TRANSFORMATION OF NEISSERIA GONORRHOEAE WITH GONOCOCCAL AND MENINGOCOCCAL DNA

by

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This thesis submitted by David Oliver Wood has been examined and approved by an appointed committee of the faculty of the School of Graduate Studies of the Medical College of Georgia.

The signatures which appear below verify the fact that all required changes have been incorporated and that the thesis has received final approval with reference to content, form and accuracy of presentation.

This thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

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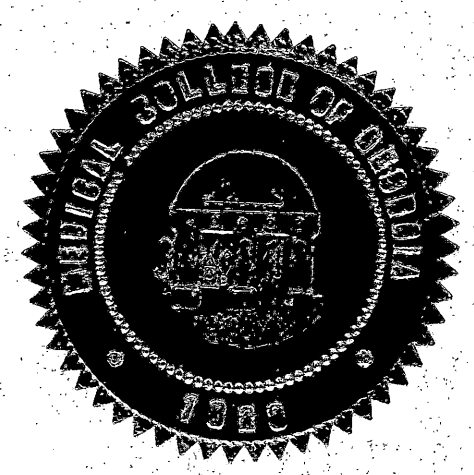

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INTRODUCTION

I. Review of Related Literature

Since Leistikow and Loeffler first isolated Neisseria gonorrhoeae in 1882, it has been the object of numerous studies. Despite extensive efforts, little was learned concerning the genetic and biochemical mechanisms of gonococcal pathogenicity. Kellogg et al. (1963) reported the existence of morphologically distinct colony types which they designated types 1 through 4 (T1, T2, T3, T4) (10). Most importantly, they showed a correlation between these types and virulence for human volunteers. T1 and T2 were the predominant types isolated from the disease state and could be maintained in vitro only by selective cloning. These types were capable of producing gonorrhoea in male volunteers. T3 and T4 arose from unselected transfer of T1 and T2 and were avirulent for human volunteers (9,10). These observations made possible the study of virulent gonococci in vitro.

Subsequent studies have identified additional differences between the virulent and avirulent colony types (8,15,18,19,20,21,22). One of special significance was provided by Sparling in 1966 (18). Prompted by Kellogg's findings, Sparling investigated the transforming potentials

of the separate colony types. This led to the initial report of the genetic transformation of N. gonorrhoeae. Sparling transformed streptomycin sensitive gonococci to streptomycin resistance with deoxyribonucleic acid (DNA) extracted from a resistant strain. The genetic transformation, however, was only successful with cells of the naturally occurring, virulent colony types 1 and 2. In addition to this observation, Sparling also determined the optimum conditions required for competence in the gonococci.

B. W. Catlin (1967) reported that gonococcal DNA could transform a Neisseria meningitidis recipient (3). Catlin not only showed the existence of a genetic transfer but also identified the gonococcal donors as methionine auxotrophs. This became evident when Catlin initiated studies on the transfer of sulfadiazine resistance. The recipient was a drug-susceptible strain of N. meningitidis which was able to grow on methionine-free media. DNA extracted from most sulfadiazine resistant meningococcal donors transferred only a high-level sulfadiazine resistance. DNA extracted from sulfadiazine resistant N. gonorrhoeae donors, however, conveyed a lower level of resistance plus a concurrent requirement for methionine. The requirement for methionine was shown to be the mechanism providing the low-level sulfadiazine resistance by end-product antagonism.

With the description of a genetic mechanism, the one essential requirement lacking for biochemical genetic studies

was a defined medium. Although several had been described, they suffered some serious disadvantages (7,11,23). Some allowed growth only of selected strains while all provided relatively slow growth rates. In 1973 Catlin conducted extensive studies on the nutritional requirements of N. gonorrhoeae, N. meningitidis, and N. lactamica. This study resulted in a completely defined medium which provided excellent growth of all strains tested (4). By omitting selected compounds from the medium, Catlin showed the existence of numerous nutritional requirements in natural isolates of the gonococci. Based on the growth responses of clinical isolates on the various deficient media, twenty-two auxotypes were identified which varied in their requirements for proline, arginine, ornithine, methionine, hypoxanthine, uracil, thiamine, and thiamine pyrophosphate (2,4). LaScolea and Young (1974) also devised a similar auxotyping system using a medium with a minimal number of nutritional requirements (12).

