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IN NEISSERIA GONORRHOEAE

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MOLECULAR STUDIES OF ARGININE AUXOTROPHY IN NEISSERIA GONORRHOEAE

By

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ABSTRACT

MOLECULAR STUDIES OF ARGININE AUXOTROPHY
IN NEISSERIA GONORRHOEAE

By

Paul Richard Martin

Neisseria gonorrhoeae is a strict human pathogen and the causative agent of gonorrhea. The development of auxotyping media for *Neisseria* led to the discovery that gonococcal strains display a diverse array of nutritional requirements. Among the more common nutritional defects found among clinical isolates is the requirement for arginine. Arginine biosynthesis from glutamate requires eight enzymatic steps. The most common defects found among gonococcal arginine auxotrophs occur at steps five and six; the conversion of acetylornithine to ornithine, and the carbamoylation of ornithine to form citrulline respectively. The fifth step is catalyzed by the enzyme ornithine acetyltransferase and is encoded by the *argJ* gene. The sixth is catalyzed by ornithine transcarbamoylase (OTCase), encoded by *argF*. I have cloned these genes by complementation of defined *E. coli* mutants, analyzed their products in minicells, and sequenced the genes. The gonococcal *argF* gene encodes a protein of 331 amino acids with a deduced molecular weight of 36,731. This gene uses a GUG codon for translation initiation, which was confirmed by N-terminal amino acid sequencing of the purified protein. The gonococcal *argF* gene contains regions of high homology with OTCase genes previously sequenced from *E. coli* and *Pseudomonas aeruginosa*, and with OTCases from eukaryotic sources, with the amino acids involved in substrate binding being particularly well conserved.

The gonococcal *argJ* gene is of particular interest because it is defective in all strains tested with the multiple requirements for arginine, hypoxanthine, and uracil (AHU strains). AHU strains are a homogeneous group that have been associated with disseminated gonococcal infections, and are believed to be clonally derived. AHU strains possess an apparently intact *argJ* gene as seen by Southern blot. The *argJ* gene encodes a protein of 406 amino acids with a deduced molecular weight of 42,879. The mutant *argJ* gene from an AHU strain was cloned, sequenced, and compared to the wild type gene. The mutant gene contained a 3 base pair deletion within a repetitive region of the gene. This deletion was restored in a naturally occuring Arg+ revertant, thus correlating this mutation with impaired enzyme activity. When other AHU strains were tested they were all found to possess this deletion, whereas other ArgJ- and wild type strains did not. These data support the theory that AHU strains are clonally derived and distinct from other strains of *N. gonorrhoeae*.

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LITERATURE REVIEW

Neisseria gonorrhoeae is a Gram negative diplococcus that has adapted a lifestyle intimately associated with our own. *N. gonorrhoeae* is an obligate human pathogen and, as its name implies, is the causative agent of gonorrhea, a sexually transmitted disease of major importance. The gonococcus was first identified in 1879, when Albert Neisser found the organism in stained smears of urethral exudates (65). This organism continues to be a serious and widespread public health problem in this country. In 1989, 733,151 new cases of gonorrhea were reported in the U.S. (18), and another million cases are estimated to have gone unreported.

Gonococcal infections occur on mucous membranes lined with nonsquamous epithelium (53). A typical infection is initiated by adherence of the bacteria to mucosal epithelial cells which is mediated by the presence of pili on the surface of the gonococcus (99). After 24-48 hours the gonococcal cells penetrate the epithelium and cause localized cell destruction (48). This damage induces the infiltration of polymorphonuclear leukocytes and local inflammation. This results in the pain and purulent discharge associated with symptomatic gonococcal infections (53).

Gonococcal infections are usually restricted to the site of introduction, but complications can occur. Among these are ascending gonococcal infections which in males can lead to colonization and inflammation of secondary sites such as the epididymis or prostate gland (53). In females ascending infection can cause pelvic inflammatory

disease, a serious complication that can result in infertility (39). The gonococcus can also be invasive causing disseminated gonococcal infection (DGI) (56). DGI is characterized by fever, skin lesions and polyarthralgia, and infrequently involves endocarditis or meningitis (56).

The gonococcus possesses a variety of outer membrane proteins that along with lipopolysaccharide interact with the environment and allow for attachment and invasion of the mucosal epithelium and evasion of host immune responses. Studies of these proteins have found that they show a high degree of antigenic diversity between strains, and that several can generate antigenic variability within a strain (87).

Pilin is the protein component of gonococcal pili which are the primary mediators of attachment to epithelial cells (100). Primary cultures of clinical isolates produce almost exclusively piliated cells, but piliation is quickly lost when subcultured (66,118,123). Non-piliated cells show a markedly reduced ability to initiate infection (66), substantiating the importance of pili to pathogenesis. The type of pilin expressed in a given gonococcal cell can spontaneously change to a structurally and antigenically different type of pilin in its progeny (52,79). This is possible due to the presence of a family of pilin genes located in the gonococcal chromosome, only one of which is typically expressed at a given time (51). The "silent" pilin genes are incomplete and lack promotors but can become expressed by homologous recombination into the expression locus (42).

Protein I (P.I) is the major outer membrane protein of the gonococcus and functions as a porin (33). A large number of structural varients of P.I exist in the gonococcal population as detected by serological reactivity. This has been exploited to develop a panel of monoclonal

antibodies against P.I to classify gonococcal strains into serovars, a tool that has been very useful for epidemiological studies (9,71,110).

Protein II (P.II) is also known as opacity protein for its noticable affect on colony morphology; cells expressing P.II develop opaque colonies while colonies from those not expressing P.II are transparent (122,133). P.II is believed to be involved in attachment to epithelial cells (78) as well as intergonococcal adherence (122). Similar to pilin, P.II is capable of phase and antigenic variation (133), though the mechanism is quite distinct (95,121). At least 11 copies of the P.II structural gene exist in the chromosome, but unlike pilin they all have functional promotors and are constitutively transcribed (121). Control of P.II expression occurs by the presence of a repeated pentameric sequence in the 5'-end of the gene encoding leader peptide. The number of pentameric repeats determines whether the remainder of the gene will be in or out of frame and thus whether it will be translated into functional P.II protein (4,95,121). The number of pentameric repeats can change by slipped-strand mispairing during DNA replication (4,95). In this way from none to several different P.II proteins can be expressed at a given time.

The gonococcus produces and secretes an extacelluar enzyme that specifically cleaves immunoglobulin A1 at the hinge region (103), impairing the ability of the antibody to effect an immune response. This enzyme, IgA1 protease, comes in two types that differ in the specificity of the site of cleavage of the antibody (94). Essentially all gonococcal strains tested produced an IgA1 protease, but no strain produced both types (94). This allowed for strains to be characterized as either type 1 or type 2 IgA1 protease producers.

Gonococci survive poorly outside their human hosts, but they have been successfully cultured *in vitro* and are actually quite nutritionally independant. The prototrophic gonococci requires cysteine, and can use glucose as a carbon and energy source (14). The development of defined media by Catlin (14) allowed for the elucidation of the nutritional requirements of clinical isolates. She found that auxotrophs were common and varied widely. Among the more common auxotrophs in the population were those that required arginine.

ARGININE BIOSYNTHESIS

In bacteria, arginine is synthesized from glutamate by an eight step pathway (25) (Fig. 1). Initially an acetyl group is transferred to glutamate from acetyl CoA to form N-acetylglutamate. This acetyl group effectively prevents the cyclization of the molecule that occurs in the proline biosynthetic pathway. The acetyl group is subsequently removed at step five when N-acetylornithine is converted to ornithine. Ornithine is combined with carbamoyl phosphate to form citrulline in the sixth step of the pathway and citrulline is converted to arginine in the last two steps.

As shown in Figure 1, arginine biosynthesis is intertwined with other other biosynthetic pathways including those for proline (glutamate is shared), pyrimidines (carbamoyl phosphate is shared), and polyamines (ornithine and arginine are precursors). These other pathways influence the availability of intermediates and can affect the regulation and activities of some arginine biosynthetic enzymes (43).

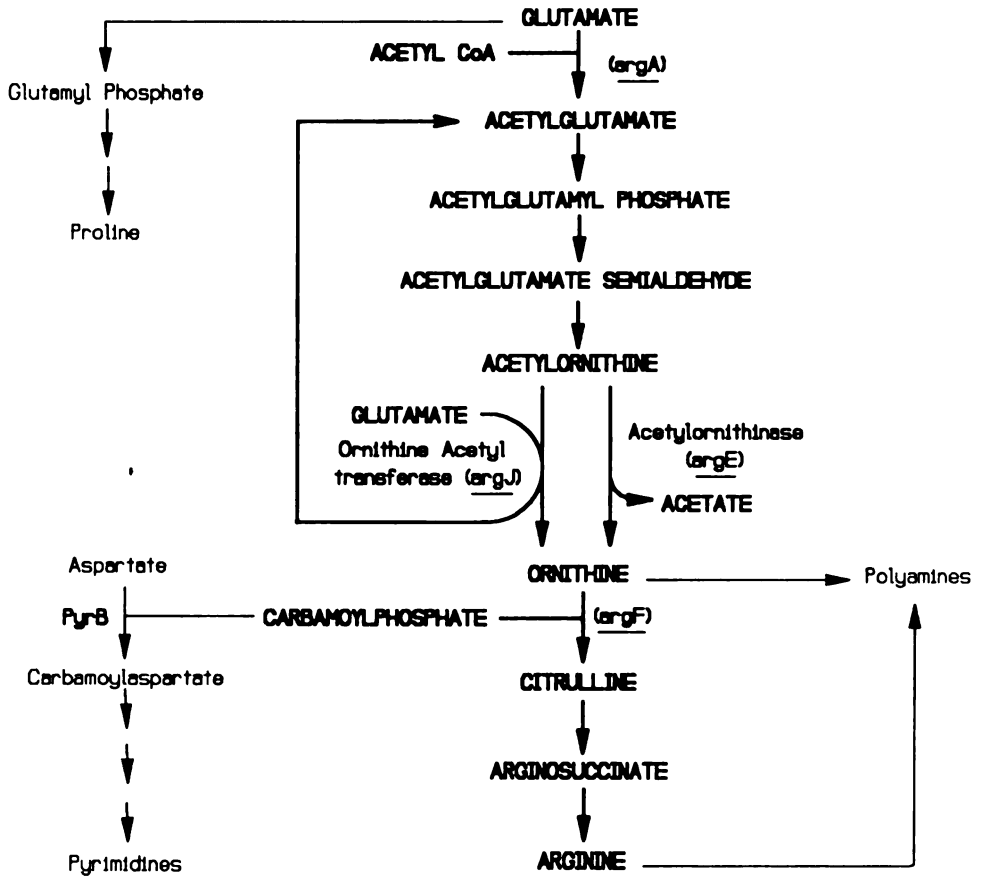

Figure 1. Arginine biosynthetic pathway. Interactions of this pathway with the proline, pyrimidine, and polyamine biosynthetic pathways are indicated.

Two independent enzymes have evolved to catalyze the conversion of acetylornithine to ornithine, and the enzyme an organism produces affects the level of control of the pathway (127). *E. coli* and other enteric bacteria catalyze this reaction by the linear pathway in which acetylornithine is hydrolyzed to form ornithine and acetate using the enzyme acetylornithinase (105,127). Bacteria of the genera *Pseudomonas*, *Thermus* and *Neisseria*, as well as cyanobacteria, methanogens and lower eukaryotes catalyze this reaction by the cyclic pathway in which the acetyl group is transfered to glutamate by the enzyme ornithine acetyltransferase (OATase)(16,28,55,86,91,127). The acetylglutamate produced from this reaction can be channeled back into the arginine biosynthetic pathway, bypassing step one. In organisms utilizing the linear pathway regulation occurs through feedback inhibition by arginine on the first enzyme of the pathway, N-acetylglutamate synthetase (127). In organisms that use the cyclic pathway, arginine feedbacks inhibits the second enzyme of the pathway, N-acetylglutamate 5-phosphotransferase (127). The cyclic pathway represents the energetically more efficient pathway in that acetyl group recycling reduces the need for input from acetyl-CoA to times of increasing activity.

Along with feedback inhibition control of arginine biosynthesis, repression of arginine biosynthetic gene expression has also been reported (81). In *E. coli*, the genes for the eight enzymes of the linear pathway have been designated sequentially *argA-argH*. The *argE,C,B* and *argH* genes are clustered and share a common operator located between *argE* and *argC*, while the remainder of the genes are independent and scattered throughout the chromosome (43,46). The operator regions of the arginine biosynthetic genes plus the genes for carbamoyl phosphate

synthesis (*carA* and *carB*) share a common regulatory sequence designated the "arg box" to which the arginine repressor protein (encoded by *argR*) binds under conditions of excess arginine (24,63). An arg box very similar to that of *E. coli* is also found in front of the *argC-A-E-B-D* gene cluster of *Bacillus subtilis* (116), and the *B. subtilis* arginine repressor was capable of regulating *E. coli arg* genes *in trans* (116). In *Pseudomonas aeruginosa* all the arginine biosynthetic genes are scattered and only one (*argF*) is repressible by arginine (50,61,132). The promotor of the *P. aeruginosa argF* gene does not contain sequences resembling an arg box (62), and the regulatory mechanism of this gene has not yet been identified. In *N. gonorrhoeae* the genes for arginine biosynthesis appear to be scattered and no evidence for repression control has been found (101,114,115,Mulks unpublished).

The gene for OATase is commonly designated *argJ*. This gene has been cloned from *N. gonorrhoeae*, and it was found that clones carrying this gene could complement *E. coli argE* and *argA* mutations (101). It was proposed that the gonococcal OATase could complement an *E. coli argA* mutation through the feedback pathway, though it was also possible that the gonococcal *argA* gene might also have been present on the same clone (101).

Bacillus subtilis was initially reported to produce acetylornithinase based on enzyme assays that found a slightly higher level of this activity than OATase activity (127). This was later supported by showing that the ability of the cloned *B. subtilis argC-A-E-B-D* gene cluster to complement *E. coli argA* and *argE* mutations could be separated (93). This assumption has recently come under question though when the *B. subtilis argC-A-E-B-D* cluster was sequenced and only one open reading frame was

found in the *argA-E* region (98). Since this region could complement *E. coli argE* and *argA* mutations, these researchers proposed that the *B. subtilis argA-E* region actually encodes only one gene, *argJ*, an OATase (98). At this writing this question remained unresolved.

The arginine biosynthetic pathway has proven to be a good model for studies of molecular evolution, and has provided several interesting examples of gene duplication (25). In *E. coli* K12 there are two copies of the *argF* gene, the second being designated *argI* (44). The *argI* gene is the copy of the gene common to all *E. coli* strains. The *argF* gene is flanked by IS1 elements and is presumed to have entered the *E. coli* chromosome by transposition from an unidentified source (59,139). The *argI* and *argF* genes share 86% amino acid sequence identity, and both genes are expressed (44,130). *E. coli* also possesses two copies of the *argD* gene (6). The second copy, *argM*, shows the interesting property of being induced, not repressed, by the arginine repressor (6), and it is thought to be part of a cryptic arginine degradative pathway. In *P. aeruginosa* there are two copies of *argF* (50,119). The second copy, *arcB*, catalyzes the reaction in the reverse direction and functions in arginine catabolism (49). Interestingly the *arcB* gene shows a significantly higher sequence identity to the *E. coli argI* and *argF* genes than does the *P. aeruginosa argF* gene (3,62).

Ornithine transcarbamoylase (OTCase, encoded for by *argF* in *N. gonorrhoeae*) has been thoroughly characterized in many prokaryotic and eukaryotic species (58,82,104,129), and the OTCase genes have been sequenced and compared among these organisms (3,5,57,60,62,83,124, 128,130,131). These comparisons revealed that all OTCases appear to have evolved from a common ancestor. All the OTCases studied function as

trimers of a peptide with a molecular weight between 33.5 and 38.5 kDa. Amino acids involved in the binding of ornithine and carbamoyl phosphate have been identified (58,80,82), and are conserved among the OTCases. The amino acids involved in carbamoyl phosphate binding are also found in a different enzyme, aspartate transcarbamoylase (ATCase)(58,80,88). ATCase is part of the pyrimidine biosynthetic pathway (*pyrB* in Fig. 1). In *E. coli*, *pyrB* and *argI* have sequence homology beyond those amino acids that interact with carbamoyl phosphate, and it is thought that *pyrB* may actually be the ancestor of the OTCase genes (58,130).

GONOCOCCAL GENETICS

The gonococcus possesses a single circular chromosome with a G+C base composition of 50%. The size of the chromosome was originally estimated to be 1,500 kb in length based on reassociation kinetics (67). Recent studies using pulse field gel electrophoresis of large chromosomal restriction fragments revealed that the chromosome was actually closer to 2,219 kb long (29), approximately half the size of the *E. coli* chromosome. The original underestimate in size is thought to be due to the existance of multiple copies of genes such as pilin and P.II, and other redundant sequences (21,22) affecting the rate of annealing.

Several plasmids have been identified in *N. gonorrhoeae* (107). The most prevalent plasmid is the 2.6 MDa cryptic plasmid which was first isolated in 1972 (38) and is found in approximately 96% of clinical isolates (109). The entire cryptic plasmid (4,207 bp) has been sequenced (75), and several open reading frames were correlated to proteins expressed in *E. coli* minicells, but no functional role has been

determined for this plasmid or its products.

Another common plasmid is the 24.5 MDa conjugal plasmid (37). This plasmid allows for the efficient transfer of itself and other smaller plasmids between gonococcal strains (35,108) and can mobilize antibiotic resistance plasmids to other *Neisseria* and to *E. coli* (41). The conjugal plasmid itself has a narrow host range and is maintained poorly in other species (41).

A group of plasmids that have been increasing in prevalence in *N. gonorrhoeae* are those that carry antibiotic resistance markers (107). These appear to have been introduced into the gonococcus either through direct acquisition from other bacteria (e.g. β-lactamase plasmid) (40), or by the transposition of antibiotic resistance genes into indigenous gonococcal plasmids (92). The appearance and spread of β-lactamase plasmids among gonococci has inevitably led to the decreased efficacy of penicillin as a first line antibiotic in the treatment of gonorrhea.

Conjugation is capable of mobilizing plasmid DNA, but chromosomal markers are not transferred by this means. Gonococci can exchange chromosomal DNA through transformation (8). Members of the genus *Neisseria* are naturally competent and can take in DNA from the environment at any stage of growth (117). The expression of pili greatly increases the transformability of the gonococcus (117), though pili do not directly participate in DNA binding or uptake (84). The importance of transformation to the gonococcus has recently been established when it was found that recombination among pilin genes occurs predominantly with DNA brought in from outside the cells rather than by gene conversion, although both processes do occur (113).

Not all DNA in the environment is taken up by the gonococcus; instead

DNA of neisserial origin is recognized and is preferentially taken into the cell (11,32,47). This also occurs in *Haemophilus influenzae*, another naturally competent organism. In *H. influenzae* selective DNA uptake is mediated by the presence of an 11 bp sequence that is present at a much higher frequency in its DNA than in DNA from other sources (26). DNA bearing this sequence is brought into the cell through specialized membrane vesicles called "transformasomes" (2,64). A similar system of DNA recognition operates in *N. gonorrhoeae*, though transformasomes have not been identified (8). In the gonococcus, the uptake sequence has been identified as a 10 bp sequence unrelated to that of *H. influenzae* (36,45). The gonococcal uptake sequence has frequently been found in inverted repeats at the ends of identified and unidentified open reading frames (45,83). This positioning suggests that uptake sequences may often be incorporated into transcriptional terminators. This could explain how the uptake sequence may have been "selected", and how it can be maintained in high copy number without disrupting coding regions (45).

