



National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service

Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

GENETICS OF ARGININE AND PROLINE BIOSYNTHESIS
IN NEISSERIA GONORRHOEAE

by

François J. Picard, M.Sc.

A thesis submitted to the School of Graduate
Studies and Research in partial fulfilment of
the requirements for the degree of
Doctor in Philosophy

Department of Biology
University of Ottawa
Ottawa, Ontario



François J. Picard, Ottawa, Canada, 1991



National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-68099-7

Canada



UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

A Michèle ainsi qu'à mes chers parents pour
leur support moral très apprécié.

ABSTRACT

A genomic library for *N. gonorrhoeae*, constructed in the lambda cloning vector EMBL4, was screened for clones carrying arginine and proline biosynthesis genes by complementation of *E. coli* mutants. Clones complementing defects in argA, argB, argE, argG, argIF, carA, carB, proA, proB and proC were isolated. An *E. coli* mutant, defective in the acetylornithine deacetylase gene (argE), was presumably complemented by the ornithine acetyltransferase gene (argJ) from *N. gonorrhoeae*. This heterologous complementation, which was confirmed by enzymatic assays, is reported for the first time.

The carAB operon from *E. coli* hybridized with the gonococcal clones carrying carA or carB genes under conditions of high stringency (i.e. detecting approximately 80% or greater similarity) suggesting that the nucleotide sequence of the carbamoylphosphate synthetase genes is very similar in these two organisms. Under these conditions for hybridization, the gonococcal clones carrying argB or argF genes did not hybridize with plasmids containing the corresponding *E. coli* gene. Hybridizations performed under conditions of lower stringency indicate that the nucleotide sequence of the argB gene is less conserved than that of argF in *E. coli* and *N. gonorrhoeae*. Co-complementation experiments established gene linkage only between carA and carB and between proA and proB.

gonococcal genes. Clones complementing a gene defect in argE were also able to complement an argA mutation. This suggests that the enzyme ornithine acetyltransferase from N. gonorrhoeae (encoded by argJ) may be able to complement both argA and argE mutations in E. coli. This hypothesis is supported by recent findings (M.H. Mulks, personal communication) showing by hybridization studies that an argA gene was apparently absent on a recombinant plasmid carrying a gonococcal genomic DNA insert which is able to complement both argA and argE mutations in E. coli. The arginine biosynthesis genes in N. gonorrhoeae appear to be scattered as in the Pseudomonadaceae.

The prevalence of specific arginine biosynthesis gene defects was studied for 319 arginine-requiring clinical isolates of Neisseria gonorrhoeae by using the ability of the strains to utilize intermediates of arginine biosynthesis. Nearly 99% of the strains were defective either in the conversion of acetylornithine to ornithine (174 strains) or in the conversion of ornithine to citrulline (141 strains). Based on a nutritional requirement for carbamoyl phosphate, only 11% of the uracil-requiring strains defective in the carbamylation of ornithine to yield citrulline were apparently defective in carAB. Three argininosuccinate-requiring strains (i.e. probably defective in argG) of auxotype PAU were identified. Analysis by hybridization of the chromosomal DNA from N.

gonorrhoeae strains defective in the CPSase gene(s) suggested that the gonococcal cryptic plasmid, which can integrate into the chromosome, does not play a role in insertional mutagenesis of that gene. A high polymorphism was observed in hybridization patterns of restricted genomic DNA from N. gonorrhoeae strains having the same auxotype and serotype with a gonococcal CPSase gene-specific probe suggesting that this probe may provide a useful epidemiological marker for N. gonorrhoeae. Some of the arginine auxotrophs of N. gonorrhoeae defective in carAB, argJ, argF or argG were complemented by genetic transformation with DNA from recombinant bacteriophages carrying characterized gonococcal arginine biosynthesis genes. Gene defects in proA (5 strains) and in proB (6 strains) were identified by gonococcal transformation assays with recombinant bacteriophages or plasmids carrying proline biosynthesis genes from N. gonorrhoeae. None of the eleven proline-requiring strains tested appears to be defective in proC.

Polymerase chain reaction (PCR) amplifications using oligonucleotides specific to conserved areas of the E. coli carAB operon yielded amplified copies of various portions of the N. gonorrhoeae CPSase genes. Amplifications using primers specific to the duplicated region of the E. coli carB gene suggest that the gonococcal carB gene contains a similar duplication.

RÉSUMÉ

Une banque génomique pour N. gonorrhoeae, construite dans le vecteur de clonage lambda EMBL4, a été criblée par complémentation de mutants de E. coli pour des clones portant des gènes gonococciques impliqués dans les biosynthèses de l'arginine et de la proline. Des clones capable de compléter des défauts dans les gènes argA, argB, argE, argG, argIF, carA, carB, proA, proB et proC ont été isolés. Une mutation dans le gène de l'acétylornithine déacétylase (argE) de E. coli a été apparemment complémentée par le gène de l'ornithine acétyltransférase (argJ) provenant de N. gonorrhoeae. Cette complémentation hétérologue, confirmée par des essais enzymatiques, est décrite pour la première fois.

L'opéron carAB de E. coli a hybridé avec les clones gonococciques portant les gènes carA ou carB dans des conditions de forte stringence (i.e. détectant une similarité d'environ au moins 80%). Ceci suggère que les gènes de la carbamoylphosphate synthétase (CPSase) chez ces deux organismes sont très similaires. Dans les mêmes conditions d'hybridation, les clones gonococciques portant les gènes argB ou argF n'ont donné aucun signal avec des plasmides contenant les gènes correspondants de E. coli. Des hybridations réalisées dans des conditions de faible stringence indiquent que la séquence du gène argB semble moins bien conservée que

celle du gène argF chez E. coli et N. gonorrhoeae. Des expériences de co-complémentation ont établi un linkage seulement entre les gènes gonococciques carA et carB ainsi que proA et proB. Les clones complétant un défaut dans le gène argE complètent aussi une mutation dans le gène argA. Ceci suggère que l'enzyme ornithine acetyltransférase codée par le gène gonococcique argJ est capable de compléter des mutations chez E. coli à la fois dans les gènes argA et argE. Cette hypothèse est appuyée par d'autres résultats (M.H. Mulks, communication personnelle) démontrant à l'aide d'hybridations qu'un gène argA était apparemment absent sur un plasmide recombinant, portant un insert d'ADN génomique de N. gonorrhoeae et capable de compléter des mutations chez E. coli à la fois dans les gènes argA et argE. Les gènes de la biosynthèse de l'arginine chez N. gonorrhoeae semblent dispersés sur le chromosome comme chez les Pseudomonadaceae.

La prévalence des défauts dans les gènes de la biosynthèse de l'arginine a été étudiée pour 319 isolats cliniques de N. gonorrhoeae ayant un besoin nutritif pour l'arginine en utilisant la capacité des souches à métaboliser des intermédiaires de la biosynthèse de l'arginine. Près de 99% des souches se sont avérées défectueuses dans la conversion de l'acetylornithine en ornithine (174 souches) ou dans la conversion de l'ornithine en citrulline (141 souches). D'après le besoin nutritif des souches en carbamoyl phosphate,

seulement 11% des souches, ayant un besoin nutritif pour l'uracile et étant déficientes dans la carbamylation de l'ornithine, étaient apparemment défectueuses dans carAB. Trois souches d'auxotype PAU, apparemment défectueuses dans le gène argG, ont été identifiées. L'analyse par hybridation de l'ADN chromosomique de N. gonorrhoeae provenant de souches défectueuses dans les gènes de la carbamoylphosphate synthetase (CPSase) suggère que le plasmide cryptique de N. gonorrhoeae, qui est capable de s'intégrer dans le chromosome, n'inactive pas ces gènes par insertion. Un polymorphisme élevé a été observé dans les patrons d'hybridation d'ADN génomique, digéré avec des endonucleases de restriction et provenant de souches de même auxotype et sérotype, avec une sonde spécifique aux gènes gonococciques de la CPSase suggérant que cette sonde pourrait représenter un marqueur épidémiologique utile pour N. gonorrhoeae. Quelques unes des souches de N. gonorrhoeae défectueuses dans les gènes carAB, argJ, argF ou argG ont été complémentées par transformation génétique avec de l'ADN de bactériophages recombinants portant des gènes gonococciques de la biosynthèse de l'arginine. Des défauts dans les gènes proA (5 souches) et proB (6 souches) ont été identifiés au moyen d'essais de transformation utilisant des bactériophages recombinants ou des plasmides portant des gènes de la biosynthèse de la proline chez N. gonorrhoeae. Aucune des 11 souches testées, ayant un besoin nutritif pour la proline, ne semble défectueuse dans le gène proC.

Des amplifications par PCR utilisant des amorces spécifiques à des régions conservées de l'opéron carAB de E. coli ont produit des copies amplifiées de différentes portions des gènes gonococciques de la CPSase. Les amplifications utilisant des amorces situées dans la région dupliquée du gène carB de E. coli suggèrent que le gène carB de N. gonorrhoeae contient une duplication similaire.

ACKNOWLEDGEMENTS

I am grateful to my supervisor Dr. Jo-Anne R. Dillon for her guidance during my Ph.D. as well as for giving me great learning experiences. I thank Drs. L. Bonen, B. Iyer and D. Kushner, the members of my advisory committee, for helpful suggestions and comments.

I would like to thank all my colleagues at Health and Welfare Canada for their help and suggestions as well as for good times off work. Special thanks to Dr. L.-K. Ng for helpful suggestions and constructive discussions throughout the course of this study.

I thank Drs. B.J. Bachmann (New Haven, CT) and N. Glansdorff (Brussel University, Belgium) for providing E. coli arginine and proline auxotrophs as well as Dr. R. Cunin (Brussel University, Belgium) for the gift of plasmids pA11, pMC7 and pMC50.

I am thankful to Dr. D. Johnson (Dept. of Biology, University of Ottawa) for providing EMBL4 arms and for his help in the construction of the genomic library. His helpful suggestions throughout the course of this study were greatly appreciated. I am also grateful to Dr. W.M. Johnson (Health and Welfare Canada, Ottawa) for her technical advice

pertaining to enzymatic assays.

I thank Dr. L. Bonen (Dept. of Biology, University of Ottawa) for the use of her digitizer (computer facilities). Je remercie Pascale Garber et Yvan Chapdelaine du groupe de L. Bonen pour leur aide dans l'utilisation du programme Microgenie et pour leurs conseils techniques.

I thank Drs. R. Pollard (deceased) and J. Campione (Health and Welfare Canada, Ottawa) for allowing me to use their thermocyclers. I am also thankful to Dr. B. Korneluk (Ottawa Children Hospital) for lending us his apparatus for electroporation.

I thank Dr. Y. Kang (Dept. Microbiology, University of Ottawa) for his collaboration in automated DNA sequencing. I am grateful to B. Mah in Y. Kang's group for her technical contributions and advice regarding automated DNA sequencing.

I would also like to thank Dr. M.P.R. Tenniswood's group (Dept. Biochemistry, University of Ottawa), Dr. B. Korneluk's group (Ottawa Children Hospital) and Dr. Y. Kang's group (Dept. Microbiology, University of Ottawa) for helpful suggestions.

Je remercie Benoit Gauthier pour ses suggestions utiles

et son aide avec les ordinateurs de même que pour son amitié et son support moral très appréciés. Je remercie également B. Gauthier pour sa collaboration au projet sur l'électroporation. Je tiens à remercier P. Ledoux pour sa contribution technique pour l'isolement et la caractérisation des bactériophages recombinants portant des gènes de la biosynthèse de la proline.

