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Cotransformation of Temperature Sensitivity and Nutritional Markers in *Neisseria gonorrhoeae*

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Cotransformation remains the only tool for establishing linkage in *Neisseria gonorrhoeae*. Because of the difficulty of inducing auxotrophic markers via mutagenesis in this species, most previous studies have utilized antibiotic resistance and naturally occurring (auxotypic) auxotrophic markers. We have succeeded in isolating auxotrophic and temperature-sensitive mutants. The temperature-sensitive mutants have been characterized by their growth on complex and defined media at 31, 37, and 40°C. Two of the mutants exhibited an unusual pattern of temperature sensitivity—growth on the defined medium but absence of growth on the complex medium at 37°C. Both mutants, however, were temperature-sensitive on the two media at 40°C. We have demonstrated linkages between markers isolated in our laboratory and the auxotypic markers of the clinical isolate RUG208. Ts-2 exhibited 85 to 95% linkage to Arg^- and *his-2* exhibited 40% linkage to Val^-. In addition weak linkages were shown between *his-2* and Arg^- (2 to 6%) and between Arg^- and Val^- (3 to 5%). Linkages among *his-2*, Arg^-, and Val^- which could be demonstrated when deoxyribonucleic acid from strain F62 was used to transform RUG208 were absent when F62 was used as recipient for RUG208 DNA. Our data are consistent with a tentative map order of *his-2*, Val^-, Arg^-, Ts-2.

Genes conferring antibiotic resistance (8, 9, 11, 15), piliation (1), nutritional independence (4, 6, 17), and temperature sensitivity (16) have been studied in *Neisseria gonorrhoeae*. The antibiotic resistance regions of the chromosome have been well characterized, and this subject has been reviewed by Sparling et al. (14). Maier et al. (8) mapped six antibiotic resistance mutations by transformation and found that they fell into three linkage groups. Group I contains loci for resistances to streptomycin, tetracycline, chloramphenicol, and erythromycin. Sarubbi et al. (11) and Sparling et al. (15) have shown that this region also includes loci that specify resistances to rifampin, spectinomycin, and fusidic acid. Group II of Maier et al. (8, 9) contains the multiple resistance locus (*mtr*) which confers a low level of resistance to a variety of agents. Group III contains a locus that specifies resistance to relatively high levels of penicillin. With the development of chemically defined media (5, 7), it became possible to isolate auxotrophs for use in mapping studies and for the study of amino acid biosynthesis and control. A number of naturally occurring auxotrophic strains (auxotypes) of *N. gonorrhoeae* have been classified on the basis of growth requirements (4, 6), but only one study has dealt with the mapping of these nutritional markers. Using auxotypes, Young et al. (17) have shown the linkage of an arginine marker to loci involved in the biosynthesis of isoleucine, valine, and hypoxanthine. A uracil marker was linked to the gene conferring rifampin resistance. Mapping studies have been restricted to these limited portions of the chromosome because of the relative refractility of the gonococcus to mutagenesis.

To adequately map the entire chromosome, it would be advantageous to have conditionally lethal mutations as well as mutations conferring auxotrophy and antibiotic resistance. Wharton and Zubrzycki (16) have reported the isolation of nine different temperature-sensitive mutants. Using one of these mutants, they have shown linkage between a marker conferring temperature sensitivity and one conferring resistance to nalidixic acid. However, there have been no studies showing the linkage of temperature-sensitive and auxotrophic markers. In this communication, we report the isolation and characterization of several temperature-sensitive mutants. Using cotransformation we have then shown linkage among several auxotrophic markers and

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the linkage of a temperature-sensitive marker to one of them.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and mutants of *N. gonorrhoeae* strain F62 that were used in this study are listed in Table 1. Strain F62 was obtained from D. S. Kellogg, and strain RUG208 was from F. E. Young. *N. gonorrhoeae* was grown on GCBA-DS (13) or on the defined gonococcal genetic medium (GGM) of La Scolea and Young (7) at 37°C in the presence of 10% CO_2. GCBA-DS and GGM (or GGM plus supplements) represent complete media and were used interchangeably. Liquid cultures were grown in the biphasic medium of Sparling (13) which consists of GCBA-DS agar and GCBB-DS broth at 37°C in a reciprocating shaker (New Brunswick, Inc., model R25).

