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Genetic Studies on *Neisseria gonorrhoeae* from Disseminated Gonococcal Infections

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Isolates from uncomplicated and disseminated gonococcal infections were analyzed by using deoxyribonucleic acid-mediated transformation. Most pairs of auxotrophs could recombine, producing independent transformants. When the constellation of arginine (Arg), hypoxanthine (Hyx), and uracil (Ura) requirements was present in donor and recipient, no recombination for these traits could be detected. Except for Arg to Hyx, no linkage between Arg, Hyx, Ura, penicillin G sensitivity, and serum resistance could be demonstrated. Some distant linkage of Ura to nalidixic acid and rifampin resistances was found. The data show that the traits associated with disseminated gonococcal infection strains are not closely linked but are identical in all strains, indicating a common origin.

By using a defined medium that she developed for the growth of *Neisseria* (5), Catlin divided gonococcal isolates into several groups according to their nutritional requirements (3). This auxotyping system is the first practical method developed to type gonococci.

Since the growth responses of gonococci have proved to be relatively stable, heritable properties, auxotyping has become an invaluable tool for epidemiological investigations. For example, auxotyping of pre- and post-treatment cultures obtained from individual patients has aided the differentiation of cases of reinfection from those representing therapeutic failure (17). At a broader level, auxotyping has proved useful in following chains of infection within a community and in establishing the distinctive distribution of different nutritional types of *N. gonorrhoeae* among isolates from different anatomic sites and syndromes (13).

Auxotyping has been of particular value in the examination of strains isolated from patients suffering from the disseminated gonococcal infection (DGI) syndrome. In Seattle (13) and elsewhere (18), gonococci that cause disseminated infections have a constellation of phenotypic characteristics that generally serve to distinguish them from most strains causing uncomplicated infections. These properties include: (i) greater susceptibility to penicillin and tetracycline (24), (ii) the requirement for arginine (Arg), hypoxanthine (Hyx), and uracil (Ura) for growth (13, 18), (iii) a small, atypical colonial morphology, usually accompanied by weak reactions on carbohydrate fermentation media (18), and (iv) resistance to the normal bactericidal action of human sera (21).

The isolation of a subgroup of gonococci from the DGI syndrome, which share common properties, raises several interesting genetic questions. It would be useful to know, for example, whether the Arg^-, Hyx^-, Ura^- phenotype, penicillin susceptibility, and serum resistance were the pleiotropic effect of a single gene or whether each determinant was encoded by a separate gene or gene cluster. Is the genetic lesion(s) present in a number of DGI gonococcal isolates at the same site(s) or are they distinguishable? Catlin has already demonstrated, by transformation, that phenotypically similar auxotrophic isolates often have distinguishable genetic defects. In some cases, however, recombination between phenotypically similar auxotrophic isolates (including a few Arg^-, Hyx^-, Ura^- strains) did not occur, indicating the presence of the identical mutation in independent isolates (6).

In this paper we present evidence to show that Arg^-, Hyx^-, Ura^-, penicillin susceptibility, and serum resistance are distinct, not closely linked genes, and that the genetic lesion present in the Arg^- gene of all DGI strains has occurred at an identical, but different, site from Arg^- mutations present in isolates from uncompli-

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cated infections. The data indicate, therefore, that the DGI strains share a common origin and were presumably derived from a single clone.

MATERIALS AND METHODS

Bacterial strains. *N. gonorrhoeae* strains, their sources, and some pertinent phenotypic characteristics are listed in Table 1. Culture identifications were verified by Gram stain, oxidase reaction, and carbohydrate fermentation. Cultures were stored at −70°C in 50% Trypticase soy broth (Difco) plus 50% GG free horse serum (GIBCO), which had been heated to 56°C for 30 min.

KH2024 and KH2025 were isolated from the same patient, and KH5730 and KH5731 were isolated from sexual contacts. All other isolates are believed to be unrelated.

KH6777 was derived from F62 (12) by three steps of ultraviolet mutagenesis and selection for proline independence, nalidixic acid resistance (Nal^r; minimum inhibitory concentration [MIC] = 15 μg/ml), and rifampin resistance (Rif^r; MIC = 15 μg/ml).

Reagents. Organic chemicals, enzymes, and antibiotics were obtained from Sigma or Calbiochem. Inorganic chemicals and soluble starch were from Fisher; medium 199 (M-199) was from GIBCO, and agarose was from Marine Colloids.

