Plasmids in *Corynebacterium diphtheriae* and Diphtheroids Mediating Erythromycin Resistance

JOHN SCHILLER,¹ NEAL GROMAN,^{1*} AND MARIE COYLE²

Departments of Microbiology and Immunology¹ and Laboratory Medicine,² School of Medicine, University of Washington, Seattle, Washington 98195

Plasmids were isolated from erythromycin-resistant *Corynebacterium diphtheriae* and skin coryneforms. Six erythromycin-resistant *C. diphtheriae* strains, isolated from cutaneous lesions, all contained a 9.5-megadalton (Mdal) plasmid. Loss of resistance was associated with the deletion of a 1-Mdal segment from the plasmid or, less frequently, with loss of the plasmid. Two erythromycin-resistant diphtheroids were isolated from similar skin lesions. One contained a 38-Mdal plasmid that was lost in the conversion to erythromycin susceptibility. The other diphtheroid contained a 30- and a 14-Mdal plasmid. Erythromycin-susceptible derivatives of this strain were not recovered. Restriction enzyme analysis indicated that the 9.5-Mdal plasmids in the *C. diphtheriae* strains are very similar, if not identical, and that each of the deleted plasmids has lost the same 1-Mdal segment. However, the restriction patterns of the plasmids in the two diphtheroids are not closely related to each other nor to the plasmids in the *C. diphtheriae* strains.

Two reports have established the presence of plasmids in plant coryneform bacteria (6, 13), but their presence has not been reported in *Corynebacterium diphtheriae* or in coryneforms associated with humans or animals. Recently, erythromycin-resistant strains of *C. diphtheriae* and other diphtheroids were isolated from skin lesions of Seattle patients. In the *C. diphtheriae* strains examined, sub-inhibitory concentrations of erythromycin induced resistance to erythromycin and to the chemically unrelated antibiotics clindamycin and veramycin B α (3). This suggests that the mechanism of resistance may be the dissociated or MLS type of resistance expressed by most clinical isolates of *Staphylococcus aureus* and *Streptococcus pyogenes* (17). Since inducible resistance to these antibiotics is known to be plasmid mediated in streptococci and staphylococci (5), we were led to investigate the Seattle isolates for plasmids. The isolation of erythromycin-resistant *C. diphtheriae* was reported once before (8), but no effort was made to determine whether the gene for resistance was carried extrachromosomally. As will be shown, the Seattle strains carried plasmids; in most cases it was demonstrated that the erythromycin resistance was plasmid mediated.

MATERIALS AND METHODS

Bacterial strains and plasmids. Eight erythromycin-resistant strains, six identified as *C. diphtheriae* and two as non-*C. diphtheriae* coryneforms (diphtheroids), were clinical isolates. Strain and plasmid designations for these isolates and for erythromycin-sus-

ceptible derivatives are given in Table 1. The *C. diphtheriae* isolates gave the same reactions as a standard strain to a set of diagnostic tests. All exhibited the coryneform morphology microscopically, produced mitis-like colonies, fermented glucose and maltose but not lactose or sucrose, were nitrate positive and urease negative, produced a halo on Tinsdale medium, and were nontoxigenic. The two diphtheroid strains deviated from this pattern. Strain S615 differed in lacking the ability to reduce nitrate, produce halos on Tinsdale medium, or ferment maltose. Strain S619 differed in being urease positive and producing gold colonies on heart infusion broth-Tween 80 agar. All the strains were susceptible to penicillin, ampicillin, cephalothin, tetracycline, chloramphenicol, and kanamycin. In addition to these strains, 22 other *C. diphtheriae* strains were taken from the stock collection of one of us (N.G.) and examined for plasmids.

Plasmids LT2 cryptic (7), RP4 (12), Sa (12), ColE1 (1), and pMB8::Tn3 were supplied by S. Falkow. Their molecular weights are ($\times 10^6$): 60, 37, 23, 4.2, and 5.0, respectively.