Isolation of mutants. Temperature-sensitive mutants were obtained after treatment with ethyl methane sulfonate (EMS) by the following procedure. *N. gonorrhoeae* strain F62 type 1 was streaked onto complex agar (GCBA-DS), and the plate was incubated at 37°C in a 10% CO_2 atmosphere for 16 h. The plate culture was harvested in 4 ml of GCBB-DS broth, and this suspension was used to inoculate biphasic medium. The cultures were grown on a reciprocating shaker (37°C, 100 rpm) to a concentration of 10^8 to 5×10^8 colony-forming units per ml. Cells were pelleted by centrifugation at 8,000 rpm for 10 min (Sorvall RC-2B, rotor SS34) and resuspended at a concentration of 10^9 CFU/ml in GGM broth with glucose omitted and containing 1% EMS (vol/vol). Cultures were incubated for 45 min at 37°C. Cells were centrifuged and resuspended in GGM broth without glucose and containing 5% sodium thiosulfate to inactivate the remaining EMS. Cells were washed once in GC broth, diluted in the same medium, and plated on GCBA-DS. The plates were incubated at 30°C in a 10% CO_2 atmosphere until the colonies just became visible under a dissecting microscope (20× magnification). The plates were then transferred to 37°C and incubated for an additional 24 h. Colonies that failed to increase in size were picked and tested for growth on GCBA-DS at 30 and 37°C. Potential mutants able to grow at 30 but not at 37°C were retested three times at the two temperatures. The procedure used for mutagenesis with EMS excludes an outgrowth step because of previous findings in our laboratory that allowance for phenotypic expression had no effect or may even have reduced the number of mutants recovered.

Auxotrophic mutants were isolated by using the following modifications of the above procedure. *N. gonorrhoeae* strain F62 type 1 was streaked onto GGM agar, and the plate was incubated at 37°C in a 10% CO_2 atmosphere for 24 to 48 h. The plate culture was harvested in GGM broth, and this suspension was used to inoculate 30 ml of GGM broth. Cells were then treated with EMS and sodium thiosulfate in the same manner as above, washed, and plated on GCBA-DS. Plates were incubated at 37°C for 48 h. Colonies were then tested for growth on GGM and GCBA-DS. Potential auxotrophs were tested by subculturing three times on the two media, and those colonies able to grow on GCBA-DS but unable to grow on GGM were characterized in terms of their nutritional requirements. Mutants were tested for growth on GGM with the addition of adenine, guanine, thymine, cytosine, tryptophan, tyrosine, phenylalanine, lysine, leucine, histidine, valine, glycine, threonine, or alanine. Using this procedure, we were able to isolate the following types of auxotrophs: Trp^-, Tyr^-, Leu^-, Ade^-, Val^-, Thr^-, and Aro^-. Mutants (both *N*-methyl-*N'*-nitro-*N*-nitrosoguanine and EMS derived) were grown routinely on GGM supplemented with 100 μg of the appropriate amino acid, purine, or pyrimidine per ml.

Isolation of DNA. DNA was routinely isolated by the Marmur procedure (10) from stationary cells of *N. gonorrhoeae* grown 18 to 24 h in liquid cultures. DNA to be used in cotransformation experiments was isolated by the Berns and Thomas procedure (2). Studies in other laboratories have indicated that this method of DNA isolation is relatively gentle, and longer pieces of DNA are recovered than by other methods. DNA was assayed by the diphenylamine method of Burton (3).