To determine the stability of these nutritional requirements and verify their hereditary basis, Catlin turned to genetic transformation. Using DNA extracted from a strain of gonococci prototrophic for the selected compounds, Catlin transformed each of the auxotrophic characters to prototrophy (5). In addition, strains with the same nutritional requirements were sometimes able to transform one another to

prototrophy. This indicated the existence of separate defects within a particular pathway.

Sparling continued his studies of N. gonorrhoeae with observations on the genetics of antibiotic resistance. It had been noted by Reyn (1961) that gonococcal isolates resistant to penicillin were likely to be resistant to tetracycline and streptomycin, indicating a common resistance mechanism (16). Manness and Sparling also reported a loss of low-level resistance to a group of drugs which included penicillin, tetracycline, erythromycin, chloramphenicol, rifampin, ethidium bromide, and acridine orange (13). Resistance to each was restored by spontaneous back mutations to any one. Manness and Sparling found, however, that single-step, spontaneous, antibiotic resistant mutants of wild-type, sensitive gonococci were ordinarily resistant only to the selected drug (13). This would indicate at least one or more genes for resistance to each of the antibiotics tested. Sparling clarified this dilemma by mapping resistance loci by genetic transformation (17). He reported the existence of linkage between genes confering high-level resistance to streptomycin, spectinomycin, and rifampin and low-level resistance to tetracycline and chloramphenicol. The gene order was rif...str...tet...chl ...spc. This proved the existence of separate genes. However, Sparling also described the existence of single-step mutations which affect resistance to multiple drugs

and occurs at a locus (env) separate from those above. Mutations of this locus increase cell envelope permeability resulting in phenotypic loss of resistance to multiple drugs (17).

II. Statement of Purpose

This study was initiated to confirm the existence of genetic transformation in Neisseria gonorrhoeae in order to investigate gene transfer from the meningococcus to the gonococcus. Documentation of this genetic transfer may lead to studies determining the extent of interaction between the two species. Until recently they were assumed to cause different diseases and occupy anatomically different niches. Recent reports of the isolation of each species in the other's habitat, coupled with Catlin's observation of the gonococcal to meningococcal genetic exchange, has dispelled this view and presented the possibility of in vivo interaction.

MATERIALS AND METHODS

I. Media

Four types of culture media were used. Two contained GC Agar Base purchased from Baltimore Biological Laboratories (BBL); one was supplemented with IsoVitaleX (BBL) and designated GCI medium, while the other contained a defined supplement (24) and was designated GCK medium. The GCI medium was used for maintenance of T1 cells and as a reconstitution medium for stock cultures. The GCK medium was used for routine cell counts and when supplemented with 100 ug of rifampin (CIBA Pharmaceutical Company) per ml, for the selection of rifampin-resistant mutants and transformants. A chemically defined medium (CDM) was used to determine the growth requirements of the strains. The composition of the CDM is provided in Table 1. The medium is similar to that described by Catlin (4). However, it was possible to exclude several components from Catlin's medium and still obtain growth of most clinical isolates. A minimal medium (MM) containing those components of the CDM indicated in Table 1 was used for the cultivation of prototrophic strains and for the selection of transformants. MM is a modification of the auxiliary A medium described by Carifo and Catlin (2). Cells for DNA extraction were grown in Mueller Hinton Broth (Difco) plus defined supplement (24).

Table 1. Complete defined medium for the cultivation of most strains of Neisseria gonorrhoeae [a]

Component	Amt (mM)
L-Aspartic acid*	3.76
L-Glutamic acid*	8.83
L-Arginine*	0.71
L-Serine*	0.48
L-Leucine	0.69
L-Isoleucine*	0.23
L-Valine	0.51
L-Tyrosine	0.39
L-Cysteine*	0.35
L-Cystine*	0.15
L-Alanine	1.12
L-Lysine	0.27
L-Proline[b]	0.43
L-Tryptophan	0.39
L-Threonine	0.42
L-Phenylalanine	0.15
L-Asparagine	0.17
L-Glutamine	0.34
L-Histidine	0.10
L-Methionine[b]	0.10
D-Glucose*	28.0
Glycine	0.33
Uracil*	0.071
Hypoxanthine*	0.024
Glutathione	0.15
Spermine tetrahydrochloride	0.25
Biotin*	0.003
Thiamine pyrophosphate[b]	0.001
Sodium acetate	25.0
Ethylenediaminetetraacetic acid*	0.01
NaCl*	100.0
K_2SO_4*	5.74
$MgCl_2\ 6H_2O$*	2.02
NH_4Cl*	4.11
K_2HPO_4*	20.0
KH_2PO_4*	20.0
$NaHCO_3$*	0.5
$CaCl_2$*	0.25
$Fe(NO_3)_3\ 9H_2O$*	0.01
Agar	20.0 g/l

