Transfer of a Gonococcal β -Lactamase Plasmid to Conjugation-deficient *Neisseria cinerea* Strains by Transformation

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(Received 12 April 1988; revised 10 August 1988)

We have previously shown that some strains of *Neisseria cinerea* can serve as recipients in conjugation (Con⁺) with *Neisseria gonorrhoeae* while others cannot (Con⁻). To determine if a replication defect contributes to the inability of certain strains of *N. cinerea* to serve as recipients in conjugation, we attempted to introduce a naturally occurring gonococcal β -lactamase plasmid into *N. cinerea* by transformation. Various methods were employed, and all proved unsuccessful. Since specific sequences are required for DNA uptake in transformation of *N. gonorrhoeae*, we constructed a number of hybrid plasmids containing *N. cinerea* chromosomal DNA inserted into the *N. gonorrhoeae*/*Escherichia coli* β -lactamase shuttle vector, pLES2. When nine randomly selected plasmids with inserts were used to transform an *N. cinerea* strain which did not accept the gonococcal β -lactamase plasmid by conjugation, transformants were observed with four of the hybrid plasmids. The presence of one of the hybrid plasmids, pCAG9, in transformants was confirmed by agarose gel electrophoresis, Southern hybridization, and β -lactamase production. When an *N. gonorrhoeae* donor strain containing pCAG9 was used in conjugation with several *N. cinerea* strains, only those strains that were previously shown to act as recipients could accept and maintain pCAG9. The ability of pCAG9 and the other three hybrid plasmids to transform Con⁻ strains demonstrates that the β -lactamase plasmid can replicate in Con⁻ strains, and, therefore, the Con⁻ phenotype is due to a block in some other stage of the conjugation process.

INTRODUCTION

Since their discovery in 1976, penicillinase-producing *Neisseria gonorrhoeae* (PPNG) have been isolated at increasing frequencies around the world (Jaffe *et al.*, 1981; Morgan, 1986). One of the potential modes for the spread of PPNG is conjugal transfer of a β -lactamase plasmid (Handsfield *et al.*, 1982). The failure to contain the spread of PPNG strains raises the possibility that a non-pathogenic indigenous bacterial species could serve as an alternative reservoir for these plasmids. We have reported previously that some, but not all, strains of commensal *Neisseria* species can serve as recipients for the β -lactamase plasmid by conjugation with *N. gonorrhoeae* (Genco *et al.*, 1984). What we observed was not simply a change in the magnitude of the conjugation frequency but rather an 'all or nothing' phenomenon. These results prompted further studies designed to define the nature of the inability of certain *Neisseria* strains to serve as recipients in conjugation with *N. gonorrhoeae*.

From studies with other conjugative plasmids (Broda, 1979) it has been shown that the process of conjugation can be divided into three events: (1) the collision event, ultimately resulting in contact formation between the donor and recipient cell, (2) transfer of plasmid DNA from the donor to the recipient cell, and (3) replication and processing of DNA in the recipient cell. Although little is known about the molecular events of the gonococcal conjugation system, we

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assume that conjugation into *Neisseria cinerea* could be blocked at any of these stages. The replication of the transferred plasmid in the new host is essential, since if it cannot replicate it will be lost during successive cell divisions. The present investigation was undertaken to determine if a replication defect contributes to the inability of some strains of *N. cinerea* to serve as recipients in conjugation with *N. gonorrhoeae* for the β -lactamase plasmid. Experiments were therefore performed to attempt to introduce gonococcal β -lactamase plasmids into *N. cinerea* conjugation deficient (Con⁻) strains by transformation. The successful maintenance of gonococcal β -lactamase plasmids in Con⁻ strains indicates that these plasmids can replicate in these strains.

METHODS

Bacterial strains and plasmids. These are listed in Tables 1 and 2. *Neisseria* strains were maintained on a daily basis by streaking colonies on GC medium base (Difco) containing Kellogg's supplement (GCK) (White & Kellogg, 1965). Large volumes were grown in GCK broth containing NaHCO₃ (420 mg l⁻¹) at 37 °C with aeration. *Escherichia coli* was grown on MacConkey agar at 37 °C or in liquid culture (LB broth) at 37 °C with aeration.