Media. GCP broth used was similar in composition to GC medium base (Difco), without agar, except that 0.5 g of soluble starch was used instead of cornstarch. The defined supplement, modified Kellogg plates (K), and HEPES (*N*-2-hydroxyethyl piperazine-*N*'2-ethanesulfonic acid)-buffered broth (GCH) used were as described earlier (16).

Proteose-peptone-saline (PPS) diluent was described (2). Soft-agar overlays used were PPS with 0.7% agar. The chemically defined medium was NEDA, described by Catlin (5), with slight modification: Tween 80 was omitted, 0.5 g of soluble starch per liter was added, and agarose was used as the solidifying agent. The minimal salts diluent was used as described (14).

Auxotyping. Nutritional requirements were deter-

TABLE 1. *N. gonorrhoeae strains, sources, and phenotypic characteristics*

Strain no.	Isolation site[a]	Patient sex	Syndrome of patient[b]	City of original isolation[c]	Colony morphology[d]	Requirement (−) or independence (+) of nutrient[e]					Penicillin G MIC (μg/ml)	Susceptibility to serum killing[f]
						Pro	Arg	Hyx	Ura	Met		
LP99	U	M	?	Sea	4	−	−	−	−	−	0.03	R
MHD316	B.W.	Catlin		Milw	1	−	−	−	−	+	0.03	R
KH1951	C	F	DGI	Sea	1	−	−	−	−	+	0.01	R
KH2031	U	M	DGI	Sea	4	−	−	−	−	+	0.06	R
KH1384	B	F	DGI	Sea	4	+	−	−	−	+	0.03	R
KH1385	B	M	DGI	Sea	4	+	−	−	−	+	0.01	R
KH1938	C	F	DGI	Sea	4	+	−	−	−	+	0.03	R
KH1950	C	F	DGI	Sea	4	+	−	−	−	+	0.03	R
KH1975	C	F	DGI	Sea	1	+	−	−	−	+	<0.01	R
KH1983	T	M	DGI	Sea	4	+	−	−	−	+	0.03	R
KH2016	?	F	DGI	Sea	1	+	−	−	−	+	0.02	R
KH2017	B	F	DGI	Sea	1	+	−	−	−	+	0.01	R
KH2020	?	F	DGI	Sea	1	+	−	−	−	+	0.01	R
KH2024	U	M	DGI	Sea	1	+	−	−	−	+	0.03	R
KH2025	J	M	DGI	Sea	1	+	−	−	−	+	0.01	R
KH2063	U	M	DGI	Sea	4	+	−	−	−	+	0.02	ND[g]
KH6305	C	F	DGI	Ind	1	+	−	−	−	+	<0.01	R
KH6356	C	F	DGI	Ind	1	+	−	−	−	+	0.01	R
F62	D.S.	Kellogg		Ind	1	−	+	+	+	+	0.5	S
KH6777	Derived from F62				4	+	+	+	+	+	0.25	S
LP53	U	M	GEN	Sea	1	−	+	+	+	+	0.5	S
KH5060	?	?	?	Tai	4	−	+	+	+	+	2.0	S
A135	C	F	GEN	Sea	1	−	−	+	+	+	0.25	S
KH5730	C	F	GEN	Sea	1	−	−	+	+	+	0.12	S
KH5731	U	M	GEN	Sea	1	−	−	+	+	+	0.12	S
KH6326	C	F	GEN	Sea	1	−	−	+	+	+	ND	S
KH6557	?	?	?	Cha	1	−	−	+	+	+	0.25	ND
KH6288	C	F	GEN	Sea	1	+	−	+	+	+	0.25	S
KH6306	C	F	GEN	Ind	1	+	−	+	+	+	ND	ND
KH6075	C	F	GEN	Sea	1	+	+	+	+	−	0.12	S
KH6327	C	F	GEN	Cha	1	+	+	+	+	−	0.12	ND
KH5767	U	M	GEN	Sea	1	+	+	+	+	+	0.1	S
KH7189	U	M	GEN	Sea	1	+	+	+	+	+	0.25	S

[a] U, Urethra; C, cervix, B, blood; T, throat; J, joint.
[b] DGI, Disseminated infection; GEN, uncomplicated genital infection.
[c] Sea, Seattle; Milw, Milwaukee; Ind, Indianapolis; Tai, Taiwan; Cha, Chapel Hill.
[d] According to Kellogg et al. (12).
[e] Pro, Proline; Arg, arginine; Hyx, hypoxanthine; Ura, uracil; Met, methionine.
[f] R, Serum titer <2; S, serum titer >4.
[g] ND, Not determined.

mined by the method described by Carifo and Catlin (3).

