ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 1983, p. 892-901
0066-4804/83/120892-10$02.00/0

Vol. 24, No. 6

Relationship Between pNG2, an Em^r Plasmid in *Corynebacterium diphtheriae*, and Plasmids in Aerobic Skin Coryneforms

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Received 15 April 1983/Accepted 12 September 1983

Erythromycin-resistant (Em^r) coryneforms from cutaneous lesions and erythromycin-susceptible (Em^s) coryneforms from normal skin sites were screened for plasmids. Approximately one-third of the 40 isolates carried one or more plasmids ranging in mass from 2.5 to 36 megadaltons, all exhibiting different restriction enzyme digest patterns. In contrast, only *Corynebacterium diphtheriae* strains comprising a single cohort of apparently identical Em^r, pNG2-carrying isolates have been identified as plasmid carriers. Homology was demonstrated between pNG2 and a number of fragments in restriction enzyme digests of plasmids from both Em^r and Em^s skin coryneforms under high-stringency conditions. However, none was detected between pNG2 and the genomic or plasmid DNAs of Em^r staphylococci or streptococci isolated concurrently with the Em^r coryneforms. One coryneform plasmid, pNG34, exhibited extensive homology with pNG2, and many comigrating fragments were observed. Very little relationship was observed between *C. diphtheriae* and the skin coryneforms when their genomic DNAs were hybridized. The origin and presence of pNG2 in Em^r *C. diphtheriae* is discussed in relation to these findings.

The presence of plasmids in isolates of erythromycin-resistant (Em^r) *Corynebacterium diphtheriae* and aerobic skin coryneforms was first reported from this laboratory (15). This successful demonstration in isolates obtained from cutaneous lesions followed failure to demonstrate any plasmids in 21 cultures of *C. diphtheriae* originating in the respiratory tract. Em^r *C. diphtheriae* organisms were recovered from cutaneous lesions between May and October 1978 in Seattle, Wash. During this same period, Em^r aerobic skin coryneforms and numerous Em^r staphylococci and streptococci were also isolated from cutaneous lesions. In some cases members of all three genera were cultured from the same lesion. Additionally it was observed that erythromycin resistance in *C. diphtheriae* was the broad-spectrum MLS type, the same type found in clinical isolates of staphylococci and streptococci (3). Thus, it seemed desirable to determine whether pNG2, the plasmid mediating erythromycin resistance in *C. diphtheriae*, shared any significant homology with genetic elements of the Em^r skin coryneforms, staphylococci, or streptococci. The paucity of plasmids in *C. diphtheriae*, in marked contrast to their presence in the first two isolates of Em^r aerobic coryneforms from cutaneous lesions (15), also suggested that the skin coryneforms might be a significant reservoir of accessory genetic material. To assess this, we have examined isolates of aerobic coryneforms from normal skin for the presence of plasmids.

In the present study, DNA sequences highly homologous to pNG2 were detected in plasmids from Em^r skin coryneforms, but not in the plasmid or genomic DNAs from Em^r staphylococci or streptococci. A high frequency of plasmid carriage was demonstrated in aerobic coryneforms from normal skin sites, and a number of these plasmids also contained sequences homologous to the DNA of pNG2.

MATERIALS AND METHODS

Bacterial strains. Clinical isolates associated with the study of erythromycin resistance were recovered at Harborview Hospital in Seattle, Wash. These isolates were identified by an "S" prefix and a number followed by a letter suffix A, B, or C. Corynebacterial isolates were identified by an "A," the staphylococci were identified by a "B," and the streptococci were identified by a "C" suffix. When different organisms were isolated from the same site or lesion, all were given the same S-number designation. Ten isolates of aerobic coryneforms from normal sites were from the

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anterior nares, and 36 isolates were from various skin sites cultured between 1974 and 1980. They were obtained from D. Nicoll and C. A. Evans, respectively, both of this department. Cultures were selected so that no more than one per subject per site of isolation was examined. In most cases the isolate was the predominant or only aerobic coryneform at that site. One other normal skin coryneform (S1008), sent to us by William Noble (Institute of Dermatology, London, England), was included in this study. Designations of other isolates are given below.

In addition to the aerobic coryneforms described above, 15 cultures of *C. diphtheriae* were screened for plasmids. Ten of these were cutaneous isolates obtained in 1980 and 1981 from Harborview Hospital, and the remaining five, nontoxinogenic clinical isolates cultured within the last 5 years, were obtained from R. Weaver (Centers for Disease Control, Atlanta, Georgia) and were probably respiratory tract isolates.

C. diphtheriae strains C7(−), C7(β), and S601A, the source of plasmids pNG2 and its deleted derivative pNG3 (15), were from our stock collection. The type strains employed were *C. diphtheriae* S1016 (ATCC 19409), *Corynebacterium ulcerans* 690 (ATCC 9015), and *Corynebacterium pseudotuberculosis* (*C. ovis*) S1019 (NCTC 3450). *Streptococcus faecalis* JH2-2(β) was received from D. Clewell (University of Michigan, Ann Arbor), and *Escherichia coli* V517 was supplied by J. Crosa (University of Oregon, School of Medicine, Portland). Susceptible derivatives of antibiotic-resistant coryneforms were recovered as previously described (15) by replica plating after growth in the absence of antibiotics.

All corynebacterial isolates were subjected to a set of tests routinely used in clinical laboratories to identify *Corynebacterium* species (13). These included fermentation of glucose, maltose, lactose, and sucrose, production of H_2S and urease, and reduction of nitrate. The Elek test was used to determine production of diphtheria toxin. Methanethiol production from methionine was also included in testing organisms from normal sites. It is used in detecting members of the genus *Brevibacterium* (16). Antibiotic susceptibility patterns were determined by the Kirby-Bauer method as described previously (2) with cephalothin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, oxacillin, penicillin, tetracycline, and vancomycin.

