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Arginine Biosynthesis in *Neisseria gonorrhoeae*: Enzymes Catalyzing the Formation of Ornithine and Citrulline

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Many of the *Neisseria gonorrhoeae* strains isolated from patients require arginine for growth in a defined medium. As a basis for genetic studies of these Arg^- strains, we examined two biosynthetic enzymes of Arg^+ (nonrequiring) gonococci. Cell-free extracts contained (i) glutamate acetyltransferase, which catalyzes the formation of L-ornithine from α-*N*-acetyl-L-ornithine, and (ii) ornithine transcarbamylase, which catalyzes the reaction between L-ornithine and carbamyl phosphate, yielding L-citrulline. Arg^- strains which were unable to utilize α-*N*-acetyl-L-ornithine for growth lacked significant activity of glutamate acetyltransferase, and activity was gained by Arg^+ clones derived by DNA-mediated transformation. Some of the Arg^- patient isolates were unable to use either α-*N*-acetyl-L-ornithine or L-ornithine in place of arginine, and two separate steps of genetic transformation were required to yield Arg^+ cells. Extracts of these doubly auxotrophic cells lacked glutamate acetyltransferase activity, but, unexpectedly, they displayed normal ornithine transcarbamylase activity. This finding illustrates the importance of identifying the products specified by *arg* loci during genetic studies of arginine auxotrophy.

Diverse nutritional requirements are found in *Neisseria gonorrhoeae* strains isolated from patients (2, 3). The patterns of requirements of these natural auxotrophs provide a system for strain differentiation and identification which has been useful in epidemiological studies (6, 10). Many strains require arginine for growth in a defined medium, and Arg^- strains often have additional requirements for hypoxanthine (Hyx^-) and uracil (Ura^-) (2). The frequent involvement of Arg^- Hyx^- Ura^- strains in disseminated gonococcal infections (10) focuses attention on the arginine biosynthetic pathway. Various genetic defects are responsible for the Arg^- phenotype as shown by the recovery of Arg^+ cells after treatment of Arg^- recipient populations with DNA extracted from other Arg^- gonococci (4, 12, 21).

Arginine biosynthesis has been extensively studied in *Escherichia coli*. The eight-step pathway proceeds from L-glutamate via four *N*-acetylated intermediates to L-ornithine, L-citrulline, and L-argininosuccinate to yield L-arginine (20). Little is known, however, about arginine synthesis in *N. gonorrhoeae*. To establish a foundation for studies of arginine auxotrophy in clinical isolates, we examined five compounds intermediate in the pathway for ability to support the growth of 212 Arg^- gonococci. The arginine requirement of three strains was satisfied by either *N*-acetyl-L-glutamate or α-*N*-acetyl-L-ornithine. Seventy percent of the 212 strains utilized L-ornithine, and all grew on defined media which replaced L-arginine with either L-citrulline or L-argininosuccinate (5). These results indicate that the intermediate compounds in the arginine pathway of *N. gonorrhoeae* are similar to those of *E. coli* (20) and *Pseudomonas aeruginosa* (8). Because practically all of our Arg^- strains appeared to have defects involving either the conversion of α-*N*-acetyl-L-ornithine to L-ornithine (step 5 in the *E. coli* pathway [20]) or the synthesis of L-citrulline from L-ornithine (step 6), we investigated these two steps in selected Arg^+ and Arg^- bacteria.

MATERIALS AND METHODS

Bacteria. *N. gonorrhoeae* strain KH1567 was isolated from synovial effusion, and UC746 came from a blood specimen. SV strains were isolated from urogenital specimens from patients in Milwaukee. Strain SS18155/71 (received from Alice Reyn, Statens Seruminstitut, Copenhagen, Denmark), SV29, and SV274 require none of the compounds used for differentiating between gonococci by auxotyping (2). Other strains had one or more nutritional requirements, as follows: proline (Pro^-), methionine (Met^-), arginine (Arg^-), hypoxanthine (Hyx^-), uracil (Ura^-). SV11 is Arg^-. SV80 is Arg^- Hyx^- Ura^- and utilizes L-ornithine in place of L-arginine for growth. UC746 (received from Majorie Bohnhoff, University of Chicago Hospitals and Clinics, Chicago, Ill.) is Arg^- Hyx^- Ura^-, and the requirement for arginine is not satisfied by L-

ornithine. KH1567 (received from King Holmes, University of Washington School of Medicine, Seattle, Wash.) is Pro$^-$ Met$^-$ Arg$^-$ Hyx$^-$ Ura$^-$, and the requirement for arginine is not satisfied by L-ornithine.

The methods used for confirming the identity of the gonococci, determining their nutritional requirements, and storing the cultures at −60°C have been described (2, 3, 6).

