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Transfer of Plasmid-Borne Beta-Lactamase in *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae strain GC82 contains a plasmid specifying a β-lactamase (β-Lam^+). Mixed incubation of strain GC82 with a penicillin-susceptible (β-Lam^-), streptomycin-resistant mutant of strain GC9 results in the expression of β-lactamase activity and streptomycin resistance in the transcipients. The frequency of transfer of the plasmid-specified resistance to penicillin seems to be proportional to the initial input ratio of the mating mixture of donor to recipient and to correlate positively with bacterial density. Cell-to-cell transmission of the deoxyribonucleic acid (DNA) appears to be by a conjugal mechanism or, alternatively, by an as yet undescribed transducing phage. Additionally, whole-cell DNA from a β-lactamase-producing strain could be used to transform streptomycin-resistant recipients, resulting in the expression of both β-lactamase activity and streptomycin resistance in the transformants, and purified gonococcal plasmid DNA transformed *Escherichia coli* but not the gonococcus. Circular DNA extracted from donor GC82 comprised three molecular species (approximately 2.7, 4.8, and 25 megadaltons [Mdal]), whereas the recipients GC9-S (Str^r) contained only the 2.7-Mdal cryptic DNA species. DNA from the GC9-S82 (Str^r, β-Lam^+) transcipient contained a 4.8-Mdal species in addition to the cryptic molecular species (2.7 Mdal). The finding that the transcipient will not retransfer β-lactamase is consistent with the hypothesis that the 25-Mdal plasmid promotes mobilization of the smaller 4.8-Mdal R plasmid.

Although *Neisseria gonorrhoeae* has been traditionally thought of as a susceptible organism, the occurrence of gonococcal strains that are relatively resistant to penicillin and other antibiotics has gradually become an important clinical problem during the past 15 years, necessitating the use of elevated dosages of penicillin. It should be noted that there have been recent indications that the minimal inhibitory concentration (MIC) for penicillin of clinical isolates has decreased somewhat (24, 31).

Resistance to penicillin in bacteria is frequently associated with the presence of β-lactamase. The literature on β-lactamase in gram-negative bacteria has been reviewed extensively (33). A number of these resistances have been specified by R plasmids. Previously, no β-lactamase has been reported in *N. gonorrhoeae*, and resistance of the gonococcus was occasioned by a change in the binding site for penicillin (34). In the last several years the isolation of β-lactamase-producing strains of *N. gonorrhoeae* has been reported (1, 2, 5, 28, 30). Recent reports have indicated the presence of plasmid-specified, β-lactamase-containing gonococci presumably associated with very high levels of resistance in these strains (13) and conjugal transfer of the gonococcal β-lactamase plasmid (12).

Evidence for plasmid deoxyribonucleic acid (DNA) in *N. gonorrhoeae* strain 2686 type 1 has been presented by Engelkirk and Schoenhard (14) and Maness and Sparling (24). The presence of plasmid DNA in each of the colonial types of gonococcus, using a laboratory strain (F62) as well as a number of other clinical isolates, was reported, and the molecular mass of this gonococcal plasmid was estimated as 2.4 megadaltons (Mdal), with 24 to 32 copies of the plasmid per cell. It was concluded that this plasmid species was phenotypically cryptic and not related to the colonial type of the organism (25). This observation was confirmed by Palchaudhuri et al. (29) and Stiffler et al. (36). The latter authors also observed a larger plasmid species with a molecular size of 24.5 Mdal present in both the virulent parent and the avirulent derivative of each of the clinical isolates studied.

In this communication we report that the

plasmid-borne β-lactamase is transferable to another strain of *N. gonorrhoeae*, and we describe experiments designed to elucidate the mechanism of transfer. Additionally, we postulate that the transfer of the 4.8-Mdal β-lactamase plasmid may be mobilized by a larger, presumably conjugative plasmid species (25 Mdal) present in the donor *N. gonorrhoeae* strain.

MATERIALS AND METHODS

Organisms. Properties of the strains of bacteria used in this study are listed in Table 1. GC9, a penicillin-susceptible gonococcal isolate that has been passed for several years in vitro, was kindly provided by D.S. Kellogg, Center for Disease Control, Atlanta, Ga. Gonococcal strains 76-061782 (GC82) and 76-073389 (GC89), both β-lactamase-producing, penicillin-resistant isolates, were provided by C. Thornsberry, Center for Disease Control. Colonies characteristic of morphological type 1 and type 4 were isolated from each strain and were selectively cloned for 40 to 80 consecutive daily subcultures on solid medium. *N. gonorrhoeae* strain GC9 was made resistant to streptomycin by serial passage in increasing concentrations of streptomycin. The final MIC for the derivative strain (GC9-S) was 1,000 μg of streptomycin per ml. Rifampin-resistant mutants of *N. gonorrhoeae* strain GC9-S were obtained after one passage of GC9-S on GC agar (Difco) (GCA) plus rifampin (100 μg/ml) as described previously (39). The initial MIC to rifampin of strain GC9-S was 0.2 μg/ml, whereas the mutant GC9-SR had an MIC to rifampin of >100 μg/ml.

Media. Daily subculture of gonococcal strains was made on GCA consisting of GC medium base (Difco) supplemented with 10 ml of filter-sterilized defined supplement (20) per liter of media. GC9-S was maintained on GCA containing 250 μg of streptomycin per ml, whereas GC82 and GC89 were maintained on GCA supplemented with 1 μg of penicillin G per ml. Liquid medium (GCB) consisted of 1.5% (wt/vol) proteose peptone no. 3 (Difco), 0.4% K_2HPO_4, 0.1% KH_2PO_4, and 0.5% NaCl. After autoclaving and immediately before use, 10 ml of Kellogg defined supplement (20), 10 ml of 4.2% $NaHCO_3$, and 10 ml of 10% soluble starch (Difco) were added per liter. *Escherichia coli* strains were grown in Penassay broth (Difco) or AB minimal broth (7) or on their respective solid media.

