JOURNAL OF BACTERIOLOGY, July 1982, p. 295-302
0021-9193/82/070295-08$02.00/0
Vol. 151, No. 1

Arginine and Pyrimidine Biosynthetic Defects in *Neisseria gonorrhoeae* Strains Isolated from Patients

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Received 24 September 1981/Accepted 23 February 1982

Neisseria gonorrhoeae strains with nutritional requirements that include arginine (Arg$^-$), uracil (Ura$^-$), and hypoxanthine have attracted attention because of their tendency to cause disseminated infections. As a basis for genetic studies of arginine and pyrimidine biosynthesis, we examined the activities of four enzymes of these pathways in cell-free extracts of both prototrophic and Arg$^-$ Ura$^-$ strains. Activities of glutamate acetyltransferase, aspartate transcarbamylase, and orotate phosphoribosyltransferase, encoded respectively by *argE*, *pyrB*, and *pyrE*, were absent in some Arg$^-$ Ura$^-$ isolates. Gonococci that were unable to utilize ornithine for growth in place of citrulline lacked activity of carbamyl phosphate synthetase (encoded by *car*). Defects of *car* imposed requirements for both citrulline (or arginine) and a pyrimidine because of the dual role of carbamyl phosphate in the two pathways. Defects of *argE*, *car*, *pyrB*, and *pyrE* were separately introduced by genetic transformation into representatives of a gonococcal strain which initially was prototrophic. Results of enzyme assays of these isogenic auxotrophic transformants confirmed the gene-enzyme relationships.

Neisseria gonorrhoeae strains freshly isolated from patients exhibit various different nutritional requirements (3, 4). Strains that require arginine (Arg$^-$) and uracil (Ura$^-$) as well as hypoxanthine and possibly other compounds frequently cause disseminated gonococcal infections of patients in some communities (17, 24). Genetic studies of these natural auxotrophs are being carried out in a number of laboratories (e.g., 5, 15, 21, 33-35, 37). Unfortunately, in many instances the genotype of an auxotrophic strain cannot be correctly inferred from the phenotype because of the presence of two or more genetic lesions affecting the same biosynthetic pathway (15). Aberrant frequencies of DNA-mediated transformation have been reported in studies employing gonococci with unrecognized defects in more than one pyrimidine gene locus (5, 21, 37). We are investigating certain gonococcal gene-enzyme relationships to obtain information on the biochemical and genetic basis of auxotrophy which has evolved in *N. gonorrhoeae* (6).

Earlier we reported (32) that many Arg$^-$ gonococci have defects of the *argE* locus, encoding the enzyme acetylornithine:glutamate acetyltransferase (GATase). Gonococci that lack GATase activity are characterized by an inability to grow on defined media which contain glutamate, acetylglutamate, or acetylornithine in place of arginine, but where the remaining enzymes of the arginine pathway are functional, the bacteria can utilize ornithine, citrulline, or argininosuccinate (Fig. 1). However, ornithine does not satisfy the requirement for arginine of some Arg$^-$ Ura$^-$ strains. In gonococci, as in other bacteria (11, 31), ornithine is converted to citrulline by ornithine transcarbamylase (OTCase). Unexpectedly, OTCase activity comparable to that of Arg$^+$ gonococci was found in various Arg$^-$ Ura$^-$ strains which utilized citrulline but not ornithine for growth (8, 32). In addition to OTCase and ornithine, carbamyl phosphate is required for the synthesis of citrulline. Mutants of *Pseudomonas aeruginosa* (11) and *Escherichia coli* (28) which lack carbamyl phosphate synthetase (CPSase) as a result of *car* lesions exhibit requirements for both arginine and uracil. Therefore, it seemed likely that activity of a corresponding synthetase might be lacking in certain Arg$^-$ Ura$^-$ strains of *N. gonorrhoeae*. In addition, some of these gonococci must possess one or more other defects of pyrimidine biosynthesis since the uracil requirement often was retained by Car$^+$ transformants selected for their ability to utilize ornithine (8, 37). It was important also to determine whether the same or other defects of pyrimidine biosynthesis are responsible for the uracil requirement of Arg$^-$ Ura$^-$ gonococci that are Car$^+$ when isolated from patient specimens.

We report here the findings with various Arg$^-$ Ura$^-$ strains of *N. gonorrhoeae* which bear lesions of *argE*, *car*, *pyrB*, and *pyrE* gene loci.