Neisseria and *E. coli* strains were screened for their plasmid content by a rapid isolation method (Davis *et al.*, 1980), and plasmids were visualized by staining with ethidium bromide.

Plasmid purification. Plasmid DNA was isolated by ethidium bromide/CsCl isopycnic density gradient centrifugation by the method of Guerry *et al.* (1973), as modified by Stein *et al.* (1983*b*).

Conjugation and transformation. Conjugations were performed by the filter mating technique as previously described (Genco *et al.*, 1984). *N. cinerea* and *N. gonorrhoeae* strains were transformed with plasmid DNA by a procedure originally designed for *N. gonorrhoeae* (Sox *et al.*, 1979) and transformants were selected on GCK agar containing 0.25 µg penicillin G ml⁻¹. Transformants and transconjugants were confirmed by detection of β -

Table 1. *Bacterial strains used in this study*

Organism	Strain	Relevant phenotype*	Source†
<i>N. gonorrhoeae</i>	CDC67	Tra ⁺ Bla ⁺	CDC, Atlanta
	CDC66	Tra ⁺	CDC, Atlanta
	RUN4062	Tra ⁺ Bla ⁺	Transformation of pCAG9 into CDC66
<i>N. cinerea</i>	RUN4029	Bla ⁻ Con ⁻	NRL 30003
	RUN4030	Bla ⁻ Con ⁺	NRL 32165
	RUN4031	Bla ⁻ Con ⁺	NRL 32824
	RUN4032	Bla ⁻ Con ⁻	NRL 33295
	RUN4033	Bla ⁻ Con ⁺	NRL 32828
<i>E. coli</i>	JM83	Lac ⁻	J. Messing

* Tra⁺ strains contain the gonococcal conjugal plasmid, pLE2451. Bla⁺ indicates that the strain produces a β -lactamase and contains either pFT1 (CDC67) or pCAG9 (CDC66). Con⁻ and Con⁺ indicate the inability or capability, respectively, of the strain to receive the β -lactamase plasmid by conjugation with *N. gonorrhoeae*. Lac⁻ indicates inability to utilize lactose as an energy source.

† NRL denotes *Neisseria* Reference Laboratory.

Table 2. *Plasmids used in this study*

Plasmid	Size (kb)	Phenotype*
pFT180	10.6	Bla ⁺
pFT1	6.7	Bla ⁺
pFT6	42.4	Bla ⁺ , conjugal
pFT300	4.8	Bla ⁺
pLE2451	37.1	Conjugal
pLES2	6.0	Bla ⁺ , <i>lacZ</i> - α -complementation
pCAG3	2.9	Cryptic
pCAG9	7.8	Bla ⁺ , contains a 1.8 kb insert of <i>N. cinerea</i> chromosomal DNA in pLES2

* Bla⁺ refers to production of a β -lactamase.

lactamase activity with the chromogenic cephalosporin nitrocefin (O'Callaghan *et al.*, 1972). *E. coli* was transformed using a CaCl₂ procedure (Cohen *et al.*, 1972).

Construction of hybrid plasmids. This was done by inserting *N. cinerea* chromosomal DNA into the lacZ- α -complementation region of the *N. gonorrhoeae*/*E. coli* shuttle vector pLES2 (Stein *et al.*, 1983*a*). Ligation mixtures were used to transform *E. coli* JM83 with selection for transformants on MacConkey agar containing 30 μ g ampicillin ml⁻¹.

Southern hybridization. DNA was transferred from agarose gels to nitrocellulose as described by Wahl *et al.* (1979). Probe DNA was labelled with ³²P-dCTP by nick-translation (Maniatis *et al.*, 1982), and hybridization was performed at 65 °C for 16 h as described by Southern (1975).

RESULTS

Transformation of N. cinerea with gonococcal β -lactamase plasmid DNA

We used *N. cinerea* in these studies of β -lactamase plasmid replication in Con⁻ strains because the β -lactamase plasmids are transferred at high frequency by conjugation and stably maintained in *N. cinerea* Con⁺ strains (Genco *et al.*, 1984). Several different gonococcal β -lactamase plasmids (pFT1, pFT180, pLES2 and pFT6) were used in attempts to transform *N. cinerea* Con⁻ strains, by a number of different methods, all of which proved unsuccessful. These included CaCl₂ transformation (Cohen *et al.*, 1972), RbCl₂ transformation (Hanahan, 1983), a transformation procedure originally described for *Haemophilus influenzae* (Stuy, 1979), and a standard procedure used for *N. gonorrhoeae* (Sox *et al.*, 1979).