Cultivation of *N. gonorrhoeae*. Gonococci were subcultured daily on GCA with and without antibiotics at 37°C in a 5% CO_2 incubator (Bellco Glass, Inc.). Colonies of GC9-S and GC82 were selected from 21-h cultures on GCA plus antibiotic and harvested into GCB. The clumped gonococci were dispersed in a Vortex mixer, and these cells provided the inocula for broth cultures that were then grown at 37°C to late log phase on a Gyrotory platform shaker (New Brunswick Scientific Co.).

Detection of β-lactamase. β-lactamase activity was detected by two methods: the quantitative microiodometric technique of Citri (9) as well as the qualitative dye technique of Novick and Richmond (27). In the staphylococcal system, the total β-lactamase activity has been shown to vary during the growth of the culture (26). For this reason, all determinations were carried out on exponentially growing cultures of gonococci that had been grown under standard conditions (37°C in the Gyrotory shaker at 180 rpm for 2 h). Using the method of Citri (9), a 10-ml portion from a turbid suspension of gonococci in GCB, which had at least doubled its original optical density, was tested. Both bound and extracellular β-lactamases were determined. In both methods, a β-lactamase-producing *Staphylococcus aureus* (S1) and a non-β-lactamase-producer *S. aureus* (S10), grown on both 0.3CY agar and GCA, were used as controls and tested for β-lactamase activity.

TABLE 1. *Bacterial strains and relevant properties*[a]

Strain	Pertinent chromosomal genotype			Pertinent properties and references
	recA	*rif*^{r}	*str*^{r}	
GC82	−	−	−	Naturally occurring β-Lam$^+$ strain; carries three plasmids
GC82-Pc^s	−	−	−	Naturally occurring β-Lam$^-$ derivative GC82
GC9	−	−	−	Isolate passed for many years in vitro: Pen^s:Str^s; carries small indigenous gonococcal plasmid
GC9-S	−	−	+	Str^r mutant of GC9
GC9-SR	−	+	+	Str^r Rif^r mutant of GC9
GC9-S82	−	−	+	Pen^r Str^r transcipient; carries two plasmid species (β-Lam$^+$)
GC89	−	−	−	Naturally occurring β-Lam$^+$; carries three plasmids
E. coli K-12 C600 *hsp* r^-m^-	−	−	−	Derivative of *E. coli* K-12 (3); *thr*$^-$, *leu*$^-$, *thi*$^-$
E. coli B HB 101	+	−	+	Derivative of *E. coli* B (8); carried the following genetic markers: *leu*$^-$, *pro*$^-$, *lac*$^-$, *gal*$^-$, *thi*$^-$, *str*^{r}, *recA*$^-$, $56r^-m^-$

[a] In the case of antibiotic genotypes, + = resistant, whereas − = susceptible. In the case of *recA*, + indicates a mutant that is recombination deficient, whereas − indicates no recombination deficiency.

Transfer procedures. Since gonococci are known to undergo rapid autolysis under normal growth conditions (17), experiments were designed that did not use the procedures classically employed in mating experiments. In typical "mating" experiments, 21-h cultures of the donor (GC82) and recipient (GC9-S) strains were grown on GCA plates with appropriate selective antibiotics, harvested, and resuspended in fresh GCB containing $CaCl_2$ (final concentration, 2 mM) to an initial cell density of approximately 10^8 cells/ml. In this final mixture, donors and recipients were mixed in the ratios of 100:1, 10:1, and 1:1 and incubated at 37°C with aeration for 3 to 6 h. At hourly intervals, portions were removed, appropriately diluted in fresh GCB, and plated on GCA plates with and without selective antibiotics. Dilutions of donor and recipient cells were plated separately as controls. Colonies were counted after 24 and 48 h of incubation; the plates were inspected for colonial type, and β-lactamase activity was assessed.

Plasmid DNA isolation. DNA was isolated from 50-ml cultures that were grown in GCB containing appropriate antibiotics to approximately 10^9 colony-forming units (CFU)/ml. Centrifuged cells were washed once in TE buffer [10 mM tris(hydroxymethyl)aminomethane (Tris)-1 mM disodium ethylene diaminetetraacetic acid (EDTA), pH 8.1], recentrifuged, and suspended in 2.5 ml of 25% sucrose in 0.05 M Tris, pH 8. Cell suspensions, quickly frozen in a dry ice-acetone bath, were subsequently lysed by a Triton X-100-deoxycholate procedure described by Engelkirk et al. (14). Cell lysates were cleared by centrifugation at 48,000 × *g* for 30 min to remove the majority of chromosomal DNA. Cleared lysates were mixed with TE buffer, cesium chloride, and 1 ml of ethidium bromide (EtBr) solution (10 mg/ml in TE buffer) to give a final EtBr concentration of approximately 1 mg/ml and $\eta_{25°} \simeq 1.3985$. Centrifugation of the above preparations was carried out in a Beckman 50 Ti rotor for 40 h at 25°C and 44,000 rpm. The covalently closed circular (CCC) DNA was fractionated from the tube bottom, extracted with isopropanol (saturated with TE-buffered CsCl) to remove the EtBr, and dialyzed exhaustively against TE buffer.

Agarose gel electrophoresis. Plasmid DNA preparations were examined directly on 0.7 and 1.0% agarose vertical slab gels (16) (Seakem, Marine Colloids, Inc.) that were subjected to electrophoresis for 4 to 6 h at 12.5 V/cm in TB buffer (pH 7.0; 89 mM Tris-hydrochloride 2.5 mM disodium EDTA-89 mM boric acid) as described previously. Gels, stained with 5 μg of EtBr per ml in water, were photographed under shortwave ultraviolet illumination with a Polaroid MP4 camera and type 55 P/N film. Reference plasmid DNAs of known size were analyzed simultaneously to extrapolate the size of the uncharacterized molecules.