Cell growth. Isolates were taken from frozen or primary cultures and grown at 37°C on 3% Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) plus 1% yeast extract (TSY) and 0.1% Tween 80 (Sigma Chemical Co., St. Louis, Mo.) (TSY-TW), or on heart infusion (Difco Laboratories, Detroit, Mich.) plus 0.2% Tween 80 agar (HITW). All cultures were grown aerobically, except streptococci, which were grown in capped bottles. The isolates from the anterior nares were taken directly from primary cultures on TSY-TW or mannitol-salt agar. To avoid repeated passages (which could lead to plasmid curing) a heavy suspension of each isolate was stored in 50% glycerol–50% TSY broth at −20°C after revival from frozen storage. Before plasmid extraction, an isolate was streaked out on TSY-TW or HITW agar from the glycerol stock and incubated aerobically at 37°C overnight. Chloramphenicol (100 μg/ml), erythromycin (1 μg/ml), kanamycin (100 μg/ml), potassium penicillin G (1 μg/ml), or tetracycline hydrochloride (10 μg/ml) was added to the medium as required.

Buffers. TE buffer consisted of 10 mM Tris and 10 mM disodium EDTA (pH 8.0). TENA buffer contained 40 mM Tris, 2 mM disodium EDTA, 18 mM NaCl, and 20 mM sodium acetate (pH 8.2). SSC contained 0.15 M NaCl and 0.015 M sodium citrate. Denhardt solution contained 0.02% each of Ficoll 400, polyvinyl pyrrolidone, and bovine serum, 0.1% sodium dodecyl sulfate (SDS), 5 mM disodium EDTA, 20 μg of sheared and denatured calf thymus DNA per ml, and 20 mM Tris (pH 7.6).

Cell lysis and genomic and plasmid DNA extraction. The procedures for cell lysis and extraction of genomic DNA followed those previously described (15). Some changes have been made in plasmid purification after cell lysis. Cells grown overnight on HITW or TSY-TW agar were inoculated in HITW broth and grown with aeration at 37°C to the midexponential phase, approximately 5×10^8 cells per ml. A routine screen involved a 100-ml broth culture, and the subsequent volumes of reagents used corresponded to an initial 100-ml culture. Penicillin was then added to a final concentration of 1 μg/ml, and the cells were reincubated as above for 2 h. More recently we have found that this step can be omitted for practically all strains without affecting the extent of cell lysis. Cells were harvested by centrifugation and washed once with 50 ml of 10 mM Tris (pH 8.0). The cells were again pelleted and suspended in 6 ml of 10 mM Tris (pH 8.0) containing 0.5 M sucrose and 5 mg of lysozyme (hen egg white; Sigma) per ml. The culture was incubated at 37°C for 1 h with shaking, after which cells were harvested by centrifugation. The pellet was suspended in 1.5 ml of 50 mM Tris (pH 8.0) and 0.5 ml of 0.25 M EDTA (pH 8.0) and chilled on ice for 5 min. Lysis was then achieved by adding 0.5 ml of SDS (20% in TE) followed by gentle inversion and rotation. Lysis was enhanced by a 15-s incubation at 55°C followed by five gentle inversions in 15 s to mix. This latter sequence was repeated eight times (8). At this point genomic DNA was extracted from the lysate by standard procedures (15).

To extract plasmid DNA the lysed cells were cleared of cell debris and membrane-chromosomal components and enriched for covalently closed circular (CCC) DNA by the following adaptation of the rapid screening method developed by LeBlanc and Lee (9). DNA in the lysate was denatured by the addition of 0.25 ml of 3 N NaOH (made fresh just before use) and 20 gentle inversions per min for 3 min. As suggested by Currier and Nester (5), the pH at this stage was kept between 12.25 and 12.40. The volume was raised to 3.0 ml with distilled water, and the lysate was quickly neutralized by adding 0.5 ml of 2 M Tris (pH 7.0) and gently inverting 10 times in 30 s. An additional 0.5 ml of 2 M Tris was added, and the lysate was mixed as above. High-molecular-weight chromosomal DNA was removed by SDS-NaCl precipitation (7) by adding 0.65 ml of 20% SDS in TE and 1.25 ml of 5 M NaCl and inverting the tubes 20 times in 1 min. The sample was stored at 4°C overnight or on ice for 1 to 2 h, after which the supernatant was collected after centrifugation at 10,000 rpm, 4°C, for 30 min in a Sorvall SS-34 rotor.

The supernatant volume was doubled with distilled water, and RNA was removed (optional) by adding RNase (Sigma; 2 mg/ml in water, heated at 100°C for

15 min) to a final concentration of 100 μg/ml and incubating for 1 h at 37°C. The remaining single-stranded DNA was removed by extraction with an equal volume of NaCl-saturated phenol (5), followed by extraction of the aqueous phase with an equal volume of chloroform-isoamyl alcohol (24:1). The DNA was precipitated by adding 0.2 volume of 2 M sodium acetate (pH 5.5) and 0.6 volume of ice-cold isopropanol; after mixing, the samples were stored at −20°C overnight. The precipitated DNA was recovered by centrifugation at 3,000 × *g* in the SS-34 rotor at −20°C for 30 min, followed by two washes with cold 95% ethanol. The DNA was dried and suspended in 200 to 300 μl (depending on the amount of precipitate) of 1 mM Tris–1 mM EDTA (pH 8.0) and stored at −20°C until examination by agarose gel electrophoresis. To obtain enough plasmid DNA for cesium chloride-ethidium bromide (CsCl-EtBr) density gradient centrifugation, this procedure was scaled up correspondingly to an initial volume of 800 ml of culture.