a The components of minimal medium (MM) are designated by an asterisk. The medium was prepared in the manner described by Catlin (4). The amino acids and uracil were obtained from Eastman Kodak Co., Rochester, N.Y.; hypoxanthine, spermine tetrahydrochloride, and biotin

Table 1, continued.

from Nutritional Biochemicals Corporation, Cleveland, Ohio; glutathione, glucose, and salts from Fisher, Pittsburgh, Pa.; thiamine pyrophosphate from Sigma, St. Louis, Mo.; and agar (Noble or Purified) from Difco, Detroit, Mich.

b Proline, methionine, and thiamine pyrophosphate must be added to the MM in pairs to isolate the remaining nutritional transformants of strain GC-31 (Pro^-, Met^-, Tpp^-)

II. Cultures

The organisms used in this investigation , together with their sources, mutant substrains, and phenotypes are given in Table 2.

III. Culture Techniques

Organisms were identified as N. gonorrhoeae or N. meningitidis on the basis of Gram stain, carbohydrate degradation, and fluorescent antibody staining. The carbohydrate fermentation reactions were observed on a serum-free medium described by Flynn and Waitkins (6). Fluorescent antibody stains were performed and identified by the Clinical Microbiology Laboratory of Talmadge Hospital, Augusta, Georgia. Stock cultures were lyophilized in skim milk (Difco) or frozen in Trypticase Soy Broth (BBL) plus 20% glycerin at -70 C. Reconstituted or thawed cultures were plated on GCI for recovery. T1 recipient gonococcal cells were identified and propagated by the methods of Kellogg et al. on GCI (9,10). Growth requirements of recipient strains were determined by plating them on CDM lacking specific metabolites. Rifampin resistance was determined by plating cells on GCK containing 100 ug/ml of the antibiotic.

IV. Donor Selection

Donor strains were developed by selecting spontaneously occurring rifampin resistant mutants from sensitive popula-

Table 2. Organisms employed in the investigation

	Organism	Source	Phenotype
N. gonorrhoeae	FG-3	Clinical isolate	$Leu^{-}Pro^{+}Met^{+}Tpp^{+}Rif\text{-}s$
	FG-3A	Mutagenic treatment	$Leu^{+}Pro^{+}Met^{+}Tpp^{+}Rif\text{-}s$
	FG-3B	Spontaneous mutant	$Leu^{-}Pro^{+}Met^{+}Tpp^{+}Rif\text{-}r$
	FG-3C	Spontaneous mutant	$Leu^{+}Pro^{+}Met^{+}Tpp^{+}Rif\text{-}r$
	GC-31	ATCC 27631	$Leu^{+}Pro^{-}Met^{-}Tpp^{-}Rif\text{-}s$
N. meningitidis	CDC-74	Center for Disease Control, Group C, 74-008509	$Leu^{+}Pro^{+}Met^{+}Tpp^{+}Rif\text{-}s$
	CDC-74A	Spontaneous mutant	$Leu^{+}Pro^{+}Met^{+}Tpp^{+}Rif\text{-}r$