Electron microscopy of DNA. Purified gonococcal plasmid DNA preparations were initially converted to the open circular form with ^{60}Co irradiation (Gammacell 220), spread on Parlodion-coated copper grids by the method of Kleinschmidt (21) as described previously (22), and rotary shadowed with platinum-palladium. Molecular measurements were made from enlargements of electron image plates with a Numonics electronic graphics calculator. Molecular sizes were determined relative to internal pSC185 reference molecules (9.2 Mdal or 13.9 kilobases) that were added to each DNA preparation. The pSC185 plasmid is a nonconjugative plasmid that carries the Tn3 translocation and mediates resistance to ampicillin and tetracycline (22).

RESULTS

Characterization of gonococcal strains. All cultures of *N. gonorrhoeae* used in this study were morphologically and biochemically typical gonococci. Additionally, the taxonomy of these cultures was verified by indirect immunofluorescence. Colonial morphology was determined as described by Kellogg and colleagues (20). *N. gonorrhoeae* strain GC9-S was cloned for >70 consecutive daily transfers as a stable colony type 1 and was used as such as a recipient in both transformation and mating experiments. *N. gonorrhoeae* strain GC82, colony type 4, was used as donor throughout our studies. Although not discussed below, it should be noted that GC89 (colony type 4) could also serve as donor in both transformation and mating procedures with equal efficiency; however, this strain did not grow as readily on GCA plates containing penicillin and was very unstable with respect to colonial morphology.

The donors, streptomycin-susceptible strains of *N. gonorrhoeae* GC82 and GC89, were β-lactamase-producing cultures with MICs to penicillin, of 8 to 16 μg/ml and MICs to streptomycin of 6.25 to 12.5 μg/ml when tested by the agar dilution method, using an inoculum of 10^3 to 10^4 CFU/ml. These cultures could be grown in GCB containing 100 μg of penicillin G per ml when an initial inoculum of about 10^8 bacteria/ml was used. The recipient *N. gonorrhoeae* strain GC9-S was a penicillin-susceptible strain (MIC to penicillin, 0.05 to 0.1 μg/ml) that was resistant to greater than 1,000 μg of streptomycin per ml.

When quantitative β-lactamase activity was assayed by the iodometric method (9), both bound and extracellular activities could be detected at levels of 155 and 10 U of β-lactamase, respectively, per 10^9 CFU of the donor strain (GC82). The enzyme was constitutive. No β-lactamase activity could be detected in the recipient GC9-S strain. One unit of β-lactamase is defined as that amount of enzyme that hydrolyzes 1 μmol of benzylpenicillin per h at 30°C.

Transfer of resistance to nonresistant cells. As shown in Table 2, when 3-h cultures of strains GC82 and GC9-S were mixed in the ratio of 100 donors to 1 recipient and incubated

TABLE 2. *Transfer of β-lactamase determinant and spontaneous loss of resistance*[a]

Time (h)	Donor[b] (CFU/ml)	Recipient[c] (CFU/ml)	Transcipients (CFU/ml)	Frequency-of-transfer ratio (transcipients/recipients)
0	6.53×10^8	5.24×10^6	0	
3	7.80×10^1	1.30×10^7	1.74×10^4	1.34×10^{-3}
6	$<1.00 \times 10^1$	2.73×10^7	5.32×10^4	1.95×10^{-3}

[a] Premating inocula were washed once and brought to the original volume, and cultures were mixed in GCB plus $CaCl_2$ (2 mM) in the absence of antibiotics at a ratio of donor to recipient of 100:1 to a total of 6.74×10^8 bacterial/ml. At hourly intervals up to 6 h, the mixtures were diluted appropriately and plated on GCA alone, GCA + penicillin (25 μg/ml), GCA + streptomycin (250 μg/ml), and GCA + penicillin (25 μg/ml) + streptomycin (250 μg/ml). All zero-time platings were made on GCA alone, whereas the 3- and 6-h platings of the mating mixture were made on GCA containing appropriate antibiotics to select for donors, recipients, and transcipients.

for 3 h as described above, penicillin resistance was transferred at a frequency of 1.34×10^{-3}. These transcipients possessed β-lactamase as indicated by the dye test (25). Figure 1 indicates β-lactamase producers and nonproducers in this population. When a ratio of 10 to 1 was used (data not shown), a frequency of 1.04×10^{-5} was observed. In several attempts, transcipients could not be detected when a ratio of 1 to 1 was employed. The frequency of transfer seems to be proportional to the initial input ratio of donors to recipients in the mating mixture and to correlate positively with increasing bacterial density.

Although not shown in Table 2, it should be noted that after 1 h of mixed incubation and plating on selective penicillin- and streptomycin-containing GCA, transcipients (8.26×10^1 CFU/ml) could be detected at a low frequency (approximately 8.63×10^{-6}). Under the conditions described above, 3 h of mixed incubation was found to be maximal for the transfer of penicillin resistance. As shown in Table 2, when longer periods of mixed incubation were employed, significantly higher frequencies of transfer were not observed.

In addition, transformation of GC9-S recipients with crude gonococcal DNA from either GC82, GC89, or GC9-S82 occurred at frequencies of 1×10^{-3}. This DNA-mediated transformation could be abolished by deoxyribonuclease (DNase) treatment. However, no transformation was observed when purified plasmid DNA from β-lactamase-producing donor or transcipient cells was used. We have been able to transform *E. coli* strains HB101 and C600 to ampicillin resistance at a frequency of about 10^{-8} by using these purified plasmid preparations; however, a marked loss of β-lactamase activity in the transformant cells was noted.

