Introduction of Cloned Genes into *Neisseria gonorrhoeae*

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There is considerable knowledge about the genetics and physiology of *Neisseria gonorrhoeae* (see references 6 and 20 for recent reviews). Recent studies with monoclonal antibodies and recombinant deoxyribonucleic acid (DNA), coupled with studies of the immunobiology of various surface antigens, have provided significant advances in our understanding of the molecular pathogenesis of the infections caused by this organism.

Molecular cloning of the DNA encoding various proteins has expedited the identification and characterization of important surface antigens. To date, a wide variety of surface antigens have been cloned in *Escherichia coli* (see reference 33 for a current review). The common thread that underlies these advances is the identification of important genes by cloning and expressing them in *E. coli*. However, many important proteins may be missed by this approach, since *E. coli* may not properly express the genes of interest. In addition, the cell signals that regulate their biosynthesis may not be recognized in *E. coli*. An alternative strategy would be to clone and identify the desired genes directly in *N. gonorrhoeae*. To introduce cloned genes into *N. gonorrhoeae*, a significant amount of research was necessary to identify what genetic tools could be used, what strains could serve as recipients, and what cloning vectors could function in this bacterium.

PLASMIDS OF *N. GONORRHOEA*

Several different naturally occurring plasmids have been isolated from *N. gonorrhoeae*. Most gonococcal strains contain a 4.2-kilobase cryptic plasmid (19), and a few also contain a 39-kilobase conjugal plasmid (18). Plasmids encoding β -lactamase were first found in *N. gonorrhoeae* in England in 1976 (16), but since then, several related plasmids have been identified elsewhere in strains isolated from patients (9, 12, 32). Recently, plasmids encoding tetracycline resistance have been described (15).

In addition to the naturally occurring plasmids that have been isolated from *N. gonorrhoeae*, researchers in several laboratories have constructed derivatives of some of these plasmids through recombinant DNA techniques. These recombinant plasmids have been exploited for studies of the mechanisms of plasmid DNA uptake during transformation (4) and conjugation (31), as well as for use in cloning gonococcal genes (28).

A bifunctional shuttle vector, pLES2, that is able to replicate in *N. gonorrhoeae* and *E. coli* has been constructed by using one of the naturally occurring β -lactamase plasmids as a base replicon (30). This shuttle vector has the ability to transform *N. gonorrhoeae* at high frequency, when it contains gonococcal DNA (28). To date, this is the only cloning vector that has been used to introduce cloned genes into *N. gonorrhoeae*, but its use is limited by the constraints of the gonococcal transformation and conjugation systems.

GENETIC MECHANISMS OF *N. GONORRHOEA*

Transformation of *N. gonorrhoeae* was first described by Sparling (23) in 1966. Unlike most bacterial transformation

systems, all piliated gonococci are competent for transformation throughout their growth cycle, and phenotypic expression of competence requires only a utilizable energy source and cations (2). Through the use of DNA-mediated transformation, a limited genetic map of *N. gonorrhoeae* has been constructed, although most of the markers included in this map involve ribosomal protein genes (24). Both plasmid and chromosomal DNAs are able to transform *N. gonorrhoeae*, but plasmid transformation is inefficient unless the plasmid contains gonococcal DNA (1). This is because gonococcal DNA is selectively taken up from the environment in a sequence-specific manner (10). Host-mediated restriction can be a barrier to transformation with either form of DNA, although it seems to act more efficiently on plasmid than on chromosomal DNA (25).

The conjugal plasmid present in certain strains of *N. gonorrhoeae* is able to mobilize gonococcal β -lactamase-encoding R factors intraspecifically as well as interspecifically (7, 18). However, this plasmid is unable to mobilize these plasmids efficiently from other bacterial species into *N. gonorrhoeae*, except for *Neisseria cinerea* (8). Piffaretti et al. (17) have shown that pUB307, a derivative of RP1, is able to mobilize gonococcal R factors from *E. coli* to *N. gonorrhoeae*. In addition, this plasmid is able to mobilize the gonococcal shuttle vector pLES2. Through the use of pUB307 and pLES2, it should be possible to use conjugation to mobilize genes cloned in *E. coli* into *N. gonorrhoeae* at high frequencies.