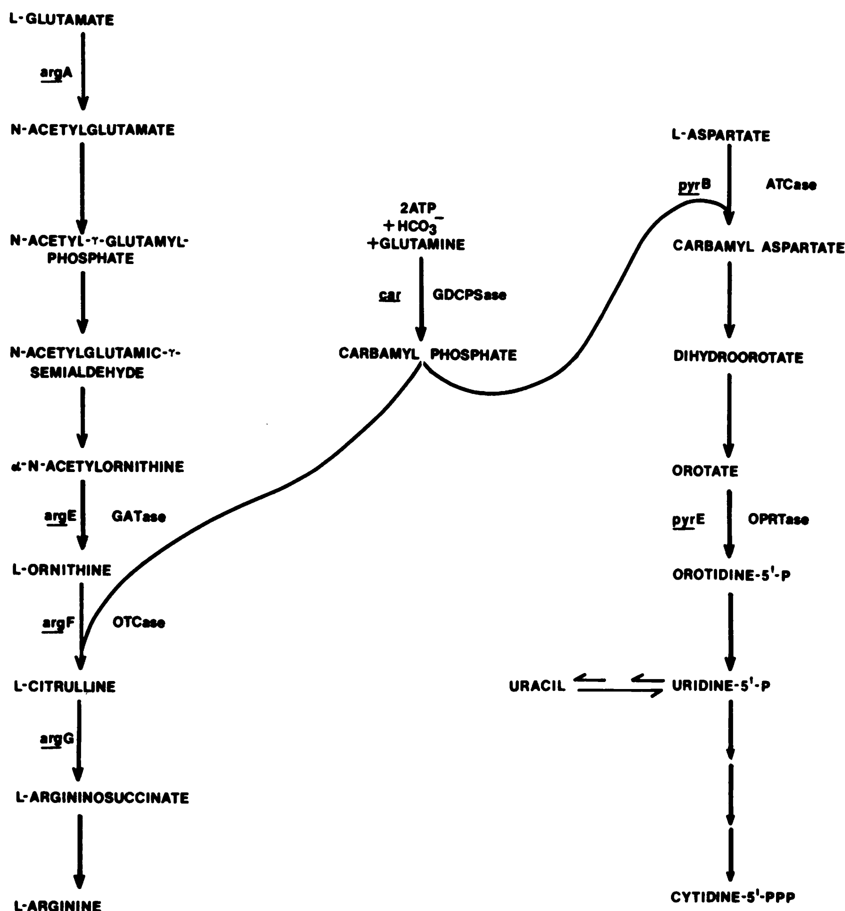

FIG. 1. Arginine and pyrimidine biosynthetic pathways and relevant gene loci. The enzymes are encoded by the following loci: GATase, *argE*; *OTCase*, *argF*; GDCPSase (glutamine-dependent CPSase), *car*; ATCase, *pyrB*; OPRTase, *pyrE*.

MATERIALS AND METHODS

Media. GC medium base (Difco) with Kellogg supplements (GCMBS) was used for routine cultures (7). The neisseria-defined agar (NEDA) was prepared by previously described methods (4, 7). The customary complete NEDA medium contains L-arginine and uracil and various other compounds, but not L-ornithine.

Gonococci. Strains of *N. gonorrhoeae* with the prefix MHD or SV were isolated from urogenital specimens from patients in Milwaukee (3, 9). Strains 89 (SS41835/43) and 13 (SS158936/69) were isolated at the Statens Seruminstitut, Copenhagen, Denmark, and were contributed by Alice Reyn. Strain LP99 (21) and strains with the prefix NRL were received from the Neisseria Reference Laboratory, U.S. Public Health Service Hospital, Seattle, Wash., through the courtesy of King Holmes and Joan Knapp. Strain PID-8 was isolated from a patient with pelvic inflammatory disease at the San Francisco General Hospital and was received from Deborah Draper. Strains UCSB185 and UC746 were provided by Josephine Morello, University of Chicago Hospitals and Clinics, Chicago, Ill. Strains designated ONT-26 and KYN-1 were received, respectively, from A. T. Hendry, Hamilton General Hospital, Hamilton, Ont., Canada, and from Jane Kenyon, Berkeley, Calif.

The methods used for confirming the identity of the *N. gonorrhoeae* strains, determining their nutritional requirements, and storing the cultures at −60°C have been described (7, 9). The relevant characteristics of the strains are given in Results.

Genetic transformation and derivation of isogenic strains. DNA was extracted from various donor gonococci and was partially purified (5). Recipient strains, maintained as T2 colonies, were treated with DNA as described for quantitative transformation or for the semiquantitative plate transformation test (5).

An isogenic set of *N. gonorrhoeae* strains was developed by introducing various genetic markers into the transformable, prototrophic strain 89 which was isolated in 1943 and has the drug susceptibilities and other traits typical of pre-penicillin era gonococci. Approximately 10^8 recipient gonococci were spread on the surface of GCMBS medium and covered with a solution of high-molecular-weight DNA (sufficient to

give 5 to 10 μg on the medium). After incubation for 6 to 8 h to obtain phenotypic expression, growth from separate areas of the nonselective medium was removed with a loop and streaked on separate sections of GCMBS containing rifampin or another selective antibiotic. After incubation for 24 h, a single transformant colony was picked from each section of the selective medium and subcultured on fresh medium of the same composition to eliminate untransformed recipients. One further single-colony transfer was made of each transformant, and the resulting 18-h cultures were screened for the introduction or elimination of unselected markers, either manually by streaking various media or mechanically by use of the Steers inocula-replicating apparatus, as described for auxotyping (7). These isogenic strains are designated by the prefix 89I.

To obtain prototrophic transformants of auxotrophic recipient strains, the gonococcal suspensions in defined fluid (NEDF-X with added uracil; 5) were treated with DNA and spread on the surfaces of appropriate defined media lacking any one essential component.

