid crosses. Heavy circles represent genetic maps of the recombining plasmids. Regions of presumed nonhomology are indicated as "blisters." Genetic structures of recombinants are indicated by light solid or dashed lines with the crossovers placed as required by the maps. Only the recombinants selected for are shown. For a description of markers, see references 14 and 18. Left: cross i. Right: cross ii. (See text.)*

TABLE 2. *Recombinants with intermediate cadmium resistance*

No. of recombinants	Markers							
	penI	*penZ*	*asa*[a]	*ero*[a]	*mcr*	*bis*	*mer*[a]	*cad*(RR)
Cross i								
PI_{258} parent	+	–	–	+	I	+	+	1
PII_{147} parent	–	+	+	–	II	ES[d]	–	125
5[b]	–	+	+	+	II	ES	–	16-32
3[b]	–	+	+	+	II	ES	–	4-8
6[b]	+	–	+	+	II	ES	+	8
Cross ii								
PI_{258} parent	+	–	+	+	I	+	–	1
PII_{147} parent	–	+	+	–	II	ES	+	125
26[b]	+	–	+	+	II	ES	+	16
1	+	–	+	+	II	ES	+	64
1[b]	–	+	+	+	II	ES	+	16
1	–	+	+	+	II	ES	+	64
1[b]	[[c]	]	+	+	–	ES	+	16

[a] Selected markers for cross i were *ero* and *asa*; for cross ii, *ero* and *mer*.
[b] Recombinants considered to have cadmium resistance levels consistent with the $cadA^-cadB^+$ genotype.
[c] Square brackets indicate deletions. See Novick (13) for a hypothesis on the occurrence of deletions in plasmid crosses.
[d] ES = epistatic sensitivity (18).

TABLE 3. *Reversion frequencies of cad mutants*

Stock no.	$Cd(NO_3)_2$ (μM)	Plasmid genotype[a]	Reversion frequency[b] $\times 10^7$		Ratio, CadR Pen$^-$/ CadR (%)
			CadR	CadR Pen$^-$	
RN966	50	*cadA114cadB*$^+$	33-45[c]	5-12	11-38
RN1467	50	*cadA114cadB*$^+$	3-25[c]	0.1-1.8	2-20
RN1422[d] (Rec$^-$)	50	*cadA114cadB*$^+$	1.4-7.7[c]	0.4-4.5	22-58
RN1424	50	*cadA114cadB1*	1.1-3.3[c]	<0.002	<0.1
	10		25		
RN1425	50	*cadA114cadB2*	0.05-0.9[c]		
	10		13		
RN1426	50	*cadA114cadB3*	0.03-2.7[c]	<0.003	<0.1
	10		67		
RN1427	50	*cadA114cadB4*	1.0-1.7[c]	<0.003	<0.2
	10		69		

[a] All plasmids listed are derivatives of PII_{147}*penI300*.
[b] Reversion frequencies are the number of colonies appearing on cadmium plates (CadR) divided by the total number of CFU plated. Pen$^-$ refers to colonies that were penicillinase-negative by the *N*-phenylnaphthylamine-azo-*o*-carboxybenzene stain procedure.
[c] Range of results for three or more different subclones.
[d] Isogenic with strain RN1467 except carrying the *recA1* allele (L. Wyman, Ph.D. thesis, New York Univ. School of Medicine, New York, N.Y., 1971).

phenotype was due to external suppression (e.g., amber, etc.) was ruled out by a transductional demonstration that the reversions were plasmid-linked. The most likely possibility would seem to be that they are intracistronic suppressors rather than same-site back mutants, but no definitive evidence in support of this possibility is available.

The other revertants (class ii), although isolated as colonies on plates containing a concentration of cadmium upon which *cadA114* was just unable to grow, showed essentially no increase in cadmium resistance over the original mutant as measured by broth dilution assay. The basis of the difference in their behavior on agar versus broth is not known. These low-level revertants could be divided into two subclasses. Members of one subclass (iib) were, surprisingly, pleiotropic, exhibiting loss of activity for various other plasmid markers concomitant with low-level cadmium reversion. The properties of these and their genetic basis are discussed below.