MIC determinations. The replicating devices described by Steers et al. (23) were used with a series of K plates containing appropriate concentrations of antibiotics to determine the MIC. Overnight growth on a K plate was swabbed into PPS and subjected to thorough Vortex mixing. The optical density at 600 nm was adjusted to 0.10, which was found to be equivalent to 1×10^8 to 2×10^8 colony-forming units (CFU) per ml. A 10^{-2} dilution of this suspension was used in the wells of the replicator. After 48-h incubation, in 5% CO_2 at 37°C, growth was compared with that on an antibiotic-free plate.

Determination of susceptibility to serum killing. The bactericidal titer was performed as previously described (21) with serum dilutions from 1:2 to 1:1,024. Transformants of Arg^-, Hyx^-, Ura^- serum-resistant strains that were selected for nutritional independence or increased resistance to penicillin G were screened for increased susceptibility to serum killing as an unselected marker. Serum dilutions of 1:4 and 1:20 were used, and the determination was usually done at least twice. The reaction mixture had a total volume of 150 μl and consisted of 1 part (50 μl) of diluted serum, 1 part of bacterial suspension, and 1 part of complement source. The bacterial suspension was prepared by suspending 18-h growth from a K plate in Trypicase soy broth (Difco) and diluting in M-199 (GIBCO) to about 5×10^3 CFU/ml. Serum from an agammaglobulinemic individual (diluted 1:2 in M-199) was the complement source. Determinations were done in triplicate. Appropriate controls of the serum and complement, in which 50 μl of M-199 was substituted for one or both components, were included in each experiment. After 75 to 90 min of incubation at 37°C in a gyratory incubator, the contents of each well were spread on a predried, warmed K plate, and these were incubated for 24 h at 37°C in a CO_2 incubator. The serum bactericidal titer was defined as the reciprocal of the greatest dilution producing 75% or more reduction in CFU when compared with the controls.

Preparation of transforming DNA. Deoxyribonucleic acid (DNA) was extracted from cells grown in GCP broth by the method described by Catlin (4). DNA preparations were stored in a 1-μg/ml concentration over a drop of chloroform. Before use, the DNA was diluted 1:10 in SSC (0.15 M NaCl and 0.01 M sodium citrate) and gently shaken for 30 min at 37°C in a loosely capped tube to remove the chloroform. Sterility of the DNA was assured by spreading 0.2 ml on a K plate.

Transformation. The transformation procedure used was a modification of that described by Sparling (22). Cells from an 18-h K plate were suspended in GCH with 1% defined supplement and $CaCl_2$ (final concentration, 2×10^{-3} M) added, and the cell suspension was adjusted to about 2×10^7 CFU/ml. The cell suspension was equally divided, and DNA was added to one while the other served as a control. DNA at 10 μg/ml was used to determine the frequency of transformation for a given trait, and DNA at a limited concentration was used (0.01 μg/ml) for linkage experiments. A CO_2-enriched atmosphere was obtained by placing the flasks in a CO_2 incubator with the caps loosened. After 5 to 10 min, the flasks were removed and the caps were immediately tightened. After 30 min of incubation in a shaking water bath at 37°C, 0.2 M $MgCl_2$ and deoxyribonuclease (1 mg/ml in 0.002 M $MgCl_2$) were added to final concentrations of 0.002 M and 50 μg/ml, respectively.

The time required for phenotypic expression was determined for each trait, as described by Sparling (22). Most markers required about 3 h for full expression, but Pen^r and Ura^+ required about 4.5 and 6 h, respectively. As Sparling reported (22), a level plateau of transformation frequency was observed in our experiments, indicating that transformants grow at the same rate as nontransformed cells and do not clump more (or less) than nontransformed cells. Therefore, it was generally possible to compare transformation frequencies of samples from different times during an experiment, so long as sufficient time was allowed for phenotypic expression before selection was applied.

When selecting for increased antibiotic resistance, the cultures were incubated for 30 min after deoxyribonuclease was added, and duplicate platings were made: one set was to be overlaid with soft agar containing antibiotic, and another was used for viable count. The overlays were done after incubation for about 1.5 times as long as was required for phenotypic expression. After 2 to 3 days of incubation these plates were compared with the controls.

