Uptake-sequence-independent DNA transformation exists in Neisseria gonorrhoeae

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INTRODUCTION

Neisseria gonorrhoeae (the gonococcus) and Neisseria meningitidis are important obligate human pathogens that are naturally competent for DNA-mediated transformation (Catlin & Cunningham, 1961; Sparling, 1966). Transformation is probably the principal means of exchange of chromosomal markers between neisserial strains, since other means of chromosomal DNA transfer have not been observed (Cannon & Sparling, 1984). The mosaic nature of certain genes, most of which encode virulence determinants (Frosch & Meyer, 1992; Halter et al., 1989; Spratt et al., 1992), suggests that transformation occurs readily in nature and is likely to play a key role in obtaining genetic diversity. In addition, evidence has been provided to support the idea that gonococcal DNA transformation has a role in promoting the diversity of pili (Gibbs et al., 1989; Norlander et al., 1979; Seifert et al., 1988), Opa proteins (Frosch & Meyer, 1992; Schwalbe & Cannon, 1986) and IgA₁ protease (Frosch & Meyer, 1992). These virulence factors are crucial in maintaining these organisms in the human population.

Gonococci are competent for DNA transformation during all phases of growth (Biswas et al., 1977; Sparling, 1966). DNA transformation in the gonococcus involves binding of DNA to cells (Dougherty et al., 1979), followed by DNase-resistant uptake (Sparling, 1966) and homologous recombination (Koomey & Falkow, 1987). Several genetic determinants involved in DNA uptake (dud-1 or pilT; Biswas et al., 1989; Koomey et al., 1994), DNA transport (comA; Facius & Meyer, 1993), processing (utr; Biswas et al., 1989) and recombination (recA; Koomey & Falkow, 1987) have been identified. Outer-membrane proteins that bind gonococcal DNA have been described (Doward & Garon, 1989), but a receptor for DNA remains undefined.

Gonococci are most efficiently transformed by genus-specific DNA (Dougherty et al., 1979) and this process is presumed to involve a receptor that recognizes a specific 10 bp gonococcal transformation uptake sequence (GCUS) (Elkins et al., 1991; Goodman & Scocca, 1988). This sequence occurs frequently on gonococcal DNA, often as a part of transcriptional terminators (Goodman & Scocca, 1988). However, evidence has also been obtained for lower efficiency gonococcal transformation by DNA that does not contain the GCUS (Stein, 1991), suggesting that the GCUS is not absolutely required for gonococcal DNA uptake. Moreover, gonococci can transport DNA molecules that do not contain this DNA sequence (Burnstein et al., 1988).

An interesting feature of gonococcal transformation is the relationship between transformation efficiency and piliation phenotype or production of pili, the major...
structural subunit of pili. Gonococci undergo phase variation, often reversibly, between piliated (P⁺) and nonpiliated (P⁻) states (Kellogg et al., 1963; reviewed by Seifert, 1992). Two general forms of P⁺ variants have been described: those that do not produce pilin and those that produce pilin monomers that are not assembled into pili (Swanson et al., 1985). Whereas all P⁺ gonococci can be efficiently transformed, nonreverting (pilin-non-producing) P⁻ variants are transformed less efficiently (Biswa et al., 1977; Sparling, 1966; Swanson et al., 1990). P⁺ variants which produce pilin monomers can be transformed at intermediate levels (Gibbs et al., 1989; Zhang et al., 1992), suggesting that the pilin protein facilitates the transformation process. The physical uptake of DNA in gonococci has been correlated with piliation, the production of the pilin monomer, and presence of the GCUS on transforming DNA; however, no direct relationship has been established between pilin production and recognition of the GCUS.

We have previously reported that cloned gonococcal DNA fragments carrying nonhomologous insertions transformed with greater efficiency when the size of the homologous fragment was increased relative to the size of the nonhomologous fragment (Boyle-Vavra & Seifert, 1993). Moreover, increased levels of input DNA also improved transformation efficiencies (Boyle-Vavra & Seifert, 1993). In this report, the dose-response of gonococcal transformation to various amounts of cloned DNA containing a heterologous cat insertion was studied. Since a saturation curve consisting of two portions was observed, we investigated the role of the GCUS and pilin phenotype in transformation at different DNA concentrations.