Members of the other subclass (iia) had sim-

TABLE 4. *Properties of CadR derivatives of various strains*

Stock no. (cadmium genotype)	CadR type	Cd^{2+} [a]	No. of isolates		RR [b]	Plasmid markers				
			Found	Tested		*penZ*	*asa*	*mer*	*bis*	*lea*
RN966 (*cadA114*)	Class i	50	44	4	125–250	+	+	+	ES	+
	Class iia	50	39[c]	6	4–16	+	+	+	ES	+
	Class iib	50	1	1	8–16	−	+	+	ES	+
	Class iib	50	8	1	8–16	−	−	+	ES	+
	Class iib	50	3	1	8–16	−	+	−	ES	+
	Class iib	50	6	1	8–16	−	−	−	ES	+
RN1424 (*cadA114cadB1*)	Full	50	4	4	125–250	+	+		ES	+
	Full	10	3	3	125–250	+	+	+	ES	+
	Intermediate	10	9	5	16–32	+	+	+	ES	+
RN223 (*cad*$^+$)	Class iia	400	6		125–250	+	+	+		
	Class iib	400	4		125–250	±[d]	+	+		
	Class iib	400	10		125–250	−	+	+		
	Class iib	400	4		125–250	−	−	+		
Parental strains										
RN966					8–16	+	+	+	ES	+
RN1424					1	+	+	+	ES	+
RN223					125–250	+	+	+	ES	+
RN1					1	NP[e]	NP	NP	NP	NP

[a] Numbers in this column signify $Cd(NO_3)_2$ concentration in micromoles/liter on which revertants were selected.
[b] RR (resistance ratio) = broth dilution end point for strain tested/broth dilution end point for plasmidless control.
[c] Total of both host-linked (>12) and plasmid-linked (<27) reversions. Transductions to distinguish these were not done for all isolates.
[d] Indicates reduced penicillinase activity (see Table 5).
[e] No plasmid.

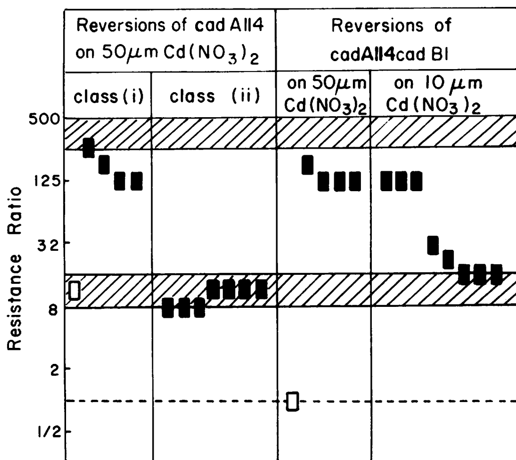

FIG. 3. *Resistance ratios of revertants. Solid blocks represent resistance ratios for revertants of mutants whose resistance ratios are indicated by open blocks. Upper and lower hatched areas indicate resistance ratios for wild-type cad*$^+$ *and cadA114 cadB*$^+$*, respectively.*

ilar low-level cadmium resistance but were not detectably pleiotropic.

When cultures of four of the second-step mutants, *cadA114 cadB*$^-$, were plated on 50 μM $Cd(NO_3)_2$ and incubated for 24 hr at 37 C, high-level revertants appeared. These were similar to those obtained with *cadA114 cadB*$^+$ and occurred at roughly the same frequency (see Table 3). When the double mutants were plated on a lower cadmium concentration (10 μM), both high- and intermediate-level revertants were obtained (see Table 4 and Fig. 3).