The plasmids from *E. coli* were extracted using the methods of Crosa and Falkow (4). Four hundred milliliters of culture was used for this preparation. The cleared lysate DNA (containing mostly plasmid DNA but with some contaminating chromosomal DNA and protein) was purified on CsCl-EtBr density gradients.

CsCl-EtBr density gradient centrifugation. To obtain purified CCC plasmid DNA for accurate determination of molecular weights, the plasmid DNA extracted from the aerobic coryneforms or from *E. coli* V517 was centrifuged to equilibrium in CsCl-EtBr density gradients (5). Gradients were prepared in Quick-Seal polyallomer centrifuge tubes (Beckman Instruments, Inc., Palo Alto, Calif.) and centrifuged in a Beckman VTi80 vertical rotor at 65,000 rpm at 15°C for 4 h. The plasmid band was removed from the gradient with a syringe, and the EtBr was extracted four to five times (one extraction past disappearance of color) with an equal volume of *n*-butanol saturated with aqueous 5 M NaCl containing 10 mM Tris and 1 mM EDTA (pH 8.0) (6). The plasmid DNA, ethanol precipitated at −20°C overnight with 2 volumes of distilled water and 6 volumes of 95% ethanol, was then collected by centrifugation, washed with ethanol, centrifuged, washed, and suspended in TE buffer.

Plasmid molecular weight determinations. Plasmid molecular weights were determined by the relative mobilities of CCC plasmids (14) or restriction fragments of plasmids when subjected to agarose gel electrophoresis (12). The CCC standards employed were a number of coliform plasmids isolated from *E. coli* V517, which contains eight plasmids of 35.8, 4.8, 3.7, 3.4, 2.6, 1.8, and 1.4 megadaltons (Mdal) (10); coliform plasmids LT2, RP4, Sa, and ColE1 of 60, 36, 23, and 4.2 Mdal, respectively; the 9.5-Mdal plasmid from *C. diphtheriae* S-601A; the 30- and 14-Mdal plasmids from the aerobic coryneform S-619A (15). The *Hin*dIII restriction fragments of bacteriophage lambda were used as linear DNA standards. A plot of the mobility of the standards versus the logarithm of their molecular mass generated a linear relationship from which the molecular masses of the plasmids or fragments were determined.

Restriction enzymes. Restriction endonucleases were purchased from Bethesda Research Laboratories, and reactions were performed according to the manufacturer's instructions. Genomic DNA was digested after extraction without additional purification. Plasmid DNA was digested after purification by CsCl-EtBr gradient centrifugation. Difficulties were encountered in digesting the plasmid DNA of the *C. diphtheriae* isolates, but not the plasmid DNA of most of the coryneforms. This problem was overcome by precipitation of the plasmid DNA in 10% polyethylene glycol containing 1 M NaCl either before or after gradient centrifugation,

In vitro labeling of DNA. Genomic and plasmid DNAs were labeled with ^{3}H or ^{32}P by nick translation (18). ^{32}P- and ^{3}H-deoxyribonucleotides and *E. coli* polymerase I were purchased from New England Nuclear Corp. (Boston, Mass.), and bovine pancreas DNase was from Worthington Diagnostics (Freehold, N.J.).

Nitrocellulose filter paper hybridizations. For dot-blot hybridizations, approximately 3 μg of each genomic DNA sample in 10 μl was spotted on a nitrocellulose filter. The DNA was denatured by floating the filter on a solution of 0.5 N NaOH in a plastic petri dish until thoroughly wetted and then neutralized by floating the filter on three changes of 1 M Tris (pH 7.4) containing 1.5 M NaCl. The filter was then processed by the procedure of Thomashow et al. (18) and then washed four times in low-stringency buffer (2× SSC–0.1% SDS–10 mM Tris–5 mM disodium EDTA, pH 7.5) at 68°C for 30 min each, followed by two washes in high-stringency buffer (0.3× SSC–0.015% SDS–1.5 mM Tris–0.75 mM disodium EDTA, pH 7.5) at 68°C for 30 min each. Under these stringent washing conditions, sequences with more than 10% mismatch would not have remained hybridized (11). After two brief rinses in 2× SSC at room temperature, the filter was allowed to dry. It was then mounted between two sheets of cellulose acetate, sandwiched between Lightning Plus intensification screens (Du Pont Co., Wilmington, Del.) with Kodak X-omat AR film, and exposed for 1 to 7 days.

Southern blots were prepared by established procedures (17, 18).

RESULTS

Characterization of Em^{r} *C. diphtheriae* from cutaneous lesions. During a 4-month period in 1978 in Seattle, 9 of 26 *C. diphtheriae* isolated from cutaneous lesions were Em^{r} as determined by the Kirby-Bauer procedure. Since then, only one additional Em^{r} isolate has been found out of a total of 144 *C. diphtheriae* isolates cultured from cutaneous sites. Biochemical tests confirmed their designation as *C. diphtheriae*. All gave the same reactions as C7, the neotype *mitis* strain (1). They fermented glucose and maltose, but not lactose or sucrose, were nitrate positive and urease negative, produced a halo on Tinsdale medium, and exhibited the coryneform morphology. All were non-toxinogenic.

To characterize the Em^{r} isolates with respect to one another, genomic DNA was extracted from all 10, digested with *Bam*HI restriction endonuclease, and subjected to agarose gel electrophoresis. The pattern of DNA fragments was visualized by UV illumination after staining with

EtBr. The banding patterns (data not shown) of all the Em^r isolates and of two Em^s *C. diphtheriae* isolates recovered during the same period were indistinguishable.