tions and by mutagenic treatment of the FG-3 wild-type strain. Rifampin resistant mutants were isolated by plating 0.1 ml of a cell suspension containing about 10^9 colony-forming units (CFU) per ml on rifampin-containing (100 ug/ml) GCK medium. The average recovery of resistant mutants was four colonies per plate. Prototrophic mutants (Leu^+) of FG-3 were obtained by treatment of a population with NTG (N-methyl-N'-nitro-N-nitrosoguanidine; Aldrich Chemical Co.) and selecting for Leu^+ mutants on MM. The FG-3 strain was inoculated into CDM broth, gassed with 10% CO_2, and incubated on a shaking incubator at 36 C until an optical density of 0.1 was reached (about 10^8 CFU/ml). NTG was then added to a final concentration of 1 ug/ml, and incubation continued until the cell population reached an optical density of 0.3. Optical densities were determined on a Baush and Lomb Spectronic 20. The cultures were then centrifuged, and the cells were resuspended in fresh CDM broth and incubated for an additional 12 h. Cell densities of about 2.0×10^7 CFU/ml were then plated on CDM plates without leucine or on MM. The NTG treatment produced an average of 10 Leu^+ mutant colonies per plate, whereas non-treated control populations failed to produce Leu^+ colonies when plated at the same cell densities.

Meningococcal donor DNAs were obtained from a strain of N. meningitidis (Group C, 74-008509) received from the Center for Disease Control in Atlanta, Georgia. This strain grew well on MM, and spontaneous rifampin resistant mutants

were selected on GCK medium containing 100 ug of rifampin per ml. The rifampin resistant mutants were tested for resistance up to levels of 400 ug/ml, and both the meningococcal and gonococcal mutants were resistant to this concentration.

V. Transforming Procedures

DNA was extracted from the donor strains by the Marmur procedure (14) and the concentration determined by the diphenylamine reaction (1). Recipient cells were cultured on GCI plates for 18 h, harvested, and suspended in CDM broth at a cell density of about 10^8 CFU/ml. The standard transforming mixture consisted of 0.9 ml of cell suspension in CDM broth and 0.1 ml of DNA in 1X SSC (0.15 M NaCl, 0.015 M $Na_3C_6H_5O_7$ $2H_2O$, pH 7). This mixture was incubated for 30 min without shaking at 36 C and then added to 20 ml of fresh CDM broth, gassed with 10% CO_2, and incubated in a shaking incubator. In some experiments the transforming activity was terminated by adding 50 ug of deoxyribonuclease (DNase I) and allowing 5 additional minutes of incubation before adding the transforming mixture to 20 ml of fresh CDM. The cell suspensions were allowed to incubate for 8 h before plating. After incubation 0.1 ml samples were removed from the flask and plated directly, or serially diluted and plated, on selective media. Appropriate dilutions were also plated on complete medium to determine total CFU plated.

Serial dilutions were made in a solution containing all salts, buffers, and inorganic ions of CDM (pH 7.2) plus the addition of cysteine as directed by Hunter et al. (7). The samples were distributed over the plate with a glass rod and the plates incubated in a candle jar at 36 C for 3 days before counting. All platings were made in duplicate and where applicable at least two dilutions were counted. Controls included DNase-treated donor DNA, no DNA, and in some cases the use of homologous non-transforming DNA. The effects of diluting donor DNA and competing with homologous DNA served to further control several experiments.

RESULTS

Initial work concentrated on the clinical isolate FG-3 as the recipient strain. It was phenotypically Leu^- Rif-s. The colonies produced by the FG-3 recipient strain on GCI were 99% colony type T1. Frequencies of transformation observed for the leucine character of gonococcal recipient FG-3 using N. gonorrhoeae donor DNAs are provided in Table 3. When 5 ug of FG-3A DNA (Leu^+) was added to the FG-3 recipient population, the transformation frequency was 3.8×10^{-2}. This amount of DNA appeared to saturate the system since a 10-fold increase in donor DNA (50 ug) failed to increase transformant recovery. The effect of diluting donor DNA was observed at the 0.005 ug level, where the frequency of transformation dropped to 1.6×10^{-4}. A competing effect was observed by mixing non-transforming FG-3 (Leu^-) DNA with Leu^+ FG-3A DNA. For example, when the transforming mixture contained 0.049 ug of FG-3A transforming DNA and 4.9 ug of non-transforming (Leu^-) FG-3 DNA (1:100), the transformation frequency was 7.5×10^{-4}. The 1:1000 ratio of DNAs produced only a frequency of 6.0×10^{-6} whereas the use of FG-3 DNA alone produced a frequency of prototrophic growth of 9.5×10^{-6}. When DNase was used to terminate transformation after 30 min of incubation, the frequency of transformation was reduced to 3.2×10^{-3}. The frequency of transformation with