Plasmid transformation is relatively inefficient in *N. gonorrhoeae* (Cannon & Sparling, 1984), and specific sequences are required for DNA uptake (Graves *et al.*, 1982). If specific uptake sequences are also required in *N. cinerea*, supplying these sequences should overcome the inability of the β -lactamase plasmid to transform this species. Hybrid plasmids (pCAG2, pCAG4, and pCAG6-12) were constructed containing *N. cinerea* chromosomal DNA inserted into the BamHI site of the *N. gonorrhoeae*/*E. coli* β -lactamase shuttle vector, pLES2 (Stein *et al.*, 1983*a*). pLES2 contains the lacZ- α -complementation region from pUC9 that allows the direct visualization of plasmids containing inserts in the BamHI site after transformation into the appropriate *E. coli* host (JM83), by the Lac⁻ phenotype. *N. cinerea* RUN4030 chromosomal DNA was inserted into pLES2, the ligated mixture was used to transform *E. coli* JM83, and Amp^r transformants were screened for the Lac⁻ phenotype. Approximately 75% of the Amp^r transformants were found to be Lac⁻.

The presence of *N. cinerea* DNA in one of the hybrid plasmids, pCAG9, was confirmed by a dot-blot procedure. pCAG9 hybridized to pCAG9, pLES2, and to DNA from two strains of *N. cinerea* and three strains of *N. gonorrhoeae*; some hybridization to *Neisseria flava* and *Branhamella catarrhalis* was also observed (data not shown). The pLES2 vector hybridized only to pCAG9, pLES2 and pCAG2, and not to the chromosomal DNA from these *Neisseria* spp. (data not shown).

When nine different hybrid plasmids containing *N. cinerea* DNA inserts (ranging from 0.2 kb to 1.9 kb) were used to transform Con⁻ *N. cinerea* RUN4029, transformants were observed with four of the plasmids, pCAG4, pCAG9, pCAG11 and pCAG12 (data not shown). The transformation frequencies varied somewhat for the four plasmids (1.5×10^{-5} to 7.0×10^{-7}), and did not correlate with the size of the DNA insert. All transformants produced significant levels of β -lactamase activity as measured by nitrocefin cleavage.

The presence of pCAG9 in the transformants was confirmed by agarose gel electrophoresis (Fig. 1*a*) and by Southern hybridization (Fig. 1*b*). The agarose gel was bi-directionally blotted onto nitrocellulose to generate two identical blots, one of which was hybridized with ³²P-labelled pCAG9 (Fig. 1*b*) and the other with ³²P-labelled pCAG3 (data not shown). pCAG3 is the resident plasmid of the host strain, *N. cinerea* RUN4029. pCAG9 hybridized to itself, to its parent plasmid pLES2, and to plasmid DNA in the *N. cinerea* RUN4029 transformants. Hybridization to chromosomal DNA from *N. cinerea* RUN4029 was not evident; significant hybridization would not be observed because of low concentrations of chromosomal DNA in the gel. In the blot probed with pCAG3, pCAG3 hybridized to plasmid DNA in the host strain and all *N. cinerea* RUN4029 transformants, at the size of pCAG3 but not of pCAG9 (data not

Fig. 1. Presence of pCAG9 in *N. cinerea* RUN4029 transformants. Plasmid DNA was isolated, as described in Methods, by CsCl density gradient centrifugation for plasmids pLES2 (lane A) and pCAG9 (lane B) or by plasmid screens for *N. cinerea* RUN4029 (lane C) and 12 transformants of RUN4029 containing pCAG9 (lanes D–O). The DNA was separated on a 1% (w/v) agarose gel, stained with ethidium bromide (a), bidirectionally blotted onto nitrocellulose and hybridized with ³²P-labelled pCAG9 (b) or ³²P-labelled pCAG3 (data not shown). The sizes (kb) of supercoiled plasmid DNA markers (Bethesda Research Laboratories) are shown on the left. The positions of open circular (OC) and closed circular (CC) forms of pCAG3 and pCAG9 prepared by the plasmid screening method are indicated on the right (Lin, linear). The mobility of pCAG9 prepared by the CsCl method is somewhat faster than what is obtained by the plasmid screening method.