Genetic stability of β-lactamase determinant in *N. gonorrhoeae* GC82 (donor) and GC 9-S82 (transcipient). It has been our experience that the β-lactamase determinant is spontaneously lost at a high frequency in a population by growth under ordinary conditions on GCA or in GCB without antibiotics, as well as after lyophilization or freezing of the strains. The spontaneous loss of the enzyme from the donor cell cultures can be calculated from Table 2. After 3 h (approximately three generations) of mixed incubation of donor and recipient populations in GCB, only 1 in 10^7 CFU retains resistance. Although it appears that the acquisition of the β-lactamase determinant in the transcipient cells increased threefold between 3 and 6 h of incubation, in fact the cloning of these transcipient colonies revealed losses of resistance comparable to that seen in the donor strain. It should be mentioned that the use of the quantitative microiodometric method (9) for studying the permanence of resistance to penicillin of β-lactamase-producing gonococci is somewhat complicated by the observation that the individual organisms making up a culture do not act uniformly in their resistance to penicillin. It is possible that in a presumably resistant culture the bulk of the cells do not produce the enzyme at the time of testing. The relatively small numbers of penicillin-hydrolyzing cells may produce sufficient β-lactamase to protect the entire culture from the inhibitory action of the antibiotic. Although not a quantitative test, the sensitive PNCB test (27) can detect the loss of β-lactamase activity in segregants of colonies grown in both the presence and absence of antibiotics. When both donor and transcipient colonies were tested by the dye method (27), only 30 to 70% of the colonies appearing on GCA containing high levels of penicillin (25 μg/ml) possessed β-lactamase activity. This loss may be seen in Fig. 1, where the dark centers of the colonies represent β-lactamase activity. Segregation of β-lactamase-negative cells may be visualized as the lighter areas at the periphery of the colony.

The high frequency of spontaneous loss was consistent with plasmid-specified resistance;

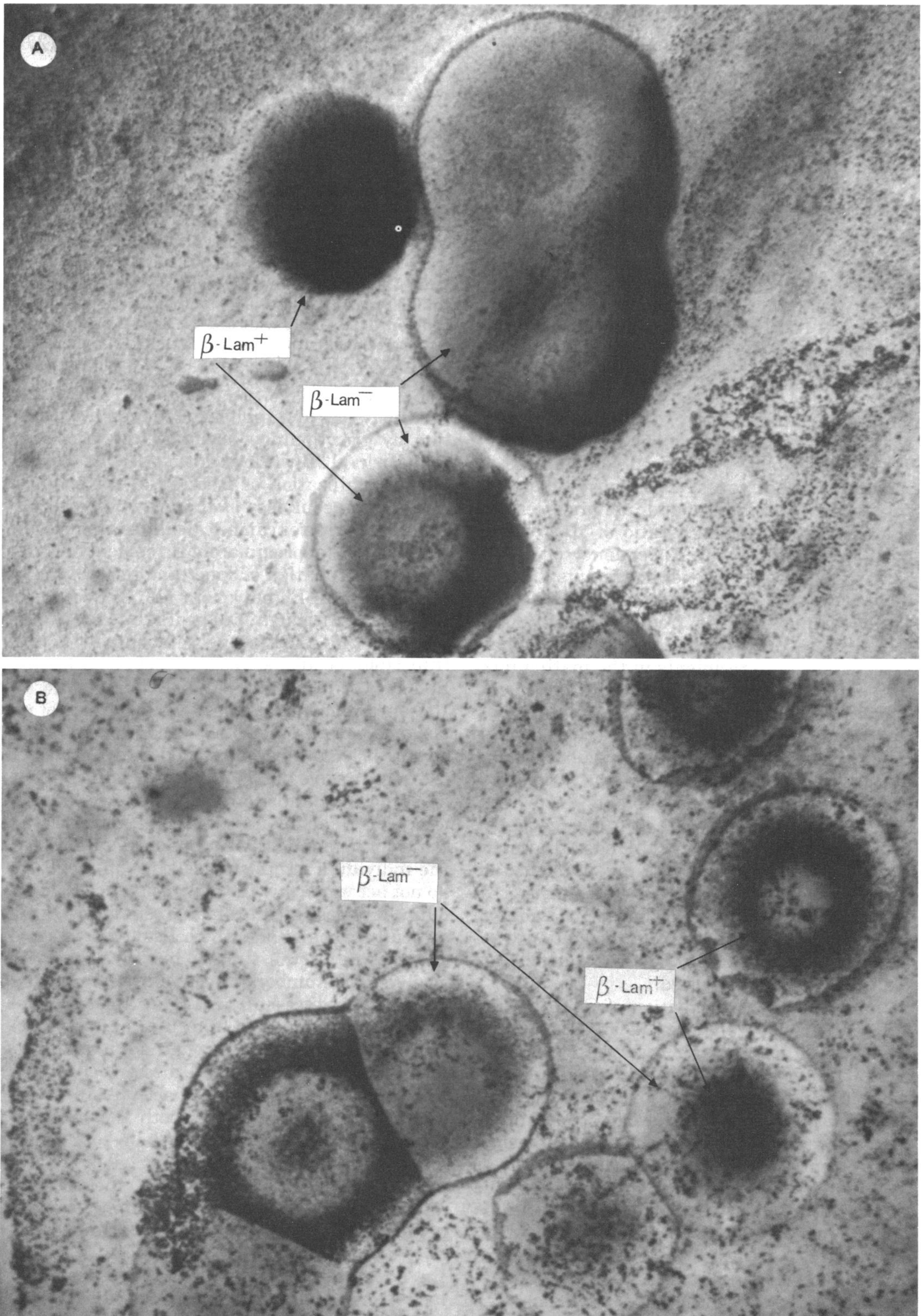


FIG. 1. *Test for β-lactamase production. (A) Colonies of donor GC82 on GCA plus penicillin (1 μg/ml) stained for β-lactamase. The dark colonies and areas represent (β-Lam+) clones, whereas the light areas represent (β-Lam−) plasmid-loss variants. (B) Colonies of transcripient GC9-S82 plated as above and stained with the PNCB indicator dye. Loss of the β-lactamase determinant can be readily detected with this sensitive technique.*

therefore, all strains used in this communication were further examined for the presence of plasmids.