Chemicals. Amino acids, diacetyl monoxime (2,3-butanedione monoxime), dithiothreitol, 5-phosphoribosyl-1-pyrophosphate, and Tris were purchased from Sigma Chemical Co. (St. Louis, Mo.). Inorganic chemicals were reagent grade obtained from Mallinckrodt Chemical Works (St. Louis, Mo.). Carbamyl phosphate and ATP were obtained from P-L Biochemicals, Inc. (Milwaukee, Wis.). Antipyrene was from Eastman-Kodak Co. (Rochester, N.Y.).

Cell-free extracts. Gonococcal cultures and suspensions were prepared as described previously (8, 32). Glycerol (10%, vol/vol) was added to the crude extracts for assay of CPSase. Extracts to be assayed for orotate phosphoribosyltransferase (OPRTase) contained both 10% glycerol and 1 mM dithiothreitol. Crude extracts from freshly harvested cultures were used for assays of GATase and OPRTase. Dense gonococcal suspensions could be frozen at −20°C for up to 1 week with no apparent loss of the activities of the other three enzymes. The gonococci were sonically disrupted, and the fragmented cell preparation was immediately subjected to centrifugation at 27,000 × *g* for 20 min at 3°C (32). The supernatant fluid (crude cell-free extract) was promptly assayed for enzyme activity at 37°C (or at room temperature, approximately 23°C, for OPRTase).

Enzyme assays. The method for determining the activity of acetylornithine:GATase of *N. gonorrhoeae* was described by Shinners and Catlin (32).

OTCase activity was assayed as described before (32), except that the reaction mixture was modified to contain 10 μmol of L-ornithine, 10 μmol of carbamyl phosphate, 5 μmol of $MgCl_2$, 100 μmol of Tris-hydrochloride (pH 7.5), and cell-free extract (0.1 to 0.5 mg of protein) in a final volume of 1.0 ml. The citrulline produced in the reaction was assayed with the diacetyl monoxime reagent, as given previously (32).

Glutamine-dependent CPSase activity was measured by coupling carbamyl phosphate formation to citrulline synthesis in the presence of OTCase as described by Levenberg (19) and modified by Piérard et al. (27). The reaction mixture, in a final volume of 1.0 ml, contained: ATP, 10 μmol; $MgCl_2$, 10 μmol; glutamine, 10 μmol; potassium bicarbonate ($KHCO_3$), 90 μmol; ornithine, 10 μmol; Tris-hydrochloride (pH 7.5), 50 μmol; OTCase from *Streptococcus faecium*, 10 to 25 U; and extract containing 0.25 to 1.0 mg of crude extract protein per ml. Appropriate controls and citrulline standards (containing 0.1 to 0.5 μmol of citrulline per ml) were run simultaneously with each assay. The diacetyl monoxime colorimetric test, as described for OTCase, was utilized to determine the amount of citrulline formed which is stoichiometric with carbamyl phosphate. Specific activity is expressed in terms of nanomoles of citrulline formed per minute per milligram of crude extract protein.

Aspartate transcarbamylase (ATCase) activity was assayed by the method of Chang et al. (10) and Adair and Jones (1). The reaction mixture, in a final volume of 1.0 ml, contained: Tris-hydrochloride, pH 8.5, 100 μmol; carbamyl phosphate, 10 μmol; L-aspartate (pH 8.5), 50 μmol; and crude extract, 0.05 to 0.1 mg of protein. Carbamyl phosphate was added to start the reaction. The reaction was terminated by adding 1.0 ml of a solution containing 2 parts of antipyrene-H_2SO_4 to 1 part of oxime reagent mixed immediately before use. The antipyrene reagent contained 0.5% (wt/vol) antipyrene in 50% (vol/vol) sulfuric acid, and the oxime reagent contained 0.8% (wt/vol) diacetyl monoxime in 5% (vol/vol) acetic acid. Upon addition of the antipyrene-oxime reagent, the contents of the reaction tubes were mixed vigorously in a Vortex mixer, and the chromophore was allowed to develop in the dark at room temperature for 16 h. The reaction mixtures were then incubated in a 45°C water bath for 75 min and cooled for 15 min in an ice-salt bath. The absorbance at 466 nm was read immediately in a Beckman model 25 spectrophotometer. Carbamyl aspartate standards (0.02 to 0.1 μmol of carbamyl aspartate per ml) and appropriate controls were run concurrently with each assay. Specific activity is expressed as nanomoles of carbamyl aspartate formed per minute per milligram of crude extract protein.

OPRTase activity was measured according to the method of Beckwith et al. (2) as modified by Potvin et al. (29). The reaction mixture, in a final volume of 1.2 ml, contained: Tris-hydrochloride (pH 8.5), 100 μmol; orotic acid, 0.25 μmol; $MgCl_2$, 2.5 μmol; 5-phosphoribosyl-1-pyrophosphate, 0.5 μmol; and crude extract protein, 0.05 to 0.5 mg. Components were added to a silica cuvette (1-cm light path, 1.2-ml volume), and the reaction was initiated by the addition of extract. The conversion of orotic acid to OMP was monitored spectrophotometrically as a function of the decrease in absorbance at 295 nm, compared with a blank containing no extract. The absorbance change at room temperature was determined from the slope of the linear portion of the curve (first 1 to 2 min), and activity was calculated with a molar absorption coefficient of 3.95 $\times 10^3$ liters mol^{-1} cm^{-1}. Specific activity is expressed as nanomoles of OMP formed per minute per milligram of crude extract protein.