shown). The presence of this plasmid confirmed the identify of the *N. cinerea* strain. Hybridization of pCAG3 to pLES2 or pCAG9 was not observed. The band in Fig. 1 (a), lane J, running just below the pCAG9 OC, did not hybridize with either pCAG9 or pCAG3.

Table 3. Transformation and conjugation frequencies for pCAG9

Donor*	Recipient†	Transformation frequency‡	Conjugation frequency‡
<i>E. coli</i> JM83(pCAG9)	RUN4029	4.0×10^{-6}	ND
<i>N. cinerea</i> RUN4029(pCAG9)	RUN4029	4.2×10^{-5}	ND
<i>N. gonorrhoeae</i> RUN4062(pCAG9)	RUN4029	1.0×10^{-6}	$< 10^{-9}$
	RUN4030	$< 10^{-8}$	7.3×10^{-5}
	RUN4031	$< 10^{-8}$	1.1×10^{-5}

ND, Not done. *E. coli* JM83(pCAG9) and *N. cinerea* RUN4029(pCAG9) were not used as donors in conjugation because they do not contain a conjugal plasmid.

* The strain from which the plasmid was isolated for transformation experiments, or the strain which contained the conjugal plasmid as well and served as a donor in conjugation.

† The *N. cinerea* strain into which pCAG9 was introduced by transformation, or the strain which interacted with a donor strain in conjugation.

‡ Transformation frequency and conjugation frequency are expressed as transformants and transconjugants, respectively, per input cell and are representative of three separate experiments.

Confirmation of unique inserts

Since four hybrid plasmids containing *N. cinerea* DNA inserts of different size were able to transform Con⁻ *N. cinerea* RUN4029, it is unlikely that this was due to the cloning of a replication origin from RUN4029. To confirm that these four inserts were unique, we hybridized pCAG9 to *Sau*3A digests of the four plasmids. pCAG9 hybridized strongly to all undigested plasmids, to vector fragments and to a band of 1.8 kb in the *Sau*3A-digested pCAG9. No hybridization of pCAG9 to the inserts of the three other hybrid plasmids was detected. The ability of pCAG9 and the three other hybrid plasmids, but not pLES2, to transform *N. cinerea* RUN4029 Con⁻ cells is therefore most likely not due to the insertion of sequences mediating replication of pLES2 in *N. cinerea*, but rather to the insertion of sequences required for DNA uptake by transformation.

Transfer of pCAG9 into *N. cinerea* recipients by transformation and conjugation

The experiments described above demonstrated that pCAG9 could be introduced into *N. cinerea* RUN4029 Con⁻ cells by transformation. To determine if pCAG9 could be introduced into *N. cinerea* Con⁻ strains by conjugation, the plasmid was used to transform *N. gonorrhoeae* CDC66, creating a donor, RUN4062, which could be used in conjugation with *N. cinerea* recipients. The Con⁻ *N. cinerea* strain RUN4029 was not able to accept pCAG9 by conjugation from the *N. gonorrhoeae* RUN4062 donor (Table 3). *N. cinerea* RUN4030 and RUN4031 (Con⁺ strains) were both able to serve as recipients in transfer of the hybrid plasmid, pCAG9, from *N. gonorrhoeae* RUN4062 by conjugation. The frequency of conjugal transfer of pCAG9 into these strains was comparable to the frequency of transfer of pFT1 (Genco *et al.*, 1984). pCAG9 DNA isolated from *E. coli* JM83, *N. cinerea* RUN4029 or *N. gonorrhoeae* RUN4062 could transform *N. cinerea* Con⁻ recipients (Table 3). The inability of pCAG9 DNA isolated from *N. gonorrhoeae* RUN4062 to transform either *N. cinerea* RUN4030 or RUN4031 may be due to restriction of plasmid DNA during transformation or to a low competence level, analogous to that of type 4 gonococci, in these strains.