Enfin, je remercie Michèle Rouleau pour ses bons conseils et son aide indispensable à la dactylographie de cette thèse ainsi que M. Rouleau, Y. Chapdelaine et S. Hasnain pour la révision de cette thèse.

TABLE OF CONTENTS

	Page
Dedication.....	i
Abstract.....	ii
Résumé.....	v
Acknowledgements.....	ix
Table of contents.....	xii
List of tables.....	xvi
List of figures.....	xviii
List of abbreviations.....	xxi
Preface.....	1
Literature review.....	2
1. Nutritional requirements in <u>N. gonorrhoeae</u>	3
1.1 Relationship between auxotrophy and gonococcal virulence.....	7
2. Arginine biosynthesis.....	9
2.1 Enzymatic steps.....	9
2.2 Genetic organization of arginine biosynthesis genes.....	15
2.3 Control of gene expression in arginine biosynthesis.....	17
3. Proline biosynthesis.....	24
3.1 Enzymatic steps and regulation of proline biosynthesis.....	24
3.2 Genetics of proline biosynthesis.....	26

4. Gene transfer in <u>N. gonorrhoeae</u>	27
4.1 Gonococcal transformation.....	27
4.2 Plasmids of <u>N. gonorrhoeae</u>	30
4.3 DNA restriction and modification systems in <u>N. gonorrhoeae</u>	33
4.4 Introduction of cloned genes in <u>N. gonorrhoeae</u> ..	34
Research objectives.....	37
Chapter one: Cloning and organization of seven arginine biosynthesis genes from <u>Neisseria gonorrhoeae</u>	40
Introduction.....	41
Materials and methods.....	43
Bacterial strains and plasmids.....	43
Media and bacterial growth conditions.....	46
DNA preparation.....	47
Restriction endonuclease digestion and electrophoresis.....	49
Fractionation of <u>N. gonorrhoeae</u> chromosomal DNA.....	49
Cloning protocol.....	50
Complementation of <u>E. coli</u> arginine auxotrophs.....	51
Probe preparation and blot hybridization.....	53
Cell-free extracts.....	54
Enzymatic assays.....	55
Results.....	57
Verification of the <u>N. gonorrhoeae</u> genomic library....	57
Isolation of <u>Arg</u> ⁺ clones.....	57
Molecular characterization of <u>Arg</u> ⁺ clones.....	58

Hybridization with cloned <u>E. coli</u> arginine genes.....	62
Co-complementation studies.....	68
Enzymatic assays.....	69
Discussion.....	74
Chapter two: Biochemical and genetic studies with arginine	
and proline auxotrophs of <u>Neisseria gonorrhoeae</u>	84
Introduction.....	85
Materials and methods.....	87
Bacterial strains, growth conditions and	
identification.....	87
Auxotype determination.....	88
DNA preparation.....	90
Restriction endonuclease digestion and	
electrophoresis.....	91
Cloning of proline genes and complementation of	
<u>E. coli</u> auxotrophs.....	91
Probe preparation and blot hybridization.....	92
Gonococcal transformation.....	93
Results.....	95
Utilization of arginine biosynthesis intermediates	
by <u>N. gonorrhoeae</u>	95
Association of auxotype with gene defects.....	97
Analysis by hybridization of genomic DNA from	
CPSase defective <u>N. gonorrhoeae</u>	99
Isolation and characterization of <u>Pro</u> ⁺ recombinant	
bacteriophages and plasmids.....	108

Transformation of <u>N. gonorrhoeae</u> arginine and proline auxotrophs.....	110
Discussion.....	118
Chapter three: Molecular characterization of the gonococcal CPSase genes.....	130
Introduction.....	131
Materials and methods.....	134
DNA preparation.....	134
Restriction endonuclease digestions and electrophoresis.....	134
Probe preparation and blot hybridization.....	134
PCR amplification.....	134
Results.....	143
Subcloning of <u>carA</u> and <u>carB</u>	143
PCR amplifications of the gonococcal CPSase genes.....	145
Discussion.....	155
Conclusions.....	159
References.....	165
Appendix A. Some characteristics of the DNAs from the recombinant bacteriophages carrying gonococcal arginine and proline biosynthesis genes.....	183
Appendix B. DNA sequence from a portion of the <u>N. gonorrhoeae carB</u> gene.....	187
Appendix C. Transformation of <u>N. gonorrhoeae</u> by electroporation.....	212

LIST OF TABLES

	Page
Table 1-1. Bacterial Strains.....	44
Table 1-2. Lytic complementation of a variety of <u>E. coli</u> K-12 arginine mutants with the <u>N. gonorrhoeae</u> genomic library.....	59
Table 1-3. Complementation of ten different <u>E. coli</u> arginine auxotrophs by recombinant bacteriophages carrying gonococcal arginine biosynthesis genes.....	70
Table 1-4. Activities of AODase, OATase and AGSase in cell-free extracts and in cell lysates of <u>E. coli</u> mutants complemented with the bacteriophage EFP30.....	71
Table 2-1. <u>E. coli</u> strains.....	89
Table 2-2. Number of arginine-requiring strains of <u>N. gonorrhoeae</u> capable of utilizing intermediates of arginine biosynthesis for growth.....	96
Table 2-3. Possible arginine biosynthesis gene defects in 319 isolates of <u>N. gonorrhoeae</u> distributed by auxotypes.....	98
Table 2-4. Sizes of the genomic DNA fragments from 9 <u>N. gonorrhoeae</u> strains which hybridized with the gonococcal cryptic plasmid or with the	

gonococcal CPSase genes probe.....	100
Table 2-5. Some properties of clones carrying proline biosynthesis genes from <i>N. gonorrhoeae</i>	109
Table 2-6. Transformable strains of <i>N. gonorrhoeae</i> having different arginine biosynthesis gene defects with recombinant bacteriophage DNA isolated from <i>E. coli</i> and carrying gonococcal arginine biosynthesis genes.....	112
Table 2-7. Transformation of proline-requiring strains of <i>N. gonorrhoeae</i> with recombinant bacteriophage DNA isolated from <i>E. coli</i> and carrying gonococcal proline biosynthesis genes.....	114
Table 3-1. Primers used for PCR amplifications of the gonococcal CPSase genes.....	137
Table A-1. Some characteristics of the DNAs from the recombinant bacteriophages carrying gonococcal arginine and proline biosynthesis genes.....	184
Table C-1. <i>N. gonorrhoeae</i> strains and plasmids used for electroporation.....	216
Table C-2. Major differences between the protocols for plasmid transformation of <i>N. gonorrhoeae</i> by electroporation used by us (protocol A) or by Kapczynski and Genco (protocol B).....	221

LIST OF FIGURES

	Page
Fig. LR-1. Arginine and proline biosynthesis.....	10
Fig. LR-2. Structure of the control region for the <u>carAB</u> operon in <u>E. coli</u>	21
Fig. 1-1. Hybridization under conditions of high stringency of the gonococcal clones EFP10 (<u>carAB</u> ⁺) and EFP20 (<u>carB</u> ⁺) with the corresponding <u>E. coli</u> genes.....	63
Fig. 1-2. Hybridization under conditions of low stringency (T _m -50°C) of the gonococcal clones EFP40 (<u>argF</u> ⁺) and EFP70 (<u>argB</u> ⁺) with the corresponding <u>E. coli</u> genes.....	65
Fig. 2-1. Hybridization of genomic DNA from six CPSase defective strains of <u>N. gonorrhoeae</u> with the cryptic plasmid and with the gonococcal <u>carAB</u> probe.....	103
Fig. 2-2. Hybridization of genomic DNA from six CPSase defective strains of <u>N. gonorrhoeae</u> with the gonococcal <u>carAB</u> probe.....	106
Fig. 3-1. Comparison of the nucleotide sequence of the four primers (A1, B1, B2 and B3) used for PCR amplifications with the correspon- ding regions of the <u>E. coli</u> , the yeast and the rat CPSase genes.....	139

- Fig. 3-2. Nucleotide positions of the four primers on the *E. coli* carAB operon used for PCR amplifications..... 141
- Fig. 3-3. Amplification by PCR of recombinant bacteriophage DNA from EFP10 (carAB⁺), recombinant plasmid pMC50 (carries the entire *E. coli* carAB operon) and *N. gonorrhoeae* chromosomal DNA from the prototrophic strain CH811..... 146
- Fig. 3-4. Amplification by PCR of recombinant bacteriophage DNA from EFP10 (carAB⁺) and recombinant plasmid pMC50 (carries the entire *E. coli* carAB operon)..... 148
- Fig. A-1. EcoRI digested DNA from some of the recombinant bacteriophages carrying gonococcal arginine biosynthesis genes..... 185
- Fig. B-1. Asymmetric amplification by PCR: Titration of the limiting primer against the non-limiting primer to determine the optimal primer ratio to yield ssDNA..... 189
- Fig. B-2. Nucleotide sequence for a portion of the gonococcal carB gene amplified from clone EFP10 (carries the gonococcal CPSase genes) using primers B2-B1..... 198
- Fig. B-3. Semi-graphical representation of the restriction sites on the 576 nucleotide sequence from the gonococcal carB gene for

	fifteen restriction endonucleases.....	200
Fig. B-4.	Alignments of the CPSase amino acid sequences from <u>N. gonorrhoeae</u> , <u>E. coli</u> , <u>S. cerevisiae</u> and the rat.....	202
Fig. B-5.	Comparison of the sequencing ladders obtained with PCR amplified ssDNA (A) and with ssDNA prepared from M13 recombinant bacteriophages (B).....	206

LIST OF ABBREVIATIONS

A	arginine
acetyl-CoA	acetyl-coenzymeA
AGSase	acetylglutamate synthetase
AODase	acetylornithine deacetylase
amp	ampicillin
BMC	Boehringer Mannheim Canada
C	citrulline
CCC	covalently closed circular
CP.	carbamoylphosphate
CPSase	carbamoylphosphate synthetase
dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytosine-5'-triphosphate
dGTP	deoxyguanosine-5'-triphosphate
dig-dUTP	digoxigenine-deoxyuridine-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ds	double-stranded
dTTP	deoxythymine-5'-triphosphate
EDTA	ethylenediaminetetraacetate
ELISA	enzyme-linked immunoassay
Fig.	Figure
GC	<u>N. gonorrhoeae</u> (i.e. gonococcus)
GCMB	GC medium base
GEB	gonococcal electroporation buffer
h	hour
H	hypoxanthine
HBS	HEPES buffered sucrose
HEPES	hydroxyethyl piperazine-ethane sulfonic acid
HPLC	high pressure liquid chromatography
kb	kilobase pair
kDa	kiloDalton
kV	kiloVolt
λ	lambda bacteriophage
L	leucine
M	methionine
Mcr	Methylated cytosines restriction
min	minute
mRNA	messenger RNA
ms	millisecond
μ Fd	microFarad
NR	non-requiring
O	ornithine
OATase	ornithine acetyltransferase
OC	open circular form
OD ₆₀₀	optical density at 600 nm
OTCase	ornithine transcarbamoylase

P	proline
PCR	polymerase chain reaction
ppGpp	guanosine tetraphosphate
r	resistance
rec	recombination
RNA	ribonucleic acid
rpm	rotation per minute
SDS	sodium dodecyl sulphate
SEB	sucrose electroporation buffer
ss	single-stranded
str	streptomycin
tc	tetracycline
TE	Tris-HCl, EDTA buffer
T _m	melting temperature
TMM	tris-maleate medium
tRNA	transfer RNA
TSA	trypticase soy agar
TSB	trypticase soy broth
U	uracil
UMP	uridylate
U.V.	ultra-violet
v	volume
w	weight

PREFACE

Neisseria gonorrhoeae, the etiological agent of the sexually transmitted disease gonorrhoeae, have accumulated spontaneous mutations in genes implicated in a variety of biosynthesis pathways. Although no direct role of auxotrophy in gonococcal virulence has been demonstrated, a possible association of gonococcal auxotrophy with virulence has been suggested by several investigators.