Cell growth. All cultures were grown aerobically at 37°C either in heart infusion broth (Difco Laboratories, Detroit, Mich.)–0.2% Tween 80 (Sigma Chemical Co., St. Louis, Mo.) (HITW) or on HITW agar (1%). When necessary, erythromycin (The Upjohn Co., Kalamazoo, Mich.) was added to HITW to a final concentration of 1 μ g/ml to give HITW-Em medium.

Minimal inhibitory concentrations of antibiotics. Agar dilution minimal inhibitory concentrations were done by a standardized method (16).

Isolation of erythromycin-susceptible derivatives. An inoculum from an isolated colony of an erythromycin-resistant strain, grown on HITW-Em agar, was transferred to a tube of HITW broth and grown to late exponential phase. In some experiments

TABLE 1. *Bacterial strains and plasmids*

Strain	Description	Phenotype ^a	Plasmid	Mol wt (× 10 ⁶)
S600	<i>C. diphtheriae</i>	Em ^r	pNG1	9.5
S601	<i>C. diphtheriae</i>	Em ^r	pNG2	9.5
S601-1	Derivative of S601	Em ^s	pNG3	8.5
S601-12	Derivative of S601	Em ^s	— ^b	—
S601-16	Derivative of S601	Em ^s	pNG4	8.5
S601-17	Derivative of S601	Em ^s	pNG5	8.5
S605	<i>C. diphtheriae</i>	Em ^r	pNG6	9.5
S605-1	Derivative of S605	Em ^s	pNG7	8.5
S606	<i>C. diphtheriae</i>	Em ^r	pNG8	9.5
S615	Diphtheroid	Em ^r	pNG9	38
S615-1	Derivative of S615	Em ^s	—	—
S615-2	Derivative of S615	Em ^s	—	—
S619	Diphtheroid	Em ^r	pNG10	30
			pNG11	14
S624	<i>C. diphtheriae</i>	Em ^r	pNG12	9.5
S625	<i>C. diphtheriae</i>	Em ^r	pNG13	9.5
S625-1	Derivative of S625	Em ^s	pNG14	9.5
S625-2	Derivative of S625	Em ^s	pNG15	8.5

^a Em^r = Erythromycin resistant; Em^s = erythromycin susceptible.

^b —, None.

10⁻⁶ M ethidium bromide (EtBr) was added to the HITW broth in an attempt to increase the rate of plasmid curing. This concentration was used because it inhibited growth. Cultures were diluted and plated for isolated colonies on HITW agar. After 36 to 48 h of incubation, the colonies were tested for resistance to erythromycin by stabbing each with a sterile toothpick and then stabbing the toothpick into plates of HITW-Em and HITW successively. Presumptive erythromycin-susceptible isolates grew on HITW but not on HITW-Em. The erythromycin susceptibility of the colonies was confirmed by their inability to produce visible growth in 48 h when streaked on HITW-Em agar.

Buffers. TE buffer consisted of 50 mM tris(hydroxymethyl)aminomethane (Tris) and 50 mM disodium ethylenediaminetetraacetate (EDTA) (pH 8.0). TES buffer contained 50 mM Tris, 5 mM disodium EDTA, and 50 mM NaCl (pH 8.0). TB buffer consisted of 89 mM Tris, 2.5 mM disodium EDTA, and 89 mM boric acid. TENA buffer contained 40 mM Tris, 2 mM disodium EDTA, 18 mM NaCl, and 20 mM sodium acetate (pH 8.2). RNase buffer contained 0.25 M Tris, 0.5 M disodium EDTA, and 1 M NaCl.

Cell lysis. The procedure employed for cell lysis is an adaptation of that described by Murai et al. (13) for lysing *Corynebacterium fascians*, a plant pathogen. It is based on the finding by Chassey (2) that certain species of oral streptococci, normally resistant to lysozyme, can be made susceptible if they are first grown under conditions that weaken the cell wall and are then suspended in a low-salt buffer.