The protein content of the extracts was determined by the method of Lowry et al. (20) as modified by Miller (25), using bovine serum albumin (fraction V; Sigma Chemical Co.) as the standard.

RESULTS

CPSase. The inability of some Arg⁻ Ura⁻ strains to utilize ornithine for growth, although they produced OTCase, suggested that these

gonococci might bear *car* defects, resulting in a failure to synthesize carbamyl phosphate. In preparation for tests of such strains, we determined CPSase activities in prototrophic *N. gonorrhoeae* strains 89 and SV274 (Table 1). The reaction was dependent on potassium and magnesium ions as well as glutamine, bicarbonate, and ATP. The activity was linear for 60 min in assays containing 0.25 mg of crude extract protein per ml.

A significant repression of CPSase activity occurs when *E. coli* is cultivated in media containing arginine and uracil (26). The gonococcal CPSase activity, however, was not affected by the concentrations of arginine (150 μg/ml, 0.71 mM) and uracil (8 μg/ml, 0.071 mM) present in the complete NEDA medium, contrary to previous indications (8). Thus, crude extracts of strain 89 cultivated for 18 h on NEDA medium lacking arginine and uracil exhibited the same activity as extracts of cells grown in parallel on the complete medium.

CPSase activity was absent in extracts of Arg^- Ura^- gonococci characterized by inability to grow on the NEDA medium containing ornithine in place of arginine. However, such Car^- strains yielded transformants which produced carbamyl phosphate and, accordingly, were able to utilize ornithine for growth. For example, Table 1 shows the CPSase activity present in extracts of transformant strains 327-1 and 996-1 (derived, respectively, from the Car^- strains SV327 and MHD996). Strains 327-1 and 327-2 were independent Car^+ transformants which differed with respect to a uracil requirement; 327-2 retained, and 327-1 lost, the *pyr* defects borne by strain SV327.

In addition to removing the *car* defects by transformation, *car* defects were introduced into isogenic representatives of strain 89. Thus, the Car^- transformant 89I-739 (Table 1) was derived by treatment of the Car^+ strain 89I-660 (His^- Met^-) with DNA from MHD996; Met^+ transformants were selected and screened for the simultaneous acquisition of requirements for uracil and arginine (not satisfied by ornithine). Also, treatment of the Car^+ recipient 89I-608 (His^- Pro^- Hyx^-) with DNA from strain 13 elicited the His^+ Car^- transformant 89I-624. The presence of *car* defects was confirmed by the lack of CPSase activity in strains 89I-739 and 89I-624.

Reciprocal crosses between strains 13 and MHD996 yielded Car^+ transformants, indicating that the absence of CPSase activity involves two classes of defects, *car-1* and *car-2*. The *car-1* defect of strain 13 was removed by recombination with the intact *car-1* DNA sequence of MHD996. In turn, the *car-2* defect of MHD996 was eliminated by transforming DNA from strain 13. Furthermore, Car^+ transformants were obtained in similar reciprocal crosses between the two Car^- strains 89I-624 and 89I-739, described above. Using eight DNA preparations in plate transformation tests of 15 additional Car^- patient isolates, we found only two recombination classes—those exemplified by strains 13 and MHD996. We have not yet determined whether these *car* defects correspond to *carA*

TABLE 1. CPSase activities and relevant phenotypes of *N. gonorrhoeae* patient isolates and transformants

Strain[a]		Relevant phenotype[b]	Sp act (nmol min^{-1} mg of $protein^{-1}$)[c]
Designation	Status		
SV274	P	Arg^+ Car^+ Ura^+ Hyx^+	13.4
SV327	P	Arg^- Car^- Ura^- Hyx^-	<0.1
327-1	T (SV274)	Arg^- Car^+ Ura^+ Hyx^-	10.0
327-2	T (SV274)	Arg^- Car^+ Ura^- Hyx^-	9.2
89	P	Arg^+ Car^+ Ura^+ Hyx^+	10.0
MHD996	P	Arg^- Car^- Ura^- Hyx^-	<0.1
996-1	T (SV274)	Arg^+ Car^+ Ura^+ Hyx^-	7.9
89I-739	T (MHD996)	Arg^- Car^- Ura^- Hyx^+	<0.1
13	P	Arg^- Car^- Ura^- Hyx^-	<0.1
89I-624	T (13)	Arg^- Car^- Ura^- Hyx^+	<0.1

[a] P, Parent strain isolated from a patient; T, transformant derived by treatment with DNA from the strain given in parentheses. Strains 89I-739 and 89I-624 were Car^- transformants of Car^+ isogenic representatives of strain 89.

[b] Requirements for arginine (Arg^-), uracil (Ura^-), and hypoxanthine (Hyx^-); Car^- indicates an arginine requirement not satisfied by ornithine.

[c] Cells were grown on NEDA medium, and activity in crude extracts was assayed in the presence of 10 mM glutamine.