The genetics of most biosynthesis pathways for N. gonorrhoeae has not yet been studied. Only two biosynthesis genes (proA and proB) have been cloned. The focus of this thesis has been on gonococcal arginine and proline biosynthesis pathways since naturally-occurring auxotrophs of N. gonorrhoeae are more frequently defective in these two pathways. This thesis deals with the cloning and molecular characterization of arginine and proline biosynthesis genes as well as with the study of arginine and proline biosynthesis gene defects naturally encountered in the gonococcus.

LITERATURE REVIEW

Neisseria gonorrhoeae, the etiological agent of the disease gonorrhea, has been relatively well characterized at the genetic level. The study of the gonococcal plasmids and of the chromosomal genes coding for common surface antigens, pili and serum resistance has been a focus of interest because of their direct role in gonococcal pathogenesis (Cannon and Sparling, 1984). However, the genetics of most biosynthesis pathways has not yet been studied in this micro-organism, despite the fact that most clinical isolates of N. gonorrhoeae have accumulated numerous mutations in genes implicated in a variety of these pathways. In fact, the only biosynthesis genes from N. gonorrhoeae which have been cloned are implicated in proline biosynthesis (proA and proB) (Stein et al., 1984). The following summarizes the current knowledge of the physiology and the genetics of arginine and proline biosynthesis pathways in N. gonorrhoeae and other procaryotes. Aspects of gonococcal genetics such as the natural occurrence of biosynthesis gene defects, gene transfer and the genetics of gonococcal plasmids are also reviewed.

1. Nutritional requirements in *N. gonorrhoeae*

Clinical isolates of *N. gonorrhoeae* have accumulated spontaneous mutations in genes encoding enzymes implicated in a variety of biosynthesis pathways (Carifo and Catlin, 1973; Catlin, 1977). Consequently, auxotyping of *N. gonorrhoeae*, which characterizes the gonococcal strains according to their nutritional requirements (Carifo and Catlin, 1973), represents a very useful epidemiological marker. The most frequently encountered auxotrophic defects are found in the arginine and the proline biosynthesis pathways (Carifo and Catlin, 1973; Copley and Egglestone, 1983; Dillon and Pauzé, 1984; Hendry and Stewart, 1979). Arginine and proline auxotrophs of *N. gonorrhoeae*, which have a worldwide distribution, account for up to 40-60% and 20-40% of all clinical isolates, respectively (Carifo and Catlin, 1973; Copley and Egglestone, 1983; Dillon and Pauzé, 1984).

The natural occurrence of auxotrophy for amino acids is not limited to *N. gonorrhoeae*. *N. meningitidis*, another pathogen from the genus *Neisseria*, also requires several amino acids for growth (Catlin, 1973). However, many nonpathogenic *Neisseria* do not possess growth requirements for amino acids (McDonald and Johnson, 1975). Naturally-occurring auxotrophs

for a number of amino acids are also found in Legionella pneumophila (Tesh and Miller, 1983), Shigella dysenteriae (Manson and Yanofsky, 1976) as well as in the genus Lactobacillus (Hall et al., 1983). A requirement for arginine has been consistently reported for all strains of L. pneumophila (Tesh and Miller, 1983). The evolutionary significance of growth requirements in bacteria is unknown, but the conservation of these phenotypes suggests that they may have survival value in their ecological niches.

Many gonococcal auxotypes have been identified and their geographical distribution extensively studied (Carifo and Catlin, 1973; Copley and Egglestone, 1983; Dillon and Pauzé, 1984; Hendry and Stewart, 1979; Knapp et al., 1978). Gonococcal isolates which have no nutritional requirement (i.e. prototrophic) and those requiring only proline are prevalent worldwide (Sarafian and Knapp, 1989). Arginine-requiring strains of N. gonorrhoeae are also widely distributed geographically but are usually less numerous; these strains are not commonly isolated in the Far East (Knapp and Holmes, 1975; Knapp et al., 1985b; Sarafian and Knapp, 1989). Strains of N. gonorrhoeae with multiple growth requirements are commonly encountered in Canada and other countries (Sarafian and Knapp, 1989; Dillon et al., 1990).

They are often of auxotype AHU (i.e. arginine-, hypoxanthine- and uracil-requiring) or PCU (i.e. proline-, citrulline- and uracil-requiring). In Canada, these two auxotypes account for one third of all clinical isolates (Dillon et al., 1990).

The AHU strains, which started to be prevalent in the mid-1970s, account for as many as 50% of isolates in cities of the United States and Denmark (Knapp et al., 1985a; Knapp et al., 1987). AHU strains have increased susceptibility to penicillin and are resistant to the bactericidal effect(s) of human sera (Eisenstein et al., 1977). These properties have been suggested by some workers to be correlated with the frequent association of these strains with disseminated gonococcal infections (Knapp and Holmes, 1975; Morello et al., 1976; Eisenstein et al., 1977).

Strains of auxotype PCU are more commonly encountered in Canada than in other countries (Sarafian and Knapp, 1989) with a frequency of 14% of total clinical isolates (Dillon et al., 1990). Although PCU isolates were initially identified in a limited number of geographical areas, they now occur relatively frequently in the United States, Europe and Japan (Copley and Egglestone, 1983; Knapp et al., 1987; Sarafian and Knapp, 1989). PCU strains are unusual since they do not carry

any plasmid including the 4.2 kilobase (kb) cryptic plasmid found in most gonococcal isolates (Dillon and Pauzé, 1981).

Naturally-occurring N. gonorrhoeae auxotrophs have been used in co-transformation studies to ascertain linkage with other chromosomal DNA markers (Steinberg et al., 1979; Spratt et al., 1980). Total genomic DNA transformations using these strains as recipients established linkage between genes marked by arginine auxotrophy and several other gonococcal chromosomal markers (i.e. the serum resistance gene, a temperature-sensitive marker and other biosynthesis genes) (Steinberg et al., 1979; Spratt et al., 1980).

The genetic map of the gonococcal chromosome remain limited despite the genetic characterization of several virulence determinants for this micro-organism (West and Clark, 1989). This is partly attributable to the fact that N. gonorrhoeae strains do not exchange naturally chromosomal DNA by conjugation or transduction (West and Clark, 1989). The only genetic system available for this micro-organism to exchange large chromosomal DNA fragments between strains is transformation which will demonstrate linkage only if genes are within about 30 to 40 kb of each other (West and Clark, 1989). Another factor limiting the development of the

gonococcal genetic map is the difficulty to generate mutants with artificial mutagens which is attributable to the absence of error-prone repair system in *N. gonorrhoeae* (Campbell and Yasbin, 1984; West and Clark, 1989). Consequently, few linkage associations between gonococcal genetic markers have been identified by transformation (West and Clark, 1989). Some genetic loci were also identified by characterization of the gene product or by cloning (West and Clark, 1989).

1.1 Relationship between auxotrophy and gonococcal virulence

There are several lines of evidence indicating a possible role of auxotrophy in gonococcal virulence. Kenyon (1978) has shown that, during passage in a mouse, a strain of *N. gonorrhoeae* initially requiring only proline had acquired additional requirements for arginine, uracil and hypoxanthine. The mouse-derived strain possessed nutritional requirements characteristic of *N. gonorrhoeae* isolates causing disseminated gonococcal infections (i.e. auxotype AHU) (Eisenstein *et al.*, 1977). This suggested that the acquisition of these nutritional requirements may be correlated with gonococcal virulence. In fact, Eisenstein *et al.* (1977) have suggested that arginine auxotrophy may enhance the gonococcal pathogenicity of AHU strains because of their higher

susceptibility to penicillins. Auxotrophy for nutrients which are in short supply in the human circulatory system may help these strains to escape penicillin therapy, which only kills growing cells. Another hypothesis, based on the fact that N. gonorrhoeae auxotrophs may be able to escape penicillin therapy (Catlin, 1975) proposes that penicillin would reversibly inhibit a surface protein which participates in the transport of an essential amino acid (proline and arginine are possible candidates). Therefore, the presence of penicillin would shut off the supply of the compound(s) required for growth and consequently the auxotrophic gonococci would be temporarily non-susceptible to the bactericidal effect of penicillin. Subsequently, surviving gonococci would re-initiate growth when the concentration of penicillin in the tissue is sufficiently low to allow the transport of the required amino acid(s).

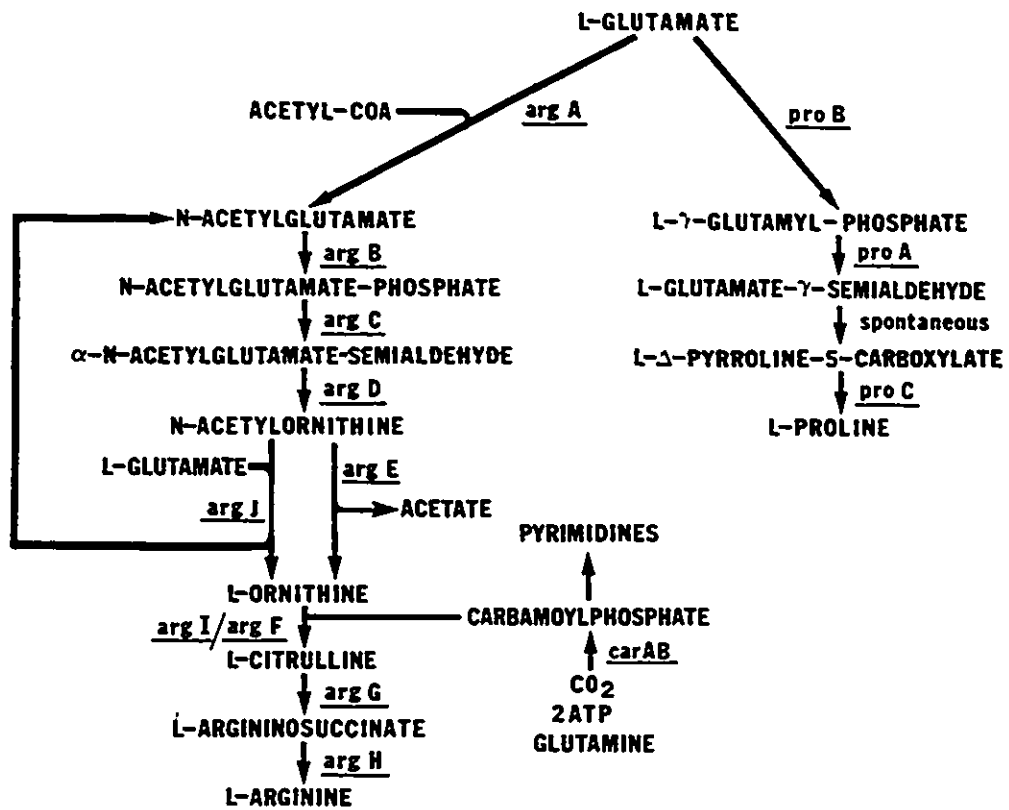
2. Arginine biosynthesis

When the operon concept was initially developed (Jacob and Monod, 1961), it was already clear that the arginine biosynthesis pathway offered a suitable model to study the molecular basis for the control of gene expression at a higher degree of complexity. This higher level of complexity results from the scattering of arginine biosynthesis genes over the chromosome in most organisms investigated and from the presence of carbamoylphosphate (CP), a precursor common to the biosynthesis of arginine and pyrimidines. Consequently, the biochemistry and the genetics of this pathway has been well studied in several procaryotes and eucaryotes over the last three decades.