Mechanism of transfer. The possibility that gonococcal plasmid DNA could be transferred by transformation during mating appeared doubtful in light of our evidence that 50 μg of DNase (pancreatic DNase, Worthington Biochemicals Corp.) per ml, when added repeatedly to the mating mixtures to inactivate any spontaneously released transforming DNA, had no effect on transfer frequencies. After 3 h of mixed incubation the frequency of transfer in the DNase-treated mixture was 1.81×10^{-3}, whereas that for the untreated control was 1.34×10^{-3}. Phage-mediated transduction could be an alternative explanation for our results; however, to date, no gonococcal phage has been described. Evidence that conjugal transfer, rather than phage-mediated transduction, was occurring was provided by experiments showing that neither cell-free filtrates of the donor strains nor chloroform-treated donor cultures were able to transfer the R plasmid to *N. gonorrhoeae* or *E. coli* recipients (data not presented).

As a consequence, since the most probable mode of transfer appeared to be conjugation, a mechanism that is known to be occasioned by R-plasmid transfer (38), the plasmid complement of all strains was analyzed.

Isolation and characterization of plasmids in donor, recipient, and transcipient cells. The recent report that the β-lactamase determinant in gonococci exists on a plasmid (13), the instability reported herein of this character in various gonococcal isolates, and the apparent transmissibility of this element between different gonococcal strains strongly suggest that a plasmid(s) is involved in the events reported in this communication. The various gonococcal isolates were examined for their plasmid composition by growing and lysing the strains as described above. CCC DNA, purified on CsCl-EtBr gradients, was dialyzed exhaustively before examining it by agarose gel electrophoresis and by direct visualization under an electron microscope.

Examination of freshly isolated CCC DNA by agarose gel electrophoresis permits a minimum estimation of the number of different molecular species present and an extrapolation of their size relative to references of known size. Figure 2 shows an agarose gel analysis of purified plasmid DNA from the donor GC82, the recipient GC9-S, and the transcipient GC9-S82. Standard plasmid DNAs, ranging in size from 63×10^6 daltons to 2.65×10^6 daltons, were used as reference molecules. DNA from the recipient GC9-5 strain contained only a single plasmid DNA species. Calculation of the size of the plasmid from agarose gel electrophoresis (Fig. 2B) as well as contour length measurements of more than 50 GC9-S plasmid molecules relative to the pSC185 reference marker gave a mean value of 2.67×10^6 daltons, a value that closely agrees with published observations on this phenotypically cryptic plasmid species (15, 25, 29, 36). No larger plasmid DNA molecules were detected in the recipient strain. Lysates of the donor GC82 show three distinct molecular species that migrate in the agarose gel at positions corresponding to molecular masses of 25 and 2.7 Mdal (which are typical of the two previously described indigenous gonococcal plasmids) as well as a unique 4.8-Mdal plasmid species (Fig. 2C). Circular DNA in the donor strain that was similar to that observed in the recipient strain had a contour length of about 4.12 ± 0.14 kilobases, equivalent to a molecular size of about 2.7 Mdal. From contour length measurements of at least 15 molecules of the intermediate plasmid species present in this strain relative to both the smaller indigenous gonococcal plasmid as well as to the internal pSC185 reference marker, a molecular mass of 4.8 Mdal was calculated. The larger plasmid DNA molecule was estimated at 25 Mdal from agarose gel electrophoresis (data not shown). Large CCC and linear forms of the presumed 25-Mdal species, but no open circular molecules, were observed when DNA preparations were examined under the electron microscope. This may indicate that this species is converted directly from the CCC state to a linear form. Lysates from the transcipient strain GC9-S82 contain the cryptic plasmid species present in the recipient (2.7 Mdal) as well as the 4.8-Mdal plasmid species present in the β-lactamase-producing donor (Fig. 2D). Since to date it has been our experience that this 4.8-Mdal plasmid species is present only in β-lactamase-producing strains, the acquisition of the 4.8-Mdal species in the transcipient is consistent with the thesis that this 4.8-Mdal species is the β-lactamase plasmid. It should be noted that, although the transfer of the determinant has regularly occurred, we have not been able to locate the 25-Mdal species in the donor strain in all instances on agarose gels or under the electron microscope. This may be due to the instability of this plasmid, e.g., that this species is rapidly converted to a linear form. It is also possible that the 25-Mdal species may not be involved in the transfer of the 4.8-Mdal β-lactamase plasmid; however, this seems unlikely since transcipients have never been found to retransfer the 4.8-Mdal plasmid and the transcipients