DISCUSSION

The transformation of *N. cinerea* RUN4029 by gonococcal β -lactamase plasmid DNA indicated that this plasmid can be replicated in a Con⁻ strain. That pCAG9 was present as a plasmid, and not integrated into the chromosome, was evident from agarose gels and Southern hybridizations. The failure to transfer the β -lactamase plasmid into *N. cinerea* RUN4029 by conjugation was not due to lack of expression of the gonococcal β -lactamase gene, as seen by measurements of β -lactamase activity in the transformants.

In other systems, the ability of a transferred plasmid to replicate in diverse bacteria appears to be the most important factor determining host range (Guiney, 1982). Conjugative plasmids differ in their ability to transfer and replicate in unrelated bacteria. Plasmids of the incompatibility group P (IncP) have a very broad host range and can be transferred to most Gram-negative organisms (Chikami *et al.*, 1985). These plasmids must possess a conjugation system that facilitates transfer between unrelated bacteria, together with a replication system that maintains the plasmid in each host. In contrast, plasmids of the IncFI and IncFII groups have a narrower host range, in that they can be transferred among the *Enterobacteriaceae* but not to *Pseudomonas aeruginosa* (Datta & Hedges, 1972). The basis for the host range limitation appears to be an inability of these plasmids to replicate in *P. aeruginosa* (Guiney, 1982).

The ability of pCAG9 to replicate in *N. cinerea* RUN4029 does not appear to be due to the unlikely event of cloning of an origin for replication. Of nine randomly selected hybrid plasmids, four could transform RUN4029. This frequency would be too high to represent cloned replication origins, unless they all represented progeny of the same clone. This was not the case, as pCAG9 did not share homology in its inserted sequence with the other three hybrid plasmids. Even if a replication origin had been cloned, it would not alter our conclusion that replication is not the stage at which conjugation is blocked, since pCAG9 still cannot be transferred to *N. cinerea* RUN4029 by conjugation.

The fact that pCAG9 isolated in an *N. gonorrhoeae* host could be transformed into *N. cinerea* RUN4029 Con⁻ organisms suggests that the inability to transfer the plasmid from this donor to this strain by conjugation is not due to restriction of gonococcal β -lactamase plasmid DNA. Some restriction of pCAG9 may occur during transformation; however, it is not great enough to explain the complete inhibition of conjugal transfer. Stein *et al.* (1988) have recently demonstrated that host-mediated restriction can prevent the gonococcus from acquiring DNA via transformation but not via conjugation.

In summary, the ability of *N. cinerea* RUN4029 to accept pCAG9 by transformation, but not by conjugation, suggests that the barrier to conjugation in *N. cinerea* RUN4029 is not the inability to replicate the gonococcal β -lactamase plasmid. The Con⁻ phenotype of the *N. cinerea* strains is also probably not due to restriction of plasmid DNA after transfer. Therefore, these Con⁻ strains must be deficient in another, probably earlier, stage in the conjugation process. Results described in the accompanying paper (Genco & Clark, 1988) suggest that these Con⁻ *N. cinerea* strains may lack a component in the outer membrane that is required for cells to serve as recipients in conjugation with *N. gonorrhoeae*.

This study was supported by US Public Health Service grant AI-11709 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

- BRODA, P. (1979). Conjugal transfer. In *Plasmids*, pp. 83–99. San Francisco: W. H. Freeman.
- CANNON, J. G. & SPARLING, P. F. (1984). The genetics of the gonococcus. *Annual Review of Microbiology* **38**, 111–133.
- CHIKAMI, G. K., GUINEY, D. G., SCHMIDHAUSER, T. J. & HELINSKI, D. R. (1985). Comparison of 10 IncP plasmids: homology in the regions involved in plasmid replication. *Journal of Bacteriology* **162**, 656–660.
- COHEN, S. N., CHANG, A. C. Y. & HSU, L. (1972). Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R factor DNA. *Proceedings of the National Academy of Sciences of the United States of America* **69**, 2110–2114.
- DATTA, N. & HEDGES, R. W. (1972). Host range of R factors. *Journal of General Microbiology* **70**, 453–460.
- DAVIS, R. W., BOTSTEIN, D. & ROTH, J. R. (1980). *A Manual for Genetic Engineering: Advanced Bacterial Genetics*, pp. 120–123. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- GENCO, C. A. & CLARK, V. L. (1988). Role of outer-membrane proteins and lipopolysaccharide in conjugation between *Neisseria gonorrhoeae* and *Neisseria cinerea*. *Journal of General Microbiology* **134**, 3285–3294.
- GENCO, C. A., KNAPP, J. S. & CLARK, V. L. (1984). Conjugation of plasmids of *Neisseria gonorrhoeae* to other *Neisseria* species: potential reservoirs for the beta-lactamase plasmid. *Journal of Infectious Diseases* **150**, 397–401.
- GRAVES, J. F., BISWAS, G. D. & SPARLING, P. F. (1982). Sequence-specific DNA uptake in transformation of *Neisseria gonorrhoeae*. *Journal of Bacteriology* **152**, 1071–1077.