INTRODUCTION OF PLASMIDS INTO *N. GONORRHOEA*

Considerable attention has been given to the transformation of *N. gonorrhoeae* with plasmids. When plasmid DNA isolated from *N. gonorrhoeae* is reintroduced into it by transformation, the plasmid is linearized during transformation by a nonspecific nuclease (3), yet it retains its ability to transform. In contrast, when the same plasmid is isolated from *E. coli* and then used to transform *N. gonorrhoeae*, it can be restricted in a site-specific manner, and no transformants are obtained (25). Whether or not it is restricted depends on the amount of endonuclease present in the recipient strain, as well as the number of recognition sites that are present on the plasmid (D. C. Stein, unpublished observations).

When *N. gonorrhoeae* is transformed with plasmid DNA that lacks homology with resident plasmids, about 25% of the transformants will contain plasmids of altered size (22). When the recombinant plasmid pLES7, encoding the proline-biosynthetic genes from *N. gonorrhoeae* KH45, was introduced into a proline auxotroph by transformation, the DNA was stably maintained as an extrachromosomal element (29). Furthermore, there was no apparent interaction between the plasmid-encoded DNA and the chromosomal genes. However, in this experiment, the primary selection was for the presence of the plasmid, and then those plasmid-containing cells were screened for the ability to grow in the absence of proline. This experiment inadvertently selected

for intact plasmids, because only cells that possessed plasmid markers were analyzed for the maintenance of gonococcal genes. Nevertheless, these data indicate that plasmid-encoded gonococcal chromosomal DNA can be stably maintained as a plasmid in *N. gonorrhoeae*. When the gene encoding pilin biosynthesis is cloned from *N. gonorrhoeae* PGH3-2 into pLES2, the plasmid is unable to transform *N. gonorrhoeae* PGH3-2 to produce stable transformants, even when properly methylated (Stein, unpublished). This indicates that not all gonococcal genes can be maintained as plasmid-encoded traits.

As an alternative method for introducing recombinant plasmids into *N. gonorrhoeae*, I have cloned a gene encoding a nalidixic acid-resistant form of the gonococcal DNA gyrase onto a plasmid that does not replicate in *N. gonorrhoeae*. When this recombinant plasmid was introduced into *N. gonorrhoeae* by transformation, nalidixic acid-resistant transformants were obtained. When these transformants were screened for the presence of vector sequences by Southern hybridization techniques, no traces were found in the cells. This indicates that plasmid-encoded DNA can interact with chromosomal DNA (Stein, unpublished). This interaction between plasmid and chromosomal sequences occurs even when host-mediated restriction prevents the acquisition of plasmids.

Host-mediated restriction has proven to be an effective barrier in preventing *N. gonorrhoeae* from acquiring plasmids via transformation (25). It is the presence of these enzymes that probably accounts for the deletions seen in plasmids that are introduced into *N. gonorrhoeae* by transformation (22). Although this restriction can be overcome by in vitro methylation (25), the lack of purified methylases has precluded their use in modifying DNA to allow for successful transformation. Host-mediated restriction does not seem to be a barrier to genetic transfer of plasmid DNA via conjugation between different *Neisseria* strains (26). By using RP1 derivatives, it should be possible to introduce any gene, cloned in pLES2, from *E. coli* into *N. gonorrhoeae* by conjugation.

FUTURE STRATEGIES

Researchers in many laboratories would like to study the role of certain cellular components in the disease process by constructing mutants that lack these factors and then using the mutants in specific test systems. Chemical mutagenesis of *N. gonorrhoeae* is inefficient because (i) the dose of most mutagens required to produce a mutation is identical to the lethal dose (5), and (ii) multiple mutations may occur. This has forced researchers in most laboratories to study the genes of interest by cloning and characterizing them in *E. coli*. An alternative strategy would be to apply the techniques of in vitro mutagenesis to the cloned genes and then introduce these mutations into the gonococcal chromosome. Koomey et al. (13, 14) have introduced two different cloned genes into the gonococcal chromosome by transformation-mediated marker rescue. In both cases, they inserted a selectable marker (*bla*) into the gene they were trying to mutagenize. One possible problem with this strategy is that DNA must be inserted into the gene under study. In many cases, the insertion of a foreign gene into the chromosome may block or interfere with the natural processes that regulate the genes expression.