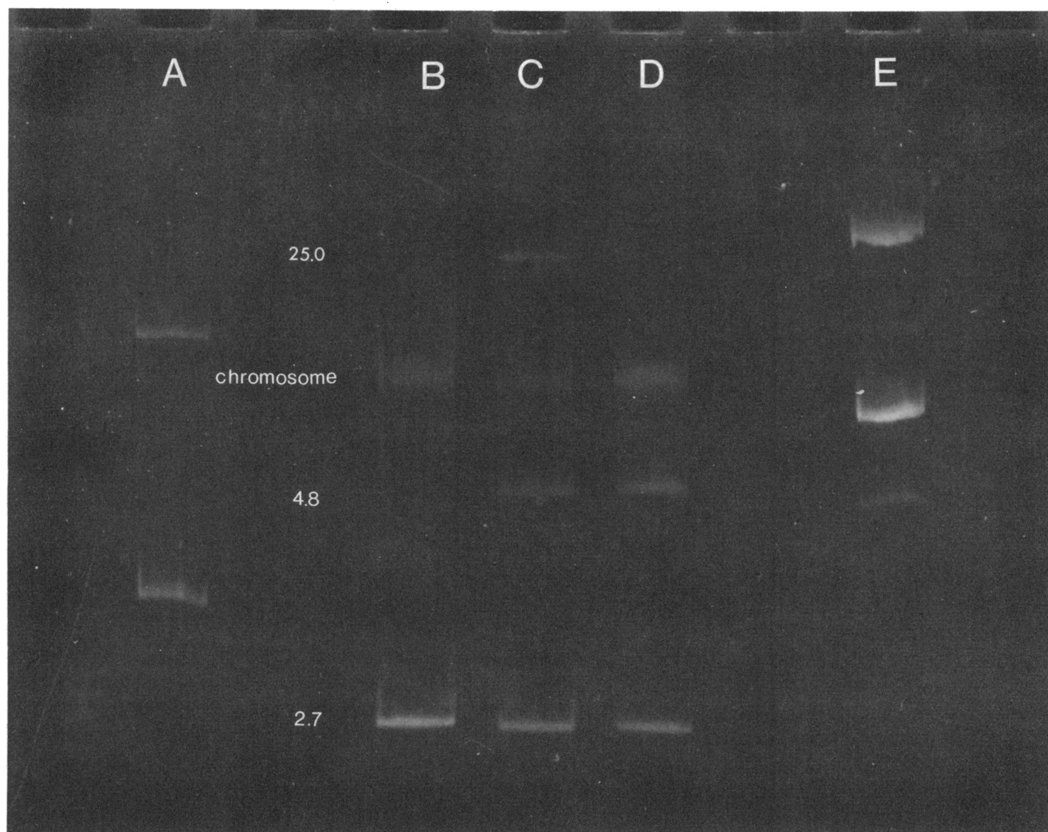


FIG. 2. *Analysis of plasmid size in a 1% agarose gel by electrophoresis. The plasmid DNA was purified by CsCl-EtBr centrifugation and fractionation. Conditions for electrophoresis are given in the text. (A) Standard plasmid DNA pVA310 (monomer molecular weight = 3.7 Mdal; dimer molecular weight = 7.4 Mdal). (B) DNA from GC9-S in which the most prominent band represents 2.7 Mdal. (C) DNA from the GC82 donor containing the 25-Mdal cryptic plasmid and the 2.7-Mdal smaller indigenous plasmid as well as a 4.8-Mdal plasmid species. (D) DNA from the GC9-S82 transcipient that received the intermediate 4.8-Mdal plasmid species and also contains the 2.7-Mdal indigenous plasmid. (E) Standard plasmid DNA pSC101 (molecular weight = 6.02 × 10⁶) in the linear, CCC, and open circular forms. Although all preparations were obtained from CsCl-EtBr gradients, a small amount of chromosomal DNA can be seen as a diffuse band on the gels as indicated. The molecular weight of plasmid species was obtained from the extrapolation from internal standard plasmid preparations and further confirmed by the direct measurement of contour length of plasmid molecules under an electron microscope.*

have never been shown to possess the 25-Mdal species (see below).

Molecular size determinations of these plasmids, obtained by electron microscopy, are summarized in Table 3. As shown in this table, the donor, recipient, and transcipient strains maintained a 2.7-Mdal plasmid that is assumed to be very similar in all strains. The 4.8-Mdal (β-lactamase) plasmid was transferred to the GC9-S recipient, giving rise to a transcipient strain that expresses β-lactamase, but which in turn can not retransfer the 4.8-Mdal R plasmid. Figure 2 shows that the lysates from strains GC82 and GC9-S differ in two respects: namely, the former contains the 25-Mdal plasmid species and the 4.8-Mdal species, whereas the latter does not. More importantly, the absence of the 25-Mdal plasmid species in the transcipient strain GC9-S82 in addition to the observation that the transcipient could not retransfer β-lactamase into GC9-SR recipients suggests that the larger 25-Mdal molecule, present in GC82 but absent in GC9-S or GC9-SR, is responsible and necessary for the transfer of the smaller 4.8-Mdal β-lactamase plasmid during mixed incubation in broth.

To determine whether the direction of transfer was from the putative donor GC82 to the

TABLE 3. *Plasmid DNA size determination*[a]

Bacterial strain	Plasmid designation	Kilobases ± SEM[a]	Mdal ± SEM[a]	Frequency of observation[b] (%)
GC82 (β-Lam$^+$) (donor)	pGC82-1	4.12 ± 0.14	2.72 ± 0.09	92
	pGC82-2	7.20 ± 0.22	4.77 ± 0.14	7
	pGC82-3[c]	37.5 ± 7.5	25.0 ± 5	1
GC9 (Str^r) (recipient)	pGC9-S-1	4.02 ± 0.12	2.67 ± 0.08	100
GC9-S82 (Str^r, β-Lam$^+$) (transcipient)	pGC9-S82-1	4.02 ± 0.14	2.66 ± 0.08	85
	pGC9-S82-2	7.42 ± 0.31	4.91 ± 0.22	15

[a] SEM, Standard error of the mean.
[b] The frequency of observation is the frequency at which a particular molecular species is observed in electron micrographs prepared from the total supercoiled circular plasmid DNA isolated from the various strains.
[c] Size calculations of pGC82-3 were estimated from agarose gel electrophoresis.

putative recipient GC9-S or vice versa, recipient cells were analyzed for the acquisition of donor plasmids. An analysis of recipients after mating revealed the absence of the larger 25-Mdal plasmid in the transcipients (Table 3), suggesting that the direction of transfer was from the donor (β-Lam$^+$) GC82 strain into the GC9-S (β-Lam$^-$) recipient.

