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Sequence-Specific DNA Uptake in Transformation of *Neisseria gonorrhoeae*

JAMES F. GRAVES,[1]* GOUR D. BISWAS,[1] AND P. FREDERICK SPARLING[1,2]

Departments of Bacteriology and Immunology[1] and Medicine,[2] University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514

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Piliated, competent gonococci are known to preferentially take up homologous transforming DNA into the cell. We examined the mechanism for DNA uptake with pFA10, a hybrid 11.5-kilobase (kb) penicillin-resistant (Pc^r) plasmid composed of heterologous DNA from a 7.2-kb Pc^r plasmid and homologous DNA from a 4.2-kb gonococcal cryptic plasmid. The presence of the gonococcal cryptic plasmid DNA in the hybrid resulted in markedly increased transformation efficiencies in isogenic crosses as compared with the parent 7.2-kb Pc^r plasmid. Uptake of ^{32}P-end-labeled *MspI* or *TaqI* restriction fragments of the hybrid was limited to fragments entirely derived from the 4.2-kb gonococcal cryptic plasmid, indicating that DNA uptake was probably dependent on the presence of a specific DNA sequence. Since *Haemophilus* DNA did not inhibit transformation by the hybrid Pc^r plasmid, the gonococcal DNA uptake sequence is different from the known sequence involved in homologous DNA uptake by *Haemophilus* spp.

Considerable attention has been given to the mechanisms of genetic transformation of *Bacillus subtilis* and *Streptococcus pneumoniae*. These bacteria have not evolved a means to distinguish between their own DNA and that of other organisms at the level of uptake (11, 22). However, Sisco and Smith (20) proved that competent *Haemophilus influenzae* organisms preferentially recognize homologous DNA for uptake by interaction with an "uptake site" on the chromosome that occurs once in about every 4 kilobases (kb). These conclusions were reached after observing the uptake of labeled chromosome fragments from a hybrid plasmid that contained cloned *Haemophilus* chromosomal DNA. Subsequently, Danner et al. (4) determined that the uptake site on *Haemophilus* DNA is probably a nucleotide sequence of 11 base pairs (bp).

The demonstration of sequence-specific DNA uptake in *Haemophilus* cells was of interest to us because of the possibility that a similar mechanism might be involved in transformation of *Neisseria gonorrhoeae* cells. Dougherty et al. (6) showed that gonococci take up homologous DNA into a DNase-resistant state, but fail to take up a variety of heterologous DNAs. This finding was confirmed by Graves et al. (7). These experiments did not examine the mechanism of preferential uptake of homologous DNA and could not exclude effects due to differences in restriction-modification systems between the donor DNAs and the competent recipient cells.

We approached this problem by studying the uptake of DNA fragments from an 11.5-kb hybrid plasmid composed of 7.2 kb of heterologous DNA and 4.2 kb of homologous gonococcal cryptic plasmid DNA. The hybrid plasmid was prepared in host cells isogenic with the recipient, eliminating any possible effect of different restriction systems on transformation. The results demonstrated that gonococci selectively took up homologous DNA fragments and strongly suggested that the gonococcal DNA uptake sequence is different from the sequence required for uptake by *Haemophilus* spp.

MATERIALS AND METHODS

Bacterial strains and plasmids. Relevant strains are described in Table 1. The 11.5-kb hybrid penicillin-resistant (Pc^r) plasmid pFA10 was isolated previously (25); its structure is discussed in this paper. The origin of the naturally occurring 7.2-kb Pc^r plasmid pFA3 is uncertain, but it is not native to the gonococcus. The 7.2-kb gonococcal Pc^r plasmids were only recently observed in gonococci (16) and are very closely related to plasmids of similar size isolated in various *Haemophilus* species (9, 18). The guanine-plus-cytosine (G+C) content of the 7.2-kb Pc^r plasmid is about 40% (18), which is similar to *Haemophilus* DNA, but unlike gonococcal chromosomal DNA and the native 4.2-kb cryptic and 36-kb conjugative plasmids, which are all about 50% G+C (18, 24).

Media. Bacterial cells were cultured on Difco GC base agar medium plus supplements as described by Biswas et al. (3). CO_2 was provided to liquid cultures by the addition of 0.04% $NaHCO_3$ to the medium at the time supplements I and II were included.