TABLE 2. Activities of ATCase and OPRTase in patient isolates and transformants

Strain[a]		Relevant genotype				Sp act (nmol min^{-1} mg of protein^{-1})	
Designation	Status	*car*	*arg*	*pyrB*	*pyrE*	ATCase	OPRTase
89	P	+	+	+	+	250	43
89I-4	T (MHD661[b])	+	*argA*	+	+	274	85
MHD316	P	+	*argE*	−	−	<20	<1
316-17	T (MHD197[c])	+	*argE*	+	+	390	42
89I-501	T (MHD316)	+	+	−	−	<20	<1
89I-500	T (MHD316)	+	+	+	−	384	<1
MHD996	P	−	*argE*	+	+	661	63
MHD872	P	+	*argE*	−	−	<20	<1
872-1	T (MHD996)	+	*argE*	+	+	437	70
89I-525	T (MHD872)	+	+	−	+	<20	43
89I-616	T (MHD872)	+	+	+	−	320	<1
13	P	−	*argE*	−	−	<20	<1
89I-629	T (13)	+	+	−	+	<20	59
89I-622	T (13)	+	+	+	−	299	<1
MHD32/69	P	+	*argE*	−	−	<20	<1
89I-528	T (MHD32/69)	+	+	−	+	<20	78
89I-505	T (MHD32/69)	+	+	+	−	300	<1

[a] P, Parent strain isolated from patient; T, transformant derived by treatment with DNA from the strain given in parentheses. The prefix 89I indicates auxotrophic derivatives of parent strain 89; some 89I strains possessed additional markers.

[b] Donor strain MHD661, *argA pro pyrB*$^+$ *pyr*E$^+$.

[c] Donor strain MHD197, *pro arg*$^+$ *pyrB*$^+$ *pyr*E$^+$.

and *carB* of *E. coli* which encode, respectively, the light (glutamine-binding) subunit and the heavy subunit of CPSase (22).

ATCase. The results of transformation tests showed that some Car⁻ patient isolates possess additional defects of pyrimidine biosynthesis; for example, the Car⁺ transformant 327-2 (Table 1) remained Ura⁻. The Arg⁻ Ura⁻ gonococci, also, must bear *pyr* defects. Therefore, we examined crude extracts of prototrophic gonococci and various Arg⁻ Ura⁻ strains for activity of ATCase, the first enzyme of pyrimidine biosynthesis which catalyzes the carbamylation of aspartic acid in other bacteria (14, 36).

The ATCase activity found in the wild-type *N. gonorrhoeae* strain 89 was 250 nmol min^{-1} mg of protein^{-1} (Table 2). Extracts of cells grown on complete NEDA medium (uracil; 8 μg/ml) exhibited ATCase activities similar to those of gonococci grown on medium lacking uracil. No significant ATCase activity was found (specific activity, <20 nmol min $^{-1}$ mg of protein^{-1}) when either carbamyl phosphate or aspartate was omitted from the reaction mixture or when boiled extract was used in place of unheated crude extract.

ATCase assays were performed on 12 Car⁺ Arg⁻ Ura⁻ strains isolated from patients. Activity was present in extracts of strain UCSB185 (Table 3), but was absent in the other 11 strains, including MHD316, MHD872, and MHD32/69 (Table 2). Also, ATCase activity was absent in some Car⁻ Arg⁻ Ura⁻ strains (e.g., strain 13), but was found in others (MHD996). Treatment of Ura⁻ strains with DNA from ATCase-positive gonococci yielded transformants which produced ATCase (e.g., strains 316-17 and 872-1, Table 2).

A group of linked chromosomal antibiotic resistance loci in *N. gonorrhoeae*, including *rif*, *str*, and *spc*, was shown by Sparling and colleagues (33) to map near a *ura* locus. We found that transformants selected for resistance to rifampin, streptomycin, or spectinomycin occasionally also incorporated the *pyrB* locus of the Ura⁻ antibiotic-resistant donor. This indirect method of deriving Ura⁻ transformants made it possible to generate an isogenic set of strains each with a single *pyr* defect introduced into the uniform matrix of strain 89. ATCase activity was absent in crude extracts of 24 Ura⁻ transformants derived in this way (Table 4).

Evidence for an additional pyrimidine defect. Other Ura⁻ transformants were derived by treating the ArgA⁻ Ura⁺ recipient 89I-4 with DNA from ArgE⁻ Ura⁻ patient isolates. Arg⁺ transformants were selected and screened for those which had received a defective *pyr* locus

TABLE 3. Patterns of enzyme activities in Arg⁻ Ura⁻ gonococci of various classes

Class	Strains	Relevant phenotype[a]	Enzyme activity[b]				
			GATase	OTCase	CPSase	ATCase	OPRTase
I	LP99, PID-8, ONT-26, KYN-1	Arg^- Car^- Ura^- Hyx^+	+	+	−	+	+
II	NRL8739, NRL8747	Arg^- Car^- Ura^- Hyx^+	−	+	−	+	+
III	MHD996	Arg^- Car^- Ura^- Hyx^-	−	+	−	+	+
IV	NRL7155, MHD530, SV327, 13	Arg^- Car^- Ura^- Hyx^-	−	+	−	−	−
V	NRL5033, NRL5035, NRL5043, MHD316, MHD32/69, MHD872	Arg^- Car^+ Ura^- Hyx^-	−	+	+	−	−
VI	UCSB185	Arg^- Car^+ Ura^- Hyx^-	−	+	+	+	−

[a] Requirements for arginine (Arg^-), uracil (Ura^-), and hypoxanthine (Hyx^-); Car^- indicates an arginine requirement not satisfied by ornithine. One or more additional requirements for proline, methionine, glutamine, histidine, branched-chain amino acids, or thiamine pyrophosphate were exhibited by some patient isolates.