Characterization of Em^r aerobic coryneforms from cutaneous lesions. During the same period that Em^r *C. diphtheriae* strains were being isolated, four Em^r skin coryneforms were also isolated from cutaneous lesions as well as a single Em^r throat isolate from a patient with cutaneous lesions. To distinguish these organisms from *C. diphtheriae*, they will be referred to as coryneforms. In some instances Em^r, coagulase-positive staphylococci or group A streptococci or both were isolated from the same lesion as the Em^r coryneforms. In addition, individual isolates of Em^r staphylococci or streptococci were recovered from other cutaneous lesions.

To examine the relationship of the Em^r coryneforms, biochemical tests normally used to characterize *C. diphtheriae* were performed as well as antibiotic susceptibility tests (Table 1). Each isolate gave a unique pattern of tests differing from that of *C. diphtheriae*. All isolates were resistant to both erythromycin and clindamycin. The individuality of the isolates was confirmed by their genomic DNA restriction patterns (Fig. 1). The patterns (Fig. 1, lanes B through F) do not resemble each other or the patterns of other *Corynebacterium* species. It is of interest that the patterns of the two *C. diphtheriae* strains (Fig. 1, lanes A and G) are similar in some regions, particularly in the lower regions of the gel, indicating along with other data we have accumulated that this technique can be quite useful in discriminating between isolates.

Plasmids from Em^r coryneforms. The screen for plasmids in Em^r coryneforms is shown in Fig. 2. Isolates S615A and S651A (Fig. 2, lanes D and F) each had one plasmid, S607A and S619A (Fig. 2, lanes A and E) had two, and the throat isolate S632A had none (data not shown). Each of the plasmids had a different mobility and also differed in mobility from the *C. diphtheriae* plasmid pNG2 (lane B) and its deleted derivative pNG3 (lane C), isolated from an Em^s revertant of S601A (15).

The molecular masses of the plasmids were determined from the electrophoretic mobilities of the whole plasmids (Table 2) and from the mobility of *Bst*EII-generated restriction fragments as well (data not shown). Both methods gave similar results. The plasmids ranged in

TABLE 1. Biochemical reactions of Em^r coryneforms[a]

Strain	Glucose	Maltose	Galactose	Fructose	H_2S	Nitrate	Urease
S601A	+	+	+	+	+	+	−
S607A	+	−	+	+	−	+	−
S615A	+	−	W+	+	−	−	−
S619A	−	−	−	+	+	+	+
S632A	−	−	−	−	+	+	+
S651A	+	+	W+	+	+	−	−

[a] S601 is *C. diphtheriae*, and the remaining isolates are coryneforms. All were cultured from cutaneous lesions, except S632A, which came from the throat of a patient with a cutaneous lesion. All were both erythromycin and clindamycin resistant and lactose and sucrose negative, with the exception of S651A, which was sucrose positive. +, Positive reaction; −, no reaction; W+, weakly positive reaction.

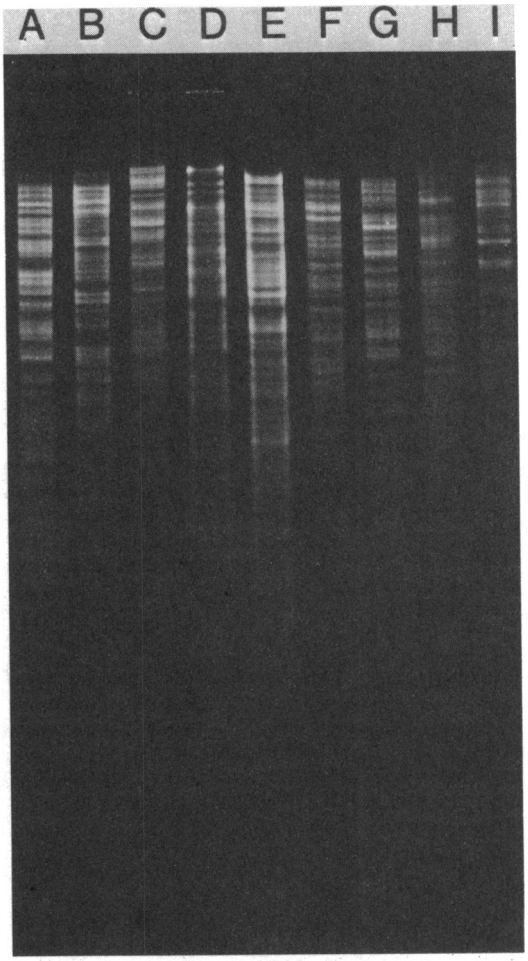

FIG. 1. Comparison of *Bam*HI cleavage products of the genomic DNA from Em^r *C. diphtheriae* and other coryneforms. *Bam*HI-cleaved genomic DNA was loaded onto a 1% agarose vertical slab gel and run in TENA buffer at 25 V for 16 h. Lanes: (A) S601A, Em^r *C. diphtheriae*; (B through F) Em^r coryneforms; (B) S607A; (C) S615A; (D) S619A; (E) S632A; (F) S651A; (G) S1016, *C. diphtheriae*; (H) 690, *C. ulcerans*; (I) S1019, *C. pseudotuberculosis* (*C. ovis*).