Up to 6 h of incubation in GCH broth was used when selecting for nutritional independence. After incubation, 2 ml were removed, and the cells were harvested by centrifugation (2,000 × *g*, 20 min). The cell pellet was suspended in 2 ml of minimal salts diluent and platings were made. Occasionally, Arg^-, Hyx^-, Ura^- recipients were suspended and diluted in PPS to provide limited amounts of growth requirements.

Growth was compared with controls as above. For pairs of strains that did not show recombination at a 10^{-1} dilution, 15-cm petri dishes with 50 ml of medium were used, and 0.5 ml of the resuspended cells were spread onto each dish.

Viable counts were made on K plates and NEDA complete medium. The efficiency of plating on defined medium compared with K plates was usually 100%. Plates from any experiment in which the efficiency of plating was less than 85% were discarded.

Since KH6777 is prototrophic for every marker examined and carries resistance to Nal and Rif, each recipient in an experiment was also transformed with KH6777 DNA. Selection of Nal^r or Rif^r transformants served as a control on the recipients' competency, and selection for a nutritional marker controlled competency, time of phenotype expression, and media quality.

Rate of spontaneous reversion. Since the minus DNA control or homologous DNA control usually had some colonies on the minus Hyx plates, an attempt was made to determine the rate of spontaneous reversion to Hyx independence and some of the other traits by the method described in Hayes (10). Hyx^- to Hyx^+ is the only change that could be readily quantified. No spontaneous changes from Ura^- to Ura^+ or Pen^s to Pen^r at 0.1 μg/ml could be seen in these experiments. Some spontaneous Pro-, Arg-, or Met-inde-

pendent and Nal- or, to a lesser degree, Rif-resistant colonies were observed occassionally, but these were infrequent.

Linkage. Transformants were passed on medium identical to that used for primary selection (i.e., 0.1 μg of penicillin G per ml, without Arg, etc.). Twenty-four-hour growth was used to test for auxotype, as above, and was streaked on antibiotic agar in a similar manner.

Additionally, double selection was done by overlaying with two antibiotics, omitting more than one component from the defined medium, or overlaying with antibiotic after 12 to 16 h of growth on a defined plate without one medium component.

RESULTS

Transformation and stability of gonococcal auxotypes. Initially, different auxotrophic clinical isolates were examined for their ability to be transformed by DNA extracted from several different strains. The clinical isolates could be transformed to prototrophy or antibiotic resistance at a frequency ranging from 0.03 to 0.91% (Table 2). The results of the transformation tests verify and extend the observations of Catlin (6) that auxotrophic gonococci possess repairable defects of the pathways involved in the biosynthesis of proline, arginine, hypoxanthine, uracil, and methionine. Transformation of the exquisitely penicillin G-sensitive (MIC, ≤0.01 to 0.03 μg/ml) DGI isolates to penicillin resistance resulted in increased levels of resistance (≥0.1 μg/ml), although these were ordinarily not equivalent to the levels exhibited by the donor strain (0.5 μg/ml). Since previous studies on the genetics of antibiotic resistance have revealed that penicillin resistance requires several discrete loci (15), this was not an unexpected finding. Nonetheless, the data do demonstrate that DGI isolates are no different than other gonococcal isolates with respect to their ability to become penicillin resistant.

The stability of the nutritional markers of the gonococci was determined for the Pro^-, Arg^-, Hyx^-, and Ura^- requirements. Spontaneous reversion from Ura^- to Ura^+ was never observed. The reversion rate of Pro^- to Pro^+ and of Arg^- to Arg^+ was found to be $\leq 3 \times 10^{-9}$ per cell per generation. However, the reversion of Hyx^- to Hyx^+ was relatively frequent and occurred at a rate of 7.3×10^{-6} per cell per generation.