TABLE 1. Gonococcal strains used

Strain	Plasmid content (kb)[a]	Description (reference)
F62	4.2 (pFA101)	Wild type
FA19	4.2 (pFA102)	Wild type
FA288	4.2 (pFA1), 7.2 (pFA3), 36 (pFA2)	Penicillinase-producing (Pc^r) clinical isolate
FA293	4.2 (pFA1), 36 (pFA2)	Penicillin-sensitive (Pc^s), cured derivative of FA288
FA514	11.5 (pFA10), 36 (pFA2)	Pc^r transformant, FA288 × FA293 (25)
FA534	None	*rif-7* (Rif^r) mutant of FA759
FA559	7.2 (pFA3)	Pc^r transconjugant, FA288 × FA534
FA675	11.5 (pFA10)	Pc^r transformant, FA514 × FA534
FA759	None	Wild type

[a] Plasmid designations are given in parentheses. pFA1, pFA101, and pFA102 are closely related cryptic plasmids; pFA2 is a conjugative plasmid; pFA3 is a naturally occurring beta-lactamase plasmid; pFA10 is a pFA3ΩpFA1 hybrid.

Preparation of DNAs and restriction endonuclease digestion. Chromosomal DNA was isolated by the method of Marmur (12). Plasmid DNA was isolated by alkali denaturation and phenol extraction of whole-cell DNA, followed by one or two cycles of ethidium bromide-cesium chloride density gradient centrifugation (1, 10). Restriction endonuclease digestions (*Hin*fI, *Msp*I, *Taq*I) were performed according to instructions supplied by the manufacturers (New England Biolabs and Bethesda Research Laboratories).

Transformation. The transformation protocol was similar to that described by Sox et al. (25).

Agarose gel electrophoresis. Electrophoresis was performed by the procedure of Meyers et al. (14) on agarose (0.7 to 2%, depending on the experiment) or polyacrylamide (5%) gels in a vertical gel box (E. C. Corporation) or on 1% agarose horizontal gels. Plasmid fragment sizes were determined by comparing their migration in gels with that of standard digests of phages (*Hae*III digestion of ϕX174; *Hin*dIII digestion of lambda).

Labeling of plasmid DNA. Samples of plasmid DNA (0.2 to 0.4 μg) were labeled with [α-^{32}P]dATP (Amersham Corp. and New England Nuclear) by nick translation (17). *Escherichia coli* DNA polymerase (Boehringer) and DP DNase (Worthington Diagnostics) were added before reaction mixtures were incubated at 18 to 22°C. After 3 to 5 h, trichloroacetic acid-precipitated samples from mixtures produced activities of 1×10^6 to 5×10^7 cpm/μg of plasmid DNA. Fractions of labeled DNA were collected from a 5-ml Sephadex G-100 column (Pharmacia), pooled, and used for hybridization.

Fragments of plasmid DNA (5 to 10 μg) produced by digestion with restriction endonucleases were end-labeled after removing the 5'-terminal phosphate with *E. coli* alkaline phosphatase (Bethesda Research) by incubation with [γ-^{32}P]ATP (New England Nuclear) and polynucleotide kinase (P-L Biochemicals) as described by Maxam and Gilbert (13). Most of the unincorporated label was removed by two ethanol precipitations and one wash of the pellet in ethanol.

DNA hybridization, fragment uptake assay, and autoradiography. DNA samples were transferred from agarose gels to sheets of cellulose-nitrate (Schleicher and Schuell) by the procedure of Southern (23). DNAs which were known to be homologous or heterologous to the probes were bound to cellulose-nitrate sheets as controls. Hybridization between the DNA on the Southern blots and the nick-translated probes was performed in a Kapek/Scotchpack heat-sealable pouch.