Table 3. Transformation of the leucine character of gonococcal recipient FG-3 with N. gonorrhoeae donor DNA[a]

Materials Tested	Total CFU Plated x 10^{-7}	Transformation Frequency
FG-3A (50 ug)	4.6 ± 1.2[b] (3)[c]	3.3×10^{-2}
FG-3A (5 ug)	1.7 ± 0.8 (3)	3.8×10^{-2}
FG-3A (5 ug); DNase terminated	0.9 ± 0.4 (3)	3.2×10^{-3}
FG-3A (0.5 ug)	7.5 ± 0.4 (2)	1.6×10^{-2}
FG-3A (0.005 ug)	4.0 ± 1.4 (2)	1.6×10^{-4}
FG-3A plus FG-3 (1:1)	9.6 (1)	2.1×10^{-3}
FG-3A plus FG-3 (1:100)	3.6 (1)	7.5×10^{-4}
FG-3A plus FG-3 (1:1000)	9.0 (1)	6.0×10^{-6}
FG-3C (5 ug)	6.6 ± 6.4 (2)	3.0×10^{-3}
FG-3 (50 ug)	6.2 ± 0.5 (2)	9.5×10^{-6}
FG-3B (5 ug)	7.4 ± 4.6 (2)	1.3×10^{-7}
DNase-treated FG-3A (5 ug)	29.0 ± 0.5 (2)	2.8×10^{-7}
No DNA	8.9 ± 5.1 (3)	3.7×10^{-7}

a The total amount of DNA used in the competition experiments was 5 ug. For the DNase-treatment, DNase was added at twice the donor DNA concentration

Table 3, continued.

to 1 ml of DNA-containing transforming broth. The DNA concentration was such that 0.1 ml gave the desired amount of DNA. This mixture was incubated 5 min before addition to recipient cells.
In the experiments terminated by DNase, 50 ug of DNase was added to the recipient cells after they had incubated for 30 min with the transforming DNA. After 5 additional min incubation, the mixture was added to the 20 ml of fresh CDM.

b Mean $\pm$ Standard Error

c Number of Experiments

Phenotype Designations: FG-3, Leu^-Rif-s; FG-3A, Leu^+ Rif-s; FG-3B, Leu^-Rif-r; FG-3C, Leu^+Rif-r.

gonococcal donor FG-3C was 3.0×10^{-3}, approximately 10-fold lower than with the same amount of FG-3A donor DNA. The absence of DNA in the transforming mixture or the use of DNase-treated donor DNA reduced the frequency of prototrophic recovery on the leucine-deficient medium to 3.7×10^{-7} and 2.8×10^{-7}, respectively.

Table 4 provides transformation frequencies obtained for the rifampin character of recipient FG-3 using N. gonorrhoeae donor DNAs. Rifampin resistant (100 ug/ml) donor DNAs (FG-3B and FG-3C) transformed the FG-3 Rif-s recipient to Rif-r at frequencies of 5.3×10^{-3} and 1.7×10^{-3}. Using homologous FG-3 donor DNA, the frequency of transformation dropped to 3.5×10^{-8}. Transformant recovery with DNase-treated donor DNA (FG-3C) also dropped to 5.0×10^{-8}, while in the absence of DNA no Rif-r colonies were observed at the cell densities plated. The recovery of cotransformed FG-3 recipients (Leu^+ Rif-r) from the FG-3C DNA was 2.9×10^{-6}, indicating that the two traits are unlinked (not shown in tables).

The transforming activity of N. meningitidis donor DNA on gonococcal recipient FG-3 is shown in Table 5. 5 ug of meningococcal DNA (CDC-74) transformed the FG-3 Leu^- recipients to Leu^+ phenotypes at a frequency of 3.4×10^{-4}. The efficiency of CDC-74 and CDC-74A DNAs to transform either the Leu^- or Rif-s traits of the FG-3 recipient was 10- to

Table 4. Transformation of the rifampin character of gonococcal recipient FG-3 with N. gonorrhoeae donor DNA

Materials Tested	Total CFU Plated x 10^{-7}	Transformation Frequency
FG-3B (5 ug)	6.9 ± 1.2[b] (2)[c]	5.3 x 10^{-3}
FG-3C (5 ug)	4.5 ± 4.5 (3)	1.7 x 10^{-3}
FG-3 (50 ug)	37.0 ± 17.0 (2)	3.5 x 10^{-8}
DNase-treated FG-3C (5 ug)	15.0 ± 14.0 (2)	5.0 x 10^{-8}
No DNA	9.9 ± 9.0 (3)	*

a The DNase treatment used the same procedures as described in Table 3.

b Mean ± Standard Error

c Number of Experiments

* No Rif-r colonies observed at cell densities plated

Phenotype Designations: Table 3.