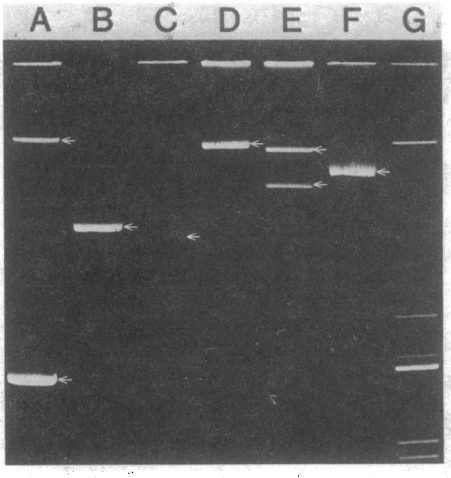

FIG. 2. Plasmids of Em^r *C. diphtheriae* and skin coryneforms. Purified plasmid DNA was loaded onto a 0.7% agarose vertical slab gel and run in TENA buffer at 60 V for 3.5 h. The designation of the isolate carrying the plasmid(s) is given below within parentheses. The *C. diphtheriae* plasmids are in lanes B and C. Lanes A, D, E and F contain plasmids from skin coryneforms. Lanes: (A) pNG17, pNG18 (S607A); (B) pNG2 (S601A); (C) pNG3 (S601A-1); (D) pNG9 (S615A); (E) pNG10, pNG11 (S619A); (F) pNG16 (S651A); (G) plasmids of *E. coli* V517. The more intense bands in the lanes (see arrows) represent CCC forms of the plasmids. Minor bands correspond to open circular, linear, or concatemeric forms of some of the plasmids.

mass from 3 to 36 Mdal, but none was similar in mass to plasmid pNG2 or pNG3.

Hybridization of pNG2 to plasmids from Em^r skin coryneforms. Homology between plasmids from Em^r skin coryneforms and *C. diphtheriae* was assessed by hybridizing ^{32}P-labeled pNG2 to purified plasmid DNA. The autoradiographs revealed (data not shown) that pNG9, pNG10, pNG16, and, to slight extent, pNG17 and pNG18 all hybridized. *Bst*EII digests of these plasmids were then hybridized with labeled pNG2 (Fig. 3). Some fragments of all of the above coryneform plasmids showed homology. The restriction digest patterns of the coryneform plasmids (Fig. 3, lanes C through F) were clearly different from each other and from those of the *C. diphtheriae* plasmids (Fig. 3, lanes A and B). In a very few instances, fragments of the coryneform plasmids appeared to comigrate. The same diversity of pattern was observed after *Hin*dIII digestion. It should be noted that each coryneform plasmid, except pNG18, had a hybridizing fragment with approximately the same molecular mass as fragment D of pNG2. In addition pNG9, pNG10, and pNG17 each had a hybridizing fragment with approximately the same molecular mass as fragment F of pNG2. Both D and F of pNG2 were absent in digests of pNG3 (Fig. 3, lane A), the plasmid from S601A-1, an Em^s derivative of S601A resulting from a 1-Mdal deletion in pNG2 (15).

TABLE 2. Molecular mass of plasmids from Em^r isolates

Isolate no.[a]	Plasmid	Molecular mass[b] (Mdal)
S601A	pNG2	9.6
S615A	pNG9	34.0
S619A	pNG10	30.0
	pNG11	14.6
S651A	pNG16	19.2
S607A	pNG17	36.2
	pNG18	3.2

[a] S601A is *C. diphtheria*, and the remaining isolates are aerobic skin coryneforms.

[b] All values are based on the average of two or three electrophoretic determinations.

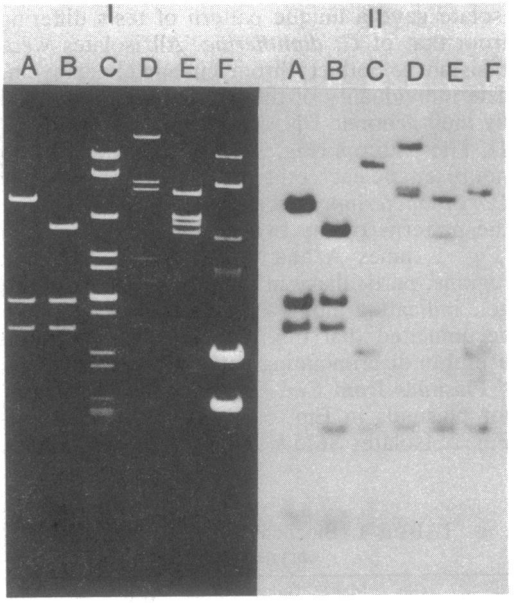

FIG. 3. *Bst*EII cleavage products of plasmids from Em^r skin coryneforms. *Bst*EII-cleaved plasmid DNA was loaded onto a 1.0% agarose vertical slab gel and run in TENA buffer at 25 V for 16 h (panel I). The DNAs were transferred to a nitrocellulose and hybridized to ^{32}P-labeled pNG2 at 68°C for 67 h. The filter was washed under stringent conditions and autoradiographed (panel II). The isolate carrying the plasmid(s) is identified below within parentheses. Strain S619A-1 is an Em^r coryneform derivative of S619A that lost plasmid pNG11. Lanes: (A) pNG3 (Em^s *C. diphtheriae* S-601A-1); (B) pNG2 (Em^r *C. diphtheriae* S601A); (C through F) Em^r skin coryneforms; (C) pNG9 (S615A); (D) pNG10 (S619A-1); (E) pNG16 (S651A); (F) pNG17, pNG18 (S607A). The two brightest fragments in lane F belong to pNG18.

Hybridization of pNG2 to DNA from Em^r staphylococci and streptococci. Em^r β-hemolytic group A streptococci and coagulase-positive or -negative staphylococci or both were often obtained from cutaneous lesions during the period when Em^r *C. diphtheriae* were being isolated. A number of these isolates were examined for plasmids by the rapid screening procedure (Table 3). Plasmids ranging in size from 4.5 to 23 Mdal were recovered from the staphylococcal isolates. All contained at least two plasmids, but none of the plasmids co-migrated with pNG2. Plasmids were not detected in streptococcal isolates. A band that comigrated with the chromosomal band of the pNG2 preparation was cut with *Bam*HI, and the complex digest pattern that resulted showed it was also chromosomal DNA.