Described below is one strategy that might be applied for the in vivo construction of mutants of *N. gonorrhoeae*. Gonococcal DNA that has been inserted into plasmids

TABLE 1. Transformation of FA5100 with plasmid-encoded chromosomal genes

DNA added ^a	No. of transformants generated ^b		
	Nal ^c	L8 ^d	Nal ^c + L8 ^e
None	$<4 \times 10^{-9}$	$<4 \times 10^{-9}$	NT ^f
pSY6	3.8×10^{-9}	$<4 \times 10^{-9}$	$<2.6 \times 10^{-5}$
pL81	$<4 \times 10^{-9}$	1.6×10^{-3}	NT
pL81 + pSY6	NT	1.6×10^{-3}	6.5×10^{-1}

^a Approximately 1 ng of plasmid DNA was added for each transformation.

^b Transformants were generated by adding DNA to ca. 5×10^7 cells and allowing cells to express transforming DNA for 6 h before plating on GCK agar.

^c Nalidixic acid-resistant transformants were detected by plating cells on GCK agar plus 1 μ g of nalidixic acid per ml.

^d L8 reactive cells were those that reacted with monoclonal antibody 2-1-L8 under the conditions described by Stein et al. (27).

^e These are nalidixic acid-resistant colonies that reacted with monoclonal antibody 2-1-L8.

^f NT, Not tested.

incapable of replication in *N. gonorrhoeae* is still able to transform *N. gonorrhoeae*. In addition, when this DNA transforms *N. gonorrhoeae*, the cloned DNA recombines with the chromosome to produce stable transformants. This recombination event happens even if the DNA has had small alterations in its coding sequence, and it also occurs in the presence of host-mediated restriction (Stein, unpublished). By altering cloned DNA through in vitro mutagenesis techniques before introducing the DNA into *N. gonorrhoeae* by transformation, it should be possible to introduce precise mutations into the gonococcal chromosome. This procedure is limited only by our ability to select for the desired transformation event.

A DNA fragment that is able to complement a lipooligosaccharide-defective strain (11, 21) of *N. gonorrhoeae*, FA5100, has been cloned in the cosmid cloning vector, pHCT9. When this plasmid (pL81) is introduced into FA5100, it is able to correct the defect and allow for the synthesis of parental LOS (E. F. Petricoin and D. C. Stein, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, D187, p. 102). Positive transformants were identified by blotting transformed cells onto nitrocellulose and determining which ones reacted with a specific monoclonal antibody. The best transformation frequencies seen in this system approached 1% (Stein, unpublished), and so several hundred colonies had to be analyzed before a single transformant with the altered trait could be isolated. It seemed probable that if a congression system could be worked out for *N. gonorrhoeae*, it would dramatically increase the likelihood of isolating the desired event.

The data presented in Table 1 illustrate how two cloned genes have been used in a congression experiment to introduce specific DNA fragments into the gonococcal chromosome, even in the absence of the ability to select directly for one of the fragments. This experiment takes advantage of the fact that a gonococcal cell is able to take up more than one piece of DNA. Plasmid pSY6, a plasmid encoding a nalidixic acid resistance gene, is able to transform *N. gonorrhoeae* FA5100 to generate nalidixic acid-resistant transformants. When the concentration of pSY6 used to transform *N. gonorrhoeae* is limiting and the concentration of a second plasmid encoding the nonselective trait is saturating, 65% of the nalidixic acid-resistant transformants also acquire the nonselective trait. In the experiment described in Table 1, hundreds of FA5100 transformants were isolated that had taken up DNA fragments from pL81. In the absence of this

type of analysis, much time was required to isolate transformants of FA5100 that were transformed with the DNA fragment, because the identification procedure was a screening procedure rather than a selection procedure and so all the cells had to be analyzed to determine which were transformants. By having many transformants to analyze, it is now possible to determine the effects of the cloned DNA on lipooligosaccharide production.

This type of experiment can be applied to introduce a variety of nonselective mutations into the gonococcal chromosome. For example, a variety of surface proteins have been cloned from various strains of *N. gonorrhoeae*. However, the mechanism by which their antigenic variation is controlled is unknown. By making specific mutations in the cloned gene before introducing it into *N. gonorrhoeae*, one can determine the role that specific DNA sequences play in regulating the antigenic variation. The usefulness of the congression experiment will be limited by the ability to clone the desired genes and the amount of mismatch, insertion, or deletion the gonococcal recombination system will tolerate.