These results were interpreted as suggesting: (i) that *cadA* by itself is responsible for essentially the entire difference in cadmium resistance between plasmid-positive and negative strains; (ii) that *cadB*, by itself, confers an intermediate level of resistance but its effects do not appear to add to those of *cadA;* and (iii) that the intermediate-level revertants of *cadA114 cadB1* involve mainly the *cadB* locus, the high-level *cadA*.

Pleiotropic effects of marginal increases in cadmium resistance. As mentioned above, many CadR reversions of PII_{147} *cadA114* were pleiotropic, having suffered the loss of activity of various other plasmid markers concomitantly with the acquisition of a marginal increase in cadmium resistance. It was readily demonstrated that not only PII_{147} *cadA* strains but also PII_{147} *cadA cadB* double mutants, as well as strains harboring the three wild-type

plasmids, PII_{147}, PI_{258}, and PI_{524}, gave rise to similar pleiotropic mutants on selection for growth on agar containing $Cd(NO_3)_2$ at a concentration just inhibitory for the strain being tested. Plasmid-negative strains, however, did not give low-level cadmium-resistant mutants at appreciable frequencies. Because of residual growth under these conditions, it was necessary to use relatively low inocula ($<10^8$ CFU/plate) to demonstrate the pleiotropic revertants.

A number of these low-level "resistant" clones were examined for plasmid markers, and the plasmid was transduced to a plasmidless recipient to test linkage of the new genotype. In Table 4 are listed the properties of two series of such isolates, from strains RN966 and RN223, respectively. In general, anywhere from 2 to 60% of revertants showing the slight increase in cadmium resistance were found to have suffered concomitant loss of one or more plasmid marker activities. With strain RN966 (carrying PII_{147} *penI300 cadA114*), patterns of marker losses included *pen*, *pen asa*, *pen mer*, and *pen asa mer*. With strain RN223 (carrying PII_{147} *penI300*), losses of *pen* and *pen asa*, but none involving *mer*, were observed; in addition, several distinct phenotypes involving partial loss of penicillinase activity were encountered. All of these were low-level constitutives (which is expected since the parental plasmid was already *penI*⁻), and it was initially thought that they might involve alterations of the second penicillinase regulatory locus, *penB*, which controls the maximal level of penicillinase production (20; K. Smith, Ph.D. thesis, Univ. of Edinburgh, Edinburgh, Scotland, 1968). However, this is evidently not the case: *penB*⁻ mutations are *trans*-recessive, and preliminary tests of the mutants in question showed that the low-level penicillinase genotype is *cis*-dominant, i.e., is fully expressed in plasmid diploids where the second plasmid is *penB*⁺. Penicillinase activities for seven such derivatives of RN223 are shown in Table 5. A reasonable but untested possibility is that they are defective in promoter activity.

Genetic basis of pleiotropic mutations. The properties of the mutants selected for slight increases in cadmium resistance can best be explained by the existence of a plasmid-linked gene locus that, in its wild-type state, results in a *ma*rginal *d*ecrease in cadmium resistance. For this reason, and out of respect for the state of mind of the senior author during her studies of this situation, we have elected to use the genotypic notation *mad* to refer to this locus. As discussed, mutations affecting this locus often result in the simultaneous loss, or partial loss, of other plasmid properties, and several such pleiotropic effects have been observed. Two possible explanations for these observations are that the product of the *mad* locus is multifunctional and central to the expression of other plasmid genes or that selection for *mad*⁻ results in deletions of the *mad* locus plus various other plasmid markers according to their linkage relationships. Since the latter hypothesis not only seemed more likely but also was readily testable, we undertook electron photomicrographic measurements of several of the pleiotropic *mad*⁻ plasmids.

TABLE 5. *Penicillinase activities of certain mad⁻ mutants of PII_{147}*

Stock no.	Plasmid genotype	Penicillinase activity[a]
RN223	PII_{147} *penI300 mad*⁺	480
RN1583	PII_{147} *penI300 mad-7*	8.5
RN1582	PII_{147} *penI300 mad-6*	21
RN1584	PII_{147} *penI300 mad-8*	24
RN1589	PII_{147} *penI300 mad-13*	22
RN1585	PII_{147} *penI300 mad-9*	160
RN1587	PII_{147} *penI300 mad-11*	200
RN1588	PII_{147} *penI300 mad-12*	180

[a] Iodometric penicillinase assays were run on samples of whole broth cultures in exponential phase. Activities are units per milligram (dry weight).