Fragments of end-labeled DNA taken up by competent cells were detected by a method similar to that used by Sisco and Smith (20). About 1.0 μg of end-labeled plasmid fragments in 0.1 or 1 ml of 10 mM Tris-hydrochloride (pH 7.6) was combined with 0.9 or 9.0 ml, respectively, of competent cells (about 10^8 colony-forming units per ml) in GC base plus 10 mM $MgCl_2$ and incubated for 5 to 15 min at 37°C to permit DNA uptake. Use of higher concentrations of competent cells often resulted in decreased uptake of DNA (unpublished data). Suspensions were then treated with DNase (100 μg/ml) on ice for 20 min, and the cells were carefully washed by centrifugation three times in cold medium. The final pellets were suspended in 0.5 ml of TES buffer (50 mM NaCl, 10 mM EDTA, 30 mM Tris, pH 8.0). Sodium lauryl sulfate was added to 1% (wt/vol), and the cells were incubated at 65°C for 10 min to complete lysis. Samples were then extracted three times with $CHCl_3$-phenol (1:1, vol/vol) before precipitation of the nucleic acid in ethanol. The nucleic acid pellets were dissolved in 25 μl of TES buffer in preparation for electrophoresis. Samples of appropriately diluted labeled fragments (control) and of labeled fragments reisolated from competent cells were electrophoresed on 1% horizontal agarose gels. Gels were blotted dry between filter paper (3MM, Whatman) with layers of paper towels under pressure of a mass of about 3 kg, similar to a protocol described by Smith and Birnstiel (21). Kodak X-Omat R film was exposed to filters (6 to 24 h) or dried gels (1 to 3 weeks) in the presence of a Cronex Lighting-Plus fast calcium tungstate X-ray screen at −70°C.

RESULTS

Structure of hybrid 11.5-kb Pc^r plasmid pFA10. Transformation of Pc^s recipient strain FA293, which contained the 4.2-kb cryptic plasmid pFA1 and the 36-kb conjugative plasmid pFA2, by the 7.2-kb Pc^r plasmid pFA3 resulted in occasional transformants with Pc^r plasmids larger than the donor plasmid, as previously reported (25). One of these (pFA10) was 11.5 kb. Introduction of pFA10 into strain FA293(pFA1, pFA2) by transformation or conjugation always resulted in loss of the recipient 4.2-kb plasmid pFA1 (data not shown). This apparent incompatibility between pFA10 and pFA1 suggested that pFA10 was a hybrid between the 7.2-kb Pc^r plasmid pFA3 and the 4.2-kb gonococcal plasmid pFA1. To test this possibility, we digested

pFA10, pFA3, and pFA1 with the restriction endonuclease *Taq*I and examined them by gel electrophoresis. Many fragments of common size were shared among these plasmids (Fig. 1). The *Taq*I digest of pFA10 exhibited fragments corresponding to all of those in pFA1, except for a 95-bp pFA1 fragment. All fragments of pFA3 apparently were present in pFA10, except for the largest 1.5-kb pFA3 fragment. We concluded that pFA10 was a hybrid between pFA3 and pFA1. The recombinational event which produced pFA10 occurred within the lost 95-bp pFA1 and 1.5-kb pFA3 *Taq*I fragments. Two fragments were present in digests of pFA10 which were not present in either pFA3 or pFA1; these fragments probably contained the junctions of pFA3 and pFA1. Use of other restriction endonucleases permitted construction of a physical map of pFA10 (Fig. 2). The structure of pFA10 was further confirmed by hybridization between either pFA1 or pFA3 and pFA10 (see Fig. 4) and by analysis of heteroduplexes formed between pFA3 and pFA10 (data not shown).

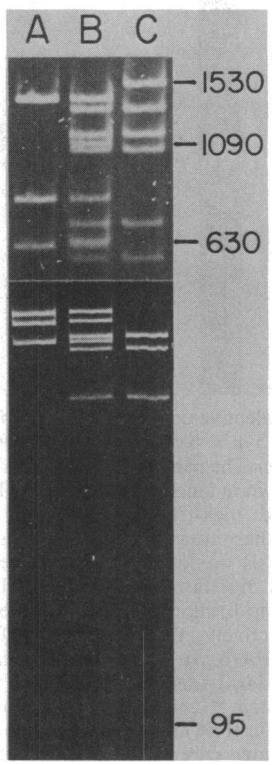

FIG. 1. *Taq*I restriction endonuclease analysis of 11.5-kb hybrid Pc^r plasmid pFA10 (pFA3ΩpFA1), 7.2-kb Pc^r plasmid pFA3, and 4.2-kb gonococcal cryptic plasmid pFA1. Plasmid digests were separated on a 1.2% agarose gel (top) and on 5% polyacrylamide (bottom) to show all fragments. Lanes: A, pFA1; B, pFA10; C, pFA3. Numbers indicate sizes of selected fragments (bp).