Transformation. Only T1 and T2 clonal types of *N. gonorrhoeae* are competent for transformation (13). Cultures of type T1 were maintained by selective transfer of individual colonies on GCBA-DS. To start a competent culture a GCBA-DS plate was streaked with T1 cells and incubated at 37°C for 16 h. When competent cells of a temperature-sensitive mutant were needed, the plate was incubated at 30°C for 40 h. The plate was harvested in GCBB. Competent cells

TABLE 1. *Strains and mutants of N. gonorrhoeae used in this study*

Strain or mutant	Genotypic or phenotypic characteristics[a]	Source
KUG1	*tyr-1*	NTG-induced mutant[b]
KUG6	*his-2*	NTG-induced mutant
KUG21	*aro-1*	EMS-induced mutant
KUG27	*trp-1* Strr	NTG-induced mutant
KUG29	*tyr-1 trp-1* Strr	Transformant of KUG1 with KUG27 DNA
KUG30	Ts-1 *aro-1*	EMS-induced mutant of KUG21
KUG31	Ts-2 *trp-1 tyr-1* Strr	EMS-induced mutant of KUG29
KUG32	Ts-3 *aro-1*	EMS-induced mutant of KUG21
KUG33	*trp-1 tyr-1 his-2* Strr	Transformant of KUG31 with KUG6 DNA
RUG208	$Arg^- Val^- Ura^- Ile^-$	F. Young
KUG101	*his-2* $Ura^- Ile^-$	Transformant of RUG208 with KUG33 DNA

[a] The genotypic or phenotypic characteristics designate the following based on growth requirements or antibiotic resistance: tyr, tyrosine; his, histidine; trp, tryptophan; Strr, streptomycin resistance (1 mg/ml); aro, tryptophan + tyrosine + phenylalanine; Ts, unable to grow at 40°C; Arg, arginine; Val, valine; Ura, uracil; Ile, isoleucine.

[b] *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG)-induced mutants were isolated by A. Siddiqui (12).

were prepared by diluting cells from the plate harvest to a concentration of 10^7 to 10^8 CFU/ml in GCBB.

Donor DNA was diluted to a specified concentration in 0.15 M NaCl–0.015 M sodium citrate, pH 8.0 (SSC). A portion (0.9 ml) of the competent culture was mixed with 0.05 ml of donor DNA and 0.05 ml of $CaCl_2$ (0.002 M final concentration) in a tube. The tubes were routinely incubated for 30 min at 37°C in the presence of CO_2. When the competent cells were temperature sensitive, the tubes were incubated for 45 min at 30°C. Pancreatic DNase (Worthington) (50 μg/ml final concentration) was added to the transformation tubes, and the tubes were incubated an additional 10 min to degrade unbound DNA. The cell suspensions were diluted in GCBB and plated for transformants and total number of recipient cells. Transformants to temperature independence were selected on GCBA-DS. Transformants to nutritional independence were selected on GGM. All plates were incubated for 40 to 48 h at 37°C in the presence of 10% CO_2.

Plate transformation. Temperature-sensitive mutants were tested for genetic identity by a plate transformation procedure. Competent cells (10^7 to 5×10^7 CFU/ml) and $CaCl_2$ (final concentration 0.002 M) were mixed, and portions (0.1 ml) were spread onto GCBA-DS plates. DNA (0.01 ml) was spotted onto these plates. The plates were then incubated for 48 h at 37°C and examined for transformants. Controls consisted of recipient cells without DNA, recipient cells spotted with DNA that had been pretreated with DNase (50 μg/ml), and recipient cells spotted with homologous DNA. The controls were all negative.

Cotransformation studies. To investigate the possible linkage between two markers, transformation experiments were performed, and the transformants were scored by an unselected marker procedure. One mutant (A^- B^+) was transformed with DNA from another mutant (A^+ B^-), and A^+ transformants were selected. Transformants were replica picked with toothpicks to the same medium for purification and then transferred by toothpicks to supplemented and nonsupplemented plates to test for the unselected marker (B^-). The purification step was necessary to separate transformants from nontransformed cells. In its absence the results were sometimes complicated by the survival and residual growth of nontransformed cells.

Plates were scored for transformants after 40 to 48 h of incubation at 37°C. Cotransformation experiments were performed at both saturating and nonsaturating levels of DNA and, whenever possible, direct selection was used for both markers. The percentage of cotransformation was determined by the following formula:

(number of double transformants/
number of single + double transformants) × 100.