**METHODS**

**Strains and media.** Gonococci were grown on GC medium base (Difco) with added supplements (Kellogg et al., 1963) (GCB) at 37°C with 5% (v/v) CO₂. Chloramphenicol (Cm) was used at 10 µg ml⁻¹. MS11-A is a P⁺ gonococcal strain which expresses the pilin gene from two pilin expression loci, pilE1 and pilE2 (Meyer et al., 1984). MS11-B2 is a nonreverting P⁻ variant of MS11-A with a 1.4 kb deletion in both pilE1 and pilE2 (see Fig. 3) (Segal et al., 1986). MS11Gm20 is a derivative of MS11-A which contains the mini-transposon mTnCm (Seifert et al., 1990) inserted in pilE2.

**Plasmids.** pNG1721CAT contains a 10.2 kb gonococcal insert from pNG1721 (Meyer et al., 1984) containing a promoterless 780 bp Cm resistance marker (encoded by the cat gene) in the MrrI site of pilE2 (Taha et al., 1988). At least one GCUS is present downstream of the pilE locus (data not shown). pNG1721CAT was prepared by either Triton lysis (Clewell & Helinski, 1969) or alkaline lysis (Birnboim & Doly, 1979) followed by CsCl/ethidium bromide purification (Sambrook et al., 1989).

**DNA transformation.** Transformation was performed as described by Seifert & So (1991). Bacteria were grown on solid GCB medium for 18 h and swabbed into unsupplemented GC liquid medium containing 5 mM MgCl₂ (GCB-Mg) at 37°C. The cell density was adjusted to about 10⁸ c.f.u. ml⁻¹ and the suspension diluted 1:10 into GCB-Mg at 37°C containing the indicated concentrations of transforming DNA and GCUS competitor. After 10 min at 37°C, the cell/DNA suspension was diluted 1:10 into pre-warmed Kellogg-supplemented liquid GCB and incubated at 37°C in 5% CO₂ for 4-5 h to allow for phenotypic expression of Cm resistance.

**RESULTS**

**Dose-response of transformation of P⁺ strain MS11-A**

Our previous experiences with the transposon shuttle mutagenesis system had suggested that higher amounts of transforming DNA led to higher levels of gonococcal transformation (Boyle-Vavra & Seifert, 1993). To investigate this further, a dose-response curve for transformation with pNG1721CAT was generated. This plasmid contains 10.2 kb of cloned gonococcal DNA consisting of pilE2 and its flanking sequences (Meyer et al., 1984) and carries a promoterless cat gene downstream of the transcriptional start point of pilE2 (Taha et al., 1988). After binding and uptake of pNG1721CAT by gonococci, the cat gene is integrated into the pilE2 region of the chromosome by homologous recombination. Since pNG1721CAT does not replicate in gonococci, Cm resistance in transformants is expressed from the chromosome.

Various amounts of plasmid DNA were used in transformations of the highly competent gonococcal strain MS11-A, which contains two pilin expression loci (Meyer et al., 1984) and demonstrates stable piliation. Surprisingly, the dose-response curve produced with pNG1721CAT in MS11-A (Fig. 1) was different from
dose-response curves previously described (Biswas et al., 1977; Sparling, 1966). At low amounts of DNA, large increases in transformation frequency were seen in response to small changes in DNA concentration (Fig. 1). This was followed by an apparent saturation of transformation between 2 and 10 μg of DNA ml⁻¹. This first level of saturation was followed by a slight decrease in frequency at 20 μg pNG1721CAT ml⁻¹ followed by a gradual increase in frequency with increasing DNA concentration (producing a gradual slope) reaching a final plateau between 40 and 70 μg DNA ml⁻¹. At this concentration of DNA, as many as 39% of the cells exposed to the plasmid became Cm resistant (CmR); to our knowledge, this represents the highest level of transformation ever reported for the gonococcus.