The mechanism behind this selective uptake is still largely unknown, but recent studies have turned up some interesting leads. While looking for gonococcal proteins that specificallly bind neisserial DNA, Dorward and Garon found an 11 kDa peptide located in the inner membrane of the gonococcal cell that bound DNA strongly and had a higher affinity for DNA containing the uptake sequence than to DNA without (30). This protein was strikingly absent in the DNA uptake deficient mutants isolated by Biswas *et al.* (7). Gonococcal inner membranes can form membrane blebs that are released from the cell and can be incorporated by other cells in the area (31). It was proposed that these blebs may

mediate chromosomal DNA transfer, functionally analogous to *H. influenzae* transformasomes (30). Blebs have been shown to carry plasmids between cells (31), but their role in transport of chromosomal DNA remains unclear.

Besides DNA discrimination during uptake, the gonococcus possesses other bariers to genetic exchange (8). Gonococcal DNA is heavily methylated at cytosine residues and in some strains adenine is methylated as well (76,77,97,125). This methylation is sequence specific and serves at least in part to protect the DNA from endogenous restriction endonucleases, several of which have been identified (19,27,97). The production of methylases and restriction enzymes is not uniform among gonococcal strains, which has been shown to limit exchange of DNA by transformation to strains with compatible restriction and modification systems (120). The methylated cytosines also interfere with cloning gonococcal DNA into *E. coli* strains that possess the McrA or McrB restriction enzymes (106,138). These enzymes recognize DNA sequences with methylated cytosines but not unmethylated sequences. Fortunately cloning strains are available that lack these enzymes.

The ability of gonococcal strains to take in chromosomal DNA by transformation has allowed for the mapping of selectable chromosomal markers by cotransformation (137). This technique involves transforming a recipient strain with chromosomal DNA from a donor, selecting for one marker (i.e. antibiotic resistance), and then testing those transformants for one or more other selectable markers. Logically, markers that are physically closer together on the chromosome will have a higher cotransformation frequency than those further apart, and thus map distances and orders can be determined. In this way a short linkage

map representing approximately 3% of the gonococcal chromosome was constructed (1,137). This map consisted mostly of antibiotic resistance markers representing genes for ribosomal proteins and cell wall synthesis, but also included a few outer membrane protein and auxotypic markers.

AUXOTYPING AND SEROTYPING

In 1973, Wesley Catlin developed a particularly useful defined medium for growth of *Neisseria* species (14). The medium was not a minimal medium but a complex of amino acids, vitamins, purines, pyrimidines, minerals and metals, with glucose provided as a carbon and energy source. The purpose of creating this type of medium was to provide a rich growth environment that could be manipulated for the rapid and reliable detection of auxotrophs. The initial study was in part intended to determine the prevalence of auxotrophs among the *Neisseria*, so as to determine if auxotyping would be useful for epidemiological studies. Three *Neisseria* species were examined; *N. meningitidis*, *N. lactamica*, and *N. gonorrhoeae*. The *N. meningitidis* strains tested were relatively homogeneous, with 5 of 57 requiring cysteine but otherwise few auxotrophs were identified. The *N. lactamica* strains were similarly homogeneous in nutritional requirements. The 74 strains of *N. gonorrhoeae* tested on the other hand contained a wide variety of auxotrophs including those with requirements for hypoxanthine, uracil, thiamine, methionine, proline, isoleucine, and arginine, forming 13 distict auxotypes.

In a follow up study, Carifo and Catlin auxotyped 325 clinical

isolates, and of these 75% had at least one requirement and the number of different auxotypes was increased to 22 (13). The auxotype of an isolate was shown to be stably maintained when transmitted to sexual partners or different anatomical sites (13). The ease in detecting auxotypic markers along with their inherent stability made auxotyping very attractive to epidemiologists, and auxotyping rapidly became a standard tool for identifying gonococcal strains.

The most common nutritional requirements detected by Carifo and Catlin were for proline and arginine, and similar patterns were later seen in other studies (20,54,102). Arginine auxotrophy was further dissected by testing the ability of arginine auxotrophs to grow in the presence of intermediates of the arginine biosynthetic pathway (16). This study revealed that the defects were not randomly distributed throughout the pathway but instead occurred predominantly at steps five and six. Of the 212 arginine auxotrophs studied, 1% had a block in the first step of the pathway (required acetylglutamate for growth), 69% had a block at step five (required ornithine), and 30% had a block at step six (required citrulline). Enzyme assays were done on a selection of these strains to determine if the growth requirements correlated to defective enzymes of the arginine biosynthetic pathway (16,114,115). It was found that strains that required ornithine did indeed lack detectable ornithine acetyltransferase activity (16,114), but strains that required citrulline unexpectedly had ornithine transcarbamoylase activity levels comparable to wild type (16,114,115). It was later shown that the citrulline requirement of these strains was due to a defect in carbamoylphosphate synthetase, which in part explains the dual requirement for uracil in all citrulline auxotrophs (115).

Shortly after the development of auxotyping, another typing system was developed based on serological reactivity. The first serotyping strategies used polyclonal antibodies against formalinized whole gonococcal cells and were able to separate gonococcal strains into three broad groups (111,134). These groups were later divided into nine with the development of polyclonal antibodies against purified gonococcal porin (P.I) proteins (10). Serotyping was further refined with the development of a serotyping system based on a set of 12 monoclonal antibodies against P.I proteins (71). This separated gonococcal strains into two serotypes (P.IA and P.IB), with each serotype being subdivided into multiple serovars (23 for P.IA, 32 for P.IB). The serovar system works well for epidemiological studies in that it recognizes the two distinct types of P.I proteins (94), as well as detecting minor variations within these groups for more detailed analyses.

HISTORY OF AHU STRAINS

At the same time that auxotyping media were being developed, other labs were looking for characteristics of gonococcal strains that may influence their ability to cause disseminated infections. It was already known that the incidence of disseminated disease was higher in some locations than others, and it was thought that strain specific factors may be involved in this discrepancy. The first correlation found among strains isolated from disseminated infections was that they had an increased sensitivity to penicillin and tetracycline (136). The basis for this correlation was unclear, but it was proposed that mutations that led to increased antibiotic resistance were achieved at the cost of

decreased virulence (34).

The next major correlation came in 1975 when Knapp and Holmes found that strains causing DGI in Seattle predominantly had the multiple requirements for arginine, hypoxanthine, and uracil (AHU auxotype) (68). This observation was followed by other reports supporting this correlation from other parts of the U.S. and Canada (72,96,126).

The discovery that AHU strains were associated with DGI stimulated further epidemiologic and biological investigations into these strains, and soon a picture of a rather unusual, homogeneous and clinically important set of strains was revealed. AHU strains were common in parts of Europe, the United States and Canada, but were not found in the Far East, where DGI was uncommon (20,54,68,72,96). Along with being associated with DGI, AHU strains were also frequently isolated from men with asymptomatic infections (23,72). The association of AHU strains with asymptomatic infection potentially explains their success in becoming widespread in so many areas, in that asymptomatic males are less likely seek treatment and more likely to spread the infection than symptomatic males.

Besides being associated with patterns of infection, AHU strains were also shown to share a wide variety of unusual phenotypic markers. AHU strains characteristically gave atypically small colonies when cultured and were more sensitive to the toxic components of agar than strains of other auxotypes (68,90). AHU strains are more sensitive to penicillin (34,68,72,126), but were found to be more resistant to the bactericidal affects of normal human serum than non-AHU strains (112). Another study found that AHUs show a decreased ability to utilize lactoferrin-bound iron than other strains, a characteristic thought to be important in

their slow growth and correlation with asymptomatic infection (89).

Several studies found that AHU strains comprise a very homogeneous group. These strains have identical plasmid profiles in that they all had the cryptic plasmid, but not the conjugal plasmid (70). AHU strains all produce type 1 IgA protease, while type 2 is more common among other strains (94). DNA methylation patterns were also shared by AHU strains, with all AHU strains possessing an adenine methylase (74), not commonly found in other strains. Serotyping studies found that AHU strains belonged almost exclusively to 2 closely related serovars in the P.IA serotype that are rarely found outside of the AHU auxotype (70,71). All of these factors supported the popular hypothesis that AHU strains may have arisen only once, with all the present day strains being clonally derived.

The hypothesis that AHU strains may be clonally derived was first proposed by Mayer *et al.* after studying the genetic defects of different gonococcal arginine auxotrophs (85). Genomic DNA from a variety of AHU and Arg- non-AHU strains was tested for the ability to transform one another to Arg+. They found that DNA from one AHU strain could not restore any other to Arg+, but it was capable of transforming other Arg- strains to form Arg+ recombinants. The Hyx- and Ura- markers from AHU strains were also shown to be incapable of recombining. From this they concluded that the mutations responsible for auxotrophy in AHU strains must be identical or overlapping, and that AHU strains were likely to be clonally derived.

Biochemical studies of AHU strains revealed that they were all defective in converting acetylornithine to ornithine (*argJ* mutants)(16). This was shown by their ability to grow on an arginine deficient medium

supplemented with ornithine, but not with supplemental acetylornithine, as well as by showing that ornithine acetyltransferase activity was lost in these strains (114). Some AHU strains had an additional lesion at the sixth step of the pathway and thus required citrulline for growth. It was found that these strains were defective in carbamoylphosphate synthetase as well as OATase.

Interest in AHU strains began with their association with DGI, but as it turns out the association of auxotype with DGI would later be dispelled. In 1977, Eisenstein *et al.* did a careful study independently analyzing the different characteristics that had been correlated with strains isolated from disseminated infections (34). They found that serum resistance and penicillin sensitivity both had a positive correlation with DGI, but the relationship of the AHU auxotype and DGI was not significant. The reports indicating a correlation were most likely detecting an artifact of the lack of diversity of AHU strains and the real association of AHU strains and penicillin sensitivity (34). Another characteristic of AHU strains that was positively correlated to DGI was the P.IA serotype (12). Gonococci of the P.IA serotype were repeatedly shown to be more common among DGI isolates than P.IB, independant of auxotype (9,12,94).

The homogeneous nature of AHU strains along with their unusual collection of phenotypic markers led several groups to search for their origin. In a retrospective study, Catlin and Reyn auxotyped a variety of gonococcal strains isolated between 1935 and 1948, a time window chosen to predate the widespread use of penicillin, which was introduced in the mid-1940s (17). In their survey, a wide variety of auxotypes were discovered, but no uracil requiring strains were found. Two hypoxanthine

requiring strains were found but they were not also arginine auxotrophs. Arginine auxotrophs were common and were found alone and with other requirements but no AHU strains were identified. Another retrospective study looked at several different phenotypes (plasmid content, auxotype, serotype, protease type, and adenine methylation) in gonococcal strains collected from 1928 - 1976 in Copenhagen, Denmark (70). This study found that AHU strains did not appear until 1946, and then increased in frequency through the seventies. This study had the potential of identifying a possible ancestor of AHU strains, but no strain was found that shared the characteristic set of phenotypes found in AHUs. The event that created AHU strains remains a mystery, but it has been speculated that a large portion of the chromosome may have been affected if several characteristics were incorporated simultaneously.

The working hypothesis of today is that AHU strains appeared early in the penicillin era, essentially complete with phenotypes that increased their ability to cause asymptomatic and disseminated infections, and changed very little over time (70). The appearance of a multiply auxotrophic strain at the time of the introduction of penicillin therapy is likely to be more than coincidental. Penicillin kills bacteria by preventing the formation of peptide cross bridges between peptidoglycan molecules in the cell wall, thus compromising the integrity of the entire cell. One consequence of this mode of action is that only actively growing cells will be killed by penicillin. Auxotrophic cells, normally at a disadvantage compared to their prototrophic counterparts in nutritionally depleted environments, would be more likely to survive a shot of penicillin due to their increased likelihood of being in stasis from nutrient starvation. This "advantage" along with their

propensity for causing asymptomatic infections probably contributed to the spread of AHU strains and to their becoming the predominant gonococcal strain in many areas (69,70,135). The incidence of recovery of AHU strains decreased in the 1980's in several areas (69,73). The secret of their initial success has never been fully explained, but is likely to be due to the pleiomorphic effects of their constellation of unique phenotypes, including arginine auxotrophy, making them particularly well adapted to their hosts, and increasing their frequency of transmission.

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CHAPTER I

(ARTICLE)

Sequence of the *argF* Gene Encoding Ornithine Transcarbamoylase from *Neisseria gonorrhoeae*

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ABSTRACT

We report the nucleotide sequence of the gonococcal ornithine transcarbamoylase gene (*argF*) and flanking sequences. This gene contained an open reading frame of 993 nt which encoded a peptide with a predicted molecular weight of 36,731. This was in close agreement with the size of the gene product as determined by minicell analysis. The translation-initiation codon used by this gene was GUG, which was confirmed by N-terminal amino acid sequencing of the purified protein. In the flanking regions of the gene were found 15 bp inverted repeats containing the neisserial DNA uptake sequence, supporting the hypothesis that these sequences may be used as transcriptional termination signals. We compared the predicted amino acid sequence to OTCase sequences previously determined from *E. coli*, *Pseudomonas aeruginosa*, and several eukaryotic species, and found that highly conserved regions in the genes from these organisms were also conserved in *N. gonorrhoeae*. Amino acids known to be important for carbamoyl phosphate and ornithine binding were also conserved in the gonococcal OTCase.

INTRODUCTION

Arginine biosynthesis from glutamate occurs by an eight step pathway (6). *Neisseria gonorrhoeae* possesses all the enzymes required for this pathway, yet arginine auxotrophs are commonly encountered among clinical isolates (4). A survey of 212 arginine auxotrophs showed that blocks in the pathway occurred almost exclusively at the fifth or sixth steps; that is, the conversion of acetylornithine to ornithine by ornithine acetyl transferase, and the conversion of ornithine and carbamoyl phosphate to citrulline by ornithine transcarbamoylase (OTCase) (5). Thirty percent of the arginine auxotrophs examined in this study required citrulline, suggesting that these would be OTCase mutants. Subsequent studies (30,31) showed that all of the citrulline auxotrophs screened showed normal levels of OTCase activity, and that the requirement for citrulline was due to a defect in carbamoyl phosphate synthesis. We have also examined a number of citrulline auxotrophs and to date none of these have been OTCase mutants (Mulks, unpublished data).

OTCase is the product of the *argF* gene in *N. gonorrhoeae* and other bacteria. *E. coli* K-12 strains have two copies of this gene, the second being designated *argI*. *ArgI* is actually the copy common to all *E. coli* strains; *argF* is thought to have been acquired in *E. coli* K-12 by transposition from an unidentified source (14). *Pseudomonas aeruginosa* also has two OTCases, though with separate functions. The pseudomonas ArgF enzyme is involved in arginine biosynthesis, while the ArcB enzyme is involved in arginine catabolism (25). All four of the above mentioned

genes have been cloned and sequenced (1,2,16,34).

The gonococcal *argF* gene and several other genes of the arginine biosynthetic pathway have been cloned (22), and gonococcal OTCase has been purified (23). The purified enzyme was shown to exist as a trimer of identical subunits of 36,500 Mr. We report here the complete nucleotide sequence of the *argF* gene from *N. gonorrhoeae*, and the predicted amino acid sequence of the gonococcal ArgF enzyme. These sequences were compared to the OTCase sequences of *E. coli*, *P. aeruginosa*, and several eukaryotic species and the relationship of these genes is discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* GM2163 (*McrA-, McrB-, ara-14, leuB6, tonA13, lacY1, tsx-78, supE44, galK2, dcm-6, hisG4, rpsL136, dam-13*::Tn9, *xyl-5, mtl-1, thi-1 hsdR2*) was used as the initial receptor of the gonococcal genomic library. *E. coli* N134 (*argI68, thi-1, spoT1, relA1, Δ(gpt-lac) 5*) was used for selection of the *argF* clone, and for complementation experiments. *E. coli* DS410 (*minA1, minB2, rpsL135, xyl-7, mtl-2, thi-1*) was used for minicell experiments. *N. gonorrhoeae* CDC50 (Arg+,Pro-,Dam+) was the source of our gonococcal *argF* clone. *E. coli* strains were routinely grown on LB medium with ampicillin added to 50 ug/ml for plasmid selection when required. *N. gonorrhoeae* strains were grown on GC base agar (Difco) with 1% Kellogg's suplement (37) at 36°C under 5% CO_2. NEDA defined medium (4), either as a broth or with 1.5% agarose (Baker), was used for auxotyping and complementation studies.

DNA manipulations. Restriction enzymes and DNA ligase were purchased from Boehringer Mannheim Biochemicals or Bethesda Research Laboratories. Genomic and plasmid DNA preparations, gel elctrophoresis, and *E. coli* transformation were all done by conventional methods (28).

Cloning and subcloning *argF*. A plasmid library of *N. gonorrhoeae* strain CDC50 was constructed by partially digesting genomic DNA with *Sau*3A, ligating fragments from 1.5-8 kb into the *Bam*HI site of the plasmid vector pUC12, and transforming this ligation reaction into *E. coli* GM2163. This library was screened for the gonococcal *argF* gene by transforming the pooled plasmids into *E. coli* N134 and selecting for the ability to grow on NEDA plates lacking arginine. The resulting clone was

restriction mapped by conventional means, and subcloned into M13mp19 for DNA sequencing.

OTCase assays. OTCase activity was assayed in cell-free extracts by measuring the production of citrulline from ornithine and carbamoyl phosphate as described by Shinners and Catlin (30).

Minicell analysis. Minicells were purified by differential centrifugation followed by filtration through a Whatman GF/F glass filter as described by Lurquin (18). Purified minicells were incubated in the presence of ^{35}S-methionine and the labelled proteins were separated on an SDS-polyacrylamide gel and visualized by autoradiography.

DNA sequencing. Nested deletions of the *argF* containing insert in M13mp19 were constructed using the Cyclone deletion system (International Biotechnologies, Inc). These deletions were sequenced using the dideoxy chain termination reagents and Sequenase 2.0 enzyme from U.S. Biochemicals. The insert was sequenced completely on both strands using the method of Sanger *et al.* (29).

Protein sequencing. The ArgF protein was isolated from *E. coli* minicells containing the gonococcal *argF* clone by band purification from an SDS-polyacrylamide gel. The minicell proteins were separated on a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon P, Schleicher and Schuell) using the method of Matsudaira (20). The proteins on the membrane were stained with Coommasie blue and the unique ArgF band was excised from the membrane. The first 10 amino acids were determined using an Applied Biosystems 477A Protein Sequencer at the Macromolecular Structural Facility at Michigan State University.