Cells grown overnight on HITW agar were inoculated into HITW broth and grown with aeration to midexponential phase, approximately 5×10^8 cells per ml. Potassium penicillin G (Pfizer Inc., New York, N.Y.) was added to a final concentration of 1 µg/ml, and the culture was incubated for 2 more h. Cells were harvested by centrifugation and washed in 10 mM Tris (pH 8.2) in a volume not less than one-half of the volume of the original suspension. The pellet was

suspended in 10 mM Tris (pH 8.2) containing 0.5 M sucrose and 5 mg of lysozyme per ml (hen egg white; Calbiochem, La Jolla, Calif.) in not less than 0.03 volumes of the original suspension. The culture was incubated at 37°C with shaking for 1 h. The cells were harvested by centrifugation, and the pellet was suspended in 10 mM Tris (pH 8.0) containing 10 mM disodium EDTA in a volume equal to 0.9 volume of the lysozyme-containing suspension. Sodium dodecyl sulfate (10% in TE buffer) was added to give a final concentration of 1%, and the contents were mixed by gentle inversion and rotation. The culture was heated at 55°C for approximately 15 min with intermittent mixing by gentle inversion and rotation, during which time lysis occurred. When the strains were screened for plasmids, 15 ml of broth culture was routinely employed in the above procedure.

Maximum lysis, i.e., a drop of over 90% in the culture's absorbance, was obtained only when the cells were grown in penicillin and then treated with lysozyme. When growth in penicillin was omitted, there was still considerable lysis. When treatment with lysozyme was omitted, there was little lysis, and when both were omitted, there was none. Penicillin treatment was retained both for the additional lysis and the fact that cells grown in penicillin were easier to suspend. Incubation was carried out at 55°C since this appeared to increase the lysis of concentrated cell suspensions.

DNA extraction. To clear the lysed culture of cell debris and membrane-chromosome complexes, the lysate was centrifuged in a Sorvall SS34 rotor at 15,000 rpm at 4°C for 30 min. The supernatant was carefully decanted and extracted at least twice with cold phenol equilibrated with 10 mM Tris-10 mM disodium EDTA (pH 8.0) and twice with chloroform-isoamyl alcohol (24:1). The nucleic acid was precipitated by adding 2 M sodium acetate (pH 5.5) to a concentration of 0.4 M, adding 0.6 volume of isopropanol and incubating at -20°C for at least 30 min. After redissolving the precipitate in a minimum amount of distilled wa-

ter, 10 μ l of RNase buffer and 10 μ l of 5-mg/ml bovine pancreas ribonuclease (Calbiochem) in 50 mM sodium acetate (pH 4.8) were added per ml of solution. After incubation at 60°C for 1 h, the RNase was removed by extraction with chloroform:isoamyl alcohol.

The deoxyribonucleic acid (DNA) at this point, designated cleared lysate DNA, contained chromosomal fragments, as well as plasmid DNA. Some of the plasmid molecules were in the covalently closed circular (CCC) form, but open circular (OC) and linear molecules were also present. This was determined by isolating the CCC plasmid DNA by centrifugation of the cleared lysate DNA in a CsCl-EtBr density gradient and comparing the electrophoretic patterns produced by the cleared lysate DNA and the gradient-purified CCC plasmid DNA. The gradient-purified plasmid DNA produced a single band on the gel; the corresponding cleared lysate DNA produced a relatively large chromosomal band, a plasmid band with the same mobility as the band produced by the gradient-purified plasmid DNA, and one or two additional slower-migrating bands. Whether one or two additional bands appeared depended on the size of the plasmid and the electrophoretic conditions. After prolonged storage, the gradient-purified plasmid preparations produced the fast-migrating band but also bands of the same mobility as the slower-migrating bands seen in the pattern produced by the cleared lysate DNA. This indicates that the band of greater mobility corresponds to the CCC form of the cleared lysate DNA and that some of the CCC molecules were converted to the OC and linear forms during isolation and produced the slower-migrating plasmid bands seen on the gel.