Electron micrographs of the various plasmid DNA species are seen in Fig. 3A through C. Although not shown, catenated dimers of the 2.7- and 4.8-Mdal species were observed occasionally; also, D-loop forms, indicative of replicative intermediates, were observed.

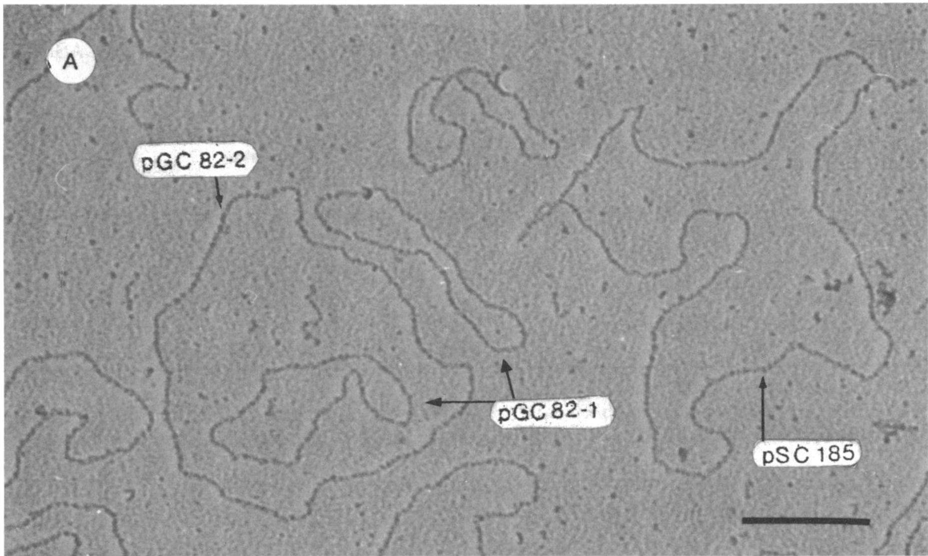
DISCUSSION

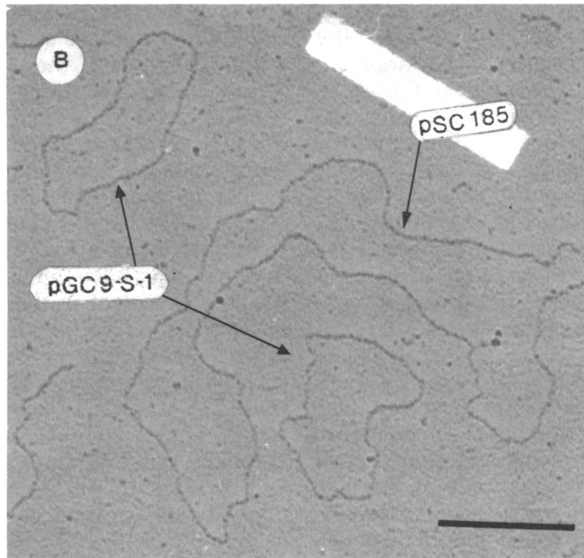
It may be inferred from the data presented that the mechanism of transfer of the presumed β-lactamase plasmid is either by conjugation or by transduction. Transformation may be ruled out since transfer in mixed cultures is insensitive to the action of DNase. It should be noted, however, that crude gonococcal DNA from the penicillinase-producing strain GC82 can be introduced into GC9-S recipients via normal transformation procedures, resulting in GC9-S82 transformants at a frequency of approximately 1×10^{-3}. Furthermore, by employing the procedure of Cohen et al. (10), using purified plasmid DNA from GC82 and GC9-S82 transcipient cells, we have been able to transform *E. coli* strains HB101 (8) and C600 r^-m^- (3), both host restriction modification mutants, to ampicillin resistance (Amp^r), and the β-lactamase activity was readily detectable by using the sensitive dye test devised (27). Curiously, however, when the same procedures were used, attempts to transform penicillin resistance with purified plasmid DNA from β-lactamase-producing gonococcal cells to penicillin-susceptible gonococcal recipients were not successful.

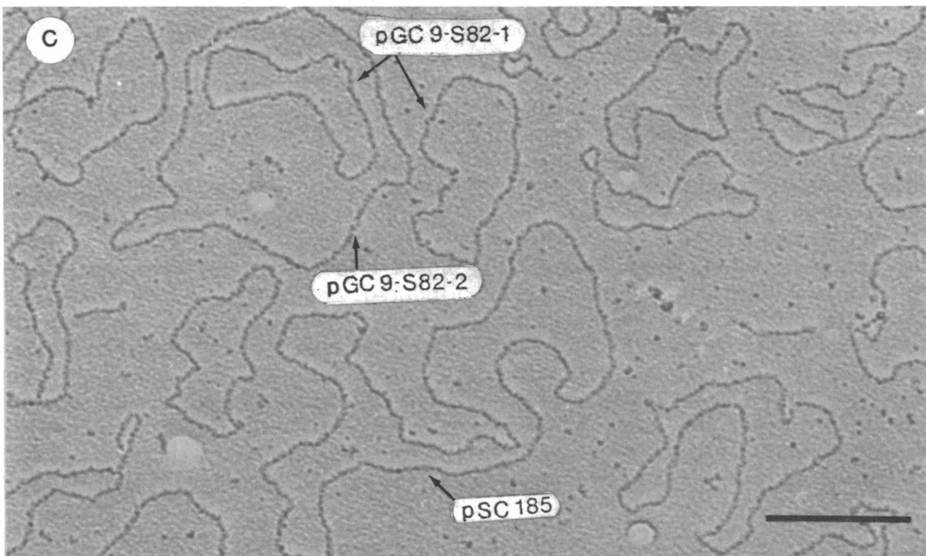
The alternative possibility of a bacteriophage acting as the β-lactamase transfer vector seems unlikely in light of our findings that neither culture filtrates of the donor cells nor chloroform-treated, nonviable donor cell cultures were effective in promoting plasmid transfer. We believe that transfer by conjugation is the more likely mechanism; however, transduction cannot be completely ruled out at this time. Numerous attempts in our laboratory as well as in others, using a variety of procedures to induce a phage, have failed. There are reports in the staphylococcal system of the efficient transfer of a plasmid conferring tetracycline resistance in mixed cultures that is dependent on the carriage of a prophage (23). It is thought that these DNA particles may be very unstable or largely bound to the donor cells. In our system no phage could be detected in the donor cells, and transducing phage particles, if they exist, might be expected to be susceptible to chloroform and pass through filters.