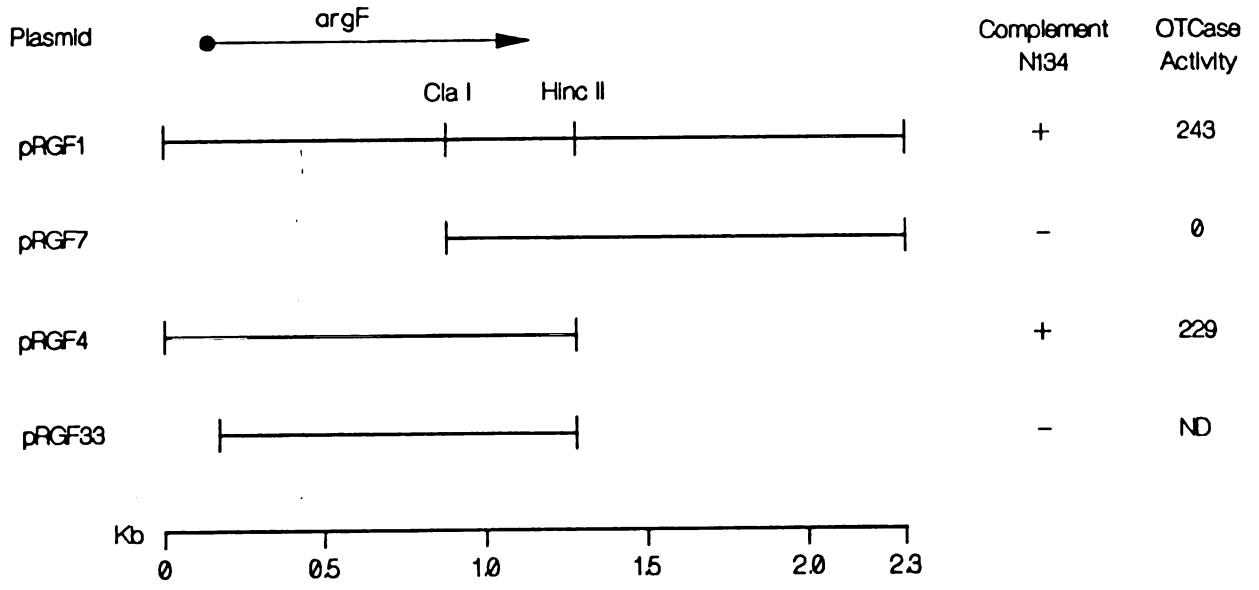
RESULTS AND DISCUSSION

Cloning and initial characterization of the gonococcal *argF* gene. The cloning of the gonococcal *argF* gene has been previously reported (22). We independently isolated the gene from *N. gonorrhoeae* strain CDC50 by constructing a genomic library of this strain in the plasmid vector pUC12. The library was initially transformed into *E. coli* GM2163 to avoid restriction of the heavily cytosine-methylated gonococcal DNA (24). This library was screened for the gonococcal *argF* gene by transformating it into *E. coli* N134 and selecting for Arg+ transformants on NEDA plates lacking arginine. A plasmid with a 2.3 kb insert complementing the *argI* mutation was isolated and designated pRGF1. The presence of the *argF* gene was confirmed by assaying cell-free extracts of *E. coli* N134 containing pRGF1 for OTCase activity. By restriction mapping and the construction of deletions, the clone was trimmed to a 1.3 kb fragment containing the active gene in the plasmid pRGF4 (Fig. 1). This plasmid was used for minicell analysis of the gene product, and the insert was subcloned in both orientations into the phage vector M13mp19 for DNA sequencing.

Minicell analysis. The protein encoded by the gonococcal *argF* clone was analyzed by transforming the minicell producing *E. coli* strain DS410 with pRGF4. Figure 2 shows that the pRGF4 containing minicells produced a unique band of 38,500 Da when compared to minicells containing only the plasmid vector. The size of this protein correlated well with the size of 36,731 Da predicted from DNA sequence analysis.

Figure 1. Restriction map and deletions of pRGF1. Plasmid deletions are shown along with their ability to complement the *argI,F E. coli* strain N134 and OTCase activity produced in *E. coli* N134 containing each plasmid. OTCase activity is given as nmol citrulline produced/minute x mg protein in cell-free extract. Deletion pRGF33 was constructed by exonucleolytic digestion of the pRGF4 insert in M13mp19 with T4 DNA polymerase and then subcloning back into pUC19 to test for complementation.

Figure 1


Plasmid
argF
Cla I
Hinc II
Complement N134
OTCase Activity
pRGF1
+
243
pRGF7
-
0
pRGF4
+
229
pRGF33
-
ND
Kb
0
0.5
1.0
1.5
2.0
2.3

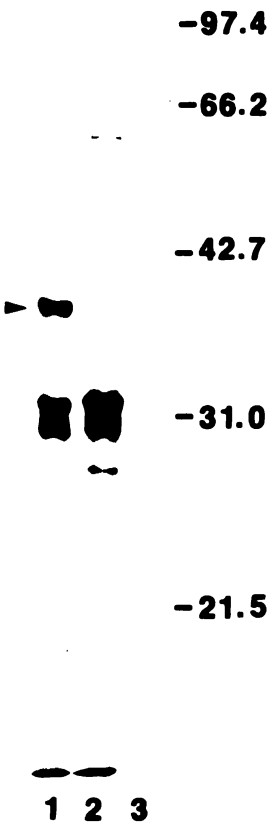


Figure 2. Minicell analysis of pRGF4. Minicells contain: Lane 1, pRGF4; Lane 2, pUC19; Lane 3, no plasmids. Molecular weight standards in kDa are indicated along the right. The arrow indicates the ArgF protein.

DNA sequence analysis. The sequence of the gonococcal *argF* gene and flanking regions is shown in Figure 3. This sequence contains an open reading frame of 993 nt preceded by a 6 nt ribosome binding site sequence complementary to the 3'-end of the gonococcal 16S rRNA (27). This gene uses the codon GUG at position 141-143 for translation initiation. This is the first report of a GUG start codon in *N. gonorrhoeae*, but GUG start codons have been found in approximately 8% of sequenced *E. coli* genes (32). The first AUG codon within the open reading frame occurs at nt 266. Several lines of evidence show that this is not the start codon for the gonococcal *argF* gene. First, previously sequenced OTCase genes from *E. coli* and *P. aeruginosa*, when aligned with the *argF* sequence of *N. gonorrhoeae*, initiate near the GUG codon, not the AUG (Fig.4). Second, in the plasmid pRGF33, the GUG codon has been deleted and the AUG codon remains, yet the plasmid does not complement *E. coli* N134 (Fig.1). Third, the molecular weight of the ArgF protein as produced in minicells (Fig.2) closely matches the predicted size of the protein transcribed beginning at the GUG codon, but would be significantly larger than the protein predicted if the start codon were the first available AUG. Also, the predicted size correlated very well to the size determined from the purified protein of 36,500 (23), but only if the GUG initiator is utilized. The most compelling evidence of all for the GUG initiator came from N-terminal amino acid sequence analysis of the ArgF protein (see below).

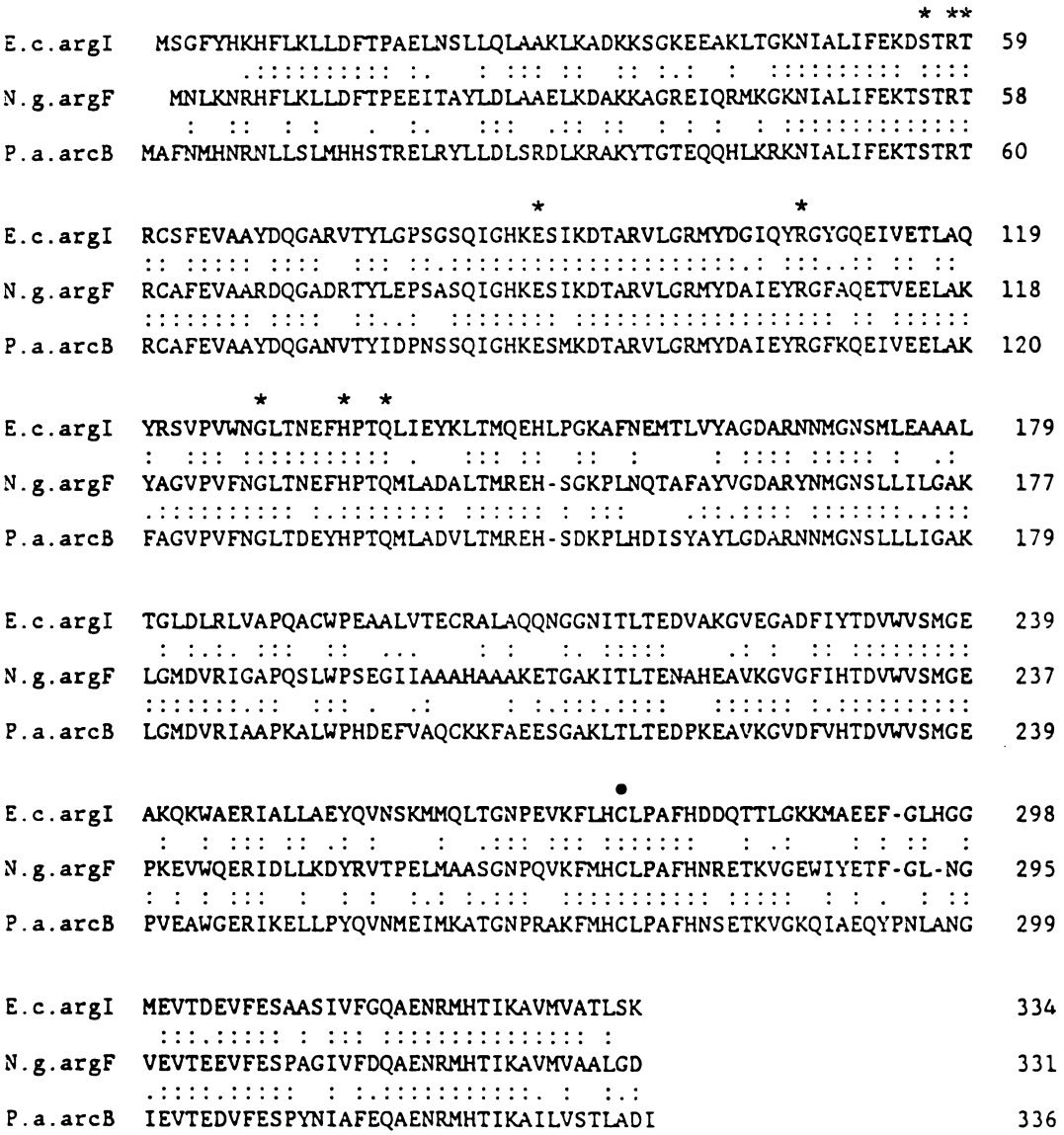
The promotor and transcription start site could not be clearly identified from the sequence of the *argF* gene since no obvious -10 (TATAAT) sequence was found upstream of the open reading frame. A

Figure 3. Nucleotide sequence of the gonococcal *argF* gene and flanking sequences. The predicted amino acid sequence is shown below the coding region. N-terminal amino acids that were sequenced are indicated in italics. Inverted repeats are underlined as is the putative ribosome binding site (RBS). Neisserial DNA uptake sequences are underlined twice.

```
                                                                    TCCCCGTCATCCGTATGGAGTAAGGGATTGACCGCAATGCCGTCTGAACAACCTTCAGACG   61

GCATTGCAACATTCCGCTAACCCTTCTTTCCGCAAACGCTGCAAATACGGCGTTCACGCCCCACATAAAGGAAACGACA  140
                                                                                S/D

GTG AAC CTG AAA AAC CGC CAT TTT CTG AAA CTT TTG GAC TTC ACG CCG GAA GAA ATC ACC  200
Met Asn Leu Lys Asn Arg His Phe Leu Lys Leu Leu Asp Phe Thr Pro Glu Glu Ile Thr

GCC TAC CTC GAC CTT GCC GCC GAG TTG AAA GAC GCC AAA AAG GCA GGG CGC GAG ATT CAG  260
Ala Tyr Leu Asp Leu Ala Ala Glu Leu Lys Asp Ala Lys Lys Ala Gly Arg Glu Ile Gln

CGG ATG AAA GGG AAA AAC ATC GCC CTG ATT TTT GAA AAA ACA TCC ACG CGC ACA CGC TGT  320
Arg Met Lys Gly Lys Asn Ile Ala Leu Ile Phe Glu Lys Thr Ser Thr Arg Thr Arg Cys

GCG TTT GAA GTC GCC GCA CGC GAC CAA GGC GCG GAT CGA ACC TAT CTG GAA CCG TCC GCC  380
Ala Phe Glu Val Ala Ala Arg Asp Gln Gly Ala Asp Arg Thr Tyr Leu Glu Pro Ser Ala

AGC CAA ATC GGG CAC AAA GAA AGC ATC AAA GAC ACG GCG CGC GTC TTA GGC AGA ATG TAC  440
Ser Gln Ile Gly His Lys Glu Ser Ile Lys Asp Thr Ala Arg Val Leu Gly Arg Met Tyr

GAT GCC ATC GAA TAT CGC GGC TTC GCT CAG GAA ACT GTC GAA GAA TTG GCA AAA TAT GCG  500
Asp Ala Ile Glu Tyr Arg Gly Phe Ala Gln Glu Thr Val Glu Glu Leu Ala Lys Tyr Ala

GGC GTA CCC GTG TTC AAC GGG CTG ACC AAC GAG TTC CAT CCC ACA CAA ATG CTT GCC GAC  560
Gly Val Pro Val Phe Asn Gly Leu Thr Asn Glu Phe His Phe Thr Gln Met Leu Ala Asp

GCA CTG ACT ATG CGC GAA CAC AGC GGC AAA CCT TTG AAC CAA ACC GCG TTT GCC TAC GTC  620
Ala Leu Thr Met Arg Glu His Ser Gly Lys Pro Leu Asn Gln Thr Ala Phe Ala Tyr Val

GGC GAC GCG CGT TAC AAC ATG GGC AAT TCC CTG CTG ATT TTA GGG GCA AAA TTG GGG ATG  680
Gly Asp Ala Arg Tyr Asn Met Gly Asn Ser Leu Leu Ile Leu Gly Ala Lys Leu Gly Met

GAC GTG CGT ATC GGC GCA CCT CAA AGC CTG TGG CCG TCT GAA GGC ATT ATC GCC GCC GCA  740
Asp Val Arg Ile Gly Ala Pro Gln Ser Leu Trp Pro Ser Glu Gly Ile Ile Ala Ala Ala

CAC GCC GCC GCC AAA GAA ACC GGT GCA AAA ATT ACC CTG ACC GAA AAC GCG CAT GAA GCC  800
His Ala Ala Ala Lys Glu Thr Gly Ala Lys Ile Thr Leu Thr Glu Asn Ala His Glu Ala

GTC AAA GGT GTC GGT TTC ATT CAT ACT GAC GTA TGG GTC AGC ATG GGC GAG CCG AAA GAA  860
Val Lys Gly Val Gly Phe Ile His Thr Asp Val Trp Val Ser Met Gly Glu Pro Lys Glu

GTC TGG CAG GAA CGC ATC GAT TTG CTG AAA GAT TAC CGC GTT ACG CCC GAA CTG ATG GCG  920
Val Trp Gln Glu Arg Ile Asp Leu Leu Lys Asp Tyr Arg Val Thr Pro Glu Leu Met Ala

GCA TCG GGC AAT CCG CAA GTC AAA TTC ATG CAC TGC CTG CCC GCC TTC CAC AAC CGC GAA  980
Ala Ser Gly Asn Pro Gln Val Lys Phe Met His Cys Leu Pro Ala Phe His Asn Arg Glu

ACC AAA GTC GGC GAA TGG ATT TAC GAA ACC TTC GGG CTG AAC GGT GTG GAA GTT ACA GAA 1040
Thr Lys Val Gly Glu Trp Ile Tyr Glu Thr Phe Gly Leu Asn Gly Val Glu Val Thr Glu

GAA GTA TTC GAA AGT CCG GCC GGC ATC GTG TTC GAT CAG GCG GAA AAC CGT ATG CAC ACG 1100
Glu Val Phe Glu Ser Pro Ala Gly Ile Val Phe Asp Gln Ala Glu Asn Arg Met His Thr

ATT AAA GCG GTA ATG GTC GCG GCT CTG GGC GAC TGA CAGAACTGTGCCTGTTTAAATTCATCCGCAA 1167
Ile Lys Ala Val Met Val Ala Ala Leu Gly Asp End

CACAGATACCGTCTGAACACGATGTTCAGACGGTATCCATATAACAAACTGCCTACACGATGTGTAGGCAGTCCCGTTT 1246

GAAAACAATCAGTTTTTGTTCTTGGTCGACT                                                  1277
```

Figure 4. Comparison of the predicted amino acid sequences of the gonococcal *argF*, *E. coli argI*, **and** *P. aeruginosa arcB* **genes. Two dots** indicate **identical residues, one dot represents conservative** substitutions. **Amino acids implicated in carbamoyl phosphate binding or** catalysis **are marked by an asterisk. The cysteine residue involved in** binding **of ornithine is indicated by o.**

Figure 4


E.c.argI MSGFYHKHFLKLLDFTPAELNSLLQLAAKLKADKKSGKEEAKLTGKNIALIFEKDSTRT 59
N.g.argF MNLKNRHFLKLLDFTPEEITAYLDLAAELKDAKKAGREIQRMKGKNIALIFEKTSTRT 58
P.a.arcB MAFNMHNRNLLSLMHHSTRELRYLLDLSRDLKRAKYTGTEQQHLKRKNIALIFEKTSTRT 60
E.c.argI RCSFEVAAYDQGARVTYLGPSGSQIGHKESIKDTARVLGRMYDGIQYRGYGQEIVETLAQ 119
N.g.argF RCAFEVAARDQGADRTYLEPSASQIGHKESIKDTARVLGRMYDAIEYRGFAQETVEELAK 118
P.a.arcB RCAFEVAAYDQGANVTYIDPNSSQIGHKESMKDTARVLGRMYDAIEYRGFKQEIVEELAK 120
E.c.argI YRSVPVWNGLTNEFHPTQLIEYKLTMQEHLPGKAFNEMTLVYAGDARNNMGNSMLEAAAL 179
N.g.argF YAGVPVFNGLTNEFHPTQMLADALTMREH-SGKPLNQTAFAYVGDARYNMGNSLLILGAK 177
P.a.arcB FAGVPVFNGLTDEYHPTQMLADVLTMREH-SDKPLHDISYAYLGDARNNMGNSLLLIGAK 179
E.c.argI TGLDLRLVAPQACWPEAALVTECRALAQQNGGNITLTEDVAKGVEGADFIYTDVWVSMGE 239
N.g.argF LGMDVRIGAPQSLWPSEGIIAAAHAAAKETGAKITLTENAHEAVKGVGFIHTDVWVSMGE 237
P.a.arcB LGMDVRIAAPKALWPHDEFVAQCKKFAEESGAKLTLTEDPKEAVKGVDFVHTDVWVSMGE 239
E.c.argI AKQKWAERIALLAEYQVNSKMMQLTGNPEVKFLHCLPAFHDDQTTLGKKMAEEF-GLHGG 298
N.g.argF PKEVWQERIDLLKDYRVTPELMAASGNPQVKFMHCLPAFHNRETKVGEWIYETF-GL-NG 295
P.a.arcB PVEAWGERIKELLPYQVNMEIMKATGNPRAKFMHCLPAFHNSETKVGKQIAEQYPNLANG 299
E.c.argI MEVTDEVFESAASIVFGQAENRMHTIKAVMVATLSK 334
N.g.argF VEVTEEVFESPAGIVFDQAENRMHTIKAVMVAALGD 331
P.a.arcB IEVTEDVFESPYNIAFEQAENRMHTIKAILVSTLADI 336

potential -35 (TTGACA) sequence of TTGCAA was found starting at nt 67 (Fig 3). This would put the transcription initiation site at approximately nt 104, but this has not yet been confirmed. It seems likely that the cloned gonococcal *argF* gene uses its own promotor for expression in *E. coli* since equivalent levels of expression were obtained when the gene was present in either orientation in pUC12 (data not shown).