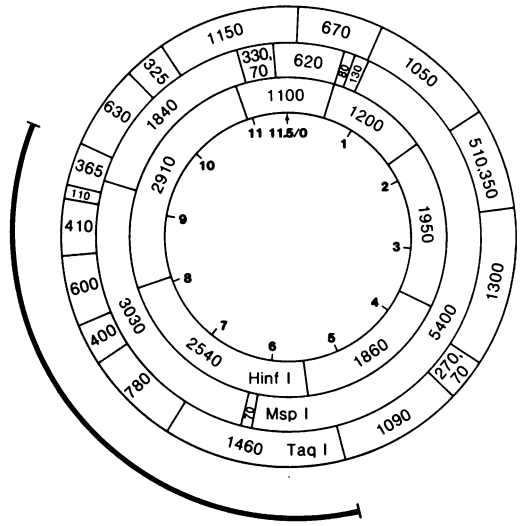

FIG. 2. Restriction endonuclease cleavage map of hybrid Pc^r plasmid pFA10 (pFA3ΩpFA1). The map is calibrated in kb units extending clockwise from the single *Hin*cII site (arrow) which is contained within the DNA of Pc^r Tn*2*. The heavy black line designates that part of pFA10 originating from pFA1 DNA; the remainder originated from pFA3 DNA. Approximately 70 bp of the hybrid were unaccounted for when fragment sizes were added. The map was composed by reference to a map of a 7.2-kb Pc^r plasmid (15) which is probably identical to pFA3 and to a map of a 4.2-kb plasmid in strain Um6601 (5). The 4.2-kb cryptic plasmid in Um6602 is very similar to pFA1 except for an additional *Msp*I site in the 400-bp *Taq*I fragment.

Increased transformation efficiency of the hybrid plasmid pFA10. Transformation efficiencies of the Pc^r plasmids pFA10 (pFA3ΩpFA1) and pFA3 were compared (Fig. 3). An isogenic strain (FA534) which did not contain any evident plasmids was used as the recipient to obviate the possible effects of marker rescue which can occur when pFA10 is introduced into recipients containing a homologous 4.2-kb plasmid (2). Transformation efficiencies (transformants per recipient per microgram of DNA) were 10- to 30-fold greater with pFA10 than with pFA3 at all concentrations of donor DNA tested.

Uptake of labeled pFA10 fragments. We postulated that the increased transformation efficiency of pfA10 as compared with pFA3 was due to the presence of the 4.2-kb pFA1 insert in pFA10; the presence of homologous pFA1 DNA in the hybrid plasmid may have provided a mechanism for increased DNA uptake. To determine whether a region of the hybrid contained an uptake site that was recognized by the competent cell, we incubated [γ-^{32}P]ATP-end-labeled restriction digest fragments of the hybrid briefly with competent cells, and DNA which resisted the addition of DNase was isolated, separated by agarose gel

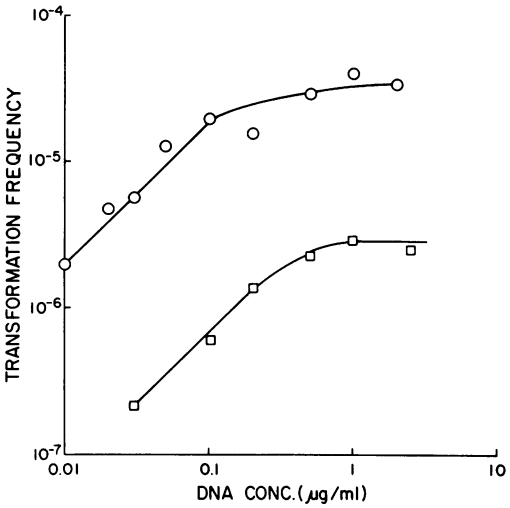

FIG. 3. Dose-response curves for parent Pc^r plasmid pFA3 and hybrid Pc^r plasmid pFA10. Various concentrations of plasmid DNA purified by one cycle of dye-buoyant density centrifugation from either strain FA559 (pFA3) or FA675 (pFA10) were used to transform 10^8 cells of FA534 per ml in GC base with 10 mM Mg^{2+}. □, pFA3 transformation; ○, pFA10 transformation.