P. aeruginosa PA0222 (received from B. W. Holloway), an Arg$^+$ strain employed as a positive control in enzyme assays, was cultivated on the same media used for *N. gonorrhoeae*.

Media and chemicals. GC medium base (Difco) with supplements (GCMBS) was used for routine cultures (6). The neisseria defined agar (NEDA) was prepared by previously described methods (2, 3). The complete NEDA medium contains L-arginine and uracil and various other compounds, but not L-ornithine or L-citrulline.

Amino acids and arginine pathway intermediates were purchased from Sigma Chemical Co. (St. Louis, Mo.) or Calbiochem (San Diego, Calif.). Inorganic chemicals were reagent grade obtained from Mallinckrodt Chemical Works (St. Louis, Mo.).

Cell-free extracts. Bacteria were cultivated for 20 h on multiple plates of the appropriate NEDA medium incubated at 36°C in an atmosphere of air with 8% carbon dioxide. Cells were swept gently from the agar surfaces with a loop, and dense suspensions were made in 4 ml of cold (2°C) standard Tris-hydrochloride buffer composed of 50 mM tris(hydroxymethyl)-aminomethane-hydrochloride, pH 7.5. When glutamate acetyltransferase (GATase) was to be assayed, cells were suspended in Tris-hydrochloride buffer (pH 7.5) modified to contain 1 mM dithiothreitol and 1 mM disodium ethylenediaminetetraacetate. The cells, kept continuously chilled, were fragmented using a microtip attachment of a Sonifier cell disruptor (model W185, Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). The disruption was carried out at 15-s intervals for a total of 3 min with 15-s intervening periods for cooling; then the preparation was centrifuged at 27,000 × *g* for 15 min. The supernatant fluid (crude cell-free extract) was promptly assayed for enzyme activity.

Assays. GATase was assayed by a method modified by Staub and Dénes (16) from that of Udaka and Kinoshita (18) with the ninhydrin reagent of Vogel and Bonner (19) for determining the concentration of ornithine produced. The reaction mixture, incubated at 37°C, contained 50 μmol of standard Tris-hydrochloride buffer (pH 7.5), 3 μmol of α-*N*-acetyl-L-ornithine, cell-free extract (0.1 to 0.6 mg of protein), and 3 μmol of L-glutamic acid (adjusted with NaOH to pH 7.5). The reaction was started by the addition of the prewarmed sodium glutamate, giving a total volume of 0.5 ml. After an appropriate time, the reaction was stopped by addition of 0.5 ml of ninhydrin reagent. This reagent was prepared immediately before use by mixing two volumes of 1.5% solution of ninhydrin in Methyl Cellosolve (ethylene glycol monomethyl ether, Mallinckrodt) with one volume of a 1.2 M aqueous solution of citric acid. The covered tubes were placed for 10 min in boiling water and cooled in water; 0.5 ml of 4.2 N NaOH was added, and the contents were immediately mixed vigorously in a Vortex mixer. After 20 min at room temperature, the intensity of the orange-yellow color was measured at 470 nm (model 25 spectrophotometer, Beckman Instruments, Inc., Irvine, Calif.). Assays were performed in triplicate and were accompanied by control incubation mixtures in which either extract and/or substrate(s) were omitted; also, a set of standards containing 0.02 to 0.10 μmol of L-ornithine was included each day. The rate of ornithine formation was linear for at least 30 min. The rates were calculated from simple linear regression analysis of the data which represent the means of triplicate determinations. Specific activity is expressed as nanomoles of ornithine formed per minute per milligram of protein in the crude cell-free extract.

Ornithine transcarbamylase (OTCase) activity was assayed by the production of citrulline by a method modified from Archibald (1) by Nakamura and Jones (15) and Jacoby (9). The reaction mixture in a final volume of 1.0 ml contained 45 μmol of L-ornithine, 23 μmol of carbamyl phosphate, 5 μmol of $MgCl_2$, 100 μmol of Tris-hydrochloride (pH 7.5), and cell-free extract (0.1 to 0.6 mg of protein). The reaction was started by addition of the extract to the prewarmed (37°C) mixture. After appropriate incubation at 37°C, a 2.0-ml volume of 2 N HCl was added to each reaction mixture, which then was boiled for 10 min. After cooling in water, 2.0 ml of a solution made up by mixing three volumes of 85% phosphoric acid with one volume of concentrated H_2SO_4 was added, followed by 0.2 ml of 3.75% diacetyl monoxime (2,3-butanedione monoxime, Sigma Chemical Co.). The tubes were blended in a Vortex mixer and placed in boiling water in a covered container; after 30 min they were cooled in water and kept well protected from light, because the colored complex which forms is light sensitive (1). The absorbances at 490 nm were determined with a Beckman spectrophotometer in a darkened room. Standards containing 0.05 to 0.80 μmol of citrulline and appropriate controls were included with each group of assays. The analysis used for calculating rates was as described for GATase. Specific activity of OTCase is expressed as nanomoles of citrulline formed per minute per milligram of protein in the cell-free extract.