Agarose gel electrophoresis. Agarose gel electrophoresis of plasmid DNA, visualization of the bands by staining with EtBr, and viewing under ultraviolet light were done by the method of Meyers et al. (12). For the initial screening of strains for plasmids, the cleared lysate DNA was run on 0.7% agarose vertical slab gels in TB buffer at 100 V for 2.5 h. To increase the separation of plasmids of similar molecular weight, 0.5% agarose horizontal gels were used (11). These gels were run at 35 V for 18 to 24 h in TENA buffer with the buffer recirculated continuously.

CsCl-EtBr density gradient centrifugation. To obtain purified CCC plasmid DNA, the DNA extracted from the cleared lysate of 1 to 2 liters of cells was centrifuged to equilibrium in a CsCl-EtBr density gradient (4). The gradient was centrifuged in a Spinco type 40 rotor at 35,000 rpm at 15°C for 48 h. The plasmid band was removed from the gradient with a syringe, and the EtBr was extracted with isopropanol equilibrated with CsCl-saturated TES. The plasmid DNA was then dialyzed at 4°C against five changes of 10 mM Tris-0.5 mM disodium EDTA (pH 8.0).

Electrophoretic elution of plasmid DNA from agarose gel. The electroelution of plasmid DNA from slices cut from agarose gels was used as an alternate method of separating plasmid from chromosomal DNA. The technique of McDonnell et al. (11) was used with the following modification. Plasmid bands, cut from 0.7% agarose vertical slab gels of cleared lysate DNA, were suspended in TB buffer and eluted at 50 V for 4 h.

Restriction enzyme analysis. Restriction endonucleases *Bst*EII, *Eco*RI, *Hinc*II, and *Hind*III were purchased from Bethesda Research Laboratories, and reactions were performed according to the manufacturer's instructions. Plasmid DNA isolated by CsCl-EtBr gradient centrifugation or electroelution from agarose gel was digested. Digestion patterns were characterized by electrophoresis in 0.7 or 1.2% agarose or 4% bis-acrylamide gels, depending upon the size of the fragments generated. Smaller fragments were resolved more clearly in 4% bis-acrylamide gels.

Plasmid molecular weight determination. Plasmid molecular weights were determined by gel electrophoresis and by electron microscopy. In the first procedure, molecular weights were calculated from the relative mobility of the CCC forms of the coryneform and standard plasmids when subjected to electrophoresis in agarose gel (12). A plot of the logarithm of the mobility of the standard plasmids versus the logarithm of their molecular weight generated a linear curve from which the molecular weights of the diphtheroid and *C. diphtheriae* plasmids were determined. In the second procedure, plasmid DNA, isolated by CsCl-EtBr gradient centrifugation, was spread by the Kleinschmidt technique (9) and examined by electron microscopy. The plasmid pMB8::Tn3 (molecular weight, 5.0×10^6) was included on the grids as a molecular weight standard. Although plasmid DNA from the CsCl-EtBr gradients was predominantly in the CCC form when first isolated, prolonged storage at 4°C converted much of it to the OC form. The OC molecules were photographed, the negatives were projected through a photographic enlarger, and the molecules were traced. Contour lengths were measured with an electronic graphics calculator (Numonics Corp., North Wales, Pa.).

RESULTS

When cleared lysate DNAs from the six *C. diphtheriae* strains were subjected to agarose gel electrophoresis, all six produced the same pattern. Three plasmid bands and a band of chromosomal fragments were visible. The patterns of three of these strains (S601, S605, S625) are shown in Fig. 1, lanes A-C. CCC plasmid DNA from the same strains, isolated by CsCl-EtBr density gradient centrifugation, produced only a single band when subjected to electrophoresis. This band had the same mobility as the fastest-migrating plasmid band of the cleared lysate DNA. From this, and the appearance of the other two bands after storing the density gradient preparation, it was concluded that the three plasmid bands seen in each sample correspond to the three conformational forms of a single plasmid, with the CCC molecules migrating faster than the OC and linear molecules.