The plasmid DNA from coagulase-positive and -negative staphylococci and chromosomal DNA extracted from the streptococcal isolate S605C by the plasmid screening method was separated by gel electrophoresis and blotted to nitrocellulose. When hybridized with ^{32}P-labeled pNG2, all test blots were negative (Table 3). Total genomic DNA of the Em^r streptococci was also probed in dot blots (Fig. 4), and there was no hybridization with pNG2. Similar results were obtained with genomic DNAs from staphylococcal isolates, as well as from *S. faecalis* strain JH2-2, which carries the wide-host-range Em^r conjugative plasmid β (Table 3).

Plasmids in coryneforms from normal sites. Isolates of aerobic coryneforms obtained from normal skin sites and from the anterior nares were examined for plasmids by a rapid screening technique. Approximately one-third of the skin coryneforms (12 of 37) carried one or more plasmids, whereas only 1 of the 10 isolates from the anterior nares did so. The plasmid-bearing isolates and the molecular masses of the plasmids are given in Table 4. Fifteen recent clinical isolates of *C. diphtheriae* were also screened for plasmids. None was found, confirming previous findings (15) that plasmid carriage by this organism was not common.

Biochemically, the aerobic coryneforms from the skin and anterior nares were a very heteroge-

TABLE 3. Homology to pNG2 of plasmid or genomic DNA (or both) from Em^r staphylococci and group A streptococci

Isolate	Coagulase[a]	Plasmids (Mdal)	Homology to pNG2[b]		
			Plasmid DNA	Genomic DNA	
				Dot-blot	Restricted (*Hin*dIII)
Staphylococci					
S601B[c]	+	10.5, 5.3, 4.5		−	−
S604B	+	10.5, 5.3, 4.5		−	
S606B[c]	+	10.5, 5.3, 4.5	−	−	
S607B[c]	+	19, 11, 4.5	−	−	−
S608B	+	10.5, 5.3, 4.5		−	
S638B[c]	+	10.5, 5.3, 4.5	−	−	
S641B$_2$[d]	−	(+)[e]		−	
S642B[c]	+	10.5, 5.3, 4.5		−	
S657B$_2$[d]	+	17.5, 12.5, 4.7	−	−	−
S665B$_1$[d]	+	23, 19	−	−	−
S665B$_2$[d]	−	11.2, 5.3, 5.7	−	−	
S796B$_2$[d]	−	18, 4.5	−	−	
Streptococci					
S601C[c]		ND[f]		−	
S602C		ND		−	−
S603C		ND		−	
S604C		ND		−	
S605C[c]		ND	−[g]	−	
S607C[c]		ND		−	
S623C		ND		−	
S625C[c]		ND		−	

[a] +, Coagulase positive; −, coagulase negative; blank, not tested.
[b] −, No homology to pNG2; blank, not tested.
[c] Isolate came from a cutaneous lesion from which one of the Em^r *C. diphtheriae* or Em^r skin coryneforms was isolated.
[d] Subscript numbers indicate that more than one isolate of the same genus was cultured from the same lesion.
[e] This isolate carried plasmids, but their molecular mass was not determined.
[f] ND, No plasmids detected.
[g] Chromosomal DNA isolated by the plasmid extraction method.

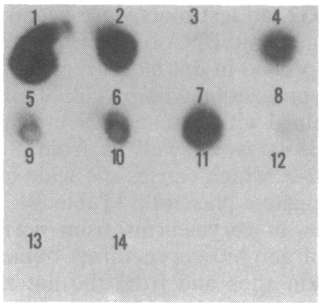

FIG. 4. Hybridization of pNG2 to genomic DNA of the Em^r coryneforms and Em^r streptococci. Genomic DNA, spotted in 10-μl samples on a nitrocellulose filter, was hybridized to ^{32}P-labeled pNG2 at 68°C for 49 h. The filter was washed under stringent conditions and autoradiographed. Dots: (1) S601A (Em^r *C. diphtheriae*), (2 and 4 through 7) Em^r skin coryneforms, (3) Em^s derivative of S615A cured of its plasmid, (2) S615A, (3) S615A-1, (4) S619A, (5) S632A, (6) S651A, (7) S607A, (8 through 14) Em^r group A *Streptococcus*, (8) S601C, (9) S602C, (10) S604C, (11) S605C, (12) S607C, (13) S623C, (14) S625C.

neous group. Twenty different reaction patterns were observed, even with the limited number of tests employed, and the plasmid-bearing isolates alone exhibited eight different patterns. Susceptibility to 10 antibiotics (see above) was determined for all 47 isolates. A correlation between antibiotic resistance and plasmid carriage was observed in two isolates. Tetracycline-penicillin-susceptible derivatives of isolate 77-51 that had lost the 12.75-Mdal plasmid were isolated after four to six passages in broth. Similarly, chloramphenicol-kanamycin-susceptible derivatives of S1008 that had lost plasmid pNG32 were isolated.

Hybridization of pNG2 to plasmids from skin coryneforms. Homology between plasmids from skin coryneforms and pNG2 was assessed. Purified plasmid DNAs were hybridized with ^{32}P-labeled pNG2 under conditions of high stringency and then autoradiographed. The results revealed (data not shown) that 8 of 20 plasmids shared some homology with pNG2, including the plasmid in S1008, the isolate from England. The plasmids sharing homology are identified in Table 4. The *Bst*EII digest patterns of these coryneform plasmids and the extent of their homology to pNG2 were determined (Fig. 5). The digest patterns of the coryneform plasmids were all very different from each other and from pNG2, but many of their fragments hybridized with pNG2 at high stringency. None of the fragments of the normal skin coryneform plasmids hybridizing with pNG2 comigrated with pNG2 fragments D and F. It appears from an examination of the molecular mass of the hybridizing fragments in lane C that, in addition to pNG26, either or possibly both pNG27 and pNG28 exhibit homology with pNG2. Plasmid pNG34 from isolate 80-34 hybridized extremely well with pNG2, all three of its fragments giving an intensity equal to that of the control. In a comparison of the *Hha*II digests of pNG2 and pNG34, which contained more fragments, many of their bands comigrated, but pNG34 also had a number of unique bands.