The protein content of the extracts was determined by a modification of the Lowry method (11, 14) with bovine serum albumin (Sigma Chemical Co.) as the standard.

Genetic transformation. DNA was extracted from various donor gonococci and was partially purified (4). Auxotrophic strains, maintained as T2 colonies, were treated with DNA, and prototrophic transformants were recovered by methods described earlier (4).

RESULTS

GATase. Ornithine formation from *N*-acetylornithine is catalyzed by acetylornithine deacetylase in *E. coli* (20). Alternatively, *P. aeruginosa* (8) and *Micrococcus glutamicus* (18) form ornithine by a GATase-catalyzed transacetylation reaction between acetylornithine and glutamate. Acetylornithine deacetylase activity was not detected in *N. gonorrhoeae* (5). However,

GATase was present in cell-free extracts of Arg$^+$ strains of *N. gonorrhoeae*. Thus, in an assay of *N. gonorrhoeae* SV29 cultivated on an arginine-free medium the GATase activity was 13.6 nmol/min per mg of protein compared to 14.0 found in a similar assay of *P. aeruginosa* PA0222.

The synthesis of various enzymes of the arginine pathway may be repressed in bacteria grown in media containing even low concentrations of arginine (20). Therefore, to find an appropriate medium for cultivating the *N. gonorrhoeae* Arg$^-$ strains to be assayed for GATase, we examined the activities of Arg$^+$ cultures. Cell-free extracts of *N. gonorrhoeae* SS18155/71 grown for 20 h on defined media, either lacking arginine or containing 0.7 mM L-arginine, had GATase activities of 10.8 and 11.0 nmol/min per mg of protein, respectively. Furthermore, the specific activities were not affected by replacing L-arginine with either L-citrulline or L-ornithine at concentrations of 0.7 mM. The GATase activity was 8.7 nmol/min per mg of protein in an extract of cells grown for 20 h on GCMBS, a peptone-containing medium. Extracts of strain SV29 grown on the same media had GATase activities ranging from 13.6 to 10.9 nmol/min per mg of protein.

OTCase. The reaction between L-ornithine and carbamyl phosphate to yield L-citrulline and phosphate is catalyzed by OTCase in *E. coli* (20) and *P. aeruginosa* (8). We found OTCase activity in *N. gonorrhoeae* as well as in *P. aeruginosa*. Kinetic analyses showed that the rates of citrulline formation were linear for at least 30 min at the protein concentrations used. Assays of extracts of Arg$^+$ strains cultivated on arginine-free medium gave OTCase activities, in terms of nanomoles of citrulline formed per minute per milligram of protein, as follows: *P. aeruginosa* PAO222, 308; *N. gonorrhoeae* SV29, 237; and *N. gonorrhoeae* SS18155/71, 303.

Repression of OTCase synthesis occurs during growth of wild-type strains of *P. aeruginosa* in the presence of arginine at concentrations of 1 to 40 mM (8). We assayed *N. gonorrhoeae* SS18155/71 to determine whether the gonococcal OTCase might be subject to repression by the L-arginine present at a concentration of 0.7 mM in the complete NEDA medium. Cultures were harvested after incubation for 20 h on four NEDA media: (i) complete, (ii) lacking arginine or arginine intermediates, (iii) arginine-free but with the addition of 0.7 mM L-citrulline, and (iv) arginine-free but with the addition of 0.7 mM L-ornithine. For comparison, a GCMBS culture was tested also. The cell-free extracts of these five populations possessed OTCase activities clustered in the range 282 to 312 nmol/min per mg of protein. The possible effect of a high concentration of arginine was not investigated. However, evidently cells grown on the complete NEDA medium are satisfactory for OTCase assays.

Enzyme activities of Arg$^-$ isolates and Arg$^+$ transformants. Having identified GATase as the enzyme in Arg$^+$ *N. gonorrhoeae* which converts *N*-acetylornithine to ornithine, we postulated that little or no GATase activity would be present in Arg$^-$ patient isolates characterized by inability to utilize α-*N*-acetyl-L-ornithine for growth. Furthermore, GATase activity should be found in Arg$^+$ cells which were derived by DNA-mediated transformation. In accordance with expectation, extracts of the Arg$^-$ strains SV11 and SV80 showed physiologically negligible GATase activity compared with the activities displayed by the respective Arg$^+$ transformants (Table 1). These findings established that defects of the structural gene encoding GATase are responsible for arginine auxotrophy in these isolates.