Screening of cleared lysates from the two diphtheroid strains revealed that they had plasmids, all of which were larger than those found in the *C. diphtheriae* strains. After isolation of the CCC plasmid DNA, it was determined that the diphtheroid strain S615 had one plasmid and

FIG. 1. Plasmids from *Em*^r *C. diphtheriae* and *Em*^s derivatives. A 35- μ l amount of DNA plus 5 μ l of bromophenol blue dye solution (10) were loaded into wells (6.5 by 1 mm) of a 0.5% agarose horizontal slab gel, dimensions 24.4 by 16.7 by 0.7 cm, and run in TENA buffer at 60 V for 18 h. Migration was from top to bottom. In lanes A through G, the large diffuse band at the top is chromosomal DNA, the two middle bands are the linear and OC forms of the plasmid, and the bottom band is the CCC form of the plasmid. In lane H, only the chromosomal band is present. (A) pNG13 (S625, *Em*^r *C. diphtheriae*); (B) pNG6 (S605, *Em*^r *C. diphtheriae*); (C) pNG2 (S601, *Em*^r *C. diphtheriae*); (D) pNG3 (S601-1, *Em*^s *C. diphtheriae*); (E) pNG4 (S601-16, *Em*^s *C. diphtheriae*); (F) pNG7 (S605-1, *Em*^s *C. diphtheriae*); (G) pNG15 (S625-2, *Em*^s *C. diphtheriae*); (H) S601-12, *Em*^s *C. diphtheriae*.

strain S619 had two. The patterns produced by clear lysates of these strains are shown in Fig. 2, lanes D and E. Distinct bands corresponding to the OC and linear molecules are not seen on this gel because they either are obscured by the chromosomal band or comigrate with the CCC form. Twenty-two *C. diphtheriae* strains, including some older and some recent isolates from various parts of the world, were also screened for plasmids, and none was found.

Isolation of erythromycin-susceptible derivatives. To determine the relationship be-

FIG. 2. Molecular weight determinations of *C. diphtheriae* and diphtheroid plasmids. A 20- μ l amount of DNA plus 5 μ l of bromophenol blue dye solution were loaded in wells (6.5 by 2 mm) of a 0.7% agarose vertical slab gel, dimensions 14.2 by 9.8 by 0.2 cm, and run in TENA buffer at 70 V for 2 h. Migration was from top to bottom. The large diffuse band between the two bars seen in each lane is chromosomal DNA. The bright SA plasmid band (third from the top in lane C) is at the upper limits of the chromosomal band. (A) pNG3 (S601-1, *Em*^s *C. diphtheriae*); (B) pNG2 (S601, *Em*^r *C. diphtheriae*). In lanes A and B the OC form of the plasmid is immediately below the chromosomal band, and the CCC form is well below the band. (C) CCC molecular weight standards from top to bottom: LT2 cryptic, 60×10^6 ; RP4, 36×10^6 ; Sa, 23×10^6 ; ColE1, 4.2×10^6 ; (D) pNG9 (S615, *Em*^r diphtheroid); (E) pNG10, pNG11 (S619, *Em*^r diphtheroid). In lane D the CCC plasmid band is above the chromosomal band, and in lane E one CCC plasmid band is above and one is below the chromosomal band.

tween plasmid content and erythromycin resistance, erythromycin-susceptible derivatives were sought from three of the six *C. diphtheriae* strains and from the two diphtheroids. The results (Table 2) show that susceptible derivatives were isolated from all three *C. diphtheriae* strains (S601, S605, S625) and from diphtheroid strain S615. None was isolated from diphtheroid strain S619. In addition to screening the latter strain by the standard procedure, 1,000 colonies were each unsuccessfully screened after growth with EtBr and after eight consecutive passages in broth.