2.1 Enzymatic steps

Arginine is synthesized from glutamate in eight enzymatic steps (Fig. LR-1) (Cunin, 1983). Five steps involving N-acetylated intermediates lead to ornithine. The acetylation of the amino group of glutamate prevents the cyclization of glutamate semialdehyde yielding the proline precursor pyrroline-5-carboxylate, thus keeping the two pathways separated (Fig. LR-1) (Vogel, 1970). The conversion of

Figure LR-1. Arginine and proline biosynthesis. The gene-enzyme relationships are as follows: argA = N-acetylglutamate synthetase, argB = N-acetylglutamate 5-phosphotransferase, argC = N-acetylglutamate 5-phosphate reductase, argD = N-acetylornithine aminotransferase, argE = acetylornithine deacetylase, argJ = ornithine acetyltransferase, argF and argI = ornithine transcarbamoylase, argG = argininosuccinate synthetase, argH = argininosuccinase, carAB = carbamoylphosphate synthetase, proB = glutamate kinase, proA = glutamate semialdehyde dehydrogenase and proC = 1-pyrroline 5-carboxylate reductase.



ornithine to arginine requires three additional steps, the first of which implicates CP utilization, an energy-rich metabolite which also participates in the biosynthesis of pyrimidines (Cunin, 1983). CP is synthesized from glutamine, 2ATP and CO₂ by the enzyme carbamoylphosphate synthetase (CPSase) which consists of a small and a large subunit encoded by two adjacent genes carA and carB, respectively (Fig. LR-1) (Mergeay et al., 1974; Cunin, 1983).

In procaryotes, three types of organization for the biosynthesis of CP are found (Cunin et al., 1986). The first type corresponds to organisms which use a single CPSase to produce CP for both the arginine and pyrimidine biosynthesis pathways. The CP produced is not channelled in separate pools and is therefore available for both biosynthesis. This strategy is commonly utilized by gram-negative bacteria and in particular by members of the families Enterobacteriaceae and Pseudomonadaceae (Cunin et al., 1986). The single CPSases from E. coli, S. typhimurium and Serratia marcescens are well characterized and appear very similar in structural, catalytic and regulatory properties. All three enzymes are subject to cumulative repression by arginine and uracil (Cunin et al., 1986). The single CPSase of P. aeruginosa shows much similarity with its enteric counterpart and is subject to

partial cumulative repression by arginine and pyrimidines (Cunin et al., 1986). For the gram-negative micro-organism N. gonorrhoeae, the high frequency of double auxotrophs for arginine and the pyrimidines suggests the existence of a single CPSase (Shinners and Catlin, 1982). A second type of organization is represented by B. subtilis which possesses one CPSase for each pathway (Potvin and Gooder, 1975). However, there is no strict channelling of the CP produced; therefore, strains defective in one CPSase can utilize the CP from the other pathway (Potvin and Gooder, 1975; Cunin, 1983). This situation resembles that encountered in the eucaryote Saccharomyces cerevisiae but not in other fungi where the two pools of CP are effectively channelled (Lacroute et al., 1965; Cunin, 1983). The third type of CP biosynthesis is used by lactic bacteria which appear to lack CPSase activity but possess the arginine deiminase pathway of arginine catabolism from which the CP seems to be derived (Hutson and Downing, 1968; Cunin et al., 1986).

In procaryotes, the pathway of arginine biosynthesis follows two alternative patterns which differ only by the strategies used for the acetylation and subsequent deacetylation of N-acetylornithine (Fig. LR-1). In members of the families Enterobacteriaceae and Bacillaceae, N-

acetylornithine is deacetylated via acetylornithine deacetylase (AODase) encoded by argE (Udaka, 1966; Vogel and Bonner, 1956). In N. gonorrhoeae, members of the family Pseudomonadaceae, cyanobacteria and photosynthetic bacteria, the acetyl group from N-acetylornithine is recycled by the enzyme ornithine acetyltransferase (OATase) encoded by argJ (Catlin and Nash, 1978; Hoare and Hoare, 1966; Udaka, 1966). This energetically more economical version of arginine biosynthesis is also utilized by eucaryotic organisms (Davis, 1983). AODase activity has not been detected in N. gonorrhoeae (Shinners and Catlin, 1978). This difference in the mechanism of glutamate acetylation is reflected in the targets for feedback inhibition of the arginine biosynthesis enzymes by arginine. Only the first enzyme of the pathway, acetylglutamate synthetase (AGSase), is feedback-inhibited by arginine in the so-called linear pathway utilized by enteric bacteria and bacilli, whereas the second enzyme of the pathway (i.e. acetylglutamate phosphotransferase) and sometimes the first two enzymes of the pathway are feedback-inhibited by arginine in the procaryotes which recycle the acetyl group (Vyas and Maas, 1963; Udaka, 1966; Haas et al., 1977).

The CPSase is a highly regulated enzyme. In E. coli and S. typhimurium, this enzyme is feedback-inhibited by uridylate

(UMP) (Piérard et al., 1965; Glansdorff, 1987). This inhibition is antagonized by ornithine and to a lesser extent by inosinate (Piérard, 1966). The antagonistic effect of UMP and ornithine ensure adequate supplies of CP for both the arginine and the pyrimidine biosynthesis pathways. The large subunit of the CPSase (encoded by carB) is the target for all known allosteric effectors for this enzyme (Trotta et al., 1971).

2.2 Genetic organization of arginine biosynthesis genes

The genes coding for the nine arginine biosynthesis enzymes are scattered over the chromosome in most bacteria (Cunin, 1983). This scattering is extreme in Pseudomonas aeruginosa where these genes are unlinked and spread over the chromosome (Haas et al., 1977). Only the genes carA and carB, encoding the CPSase, are closely linked in P. aeruginosa as in all procaryotes investigated to date (Abdelal and Ingraham, 1975; Gigot et al., 1980; Haas et al., 1977; Mountain et al., 1984). In E. coli, the four genes argE, argC, argB and argH are clustered (Glansdorff, 1965). This gene cluster seems characteristic of enteric bacteria since it is also present in Klebsiella pneumonia and Salmonella typhimurium (Cunin, 1983). As in E. coli, several genes of the arginine biosynthesis

pathway are clustered in Proteus species and Serratia marcescens, but in addition to argECBH, they include the gene argG (i.e. gene cluster argECBGH) (Prozesky et al., 1973; Matsumoto et al., 1975). Arginine biosynthesis genes are highly clustered in B. subtilis, for which eight arginine genes have been cloned on a 12 kb DNA fragment (Mountain et al., 1984). Furthermore, the two other arginine genes (i.e. argG and argH) are also closely linked (Mountain et al., 1984). In E. coli K-12 strains, two genes code for OTCase (i.e. argF and argI). These genes may have originated by duplication of the ancestor of argI, the only allele present in other E. coli strains and in other enteric bacteria (Cunin, 1983). The presence of two IS-1 elements flanking the argF gene in E. coli K-12, opens the possibility that this gene was translocated from a related species into E. coli K-12 (York and Stodolsky, 1981).

These findings show clearly that arginine biosynthesis genes exhibit a wide variety of patterns of gene arrangements in procaryotes. This situation contrasts with histidine or tryptophan biosynthesis pathways, in which operons have been highly conserved (Somerville, 1983; Crawford, 1975). Are evolutionary advantages provided by either of these two types of genetic organization? It has been suggested that a

scattered regulon, such as in E. coli, allows a finer tuning of gene control than clustering into a single operon (Abdelal, 1979; Cunin et al., 1986). This may be advantageous when one considers the potential interactions of arginine biosynthesis with other biosynthesis pathways and with arginine catabolism (Abdelal, 1979). It may be necessary for the bacterial cell to adjust separately the rate of synthesis of enzymes working at metabolic branch points (e.g. the single CPSase of Enterobacteriaceae) or involved in both catabolism and anabolism (e.g. the OTCase or the succinylornithine aminotransferase of Pseudomonadaceae).

2.3 Control of gene expression in arginine biosynthesis

Based on available information, procaryotes appear to be divided into two categories according to the strategy used to regulate their arginine biosynthesis (Udaka, 1966; Cunin, 1983). In Enterobacteriaceae and Bacillaceae, which do not recycle the acetyl group, arginine represses the synthesis of all arginine biosynthesis enzymes and inhibits AGSase, the first enzyme of the pathway. Bacteria which possess an OATase and recycle the acetyl group from acetylornithine to glutamate appear to rely mostly on feedback inhibition of the second enzyme (i.e. acetylglutamate phosphotransferase) by arginine

to control the pathway. In these bacteria, control of gene expression by repression appears to be absent (Cunin et al., 1986). *N. gonorrhoeae*, which is a member of this group, possesses a non-repressible CPSase (Shinners and Catlin, 1982). The other enzymes of the arginine pathway have not yet been examined in this microorganism.

In *E. coli*, the coordinate expression of the arginine biosynthesis genes, which are scattered around the chromosome (Bachmann, 1990), is achieved by transcriptional control via the arginine repressor (Cunin, 1983). The repressor (the argR gene product) binds in the presence of arginine to similar operator sequences which are found at every arg locus investigated to date (Cunin, 1983; Glansdorff, 1987). Such organizational units, in which one regulatory gene controls the expression of unlinked but functionally related structural genes, are called regulons (Maas and Clark, 1964). It is not yet known whether argS, the structural gene for arginyl-transfer RNA (tRNA) synthetase, belongs to the regulon (Cunin et al., 1986). The genes implicated in arginine transport are apparently not part of the *E. coli* regulon (Cunin et al., 1986).

Available DNA sequences for *E. coli* K-12 arginine

biosynthesis genes include the complete sequence of genes carAB (Piette et al., 1984; Nyunoya and Lusty, 1983), argI (Bencini et al., 1983), argR (Cunin et al., 1983; Glansdorff, 1987) and argA (Brown et al., 1987), as well as the sequence for the control regions of genes argECBH (Piette et al., 1982), argD and argG (Cunin et al., 1986). A feature common to all E. coli K-12 arginine biosynthesis genes sequenced so far is the presence of one (in argR) or two adjacent (in argE, argI, argECBH, argD, argG and carAB) conserved 18 base-pair-long sequences showing dyad symmetry and overlapping the promoters to various extents (Cunin et al., 1983; Glansdorff, 1987). These sequences called "ARG boxes" were shown to constitute operator sites (Cunin et al., 1983; Van Vliet et al., 1984). Steric hindrance between the repressor and the RNA polymerase appears to be the basis of the repression response (Cunin et al., 1986). Factors such as the base composition of ARG boxes, their number as well as their position relative to the promoter are thought to be at the basis of differential repressor effectiveness in the arginine regulon (Cunin, 1983).