Isolation and measurement of plasmid DNA molecules. The plasmids from four different pleiotropic *mad*⁻ derivatives (PII_{147} *penI300 cadA114*) were transduced into strain RN450, which is plasmidless and free from detectable prophages. Open circular plasmid DNA was then isolated from each of these four strains and from strain RN996 (carrying the parental PII_{147} *cadA114* plasmid), and was measured by electron microscopy. The contour length measurements shown in Table 6 revealed that large sections of plasmid DNA have been deleted in three cases and that the size of the deletion in each case is a reflection of the number of markers lost.

In one case, that of the *mad*⁻ *pen*⁻ deletion, the contour length was not significantly different from that of the parental plasmid. The interpretation of this latter result is limited by the fact that, although the internal reproducibility within a set of measurements from one spreading was good ($<5\%$ error), and the values obtained for the contour length of PM2 DNA were similar to the 3.02 nm $\pm$ 0.11 ob-

TABLE 6. *Contour lengths of plasmids with mad deletions*

Plasmid deletion	No. of molecules measured	Mean contour length ± SD (μm)	Molecular wt × 10^{-6}
None[a]	14	10.9±0.5	20.4±0.9
[*pen*]	20	10.8±0.2	20.2±0.4
[*cadA pen asa*]	8	5.5±0.2	10.3±0.4
[*mer cadA pen*]	30	5.3±0.1	9.9±0.2
[*mer cadA pen asa*]	10	3.43±0.04	6.42±0.07
Phage PM2 DNA	39	3.16±0.12	5.91[b]

[a] The parental plasmid for this set of derivatives is PII_{147}*penI300 cadA114* (see Table 4: class iib revertants of strain RN966). The measurements, however, are of PII_{147}, the original wild type.

[b] The published value for PM2 (4). With this value, we obtained a figure of 1.87 × 10^6 daltons/nm for the mass-to-length ratio of circular DNA under our conditions of spreading, etc. Molecular weights in the last column are calculated with this value.

tained by Espejo and co-workers (4), we had difficulty controlling the degree of stretching of the molecules during spreading. This resulted in variations in contour length of up to 15% for different spreadings of the same sample. Although it was possible to assess roughly the degree of stretching from the appearance of the molecules and to discard badly stretched preparations, it would have been necessary to include an internal contour length standard with each spreading to compare accurately molecules of similar length. Thus, one can say no more than that *pen* is closer to *mad* than either *asa* or *mer*.

Genetic map of PII_{147}. Because the various plasmid deletions occurred with roughly similar frequencies and were spontaneous accompaniments of selection for *mad*$^-$, it seems probable that each represents an *en bloc* deletion of all of the markers involved. On this basis, one can deduce the gross genetic structure of the PII_{147} plasmid as shown in Fig. 4, where genetic distances are approximated by deletion contour lengths. As indicated in Table 4, none of the *mad*$^-$ deletions affected *bis*, *lea*, or *cadB*. All were still autonomous plasmids, and so the deletions could not have affected the *mcr* region (10). This argument, together with the recombination data, suggests that *cadB*, *lea*, *bis*, and *mcr* must be relatively closely linked, and it seems more likely that *lea* and *bis* are between *cadB* and *mcr* than elsewhere, since the conditions for selection of *mad*$^-$ mutant plasmids from strain RN966 required the presence of the latter two loci but not of the former two.