Linkage relationship. The difference in the frequency of transformation for the Arg^-, Hyx^-, and Ura^- markers shared by DGI isolates, as well as the difference in the reversion of each nutritional requirement, suggested that they represented separate independent loci rather than a pleiotropic effect of a single gene. This conclusion was supported by picking 50 to 160 transformant colonies for a single marker and testing them for the inheritance of an unselected genetic trait. Each transformant colony was restreaked on the same medium used for primary selection and then tested on plates of defined media, each of which lacked a nutritionally essential compound or an appropriate antibiotic. Most Arg^+ transformants of DGI isolates retained the unselected Hyx^- and Ura^- marker. However, roughly 5% of Arg^+ transformants (7/160) were found to be Hyx^+, but in no instance tested (0/160) was Ura^+ present as an unselected marker. Minimal linkage between Ura^+ and both the Nal^r and Rif^r was noted. Ura^+ and Nal^r were co-inherited about 4% of the time, whereas Ura^+ and Rif^r were co-transformed 0.25% of the time. None of the Arg^+ (160 transformants tested) or Ura^+ (73 transformants tested) showed increased resistance (MIC, 0.1 μg/ml) when transformed with an Arg^+, Pen^r (MIC, 0.5 μg/ml) donor strain. Similarly, the transformation of an Arg^-, Hyx^-, Ura^-, Pen^s recipient to penicillin resistance (MIC, 0.1 μg/ml) failed to reveal linkage in the 268 transformants that were tested.

We also examined 12 to 50 Arg^+, Ura^+, Hyx^+, and Pen^r transformants of DGI isolates for any change in their susceptibility to serum killing. All of the independent transformants retained their serum resistance as assayed in the serum bactericidal reaction, as did the Pen^r transformants that were tested. Although these studies failed to disclose a relationship between penicillin susceptibility and serum resistance, subsequent studies, to be reported elsewhere, have shown a complex genetic relationship between these two properties. Nevertheless, the transformation and linkage studies reported here indicate that the auxotrophic markers found frequently in DGI strains of *N. gonorrhoeae* are present as discrete, independent genes that show

TABLE 2. *Transformation frequencies*

Marker[a]	No. of donors	No. of recipients	% Transformation
Nal^r	1	21	0.43
Rif^r	1	21	0.53
Pen^r	2	7	0.21
Pro^+	5	8	0.91
Arg^+	5	8	0.54
Hyx^+	4	2	0.5[b]
Ura^+	7	7	0.03
Met^+	30	2	0.74

[a] Selection of Nal or Rif resistance was at 1 μg/ml.

[b] Due to the high reversion rate of this trait, the frequency is estimated by [(no. of transformants − no. on control at same dilution)/CFU] × 100% = estimated % of transformation.

little or no genetic linkage to each other. These results are similar to those published previously by Young et al. (25).

Recombination among Arg⁻ and Pro⁻ auxotrophs. The finding that gonococci isolated from patients frequently (about 75% of the time) possess one or more nutritional requirements is not only useful for epidemiological studies, but also raises the question whether a particular or preferred genetic block in some biosynthetic pathway is found selectively. In the case of the DGI isolates, it is also of interest whether their multiple genetic lesions are distinguishable at the fine-structural level. Provided that the sensitivity of the genetic system is adequate, transformation between phenotypically similar auxotypes can serve to distinguish identical or overlapping genetic defects from nonidentical mutations. Thus, the ability of pairs of phenotypically similar auxotrophs to produce prototrophic recombinants may be reasonably taken to indicate genetic nonidentity of the mutations. On the other hand, if such crosses are nonproductive and fail to produce recombinants, it is assumed that the strains possess a mutation at the same localized site. In a limited series of such experiments, Catlin (6) was, in fact, able to verify that different genetic defects were involved in independent isolates with phenotypically similar requirements.

To determine whether the Arg⁻ auxotrophic mutants from DGI isolates could be differentiated from each other or from other Arg⁻ isolates from uncomplicated infections, 25 donor DNA preparations extracted from 18 Arg⁻ DGI isolates and 7 Arg⁻ non-DGI isolates were employed to transform 16 recipient strains. The results of some homologous and heterologous crosses are shown in Table 3. The data show that the DGI strains, regardless of source, possess identical or overlapping genetic defects. In contrast, DNA preparations extracted from DGI strains were capable of transforming Arg⁻ strains isolated from uncomplicated infections to arginine independence, albeit at a lower frequency than usually observed with an Arg⁺ transforming DNA preparation. Yet, even among Arg⁻ non-DGI strains, independent isolates often harbored a mutation at the same localized site. Thus, strains A135 and KH5730, in reciprocal reactions, could not transform one another to prototrophy. The strains KH6288, KH6326, and KH6557 formed a similar homologous group. The Arg⁻ strain KH6306 could, however, transform all other strains tested to Arg⁻. In all, therefore, four genetically distinct Arg⁻ mutations were identified.