By analogy with the conjugal transfer of R plasmids among members of the *Enterobacteriaceae*, one would expect that cell-to-cell contact between a donor and a recipient cell is essential for conjugation (38). Such cell-to-cell contact may be mediated by a mechanism similar to that of *Streptococcus faecalis* (11, 18, 19, 37); however, we have no further information indicating the precise mechanism.

In our hands, in all instances, when plasmids, either naturally occurring or laboratory induced, are introduced into gonococci as well as into cultures of *E. coli*, the plasmid specifying β-lactamase is very unstable, and as shown in Table 2, is lost at a very high frequency. Although not presented, similar losses occur in the *E. coli* HB101 and C600 strains used herein. These observations are consistent with reports by Barber (6), Asheshov (4), and Richmond (32)


A
pGC 82-2
pGC 82-1
pSC 185


B
pSC 185
pGC 9-S-1


C
pGC 9-S82-1
pGC 9-S82-2
pSC 185

of the loss of the β-lactamase determinant in cultures of staphylococci at frequencies as high as 1 in 700 cells. Although cultures of GC82 and GC9-S82 are capable of growth in 50 to 100 μg of penicillin per ml, it is curious that, despite the rapid loss of the β-lactamase determinant, the stability of the plasmid could be promoted by growth of the strains in low levels of penicillin. When penicillin, at a concentration of 1 μg/ml, was incorporated into GCA, it stabilized β-lactamase-producing bacterial strains. We have no ready explanation for this apparent stabilization. This procedure was used routinely for daily subculturing of β-lactamase-producing bacterial strains.

The data presented here demonstrate the ability of the β-lactamase plasmid of *N. gonorrhoeae* strain GC82 to transfer and become established in a new gonococcal host. We have shown that the penicillin resistance was not transferable from the primary recipient to a second one. As a result of mating experiments between the GC9-S82 transcipient and GC9-SR, where selection was carried out for the penicillin resistance and further characterized on GCA plus rifampin, we conclude that strains harboring only the smaller cryptic 2.7- and 4.8-Mdal plasmid species are incapable of acting as donors in the conjugal transfer of penicillin resistance. At this time, since no phenotypic trait(s) has been associated with the indigenous gonococcal plasmids, it is difficult to determine whether the 25- or 2.7-Mdal species is capable of being transferred separately. It is logical to hypothesize that a larger cryptic plasmid may mobilize the smaller plasmid species.

An increasing concern regarding the control and chemotherapy of gonorrheal infections has emerged with reports on the incidence of antibiotic resistance, in particular to penicillin, in the gonococcus. Recently, numerous reports have appeared in the literature on the isolation of penicillinase-producing *N. gonorrhoeae* (1, 2, 5, 28, 30). β-lactamase-producing gonococcal strains have been identified in 11 different countries, and spread of the organism within the civilian U.S. population has been confirmed in several areas. These reports have aroused widespread concern. However, reports on the prevalence of antibiotic resistance in the gonococcus in fact suggest a significant decrease in resistance since 1968 (31), with few reports of MICs greater than 1 μg/ml and none in excess of 8 μg/ml. It should be noted that the vast majority of strains currently resistant to benzylpenicillin do not produce β-lactamase. Epidemiological reports issued by the Center for Disease Control (1) confirm only 94 instances of penicillinase-producing *N. gonorrhoeae* isolated during the period from March 1976 to January 1977. In this communication, it has been demonstrated that such plasmid-containing strains lose the β-lactamase plasmid at a very high rate and revert to benzylpenicillin susceptibility. Furthermore, since in numerous other gram-negative organisms β-lactamase does not appear to play a major role in resistance, it would perhaps be wiser to be conservative in predictions that the newly isolated strains will become the predominant type of gonococcus in the future. Obviously, when treatment failure parallels β-lactamase production, another appropriate antibiotic therapy should be considered (e.g., spectinomycin) if the isolated organism is susceptible to such compounds.

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ADDENDUM

After submission of this manuscript a short communication that reported the finding of similar plasmids and conjugal transfer of R plasmids in *N. gonorrhoeae* appeared (M. Roberts, and S. Falkow, Conjugal transfer of R plasmids in *Neisseria gonorrhoeae*, Nature [London] 266:630–631, 1977).

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FIG. 3. *Electron photomicrographs of open-circular molecules of plasmid DNAs with pSC185 used as reference. (A) Nicked DNA of donor GC82 (×56,915). Shown are two plasmid species, in addition to the reference marker pSC185, corresponding to molecular masses of 2.72 Mdal (pGC82-1) and 4.77 Mdal (pGC82-2). (B) Nicked recipient GC9-S (×60,680) plasmid DNA containing a single molecular species (pGC9-S-1) of 2.67 Mdal. (C) Nicked transcipient GC9-582 (×62,840) DNA contained two plasmid species: pGC9-S82-1 and pGC9-S82-2 with molecular sizes of 2.66 and 4.91 Mdal, respectively. The bar in each micrograph = 0.3 μm.*

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