RESULTS

Isolation and characterization of temperature-sensitive mutants. Figure 1 shows the survival of two different mutants, KUG21 and KUG29, of *N. gonorrhoeae* after different times of exposure to 1% EMS. Approximately 0.1% of the bacteria survived after 45 min of exposure to EMS. These bacteria were plated for the isolation of mutants. Three temperature-sensitive mutants were isolated. These mutants were shown to differ from one another genetically by means of plate transformation. DNA from KUG30 (Ts-1) transformed KUG31 (Ts-2) and KUG32 (Ts-3) to temperature independence; DNA from KUG31 transformed the other two temperature-sensitive mutants, as did DNA from KUG32.

The temperature-sensitive markers were tested for linkage with several auxotrophic markers by means of transformation. Ts-1 and Ts-3 were tested for linkage with *aro-1*; Ts-2 was tested for linkage with *trp-1* and *tyr-1*. Linkage was calculated from two kinds of data. The first was obtained by direct selection of both single and double transformants. Single Ts^+ transformants were selected on complex medium (GCBA-DS) incubated at 37°C; Ts^+ Aro^+ (or Ts^+ Trp^+ or Ts^+ Tyr^+) transformants were selected on supplemented defined GGM medium incubated at 37°C. Calculations based on these data indicated that Ts-3 and *aro-1* were not linked; Ts-1 and *aro-1* exhibited 18 to 19% linkage; Ts-2 and *trp-1* exhibited 32 to 46% linkage; and Ts-2 and

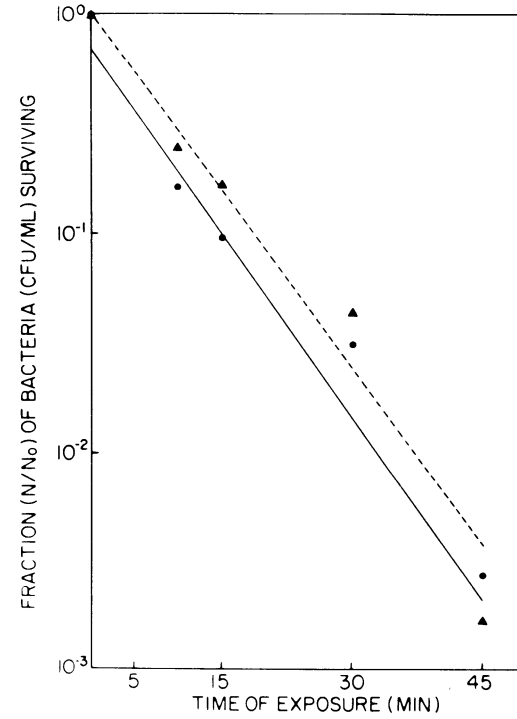

FIG. 1. *Survival of N. gonorrhoeae after exposure to EMS (1%). Symbols: ●, KUG21; ▲, KUG29. See Isolation of mutants under Materials and Methods for the procedure.*

tyr-1 exhibited 25 to 27% linkage. Linkage was also calculated by determining the percentage of Ts^+ transformants receiving the unselected marker (Aro^+, Trp^+, or Tyr^+). Calculations based on these results indicated little if any linkage between the temperature-sensitive and auxotrophic markers tested.

The discrepancies noted above could have resulted if the Ts mutants had exhibited temperature sensitivity on complex but not on defined media at 37°C. Cells of both mutants and of the wild type were harvested from GCBA-DS plate cultures. The suspensions were diluted, plated for viable cell counts on GCBA-DS and GGM with appropriate supplements, and incubated at 31 and 37°C. A permissive temperature of 31°C rather than 30°C was used because GGM will not support the growth of *N. gonorrhoeae* F62 below 31°C. Both Ts-1 and Ts-2 were able to grow on GGM at both 37 and 31°C and on GCBA-DS at 31°C but not on this latter medium at 37°C.

To test the possibility that the mutants would exhibit the temperature-sensitive phenotype on defined as well as complex media when tested at a higher temperature, the growth of the wild-type F62 and the two mutants was compared on GGM and GCBA-DS at 31, 37, and 40°C (Table 2). The wild type grew at all three temperatures. Ts-1 and Ts-2 were unable to grow at 40°C on either medium.