[b] Enzyme activity present (+) or absent (−) in crude cell-free extract (compared with control extract from a prototrophic strain).

from the DNA donor together with the $argA^+$ allele. Assays of 42 Ura^- strains obtained by this means showed the presence of ATCase activity in 39 of the strains (Table 4). Their requirement for uracil evidently was due to defects of a different locus, *pyr-2*. As expected, Ura^+ transformants were obtained in reciprocal crosses between donors and recipients bearing *pyrB* and *pyr-2*. No Ura^+ transformants were recovered in any cross when both participants were *pyr-2* strains or both were *pyrB*.

OPRTase. In considering which locus might correspond to *pyr-2*, we noted that the chromosome map of *P. aeruginosa* PAO showed linkage between *argA* and *pyrE* and between *pyrB*, *rifA*, and *strA* (13). This order was compatible with the overall results presented in Table 4. Genetic mapping experiments (to be presented elsewhere) also had revealed tight linkage between *argA* and *pyr-2* of *N. gonorrhoeae*. Therefore, we investigated the possibility that *pyr-2* might encode OPRTase, the *pyrE* product (Fig. 1).

OPRTase activity was present in crude extracts of Ura^+ gonococci. As for other bacteria (2, 14), orotate, phosphoribosylpyrophosphate, and $MgCl_2$ were absolute requirements for the reaction. Strains that carried *pyr-2* lesions as determined by genetic crosses lacked OPRTase activity, as did seven Car^+ Arg^- Ura^- strains that were assayed (Tables 2 and 3). The ATCase and OPRTase activities were either both present or both absent in the Car^- Arg^- Ura^- strains examined thus far (Table 3).

DISCUSSION

The development of a chemically defined medium for cultivation of *N. gonorrhoeae* (4) and its use for auxotyping patient isolates (3) led to the detection of a variety of nutritional requirements (7, 17, 37; and references therein). Strains with requirements that include arginine, uracil, and hypoxanthine are geographically widespread (12, 18, 23, 24) and numerous in some communities (17, 18, 23). Furthermore, this Arg^- Ura^- Hyx^- phenotype appears to be an indicator of strains which possess one or more properties conducive to the development of dis-

TABLE 4. Presence or absence of ATCase activity in isogenic Ura^- transformants[a]

Strain		Medium for selection	Ura^- transformants assayed	
Ura^+ recipient[b]	Ura^- donor[c]		No. ATCase positive[d]	No. ATCase negative
891-4	MHD872	NEDA-ARG	6	2
	MHD316		1	1
	MHD32/69		11	0
	MHD510		8	0
	MHD446		7	0
	13		6	0
891-1	MHD872	GCMBS	0	5
	MHD32/69		0	1
	13		0	5
891-618	UCA62	GCMBS + RIF	0	5
891-618	13	GCMBS + SPC	1	8

[a] The Ura^- transformants were derived indirectly in plate transformation tests, described in the text. The Arg^+ or streptomycin-nondependent transformants formed colonies during incubation on the selection media; the transformants resistant to rifampin (RIF; 5 μg/ml) or to spectinomycin (SPC; 25 μg/ml) were incubated initially on drug-free medium for phenotypic expression. Transformants picked from the selection medium were purified by subculture, screened for the unselected Ura^- trait, and subsequently assayed for ATCase activity.

[b] Derivatives of prototrophic strain 89 transformed to $ArgA^-$ (891-4), streptomycin dependence (891-1), or His^- Met^- (891-618; 618 was susceptible to rifampin and spectinomycin).

[c] All had requirements that included arginine (*argE*) and uracil; also MHD446, 13, and UCA62 were Car^-. Strains UCA62, MHD872, MHD32/69, and 13 were resistant (by transformation) to rifampin, streptomycin, and spectinomycin.

[d] ATCase activity was similar to that of Ura^+ strain 89.

seminated gonococcal infections (17, 24). Interest in the evolution of auxotrophy in *N. gonorrhoeae* (6) led us to examine the nutritional requirements and enzyme defects of strains isolated over the past 45 years.

Seventy *N. gonorrhoeae* isolates from the years 1935 to 1948 exhibited requirements for one or more of eight compounds, including arginine. Defects of *argA* and *argG* were found in one strain each, and eight strains contained *argE* lesions. None of the 70 strains required uracil, although 13 of them were from disseminated gonococcal infections (B. W. Catlin and A. Reyn, manuscript in preparation). However, requirements for uracil as well as for arginine and other compounds were displayed by gonococci isolated in 1955 (J. Knapp, personal communication). Among these strains are NRL8739 and NRL8747 which, according to our findings, lack activities of GATase and CPSase, but possess functional ATCase and OPRTase enzymes (class II, Table 3). Strains NRL5033, NRL5035, and NRL5043, isolated in about 1963, possess CPSase activity, but lack ATCase, OPRTase, and GATase activities (class V), as do many of the Arg^- Ura^- strains isolated more recently. Classes III and VI are each represented by a single strain thus far. Arg^- Ura^- Hyx^+ gonococci are not commonly reported in the United States, but in Ontario, Canada, 32% of a large group of isolates exhibited requirements for arginine (satisfied by citrulline but not ornithine), uracil, and proline (12); ONT-26 is one of these Canadian strains (class I).