Although the series of cultures is not extensive, the data with the Arg⁻ strains indicate that although *N. gonorrhoeae* strains isolated from patients often possess mutant genes, it is possible that the distribution of such mutations among isolates is nonrandom. This possibility was further strengthened by examining a series of 12 independently isolated Pro⁻ strains of gonococci (Table 4). Four of the strains represented DGI

TABLE 3. *Recombination between arginine auxotrophs (Arg⁻)*[a]

Arg⁻ donor	Arg⁻ recipient									
	I					II	III		IV	
	316	1951	1975	6305	6356	6306	A135	5730	6288	6326
LP99	−	−	−	−	−	+	++	++	++	++
MHD316	0	−	−	−	−	+	++	++	++	++
KH1951	−	0	−	−	−	+	++	++	++	++
KH2031	−	−	−	−	−	+	++	++	++	++
KH1384	−	−	−	−	−	+	++	++	++	++
KH1938	−	−	−	−	−	+	++	++	++	++
KH1950	−	−	−	−	−	+	++	++	++	++
KH1975	−	−	0	−	−	+	++	++	++	++
KH6305	−	−	−	0	−	+	++	++	++	++
KH6356	−	−	−	−	0	+	++	++	++	++
KH6306	+	+	+	+	+	0	+	+	++	++
A135	++	++	++	++	++	+	0	−	++	++
KH5730	++	++	++	++	++	+	−	0	++	++
KH6288	++	++	++	++	++	++	++	++	0	−
KH6326	++	++	++	++	++	++	++	++	−	0
KH6557	++	++	++	++	++	++	++	++	−	−

[a] Members of each group produce no Arg-independent transformants in crosses within their own group, but crosses with other groups result in Arg-independent transformants. 0, Homologous crosses; −, no Arg-independent transformants obtained (lower limit ranged from 8×10^{-6} to 10^{-7}%, with an average of 2.4×10^{-6}%); +, recombinants observed at a frequency of 10^{-3} to 10^{-5}%; ++, recombinants observed at a frequency of 10^{-2} to 10^{-1}%.

TABLE 4. *Recombination between proline auxotrophs (Pro⁻)*[a]

Pro donor		Pro⁻ recipient							
		I				II	IV	V	VI
Group	Strain	F62	A135	6326	6557	LP53	5730	MHD316	1951
	F62	0	−	−	−	+	+	++	+++
	A135	−	0	−	−	+	+	++	+++
I	KH6326	−	−	0	−	+	+	++	+++
	KH6557	−	−	−	0	+	+	++	+++
	LP53	+	+	+	+	0	+	++	+++
III	KH5060	+	+	ND[b]	+	+	+	++	+++
IV	KH5730	+	+	+	+	+	0	++	+++
	MHD316	++	++	++	++	++	++	0	+++
V	LP99	++	++	++	++	++	++	−	+++
	KH2031	++	++	++	++	++	++	−	+++
VI	KH1951	+++	+++	+++	+++	+++	+++	+++	0

[a] Members of each group produce no Pro-independent transformants in crosses within their own group, but crosses with other groups result in Pro-independent transformants. 0, Homologous crosses arbitrarily set at 0; −, no Pro-independent transformants obtained (lower limits range from 2×10^{-5} to 10^{-7}%, with an average of 2.3×10^{-6}%); +, recombinants observed at a frequency of 10^{-3} to 10^{-5}%; ++, recombinants observed at a frequency of 10^{-2} to 10^{-1}%; +++, recombinants observed at a frequency of 10^{-1}%.

[b] ND, Not determined.

isolates. Three of the four (MHD316, LP99, and KH2031) possessed identical or overlapping Pro⁻ mutations; one, strain KH1951, was capable of transforming all other Pro⁻ strains to Pro⁺ at a frequency of 4.5×10^{-1}%, which is very nearly the frequency of transformation seen when DNA from a proline-independent strain is used. Similarly, strains LP53, KH5730, and KH5730, of independent origin, could transform all other Pro⁻ strains to Pro⁺. However, a group of four independent non-DGI Pro⁻ isolates, A135, KH6326, and KH6557, as well as the used laboratory strain F62, were found to contain identical genetic Pro⁻ lesions, as evidenced by their inability to transform one another to Pro⁺.