Gonococcal cells are naturally competent for DNA transformation during all stages of growth, though only DNA of neisserial origin will bind to the cells (8). This selectivity is due to the higher occurence of the 10bp sequence 5'-GCCGTCTGAA-3' in neisserial DNA than in DNA from other sources (9). This DNA uptake sequence, in at least a 9 out of 10 bp match, is present 5 times in the *argF* sequence, twice as part of 15 bp inverted repeats in the flanking regions of the gene, and once unrepeated within the coding region. The presence of the uptake sequence within these flanking inverted repeats supports the hypothesis of Goodman and Scocca that the uptake sequence may be maintained in high frequency in *Neisseria* by being incorporated into transcription termination sequences, though the presence of the uptake sequence within the coding region of the gene suggests that this is not the only means of preserving this sequence. Downstream from the proposed *argF* transcriptional terminator containing the uptake sequences, which has a ΔG value of -25.6 kcal, is a second 11 bp inverted repeat (ΔG = -19.2 kcal). Both sets of inverted repeats resemble rho-dependent transcriptional terminators (11), but which, if either, of these inverted repeats actually functions in termination of the *argF* gene has not yet been determined. It should be noted that the inverted repeat

upstream of the *argF* gene (ΔG = -30.8 kcal) overlaps the putative -35 promoter sequence. Whether there is an actual gene upstream of *argF* that utilizes this inverted repeat as a terminator, or whether transcription of that gene might interfere with initiation of the *argF* gene, is not yet known.

The codon usage data for the gonococcal *argF* gene is shown in Table I. This data is in good agreement with the codon usage frequencies compiled by West and Clark (36) for previously sequenced gonococcal genes. Codons infrequently used in gonococcal genes were either not used or used only once in *argF* with the exception of GGG (Gly) which was used 7 times. Codons infrequently used in highly expressed *E. coli* genes (10) were also used no more than once in *argF*, again with the exception of GGG.

N-terminal amino acid sequence analysis. N-terminal amino acid sequencing of the ArgF protein was performed in order to confirm the translation initiation site of the gene. The seventh amino acid could not be determined with certainty, but the remaining amino acid sequence data supported the conclusion that the GUG codon at nt 141-143 is the initiation codon for the gonococcal *argF* gene.

Comparison to OTCases from other species. The nucleotide sequences of OTCases from other bacteria (*E. coli argF* and *argI*, *P. aeruginosa argF* and *arcB*), fungi (*Aspergillus niger* and *Saccharomyces cerevisiae*), and mammals (human, mouse, and rat) have been previously reported (1,2,3,12,15,16,33,34,35). The DNA sequence and predicted amino acid sequence of the gonococcal *argF* gene were compared to these other OTCases (Table II). The gonococcal *argF* gene showed 43.4 to 66.6% DNA

TABLE I

Codon usage in the *N. gonorrhoeae argF* gene[a]

Codon	Amino Acid	used in *argF*	Codon	Amino Acid	used in *argF*	Codon	Amino Acid	used in *argF*	Codon	Amino Acid	used in *argF*
TTT	Phe	4	TCT	Ser	1	TAT	Tyr	3	TGT	Cys	1
TTC	Phe	10	TCC	Ser	3	TAC	Tyr	6	TGC	Cys	1
TTA	Leu	2	TCA	Ser	0	TAA	***	0	TGA	***	1
TTG	Leu	6	TCG	Ser	1	TAG	***	0	TGG	Trp	4
CTT	Leu	3	CCT	Pro	2	CAT	His	4	CGT	Arg	3
CTC	Leu	1	CCC	Pro	4	CAC	His	6	CGC	Arg	11
CTA	Leu	0	CCA	Pro	0	CAA	Gln	6	CGA	Arg	1
CTG	Leu	15	CCG	Pro	6	CAG	Gln	4	CGG	Arg	1
ATT	Ile	8	ACT	Thr	3	AAT	Asn	2	AGT	Ser	1
ATC	Ile	9	ACC	Thr	9	AAC	Asn	11	AGC	Ser	5
ATA	Ile	0	ACA	Thr	4	AAA	Lys	20	AGA	Arg	1
ATG	Met	11	ACG	Thr	5	AAG	Lys	1	AGG	Arg	0
GTT	Val	2	GCT	Ala	2	GAT	Asp	5	GGT	Gly	4
GTC	Val	11	GCC	Ala	18	GAC	Asp	10	GGC	Gly	14
GTA	Val	4	GCA	Ala	9	GAA	Glu	26	GGA	Gly	0
GTG	Val	5	GCG	Ala	11	GAG	Glu	4	GGG	Gly	7

[a] Total number of codons in the *argF* gene is 331; *** indicates stop codons.

Table 2. DNA and amino acid sequence comparison of the OTCase of *Neisseria gonorrhoeae* and OTCases and ATCases from other species[a]

Organism	% Identity DNA	% Identity Amino Acid
E. coli OTCase *(argF)*	64.0	64.0
E. coli OTCase *(argI)*	62.5	61.6
P. aeruginosa OTCase *(arcB)*	66.6	68.0
P. aeruginosa OTCase *(argF)*	50.1	37.5
A. niger OTCase	43.7	37.1
S. cerevisiae OTCase	43.4	33.5
Human OTCase	48.5	39.8
Mouse OTCase	47.9	40.5
Rat OTCase	47.7	40.1
E. coli ATCase *(pyrB)*	42.6	30.5
S. typhimurium ATCase *(pyrB)*	42.1	30.1
B. subtilis ATCase *(pyrB)*	41.8	30.3

[a] % identity for each pair of sequences was calculated using the GAP program from the University of Wisconsin Genetics Computer Group (Devereux et al., 1984), which makes use of the algorithm of Risler et al., (1988).

sequence identity and 33.5 to 68.0% amino acid sequence identity with these bacterial, fungal, and mammalian OTCases, suggesting that these genes may have all arisen from a common origin. However, the data suggest that the gonococcal *argF* gene is more closely related to *P. aeruginosa arcB* (66.6% identity) and *E. coli argF* (64.0%) and *argI* (62.5%) than to *P. aeruginosa argF* (50.1%) or the fungal or mammalian OTCases (43.4-48.5%). These data correlate well with the results of Itoh et al., (16) showing a closer relationship of *P. aeruginosa arcB* to *E. coli argF* and *argI* than to *P. aeruginosa argF*.

The nucleotide sequences of three bacterial aspartate trans-carbamoylases (ATCases), which catalyze the conversion of aspartate and carbamoyl phosphate to carbamoyl aspartate, have been reported (13,17,21). Comparison of the DNA and predicted amino acid sequences of these genes to that of gonococcal *argF* (Table II) indicates ~42% identity of the DNA and ~30% identity of the protein sequences and supports the conclusion of Houghton et al. (13) that these two classes of enzymes share a common evolutionary origin.

Figure 4 compares the predicted amino acid sequences of the gonococcal *argF*, the *E. coli argI*, and the *P. aeruginosa arcB* genes. Long stretches of near identity were found throughout the length of the sequences. The regions from amino acids 55-59 (Ser-Thr-Arg-Thr-Arg) and from 133-136 (His-Pro-Thr-Gln) are highly conserved in all OTCases as well as ATCases sequenced to date from either prokaryotic or eukaryotic sources. Within and between these regions are the amino acids involved in carbamoyl phosphate binding (13,17), and these are all present in the gonococcal OTCase sequence (Fig. 4). Another strongly conserved region common to OTCases occurs at amino acids 262-268 (Phe-X-His-Cys-Leu-Pro).

The cysteine residue in this sequence is required for ornithine binding in OTCases, (19), is not found in ATCases, and is conserved in the gonococcal *argF* protein.

Unlike *E. coli* and *P. aeruginosa*, a gonococcal strain lacking OTCase activity has not yet been identified. Experiments are in progress to construct defined *argF* mutants, which should help determine if the lack of *argF* mutants among arginine auxotrophs is due to a selective dissadvantage of this type of mutation.

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CHAPTER II

(ARTICLE)

Sequence analysis and complementation studies of the *argJ* gene from *Neisseria gonorrhoeae*

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ABSTRACT

Clinical isolates of *Neisseria gonorrhoeae* frequently are deficient in arginine biosynthesis. These auxotrophs often have defects in the fifth step of the arginine biosynthetic pathway, the conversion of acetylornithine to ornithine. This reaction is catalyzed by the enzyme ornithine acetyltransferase which is a product of the *argJ* gene. We have cloned and sequenced the gonococcal *argJ* gene and found that it contains an open reading frame of 1,218 nucleotides and encodes a peptide with a deduced M_r of 42,879. This predicted size was supported by minicell analysis. This gene was capable of complementing *E. coli argE* and *argA* mutations, and of transforming an ArgJ- strain of *N. gonorrhoeae* to Arg+. Southern blots were able to detect bands that specifically hybridized to the gonococcal *argJ* gene in genomic DNA from *Pseudomonas aeruginosa*, but not *Escherichia coli*, a result that reflects the divergent nature of the arginine biosynthetic pathway in these organisms.

INTRODUCTION

Arginine biosynthesis in prokaryotes occurs by an eight step pathway (6) (Fig. 1). The first four steps of the pathway involve N-acetylated intermediates, beginning with the acetylation of glutamate and ending with acetylornithine. N-acetylornithine is converted to ornithine in the fifth step of the pathway, and two separate enzymes have evolved to catalyze this reaction. In the *Enterobacteriaceae*, N-acetylornithine is hydrolyzed to ornithine and acetate by the ArgE enzyme N-acetyl-ornithinase (EC 3.1.5.16) (35). In other bacteria including the methanogens, cyanobacteria, pseudomonads, and *N. gonorrhoeae*, the acetyl group of N-acetylornithine is transferred to glutamate by the ArgJ enzyme ornithine acetyltransferase (OATase, EC 2.3.1.35) (19,13,34,28). The N-acetylglutamate produced from this reaction can be cycled back into the arginine biosynthetic pathway, bypassing step one. N-acetylornithinase has been purified from *E. coli* and appears to function as a monomer with a M_r of 62,000 (6). In contrast, no OATase from any source has been characterized and little is known of the physical properties of this enzyme. While the biosynthesis of arginine by *E. coli* has been extensively studied as a model of gene regulation, few of the genes encoding the enzymes involved in this pathway have been sequenced. Nucleotide sequence data is available for *E. coli argA*, *argF*, *argI*, and *carAB*, and the regulatory region of the *argECBH* operon, and for *Pseudomonas aeruginosa argF* and *arcB*. Neither *E. coli argE* nor *Pseudomonas aeruginosa argJ* have been sequenced.

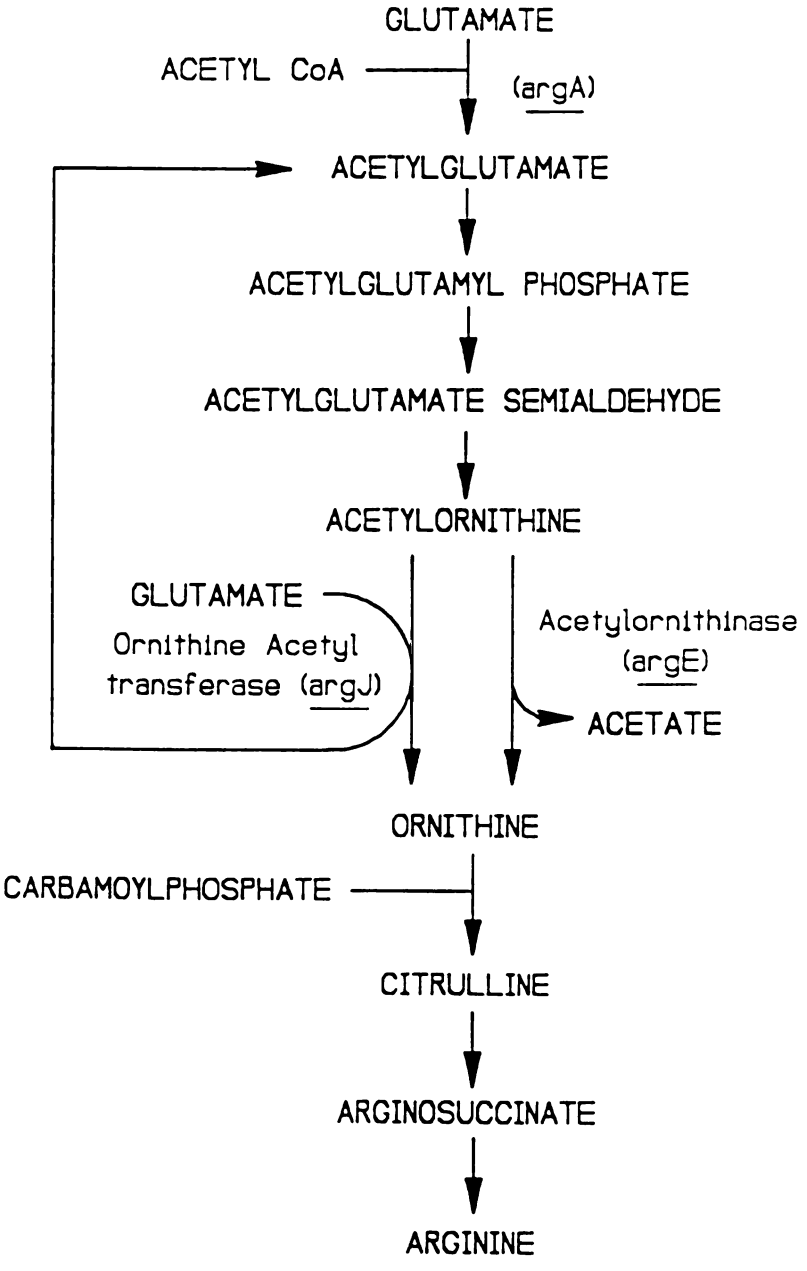


Figure 1. Arginine biosynthetic pathway. The two alternative pathways for the conversion of acetylornithine to ornithine are indicated.

Although *N. gonorrhoeae* possesses all the enzymes required for arginine biosynthesis, arginine auxotrophs are commonly encountered among clinical isolates of this pathogen (3). In a survey of 212 arginine auxotrophs, 69% were found to have blocks at the fifth step of the pathway (4). Gonococcal strains with combined auxotrophy for arginine, hypoxanthine and uracil (AHU strains) are of particular interest due to their prevalence among isolates from asymptomatic and disseminated gonococcal infections (4,14). AHU strains have repeatedly been shown to be a highly homogeneous group and are believed to be clonally derived (15,18,21). All AHU strains tested were found to be *argJ* mutants (28), and AHU strains were shown to have identical or overlapping *argJ* mutations in that DNA from one AHU strain could not restore any other to Arg+ in paired transformation experiments (4,18).

At least four of the genes of the arginine biosynthetic pathway (*argB*, *argF*, *argG*, and *argJ*) plus the genes for carbamoyl phosphate synthetase (*carA* and *carB*) have been cloned from *N. gonorrhoeae* (23). In that report, Picard and Dillon found that a single lambda clone containing the gonococcal *argJ* gene could complement both *E. coli argE* and *argA* mutations. They proposed that the gonococcal OATase may be able to complement the *argA* mutation through the feedback pathway, though it was also possible that the gonococcal *argA* gene was present on the same clone.

In this paper we report the cloning and sequencing of the gonococcal *argJ* gene, and investigate its potential to complement *E. coli argE* and *argA* mutations. This represents the first reported sequence of an ornithine acetyltransferase from any source. Along with providing new information on the properties of the OATase enzyme, this study will

provide a foundation for further investigations into the molecular basis of arginine auxotrophy and on the regulation of gene expression in *N. gonorrhoeae*.

MATERIALS AND METHODS

Bacterial strains. The *mcrA,B E. coli* strain GM2163 (*McrA-, McrB-, ara-14, leuB6, tonA13, lacY1, tsx-78, supE44, galK2, dcm-6, hisG4, rpsL136, dam*-13::Tn9, *xyl-5, mtl-1, thi-1, hsdR2*) was used for the initial construction of the genomic library. The *argE E. coli* strain AT2538 (*thr-1, ara-14, leuB6, Δ(gpt-proA)62, lacY1, supE44, galK2, λ-, hisG4, rpsL31, xyl-5, mtl-1, pyrE60, argE3, thi-1)* and *argA E. coli* strain W3421 (*argA21, galT23, IN(rrnD-rrnE)1, λ-)* were used for cloning and complementation studies. The minicell producing *E.coli* strain DS410 (*minA1, minB2, rpsL135, xyl-7,mtl-2, thi-1*) was used for minicell isolation and protein labelling experiments. *E. coli* DH1 (Arg+), *E. coli* N134 (*Δ(gpt-lac)5, relA1, spoT1, thi-1, argI68)* and *Pseudomonas aeruginosa* ATCC 27853 were used for Southern blots. *N. gonorrhoeae* strain CDC50 (Pro-) was the source of the *argJ* gene. *N. gonorrhoeae* strain 30465 (ArgJ-,Hyx-,Ura-) was used as a transformation recipient.

Media and growth conditions. All *E. coli* strains were grown on LB medium and, when needed, ampicillin was added to 50 ug/ml for plasmid selection. All *N. gonorrhoeae* strains were routinely grown on GC base medium (Difco) plus 1% Kellogg's supplement (39) at 36°C under 5% CO_2. NEDA defined medium for gonococci (4), either as a broth or with 1.5% agarose (Baker), was used for growth of all bacteria in auxotyping or

complementation experiments.

DNA manipulations. Restriction enzymes and DNA ligase were purchased from Boehringer Mannheim Biochemicals or Bethesda Research Laboratories and used according to the manufacturer's instructions. Genomic and plasmid DNA preparations, gel electrophoresis, and *E. coli* transformation were all performed by conventional methods (26).

Cloning and sequencing of the gonococcal *argJ* gene. A genomic library of *N. gonorrhoeae* CDC50 was constructed by partially digesting genomic DNA with *Sau*3A and ligating fragments from 1.5 - 8 kb into the *Bam*HI site of the plasmid vector pUC12. A recombinant plasmid containing the gonococcal *argJ* gene was isolated from this library by complementation of the *argE E. coli* strain AT2538. This isolate was subcloned into M13mp19, and nested deletions were constructed using the Cyclone deletion kit from International Biotechnologies, Inc. These deletions were sequenced using the dideoxy chain termination reagents and Sequenase 2.0 enzyme from U.S. Biochemicals, by the method of Sanger et al. (27). The sequence was analyzed using GENEPRO software (Riverside Scientific Enterprises), and this data was used to determine the probable *argJ* open reading frame. Second strand sequencing was done from identified restriction sites in M13 or pUC constructs.

OATase assays. Ornithine acetyltransferase activity was assayed by measuring the production of ornithine from acetylornithine and glutamate using the ninhydrin reaction as described by Shinners and Catlin (28).

Gonococcal transformation. Transformation of *N. gonorrhoeae* strain 30465 was done as previously described (1). Positive transformation was scored as growth of GC30465 on NEDA plates lacking arginine after 48 hrs.