The frequency of isolation of susceptible derivatives appeared to be similar for the three *C. diphtheriae* strains and was not significantly

TABLE 2. Isolation of spontaneous erythromycin-susceptible strains

Parental strain ^a	Colonies tested ^b	No. of independent isolates	Em ⁺ strain designation
S601	5/2,576	3	S601-12 S601-16 S601-17
S605	1/960	1	S605-1
S625	2/1,924	2	S625-1 S625-2
S615	22/208	2	S615-1 S615-2
S619	0/1,000	0	—

^a Strains S601, S605, and S625 are *C. diphtheriae*, and S615 and S619 are diphtheroids.

^b No. of erythromycin-susceptible (Em⁺) colonies/total number tested.

different in other experiments in which EtBr was added to the growth medium. The frequency of isolation of antibiotic-susceptible strains from diphtheroid strain S615 was at least 10 to 20 times greater, e.g., in one experiment almost 50% of the colonies were erythromycin susceptible after only two successive passages in broth.

Correlation of plasmids and erythromycin resistance. The plasmid content of a number of erythromycin-susceptible derivatives of the *C. diphtheriae* strains was examined. Representative results are shown in Fig. 1. Five independent isolates of *C. diphtheriae* strain S601 were examined, of which three are identified in Table 2 and two were isolated in experiments in which EtBr was present. One strain was completely cured of its plasmid (lane H). In the other four, two of which are shown in lanes D and E, plasmids were still present. However, all three forms of the plasmid in each of these strains migrated more rapidly than the corresponding forms of the parental plasmid. The change in mobility was the same for each of the four strains. The constancy of this difference in mobility between parental and derived plasmids was demonstrated by examining replicate clones, seven from one and four from another, from two of the independent S601 derivatives. All contained a plasmid with the same increased mobility. The erythromycin-susceptible derivative of *C. diphtheriae* S605 contained a similarly altered plasmid (lane F) as did one of the derivatives from *C. diphtheriae* strain S625 (lane G). The other susceptible derivative of S625 contained a plasmid that had the same mobility as the parental plasmid common to all of the orig-

inal erythromycin-resistant *C. diphtheriae* isolates. So, with the exception of one isolate, loss of resistance was accompanied by loss of either the entire plasmid or a fragment thereof. The fragment lost was a constant size as determined by electrophoretic mobility. When the susceptibilities of a plasmidless (S601-12) and plasmid-containing (S601-1) derivative were tested, both proved to be susceptible to erythromycin, as well as clindamycin, with minimal inhibitory concentrations of 0.015 μ g of erythromycin per ml and 0.07 μ g of clindamycin per ml. We conclude that the MLS type of erythromycin resistance is plasmid mediated in the three *C. diphtheriae* strains examined and probably in all six clinical isolates of *C. diphtheriae*. It is likely that a point mutation occurred in the one susceptible derivative of *C. diphtheriae* S625 in which there was no apparent change in plasmid content. Further evidence supporting this conclusion is given below in an examination of restriction endonuclease digest patterns.

The plasmid contents of three independently isolated erythromycin-susceptible derivatives of diphtheroid strain S615 were also examined. Two of the strains are identified in Table 2. Plasmids were completely eliminated in all three derived strains, including seven replicate clones of one, four from the second, and three from the third. As a control on the correlation of plasmid loss with acquisition of susceptibility, some of the resistant colonies from the same plates yielding susceptible isolates were examined. As expected, they retained the parental plasmid. We conclude that erythromycin resistance in this diphtheroid is also plasmid mediated.

Molecular weights of plasmids. The molecular weights of plasmids from a representative erythromycin-resistant and a derived, susceptible strain of *C. diphtheriae* and of plasmids present in the two resistant diphtheroid strains were determined by their mobility relative to a set of standard plasmids (Fig. 2). The calculated molecular weights were 9.4×10^6 and 8.4×10^6 for the plasmids from the resistant (lane B) and derived, susceptible (lane A) *C. diphtheriae*, respectively; they were 38×10^6 for the single plasmid in diphtheroid S615 (lane D) and 14×10^6 and 30×10^6 for the two plasmids in diphtheroid strain S619 (lane E).