In E. coli, the promoter-operator interaction is complex with the argECBH gene cluster and with the carAB operon. The argECBH cluster is a divergent operon consisting of two facing arms, argE and argCBH, with an internal operator region

flanked by two promoters (Glansdorff, 1987). The binding of the repressor to the single operator site prevents the binding of the RNA polymerase at both promoters thereby allowing repression of both arms of the divergent operon (Cunin *et al.*, 1986; Glansdorff, 1987). For the carAB operon, which is cumulatively repressed by arginine and the pyrimidines, transcription is initiated at two tandem promoters (P1 and P2) which are regulated differently (Fig. LR-2); expression from P1, the upstream promoter, is prevented in the presence of an excess of pyrimidines, while P2 is repressed by arginine (Piette *et al.*, 1984). In *E. coli*, the mechanism of regulation for the upstream promoter P1 by the pyrimidines is unknown (Glansdorff, 1987). P2, the downstream promoter, is regulated specifically by arginine. P2 overlaps a tandem of two ARG boxes separated by three nucleotides which act as an operator site as for the other genes of the arginine regulon (Fig. LR-2) (Glansdorff, 1987). The transcription start points of P1 and P2 are only 67 bp apart which corresponds approximately to the length of DNA actually protected by RNA polymerase against DNAase I (Fig. LR-2) (Charlier *et al.*, 1988). Nuclease S1 mapping experiments have shown that transcription initiated upstream at P1 is able to proceed into the P2 region (and beyond) even when arginine represses P2 (Piette *et al.*, 1984). It has been suggested that P2 operator-arginine repressor

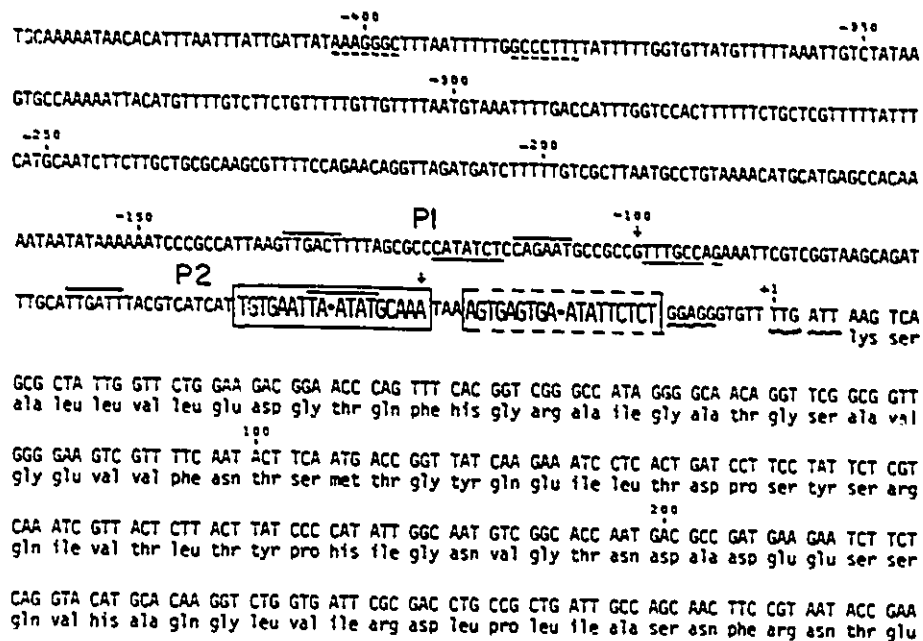


Figure LR-2. Structure of the control region for the carAB operon in E. coli. The -35 and -10 regions of the two promoters (P1 and P2) are indicated by a solid overline. Transcriptional starts are shown by the arrows. Operator modules (ARG boxes) are framed. Wavy lines indicate Shine-Dalgarno sequences and possible translational starts for carA.

complex is destabilized by RNA polymerase binding at P1 or transcription from P1 (Charlier *et al.*, 1988). Other interesting features of the *E. coli carAB* operon cumulative repression have been observed: (i) Repression by arginine at P2 is more intense when P1 is also repressed by pyrimidines (Charlier *et al.*, 1988). (ii) Under conditions of pyrimidine shortage, the synthesis of CPSase is only weakly repressed by arginine (Piérard *et al.*, 1976). This situation could be explained by an unknown pyrimidine-specific factor which cooperates with the arginine repressor to enhance repression at P2 (Glansdorff, 1987). The *S. typhimurium carAB* operon is also regulated via two tandem promoters in an apparently similar fashion (Kilstrup *et al.*, 1988). The physiological significance of dual promotor structure is particularly clear for the *carAB* operon (Glansdorff, 1987). At the level of gene expression it complements the regulatory effects exerted on the enzyme itself which were described earlier (Literature review, Section 2.1). It constitutes an elegant alternative to the existence of independently controlled CPSases as in fungi and *B. subtilis* (Glansdorff, 1987).

Besides *argR*-mediated control of DNA transcription, the nucleotide ppGpp, chemical messenger of the stringent response, influences in a positive way the expression of *arg*

genes (Cunin et al., 1986). The positive effects of ppGpp, which have also been observed for other amino acid biosynthesis genes, would be exerted at the level of translation (Gallant, 1979; Cunin et al., 1986). Sequence data available for most E. coli K-12 arginine biosynthesis genes indicates that attenuation control, which is widely used in amino acids regulation (Yanofsky and Kolter, 1982), does not seem to play a role in the arg regulon (Cunin, 1983; Glansdorff, 1987).

It appears that an extensive repression control by arginine comparable to that encountered in E. coli is also present in the enteric bacteria S. typhimurium, Klebsiella aerogenes, Serratia marcescens and Proteus sp as well as in several Bacillus species (Cunin et al., 1986). In S. typhimurium, the operator sites for the arginine biosynthesis genes are necessarily similar to those from E. coli since normal repression is restored in S. typhimurium by the E. coli repressor (Gardner et al., 1983). In B. subtilis, some sequence similarity was detected between the argC regulatory region and the E. coli ARG boxes (North et al., 1989). This similarity was shown to be functional in vivo since the B. subtilis repressor is able to regulate the expression of E. coli arginine biosynthesis genes (Smith et al., 1989).

3. Proline biosynthesis

Only little information on proline biosynthesis accumulated during the 1960s and 1970s, when studies on the biochemistry and regulation of amino acids biosynthesis flourished (Leisenger, 1987). This situation, which contrasts with arginine biosynthesis, is attributable to difficulties in assaying the first two enzymes of proline biosynthesis (Csonka and Baich, 1983; Leisenger, 1987). Proline biosynthesis was therefore not suitable for studies on gene expression and the main challenge in this pathway was the understanding of its enzymology.

3.1 Enzymatic steps and regulation of proline biosynthesis

In bacteria, the proline biosynthesis pathway from glutamate involves four steps of which only three are enzymatically catalyzed (Csonka and Baich, 1983; Fig. LR-1). Initially, glutamate is phosphorylated in an ATP-dependent reaction by the γ -glutamate kinase (Csonka and Baich, 1983; Leisenger, 1987). Subsequently, glutamyl-phosphate is reduced yielding glutamate-semialdehyde. The third reaction of the pathway which is the cyclization of glutamate semialdehyde proceeds spontaneously (Csonka and Baich, 1983). The final

step consists in the reduction of pyrroline-5-carboxylate to produce L-proline (Csonka and Baich, 1983; Leisenger, 1987). The first two reactions in proline biosynthesis resemble the initial steps of arginine biosynthesis in which N-acetylglutamate is converted to N-acetylglutamate semialdehyde (Adams and Frank, 1980; Csonka and Baich, 1983). The acetylation of glutamate in arginine biosynthesis prevents the cyclization of glutamate semialdehyde required in proline biosynthesis, thus segregating both pathways immediately at the first step. Feedback inhibition of γ -glutamate kinase, the first enzyme of the pathway, appears to be the major control mechanism for proline biosynthesis in *E. coli* (Csonka and Baich, 1983; Leisenger, 1987). Available data indicate the absence of repression and attenuation control in this pathway (Leisenger, 1987).

Proline synthesis from glutamate has also been demonstrated in a variety of animal tissues and cell lines (Csonka and Baich, 1983). In a number of Gram-positive bacteria, fungi and animal tissues, proline can also be synthesized from arginine through ornithine (Adams and Frank, 1980; Csonka and Baich, 1983). In many cells, both glutamate and ornithine are precursors of proline (Csonka and Baich, 1983).

3.2 Genetics of proline biosynthesis

In E. coli, the genes encoding the three enzymes implicated in proline biosynthesis are proB, proA and proC (Fig. LR-1). The proB and proA genes are located at minute 6 and proC at minute 9 of the E. coli linkage map (Bachmann, 1990). In S. typhimurium, the three proline genes are arranged in a similar fashion at the same position on the linkage map (Sanderson and Hartman, 1978).

The E. coli proBA genes have been cloned on a 3.0 kb PstI fragment which has been entirely sequenced (Deutch et al., 1984). The DNA sequence analysis revealed that the two genes were organized in an operon in which proB precedes proA. The E. coli proC gene has also been cloned and sequenced (Deutch et al., 1982). This gene has not yet been cloned in other procaryotes. The proBA cluster has been cloned in other procaryotes including N. gonorrhoeae by interspecies complementation of E. coli mutants (Maham and Csonka, 1983; Stein et al., 1984; Lee et al., 1985; Datta et al., 1987). P. aeruginosa represents the only procaryote investigated to date in which proA and proB genes are unlinked (Holloway et al., 1979).

4. Gene transfer in *N. gonorrhoeae*

N. gonorrhoeae possesses transformation and conjugation systems to exchange DNA (Cannon and Sparling, 1984). In nature, transformation represents the primary means of transfer of chromosomal genes between gonococcal strains (Cannon and Sparling, 1984; Biswas et al., 1989b). It has been suggested that transformation may be the genetic system that results in the antigenic variation of several gonococcal surface components (Meyer, 1987; Seifert et al., 1988). Conjugation in *N. gonorrhoeae* results in the mobilization of antibiotic resistance plasmids (Biswas et al., 1989b). However, chromosomal genes cannot be transferred by conjugation (Cannon and Sparling, 1984). No bacteriophage-mediated mechanisms of gene transfer between gonococci have been reported.

4.1 Gonococcal transformation

Many bacteria are naturally competent for the adsorption and uptake of free DNA into a deoxyribonuclease-resistant form (Smith et al., 1981). However, *N. gonorrhoeae* cells are unique in that they are competent for transformation throughout their

entire growth cycle (Biswas et al., 1989b). The phenotypic expression of gonococcal competence, which is restricted to piliated cells (Sparling, 1966), requires a utilizable energy source and cations (Biswas et al., 1977). Gonococci take up only homologous chromosomal DNA into a deoxyribonuclease-resistant form (Dougherty et al., 1979). This occurs since gonococcal genomic DNA is selectively taken up in a sequence-specific way (Graves et al., 1982). Uptake of gonococcal chromosomal DNA was shown to be more efficient than that of gonococcal plasmid DNA (Graves et al., 1982). This suggests that gonococcal chromosomal DNA contains either a more efficient uptake sequence or a higher frequency of uptake sequences than the gonococcal plasmids. Putative surface receptors which may be implicated in the specific binding of homologous DNA have not yet been identified (Biswas et al., 1989b). The correlation between the presence of pili and competence for transformation suggests that pili may be involved in DNA uptake although there is no evidence that pili bind DNA in vitro (Mathis and Scocca, 1984). The isolation of transformation-deficient pilated *N. gonorrhoeae* (Biswas et al., 1989a) may indicate that pili may not be sufficient for DNA uptake in gonococci. In addition to the preferential internalization of homologous chromosomal DNA, some gonococcal DNA sequences are taken up more efficiently than others.