The ability of pNG1721CAT to produce two levels of saturation for transformation was different from the single level of saturation previously reported when chromosomal DNA containing a streptomycin (Str) resistance marker (Biswas et al., 1977; Sparling, 1966) was used. Such differences in results could be due to the selectable marker used to produce transformants or the physical form of the transforming DNA, i.e. chromosomal vs plasmid. To distinguish between these possibilities a dose-response curve of MS11-A transformation was done by transforming MS11-A with pNG1721CAT at concentrations from different portions of the transformation dose-response curve in the presence of a 1000-fold molar excess of a competing oligomer encoding the GCUS. Transformants were selected on 10 μg Cm ml⁻¹. The data for MS11-A transformation without the GCUS also appear in the dose-response curve in Fig. 1. Transformations of MS11-A were repeated at least three times and transformations of MS11-B2 were repeated twice. The data for transformation of MS11-B2 at 1 μg DNA ml⁻¹ represent transformants obtained in one of two experiments. All measurements of c.f.u. were performed in triplicate. Error bars indicate SEM.

**Fig. 1.** Dose-response curve for gonococcal transformation with a pil-cat marker. The P⁺ gonococcal strain MS11-A was transformed with varying amounts pNG1721CAT plasmid DNA (triangles) or chromosomal DNA (circles) isolated from MS11Cm20 and transformants were selected on 10 μg Cm ml⁻¹. Each data point represents the mean of between three and seven experiments. Error bars indicate SEM.

levels of saturation (Fig. 1). The ability of plasmid and not chromosomal DNA to produce a second rise in transformation frequency is attributed to the homogeneous single gonococcal fragment in the plasmid DNA as opposed to heterogeneous chromosomal DNA, which has a low molar ratio of the transforming fragment relative to other competing chromosomal fragments.

**Competition for transformation of P⁺ cells by the GCUS**

Since the GCUS has been shown to be utilized for efficient DNA uptake during transformation (Elkins et al., 1991; Goodman & Scocca, 1988) we tested whether both portions of the transformation dose-response curve were dependent on uptake promoted by the GCUS. This was done by transforming MS11-A with pNG1721CAT at concentrations from different portions of the transformation dose-response curve in the presence of a 1000-fold molar excess (over transforming DNA) of double-stranded oligomer carrying two inverted copies of the GCUS. Using 1 and 10 μg DNA ml⁻¹, the GCUS-oligomer was able to partially inhibit transformation of the P⁺ strain, by 100-fold and 10-fold, respectively (Fig. 2). This is similar to results previously obtained in identifying and characterizing the GCUS (Elkins et al., 1991; Goodman & Scocca, 1988). However, at 40 μg pNG1721CAT ml⁻¹, the presence of excess GCUS produced transformation frequencies similar to those obtained without the GCUS (Fig. 2). Assuming that 1000-fold molar excess of the competitor was sufficient to
inhibit the GCUS-dependent process, the transformants obtained in the presence of the GCUS-containing oligomer were produced mainly by a GCUS-independent mechanism. These data suggest that two distinct processes were occurring in different portions of the transformation dose-response curve. One process, showing apparent saturation at approximately 2 μg DNA ml⁻¹, was inhibited by the GCUS. The second process, showing saturation at higher levels of DNA, was not inhibited by the GCUS.