The molecular weights of plasmids from one resistant and one derived, susceptible *C. diphtheriae* strain were also determined by contour length measurement. Plasmid DNA together with a standard was spread by the Kleinschmidt technique and photographed in the electron microscope. An example of an OC molecule of the parental plasmid and of the standard is given in Fig. 3. The mean contour length of 20 OC mol-

FIG. 3. *C. diphtheriae* plasmid. The larger molecule is an OC molecule of the *C. diphtheriae* plasmid pNG2 (molecular weight, 9.5×10^6) and the smaller molecule is the standard pMB8::Tn3 (molecular weight, 5.0×10^6) spread by the Kleinschmidt procedure.

ecules of each of the *C. diphtheriae* plasmids, compared with the mean contour length of 30 OC molecules of the standard, yielded molecular weights of $9.5 \pm 0.3 \times 10^6$ and $8.5 \pm 0.2 \times 10^6$ for the plasmids from the resistant and derived, susceptible strains. These values compare well with those obtained by relative mobility measurements and indicate that the plasmids with increased mobility have lost a 1-megadalton (Mdal) fragment.

Restriction endonuclease digest patterns. The banding patterns for a number of plasmids were determined after digestion with each of four restriction enzymes, *Bst*EII, *Eco*RI, *Hinc*II, and *Hind*III. The patterns generated by *Bst*EII are shown in Fig. 4. The pattern for plasmids from three representative erythromycin-resistant *C. diphtheriae* strains (lanes C-E)

were indistinguishable as were their patterns after digestion with the other three enzymes (not shown). The results show that these plasmids are closely related. The *Bst*EII patterns of plasmids from three independently isolated susceptible derivatives of S601 (one of which is shown in lane G) were the same, and three of the bands corresponded to those from the resistant plasmid (compare lanes E and G). However, at least one band was different in each case, and one band was absent. Digestion with the other three enzymes gave similar results. The same relationship between parental resistant and derived, susceptible plasmid patterns was observed for *C. diphtheriae* strain S605 (compare lanes D and F) and strain S625 and one of its derived, susceptible strains S625-2. The similarities in these patterns, coupled with the molecular

FIG. 4. Restriction endonuclease *BstEII* cleaved products of diphtheroid and *C. diphtheriae* plasmids. A 20- μ l amount of DNA cleaved by *BstEII* plus 5 μ l of bromophenol blue dye solution were loaded in wells (6.5 by 1.0 mm) of a 4% bis-acrylamide vertical slab gel, dimensions 14.2 by 9.8 by 0.1 cm, and run in TB buffer at 100 V for 4 h. Migration was from top to bottom. (A) pNG10 and pNG11 (S619, *Em*^r diphtheroid); (B) pNG9 (S615, *Em*^r diphtheroid); (C) pNG13 (S625, *Em*^r *C. diphtheriae*); (D) pNG6 (S605, *Em*^r *C. diphtheriae*); (E) pNG2 (S601, *Em*^r *C. diphtheriae*); (F) pNG7 (S605-1, *Em*^r *C. diphtheriae*); (G) pNG3 (S601-1, *Em*^r *C. diphtheriae*).

weight determinations, indicate that in all cases the plasmids from derived, susceptible strains were formed by deletion of the same 1-Mdal fragment from the parental plasmids.

The only plasmid in a derived, susceptible strain for which an altered pattern could not be demonstrated was from the *C. diphtheriae* S625-1 isolate. This was not only true for digests by the four enzymes already described, but also for restriction enzyme *HhaI*. This enzyme cut the plasmid into more than 20 fragments, but no differences were seen between the parental and derived plasmids. This supports the conclusion given previously that, in this case, a point mutation was probably responsible for the change from erythromycin resistance to susceptibility.

The plasmids of the erythromycin-resistant diphtheroids S615 and S619 were also digested with restriction enzymes, and the patterns generated were compared with those in the erythromycin-resistant strains of *C. diphtheriae*. The DNAs of the two plasmids in S619 were not

separated before digestion so that their banding patterns are superimposed. In the *BstEII* digests (Fig. 4), few of the bands from the S615 plasmid aligned with bands of the *C. diphtheriae* digest (compare lanes B and C), and there was little similarity in the patterns of the two diphtheroids (compare lanes A and B).