Location of cadA. The presence of *cadA114* was presumably not required for the selection of *mad*$^-$ mutants, so it is possible that some of the deletions may have included this locus. Since only those that retain *cadA114* should give rise to high-level CadR reversions, reversion tests should reveal the presence or absence of this locus. The four *mad* plasmids whose contour lengths were measured were therefore tested for reversion, with the results given in Fig. 5. None of the three larger deletions gave rise to high-level CadR reversions, whereas the smallest one, *mad*$^-$ *pen*$^-$, did. This result suggests that CadA is fairly close to the *mad pen* region. CadA can be further localized by a consideration of the pattern of deletions obtained from strain RN223 (which is *cadA*$^+$), where the selection technique precludes the loss of *cadA*. No *mer* deletions were found among the 24 *mad* isolates examined (see Table 4), which is the result expected if *mer* is between *cadA* and *mcr*. Moreover, since *mad*$^-$ *pen*$^-$ *asa*$^-$ deletions *were* obtained from

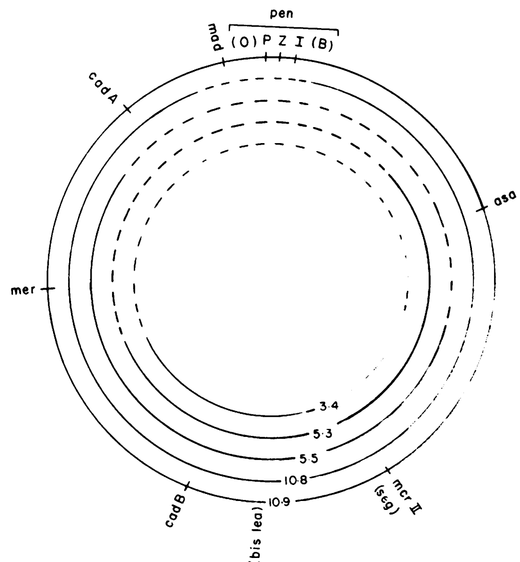

FIG. 4. *Deletion map of PII_{147}. In addition to cadmium resistance loci, cadA and cadB, markers indicated are resistances to arsenate (asa), bismuth (bis), lead (lea), and mercury (mer), maintenance, compatibility, and replication (mcr), plasmid segregation (seg), marginal decrease in cadmium resistance (mad), and penicillinase (pen). Markers in parentheses have not been ordered within the local region where they a.e placed. Solid lines represent segments of plasmid genome remaining after various mad deletions (see Table 6). Figures given with each deletion represent contour lengths in micrometers of remaining plasmid DNA.*

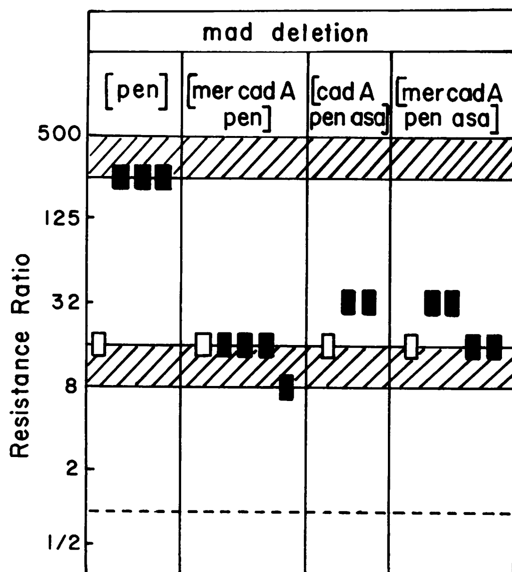

FIG. 5. *Resistance ratios for CadR revertants of mad deletions. Solid blocks represent resistance ratios of cadmium-resistant revertants derived from mad deletions indicated above. Open blocks indicate resistance ratios for the mad deletions themselves. Upper and lower hatched areas represent resistance ratios for wild-type cad⁺ and cadA114 cadB⁺, respectively.*

this strain, the *mad pen* region must be between *cadA* and *asa*.