**Association between piliation (or pilin production) and recognition of the GCUS**

To test whether a relationship exists between recognition of the GCUS and production of pilus or pilus fibres in recipient bacteria, a pilin-nonproducing strain was transformed in the absence or presence of the GCUS oligomer. The P⁻ strain, MS11-B2, is nonreverting due to 1.4 kb deletions in both pilin expression loci (Segal et al., 1986) and therefore is a stable pilin-nonproducing variant. DNA transformation of MS11-B2 with 1, 10 and 40 μg pNG1721CAT ml⁻¹ in the absence of the GCUS competitor produced a dose-response with frequencies increasing from 10⁻⁸ to 10⁻⁷ CmR transformants per c.f.u. (Fig. 2) with increasing amounts of DNA. These low transformation frequencies are not attributed to poor recombination at the deleted chromosomal pil loci of strain B2 since this strain shows similar frequencies when transformed by DNA containing a point mutation that confers spectinomycin resistance (H. S. Seifert, unpublished). Also, these frequencies are similar to those previously reported for other nonreverting P⁻ strains (Biswas et al., 1977; Sparling, 1966). In competition experiments with the GCUS, a 1000-fold molar excess of the GCUS competitor did not inhibit transformation of MS11-B2 by pNG1721CAT at any DNA concentration tested. These data indicate that in the absence of pilin production or elaboration of pilin fibres transformation can occur by a GCUS-independent route.

Since some of the CmR transformants of MS11-B2 were obtained at a frequency that approached the spontaneous mutation rate (although CmR colonies were not observed at this level without added DNA), Southern analysis was performed with pil and cat gene probes to test whether the CmR colonies obtained were true transformants (Fig. 3). In blots probed with a pil gene probe, MS11-A contains the two expected bands (Meyer et al., 1984) containing pilin expression at loci at 4.2 (pilE2) and 4.0 kb (pilEI) (Fig. 3a). The additional hybridizing bands in MS11-A represent partial, unexpressed, silent pil loci (PilS) (Haas & Meyer, 1986; Haas et al., 1992). MS11-B2 contains deletions in both pilin expression loci and exhibits pil-hybridizing bands at 2.6 and 2.8 kb which correspond to deleted forms of pilE2 and pilEI, respectively (Fig. 3a). The additional hybridizing bands in MS11-A represent partial, unexpressed, silent pil loci (pilS) (Haas & Meyer, 1986; Haas et al., 1992). MS11-B2 contains deletions in both pilin expression loci and exhibits pil-hybridizing bands at 2.6 and 2.8 kb which correspond to deleted forms of pilE2 and pilEI, respectively (Fig. 3a). The deleted form of pilE2 in the MS11-B2 parent comigrates with pilS, producing an apparently darker pil band at the 2.8 kb position. In contrast, the MS11-B2 transformants in lanes a, b, c and d do not exhibit the darker band at 2.8 kb, indicating that cat was inserted into the pilE2 locus of the 2.8 kb band, which altered the band’s mobility. Probing with the cat gene revealed that each transformant contains a cat gene in a band of a size consistent with a restored pilin expression locus migrating with or close to pilS (Fig. 3b). The missing fragments corresponding to pilS (Haas et al., 1992) in lanes a, b and

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**Fig. 3.** Southern blot of MS11-B2 transformants of pNG1721CAT. Chromosomal DNA was isolated from MS11-A (M), MS11-B2 (B2), and four MS11-B2 CmR transformants (a–d) and digested with BspDl (a Clal isoschizomer). Blots were probed as described in Methods with either (a) a pilin gene probe, or (b) a cat gene probe. Hybridizing silent pilin copies (pilS) or pilin expression loci (pil/ε) are indicated on the left with their corresponding sizes in kb. The deleted pilEl (ΔpilEl) and deleted pilE2 (*) bands appear in all the MS11-B2 variants at 2.6 and 2.8 kb, respectively, with deleted pilE2 co-migrating with pilS5. pilE2-cat (#) comigrates with pilSl. pilS7 is not shown but hybridizes equally well in all lanes. The band appearing at the top (>) of lanes with DNA from MS11-B2 variants is of unknown origin and variably appears in BspDl digests of gonococcal chromosomal DNA.
indicating that pil sequences in pilS6 were involved in homologous pairing during the recombination reaction which resulted in the insertion of cat into pilE2. All of pilS6 is upstream of pilE2 in the gonococcal chromosome and a portion is carried on pNG1721CAT (Haas et al., 1992; Meyer et al., 1984). The transformants in lanes a, b and d probably arose by homologous recombination between the silent sequences upstream of pilE2 in pNG1721CAT and homologous sequences in the chromosomal pilS6 locus resulting in crossing out of the intervening DNA between pilS6 and the upstream silent copies of pilE2, including the BspDl site. We have previously noted similar pairing reactions between partial pilin copies of pilS1 and pilE1 (Seifert et al., 1988). A simpler transformant is represented in Fig. 3(b), lane c, where the cat gene inserted into pilE2 as a result of recombination between pilE2 sequences in pNG1721CAT and the chromosome. These patterns are consistent with the proposed locations of homologous pairing and each transformant tested was therefore a true recombinant that had repaired the deletion of pilE2 with input pNG1721CAT DNA. While the frequency of transformation of this P+ variant was lower than that of the P- variant, the transformants are the result of homologous recombination representative of true transformation events.