CONCLUSIONS

The current data indicate that introduction of cloned genes into *N. gonorrhoeae* is not as simple as was initially thought. Owing to the presence of efficient restriction systems, plasmids may be introduced only into strains that lack the enzyme or that do not possess the recognition sequence in their DNA. These problems can be overcome by a judicious choice of strains or through in vitro DNA methylation. Most of the DNA methylases are not commercially available, and their purification is difficult. In addition, no true restriction-negative strains have been described. The transformation-congression experiment has great promise for the introduction of specific mutations, but more work must be done to determine the limits of the system. The final limitation will be in our ability to clone the desired genes.

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LITERATURE CITED

- Biswas, G. D., J. Graves, T. E. Sox, F. C. Tenover, and P. F. Sparling. 1982. Marker rescue by homologous recipient plasmid during transformation of gonococci by a hybrid *Pc'* plasmid. *J. Bacteriol.* **151**:77-82.
- Biswas, G. D., T. Sox, E. Blackman, and P. F. Sparling. 1977. Factors affecting genetic transformation of *Neisseria gonorrhoeae*. *J. Bacteriol.* **129**:983-992.
- Biswas, G. D., and P. F. Sparling. 1981. Entry of double-stranded deoxyribonucleic acid during transformation of *Neisseria gonorrhoeae*. *J. Bacteriol.* **145**:638-640.
- Burnstein, K. L., and P. F. Sparling. 1988. Specificity of DNA uptake by gonococci, p. 231-234. In J. T. Poolman, H. C. Zanen, T. F. Meyer, J. E. Heckels, P. R. H. Makela, H. Smith, and E. C. Beuvery (ed.), *Meningococci and gonococci*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Campbell, L. A., and R. E. Yasbin. 1984. Mutagenesis of *Neisseria gonorrhoeae*: absence of error-prone repair. *J. Bacteriol.* **160**:288-293.
- Cannon, J. G., and P. F. Sparling. 1984. Genetics of the gonococcus. *Annu. Rev. Microbiol.* **38**:111-113.
- Flett, F., G. O. Humphreys, and J. R. Saunders. 1981. Intra-specific and intragenic mobilization of non-conjugative resistance plasmids by a 24.5 megadalton conjugative plasmid of *Neisseria gonorrhoeae*. *J. Gen. Microbiol.* **125**:123-129.
- Genco, C. A., J. S. Knapp, and V. L. Clark. 1984. Conjugation of plasmids of *Neisseria gonorrhoeae* to other species: potential reservoirs for the beta-lactamase plasmid. *J. Infect. Dis.* **150**:397-401.
- Gouby, A., G. Bourg, and M. Ramuz. 1986. Previously undescribed 6.6-kilobase R plasmid in penicillinase-producing *Neisseria gonorrhoeae*. *Antimicrob. Agents Chemother.* **29**:1095-1097.
- Graves, J. F., G. D. Biswas, and P. F. Sparling. 1982. Sequence-specific DNA uptake in transformation of *Neisseria gonorrhoeae*. *J. Bacteriol.* **152**:1071-1077.
- Guymon, L. F., M. Esser, and W. M. Shafer. 1982. Pyocin-resistant mutants of *Neisseria gonorrhoeae*: alterations in sensitivity of normal human serum and polymyxin B. *Infect. Immun.* **36**:541-547.
- Johnson, S. R., B. E. Anderson, J. W. Biddle, G. H. Perkins, and W. E. DeWitt. 1983. Characterization of concatemeric plasmids of *Neisseria gonorrhoeae*. *Infect. Immun.* **40**:843-846.
- Koomey, J. M., and S. Falkow. 1987. Cloning of the *recA* gene of *Neisseria gonorrhoeae* and construction of gonococcal *recA* mutants. *J. Bacteriol.* **169**:790-795.
- Koomey, J. M., R. E. Gill, and S. Falkow. 1982. Genetic and biochemical analysis of gonococcal IgA1 protease: cloning in *Escherichia coli* and construction of mutants of gonococci that fail to produce the activity. *Proc. Natl. Acad. Sci. USA* **79**:7881-7885.