Minicell analysis. The minicell producing *E. coli* strain DS410, transformed with pUC19 or pRGE clones, was grown to high density in one liter LB broth cultures and minicells were harvested by differential centrifugation followed by sucrose density gradient centrifugation as described by Dougan and Kehoe (8). The minicells were resuspended in 1 ml of NEDA broth lacking methionine to which 50 μCi ^{35}S-methionine (Amersham) was added and labelled at 37°C for 1 hr. Labelled proteins were separated by SDS-PAGE and visualized by autoradiography.

Southern blots. Genomic DNA from *N. gonorrhoeae* CDC50 and 30465, *E. coli* DH1, N134, and AT2538, and *P. aeruginosa* ATCC 27853 was digested to completion with either *Cla*I or *Eco*RI, separated on an 0.8% agarose gel, and transferred to nitrocellulose using the method of Southern (31). Blots were probed with either the entire 3.4 kb insert of pRGE5 or with the 0.6 kb *Hinc*II fragment of pRGEB labelled with digoxigenin using the Genius kit from Boehringer Mannheim Biochemicals. Hybridizations were performed at both low and high stringency. High stringency conditions included hybridization at 60°C in 5X SSC followed by washes in 0.1X SSC plus 0.1% SDS at 60°C. Low stringency included hybridization at 50°C in 5X SSC followed by washes in 2X SSC plus 0.1% SDS at 50°C.

Nucleotide sequence accession number. The nucleotide and amino acid sequences for the *Neisseria gonorrhoeae argJ* gene have been submitted to GenBank and assigned the accession number M65216.

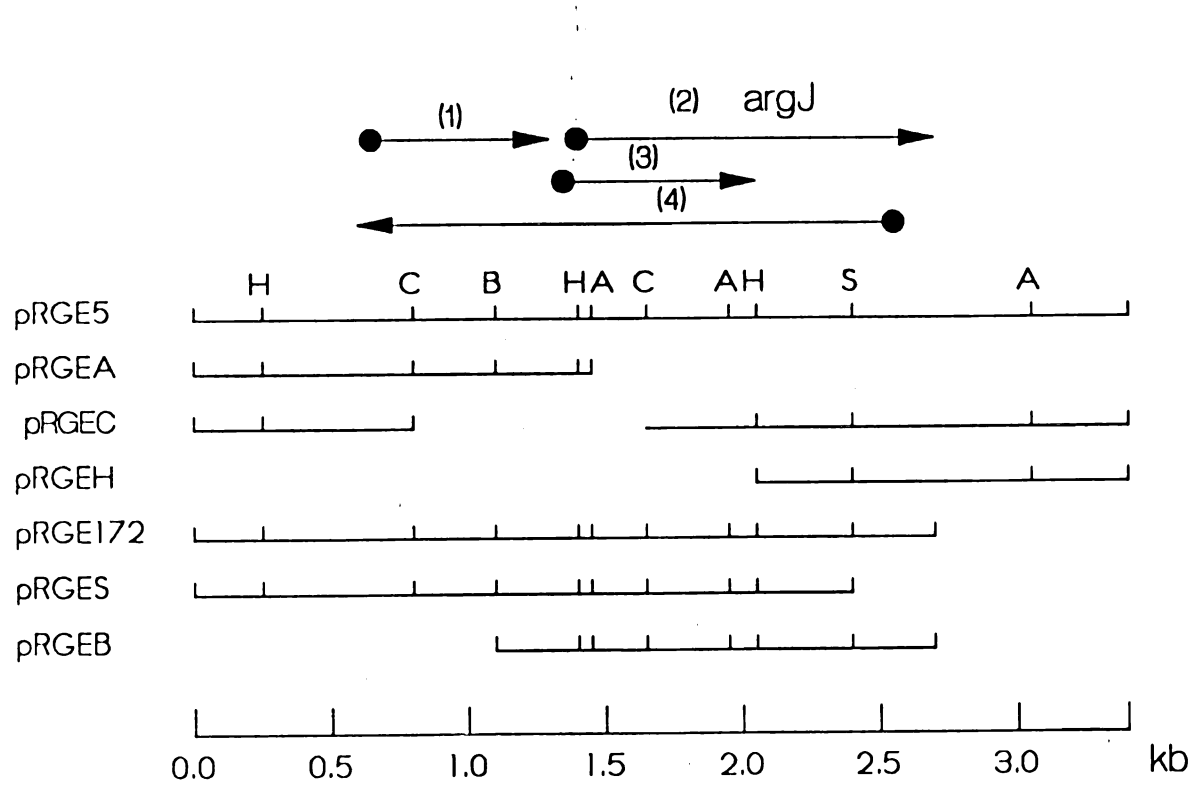
RESULTS

Cloning of the *Neisseria gonorrhoeae argJ* gene. A genomic library of *N. gonorrhoeae* strain CDC50 DNA was constructed in pUC12 and used to transform *argE E. coli* strain AT2538 to arginine prototrophy. A recombinant plasmid, designated pRGE5, was isolated from an Arg+ transformant. Restriction enzyme analysis revealed that pRGE5 contained a 3.4 kb insert of gonococcal DNA (Fig. 2). OATase assays of *E. coli* AT2538 cells containing pRGE5 confirmed that this plasmid encoded active OATase enzyme (Table 1). Deletion mutants were constructed using *Ava*I, *Cla*I, and *Hinc*II restriction sites identified in pRGE5; these were tested for *argE* complementation (Fig. 2) and for production of OATase (Table 1). All three deletion mutants were negative for OATase activity and were unable to complement *E. coli argE*, indicating that at least part of the gonococcal *argJ* gene is encoded by the 0.19 kb *Ava*I-*Cla*I fragment deleted in all three constructs (Fig. 2).

Sequencing and identification of the *argJ* gene. The entire 3.4 kb insert in pRGE5 was subcloned into M13mp19 for DNA sequencing. Nested deletions of the 3.4 kb insert were generated using the 3'-5' exonuclease activity of T4 polymerase provided in the Cyclone deletion kit from IBI. These deletions were sequenced and provided a 3.01 kb stretch of continuous nucleotide sequence which when analyzed revealed four large open reading frames (Fig. 2). It was clear that ORF 1 could not encode ArgJ, since pRGEA could neither complement *argE* nor specify production of active OATase. To determine which of the three remaining ORFs encoded *argJ*, three more deletion mutants were constructed. In

Figure 2. Restriction map and analysis of deletions of the gonococcal *argJ* gene. Restriction sites shown are; A, AvaI; B, BsmI; C, ClaI; H, HincII; S, SacII. Arrows represent open reading frames. ArgE complementation was tested in *E. coli* AT2538; ArgA complementation was tested in *E. coli* W3421. Gonococcal transformation to Arg+ was tested in strain 30465. Asterisk indicates a 10-fold decreased transformation efficiency relative to pRGE5.

Figure 2



COMPLEMENT		TRANSFORM
ArgE	ArgA	AHU to Arg+
+	+	+
−	−	−
−	−	+ *
−	−	−
+	+	+
−	−	+
+	+	+

Table 1. Ornithine acetyltransferase activity

Strain	Enzyme Units[a]
E. coli AT2538	<0.1
E. coli AT2538 + pRGE5	6.54
E. coli AT2538 + pRGEA	<0.1
E. coli AT2538 + pRGEC	<0.1
E. coli AT2538 + pRGEH	<0.1

[a] One unit of enzyme activity produces one nanomole of ornithine from glutamate + N-acetylornithine per minute per mg protein in the enzyme sample (3).

pRGE172 (Fig. 2), the nested deletion closest to the 3' end of ORF 2 was subcloned into pUC19; this plasmid, which contained all four ORFs intact, retained the ability to complement *E. coli* AT2538. pRGE172 was trimmed to the *Bsm*I site to construct pRGEB, and also to the *Sac*II site to construct pRGES. pRGEB contained two complete overlapping ORFs, ORF 2 and ORF 3, as well as a truncated ORF 4, and was able to complement *argE*. The ability to complement *E. coli argE*, which requires an intact *argJ* gene, was lost in pRGES, a deletion that truncates ORF 2 but does not enter ORF 3. These data indicate that *argJ* is encoded by ORF 2.

Minicell analysis. Plasmid pRGE5 and its deletions were transformed into the minicell producing *E. coli* strain DS410 and the protein products of these plasmids were labelled with ^{35}S-methionine, separated by SDS-PAGE, and visualized by autoradiography (Fig. 3). When compared to the pUC19 vector, the plasmids containing the complete ORF 2 coded for the production of a unique peptide with an apparent Mr of 46,000. In pRGES this peptide was truncated to an apparent Mr of 38,000, which is consistant with the conclusion that this peptide is encoded by the *argJ* ORF, ORF 2. A smaller peptide of 23,000 Mr was also produced from plasmids pRGE5, pRGEB, and pRGES. Although this peptide was produced only by plasmids containing the *argJ* ORF, it is unlikely to represent the intact ArgJ peptide since it appeared unaltered in pRGES, a plasmid in which ArgJ activity was lost. We were unable to identify peptides that correlated with either ORF 1 or ORF 4.

Nucleotide sequence analysis. Second strand sequencing was performed to confirm the sequence of the *argJ* gene and its flanking regions. The nucleotide sequence and corresponding amino acid sequence of *argJ* are shown in Figure 4. The gonococcal *argJ* gene consists of an

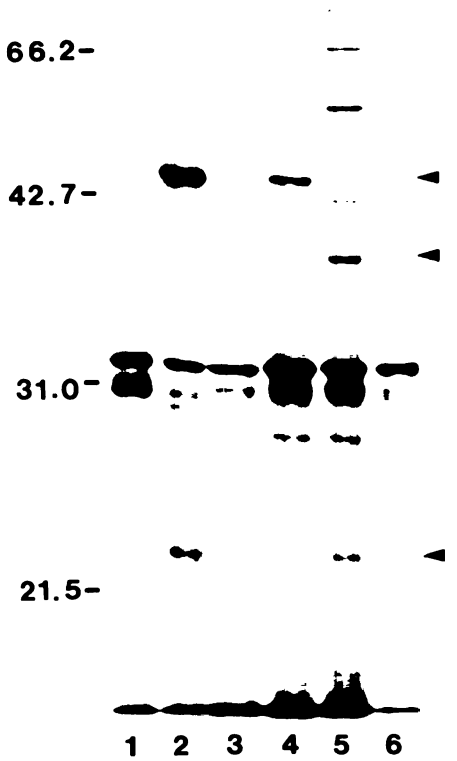
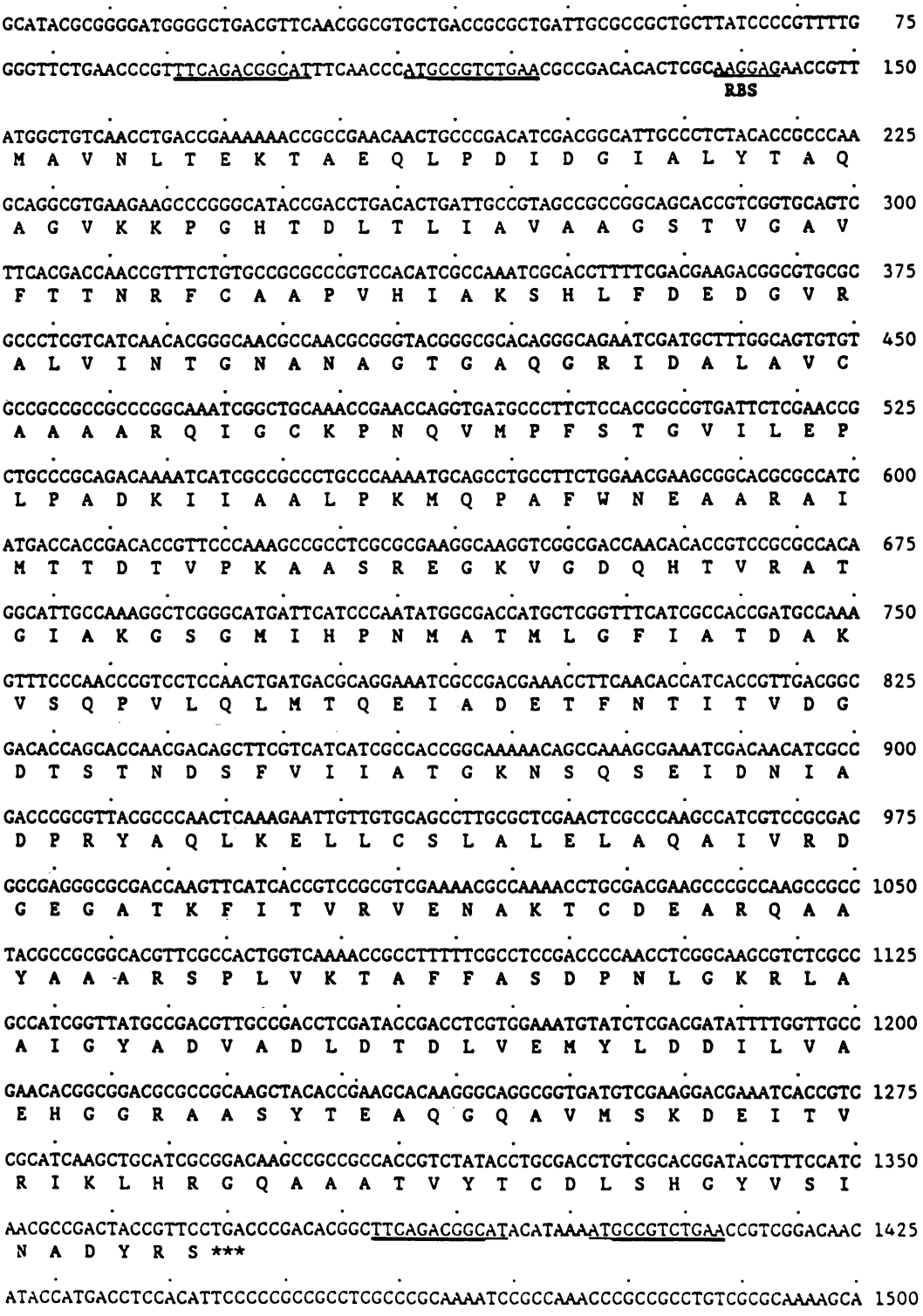


Figure 3. Minicell analysis of pRGE5 and deletions. Minicells contained; lane 1, pUC19; lane 2, pRGE5; lane 3, pRGEA; lane 4, pRGEB; lane 5, pRGES; lane 6, pRGEH. Molecular weight standards are indicated on the left. Arrows on the right indicate peptides produced by recombinant plasmids; 46 kDa ArgJ peptide, 38 kDa truncated ArgJ peptide, and 23 kDa peptide.

Figure 4. Nucleotide sequence of the gonococcal *argJ* gene and flanking regions. The predicted amino acid sequence is shown below the coding region. Inverted repeats are underlined as is the putative ribosome-binding site (RBS). Neisserial DNA uptake sequences are double-underlined.

Figure 4


GCATACGCGGGGATGGGGCTGACGTTCAACGGCGTGCTGACCGCGCTGATTGCGCCGCTGCTTATCCCCGTTTTG 75
GGGTTCTGAACCCGTTTCAGACGGCATTTCAACCCATGCCGTCTGAACGCCGACACACTGGCAAGGAGAACCGTT 150
RBS
ATGGCTGTCAACCTGACCGAAAAAACCGCCGAACAACTGCCCGACATCGACGGCATTGCCCTCTACACCGCCCAA 225
M A V N L T E K T A E Q L P D I D G I A L Y T A Q
GCAGGCGTGAAGAAGCCCGGGCATACCGACCTGACACTGATTGCCGTAGCCGCCGGCAGCACCGTCGGTGCAGTC 300
A G V K K P G H T D L T L I A V A A G S T V G A V
TTCACGACCAACCGTTTCTGTGCCGCGCCCGTCCACATCGCCAAATCGCACCTTTTCGACGAAGACGGCGTGCGC 375
F T T N R F C A A P V H I A K S H L F D E D G V R
GCCCTCGTCATCAACACGGGCAACGCCAACGCGGGTACGGGCGCACAGGGCAGAATCGATGCTTTGGCAGTGTGT 450
A L V I N T G N A N A G T G A Q G R I D A L A V C
GCCGCCGCCGCCCGGCAAATCGGCTGCAAACCGAACCAGGTGATGCCCTTCTCCACCGCCGTGATTCTCGAACCG 525
A A A A R Q I G C K P N Q V M P F S T G V I L E P
CTGCCCGCAGACAAAATCATCGCCGCCCTGCCCAAAATGCAGCCTGCCTTCTGGAACGAAGCGGCACGCGCCATC 600
L P A D K I I A A L P K M Q P A F W N E A A R A I
ATGACCACCGACACCGTTCCCAAAGCCGCCTCGCGCGAAGGCAAGGTCGGCGACCAACACACCGTCCGCGCCACA 675
M T T D T V P K A A S R E G K V G D Q H T V R A T
GGCATTGCCAAAGGCTCGGGCATGATTCATCCCAATATGGGCGACCATGCTCGGTTTCATCGCCACCGATGCCAAA 750
G I A K G S G M I H P N M A T M L G F I A T D A K
GTTTCCCAACCCGTCCTCCAACTGATGACGCAGGAAATCGCCGACGAAACCTTCAACACCATCACCGTTGACGGC 825
V S Q P V L Q L M T Q E I A D E T F N T I T V D G
GACACCAGCACCAACGACAGCTTCGTCATCATCGCCACCGGCAAAAACAGCCAAAGCGAAATCGACAACATCGCC 900
D T S T N D S F V I I A T G K N S Q S E I D N I A
GACCCGCGTTACGCCCAACTCAAAGAATTGTTGTGCAGCCTTGCGCTCGAACTCGCCCAAGCCATCGTCCGCGAC 975
D P R Y A Q L K E L L C S L A L E L A Q A I V R D
GGCGAGGGCGCGACCAAGTTCATCACCGTCCGCGTCGAAAACGCCAAAACCTGCGACGAAGCCCGCCAAGCCGCC 1050
G E G A T K F I T V R V E N A K T C D E A R Q A A
TACGCCGCGGCACGTTCGCCACTGGTCAAAACCGCCTTTTTCGCCTCCGACCCCAACGCTCGGCAAGCGTCTCGCC 1125
Y A A A R S P L V K T A F F A S D P N L G K R L A
GCCATCGGTTATGCCGACGTTGCCGACCTCGATACCGACCTCGTGGAAATGTATCTCGACGATATTTTGGTTGCC 1200
A I G Y A D V A D L D T D L V E M Y L D D I L V A
GAACACGGCGGACGCGCCGCAAGCTACACCGAAGCACAAGGGCAGGCGGTGATGTCGAAGGACGAAATCACCGTC 1275
E H G G R A A S Y T E A Q G Q A V M S K D E I T V
CGCATCAAGCTGCATCGCGGACAAGCCGCCGCCACCGTCTATACCTGCGACCTGTCGCACGGATACGTTTCCATC 1350
R I K L H R G Q A A A T V Y T C D L S H G Y V S I
AACGCCGACTACCGTTCCTGACCCGACACGGCTTCAGACGGCATACATAAAATGCCGTCTGAACCGTCGGACAAC 1425
N A D Y R S ***
ATACCATGACCTCCACATTCCCCCGCCGCCTCGCCCGCAAAATCCGCCAAACCCGCCGGCCTGTCGCGCAAAAGCA 1500

ORF of 1,218 bp encoding a peptide with a predicted mol wt of 42,879. This is in excellent agreement with the Mr of 46,000 for the ArgJ enzyme determined by minicell analysis. The *argJ* ORF is preceeded by a potential ribosome binding site containing a 6 bp sequence, AAGGAG, complementary to the 3' end of the gonococcal 16S rRNA (25). No consensus promoter sequence of either the -35/-10 (12) or -24/-12 (33) type was found. It is possible that this gene could be cotranscribed from a promoter upstream of ORF 1, although the fact that pRGEB, which has the upstream portion of ORF 1 deleted, can complement *E. coli argE* argues against this assumption. We also found no evidence of an "*arg* box" operator sequence, the binding site for the arginine repressor that has been described in *E. coli* (5) and *Bacillus* (29), upstream of the *argJ* open reading frame.