Table 5. Transforming activity of N. meningitidis donor DNA on gonococcal recipient FG-3[a]

Materials Tested	Total CFU Plated x 10^{-7}		Transformation Frequency Leu$^+$	Rif-r
CDC-74 (50 ug)	8.3 ± 2.9[b]	(3)[c]	1.6 x 10^{-4}	
CDC-74 (5 ug)	6.4 ± 0.7	(2)	3.4 x 10^{-4}	
CDC-74A (5 ug)	12.0 ± 7.5	(3)	4.2 x 10^{-4}	3.4 x 10^{-4}
CDC-74A (5 ug); DNase-terminated	2.3 ± 1.3	(2)	9.8 x 10^{-5}	-
CDC-74A (0.5 ug)	9.6 ± 8.4	(2)	1.6 x 10^{-5}	6.7 x 10^{-5}
CDC-74A (0.05 ug)	3.7	(1)	1.9 x 10^{-6}	3.5 x 10^{-6}
CDC-74A (0.005 ug)	22.0	(1)	3.2 x 10^{-7}	4.1 x 10^{-7}
DNase-treated CDC-74A (5 ug)	4.1 ± 2.9	(2)	1.8 x 10^{-7}	-
No DNA	5.0 ± 1.5	(3)	*	-
CDC-74A (5 ug); DNase-terminated	13.0 ± 4.4	(2)	-	7.0 x 10^{-5}
DNase-treated CDC-74A (5 ug)	17.0 ± 8.3	(2)	-	2.0 x 10^{-8}
No DNA	14.0 ± 8.5	(3)	-	1.1 x 10^{-8}

a DNase treatment of donor DNA and DNase termination procedures as in Table 3.

b Mean ± Standard Error

c Number of Experiments

Table 5, continued.

* No Leu^+ colonies observed at cell densities plated

Phenotype Designations: FG-3, Leu^-Rif-s; CDC-74, Leu^+ Rif-s; CDC-74A, Leu^+Rif-r

100-fold less than that of the gonococcal donors (FG-3A, FG-3B, and FG-3C, Tables 3 and 4). As in the case of the gonococcal DNA, 5 ug of CDC-74 DNA saturated the system since 50 ug failed to increase the frequency of transformation. 5 ug of Rif-r donor DNA (CDC-74A) transformed the FG-3 recipient to Rif-r at a frequency of 3.4×10^{-4}. Lower concentrations of donor DNA reduced the frequency of transformation as did DNase treatment of the donor DNAs and the absence of DNA from the transforming mixtures. Termination of transforming activity with 50 ug of DNase after 30 min reduced the frequency of transformation in this case only slightly (to 9.8×10^{-5} for Leu^+ and 7.0×10^{-5} for Rif-r.

To determine if additional auxotrophic markers could be transformed with meningococcal DNA, a strain of N. gonorrhoeae auxotrophic for proline, methionine, and thiamine pyrophosphate (GC-31) was selected for further study. This strain is available through the American Type Culture Collection and Catlin has employed it in previous transformation studies. The transforming activity of gonococcal DNA from an FG-3 mutant substrain (FG-3C) is provided in Table 6. When 10 ug of FG-3C DNA was added to the GC-31 recipient population, the frequency of transformation was 3.4×10^{-4} for proline, 1.4×10^{-4} for methionine, and 1.9×10^{-4} for thiamine pyrophosphate. Since transforming activity in this series of experiments was terminated with DNase after 30 min, these results correspond closely to

Table 6. Transforming activity of N. gonorrhoeae donor DNA on gonococcal recipient GC-31[a]