electrophoresis, and analyzed by autoradiography. An *Msp*I digest of the hybrid plasmid was particularly useful for performing these experiments because its second-largest (3.0-kb) fragment consisted entirely of DNA from the pFA1 insert; the first and third fragments consisted of DNA from both pFA3 and pFA1; and the other smaller fragments were derived entirely from pFA3 (Fig. 2 and 4). When ^{32}P-labeled *Msp*I fragments of pFA10 were reisolated from competent cells, the only fragment visible on the autoradiograms migrated identically with the 3.0-kb *Msp*I fragment (Fig. 4, lanes G and H). Therefore, a 3.0-kb fragment of the hybrid that consisted entirely of homologous gonococcal pFA1 DNA was apparently taken up by the recipient cells in preference to DNA originating from the remaining 1.2 kb of pFA1 DNA or the 7.2 kb of pFA3 DNA. These experiments also indicated that DNA taken up by the cell remained double stranded for at least 5 min after the cell was exposed to it.

Further evidence for specific uptake of DNA from pFA10 was provided by use of ^{32}P-end-labeled *Taq*I fragments (Fig. 5). A *Taq*I fragment of 1.5 kb derived entirely from the pFA1 portion of pFA10 was taken up in preference to other fragments of similar size derived from the pFA3 portion of pFA10. This fragment shares 640 bp with the 3.0-kb *Msp*I fragment (Fig. 2), indicating that there is probably a sequence of DNA within this 640-bp region of pFA1 which is involved in DNA uptake by competent gonococci. Background radioactivity at the bottom of the gel prevented determination of whether smaller (<400 bp) fragments were taken up in this experiment.

Nonidentity of DNA uptake sequences in *Haemophilus* and *Neisseria*. If the nucleotide sequence used for chromosomal DNA uptake by the transformation system in *Haemophilus* spp. has a counterpart in the DNA of *N. gonorrhoeae*, *Haemophilus* DNA should competitively inhibit transformation by the 11.5-kb hybrid gonococcal Pc^r plasmid pFA10. However, there was no evidence for inhibition of the transforming activity of 1 μg of pFA10 per ml by up to 10 μg of *H. influenzae* (FA6026) or *Haemophilus parainfluenzae* (FA6027) DNA per ml (Fig. 6). When pFA1 DNA was used as the competing DNA in similar experiments, there was significant inhibition of transformation by pFA10 (Fig. 6). Curiously, gonococcal chromosomal DNA

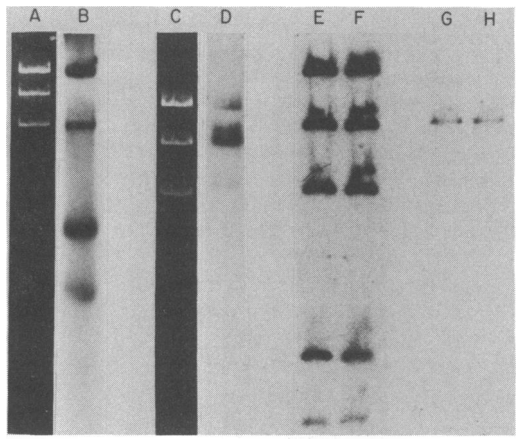

FIG. 4. Selective uptake of a 3.0-kb *Msp*I fragment of the 11.5-kb hybrid Pc^r plasmid pFA10 (pFA3ΩpFA1). The parental origin of pFA10 *Msp*I fragments is shown in lanes A through D by hybridization of ^{32}P-labeled, nick-translated pFA3 or pFA1 probe DNA to Southern transfers of *Msp*I digests of pFA10. Electrophoresis was on 1.2% agarose gels. Lanes: A and C, *Msp*I digests of pFA10; B and D, autoradiograms of hybridization of nick-translated pFA3 and pFA1, respectively, to Southern transfers of *Msp*I digests of pFA10. In uptake experiments (lanes E through H), *Msp*I fragments of pFA10 were labeled with ^{32}P at their 5′ ends before being incubated with competent cells for 5 min. After treatment of cells with DNase, repeated careful washes, and phenol extraction of the DNA, samples were separated on 1% agarose horizontal gels, and the gels were dried and autoradiographed. Lanes: E and F, autoradiogram of dilute samples of the end-labeled *Msp*I fragments of pFA10 DNA used in the uptake experiments; G and H, DNA fragment recovered after uptake by cells. The 3.0-kb *Msp*I fragment is derived entirely from the pFA1 (gonococcal plasmid) portion of pFA10.