N. gonorrhoeae SV11 and SV80 utilized L-ornithine for growth and possessed OTCase activity, as expected. We initially surmised that OTCase activity might be lacking in isolates such as UC746 and KH1567 that failed to multiply on the −ARG+ORN medium (containing L-ornithine in place of L-arginine). Contrary to expection, OTCase activity was found in extracts of *N. gonorrhoeae* UC746 (Table 1) and also KH1567. UC746 was transformed to Arg$^+$ in two steps as other investigators have reported (21) for patient isolates that are unable to utilize L-ornithine in place of L-arginine. UC746-4, the transformant selected on −ARG+ORN medium, retained the GATase defect, but gained the capacity to synthesize some still unidentified product which enabled the cells to use L-ornithine for growth. GATase activity was present, and OTCase activity was unchanged in the second-step transformant UC746-46. Arg$^+$ transformants derived from UC746 by a single exposure to a given DNA preparation occurred only at a very low frequency. Transformant clones UC746-46 and SV80-1 remained Ura$^-$, indicating that the uracil requirement was due to mutation in a separate gene.

DISCUSSION

Arginine auxotrophy is found in many *N. gonorrhoeae* strains isolated from patients with gonorrhea (5). Arg$^-$ strains are readily subjected to genetic analysis by DNA-mediated transformation and various *arg* recombination groups have been found (4, 12, 21). At this early stage of the development of gonococcal genetics, it is important to identify the polypeptides absent or de-

TABLE 1. *Growth on defined media and enzyme activities of N. gonorrhoeae Arg⁻ clinical isolates and their transformants*

Strain	Colony formation on defined medium:[a]				Activity (nmol/min per mg of protein)[b]	
	−ARG+AO	−ARG+ORN	−ARG	−URA	GATase	OTCase
SV11	No	Yes	No	Yes	<0.2	303
SV11-1[c]	Yes	Yes	Yes	Yes	8.6	300
SV80	No	Yes	No	No	<0.2	260
SV80-1[c]	Yes	Yes	Yes	No	8.7	264
UC746	No	No	No	No	<0.2	235
UC746-4[d]	No	Yes	No	No	<0.2	282
UC746-46[e]	Yes	Yes	Yes	No	9.6	276

[a] NEDA media in which L-arginine was replaced by either α-*N*-acetyl-L-ornithine (−ARG+AO) or L-ornithine (−ARG+ORN) or L-arginine or uracil were omitted (−ARG or −URA).

[b] GATase and OTCase activities in extracts of cells grown on NEDA medium containing L-arginine (0.7 mM).

[c] Arg⁺ transformant selected on −ARG medium after treatment of Arg⁻ cells with DNA from SV274, Arg⁺ Ura⁺; GATase activity of SV274, 8.7 nmol/min per mg of protein.

[d] Transformant selected on −ARG+ORN medium after treatment of UC746 with DNA from *N. gonorrhoeae* SS23157/45, an Arg⁻ Pro⁻ strain that utilizes *N*-acetylglutamate and α-*N*-acetyl-L-ornithine for growth and possesses GATase activity, 10.3 nmol/min per mg of protein.

[e] Arg⁺ transformant selected on −ARG medium after treatment of UC746-4 with DNA from SS23157/45.

fective in the Arg⁻ mutants that have arisen in infected humans.

Most of our Arg⁻ patient isolates lack GATase activity as assayed colorimetrically by the amount of ornithine formed. Although relatively specific for ornithine (7), this method is not sufficiently sensitive to exclude the possibility that Arg⁻ cells may possess slight GATase activity.

OTCase activity is necessary but not sufficient to enable a cell to form citrulline from ornithine. Although possessing OTCase activity, some Arg⁻ Hyx⁻ Ura⁻ strains fail to multiply on the medium that contains L-ornithine in place of L-arginine (e.g., UC746, Table 1). The missing function has not yet been identified. Two possibilities which will be examined are inability to transport L-ornithine into the cell or inability to produce carbamyl phosphate. In intact gonococci the formation of citrulline by the OTCase-catalyzed reaction of L-ornithine with carbamyl phosphate requires the activity of carbamyl phosphate synthetase, a glutamine-dependent enzyme present in extracts of Arg⁺ cells (5). *E. coli* mutants which lack carbamyl phosphate synthetase have a dual requirement for arginine and uracil because carbamyl phosphate is needed for synthesis of both arginine and pyrimidines (13). However, even if the unusual phenotype of *N. gonorrhoeae* UC746 is due in part to a defective carbamyl phosphate synthetase, it is clear that the cells carry additional mutations of *ura* and *arg* genes which increase the stability of the auxotrophic traits. Apparently, auxotrophy has some special pathobiological importance for these pathogenic bacteria.

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