Cotransformation of Ts and auxotrophic markers. RUG208 is a clinical auxotroph (auxotype) with requirements for uracil, arginine, and valine. The original strain had a requirement for hypoxanthine, but this marker has reverted in our isolate of RUG208. Our linkage studies were done by means of interstrain crosses because of the difficulties that we and others have encountered in attempts to construct multiply marked mutants via congression (unpublished data, 14). In the following experiments, KUG31 was transformed with DNA isolated from RUG208. Single Ts^+ transformants were selected, and these were tested for cotransformation of Ts-2 and Val^-, Ts-2 and Arg^-, and Ts-2 and Ura^-. Figure 2 shows the comparison of the frequency of single Ts^+ transformants and Ts^+ Arg^- double transformants. Saturation occurred at 1 μg of DNA per ml for both single and double transformants. The parallel dose-response curves indicate that the ratio of double to single transformants was independent of DNA concentration at levels of DNA well below saturating and, thus, that the markers were indeed linked. Ts-2 and Arg^- were linked by cotransformation of 85 to 95%. There was no linkage of Ts-2 and Val^- or Ura^- (<1%).

TABLE 2. *Growth of temperature-sensitive mutants on complex and defined media*[a]

Organism	Temp (°C)	CFU/ml on:	
		GCBA-DS	GGM[b]
Wild type	31	6.3×10^6	1.3×10^7
Wild type	37	7.6×10^6	1.8×10^7
Wild type	40	3.0×10^6	1.7×10^7
Ts-1 (KUG30)	31	3.1×10^7	3.0×10^7
Ts-1 (KUG30)	37	<10	3.2×10^7
Ts-1 (KUG30)	40	<10	<10
Ts-2 (KUG31)	31	6.7×10^7	8.3×10^7
Ts-2 (KUG31)	37	<10	6.0×10^7
Ts-2 (KUG31)	40	<10	<10

[a] Cells of both mutants and wild-type F62 were streaked on GCBA-DS plates from frozen preparations and incubated for 40 h in the presence of 10% CO_2. The plate cultures were harvested in 4 ml of GCBB-DS, and the harvests were diluted and plated on GCBA-DS and GGM with the appropriate supplements. Plates were incubated for up to 5 days at the above specified temperatures. Colonies were counted after 2 days of incubation with the exception of those of Ts-1 plated on GGM at 31 and 37°C, which were counted after 3 days.

[b] Wild-type cells were plated on GGM; KUG30 was plated on GGM-tryptophan-tyrosine-phenylalanine (100 μg of each per ml); KUG31 was plated on GGM-tryptophan-tyrosine (100 μg of each per ml).

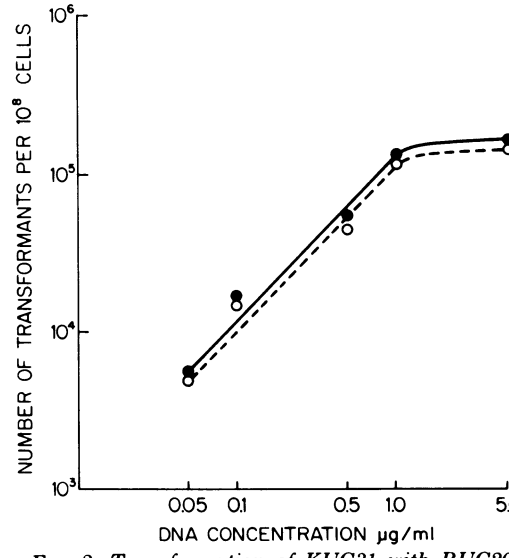

FIG. 2. *Transformation of KUG31 with RUG208 DNA. Symbols: ●, Ts^+ single transformants; ○, Ts^+ Arg^- double transformants.*

Additional auxotrophic markers of F62 were tested for linkage with auxotrophic markers of RUG208. DNA was isolated from KUG33, which

requires tryptophan, tyrosine, and histidine. RUG208 was transformed with this DNA, and Arg^+, Val^+, and Ura^+ single transformants were selected. The results are shown in Fig. 3. Parallel curves were obtained for Arg^+ and Val^+ single transformants, and again saturation occurred at about 1 μg/ml of DNA. The curve for Ura^+ single transformants was not parallel to the curves for Arg^+ and Val^+ single transformants and saturation did not occur with the levels of DNA tested. No Ura^+ transformants were obtained with levels of DNA below 1 μg/ml.