Penicillinase locus. The plasmid-linked penicillinase locus consists of a structural cistron, Z, and at least two *trans*-dominant regulatory cistrons, I and B, which control, respectively, the inducibility and the maximal level of expression of the structural gene (20; K. Smith, Ph.D. thesis, Univ. of Edinburg, Edinburgh, Scotland, 1968). Of necessity, there is also a promoter-operator (P-O) region, although mutants affecting it have not been described to date. A combination of several bits of evidence suggests the order P-Z-I (see Fig. 4) for these three of the component elements (by analogy with the *lac* operon, O would be to the right of P). Recombination mapping (Novick and Brodsky, *unpublished data*) has yielded the order *cadA penZ penI asa* for PI_{258}. Additional experiments on the isolation of *mad*⁻ mutants from PII_{147} *penI*⁺ *penZ*⁺, have yielded *mad* [*pen*] genotypes but not *mad* [*penI*]; if *mad* is to the left of *pen*, as appears to be the case, then these results support the order *cadA mad penZ penI asa*, and, if the deletions analyzed in Tables 5 and 6 are, indeed, promoter-defectives, i.e., [*mad penP*], then the overall region is most likely *cadA mad penP penO penZ penI asa*. There is no evidence from these experiments for the location of *penB*, but the senior author has found previously that for PI_{524} it is close to *penI*, possibly between *penI* and *penZ* (Smith, Ph.D. thesis, 1968).

Effect of host rec function on frequency of mad deletions. As shown by Franklin (5), Inselburg (6) and Anderson (1), if deletions arise by a type of internal recombination, it is unusual in that it occurs at the same frequency in *rec*⁺ and *rec*⁻ strains in *Escherichia coli*. Similarly, in *S. aureus*, *mad*⁻ plasmid deletions were also found to be independent of the host *rec* function. This independence was demonstrated in an experiment in which PII_{147} *penI300 cadA114* was transduced into strain RN1030, a recombination-deficient mutant similar to the *recA* mutants of *E. coli* (L. Wyman, Ph.D. thesis, New York Univ. School of Medicine, New York, N.Y., 1971), and unable to support normal plasmid recombination. As shown in Table 3, the frequency of *mad*⁻ *pen*⁻ deletions was essentially the same in the *rec*⁺ and *rec*⁻ hosts.

Physiology of mad resistance. As the increase in cadmium resistance is detectable on agar but not in liquid medium, it was possible that a contaminant in agar, such as another metal ion, was responsible for the difference. Chromous and ferric ions are present at concentrations of 10^{-4} to 10^{-5} M in 1.5% Difco agar medium (3). In preliminary experiments, the usual Difco agar was replaced with ethylenediaminetetraacetate-washed agar or Oxoid Ionagar No. 2, and Fe^{3+}, Cr^{3+}, Mg^{2+}, and Mn^{2+} were added separately or together at 10^{-4} M concentrations. The resistance differential between parent (strain RN966) and *mad*⁻ deletions was maintained in all cases, although all strains were more sensitive to Cd^{2+} in the absence of other ions. Thus, although the function of the *mad* locus remains unclear, possibilities include involvement in general ion transport or in oxygen utilization.

DISCUSSION

The results presented in this paper suggest that there are at least two distinct patterns of plasmid-linked cadmium resistance in *S. aureus*. One of these is characterized by a single *cad* locus responsible for the entire difference in cadmium resistance between plasmid-positive and -negative strains. In the one plasmid showing this pattern that has been mapped,

PI_{258} (Novick and Brodsky, *unpublished data*), the *cadA* locus is between *mer* and *pen* and is closely linked to *bis* and *lea* (see Fig. 2 and also reference 14). This linkage seems to have at least some functional significance since $cadA^-$ point mutations often result in decreased resistance to lead or mercury or both (16). A second *mcrI* plasmid, PI_{524}, shows the same pattern of cadmium resistance but has not yet been mapped. The other pattern, that shown by PII_{147} (see Fig. 4), is characterized by two distinct *cad* loci, *cadA* and *cadB*. Again, *cadA* is responsible for the CadR phenotype, whereas *cadB* is phenotypically demonstrable only in the absence of *cadA* function and, moreover, does not appear to act additively with *cadA*. As with PI_{258}, the *cadA* locus of PII_{147} is between *mer* and *pen*, but unlike the situation with PI_{258} (16), *cadA* point mutations of PII_{147} have not so far been found to affect *mer* or *lea*. The *cadB* locus seems to be located a considerable distance from *cadA* and, moreover, is linked to *bis* and *lea*.