- Morse, S. A., S. R. Johnson, J. W. Biddle, and M. C. Roberts. 1986. High-level tetracycline resistance in *Neisseria gonorrhoeae* is due to the acquisition of the streptococcal *TetM* determinant. *Antimicrob. Agents Chemother.* **30**:664-670.
- Phillips, I. 1976. Beta-lactamase producing penicillin-resistant gonococcus. *Lancet* **ii**:656-657.
- Piffaretti, J. C., A. Arini, and J. Frey. 1988. pUB307 mobilizes resistance plasmids from *Escherichia coli* into *Neisseria gonorrhoeae*. *Mol. Gen. Genet.* **212**:215-218.
- Roberts, M., and S. Falkow. 1977. Conjugal transfer of R plasmids in *N. gonorrhoeae*. *Nature (London)* **266**:630-631.
- Roberts, M., P. Piot, and S. Falkow. 1979. The ecology of gonococcal plasmids. *J. Gen. Microbiol.* **114**:491-494.
- Seifert, H. S., and M. So. 1988. Genetic mechanisms of bacterial antigenic variation. *Microbiol. Rev.* **52**:327-336.
- Shafer, W. M., K. Joiner, L. F. Guymon, M. S. Cohen, and P. F. Sparling. 1984. Serum sensitivity of *Neisseria gonorrhoeae*: the role of lipopolysaccharide. *J. Infect. Dis.* **149**:175-183.
- Sox, T. E., W. Mohammed, and P. F. Sparling. 1979. Transformation-derived *Neisseria gonorrhoeae* plasmids with altered structure and function. *J. Bacteriol.* **138**:510-518.
- Sparling, P. F. 1966. Genetic transformation of *Neisseria gonorrhoeae* to streptomycin resistance. *J. Bacteriol.* **92**:1364-1371.
- Sparling, P. F., G. D. Biswas, and T. E. Sox. 1977. Transformation of the gonococcus, p. 155-176. In R. R. Roberts (ed.), *The gonococcus*. John Wiley & Sons, Inc., New York.
- Stein, D. C., S. Gregoire, and A. Piekawicz. 1988. Restriction of plasmid DNA during transformation but not conjugation in *Neisseria gonorrhoeae*. *Infect. Immun.* **56**:112-116.
- Stein, D. C., S. A. Gregoire, and A. Piekawicz. 1988. Role of restriction and modification on genetic exchange in *Neisseria gonorrhoeae*, p. 323-327. In J. T. Poolman, H. C. Zanen, T. F. Meyer, J. E. Heckels, P. R. H. Makela, H. Smith, and E. C. Beuvery (ed.), *Meningococci and gonococci*. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Stein, D. C., E. F. Petricoin, J. M. Griffiss, and H. Schneider. 1988. Use of transformation to construct *Neisseria gonorrhoeae* strains with altered lipooligosaccharides. *Infect. Immun.* **56**:762-765.
- Stein, D. C., L. E. Silver, V. L. Clark, and F. E. Young. 1983. Construction and characterization of a new shuttle vector, pLES2, capable of functioning in *Escherichia coli* and *Neisseria gonorrhoeae*. *Gene* **25**:241-247.
- Stein, D. C., L. E. Silver, V. L. Clark, and F. E. Young. 1984. Cloning genes for proline biosynthesis from *Neisseria gonor-*

- rhoeae*: identification by interspecific complementation of *Escherichia coli* mutants. J. Bacteriol. **158**:696-700.
30. Stein, D. C., F. E. Young, F. C. Tenover, and V. L. Clark. 1983. Characterization of a chimeric β -lactamase plasmid of *Neisseria gonorrhoeae* which can function in *Escherichia coli*. Mol. Gen. Genet. **189**:77-84.
31. Tenover, F. C., D. C. Stein, F. E. Young, and V. L. Clark. 1985. Construction and characterization of chimeric β -lactamase plasmids of *Neisseria gonorrhoeae* with altered ability to be mobilized during conjugation. Sex. Transm. Dis. **12**:76-82.
32. Van Embden, J. D. A., M. Dessen-kroon, and B. Van Klingeren. 1985. A new β -lactamase plasmid in *Neisseria gonorrhoeae*. J. Antimicrob. Chemother. **15**:247-258.
33. West, S. E. H., and V. L. Clark. 1989. Genetic loci and linkage associations in *Neisseria gonorrhoeae* and *Neisseria meningitidis*. Clin. Microbiol. Rev. **2**(Suppl.):S92-S103.