Auxotrophy has been evolving for some time in *N. gonorrhoeae*, a species which occurs naturally only in human beings. Possible relations between auxotrophy and persistence of gonococci in penicillin-treated patients were discussed earlier (6, 21). The tight shut-off of arginine and pyrimidine biosynthesis due to the progressive accumulation of multiple genetic defects (e.g., classes IV and V, Table 3) apparently gives these penicillin-susceptible gonococci a selective advantage. The presence of OTCase activity in all *N. gonorrhoeae* strains we have assayed (Table 3; 8, 32) is notable and suggests that defects of specific loci have survival value for gonococci. A challenge for future research will be determining the mechanisms of selection of auxotrophic mutants in patients. The occurrence of a mutation leading to auxotrophy was detected during passage in mice of *N. gonorrhoeae* strain F62 (Car^+ Pro^-). The mutant strain retained various characteristics of F62, but exhibited requirements for arginine and uracil (16). Our tests of this strain (KYN-1) demonstrated the absence of CPSase activity and normal levels of the other enzymes assayed (class I, Table 3).

A set of *N. gonorrhoeae* strains bearing seven separate auxotrophic markers was developed by Juni and Heym (15) for use as recipients in tests with DNA preparations from patient isolates. Their results independently illustrate the value of isogenic strains for detecting the multiple defects of Arg^- Ura^- strains which had created difficulties for interpretation of earlier genetic studies (e.g., 5, 21, 30, 37). However, in the absence of knowledge of the defective gene products of auxotrophs, the results of recombination tests may lead to erroneous inferences. Thus, the defective *car* locus of *N. gonorrhoeae* strain ATCC 27633 was not recognized because Car^+ transformants were obtained in a cross with a recipient presumed to be Car^- (15). We identified a *car-2* defect in ATCC 27633; this strain (not tabulated) lacks CPSase activity and gives rise to Car^+ transformants in reciprocal tests with nine *car-1* strains but not with any of eight *car-2* strains. It is evident that future genetic studies will be facilitated by the use of isogenic strains bearing defects of loci that encode known enzymes.

ACKNOWLEDGMENT

This work was supported by Public Health Service research grant AI-02353 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Adair, L. B., and M. E. Jones.** 1978. Aspartate carbamyltransferase. Methods Enzymol. **51**:51–58.
2. **Beckwith, J. R., A. B. Pardee, R. Austrian, and F. Jacob.** 1962. Coordination of the synthesis of the enzymes in the pyrimidine pathway of *E. coli*. J. Mol. Biol. **5**:618–634.
3. **Carifo, K., and B. W. Catlin.** 1973. *Neisseria gonorrhoeae* auxotyping: differentiation of clinical isolates based on growth responses on chemically defined media. Appl. Microbiol. **26**:223–230.
4. **Catlin, B. W.** 1973. Nutritional profiles of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and *Neisseria lactamica* in chemically defined media and the use of growth requirements for gonococcal typing. J. Infect. Dis. **128**:178–194.
5. **Catlin, B. W.** 1974. Genetic transformation of biosynthetically defective *Neisseria gonorrhoeae* clinical isolates. J. Bacteriol. **120**:203–209.
6. **Catlin, B. W.** 1976. Evolution of *Neisseria gonorrhoeae*—1975, p. 453–456. *In* D. Schlessinger (ed.), Microbiology—1976. American Society for Microbiology, Washington, D.C.
7. **Catlin, B. W.** 1978. Characteristics and auxotyping of *Neisseria gonorrhoeae*, p. 345–380. *In* T. Bergan and J. R. Norris (ed.), Methods in microbiology, vol. 10. Academic Press, London.
8. **Catlin, B. W., and E. H. Nash.** 1978. Arginine biosynthesis in gonococci isolated from patients, p. 1–7. *In* G. F. Brooks, E. C. Gotschlich, K. K. Holmes, W. D. Sawyer, and F. E. Young (ed.), Immunobiology of *Neisseria gonorrhoeae*. American Society for Microbiology, Washington, D.C.
9. **Catlin, B. W., and P. J. Pace.** 1977. Auxotypes and penicillin susceptibilities of *Neisseria gonorrhoeae* isolated from patients with gonorrhea involving two or more sites. Antimicrob. Agents Chemother. **12**:147–156.
10. **Chang, T. Y., L. M. Prescott, and M. E. Jones.** 1978.

Aspartate carbamyltransferase. Methods Enzymol. 51:41–50.