Linkage was determined by cotransformation. As shown in Figure 3, the dose-response curves obtained for Val^+ His^-, Arg^+ His^-, and Val^+ Arg^+ double transformants were parallel to those for the single transformants Arg^+ and Val^+. Val^- and *his-2* were linked by 40 to 41%; Val^- and Arg^- were linked by 3 to 5%, and Arg^- and *his-2* were linked by 2 to 6%. The low efficiencies of congression (0.02 to 0.2%) in the gonococcus (unpublished data, 14) ensures the validity of these low-linkage values. At most concentrations of DNA, there were fewer Arg^+ His^- than Arg^+ Val^+ double transformants. No linkages of *trp-1* or *tyr-1* to Val^- or Arg^- were seen. This absence of linkage (<0.7%) lends further support to the validity of the linkages of *his-2* to Arg^- and Arg^- to Val^-.

In each individual experiment, Val and Arg were more closely linked than were Arg and *his-2*. Because the two linkages were very similar, an additional experiment was done to distinguish between the two most probable orders for the markers: *his-2*, Val^-, Arg^- and Val^-, *his-2*, Arg^-. To determine the order, RUG208 was transformed with DNA from KUG33, and Arg^+ Val^+ double transformants were selected. These transformants were then picked to determine whether they were His^+ or His^- (Table 3). If the order were Val^-, *his-2*, Arg^-, then the overwhelming majority of Val^+ Arg^+ transformants would be His^-. If the order were *his-2*, Val^-, Arg^-, then some Val^+ Arg^+ transformants would be His^+ and some would be His^-. As seen in the first two lines of Table 3, the Arg^+ Val^+ double transformants were 47 to 67% His^+ and 33 to 53% His^-. The most likely order is therefore *his-2*, Val, Arg, Ts-2.

The reciprocal transformation cross was done with RUG208 DNA to transform KUG 33 (His^- Trp^- Tyr^-). His^+, Trp^+, and Tyr^+ single transformants were selected. These single transformants occurred at about the same frequency as the Val^+ and Arg^+ single transformants shown in Fig. 3. However, double transformants His^+ Val^- or His^+ Arg^- were not seen (<0.7%) (data not shown). Likewise, *his-2*, *trp-1*, and *tyr-1* were also unlinked to one another.

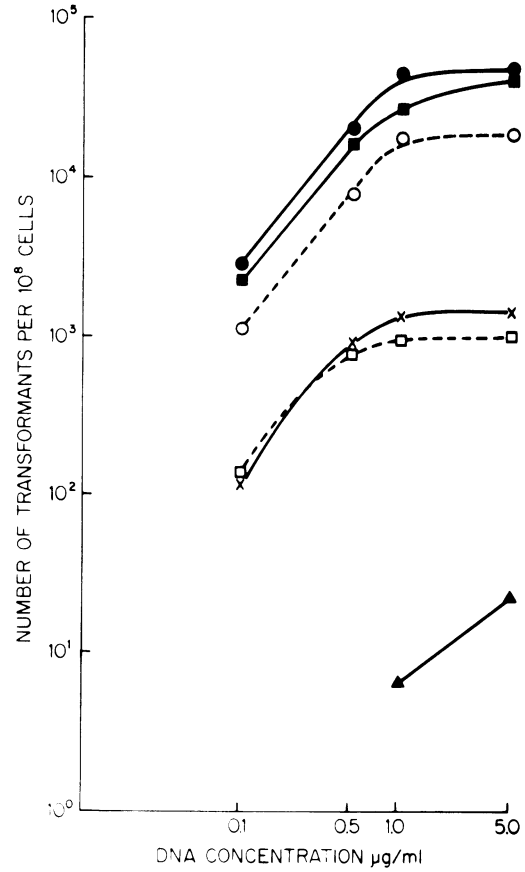

FIG. 3. *Transformation of RUG208 with KUG33 DNA. Single transformants:* ●, *Val⁺*; ■, *Arg⁺*; ▲, *Ura⁺*. *Double transformants:* ○, *Val⁺ His⁻*; □, *Arg⁺ His⁻*; ×, *Val⁺ Arg⁺*.