Materials Tested	Total CFU Plated x 10^{-8}		Transformation Frequency Pro$^+$	Met$^+$	Tpp$^+$
FG-3C (10 ug)	5.5 ± 2.6[b]	(3)[c]	3.4 x 10^{-4}	1.4 x 10^{-4}	1.9 x 10^{-4}
FG-3C (1 ug)	3.4 ± 0.8	(2)	6.2 x 10^{-5}	2.2 x 10^{-5}	5.2 x 10^{-5}
FG-3C (0.1 ug)	8.1	(1)	1.4 x 10^{-5}	2.7 x 10^{-6}	1.0 x 10^{-5}
FG-3C (0.01 ug)	3.8 ± 0.8	(3)	8.6 x 10^{-6}	2.3 x 10^{-6}	4.6 x 10^{-6}
FG-3C (0.001 ug)	8.0	(1)	1.3 x 10^{-7}	6.3 x 10^{-8}	7.5 x 10^{-8}
FG-3C; Fragmented[d]	3.8	(1)	1.2 x 10^{-6}	7.9 x 10^{-8}	2.2 x 10^{-6}
DNase-treated FG-3C (10 ug)	4.4 ± 1.3	(3)	3.0 x 10^{-7}	5.3 x 10^{-7}	3.9 x 10^{-7}
No DNA	6.3 ± 1.2	(5)	*	*	1.2 x 10^{-8}

a Transforming activity was terminated after 30 min incubation by the addition of 50 ug of DNase.
DNase treatment of donor DNA as in Table 3.

b Mean ± Standard Error

c Number of Experiments

d DNA fragmentation described in text.

* No prototrophic colonies observed at cell densities plated.

Phenotype Designations: GC-31, Pro$^-$Met$^-$Tpp$^-$; FG-3C, Pro$^+$Met$^+$Tpp$^+$

the transformant recovery for leucine using FG-3C DNA (Table 3). Once again a progressive decrease in the frequency of transformation is observed with decreasing amounts of donor DNA. The use of DNase-treated donor DNA reduced the transformation frequency to between 3 and 6 x 10^{-7} for each character. In the absence of DNA, no prototrophic colonies were observed at the cell densities plated for either proline or methionine. The frequency of prototrophic colony growth on thiamine pyrophosphate-deficient medium was 1.2 x 10^{-8}. An additional control of fragmented FG-3C DNA (sheared by sonication with a Sonifier Cell Disrupter, Model Wi85, Heat-Systems Ultrasonics, Inc. Plainview, L.I., N.Y.) reduced transformation frequency for proline, methionine, and thiamine pyrophosphate to 1.2 x 10^{-6}, 7.9 x 10^{-8}, and 2.2 x 10^{-6}, respectively.

The transforming activity of the N. meningitidis donor strain on gonococcal recipient GC-31 is shown in Table 7. 10 ug of meningococcal DNA (CDC-74A) gave transformation frequencies of 6.3 x 10^{-5} for proline, 4.9 x 10^{-6} for methionine, and 2.2 x 10^{-5} for thiamine pyrophosphate. Once again the efficiency of CDC-74A DNA to transform the Pro^-, Met^-, and Tpp^- traits of the GC-31 recipient was 10- to 100-fold less than that of the gonococcal DNA (FG-3C, Table 6). Transformant recovery dropped with decreasing amounts of DNA, with methionine showing a sharper decrease. At the 0.001 ug level of CDC-74A donor DNA, no transformant colonies were

Table 7. Transforming activity of N. meningitidis donor DNA on gonococcal recipient GC-31[a]

Materials Tested	Total CFU Plated x 10^{-8}		Transformation Frequency Pro^+	Met^+	Tpp^+
CDC-74A (10 ug)	2.2 ± 1.1[b]	(4)[c]	6.3×10^{-5}	4.9×10^{-6}	2.2×10^{-5}
CDC-74A (0.1 ug)	7.0	(1)	7.3×10^{-6}	8.9×10^{-7}	5.0×10^{-6}
CDC-74A (0.01 ug)	4.3	(1)	2.0×10^{-6}	5.8×10^{-8}	3.5×10^{-7}
CDC-74A (0.001 ug)	5.8	(1)	1.6×10^{-7}	*	1.7×10^{-7}
DNase-treated CDC-74A (10 ug)	4.8 ± 0.8	(4)	1.8×10^{-8}	1.6×10^{-8}	2.1×10^{-8}
No DNA	6.0 ± 1.5	(4)	6.8×10^{-8}	*	3.0×10^{-8}

a Transforming activity was terminated after 30 min incubation by the addition of 50 ug of DNase.
DNase treatment of donor DNA as in Table 3.

b Mean ± Standard Error

c Number of Experiments

* No prototrophic colonies observed at cell densities plated.