Graves et al. (1982) showed that a portion of the gonococcal cryptic plasmid was preferentially internalized suggesting the presence of specific uptake sequences as in Haemophilus influenzae (Sisco and Smith, 1979). More recently, Goodman and Scocca (1988) identified a 10-bp sequence (5'-GCCGTCTGAA-3') common to chromosomal DNA fragments of N. gonorrhoeae which was able to competitively inhibit transformation. Furthermore, this 10-bp sequence was sufficient to competitively inhibit transformation when cloned into the non-competing pBR322 (Goodman and Scocca, 1988). These findings suggest that this gonococcal sequence plays a role similar to the 11-bp recognition sequence responsible for DNA uptake in H. influenzae. Interestingly, the gonococcal recognition sequences for transformation are contained within the transcriptional termination sequences of all gonococcal genes investigated to date (Goodman and Scocca, 1988). This may be a general phenomenon by which the DNA uptake apparatus is efficiently preserved.

Transforming chromosomal DNA enters the gonococcal cell as a double-stranded molecule and remains principally double stranded inside the cell until homologous recombination results in transformation (Biswas and Sparling, 1981). When circular plasmid DNA from an isogenic strain is used in

transformation, a significant proportion of the transformants contains plasmids which are either larger or smaller than the parental plasmid (Biswas et al., 1989b). Entering plasmid DNA is linearized and cleaved randomly to smaller fragments by endonucleases. The cleavage of entering DNA is not mediated by a restriction endonuclease since it is observed in entirely isogenic backgrounds (Biswas et al., 1989b). These fragments are then re-ligated to form deleted or larger plasmids (Biswas et al., 1982; Biswas et al., 1986). However, if the recipient cell contains an homologous replicon, plasmid transformation is more efficient and no deletions or insertions are observed among plasmids recovered from transformants (Biswas et al., 1982; Biswas et al., 1986). This is thought to be due to marker rescue by homologous recombination of fragmented plasmids by the resident replicon. This phenomenon probably requires the gonococcal Rec function, since Rec⁻ strains of N. gonorrhoeae are non-transformable with plasmid or chromosomal DNA (Kooimey and Falkow, 1987).

4.2 Plasmids of N. gonorrhoeae

With the exception of PCU isolates and some wild-type strains, N. gonorrhoeae strains contain a 4.2 kb cryptic plasmid of unknown function (Roberts et al., 1979; Dillon and

Pauzé, 1981). It has been shown that large segments of the 4.2 kb cryptic plasmid or the entire plasmid can be integrated into the gonococcal chromosome (Hagblom et al., 1986). It has been suggested that repeated sequences identified in the plasmid were involved in site-specific recombination with the chromosome (Hagblom et al., 1986).

Isolates of N. gonorrhoeae with plasmids encoding β -lactamase were simultaneously reported for the first time in 1976 in several countries (Ashford et al., 1976; Bowmer, 1976; Phillips, 1976; Percival et al., 1976). However, these plasmids are now endemic in isolates from certain regions of North America, the Caribbean, Europe, Africa and Asia (Roberts, 1989). Originally, two plasmids of 7.2 and 5.1 kb were recognized; each carried the TEM β -lactamase gene and 40% of Tn2 transposon (Roberts et al., 1977). A number of naturally-occurring deletion- or insertion-derivatives of the 7.2 kb plasmid have been described (Dillon and Yeung, 1989).

A small percentage of gonococcal isolates carries a larger 39.2 kb conjugative plasmid (Roberts and Falkow, 1977). The plasmid efficiently mobilizes itself and the smaller β -lactamase plasmids between strains of N. gonorrhoeae. However, it does not appear to mobilize the transfer of chromosomal

markers (Biswas et al., 1989b).

Recently, *N. gonorrhoeae* strains resistant to high concentrations of tetracycline were isolated and were shown to carry the streptococcal tetM determinant on a 40.3 kb plasmid (Morse et al., 1986). Although it was initially proposed that this plasmid arose from the insertion of the tetM determinant into the 39.2 kb gonococcal conjugative plasmid (Morse et al., 1986), recent evidence shows that the conjugative plasmid is not of gonococcal origin (Gascoyne et al., 1990).

In addition to the naturally-occurring plasmids which have been isolated from *N. gonorrhoeae*, several derivatives of some of these plasmids have been constructed through recombinant DNA techniques. These recombinant plasmid DNAs have been used to study their replication (Dillon and Yeung, 1989), the mechanisms of plasmid DNA uptake during transformation (Burnstein et al., 1988) and conjugation (Tenover et al., 1985) as well as for use in cloning gonococcal genes (Stein et al., 1984). A bifunctional shuttle vector pLES2, which is able to replicate in *N. gonorrhoeae* and *E. coli* has been constructed by using one of the gonococcal naturally-occurring β -lactamase plasmids (Stein et al., 1983). This shuttle vector transforms *N. gonorrhoeae* efficiently when

it contains gonococcal genomic DNA (Stein et al., 1984). To date, this is the only cloning vector which has been used to introduce cloned genes (i.e. proline biosynthesis genes) into N. gonorrhoeae.

4.3 DNA restriction and modification systems in N. gonorrhoeae

N. gonorrhoeae can efficiently exclude foreign DNA because it possesses multiple restriction barriers (Davies, 1989). In fact, gonococci possess at least seven restriction modification systems and it appears that most individual gonococcal strains possess at least six of these systems (Davies, 1989). Any variations in these systems, within the species, may limit genetic exchange between gonococcal strains. One such barrier to intraspecies genetic exchange, attributable to the lack of one restriction modification system, has been directly demonstrated with DNA transferred by transformation (Stein et al., 1988). The major variation in the restriction modification systems between strains of N. gonorrhoeae seems to be in the ability to produce the adenine methylase which recognizes the sequence 5'-GATC-3' (Duff and Davies, 1988; Davies, 1989). It is estimated that approximately 30% of gonococcal strains have the ability to produce the adenine methylase. The existence of a mutually

exclusive set of restriction systems which recognize methylated or unmethylated versions of the sequence 5'-GATC-3' appears to be the most important barrier to the free flow of genetic information within the species (Davies, 1989).

4.4 Introduction of cloned genes in N. gonorrhoeae

Most studied gonococcal genes have been cloned and characterized in E. coli mainly because the yield of N. gonorrhoeae mutants obtained by chemical mutagenesis is very poor (Campbell and Yasbin, 1984). An alternative strategy to create N. gonorrhoeae mutants is to apply the technique of in vitro mutagenesis using cloned gonococcal genes and subsequently to introduce the mutated genes into the gonococcal chromosome by homologous recombination (Biswas et al., 1989b; Stein, 1989). Koomey and colleagues used insertions of the β -lactamase gene to inactivate the gonococcal IgA protease (Koomey and Falkow, 1987) and the recA genes (Koomey et al., 1982). The ampicillin resistant transformants carried the mutated cloned gene which had replaced the wild-type gene by transformation-mediated marker rescue (Koomey et al., 1982). Recently, Seifert et al. (1989) have developed a system of shuttle mutagenesis for introducing transposon insertions into gonococcal genes cloned into E.

coli. The transposon-mutagenized gonococcal DNA, which carries a chloramphenicol resistance gene, is introduced into the gonococcal chromosome by transformation followed by homologous recombination. This technique allows the construction of isogenic strains differing only in the gene of interest.

Conjugation appears to be more suitable than transformation to introduce recombinant plasmids isolated from E. coli into N. gonorrhoeae (Stein et al., 1988). This is due to differing susceptibilities to the restriction barrier of the incoming plasmid DNA during these two genetic exchange processes (Davies, 1989). DNA is single-stranded as it enters the recipient cell during conjugation and is therefore resistant to the action of restriction endonucleases. On the other hand, DNA taken up as double-stranded molecules during transformation can be efficiently restricted. In fact, the shuttle vector pLES2 is unstable in most N. gonorrhoeae strains when introduced into the cell by transformation (V.L. Clark, personal communication). Piffaretti et al. (1988) have shown that a plasmid (pUB307) is able to mobilize gonococcal β -lactamase plasmids as well as the cloning vector pLES2 from E. coli to N. gonorrhoeae. Therefore, through the use of pUB307 and pLES2, it should be possible to efficiently mobilize by conjugation genes cloned in E. coli into N.

gonorrhoeae. An improved N. gonorrhoeae-E. coli shuttle vector, which is derived from a gonococcal β -lactamase plasmid and also mobilizable by pUB307, has recently been developed in our laboratory (Gauthier and Dillon, unpublished data).

RESEARCH OBJECTIVES

Most Neisseria gonorrhoeae strains have accumulated spontaneous mutations in genes implicated in a variety of biosynthesis pathways. A possible association of auxotrophy with gonococcal virulence has been suggested by several investigators. However, the knowledge about the genetics of these pathways in N. gonorrhoeae is very limited. Only two gonococcal biosynthesis genes (i.e. the proline biosynthesis genes proA and proB) have been cloned. Since the most frequently encountered auxotrophic defects are found in the arginine and the proline biosynthesis pathways, the focus of this study has been on these two pathways.

The first objective of this study was to characterize, at the molecular level, the genes involved in arginine and proline biosynthesis in N. gonorrhoeae. The complementation of E. coli mutants defective in the various genes of arginine and proline biosynthesis with clones of N. gonorrhoeae genomic DNA should provide fundamental knowledge about these two biosynthesis pathways and would elucidate the following questions: Are the gonococcal arginine biosynthesis genes expressed in E. coli? Can an AODase gene defect in E. coli be complemented by the OATase gene from N. gonorrhoeae? Is the

OATase gene from N. gonorrhoeae able to complement a defect in the E. coli AGSase gene?

The comparison of the genetic organization and of the nucleotide sequence of arginine and proline biosynthesis genes with that encountered in other bacterial species should provide valuable information on the evolution of N. gonorrhoeae thereby contributing to an almost non-existent data base. The comparison of the gene arrangement for the arginine biosynthesis genes is of particular interest since this pathway exhibits a wide variety of patterns of gene arrangements in procaryotes. Furthermore, this information should contribute to the knowledge of the limited genetic map for the chromosome of N. gonorrhoeae.

The third objective of this study was to examine the arginine and proline biosynthesis gene defects in naturally-occurring auxotrophs of N. gonorrhoeae. Gonococcal strains exhibit considerable diversity in response to environmental pressures (e.g. antibiotics). The effect of these pressures is difficult to evaluate due to the limitations of the typing systems available for N. gonorrhoeae. Therefore, the characterization of the mutations in the arginine and the proline biosynthesis genes for a collection of N. gonorrhoeae

strains from Canadian and other sources may uncover further heterogeneity among populations of *N. gonorrhoeae* auxotrophs. Furthermore, specific mechanisms of mutagenesis have not yet been described in the gonococcus. The possible role of the gonococcal cryptic plasmid in the inactivation of arginine biosynthesis genes is investigated.

Finally, the potential use of electroporation as a novel method to introduce plasmid DNA into gonococcal cells is examined since natural transformation in *N. gonorrhoeae* is very inefficient with plasmid DNA.

CHAPTER ONE

CLONING AND ORGANIZATION OF SEVEN ARGININE
BIOSYNTHESIS GENES FROM NEISSERIA GONORRHOEAE

INTRODUCTION

Arginine is synthesized from glutamate in eight enzymatic steps (Fig. LR-1) (Cunin, 1983). In procaryotes, the pathway of arginine biosynthesis follows two alternative patterns which differ only by the strategies used for the acetylation and subsequent deacetylation of acetylornithine (Fig. LR-1, step 5). In Enterobacteriaceae and Bacillaceae, acetylornithine is deacetylated via acetylornithine deacetylase (AODase) encoded by argE (Vogel and Bonner, 1956; Udaka, 1966). In N. gonorrhoeae, Pseudomonadaceae, cyanobacteria and photosynthetic bacteria, the acetyl group from acetylornithine is recycled by ornithine acetyltransferase (AOTase) encoded by argJ (Hoare and Hoare, 1966; Udaka, 1966; Catlin and Nash, 1978). This energetically more economical version of arginine biosynthesis is also present in eucaryotic organisms (Davis, 1983).