Other gram-negative bacteria have three genes associated with the biosynthesis of proline (1, 19). Two of the genes are closely linked, and the third is a short distance away, with several genes mapping in the region between it and the two clustered loci.

The data presented are consistent with the hypothesis that proline genes of gonococci are located in the genome in a manner similar to other gram-negative bacteria. The division of mutants into more than three groups suggests that transformation in gonococci is sensitive enough to detect recombination within a single cistron. More quantitative genetic and biochemical experiments will be required to prove this suggestion, however.

Hyx⁻ and Ura⁻ recombination. Due to the reversion frequency of the Hyx⁻ phenotype, Hyx⁻ × Hyx⁻ recombination could not be examined.

The low transformation frequency to Ura⁺ suggests that recombination, if present, between two Ura⁻ strains may be below the sensitivity of the method. Recombination to Ura independence was looked for but was never observed, suggesting that, similar to the Arg⁻ lesion in all Arg⁻, Hyx⁻, Ura⁻ strains, the lesion producing the Ura requirement is identical in every strain examined.

DISCUSSION

The lesions producing requirements for Arg, Ura, and probably Hyx appear to be identical to all the strains we examined; this indicates a common origin for strains with the Arg⁻, Hyx⁻, Ura⁻ phenotype. Even though this constellation remains unchanged, requirements other than Arg, Hyx, and Ura can be different. An additional requirement for methionine is exhibited by LP99. Differences are also seen in several other requirements, such as proline dependence (5). Our data show that two different Pro⁻ lesions are seen in Arg⁻, Hyx⁻, Ura⁻ strains, one of which is different from every other Pro⁻ lesion examined.

Eight auxotypes containing the Arg⁻, Hyx⁻, Ura⁻ constellation are known (3). The genetics of gonococci is not well enough understood to explain this diversity of other requirements while Arg⁻, Hyx⁻, and Ura⁻ remain constant. Arg⁻, Hyx⁻, and Ura⁻ are not closely linked to themselves, or to Pen and serum resistance. The co-transformation of Arg and Hyx is probably more coincidental than significant.

In the study by Carifo and Catlin, the Arg requirement was commonly found as the only requirement or in combinations other than the

Arg$^-$, Hyx$^-$, Ura$^-$ constellation (i.e., Arg$^-$ plus Pro$^-$, Arg$^-$ plus Met$^-$, etc.) (3). In contrast, the Ura requirement was never found and the Hyx requirement was only rarely found (4 of 251 strains examined), except in the Arg$^-$, Hyx$^-$, Ura$^-$ combination (3). This uniqueness of the combined Arg, Hyx, and Ura requirements could suggest some involvement in the virulence of these strains.

We believe, however, that our data argue an opposite possibility. If the Arg, Hyx, and Ura requirements were important virulence factors that arose from random mutations, we would expect to see several lesions producing these requirements. There are several lesions producing most requirements that have been examined thus far (including proline, methionine, arginine, and thiamine pyrophosphate) (6). The Arg, Hyx, and Ura constellation is the only case in which identical lesions have been demonstrated. Therefore, we feel the evidence indicates that all strains requiring Arg, Hyx, and Ura are derived from a single ancestor.

Although there is no other evidence about the existence of this hypothesized ancestral clone, we may be able to speculate about the origin of nutritionally defective gonococci in general. This speculation is based on the penicillin selection method of producing auxotrophic mutants in vitro (17). The method exploits the fact that penicillin affects only growing cells. Auxotrophic mutants will not grow when placed in a medium lacking the growth factors they require. Since the auxotrophs cannot grow in deficient medium, they are not killed by the penicillin. In contrast, any nutritionally independent cells are killed by the penicillin, thereby enriching for the auxotrophs and making it possible to eventually isolate the mutants. It is possible that similar selection for auxotrophic gonococci has been taking place in vivo for as long as penicillin has been used to treat gonorrhea. Catlin has proposed that this method of selection may explain the high proportion of proline auxotrophs (7). Other cases in which nutritionally defective mutants have had the advantage of persistence have been described (8, 9). This hypothesis could be tested by examining *N. gonorrhoeae* strains that were isolated before the use of penicillin was begun. The hypothesis predicts that the majority of these strains would be nutritionally independent or the zero auxotype. Only about 25% of strains isolated recently are the zero auxotype. It is not known to what extent any culture collections of *Neisseria* contain gonococcal isolates from the pre-penicillin era, but some strains are available and a study of these early isolates is under way.

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