Since on PI_{258} it is *cadA* that is closely linked to *bis* and *lea*, whereas on PII_{147} it is *cadB*, it seems reasonable to suppose that the dual representation of cadmium resistance on PII_{147} is the result of a duplication and that the relatively low level of resistance determined by *cadB* is the result of random genetic drift. Alternatively, *cadB* may have some other function, such as resistance to another ion, and may be involved only incidentally in cadmium resistance. Examination of $cadA^-cadB^+$ strains for other resistances might be helpful here, as might a study of genetic homology between *cadA* and *cadB*. The linkage of *cadB* to *bis* and *lea* is consistent with the former alternative, as is our finding that the PII_{147} molecule is about 2×10^6 daltons larger than the PI_{258} (21) which evidently does not have the duplication. A key remaining question in this analysis is whether *cadA* is associated with a second set of *lea* and *bis* loci. Thus far, there is no evidence bearing on this question, since no mutations or deletions affecting *cadB* but not *cadA* have been isolated. Evidently the *mer* marker does not share in the apparent *cad* duplication, since point mutations to full mercury sensitivity have been readily isolated for PII_{147} and they occur approximately as frequently as for PI_{258} (R. Novick, *unpublished data*).

As has been described, all of some 30 penicillinase plasmids so far examined (18) belong to either of two incompatibility sets, I and II (10). In addition to their determinant of incompatibility specificity, members of set II differ from those of set I in the way they affect the resistance of the host organism to bismuth: the former make the host more sensitive to bismuth ions, whereas most of the latter make it more resistant.

Whether or not *bis* and *lea* are involved in the *cad* duplication, the latter may turn out to be yet another genotypic distinction between PI and PII plasmids. A definite conclusion on this possibility must await the testing of more naturally occurring plasmids for this duplication.

One aspect of the comparative genetic analysis of members of the two plasmid incompatibility sets has been the hope that it would be possible to reconstruct the evolutionary pathway(s) leading to the extant species and thus to arrive at some notion of plasmid origin. One hypothesis in this connection is that, since all of the penicillinase plasmids so far tested recombine quite readily with one another, all are descended from a common ancestor. If this is so, the most important evolutionary divergence among the penicillinase plasmids would be the development of incompatibility classes. Thus, in attempting to reconstruct a possible evolutionary pathway, one would start by considering characters that are correlated with incompatibility class. As has been shown previously, there is such a correlation involving response to bismuth: all of 8 *mcrII* plasmids (but none of 22 *mcrI* plasmids) determine epistatic bismuth sensitivity (18). Therefore, the apparent evolutionary divergence involving bismuth and that involving incompatibility are not separable in relative time. However, among the *mcrI* plasmids, some confer bismuth resistance and others have no effect on the host's bismuth sensitivity. This pattern would represent an evolutionary divergence that occurred *later* than that of the incompatibility classes. Cadmium resistance patterns may also be useful in this connection since, in a very small sample of three plasmids, they are correlated with incompatibility class.

We have provided also in this paper evidence for the existence of a third plasmid locus affecting cadmium resistance, *mad*, which is separate from the other two. Deletion of this locus (and presumably point mutation also) leads to slightly increased resistance to cadmium and so has provided a useful tool for genetic dissection of the plasmid. This dissection has so far yielded the plasmid map shown in Fig. 4 and promises to yield a good deal more in respect to plasmid fine structure and

the control of plasmid replication and function.

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