DISCUSSION

We present several lines of evidence to indicate that two processes of transformation, which differ in their dependence on the GCUS, might function in gonococci. First, a dose-response study of plasmid DNA transformation of P+ gonococci showed saturation of transformation at low and high DNA concentrations, indicating two processes occurring in P+ cells. Second, as evidenced by the ability of the GCUS to reduce transformation frequency, a GCUS-dependent process was only evident in P+ cells and only at DNA concentrations from the first portion of the dose-response curve. Third, the GCUS-competition results indicated that transformation by a GCUS-independent process was present in both P+ and P-, pilin-nonproducing cells at all levels of transforming DNA.

Only the GCUS-independent mechanism was apparent in both P+ and P- cells. In the P+ background, if the GCUS-independent transformation does not require a threshold level of DNA, the transformants obtained at lower DNA concentrations in the presence of the GCUS represent those obtained by the GCUS-independent mechanism (Fig. 2). Thus, at these low levels of DNA in the absence of competitor, both the GCUS-dependent and GCUS-independent mechanisms might function concurrently. The slight decrease in transformation frequency of the P+ strain observed at 20 µg pNG1721CAT ml⁻¹ (Fig. 1) indicates that transformation using the putative high-affinity receptor is inhibited at concentrations above the saturation point, at which time transformation by the second mechanism is apparent.

Since the GCUS did not decrease transformation frequencies of the P+ strain to levels as low as that of the P- strain at any level of pNG1721CAT, it can be assumed that the GCUS-independent mechanism is more efficient in the P+ background. Although the GCUS-independent mechanism appears less efficient in P- cells than in P+ cells, it still results in very high transformation frequencies at high DNA concentrations in P- cells. Since we have also observed that transformation of the P- population in a reverting P- gonococcal strain can be inhibited by the GCUS-containing oligomer (data not shown), it is likely that both pathways also operate in P-, pilin-producing backgrounds.

The GCUS-dependent mechanism was associated with a steep rise in the dose-response curve and apparent saturation at low amounts of DNA. The GCUS-independent mechanism in the P- background was associated with a more gradual slope. Therefore, we hypothesize that the GCUS-dependent process could utilize a higher-affinity receptor. It seems likely that the GCUS-independent mechanism involves binding of DNA to a specific lower-affinity receptor rather than a non-specific process since this form of transformation is saturable in P+ cells. Also, it is difficult to envision that large DNA molecules could penetrate two membranes and the peptidoglycan layer simply by passive diffusion. We cannot determine from these data whether these two mechanisms differ from each other at the stage of transformation which involves binding of DNA to the cell or at a subsequent stage.

Consistent with our hypothesis for a GCUS-independent mechanism, in addition to the well-recognized GCUS-dependent mechanism of DNA uptake, is the residual level of DNA uptake seen in the presence of competing GCUS-containing plasmid DNA observed by Elkins et al. (1991). Also in support of two pathways of gonococcal DNA transformation are earlier observations of biphasic curves both for the rate of DNase-resistant uptake of DNA (Sparling, 1966) and for the DNA-concentration-dependent dose-response of gonococcal transformation (Sparling et al., 1977). Our ability to obtain transformants at 70 µg DNA ml⁻¹ is also consistent with binding studies in gonococci which show saturation of binding of gonococcal DNA at this level (Dougherty et al., 1979). In Bacillus subtilis, similar to results in Fig. 1, the presence of low- and high-affinity receptors for DNA was demonstrated for binding of DNA to membrane vesicles at three levels of saturation at 1, 10 and 70 µg DNA ml⁻¹ (Joenje et al., 1975).