Relationship of *C. diphtheriae* to the plasmid-carrying coryneforms. Genomic DNA was extracted from all aerobic coryneforms carrying plasmids with homology to pNG2 and digested with *Bam*HI, and the fragments were hybridized with labeled DNA from *C. diphtheriae* type strain S1016. DNA from *C. diphtheriae* S601A, the source of pNG2, and from type strains of *C. ulcerans* and *C. pseudotuberculosis* (*C. ovis*), both capable of producing diphtheria toxin (1), were also included in this test. The results (Fig.

TABLE 4. Plasmids in aerobic skin coryneform bacteria

Isolate no.	Site of isolation[a]	Plasmid no.	Plasmid mol mass[b] (Mdal)	Homology to pNG2[c]
74-801	Forehead	pNG35	32.0	−
		pNG36	27.5	−
77-23[d]	Forehead	pNG25	16.5	+
76-206[d]	Axilla	pNG24	10.05	+
77-51	Forehead	pNG37	18.0	−
		pNG38	12.75	−
77-249	Antecubital fossa	pNG26	18.0	+
		pNG27	7.0	−
		pNG28	2.7	−
77-251	Toe webs	pNG39	18.5	−
77-272[d]	Axilla	pNG40	3.6	−
77-290[d]	Forehead	pNG29	8.2	+
		pNG30	5.3	+
77-292	Forehead	pNG31	17.0	+
		pNG32	6.2	−
		pNG33	2.45	−
77-294[d]	Forehead	pNG41	17.5	−
80-34	Antecubital fossa	pNG34	8.2	+
80-47[d]	Anterior nares	pNG42	20.5	−
S1008	Skin	pNG23	26.0	+

[a] The frequency of plasmid carriage at the various sites was as follows: forehead (6 of 12), axilla (2 of 12), antecubital fossa (2 of 4), toe webs (1 of 8), and anterior nares (1 of 10).

[b] Molecular mass determinations are based on the relative mobility of CsCl-purified CCC plasmid DNA with that of a series of *E. coli* plasmids in agarose gels.

[c] Performed on purified plasmid preparations. There is some suggestion from other data that pNG27 and/or pNG28 may also exhibit some homology (see the text and Fig. 5).

[d] Methanethiol producer.

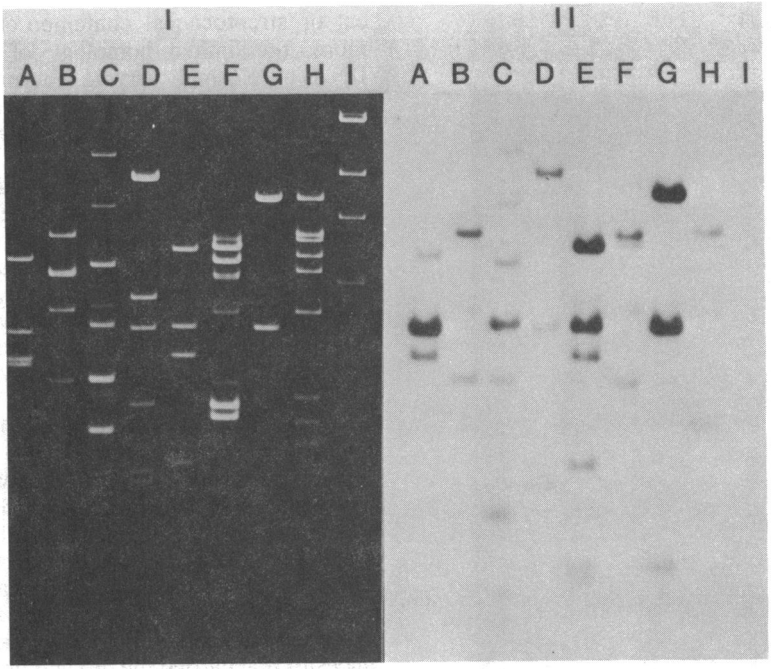

FIG. 5. *Bst*EII cleavage products of coryneform plasmids with homology to pNG2. *Bst*EII-digested plasmid DNA was loaded onto a 1.0% agarose vertical slab gel and run in TENA buffer at 25 V for 16 h (panel I). The DNAs were transferred to nitrocellulose and hybridized to ^{32}P-labeled pNG2 at 68°C for 67 h. The filter was washed under stringent conditions and autoradiographed (panel II). The designation of the isolate carrying the plasmid(s) is given below within parentheses. The boldfaced plasmids share homology with pNG2. Lanes: (A) **pNG24** (76-206); (B) **pNG25** (77-23); (C) **pNG26**, pNG27, pNG28 (77-249); (D) **pNG29**, **pNG30** (77-290); (E) pNG2 (S601A); (F) **pNG31**, pNG32, pNG33 (77-292); (G) **pNG34** (80-34); (H) **pNG23** (S1008); (I) lambda DNA digested with *Hin*dIII.

6) show that at high stringency both *C. diphtheriae* strains hybridized extensively over the entire range of fragment sizes, but that the Em^r coryneforms and the *C. ulcerans* and *C. pseudotuberculosis* strains displayed only limited homology to the *C. diphtheriae* probe. In each case a small number of fragments appeared to hybridize, suggesting that these isolates have limited, but distinct, regions of homology with *C. diphtheriae*. Similar results were obtained when the coryneforms isolated from normal sites and carrying plasmids with some homology to pNG2 were probed with S1016.