- GUERRY, P., LEBLANC, D. J. & FALKOW, S. (1973). General method for the isolation of plasmid deoxyribonucleic acid. *Journal of Bacteriology* **116**, 1064–1066.
- GUINEY, D. G. (1982). Host range of conjugation and replication functions of the *Escherichia coli* sex plasmid FLac: comparison with the broad host-range plasmid RK2. *Journal of Molecular Biology* **162**, 699–703.
- HANAHAN, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *Journal of Molecular Biology* **166**, 557–580.
- HANDSFIELD, H. H., SANDSTROM, E. G., KNAPP, J. S., PERINE, P. L., WHITTINGTON, W. L., SAYERS, D. E. & HOLMES, K. K. (1982). Epidemiology of penicillinase-producing *Neisseria gonorrhoeae* infections. Analysis by auxotyping and serotyping. *New England Journal of Medicine* **306**, 950–954.
- JAFFE, H. W., BIDDLE, J. W., JOHNSON, S. R. & WIESNER, P. J. (1981). Infections due to penicillinase-producing *Neisseria gonorrhoeae* in the United States: 1976–1980. *Journal of Infectious Diseases* **144**, 191–197.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MORGAN, R. (1986). PPNG – United States, Florida. *Journal of the American Medical Association* **225**, 1674–1679.
- O'CALLAGHAN, C. H., MORRIS, C. H., KIRBY, S. M. & SHINGLER, A. H. (1972). A novel method for detection of beta-lactamase by using a chromogenic cephalosporin substrate. *Antimicrobial Agents and Chemotherapy* **1**, 283–288.
- SOUTHERN, E. M. (1975). Detection of specific sequences among fragments separated by gel electrophoresis. *Journal of Molecular Biology* **90**, 503–517.
- SOX, T. E., MOHAMMED, W. & SPARLING, P. F. (1979). Transformation-derived *Neisseria gonorrhoeae* plasmids with altered structure and functions. *Journal of Bacteriology* **138**, 510–516.
- STEIN, D. C., SILVER, L. E., CLARK, V. L. & YOUNG, F. E. (1983a). Construction and characterization of a new shuttle vector, pLES2, capable of functioning in *Escherichia coli* and *Neisseria gonorrhoeae*. *Gene* **25**, 350–354.
- STEIN, D. C., YOUNG, F. E., TENOVER, F. C. & CLARK, V. L. (1983b). Characterization of a chimeric beta-lactamase plasmid of *Neisseria gonorrhoeae* which can function in *Escherichia coli*. *Molecular and General Genetics* **189**, 77–84.
- STEIN, D. C., GREGOIRE, S. & PIEKAROWICZ, A. (1988). Restriction of plasmid DNA during transformation but not conjugation in *Neisseria gonorrhoeae*. *Infection and Immunity* **56**, 112–116.
- STUY, G. H. (1979). Plasmid transfer in *Haemophilus influenzae*. *Journal of Bacteriology* **139**, 520–529.
- WAHL, G. F., STERN, M. & STARK, G. R. (1979). Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 3683–3687.
- WHITE, L. A. & KELLOGG, D. S. (1965). *Neisseria gonorrhoeae* identification in direct smears by a fluorescent antibody-counterstain method. *Applied Microbiology* **13**, 171–174.