TABLE 3. *Cotransformation of His^- with Arg and Val*[a]

DNA		Selected markers	Unselected markers	% Cotransfer of unselected markers
Source	Concn (μg/ml)			
KUG33	1	Arg^+ Val^+	His^+	47
			His^-	53
KUG33	0.1	Arg^+ Val^+	His^+	67
			His^-	33
KUG101	1.0	Arg^+ Val^+	His^+	50
			His^-	50
KUG101	0.1	Arg^+ Val^+	His^+	71
			His^-	29

[a] RUG208 was transformed with DNA from His^- strains. Double transformants (Arg^+ Val^+) were selected and then transferred by toothpicks to media with and without histidine.

Because of this apparent absence of reciprocity, the linkages were re-examined by using crosses between isogenic strains of *N. gonor-*

TABLE 4. *Cotransformation of auxotrophic markers*[a]

Recipient	DNA		Transformants				% Cotransformation	
			Single		Double			
	Source	Concn (μg/ml)	Class	No./10^7 recipients	Class	No./10^7 recipients	Direct selection[b]	Unselected marker procedure
RUG208	KUG101	1.0	Val^+	1.0×10^4	Val^+ His^-			45.6
					Val^+ Arg^+	1.4×10^2	1.4	<0.6
			Arg^+	9.4×10^3	Arg^+ Val^+	1.4×10^2	1.4	7.5
					Arg^+ His^-			1.9
RUG208	KUG101	0.1	Val^+	1.0×10^3	Val^+ His^-			37.7
					Val^+ Arg^+	1.4	0.2	<1.0
			Arg^+	2.8×10^2	Arg^+ Val^+	1.4	0.2	1.8
					Arg^+ His^-			1.1

[a] Cotransformation studies were done as specified in the text.

[b] Calculated as number of double transformants (Arg^+Val^+)/{number of double (Arg^+Val^+) + [(single (Arg^+) + single (Val^+) transformants)/2]}.

rhoeae. Strain RUG208 was transformed with DNA from KUG101. Val^+ and Arg^+ single transformants and Val^+ Arg^+ double transformants were selected. These transformants were then picked to determine whether they contained the unselected marker (His^-). As shown in Table 4, Val^- and Arg^- exhibited 0.2 to 7.5% linkage, Val^- and His^- exhibited 38 to 46% linkage, and Arg^- and His^- exhibited 1.1 to 1.9% linkage. When Val^+ Arg^+ double transformants were tested to determine whether they were His^+ or His^- (Table 3), 29 to 50% were His^- and 50 to 71% were His^+. This evidence suggests that the markers *his-2*, Val^-, and Arg^- are in fact linked in RUG208 and the suggested order is as specified above.

DISCUSSION

A number of auxotrophic and temperature-sensitive mutants have been isolated in our laboratory, and many of these have been tested for linkage by cotransformation. We have shown cotransformation of *his-2* and Val^-, *his-2* and Arg^-, and Ts-2 and Arg^- and confirmed the linkage of Val^- and Arg^- previously reported by Young et al. (17). Young et al., however, have shown a greater degree of linkage between Arg^- and Val^- (10.2%) than we have obtained (3 to 5%). They have also demonstrated linkages between Arg^- and Ile^- (8.5%), Arg^- and Hyx^- (44.5%), and Ura^- and Rif^r (33%).

The Ura^- requirement in RUG208 is relatively difficult to transform; we were only able to transform with super-saturating levels of DNA. This mutation may represent a large deletion in the uracil region of the chromosome (Young, personal communication), or it may be the result of more than one mutation. Further experiments must be done to determine which is the case.

At the present time, we have no explanation for the linkage of three markers, Arg^-, Val^-, and *his-2* when strain RUG208 is used as a recipient and the total absence of linkage of the same three markers when strain F62 is used as a recipient. In reciprocal crosses, one would expect approximately the same linkage among *his-2*, Arg^-, and Val^- independent of direction. Because we are dealing with a single linkage group in two nonisogenic strains (F62 and RUG208), we do not know if this dislinkage is strain specific or marker specific. Further studies are necessary to clarify this phenomenon.

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