DISCUSSION

Plasmids were demonstrated in six erythromycin-resistant *C. diphtheriae* and two erythromycin-resistant diphtheroid strains isolated from skin lesions of patients. These strains were isolated after an outbreak of diphtheria (15) during which erythromycin was used in treating patients. All six *C. diphtheriae* strains contained a 9.5-Mdal plasmid, and when treated with four restriction enzymes, the banding patterns of the plasmids from the three strains examined were identical. It is likely, therefore, that the plasmids in the six strains are at least closely related and more probably are identical. Loss of erythromycin resistance is usually associated with the loss of a specific 1-Mdal segment of the plasmid or more rarely with loss of the entire plasmid. This high correlation between a change in the plasmid content and reversion to erythromycin susceptibility leads to the conclusion that erythromycin resistance is plasmid mediated in these strains.

Deletion of a specific segment of DNA with concomitant loss of antibiotic resistance is characteristic of at least two transposons. Excision of the *S. aureus* transposon Tn551 results in the deletion of a constant 3.5-Mdal segment and loss of erythromycin resistance (14). A deletion of approximately 1 Mdal is often associated with the loss of Tn9-mediated chloramphenicol resistance (10). We have been unable to determine whether erythromycin resistance in these *C. diphtheriae* strains is transposable. The absence of a known system of genetic exchange for this bacterium has limited the approaches we have been able to take. Attempts to transfer resistance to cured derivatives or other strains of *C. diphtheriae* have not been successful.

The two erythromycin-resistant diphtheroids examined in this study also contained plasmids. One contained a single 38-Mdal plasmid. This strain produced erythromycin-susceptible derivatives at a high rate. All of the derivatives examined had lost the plasmid, indicating that the plasmid carries a gene required for erythromycin resistance. The other diphtheroid contained a 30-Mdal plasmid and a 14-Mdal plasmid. Because neither an erythromycin-susceptible nor a plasmidless derivative of this strain was isolated, we do not know whether erythromycin resistance is plasmid mediated in this strain.

The isolation of erythromycin-resistant *C. diphtheriae* is a rare event, and the six strains examined in this study could not be distinguished by colonial morphology, toxigenicity, biochemical tests, or plasmid pattern. Thus, it seems likely that this outbreak of erythromycin-resistant *C. diphtheriae* was due to the dissemination of a resistant strain from a single source. However, the possibility that several similar strains acquired the 9.5-Mdal plasmid independently cannot be ruled out.

The origin of the plasmid in the erythromycin-resistant *C. diphtheriae* strains is open to speculation. It may have been the result of an endogenous plasmid acquiring the gene(s) for erythromycin resistance. If this were the case, one might expect to find a similar plasmid in other strains of *C. diphtheriae*. None was found in 21 other strains examined, including strains isolated during the epidemic that preceded the appearance of the resistant strains. It is also possible that all or part of an erythromycin resistance plasmid from organisms in other species or genera was transferred to *C. diphtheriae*. The two erythromycin-resistant diphtheroids were considered to be possible sources of the plasmid since they were isolated from similar lesions and contained plasmids which were at least as large as the *C. diphtheriae* plasmid. However, a comparison of restriction patterns showed that there was no extensive similarity in the banding pattern between any of them and the *C. diphtheriae* plasmid. This indicates that most of the *C. diphtheriae* plasmid was not recently derived from one of the diphtheroid plasmids. Therefore, it appears that in our isolates at least three distinct elements carry the genes for erythromycin resistance: the 9.5-Mdal plasmid in the *C. diphtheriae* strains, the 38-Mdal plasmid in one of the diphtheroids, and a third undetermined element in the other diphtheroid. The possibility that the *C. diphtheriae* and diphtheroid plasmids contain short regions of homology and therefore carry homologous erythromycin-resistant genes has not been eliminated.

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