Codon usage for the gonococcal *argJ* gene is listed in Table 2. We compared the codon usage pattern for this gene with reference codon usage data for *N. gonorrhoeae* (37), *Pseudomonas aeruginosa* (38), and both highly and weakly expressed *E. coli* genes (10,11). Codon usage in *argJ* most closely resembled that seen in *P. aeruginosa*, with 19 of 21 rarely used codons and 10 of 14 most commonly used codons found in *argJ* matching the codon usage reference table for *P. aeruginosa*. There was a distinct bias in the choice of nucleotide in the wobble (third) position in degenerate codons, with 58.9% containing cytosine and 76.1% containing either cytosine or guanine in the this position. This is similar to the data for *P. aeruginosa*, where the 52.6% cytosine and 85.6% cytosine or guanine in the third position reflects an overall guanine-plus-cytosine ratio of 67%. In contrast, the *N. gonorrhoeae* reference table indicates 34.3% of codons contain cytosine and 52.1%

Table 2. Codon usage of the *Neisseria gonorrhoeae argJ* gene

Codon	Amino Acid	Used in argJ	Codon	Amino Acid	Used in argJ	Codon	Amino Acid	Used in argJ	Codon	Amino Acid	Used in argJ
TTT	Phe	1	TCT	Ser	0	TAT	Tyr	3	TGT	Cys	2
TTC	Phe	10	TCC	Ser	5	TAC	Tyr	6	TGC	Cys	4
TTA	Leu	0	TCA	Ser	0	TAA	***	0	TGA	***	1
TTG	Leu	4	TCG	Ser	6	TAG	***	0	TGG	Trp	1
CTT	Leu	2	CCT	Pro	1	CAT	His	2	CGT	Arg	4
CTC	Leu	13	CCC	Pro	10	CAC	His	5	CGC	Arg	10
CTA	Leu	0	CCA	Pro	1	CAA	Gln	12	CGA	Arg	0
CTG	Leu	11	CCG	Pro	3	CAG	Gln.	5	CGG	Arg	1
ATT	Ile	6	ACT	Thr	0	AAT	Asn	1	AGT	Ser	0
ATC	Ile	21	ACC	Thr	28	AAC	Asn	14	AGC	Ser	7
ATA	Ile	0	ACA	Thr	2	AAA	Lys	12	AGA	Arg	1
ATG	Met	10	ACG	Thr	4	AAG	Lys	6	AGG	Arg	1
GTT	Val	6	GCT	Ala	2	GAT	Asp	4	GGT	Gly	4
GTC	Val	15	GCC	Ala	47	GAC	Asp	24	GGC	Gly	20
GTA	Val	1	GCA	Ala	9	GAA	Glu	17	GGA	Gly	3
GTG	Val	7	GCG	Ala	8	GAG	Glu	1	GGG	Gly	2

contain cytosine or guanine in the third position, which would be expected of an organism with a 49.5% guanine-plus-cytosine ratio.

The 10 bp neisserial DNA uptake sequence, 5'-GCCGTCTGAA-3', (9) occurs four times in this sequence, but only as part of a larger 12 bp sequence, 5'-ATGCCGTCTGAA-3', in closely spaced inverted repeats in the flanking regions of the gene and not in the coding region. The downstream inverted repeats could form a stable stem and loop structure with a ΔG of -23.4 kcal/mol that may act as a transcriptional terminator. The upstream inverted repeats could also form a stable stem and loop structure with a ΔG of -22.4 kcal/mol. This structure follows the unidentified ORF 1 upstream of the *argJ* ORF, and overlaps the region where a promoter for *argJ* would be expected to be located.

We reviewed 18 different *N. gonorrhoeae* DNA sequences available in GenBank release 68, June/1991. Of these, 12 contained at least one copy of the gonococcal uptake sequence, either singly or in inverted repeats. When we aligned these uptake sequences and their flanking sequences, we found that 16 of 22 copies (12 inverted repeats and 10 single copies) of the 10 bp uptake sequence also contained the 5'-AT extension. This occurred more frequently in the inverted repeats than in single copies, suggesting that this extension may contribute to the proposed terminator function of these repeats. We also found this 12 bp sequence in three *N. meningitidis* genes, both in single copies within the genes and in inverted repeats at the end of the open reading frames.

Analysis of the predicted ArgJ protein. The *argJ* open reading frame encodes a protein with a predicted molecular weight of 42,879 and an isoelectric point of 5.18. A hydrophobicity plot, using the algorithm of Kyte and Doolittle (16), indicated no pronounced hydrophobic regions

in the *argJ* gene product, suggesting that this protein is soluble rather than membrane-bound. We could find no recognizable signal peptide sequence at the N-terminus (36), suggesting that this protein is not secreted. We therefore conclude that ArgJ is a soluble cytoplasmic protein, as would be expected for an enzyme involved in amino acid biosynthesis.

Southern blots. Genomic DNA from *E. coli* and *P. aeruginosa* were tested for homology to the cloned gonococcal *argJ* gene by Southern blot hybridization (Fig. 5). Under high stringency conditions, neither *E. coli* nor *P. aeruginosa* DNA hybridized with either the 0.6 kb (Fig. 5c) or the 3.4 kb (data not shown) gonococcal *argJ* probes. Under low stringency conditions, specifically hybridizing bands were apparent above background in *N. gonorrhoeae* and *P. aeruginosa* lanes, but not *E. coli* lanes, with both the 0.6 kb and 3.4 kb *argJ* probes (Fig. 5b, 5a).

Homology to other sequenced genes. Comparison of the *argJ* nucleotide and predicted amino acid sequences with the GenEMBL database, using the FASTA and TFASTA programs of the University of Wisconsin Genetics Computer Group software (7), yielded no listed DNA or protein sequences with significant homology to the gonococcal *argJ* gene.

We carefully compared the gonococcal *argJ* gene and its product to *E. coli argA* (2), *Neisseria gonorrhoeae argF* (17), and to a gene from *Leptospira biflexa* which complements *argE* in *E. coli* (40), using a variety of nucleotide and amino acid sequence analysis and protein structure analysis programs, and found no significant sequence or structural similarity between *argJ* and any of these genes. We were also able to compare our sequence with the proposed sequence of the

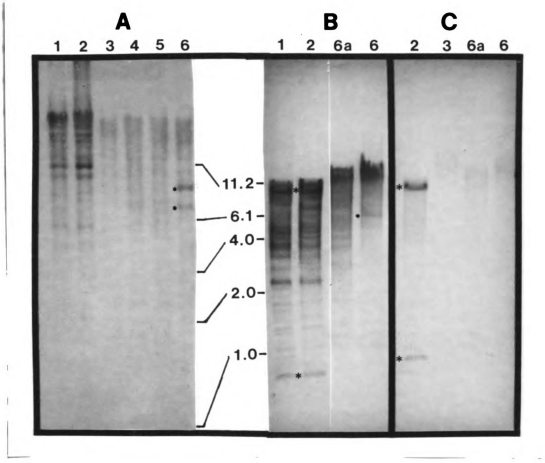


Figure 5. Southern blot of gonococcal, *E. coli*, and *P. aeruginosa* DNA probed with the gonococcal *argJ* gene. DNA size markers are indicated in kilobases. Genomic DNA used were from; Lane 1 - *N. gonorrhoeae* 30465, Lane 2 - *N. gonorrhoeae* CDC50, Lane 3 - *E. coli* DHI, Lane 4 - *E. coli* AT2538, Lane 5 - *E. coli* N134, Lane 6 - *P. aeruginosa* ATCC 27853 digested with *Eco*RI, Lane 6a - *P. aeruginosa* ATCC 27853 digested with *Cla*I. Panel A: *Eco*RI digested DNA probed with the 3.4 kb insert from pRGE5 under low stringency (see methods). Panels B & C: *Cla*I digested genomic DNA (except lanes 6) probed with the 0.6 kb *Hinc*II fragment from pRGEB. Panel B: low stringency conditions, Panel C: high stringency conditions. Solid circles mark *P. aeruginosa* bands that hybridize to the gonococcal probes under low stringency. Asterisks mark *N. gonorrhoeae* bands that hybridize under high stringency.

putative *argJ* gene from *Bacillus subtilis* (22). Significant identity was found between the predicted amino acid sequences of these two genes, which included a region with 23 of 26 identical amino acid residues (88.5% homology). We also used this conserved 26 amino acid sequence, and a larger 84 amino acid conserved sequence containing this region, to search the GenBank and EMBL databases, but found no other published sequences with significant homology to this sequence.

***E. coli* complementation and *N. gonorrhoeae* transformation.** The original pRGE5 clone and subsequent deletions were tested for their ability to complement *argE* and *argA* mutations in *E. coli*, and for the ability to transform the *argJ N. gonorrhoeae* strain 30465 to Arg+ (Fig. 2). These experiments demonstrated that the *argE* and *argA* complementing activities were inseparable, and dependent on the presence of an intact *argJ* ORF. In contrast, transformation of GC 30465 to Arg+ was not dependent on an intact *argJ* ORF. Both plasmid pRGES, which lacks the *argJ* gene downstream of the *Sac*II site, and plasmid pRGEC, which lacks the 5′ end of the *argJ* gene, were able to transform GC 30465 to Arg+, while plasmids pRGEA and pRGEH could not. These data imply that the lesion in the *argJ* gene of GC 30465 lies at least partially within the 0.39 kb *Cla*I - *Hinc*II fragment in the center of the *argJ* ORF and entirely within the 0.63 kb *Cla*I - *Sac*II fragment (Fig. 2).

DISCUSSION

In this paper we report the cloning and complete nucleotide sequence of the *Neisseria gonorrhoeae argJ* gene, which encodes ornithine acetyltransferase, the enzyme that catalyzes the conversion of acetylornithine plus glutamate to ornithine plus acetylglutamate at the fifth step of the arginine biosynthesis pathway. The *argJ* clone was identified by its ability to complement an *E. coli argE* mutant, and its identity was confirmed by demonstrating that this clone encoded OATase activity and that it could transform a gonococcal *argJ* mutant to Arg+.

We sequenced most of the 3.4 kb insert in the original *argJ* clone, and found four potential open reading frames in this sequence. The ORF encoding *argJ* was identified by construction of deletion mutants and analysis of their ability to produce active OATase and to complement *E. coli argE* mutants. The gonococcal *argJ* gene contained an open reading frame of 1,218 bp which encoded a peptide with a predicted molecular weight of 42,879. There is a good ribosomal binding site in the appropriate position upstream from the putative start codon. However, we were unable to identify a sequence homologous to either the -35/-10 or -24/-12 *E. coli* consensus promoters. This was also the case when we studied the gonococcal *argF* gene (17). While *E. coli*-type promoters have been found associated with some neisserial genes, such as the *opaE1* and IgA1 protease genes (32,24), little is known about promoter structure in *Neisseria* spp., particularly for housekeeping genes such as those involved in arginine biosynthesis.

In both *E. coli* and *Bacillus subtilis*, many of the genes involved in

arginine biosynthesis are repressed by arginine (6, 30). In *E. coli*, this repression occurs when the arginine repressor protein binds to the "arg box" operator sequence overlapping the promoter of arginine repressible genes (5). One or two copies of the arg box consensus sequence have been found upstream of several *E. coli* arginine biosythetic genes (5), as well as in the *B. subtilis argC* promoter (29). We found no evidence of an arg box sequence upstream of the gonococcal *argJ* gene. Previous studies in Catlin's laboratory (28), as well as preliminary studies in this lab have found no evidence of repression of the gonococcal *argJ* or *argF* genes by arginine.

The *N. gonorrhoeae argJ* ORF is closely flanked by inverted repeats of the neisserial DNA uptake sequence. In their study that elucidated the uptake sequence (9), Goodman and Scocca found that this sequence was often present within inverted repeats at the end of undefined ORFs and proposed that the uptake sequence may also be involved in transcription termination. The presence of the uptake sequence in inverted repeats at the end of the *argJ* ORF supports this hypothesis. This inverted repeat was also found upstream of the *argJ* ORF. Whether this sequence is involved in termination of a gene upstream of *argJ*, or in regulation of *argJ* expression, has not yet been determined. An interesting observation of the uptake sequences in the *argJ* gene is that they occur only within the longer 12 bp sequence, 5'-ATGCCGTCTGAA-3'. This 12 bp extended sequence is also present in 16 of the 22 occurrences of the uptake sequence that we analyzed in 12 different gonococcal genes, as well as in 3 meningococcal genes, and is more frequently observed in copies of the uptake sequence found as inverted repeats at the end of open reading frames than in single copies of the sequence. These data

suggest that the extended uptake sequence may contribute to a more stable stem-and-loop terminator structure.

This is the first ornithine acetyltransferase sequence to be reported from any source. The sequence of a gene complementing an *E. coli argE* mutation from the spirochete *Leptospira biflexa* has been previously reported, but the nature of the enzyme activity encoded by that gene, was not defined (40). We compared this sequence with the gonococcal *argJ* gene and found no significant similarity at either the DNA or the predicted amino acid sequence level, suggesting that these two genes are unrelated. We were also able to compare our sequence to the putative *argJ* gene from *Bacillus subtilis* (22). Significant identity was found between the predicted amino acid sequences of these two genes. This comparison revealed that these genes appear to be distantly related with several regions being highly conserved.

Picard and Dillon (23) found that their gonococcal *argJ* lambda clone could complement both *E. coli argE* and *argA* mutations. They proposed that this may be the result of the gonococcal ArgJ enzyme producing the required acetylglutamate for the pathway, but due to the size of their clone, they could not rule out the possibility that the gonococcal *argA* gene might also be present. Our findings strongly support the former theory. We have shown that *argA* complementation is dependent on having an intact *argJ* gene, and that no extra DNA outside the *argJ* gene is required for *argA* complementation. In addition, we have isolated a clone which complements *argA* in *E. coli* without complementing *argE*. This clone likely contains the gonococcal *argA* gene and has no homology to pRGE5 when compared by restriction mapping (20).

Our cloned *argJ* gene was capable of transforming the gonococcal AHU

strain 30465 to Arg+. These transformants remained Ura-, Hyx- as expected since previous cotransformation studies have shown that these markers are not closely linked (1,18). These previous studies also reported that naturally occuring Arg+ revertants of AHU strains do arise, but at a very low frequency. The occurrence of revertants makes it unlikely that the *argJ* gene has been deleted in AHU strains. In this study it was found that *N. gonorrhoeae* 30465 contains an apparently intact *argJ* gene as seen on Southern blot. The ability of deletions of the *argJ* gene to transform 30465 to Arg+ revealed that the *argJ* mutation in this strain is apparently located between the internal *Cla*I and *Sac*II sites. Experiments are in progress to determine the nature of the *argJ* mutation in AHU strains of *N. gonorrhoeae*. These experiments will lay the groundwork for future studies on arginine biosynthesis and on the regulation of housekeeping genes in *Neisseria gonorrhoeae*.

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CHAPTER III

(ARTICLE)

Molecular characterization of the *argJ* mutation in AHU strains of *Neisseria gonorrhoeae*

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ABSTRACT

Arginine auxotrophs are commonly encountered among clinical isolates of *Neisseria gonorrhoeae*. Arginine auxotrophs which also require hypoxanthine and uracil (AHU strains) comprise a unique set of strains that are highly homogeneous and are believed to be clonally derived. The Arg- phenotype of these strains is due to a lesion in the *argJ* gene encoding ornithine acetyl-transferase. We have cloned the mutant *argJ* gene from an AHU strain, and compared the sequence of this gene to the wild type *argJ* gene. The mutant gene contained a 3 bp deletion within a repetitive region of the *argJ* gene. This mutation was restored to the wild type sequence in a naturally occuring Arg+ revertant of the AHU strain. This deletion was detected in a wide variety of other AHU strains, but not in other ArgJ- strains or in ArgJ+ strains, supporting the hypothesis that AHU strains are clonally derived.

INTRODUCTION

Auxotyping, the classification of strains by nutritional requirements, has been used to identify isolates of *Neisseria gonorrhoeae* since the development of defined media for *Neisseria* by Catlin (5). The auxotype of a gonococcal strain has proven to be a stable, easily identifiable marker, and auxotyping has been used effectively to follow strains throughout a population (4,11,14).

An early epidemiological study comparing auxotype to pattern of gonococcal infection by Knapp *et al.* (11) found that strains with requirements for arginine, hypoxanthine, and uracil (AHU strains) were predominant among isolates from disseminated gonococcal infections (DGI) in Seattle. This observation prompted further studies of AHU strains which led to the discovery that AHU strains are quite homogeneous and distinctly different from other gonococcal strains. Besides sharing auxotypic markers, AHU strains predominantly belong to two closely related Protein I serovars (13), are resistant to the bactericidal action of normal human serum (25,14) and show increased sensitivity to penicillin (11,14,20,25). They produce type I IgA1 protease and Dam methylase, both of which are uncommon among gonococci of other auxotypes (21,16). The correlation of AHU strains with DGI has been shown to be an artifact of their homogeneity, with PI serotype, serum resistance and penicillin sensitivity showing a higher correlation with DGI than auxotype (3,10,2).

Genetic analysis of AHU strains revealed that the genes involved in

the AHU auxotype, serum resistance, and penicillin sensitivity are independent and not closely linked (6,19,1). These studies also found that the Arg- lesions in AHU strains are identical or overlapping, in that DNA from one AHU strain could not transform any other to Arg+ (19), and that this lesion is different from that of Arg- strains not of the AHU auxotype. These observations led to the hypothesis that AHU strains are clonally derived.

Biochemical analysis of AHU strains revealed that the Arg- phenotype of these strains was at least partially due to the inability to convert N-acetylornithine to ornithine (7). This reaction is catalyzed by the enzyme ornithine acetyltransferase, encoded by the gene *argJ*, which converts acetylornithine and glutamate to ornithine and acetylglutamate. The *N. gonorrhoeae argJ* gene has been cloned by complementation of an *E. coli argE* mutant (23,18) and has been sequenced (18). The gonococcal *argJ* gene contains an open reading frame of 1,218 bp and encodes a protein of 42,879 M_r. The cloned gene was capable of transforming an AHU strain to Arg+. The gonococcal *argJ* gene was also able to complement an *E. coli argA* mutant (18,23), which presumably occurs by feedback of the acetylglutamate produced by the ArgJ enzyme back into the arginine biosynthetic pathway.