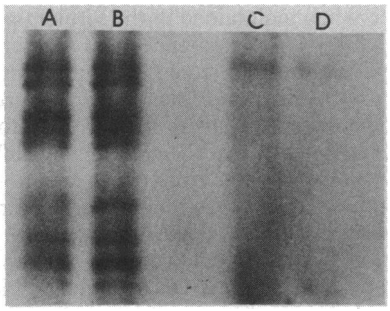

FIG. 5. Selective uptake of a 1.5-kb *Taq*I fragment of the 11.5-kb hybrid Pc^r plasmid pFA10 (pFA3ΩpFA1). The parental origin of pFA10 *Taq*I fragments is shown in Fig. 2. Uptake experiments were performed as described in the legend to Fig. 4, except the fragments were incubated with competent cells for 15 min. Lanes: A and B, autoradiogram of dilute samples of the end-labeled *Taq*I fragments of pFA10 DNA used in the uptake experiments; C and D, DNA fragment recovered after uptake by cells. The 1.5-kb *Taq*I fragment is derived entirely from the pFA1 portion of pFA10.

was a much more efficient inhibitor of transformation by pFA10 than was pFA1 DNA; the slope of the curve of inhibition by pFA1 was 0.7, but was greater than 10.0 when chromosomal DNA was used (Fig. 6).

DISCUSSION

The 11.5-kb Pc^r plasmid pFA10 isolated by Sox et al. (25) is a stable cointegrate composed of the 7.2-kb Pc^r plasmid pFA3 and the 4.2-kb gonococcal cryptic plasmid pFA1 (Fig. 1, 2, and 4). The DNA uptake experiments were undertaken after it was learned that pFA10 was considerably more active than pFA3 was in transformation of an isogenic Pc^s recipient to Pc^r (Fig. 3), which suggested that pFA10 might be useful as a probe to uncover mechanisms of gonococcal transformation.

In other experiments, we found that the increased transformation activity of pFA10 was not due to preferential formation of multimeric plasmids; rather, nearly all of the transformation activity of pFA10 was associated with monomeric plasmid DNA (unpublished data). We also showed that transformation by pFA10 was relatively more resistant than was that by pFA3 to the effects of competition by gonococcal chromosomal DNA, and that *E. coli* DNA failed to compete significantly against transformation by pFA3, pFA10, or gonococcal chromosomal DNA (7). These observations suggested that pFA10 might be more active than pFA3 in transformation because of the presence in pFA10 of the gonococcal plasmid pFA1; there might be one or more sequences on pFA1 required for DNA uptake by competent gonococci.

This idea was tested by examining the ability of gonococcal cells to take up end-labeled restriction endonuclease digest fragments of pFA10 DNA. The results (Fig. 4 and 5) showed that competent gonococci selectively took up into a DNase-resistant state a single *Msp*I or *Taq*I fragment. Each of these fragments was composed entirely of DNA from the pFA1 portion of pFA10. Thus, gonococcal DNA was apparently taken up preferentially from a mixture of fragments of homologous (pFA1) and heterologous (pFA3) DNA. We cannot formally exclude an alternative explanation for equal uptake of all fragments coupled with rapid and extensive degradation of all but the 3.0-kb *Msp*I or 1.5-kb *Taq*I fragments, but this seems to be