Swanson et al. (1990) previously characterized nonreverting P- gonococcal strains as noncompetent. The transformation protocol used by Swanson's group did not include an incubation period for the transformed cells to express antibiotic resistance, which could explain the lower frequencies they also observed for their P+ strains (10⁻⁴ transformants per c.f.u.; Swanson et al., 1990). The difference between this previous report and our findings could not be attributed to use of chromosomal DNA since we have observed similar transformation frequencies of a P+, nonreverting strain with chromosomal DNA (data not shown).
Since gonococci are highly autolytic (Hebeler & Young, 1975) and competent for DNA transformation (Sparring, 1966), it was suggested (Norlander et al., 1979) and subsequently shown (Gibbs et al., 1989; Seifert et al., 1988) that uptake of DNA released by lysed cells could be the source of DNA used in nonreciprocal pilin gene recombination reactions responsible for phase and antigenic variation of pili. The putative GCUS-independent mechanism we propose provides a mechanism by which P+ gonococci could be transformed in vivo by chromosomal DNA released from neighbouring cells, resulting in repair of a deleted pilin expression locus by recombination with an intact pilin expression locus in the transforming DNA. The involvement of transformation in pilin recombination reactions is more likely to occur if local DNA concentrations are high and the resultant P+ cells show a selective advantage over pilin-nonproducing, P− cells. We can not know what the biologically relevant concentrations of DNA are in a human but presume that in a colony there could be local DNA concentrations that match these relatively high concentrations.

Although pilin production has been associated with transformation competence in gonococci, neither pilin monomers nor pil have been shown to bind DNA or inhibit DNA transformation (Mathis & Scocca, 1984; Sparring et al., 1977). Thus, it has been suggested that other features of P+ cells, besides pil, are involved in the transformation process, such as pilus biogenesis components or pilus-associated proteins. Supporting this notion is the finding that several components involved in pilus biogenesis in *Pseudomonas*, which produce a similar pilin type (type 4) as gonococci, are homologous to competence genes in *Bacillus subtilis* and *Haemophilus influenzae* (Hobbs & Mattick, 1993). The mutated gene in *dad1* mutants (Biswas et al., 1989) responsible for decreased DNA uptake and transformation competence was recently identified as *pilT* (Koomey et al., 1994), a gene which also affects twitching motility. Although *dad1* mutants were originally identified as P+ (Biswas et al., 1989), quantitative and qualitative changes were noted in pilus expression of *pilT* mutants (Koomey et al., 1994). *pilC*, a pilus biogenesis component (Jonsson et al., 1991) that can be found as a component of pili (Rudel et al., 1995b), is essential for efficient gonococcal transformation, as shown by unusual *pilC* mutants that are capable of producing pili yet are not competent for transformation (Rudel et al., 1995a). Taken together with these previous studies, our results suggest that only wild-type expression of the pilus fibre and its biogenesis components confers the high level of DNA transformation efficiency that corresponds to the first portion of the dose-response curve.

We propose two models to explain the relationship between GCUS-dependent (and possibly GCUS-independent) DNA transformation and pilin production or pilus biogenesis: (1) the pilus or components of the pilus transport apparatus are required to present the GCUS receptor on the cell surface, or (2) the pilus fibre or pilus transport machinery is used to transport DNA into the cell once DNA has specifically bound to the cell surface. It is plausible that both models are correct and that full competence for transformation only occurs when both conditions are met. The identification of the GCUS receptor will help predict how the binding and transport of transforming DNA interacts with the pilus biogenesis apparatus.

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