In this paper we report the cloning of the *argJ* gene from an AHU strain, and identify a mutation in this gene that correlates to the Arg- phenotype. We also show that this mutation is common to other AHU strains, but not Arg+ strains or ArgJ- strains that are not Hyx-, Ura-.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* AT2538 (*argE3, thi-1, thr-1, ara-14, leuB6, Δ(gpt-proA)62, lacY1, supE44, galK2, λ-,hisG4, rpsL31, xyl-5, mtl-1, pyrE60*), and W3421 (*argA21, galT23, λ-, IN(rmD-rrnE)*1) were used for complementation studies, *E. coli* GM2163 (McrA-, McrB-, *ara-14, leuB6, TonA13, LacY1, tsx-78, supE44, galK2, λ-, dcm-6, hisG4, rpsL136, dam-13:*Tn*9, xyl-5, mtl-1, thi-1, hsdR2*) was used as the initial recipient in the cloning of the mutant gonococcal *argJ* gene. *N. gonorrhoeae* strains used in this study are listed in Table 1. All *E. coli* strains were grown on LB medium; when needed ampicillin was added to 50 ug/ml for plasmid selection. *N. gonorrhoeae* strains were grown on GC base (Difco) plus 1% Kellogg's supplement (29) at 36°C under 5% CO_2. NEDA defined medium for gonococci (4) with 1.5% agarose (Baker) was used for growth of all bacteria in auxotyping or complementation experiments.

DNA manipulations. Restriction enzymes, DNA ligase, and alkaline phosphatase were purchased from Boehringer Mannheim Biochemicals (BMB) or Bethesda Research Laboratories and used according to manufacturers recomendations. Genomic and plasmid DNA preparations, gel electrophoresis, and *E. coli* transformations were all done by conventional methods (17). DNA probes were labelled with either digoxygenin using the Genius kit from BMB, or with ^{32}P-dCTP using the random priming kit from BMB. Southern blots were done by the method of Southern (27).

Table 1. Gonococcal strains and phenotypes.

	Strain	Auxotype[a]	Serovar	Source[b]
1.	CDC50	Pro	IB1	Fla.
2.	NRL30465	AHU (ArgO)	IA1	Japan
3.	NRL905	AHU (ArgO)	IA1	*
4.	CDC22	AHU (ArgO)	IA1	Fla.
5.	CDC6	AHU (ArgC)	IA1	Iowa
6.	CDC54	AHU Ser (ArgO)	IB2	Iowa
7.	CDC208	AHU (ArgO)	IB2	Ohio
8.	CDC1	ArgO	IB24	Fla.
9.	CDC70	ArgO	IB1	N.J.
10.	CDC87	ArgO	IB2	Iowa
11.	CDC34	ArgA Pro Leu Met	IB1	Iowa
12.	CDC9	Pro	IA3	Mass.
13.	CDC99	Pro	IA1	Virginia
14.	MS-11	WT	IB9	*
15.	NRL9396	AHU	IA1	Copen.

[a] Auxotype: WT = prototrophic; Arg = requires arginine; ArgC = arginine requirement can be satisfied by citrulline; ArgO = arginine requirement can be satisfied by citrulline or ornithine; ArgA = arginine requirement can be satisfied by citrulline, ornithine or acetylglutamate; AHU = requires arginine, hypoxanthine and uracil; Pro = requires proline; Ser = requires serine; Leu = requires leucine, Met = requires methionine.

[b] Source: Copen. = Copenhagen, Denmark; others include Florida, Iowa, Massachusetts, New Jersey, Ohio, and Virginia states. Asterisk indicates source location not available.

Cloning of the mutant *argJ* gene. Genomic DNA from *N. gonorrhoeae* strain NRL30465 was completely digested with *Sau*3A and fragments from 3.2 to 3.6 kb were band purified from a 0.8% agarose gel. These were ligated into the *Bam*HI site of pUC19 and transformed into *E. coli* GM2163. Transformants were screened for plasmids containing the *argJ* gene by probing colony blots with the 0.8 kb *Cla*I fragment of the wild type gonococcal *argJ* gene using methods described by Maniatis *et al* (17).

Enzyme assays. Ornithine acetyltransferase assays were done on cell-free extracts using the ninhydrin reaction to measure the production of ornithine from acetylornithine and glutamate as described by Shinners and Catlin (26).

Construction of chimeric *argJ* clones. Chimeras of the wild type and mutant *argJ* genes were constructed by exchanging restriction fragments between the wild type clone (pRGE5) and the mutant clone (pREM1). Restriction endonuclease *Cla*I was used to release a fragment containing the 5′ end of the *argJ* gene, and *Sac*II and *Hin*dIII were used to release a fragment containing 3′ end of the gene. These fragments were separated from the remainder of the plasmid in an 0.8% agarose gel, and the fragments and remaining plasmids were band purified. Chimeras were constructed by ligating the fragments back into the plasmid from which they did not originate. Chimeric clones were tested for proper insertion and ArgE complementing activity.

DNA sequencing. The mutant *argJ* gene was subcloned into M13mp18 and M13mp19 and sequenced on both strands in the region shown to contain the *argJ* mutation. Subclones were constructed from identified restriction sites. Sequencing was done using the Sequenase enzyme from U.S. Biochemicals, by the method of Sanger *et al. (24)*.

Isolation of Arg+ revertant. *N. gonorrhoeae* strain NRL30465 was grown 16-18 hr on GC base plates and resuspended to $>10^9$ cfu/ml in NEDA broth. 100 μl of this suspension was spread onto multiple NEDA plates lacking arginine and incubated at 36°C. Revertant colonies were isolated after 48 hr and streaked for isolation on GC base. Revertants were auxotyped prior to further use.

Detection of 3 bp deletion. Gonococcal genomic DNA was digested to completion with *Hin*fI and the reaction was stopped by adding 0.8 volumes of Sequenase stop buffer (U.S. Biochemicals). The digested DNA was denatured at 80°C for 3 minutes and loaded onto an 8% polyacrylamide sequencing gel. ^{35}S-labelled sequencing reactions were used as size markers. The gel was run at 60 W for 2.5 hr, disassembled, and lifted onto 3MM paper. A 7.5 x 15 cm piece of Nytran (Schleicher and Schuell), presoaked in transfer buffer (25 mM sodium phosphate, pH 6.0), was placed over the region of the gel to be transferred. This area was cut away from the remainder of the gel, sandwiched between transfer buffer soaked 3MM paper, and placed into a western blotting chamber (Hoefer Scientific) filled with transfer buffer. The DNA was transferred to the nytran membrane at 40 V (1.5 amp) for 1 hr. The membrane was allowed to air dry and then baked under vacuum at 80°C for 2 hr. This blot was prehybridized and probed with the 0.6 kb *Hinc*II fragment of the wild type *argJ* gene by conventional means (17). Hybridization and washings were done at a reduced stringency (56°C) to increase sensitivity.

RESULTS

Initial characterization of the *argJ* gene in AHU strains. Genomic DNA from 6 different AHU strains of *N. gonorrhoeae* were checked for the presence of the *argJ* gene by comparing Southern blots of these strains to the Arg+ strain CDC50 (Fig. 1). In blots probed with the internal 0.6kb *Hin*cII fragment of pRGE5, the AHU strains possessed a 3.4 kb band apparently identical to the wild type when digested with *Sau*3A. Digests with other enzymes (*Cla*I, *Ava*I) also revealed no differences in restriction pattern in the *argJ* gene between AHU strains and CDC50 (data not shown).

Cloning of the *argJ* gene from an AHU strain. We cloned the mutant *argJ* gene from the AHU *N. gonorrhoeae* strain NRL30465 by digesting genomic DNA from this strain with *Sau*3A, band purifying fragments of approximately 3.4 kb, and cloning these fragments into the *Bam*HI site of pUC19. Recombinant plasmids containing the *argJ* gene were identified by probing colony blots with the 0.8 kb *Cla*I fragment from the wild type *argJ* gene previously cloned in this laboratory. A recombinant plasmid with a 3.4 kb insert was isolated in this way and designated pREM1. The mutant *argJ* clone appeared identical to the wild type clone pRGE5 by restriction mapping, but did not show ArgJ activity either by enzyme assay or by ability to complement *E. coli* AT2538. The mutant *argJ* clone was also unable to complement the *argA E. coli* strain W3421, while the wild type clone could complement this strain.

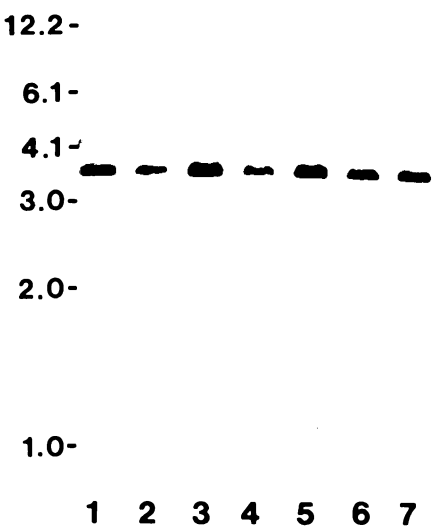


Figure 1. Southern blot of wild type and AHU strains of *N. gonorrhoeae* probed with the *argJ* gene. Lane 1, CDC50; Lane 2, NRL30465; Lane 3, NRL9396; Lane 4, CDC6; Lane 5, CDC22; Lane 6, CDC54; Lane 7, CDC208.

Analysis of chimeric *argJ* genes. Chimeras of the wild type and mutant gonococcal *argJ* genes were constructed and tested for the ability to complement the *argE E. coli* strain AT2538 (Fig. 2). When the 5'-end of the mutant *argJ* gene upstream of the *Cla*I site (pRGM15) or the 3'-end of the mutant gene downstream of the *Sac*II site (pRGSM5) was substituted into the wild type clone, *argE* complementing activity was maintained. These results suggest that these regions of the mutant gene do not contain mutations that impair ArgJ activity, and that the *argJ* lesion is located between the internal *Cla*I and *Sac*II sites.

Identification of the *argJ* mutation. The mutant *argJ* gene was subcloned into M13mp18 and M13mp19 and the region between the *Cla*I and *Sac*II sites was sequenced (Fig. 3). When compared to the wild type sequence, only one difference was found: a 3 bp deletion just downstream of the *Cla*I site. This deletion occurred within a region in which the sequence -GCC- is repeated four times in the wild type gene. This deletion would leave the remainder of the mutant gene in frame with respect to the wild type, and would result in the loss of one alanine from the predicted amino acid sequence.

In order to confirm the importance of this deletion to ArgJ activity, a spontaneous Arg+ revertant of the AHU gonococcal strain NRL30465 was isolated by plating approximately 10^9 cfu of *N. gonorrhoeae* NRL30465 onto NEDA plates lacking arginine, and checking for the appearance of colonies after 48 hr. A single Arg+ colony was recovered. This isolate remained Hyx- and Ura-. Genomic DNA was extracted from this revertant, and the *argJ* gene was cloned into pUC19 by the same methods used to clone the mutant *argJ* gene. The revertant clone allowed growth of *E. coli* AT2538 on Arg- NEDA equally as well as the wild type *argJ* clone.

Figure 2. Chimeras of wild type and mutant *argJ* genes. Ability to complement the *argE E. coli* strain AT2538 is indicated. Narrow lines represent DNA that originated from the wild type *argJ* clone; boxed lines represent DNA from the mutant *argJ* clone. Restriction sites indicated are; A, *Ava*I; C, *Cla*I; H, *Hin*cII; S, *Sac*II.

Figure 2

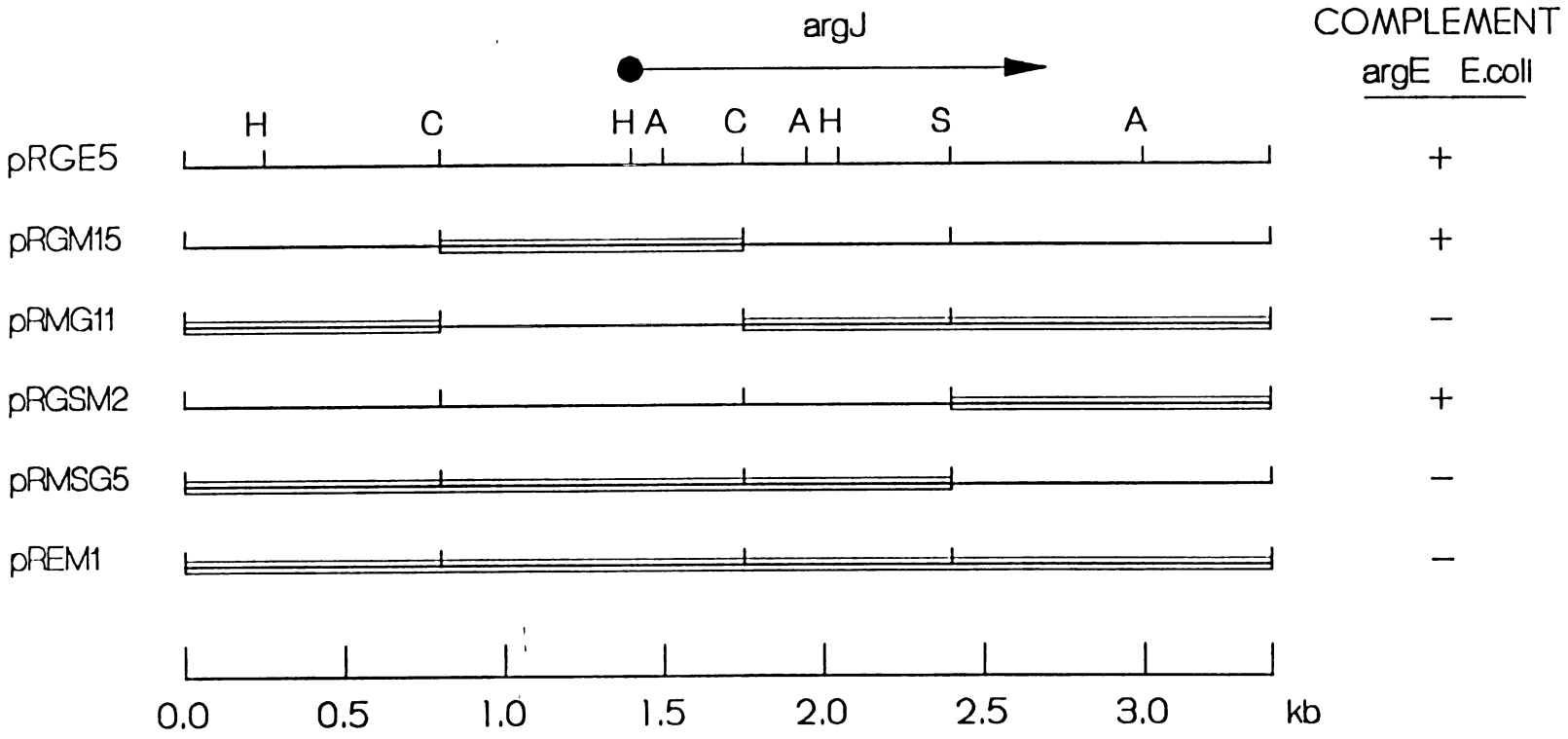
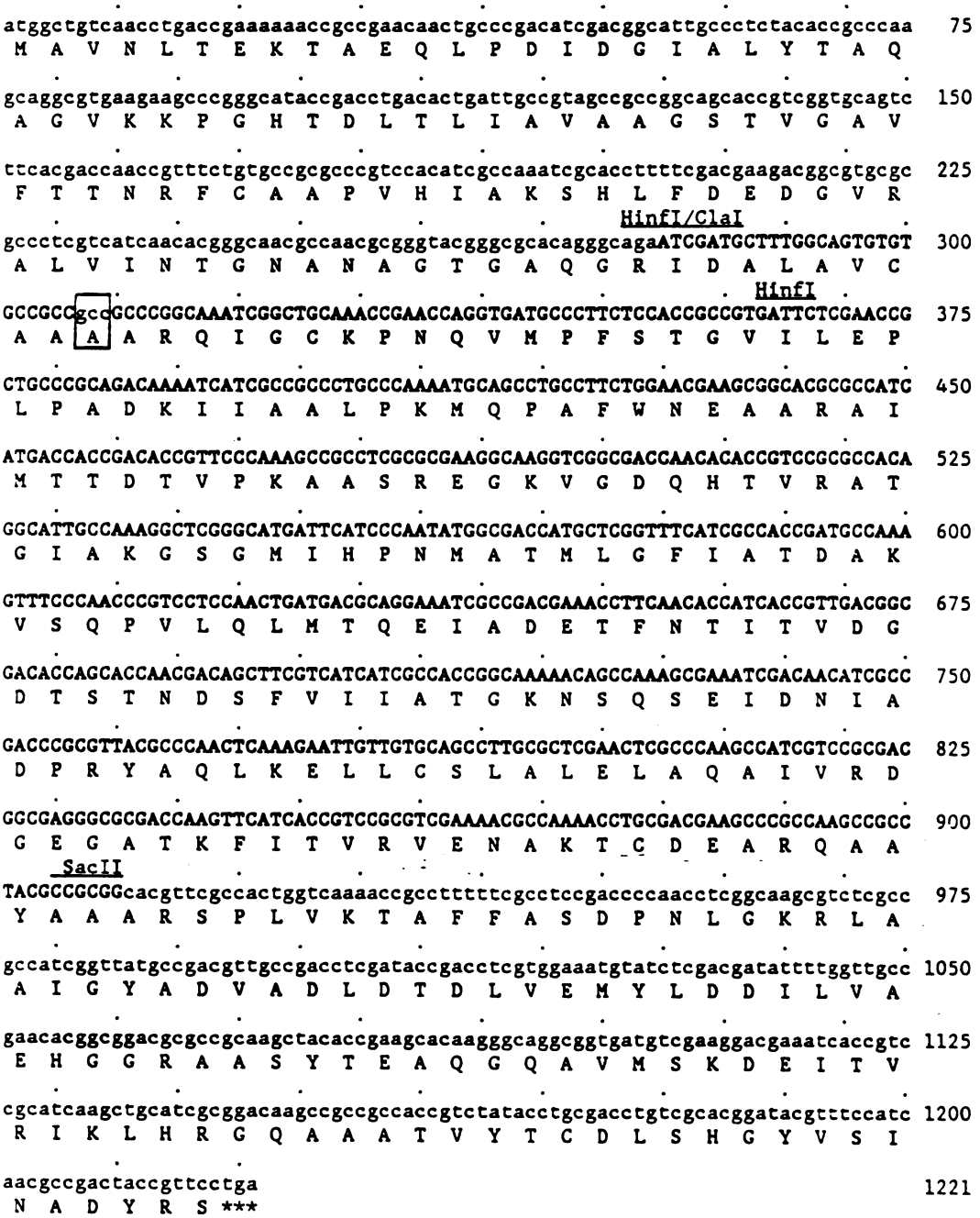

argJ
COMPLEMENT
argE E.coli
H C H A C A H S A
pRGE5 +
pRGM15 +
pRMG11 −
pRGSM2 +
pRMSG5 −
pREM1 −
0.0 0.5 1.0 1.5 2.0 2.5 3.0 kb

Figure 3. Comparison of the wild type and mutant *argJ* sequences. The predicted amino acid sequence is shown below the DNA sequence. The DNA sequenced in the mutant *argJ* gene and shown to be identical to the wild type sequence is capitalized. The 3 bp deletion of the mutant gene is boxed. The *Hin*fI sites flanking this deletion are indicated.


HinfI/ClaI
HinfI
SacII

The revertant gene was subcloned into M13mp19 and sequenced over the region containing the 3 bp deletion (Fig. 4). The wild type sequence was restored in the revertant, providing a strong correlation between ArgJ+ phenotype and absence of a 3 bp deletion in this part of the gonococcal *argJ* gene.