DISCUSSION

We have found that approximately one-third of a diverse group of aerobic coryneform bacteria isolated from various normal skin sites and cutaneous lesions carried plasmids. The plasmids ranged in mass from 2.5 to 36 Mdal, and all exhibited different restriction enzyme digest patterns. The phenotypes coded for by these plasmids are largely unknown, but a correlation between plasmid carriage and resistance to various antibiotics was demonstrated in some of them. The high frequency of plasmid carriage among the skin coryneforms was in marked contrast to the absence of plasmids in *C. diphtheriae*, the exception being a single cohort of apparently identical isolates carrying plasmid pNG2, which mediates erythromycin resistance (15).

The origin of plasmid pNG2 and its erythromycin resistance determinant is a question of some interest. Em^r *C. diphtheriae* and skin coryneforms were isolated during a 4-month period in 1978. Their appearance coincided with the administration of oral erythromycin during the years 1972 through 1978 in Seattle. The antibiotic was administered to patients involved in the epidemic and postepidemic period of an outbreak of cutaneous diphtheria (3). The presence of pNG2 in Em^r *C. diphtheriae*, in contrast to the absence of plasmids in any clinical Em^s isolate, suggests that pNG2 carriage was established during the period of antibiotic therapy. Homology between pNG2 and a number of fragments of some plasmids from both Em^r and Em^s skin coryneforms was detected under high-stringency conditions. This included homology with two fragments found only in plasmids from Em^r coryneforms, fragments that comigrated

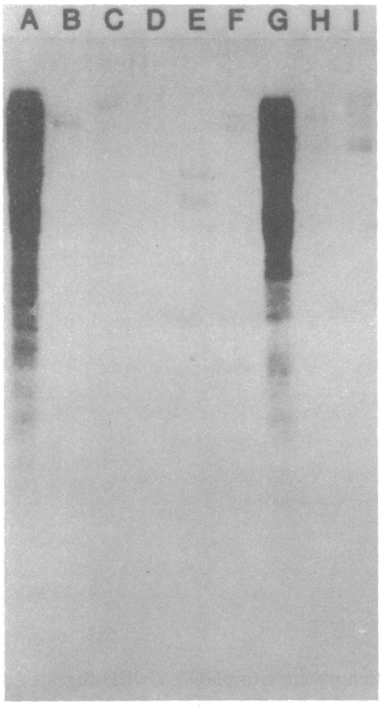

FIG. 6. Hybridization of *C. diphtheriae* S1016 genomic DNA to genomic DNA from the Em^r coryneforms. *Bam*HI-digested genomic DNA was loaded onto a 0.7% agarose vertical slab gel and run in TENA buffer at 60 V for 3.5 h. The DNA was transferred to nitrocellulose and hybridized to ^{32}P-labeled S1016 genomic DNA at 68°C for 48 h. The filter was washed under stringent conditions and autoradiographed. Lanes: (A) S601A, Em^r *C. diphtheriae*; (B through F) Em^r coryneforms; (B) S607A; (C) S615A; (D) S619A; (E) S632A; (F) S651A; (G) S1016, *C. diphtheriae*; (H) 690, *C. ulcerans*; (I) S1019, *C. pseudotuberculosis* (*C. ovis*).

with two in pNG2 whose presence was correlated with the erythromycin resistance phenotype in *C. diphtheriae* (15). In contrast, neither the plasmid nor genomic DNAs of Em^r staphylococci or streptococci isolated concurrently with the Em^r coryneforms and *C. diphtheriae* displayed any homology with pNG2. It was also recently reported by others (S. G. Wilson and R. K. Holmes, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 22nd, Miami Beach, Fla., abstr. no. 873, 1982) that there was no homology between pNG2 and previously characterized staphylococcal or streptococcal Em^r plasmids or transposons Tn*551* and Tn*917*. Thus, the plasmid and plasmid elements that correlate with erythromycin resistance in *C. diphtheriae* are closely related to plasmid sequences in skin coryneforms and do not appear to have been transferred from their staphylococcal or streptococcal contemporaries. Furthermore, the limited homology of the genomic DNA of plasmid-carrying coryneforms to *C. diphtheriae* at high stringency rules out any immediate precursor relationship between these organisms.

The high frequency of plasmid carriage by skin coryneforms in contrast to the low frequency in *C. diphtheriae* and the extensive homology between pNG2 in *C. diphtheriae* and a number of plasmids in skin coryneforms, particularly pNG34, suggests a connection between them. A number of explanatory hypotheses can be formulated. The simplest consonant with the observations is that there is an active exchange of plasmid DNA between skin coryneforms, and given the opportunity, for example in cutaneous lesions, exchanges can occur between coryneforms and *C. diphtheriae*. A second possibility is that an unidentified species shuttles DNA between these organisms or contributes its own DNA, including the Em^r determinant (or both). A third, less likely, possibility is that the skin coryneforms and *C. diphtheriae* had a common ancestor that carried the progenitor of pNG2 and acquired an Em^r determinant independently. At the present time there is no direct evidence for exchange of plasmid DNA between corynebacteria. Given the size of the coryneform plasmids, both transductional and conjugational mechanisms are possible. So far efforts to demonstrate transfer of plasmids have been unsuccessful.

ACKNOWLEDGMENTS

We acknowledge the technical assistance of Jennie Russell and Myron Rabin in this work.

This investigation was supported by Public Health Service research grant AI-10492 from the National Institute of Allergy and Infectious Diseases, and by National Research Service award GM-07270 from the National Institutes of Health.

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