The gonococcus has accumulated spontaneous mutations in genes encoding enzymes implicated in a variety of biosynthesis pathways (Carifo and Catlin, 1973; Catlin, 1977). Strains of N. gonorrhoeae having defects in the arginine biosynthesis pathway can account for 40 to 60% of all clinical isolates (Carifo and Catlin, 1973; Copley and Egglegstone, 1983; Dillon

and Pauzé, 1984). In *N. gonorrhoeae*, arginine and uracil auxotrophy are frequently associated (Copley and Egglestone, 1983; Dillon and Pauzé, 1984). A defect in the carbamoylphosphate synthetase (CPSase) genes (*carA* and *carB*) may account for this dual requirement since carbamoylphosphate (CP) is an intermediate in both the arginine and the pyrimidine biosynthesis pathways (Shinners and Catlin, 1982). Several investigators have shown that arginine-requiring strains of *N. gonorrhoeae* can be differentiated on the basis of growth requirements, genetic complementation and enzyme defects (Catlin and Nash, 1978; Shinners and Catlin, 1978; Steinberg *et al.*, 1979; Spratt *et al.*, 1980; Shinners and Catlin, 1982). However, the genes involved in arginine biosynthesis have never been studied at the molecular level in this microorganism.

The following experiments report the cloning of seven arginine biosynthesis genes from *N. gonorrhoeae* by complementation of *E. coli* mutants. Clones complementing defects in *argA*, *argB*, *argE*, *argG*, *argIF*, *carA* and *carB* were isolated. The organization of those genes and their ability to hybridize to the corresponding *E. coli* gene were investigated.

MATERIALS AND METHODS

Bacterial strains and plasmids. E. coli and N. gonorrhoeae strains are listed in Table 1-1. A non arginine-requiring strain of Pseudomonas aeruginosa was kindly supplied by E.P. Ewan (Laboratory Centre for Disease Control, Ottawa, Ontario).

The lambda lysogen MI178 (defective in carA) was cured by U.V. irradiation as previously described (Lederberg and Lederberg, 1952) in order to obtain a derivative sensitive to infection by the bacteriophage lambda. After irradiation, cells were grown for 2 h at 37°C with gentle shaking in tryptic soy broth (TSB) (Difco Laboratories, Detroit, Michigan) containing Lambdasorb phage adsorbant (Promega Biotec, Madison, WI) to neutralize phages released during induction. After growing the cells on tryptic soy agar (TSA), several thousand colonies were tested for sensitivity to bacteriophage lambda (Lederberg and Lederberg, 1952). Strain FP178, susceptible to lambda infection, was isolated. This strain was phenotypically identical to the parental strain MI178 except that it was unable to utilize maltose as determined by the ability of strain FP178 to grow on a set of minimal media with different contents of amino acids,

Table 1-1. Bacterial strains.

^a For all E. coli arginine auxotrophs, the arginine biosynthesis gene defect was verified by growing the cells on a set of media containing the various intermediates of arginine biosynthesis.

^b Strain N166 is defective in both E. coli K-12 ornithine transcarbamoylase genes (argI and argF). For this reason, the arginine gene defect of this strain was designated argIF.

Organisms and strains	Relevant genotype	Source
<u>E. coli</u> arginine auxotrophs ^a		
W3421	F ⁻ , <u>argA21</u> , λ^- , λ^s	B.J. Bachmann
N166 ^b	Hfr(H), <u>argI67</u> , $\Delta(\text{proB-argF-lac})$, λ^- , λ^s	B.J. Bachmann
UQ27	F ⁻ , <u>argC75</u> , <u>proS127</u> , λ^- , λ^s	B.J. Bachmann
NK5992	F ⁻ , <u>argA81::Tn10</u> , λ^- , λ^s	B.J. Bachmann
30SOMA4	Hfr(H), <u>argB62</u> , λ^- , λ^s	N. Glansdorff
JEF8	Hfr(P4X), <u>carB8</u> , <u>thr-31</u> , <u>metB1</u> , λ^- , λ^s	B.J. Bachmann
AT753	F ⁻ , <u>argH1</u> , <u>ilv-1</u> , <u>metB1</u> , λ^- , λ^s	B.J. Bachmann
AB663	Hfr(AB312), <u>argH1</u> , <u>purF1</u> , λ^- , λ^s	B.J. Bachmann
AB1157	F ⁻ , <u>argE3</u> , <u>thr-1</u> , <u>leuB6</u> , $\Delta(\text{gpt-proA})$ 62, <u>hisG4</u> , λ^- , λ^s	B.J. Bachmann
W3679	F ⁻ , <u>argC24</u> , λ^- , λ^s	B.J. Bachmann
AT3141	Hfr(AB312), <u>argD37</u> , <u>leuB6</u> , <u>proA30</u> , <u>thr-1</u> , λ^- , λ^s	B.J. Bachmann
MI178	Hfr(P4X), <u>carA178</u> , <u>thr-31</u> , <u>metB1</u> , λ^- , λ^r	B.J. Bachmann
FP178	Hfr(P4X), <u>carA178</u> , <u>thr-31</u> , <u>metB1</u> , λ^- , λ^s	This work
MN42	Hfr, <u>metB</u> , $\Delta(\text{ppc-argECBH})$, λ^- , λ^s	N. Glansdorff
Other <u>E. coli</u>		
NM539	<u>supF</u> , <u>hsdR</u> , (P2 <u>cox3</u>)	A.M. Frischauf
JM240	<u>proC47</u> , <u>cys-54</u> , λ^- , λ^s	B.J. Bachmann
<u>N. gonorrhoeae</u>		
CH811	Prototrophic, plasmid-free	J.R. Dillon

arginine biosynthesis precursors and sugar. Three plasmids, containing cloned *E. coli* arginine biosynthesis genes, were kindly provided by R. Cunin (Department of Microbiology, University of Brussels, Belgium). Plasmid pMC7 (Tc^r) contained the argECBH gene cluster on a 9.5 kb EcoRI fragment (Crabeel *et al.*, 1979), pMC50 (Ap^r) carried the carAB operon on a 5.6 kb HindIII fragment (Piette *et al.*, 1984) and pAI1 (Ap^r) contained the argI gene on a 2.5 kb EcoRI-HindIII fragment (Piette *et al.*, 1982). The identity of the three plasmids was verified by a limited restriction endonuclease analysis and by their ability to complement by transformation arginine gene defects in *E. coli*.

Media and bacterial growth conditions. *N. gonorrhoeae* strain CH811 was grown on GC medium base (GCMB) (Difco) supplemented with Kellogg's defined supplement as modified by Dillon (1983) in a humid environment in the presence of 5% CO₂ at 35°C for 18-24 h. *E. coli* was grown in TSB or agar using TSA or NZY medium (10 g/L casamino acids (Difco), 5 g/L yeast extract (Difco), 10 mM MgCl₂ and 85 mM NaCl) at 37°C for 18-24 h.

The basic Tris-maleate medium (TMM) (Paranchych, 1966) supplemented with 0.5% glucose, 10mM MgSO₄, 100 mg/L of

required amino acids or other nutrients and with 5 mg/L of thiamine was used as synthetic growth medium for E. coli. Minimal agar medium contained 1.5% of Bacto agar (Difco). Broth cultures of E. coli were grown in a water bath at 37°C with vigorous shaking. Prior to infection with bacteriophages, E. coli cells were grown to mid-log phase in NZY broth or liquid minimal media, both supplemented with 0.5% maltose. Antibiotics (Sigma Chemical Co., St-Louis, MO) were used, as appropriate, for the maintenance of plasmids in the following concentrations: ampicillin, 100 mg/L and tetracycline, 15 mg/L.

DNA preparation. N. gonorrhoeae and E. coli cells were lysed with the detergent Triton-X100 (Sigma Chemical Co., St. Louis, Missouri) as described by Dillon et al. (1985). Bacterial cells were harvested from eight overnight plate cultures on GCMB for N. gonorrhoeae or from five overnight plate cultures on TSA for E. coli. Cells were suspended in 10 mL of 30 mM Tris-HCl [pH 8.0], 50 mM EDTA, 5 mM NaCl. Both chromosomal and plasmid DNA were purified from the lysates by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients (Maniatis et al., 1982). The tubes were centrifuged at 55,000 rpm for 20 h at 10°C using a Beckman Type-65 rotor in a L8-M ultracentrifuge (Beckman). Fractions

containing chromosomal or plasmid DNA were collected through an 18 gauge needle (Maniatis et al., 1982). After removing the ethidium bromide with three successive isobutanol extractions, the DNA was precipitated with 95% ethanol and then washed once with 70% ethanol. The DNA preparations were digested with proteinase K (Boehringer Mannheim Biochemicals, Dorval, Québec), extracted with phenol-chloroform and precipitated with ethanol (Maniatis et al., 1982). After two ethanol washes, the DNA was resuspended in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and stored at 4°C or -20°C. Genomic DNA was isolated from N. gonorrhoeae strain CH811 and from E. coli strain JM240. Recombinant DNA was isolated from phage lysates by using Lambdasorb phage adsorbent (Promega Biotec) using the protocol provided by the manufacturer. Phage lysates were prepared using the plating procedure described by Davis et al. (1980). After confluent lysis, plates were overlayed with 5 mL of cold lambda diluent (10 mM Tris [pH 7.5] and 10 mM MgSO₄) and kept overnight at 4°C. The overlay solution free of cellular debris was used as phage stock. When necessary, lambda DNA was further purified by centrifugation in cesium chloride-ethidium bromide gradients (Maniatis et al., 1982). DNA concentrations were determined by ultraviolet absorption spectroscopy at 260 nm using a Bausch and Lomb Spectronic 1001 spectrophotometer (Fisher Scientific Co., Ottawa, Ontario).

Restriction endonuclease digestion and electrophoresis. Restriction endonucleases were obtained from Boehringer Mannheim or Bethesda Research Laboratories (Burlington, Ont., Canada) and were used according to the instructions of the manufacturer. Electrophoresis of DNA was performed in tris-acetate buffer (Dillon et al., 1985) using 0.8% agarose (Pharmacia Agarose NA, Dorval, Québec, Canada) gels in a horizontal submarine apparatus at approximately 15 mA/cm. For restriction analysis, HindIII and XhoI fragments of lambda DNA were used as molecular weight standards.

Fractionation of N. gonorrhoeae chromosomal DNA. Conditions to achieve the maximum yield of Sau3A fragments of N. gonorrhoeae chromosomal DNA ranging in size from 10 to 20 kb were determined (Maniatis et al., 1982). The optimal Sau3A concentration was of approximately 0.1 unit/ μ g of genomic DNA for digestions performed during 1 h at 37°C. For the large-scale preparation, 60 μ g of gonococcal genomic DNA was partially digested under those conditions. Sau3A-digested DNA was dephosphorylated by treatment with calf intestinal alkaline phosphatase (Boehringer Mannheim) (Frischauf et al., 1983). The DNA preparation was extracted with phenol-chloroform, precipitated with ethanol and resuspended in TE buffer. Subsequently, genomic DNA was fractionated by

centrifugation through potassium acetate gradients (Maniatis et al., 1982). The gradients were centrifuged at 15,000 rpm for 16 h at 15°C in a Beckman SW41 rotor. Fractions of 0.25 mL were collected through an 18 gauge needle inserted into the bottom of the tube. Samples of 15 μ L from each of these fractions were analyzed by agarose gel electrophoresis. Fractions containing fragments ranging in size from 10 to 20 kb were pooled, precipitated with ethanol, washed twice with 70% ethanol and resuspended in TE buffer at a concentration of approximately 500 ng/ μ L.