Prevalence of 3 bp deletion among AHU strains. Previous studies showing that AHU strains could not be transformed to Arg+ by genomic DNA from other AHU strains suggested that the *argJ* mutation in these strains was identical or overlapping (19). To test this hypothesis, a variety of AHU strains, ArgJ- non-AHU strains, and Arg+ strains were screened for the presence or absence of the 3 bp deletion described above. The deletion occurs within a *Hin*fI restriction fragment which is 85 bp in the wild type gene, but only 82 bp in the mutant (Fig. 3). This difference in restriction fragment length was used to screen gonococcal strains for the deletion. *Hin*fI digests of genomic DNA were separated on an 8% polyacrylamide sequencing gel and fragments in the range of approximately 75-100 nt were electrotransferred to Nytran. This blot was probed with the ^{32}P-dCTP labelled 0.6 kb *Hin*cII fragment of the wild type *argJ* gene, and hybridizing bands were visualized by autoradiography (Fig. 5). All AHU strains tested possessed a *Hin*fI fragment of 82 nt, while all wild type strains had an 85 nt band. The ArgJ- non-AHU strains tested had either an 85 nt fragment, or no *Hin*fI fragment in the 75-100 nt range, indicating that the *argJ* lesion of these strains was not identical to that in AHU strains.

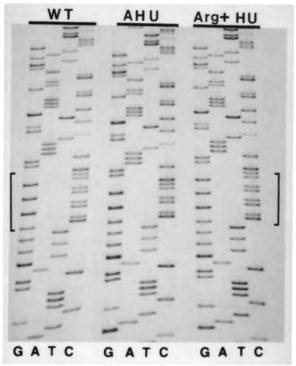


Figure 4. Presence of deletion in wild type, mutant, and revertant *argJ* genes. The 3 bp sequence -GCC- is repeated 4 times in the wild type (WT) and revertant (Arg+ HU) genes, but is present only 3 times in the *argJ* mutant (AHU). This repeated region is indicated by brackets.

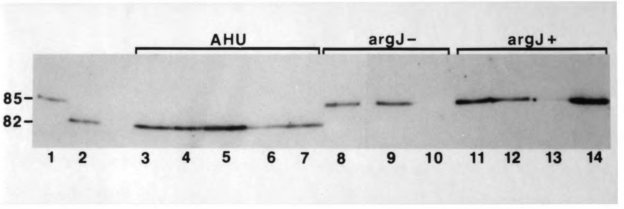


Figure 5. Presence of deletion in AHU, ArJ-, and ArgJ+ strains of *Neisseria gonorrhoeae*. *Hin*fI digested gonococcal genomic DNA was separated on an 8% sequencing gel and probed with the ^{32}P-labelled 0.6 kb *Hin*cII fragment of the wild type *argJ* gene. Hybridizing bands were either 85 or 82 nucleotides in length. Lanes 1 and 2 contain DNA from the wild type strain (CDC50) and AHU strain (NRL30465) in which the sizes of the *Hin*fI fragments were predetermined by DNA sequencing. DNA from AHU strains, ArgJ- strains, and ArgJ+ strains are indicated. Lane numbers correspond to strain numbers given in Table 1.

DISCUSSION

The development of auxotyping media for *Neisseria* in the early 1970's allowed for the discovery of the unusually invariant set of strains with multiple requirements for arginine, hypoxanthine and uracil. Retrospective studies of *N. gonorrhoeae* clinical isolates revealed that AHU strains did not appear until after the introduction of penicillin therapy in the mid 1940's (8,12). Since that time AHU strains have been spread widely and have become the predominant auxotype recovered in some locations (12,14). Though defined by auxotype, these strains also share other characteristics including PI serotype, serum resistance, and penicillin sensitivity, traits that are positively correlated to occurrence of disseminated gonococcal infection (3,10).

Genetic analysis of AHU strains revealed that the mutation responsible for the Arg- phenotype of these strains was identical (19), while biochemical analysis found that this mutation affected the *argJ* gene (7). In this study we have defined the *argJ* mutation in AHU strains at the molecular level.

The cloning and sequencing of the wild type *argJ* gene have been previously reported (18,23). We used this cloned gene to probe Southern blots of genomic DNA from AHU strains and found that AHU strains possess an apparently intact *argJ* gene with a restriction pattern comparable to the wild type gene. This information was used to clone the mutant *argJ* gene from the AHU strain NRL30465 on an identical restriction fragment as our wild type clone. The mutant clone appeared identical to the wild type by restriction mapping, but had no detectable ArgJ activity. The

ability to complement an *argA* mutation in *E. coli* was also lost in the mutant *argJ* clone. This supports our previous study in which *argA* complementation was found to be dependent on ArgJ activity and was not a separate function on our wild type *argJ* clone (18).

The lesion in the mutant *argJ* gene was identified by first narrowing down the location of the lesion by constructing chimeras of the mutant and wild type genes. This localized the lesion to a 630 bp region between the internal *Cla*I and *Sac*II sites. This region was completely sequenced and revealed only one difference between the mutant and wild type genes: a 3 bp deletion in the mutant gene. This deletion occurred within a region of the *argJ* gene in which the 3 bp sequence GCC is repeated several times.

The importance of this 3 bp deletion was analyzed by examining this region in a naturally occurring Arg+ revertant of NRL30465. It would be expected that the restoration of even a small deletion to the wild type sequence would be an excedingly rare event. Previous studies have shown that other AHU strains revert to Arg+ at the rate of approximately 3 x 10^{-9} per cell per generation (19). We were also able to obtain an Arg+ revertant of NRL30465, and the *argJ* gene was cloned from this revertant. The revertant *argJ* clone showed ArgJ activity equivalent to the wild type, and when sequenced revealed that the 3 bp deletion of the mutant gene had reverted to the wild type sequence. It seems unlikely that this type of reversion event would have occurred if the 3 bp deletion had not been a part of tandem direct repeats of this deleted sequence. The repeated nature of this sequence may allow for an occasional slippage of the DNA polymerase during replication, resulting in an insertion or deletion of the 3 bp repeat. A similar mechanism has been proposed to

explain the phase variation of expresion of the gonococcal opacity proteins (28,22). In that instance there is a 5 bp coding repeat in the 5' end of the gene encoding the leader peptide. The number of 5 bp tandem repeats in this region determines whether the rest of the gene will be in or out of frame, thus controlling expression at the translational level. The insertion or deletion of the coding repeat of the opacity genes occurs much more frequently than that of the mutant *argJ* gene. This is most likely due to the higher copy number of the repeats in the opacity genes (7-28 copies in the different opa genes), though differences in the length and base composition of the repeat may also influence the probability of slipped-strand mispairing events.

The 3 bp deletion in the mutant *argJ* gene would not affect the reading frame of the gene downstream of the deletion, but it would result in the loss of one alanine residue from the predicted amino acid sequence. The assumption that a minor change in the coding region of the gene is responsible for the lack of ArgJ activity was supported by several pieces of data. First, when the products of the mutant *argJ* gene were labelled with ^{35}S-methionine in *E. coli* minicells and compared to the wild type clone, the mutant clone produced an apparently identical 46 kDa protein (data not shown). This implies that the promotor was still functional, and the reading frame was not substantially disrupted in the mutant clone. Second, the construction of chimeras of the mutant and wild type *argJ* genes revealed that both the promotor end and the 3'-end of the mutant gene could be substituted into the wild type clone without impairing ArgJ activity (Fig. 2). Chimeras which contained the central portion of the mutant gene between the internal *Cla*I and *Sac*II sites remained ArgJ-. This, along with sequencing data of the mutant and

revertant *argJ* genes, support the conclusion that the 3 bp deletion is responsible for the Arg- phenotype of the AHU strain NRL30465.

The 3 bp deletion would result in the net loss of one alanine residue from the predicted amino acid sequence in the mutant protein. The amino acid sequence was analyzed using the Plotstructure program of the sequence analysis package from the University of Wisconsin Genetics Study Group (9). The deletion is predicted to occur within an alpha helical region of the protein which is followed by a turn. This region is flanked by cysteine residues (Fig. 3), making it possible that the deletion affects disulfide bond formation.

The fact that the mutant *argJ* gene contained a small deletion when compared to the wild type gene should allow the detection of this mutation by differences in restriction digest patterns. We used this approach to screen genomic DNA from a variety of AHU and non-AHU strains for the presence of this deletion. To detect a 3 bp deletion a restriction enzyme was chosen that closely flanked the site of the deletion (*Hin*fI, Fig. 3), and digests were separated on gels capable of distinguishing the 85 and 82 nt bands produced. When these digests were blotted and probed with a fragment of the *argJ* gene, all AHU strains tested were found to possess a 3 bp deletion in this area (82 nt band) while all ArgJ+ strains tested were intact (85 nt band, Fig. 5). The ArgJ- strains that were not Hyx- or Ura- either had an 85 nt band or no detectable band in the size range of 75 - 100 nt. These strains probably possess lesions in the *argJ* gene different from that of AHU strains and not identifiable by this methodology.

This experiment revealed that the 3 bp deletion truly exists in the gonococcal chromosome of strain NRL30465 and was not simply an artifact of

cloning. An identical or very similar deletion was also found in a variety of other AHU strains from different sources and of differing serovars. Though we did not screen all the AHU strains in our collection in this way, our sampling does support the theory that AHU strains arose from a single clone. This also provided further evidence that the 3 bp deletion is real and important to the Arg- phenotype of AHU strains.

ACKNOWLEDGMENTS

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SUMMARY AND CONCLUSIONS

In *N. gonorrhoeae*, the arginine biosynthetic pathway has been of interest primarily due to the prevalence of naturally occuring arginine auxotrophs. Initial epidemiological studies found that arginine auxotrophs were common and occurred singly or in combination with other requirements. These studies uncovered an unusual set of strains with multiple requirements for arginine, hypoxanthine, and uracil (AHU strains). These strains shared a variety of unusual phenotypes and were associated with asymptomatic and disseminated gonococcal infections.

These epidemiological studies spurred further investigations into arginine biosynthesis in *N. gonorrhoeae*. It was found that defects in arginine biosynthesis were not scattered throughout the eight step pathway, but occurred predominantly at steps five or six, the conversion of acetylornithine to ornithine and the combination of ornithine with carbamoylphosphate to form citrulline respectively. AHU strains all had defects in the fifth step of the pathway (ArgJ mutants). These defects were presumed to be identical due to their inability to recombine. Strains with defects at step six (citrulline auxotrophs) were shown to have defects in carbamoylphosphate synthetase (encoded by *carA* and *carB*), and not ornithine carbamoyltransferase (encoded by *argF*). A naturally occuring *argF* mutant has not yet been identified.

We have further examined arginine auxotrophy in *N. gonorrhoeae* by cloning and sequencing the *argJ* and *argF* genes, and identifying the *argJ* lesion common to AHU strains. The gonococcal *argF* gene was quite similar to the *argF* genes previously sequenced from *E. coli* and *P. aeruginosa*

with regions involved in ornithine and carbamoylphosphate binding being particularly well conserved. This gene was unusual in that it used a GUG initiation codon, which had been not previously reported in gonococcal genes. An internal deletion mutant of the cloned *argF* gene has been constructed, and attempts were made to introduce this mutation back into the gonococcus, but they have not yet been successful. This of course does not prove that *argF* mutations are lethal to the gonococcus, but this possibility will remain until an *argF* mutant can be isolated.

The gonococcal *argJ* gene was the first ornithine acetyltransferase gene to be sequenced from any source. We were able to compare our sequence to a preliminary sequence of a putative *argJ* gene from *Bacillus subtilis*, and distinct regions of high homology were found. As this enzyme has not yet been purified or characterized we cannot report if these conserved regions contain residues important to substrate binding or catalysis, though these sequences will be of predictive value for future biochemical investigations of this enzyme.

Perhaps the most interesting outcome of our molecular investigations of arginine auxotrophy in *N. gonorrhoeae* was the elucidation of the lesion in the *argJ* gene of AHU strains. These strains had previously been proposed to be clonally derived, and our study strongly supports this theory. All AHU strains tested in our study contained a 3 bp deletion within a repetitive region of the *argJ* gene. This deletion was not found in Arg+ strains or in ArgJ- strains that were not AHUs. The most likely explanation for these findings is that the AHU strains tested inherited the deletion from a common ancestor, and that the other ArgJ- strains were independently derived.

The fact that this deletion occurred within tandem direct repeats of

the deleted sequence gives a clue to how this mutation may have originated. This direct repeat arrangement can result in slipped-strand mispairing during DNA synthesis, leading to the insertion or deletion of the repeated unit. This model was supported by finding that a naturally occurring Arg+ revertant of an AHU strain had regained this deleted sequence. This same model has been used to explain the phase variation of expression of gonococcal P.II genes.

The 3 bp deletion in the gonococcal *argJ* gene would result in the net loss of one alanine residue from the predicted amino acid sequence. This implies that this alanine residue may be directly involved in the catalytic properties of the enzyme; or the loss of one amino acid in this region may alter the folding of the molecule and thus impair its enzymatic activity. This data should be quite useful to future studies on the characterization of the ornithine acetyltransferase enzyme.

APPENDIX

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Sequence of the *argF* gene encoding ornithine transcarbamoylase from *Neisseria gonorrhoeae*

(Arginine biosynthesis; DNA uptake sequence; start codon; sequence homology; recombinant DNA)

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SUMMARY

The gonococcal *argF* gene encoding ornithine transcarbamoylase (OTCase) contains an open reading frame of 993 nucleotides which starts with a GUG codon and encodes a peptide with a deduced M_r of 36 731. We compared the predicted amino acid (aa) sequence to OTCase sequences previously determined for *Escherichia coli* and *Pseudomonas aeruginosa* and found that highly conserved regions in the genes from these organisms were also conserved in *Neisseria gonorrhoeae*, including those aa known to be important for carbamoyl phosphate and ornithine binding. In the flanking regions of the gene were found 15-bp inverted repeats that may serve as transcriptional termination signals, and which contain the neisserial DNA-uptake sequence.

Arginine biosynthesis from glutamate occurs by an eight-step pathway (Cunin et al., 1986). The sixth step of the pathway involves the conversion of ornithine and carbamoyl phosphate to citrulline and is catalyzed by the enzyme OTCase. Arginine auxotrophs are commonly encountered in *N. gonorrhoeae*, comprising 40–60% of clinical isolates (Catlin and Nash, 1978; Picard and Dillon, 1989). While 30% of these auxotrophs require citrulline for growth, the lesion in these strains is in carbamoyl phosphate synthetase, not OTCase (Catlin and Nash, 1978). No strains of *N. gonorrhoeae* deficient in OTCase production have been identified.

The cloning of the *argF* gene from *N. gonorrhoeae* has been previously reported (Picard and Dillon, 1989). We independently cloned this gene in the plasmid pUC12 by complementation in *E. coli*, confirmed that the clone produced active OTCase using the procedure of Catlin and Nash (1978), and sequenced the gene (both strands) in M13mp19 (Fig. 1). The gonococcal *argF* gene contains a 993-nt ORF initiating at nt 141 with a GUG codon, and coding for a 36 731-Da peptide. This was supported by minicell analysis and direct N-terminal aa sequence determination showing that the GUG start codon was being utilized. The codon usage data for the *argF* gene are in good agreement with the data for previously sequenced gonococcal genes (West and Clark, 1989).

Comparison of the predicted nt and aa sequences of the gonococcal OTCase revealed that this gene is more closely related to the *E. coli* OTCases encoded by *argF* and *argI* (64.0 and 62.5% DNA and 64.0 and 61.6% aa identity, respectively) (Bencini et al., 1983; Van Vliet et al., 1984) and the *P. aeruginosa* catabolic OTCase (*arcB*, 66.6% DNA and 68% aa identity) (Baur et al., 1987) than to the *P. aeruginosa* anabolic OTCase (*argF*, 50.1% DNA and 37.5% aa identity) (Itoh et al., 1988). The gonococcal OTCase also had significant DNA (43.4–48.5%) and aa (33.5–40.5%) identity with eukaryotic OTCases from fungi, rodents, and humans (Huygen et al., 1987). Those aa involved in interactions with carbamoyl phosphate

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Abbreviations: aa, amino acid(s); bp, base pair(s); kb, kilobase(s) or 1000 bp; nt, nucleotide(s); ORF, open reading frame; OTCase, ornithine transcarbamoylase.

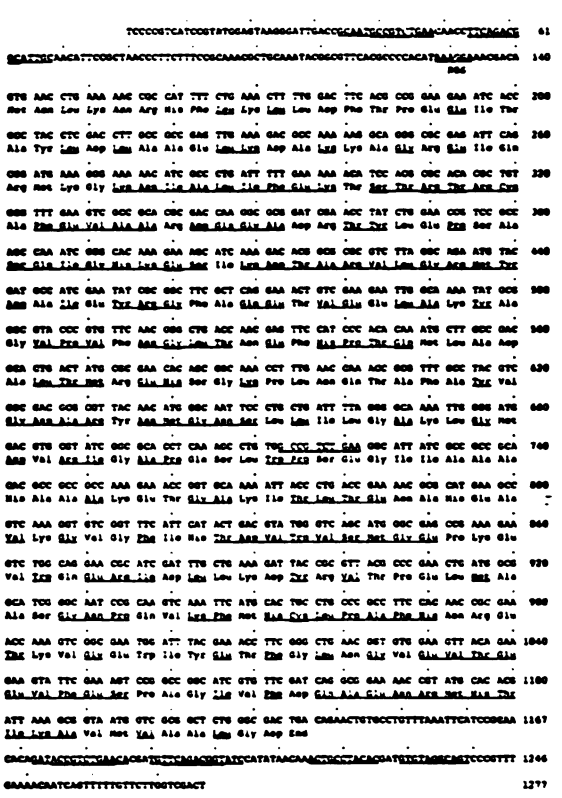

Fig. 1. The nt sequence of the gonococcal *argF* gene and flanking regions. The predicted aa sequence is shown below the coding region. N-terminal aa that were sequenced are in italics. Inverted repeats are underlined as is the putative ribosome-binding site (RBS). Neisserial DNA uptake sequences are double-underlined. The aa identical in *E. coli argI*, *P. aeruginosa arcB*, and *N. gonorrhoeae argF* are underlined. The aa implicated in carbamoyl-phosphate binding or catalysis and ornithine binding are double-underlined. The sequence data have been submitted to GenBank and assigned the accession number M34930.

(Houghton et al., 1984) and ornithine (Marshall and Cohen, 1980) were all conserved in the gonococcal OTCase (Fig. 1).

Gonococcal cells are naturally competent for DNA transformation during all stages of growth, though only DNA of neisserial origin will bind to the cells (Dougherty et al., 1979). This selectivity is due to the higher occurrence of the 10-bp sequence, 5'–GCCGTCTGAA–3', in neisserial DNA than in DNA from other sources (Goodman and Scocca, 1988). This DNA-uptake sequence, with no more than one mismatch, is present five times in the *argF* sequence: twice as part of 15-bp inverted repeats in the flanking regions of the gene, and once within the coding region. The presence of the uptake sequence within these flanking inverted repeats supports the hypothesis of Goodman and Scocca (1988) that the uptake sequence may be maintained in high frequency in *Neisseria* in part by being incorporated in transcription termination sequences.

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