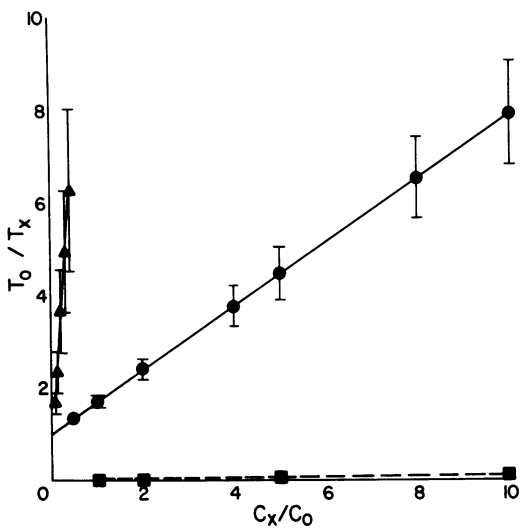

FIG. 6. Competition for transformation between the hybrid Pc^r plasmid pFA10 and gonococcal chromosome, gonococcal 4.2-kb cryptic plasmid, or *H. parainfluenzae* chromosome. Pc^r transformants were selected after exposure of strain FA534 to 1.0 μg of pFA10 DNA per ml. The effects of added species of competing DNA are plotted as a reciprocal plot of T_0/T_x versus C_x/C_0. T_0 is the transformation efficiency (transformants per cell per microgram of DNA) in the presence of 1.0 μg of donor DNA per ml; T_x is the transformation efficiency in the presence of added competing DNA; C_x is the concentration of competing DNA (μg/ml); C_0 is the concentration of transforming DNA. Symbols: ▲, Competition by *N. gonorrhoeae* FA534 chromosomal DNA; ●, competition by pFA101 DNA, prepared by two cycles of dye-buoyant density centrifugation from strain F62; ■, competition by *H. parainfluenzae* FA6027 chromosomal DNA. pFA101 is structurally identical to pFA1 except it contains an *Msp*I site which is missing from pFA1. pFA101 was used for these experiments because of difficulty in preparing sufficient quantities of pure pFA1 from host strain FA293.

less likely than the selective uptake model. Selective degradation of pFA3 DNA could account for the low transformation frequencies and relatively frequent plasmid deletions (20%) noted after transformation by pFA3; however, transformation by pFA10 results in a similar frequency of deletion (17% [2]), indicating that the increased transformation efficiency of pFA10 probably is not due to differences in selective DNA degradation.

The precise basis for selective DNA uptake is uncertain. Presumably, competent gonococci possess a surface receptor which recognizes a particular DNA sequence, common on homologous DNA but rare on heterologous DNA, which is analogous to the 11-bp sequence required for DNA uptake by *Haemophilus* spp. (4, 20). Since *H. influenzae* and *H. parainfluenzae* chromosomal DNA failed completely to inhibit gonococcal transformation by pFA10, the gonococcal DNA uptake sequence is apparently different from the *Haemophilus* sequence (Fig. 6). Mathis and Scocca (Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, H14, p. 116) also recently concluded that the gonococcal and *Haemophilus* chromosomal uptake sequences are different.

The number and distribution of sites involved in selective DNA uptake by gonococci are also uncertain. There may be only one uptake-specific site on the 4.2-kb plasmid pFA1, since only one fragment was taken up from either *Msp*I or *Taq*I digests and these fragments shared 640 bp; a DNA uptake sequence must occur within the 640-bp overlap. The presence of one gonococcal uptake sequence in a plasmid of 4.2 kb is consistent with the estimated occurrence of one 11-bp uptake sequence in approximately every 4,000 bp of *Haemophilus* DNA (4, 20).

The markedly increased efficiency of gonococcal chromosomal DNA as compared with pFA1 DNA in inhibition of pFA10 transformation (Fig. 6) was surprising. There are several possible explanations for this observation. There might be many more uptake sites per unit length of chromosomal DNA. The uptake sequence on chromosomal DNA might be slightly different from and bound more avidly by competent cells than that on pFA1. Other factors, such as the conformation of DNA around the uptake site, might affect DNA uptake. Further study is required to determine whether any of these models is correct.

Transformation is probably the principal means of transfer of chromosomal genes between gonococci in nature. There are at present no systems for chromosomal transfer between gonococci by conjugation or transduction. Among the transformable bacteria gonococci are unusual in the uniform competence of every isolate and the constitutive nature of their competence when maintained in the virulent piliated phase, although competence (and virulence) is rapidly lost after unselected tranfer in vitro (26). They are highly autolytic and freely release biologically active transforming DNA into their environment (19); mixed infection by different gonococcal strains is relatively common (8). Sequence-specific DNA recognition as a means for discrimination at the earliest stages of transformation between genetically useful (homologous) and heterologous DNA might be biologically advantageous in an organism which depends in part on transformation for genetic diversity.

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