11. **Haas, D., B. W. Holloway, A. Schamböck, and T. Leisinger.** 1977. The genetic organization of arginine biosynthesis in *Pseudomonas aeruginosa*. Mol. Gen. Genet. 154:7–22.
12. **Hendry, A. T., and I. O. Stewart.** 1979. Auxanographic grouping and typing of *Neisseria gonorrhoeae*. Can. J. Microbiol. 25:512–521.
13. **Holloway, B. W., V. Krishnapillai, and A. F. Morgan.** 1979. Chromosomal genetics of *Pseudomonas*. Microbiol. Rev. 43:73–102.
14. **Isaac, J. H., and B. Holloway.** 1968. Control of pyrimidine biosynthesis in *Pseudomonas aeruginosa*. J. Bacteriol. 96:1732–1741.
15. **Juni, E., and G. A. Heym.** 1980. Studies of some naturally occurring auxotrophs of *Neisseria gonorrhoeae*. J. Gen. Microbiol. 121:85–92.
16. **Kenyon, J. E.** 1978. *Neisseria gonorrhoeae*: acquisition of auxotrophy in the mouse. Curr. Microbiol. 1:253–256.
17. **Knapp, J. S., and K. K. Holmes.** 1975. Disseminated gonococcal infections caused by *Neisseria gonorrhoeae* with unique nutritional requirements. J. Infect. Dis. 132:204–208.
18. **Knapp, J. S., C. Thornsberry, G. A. Schoolnik, P. J. Wiesner, K. K. Holmes, and the Cooperative Study Group.** 1978. Phenotypic and epidemiologic correlates of auxotype in *Neisseria gonorrhoeae*. J. Infect. Dis. 138:160–165.
19. **Levenberg, B.** 1962. Role of L-glutamine as donor of carbamyl nitrogen for the enzymatic synthesis of citrulline in *Agaricus bisporus*. J. Biol. Chem. 237:2590–2598.
20. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
21. **Mayer, L. W., G. K. Schoolnik, and S. Falkow.** 1977. Genetic studies on *Neisseria gonorrhoeae* from disseminated gonococcal infections. Infect. Immun. 18:165–172.
22. **Mergeay, M., D. Gigot, J. Beckmann, N. Glansdorff, and A. Piérard.** 1974. Physiology and genetics of carbamoylphosphate synthesis in *Escherichia coli* K-12. Mol. Gen. Genet. 133:299–316.
23. **Moberg, I.** 1975. Auxotyping of gonococcal isolates, p. 271–273. *In* D. Danielsson, L. Juhlin, and P.-A. Mårdh (ed.), Genital infections and their complications. Almqvist and Wiksell International, Stockholm.
24. **Morello, J. A., S. A. Lerner, and M. Bohnhoff.** 1976. Characteristics of atypical *Neisseria gonorrhoeae* from disseminated and localized infections. Infect. Immun. 13:1510–1516.
25. **Miller, G. L.** 1956. Protein determination for large numbers of samples. Anal. Chem. 31:964.
26. **Piérard, A., N. Glansdorff, M. Mergeay, and J. M. Wiame.** 1965. Control of the biosynthesis of carbamoyl phosphate in *Escherichia coli*. J. Mol. Biol. 14:23–36.
27. **Piérard, A., N. Glansdorff, and J. Yashphe.** 1972. Mutations affecting uridine monophosphate pyrophosphorylase or the *argR* gene in *Escherichia coli*. Mol. Gen. Genet. 118:235–245.
28. **Piérard, A., and J. M. Wiame.** 1964. Regulation and mutation affecting a glutamine dependent formation of carbamyl phosphate in *Escherichia coli*. Biochem. Biophys. Res. Commun. 15:76–81.
29. **Potvin, B. W., R. J. Kelleher, Jr., and H. Gooder.** 1975. Pyrimidine biosynthetic pathway of *Bacillus subtilis*. J. Bacteriol. 123:604–615.
30. **Roberts, M., and S. Falkow.** 1978. Plasmid-mediated chromosomal gene transfer in *Neisseria gonorrhoeae*. J. Bacteriol. 134:66–70.
31. **Rogers, P., and G. D. Novelli.** 1959. Formation of ornithine transcarbamylase in cells and protoplasts of *Escherichia coli*. Biochim. Biophys. Acta 33:423–436.
32. **Shinners, E. N., and B. W. Catlin.** 1978. Arginine biosynthesis in *Neisseria gonorrhoeae*: enzymes catalyzing the formation of ornithine and citrulline. J. Bacteriol. 136:131–135.
33. **Sparling, P. F., T. E. Sox, W. Mohammed, and L. F. Guymon.** 1978. Antibiotic resistance in the gonococcus: diverse mechanisms of coping with a hostile environment, p. 44–52. *In* G. F. Brooks, E. C. Gotschlich, K. K. Holmes, W. D. Sawyer, and F. E. Young (ed.), Immunobiology of *Neisseria gonorrhoeae*. American Society for Microbiology, Washington, D.C.
34. **Spratt, S. K., F. Jones, T. E. Shockley, and J. H. Jackson.** 1980. Cotransformation of a serum resistance phenotype with genes for arginine biosynthesis in *Neisseria gonorrhoeae*. Infect. Immun. 29:287–289.
35. **Steinberg, V. I., A. C. Fenton, E. H. Baker, and I. D. Goldberg.** 1979. Cotransformation of temperature sensitivity and nutritional markers in *Neisseria gonorrhoeae*. J. Bacteriol. 140:206–212.
36. **Yates, R. A., and A. B. Pardee.** 1957. Control by uracil of formation of enzymes required for orotate synthesis. J. Biol. Chem. 227:677–692.
37. **Young, F. E., V. Ploscowe, and H. Short.** 1977. The application of DNA-mediated transformation to elucidation of the pathobiology of *Neisseria gonorrhoeae*, p. 307–320. *In* A. Portolés, R. López, and M. Espinosa (ed.), Modern trends in bacterial transformation and transfection. North Holland Publishing Co., Amsterdam.