Phenotype Designations: GC-31, $Pro^-Met^-Tpp^-$; CDC-74A, $Pro^+Met^+Tpp^+$

observed at the cell densities plated on methionine-deficient medium. The absence of DNA or the use of DNase-treated donor DNA reduced the frequency of prototrophic recovery to between 1.8×10^{-8} and 6.8×10^{-8} for proline and thiamine pyrophosphate. At the cell densities plated, no methionine prototrophs were observed in the absence of DNA.

DISCUSSION

The results show the transformation of the leucine, proline, methionine, thiamine pyrophosphate, and rifampin traits of N. gonorrhoeae with meningococcal as well as gonococcal DNAs. The efficiency of meningococcal DNA to transform these traits was 10- to 100-fold less than with gonococcal DNA.

The transforming procedures used differ from those of other studies (5,17,18). In this investigation the transforming mixture was essentially CDM broth, which after 30 min incubation was diluted into 20 ml of fresh CDM broth and allowed to incubate for 8 h before plating. Since the initial experiments were intended to determine whether meningococcal DNA could transform gonococcal traits, the earlier transforming mixtures were not treated with DNase to terminate the transforming activity. In most cases the dilution of the transforming mixture with 20 ml of CDM broth did not dilute the transforming DNA to a non-transforming level. Indeed, when samples of the transforming mixtures were plated for selection of transformants at intervals between 5 min and 8 h, transformant recovery increased for the first hour of incubation. After 1 h of incubation, transformant recovery per total CFU remained constant. Since there was considerable growth during this period of incuba-

tion, there does not appear to have been extensive differential multiplication of the transformed cells. When DNase was used to terminate the transforming activity after 30 min incubation, the recovery of Leu^+ transformants was reduced about 10-fold with gonococcal DNA (Table 3). In this system the recipients are able to grow in the transforming mixture, whereas other investigators used either GC Base Medium (18) or an incompletely supplemented, defined broth (5) that would not support cell growth. This transforming system was chosen to allow the growth and isolation of the maximum number of transformants.

Two of the most frequently encountered problems with the transforming procedures were agar toxicity and cell clumping. Although Noble Agar (Difco) or Purified Agar (Difco) were used as solidifying agents in the CDM or MM plates, inhibitory agar was frequently encountered. All attempts to wash inhibitory agar with various organic solvents to remove toxic components were unsatisfactory. Finally, the addition of cornstarch (Fisher) at a 1:1 ratio with some agar restored growth capabilities. Cell clumping, which is prevalent in T1 gonococci, occurred during incubation in the CDM broth. Although the initial cell suspensions were subjected to mild sonication in an ultrasonic cleaner (Cole-Palmer, Chicago, Ill.) until a nearly homogeneous diplococcal population was observed, clumping or clustered growth developed during incubation in th CDM broth. The

clusters appeared well suspended and relatively uniform in size. Dilutions and duplicate platings for either cell counts or transformant selection showed good agreement within a particular experiment. However, the amount of clumping varied from one experiment to another, which could be responsible for some variation in the frequency of transformation between experiments. Despite clumping, the results proved to be reproducible for each marker.

Although linkage has been observed between genes conferring resistance to antibiotics, linkage was not observed between any of the traits employed in this study. Each trait, however, was capable of being transferred from the meningococcus to the gonococcus, confirming the relatedness of the species.

SUMMARY

Two strains of Neisseria gonorrhoeae auxotrophic for leucine or proline, methionine, and thiamine pyrophosphate and sensitive to rifampin have been genetically transformed to prototrophy and antibiotic resistance with meningococcal as well as gonococcal DNA. The transforming efficiency of the meningococcal DNA was 10- to 100-fold less than that of the gonococcal DNA. A chemically defined medium that would support growth of most gonococcal isolates was used as a complete medium. A minimal medium was used for the selection of prototrophic transformants.

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