Cloning protocol. Fragments of 10 to 20 kb of N. gonorrhoeae chromosomal DNA (0.5 μ g) were ligated into the BamHI site of the lambda cloning vector EMBL4 (1 μ g) (kindly provided by Dr. D. Johnson, Dept. of Biology, University of Ottawa, Ontario) using T4 DNA ligase (Frischauf et al., 1983). The ligation was performed at 16°C overnight in 10 μ L of ligation buffer (0.66 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 5 mM dithiothreitol and 1 mM ATP) with 2 units of ligase. The extent of ligation was monitored by agarose gel electrophoresis (Rodriguez and Tait, 1983). The ligation was considered complete when the 2 bands corresponding to the arms of the vector had disappeared. Subsequently, the ligation mixture was in vitro packaged using commercially available

packaging extracts (Packagene from Promega Biotec). The packaging was performed according to instructions provided by the manufacturer. The packaged DNA was titrated on the restrictive host E. coli NM539. Several dilutions of the bacteriophage in lambda diluent were tested on a single NZY agar plate. Drops of 10 μ L were spotted onto plates inoculated with the host cells in soft agar. Subsequently, the library of recombinant bacteriophages was amplified as described by Maniatis et al. (1982). Host cells were infected with approximately 1000 recombinant bacteriophages and plated onto large (15 x 150 mm) NZY agar plates. Twelve bacteriophage stocks were prepared with the $\approx$ 12,000 bacteriophages obtained from the library. All stocks were pooled and kept frozen in 15% glycerol at -70°C (Davis et al., 1980). This material provides a total of approximately 25 N. gonorrhoeae genomic libraries (N. gonorrhoeae genome size is estimated at 1500 kb (Kingsbury, 1969)).

Complementation of E. coli arginine auxotrophs. Recombinant bacteriophages containing arginine biosynthesis genes were selected by lytic complementation of E. coli K-12 arginine auxotrophs sensitive to lambda bacteriophage infection as described by Davis et al. (1980). The lytic selection was performed with plates of minimal agar medium,

lacking arginine but supplemented with all other required nutrients and 10 mM MgSO_4 . Cells (2×10^9) of the E. coli K-12 arginine defective mutant, grown in liquid minimal medium, were infected with 2×10^5 and 2×10^6 recombinant bacteriophages. The cell concentration was measured with a Bausch and Lomb Spectronic 1001 spectrophotometer at 600nm. Recombinant bacteriophages from the genomic library carrying the arginine gene which complemented the host deficiency allowed the cell to both grow and support multiplication of the bacteriophages. The cell was ultimately lysed by the infecting bacteriophage and a plaque was produced. The large amount of E. coli cells used to inoculate the minimal agar plates caused a slight turbidity which permitted the visualization of the plaques.

Two control experiments were performed simultaneously with each lytic selection. The first control was to verify the arginine requirement of the strain. Two plates of minimal agar medium (one lacking arginine and the other supplemented with arginine) inoculated with 2×10^9 cells served as negative and positive growth controls, respectively. The second control was to verify the sensitivity of the E. coli arginine auxotroph to lambda infection. A plate of minimal agar medium containing arginine was inoculated with 2×10^9 cells infected with

approximately 1000 recombinant bacteriophages.

The putative selected recombinants were plaque-purified and retested for their ability to complement the arginine biosynthesis gene defect of the host (Davis et al., 1980). Bacteriophage stocks for each purified recombinant were prepared, titrated and stored in 15% glycerol at -70°C (Davis et al., 1980). Amino acids and pyrimidines, purchased from Sigma Chemical Co., were added to synthetic medium at a final concentration of $100\text{ }\mu\text{g/mL}$. Thiamine (Sigma Chemical Co.) was supplemented at a final concentration of $5\text{ }\mu\text{g/mL}$.

Probe preparation and blot hybridization. Plasmid DNA or chromosomal DNA was labelled by the nick translation procedure of Rigby et al. (1977) using [α - ^{32}P] dATP (ICN Biomedicals, Inc., Lisle, IS). Prior to labelling, chromosomal DNA was digested to completion with the restriction enzyme EcoRI and then extracted with phenol-chloroform. Plasmid fragments used as probes were isolated from low-melting-temperature agarose (Bethesda Research Laboratories) (Weislender, 1979). Nick-translated DNA was separated from unincorporated labels by two successive ethanol precipitations. The specific activity of the probes which was measured with an LKB Wallac liquid scintillation counter (Model 1217 Rackbeta, Turku,

Finland) was approximately 1×10^7 cpm/ μ g of DNA. DNA was transferred from agarose gels to nitrocellulose membranes (Hybond-C from Amersham, Arlington Heights, IL) by the method of Southern (1975). Plaque hybridization was performed as described by Maniatis *et al.* (1982). Hybridization under conditions of high stringency was carried out at $T_m - 30^\circ\text{C}$ ¹ as described by Maniatis *et al.* (1982). Hybridizations were performed in 5 x SSC without formamide at 68°C. The filters were washed 3 times in 2 x SSC plus 0.1% SDS (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 68°C for 15 min. For hybridization under conditions of low stringency ($T_m - 50^\circ\text{C}$)¹, the filters were hybridized at 50°C in 5 x SSC without formamide and then washed 3 times in 2 x SSC plus 0.1% SDS at 50°C for 15 min. Autoradiography was performed using intensifying screens at -70°C using Cronex 4 X-ray films (Du Pont, Mississauga, Ontario, Canada). Films were developed using a Du Pont film processor (Model QC1-R/T).

Cell-free extracts. Bacteria were grown in 200 mL of TMM synthetic medium to late log phase. Cells were harvested and resuspended in 5 mL of 100 mM Tris-HCl [pH 7.8], 5 mM 2-mercaptoethanol. Cells were disrupted with a Braun-Sonic

¹A (%G+C) of 50% (West and Clark, 1989) was used for melting temperature (T_m) calculations.

1510 sonicator. The suspension was placed on ice and treated for five interrupted periods of one min. Cell debris was removed by centrifugation ($25,000 \times g$ for 15 min). The supernatant was filtered through a $0.2 \mu\text{m}$ filter and stored in aliquots at -20°C .

For the preparation of bacteriophage lysates used for enzymatic assays, cells were infected in minimal media lacking arginine (Davis *et al.*, 1980) with approximately 10^7 complementing recombinant bacteriophages in order to produce a confluent lysis. Ten plates ($15 \times 100 \text{ mm}$) prepared for each lysate were each flooded with 10 mL of 100 mM Tris-HCl [pH 7.5], 10 mM MgSO_4 , 5 mM 2-mercaptoethanol and kept at 4°C overnight. The overlay solution was collected and filtered through a $0.2 \mu\text{m}$ filter. The phage lysate was then concentrated 20 times by ultrafiltration through a diaflo ultrafilter PM-10 membrane (Amicon Division, Danvers, MA). Bacteriophages were pelleted by ultracentrifugation ($30,000 \times g$ for 3 h) using a Beckman SW 50.1 rotor. The supernatant was filtered through a $0.2 \mu\text{m}$ filter and stored in aliquots at -20°C .

Enzymatic assays. Acetylornithine deacetylase (AODase) was assayed by the method of Vogel and McLellan (1970) in

which the concentration of ornithine produced was determined through reaction with ninhydrin. Ornithine acetyltransferase (OATase) was assayed colorimetrically with ninhydrin as described by Dénes (1970). The intensity of the orange-yellow color was measured at 470 nm for both assays using a Spectronic 1001 spectrophotometer (Bausch and Lomb). A set of standards containing 0.01 to 1.56 μ mole of L-ornithine were used to construct a standard curve necessary for enzyme activity determinations. Acetylglutamate synthetase (AGSase) was assayed by the method of Haas *et al.* (1972) in which the formation of N-acetylglutamate from 14 C-labelled glutamic acid (ICN Biomedicals) was measured. Chemicals used for the enzymatic assays were purchased from Sigma Chemical Co. or from Bio-Rad Laboratories (Mississauga, Ont., Canada).

The protein content of the extracts was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories) according to the instructions provided by the manufacturer.

RESULTS

Verification of the N. gonorrhoeae genomic library. The gonococcal origin of the DNA inserted into the recombinant bacteriophages was verified by a plaque hybridization assay (Maniatis et al., 1982). The nitrocellulose filter was hybridized, under conditions of high stringency, with a ³²P-labelled N. gonorrhoeae total genomic DNA probe prepared from the prototrophic strain CH811. Nearly 100% of the plaques hybridized with the gonococcal probe, thereby indicating the presence of N. gonorrhoeae genomic DNA into the recombinant bacteriophage genomes. In order to further verify the gonococcal origin of the DNA inserts, bacteriophage DNA was isolated from eight randomly selected recombinants. These DNAs were digested with EcoRI to cut out the inserted DNA from both arms of the vector and resolved by electrophoresis on an agarose gel. DNA fragments were transferred to nitrocellulose and hybridized under conditions of high stringency with the N. gonorrhoeae total genomic DNA probe. Only fragments corresponding to insert DNAs (ranging in size from 12 to 16 kb) hybridized, thereby suggesting their gonococcal origin.

Isolation of Arg⁺ clones. Recombinant bacteriophages complementing arginine biosynthesis gene defects in E. coli,

were selected from the *N. gonorrhoeae* genomic library by lytic complementation. Clones complementing *E. coli* gene defects in argA, argB, argE, argG, argIF, carA and carB genes were isolated (Table 1-2). Several recombinant bacteriophages (i.e. from 3 to 17) were isolated and purified for each arginine biosynthesis gene defect complemented (Table 1-2). A defect in argE was presumably complemented by the argJ gene from *N. gonorrhoeae* since the OADase enzyme encoded by argE is absent in *N. gonorrhoeae* (Shinners and Catlin, 1982). Clones complementing defects in argC, argD or argH were not isolated. Recombinant bacteriophages complementing the argD mutation could not be isolated mainly because *E. coli* K-12 argD mutants are able to synthesize arginine in growth-rate-limiting amounts (Itikawa *et al.*, 1968). This low level of arginine synthesis is presumably attributable to the activity of transaminase other than N-acetylornithine aminotransferase (encoded by argD) (Itikawa *et al.*, 1968). The failure to isolate clones complementing argC or argH gene defects may be explained by the absence of intact genes in the library. Finally, no recombinant complemented the strain MN42 having a deletion in the gene cluster argECBH (Table 1-2).

Molecular characterization of Arg⁺ clones. DNA was isolated from all purified recombinant bacteriophages and

Table 1-2. Lytic complementation of a variety of *E. coli* K-12 arginine mutants with the *N. gonorrhoeae* genomic library.

Arginine auxotrophs	Selected marker	No. of recombinants purified
NK5992	<u>argA</u> ⁺	5
W3421	<u>argA</u> ⁺	5
30SOMA4	<u>argB</u> ⁺	5
W3679	<u>argC</u> ⁺	0
AT3141	<u>argD</u> ⁺	0
AB1157	<u>argE</u> ⁺	17
UQ27	<u>argG</u> ⁺	5
AT753	<u>argH</u> ⁺	0
AB663	<u>argH</u> ⁺	0
N166	<u>argIF</u> ⁺	6
FP178	<u>carA</u> ⁺	3
JEF8	<u>carB</u> ⁺	10
MN42	<u>argECBH</u> ⁺	0

characterized by restriction endonuclease analysis using the enzymes EcoRI and SalI. The restriction endonucleases EcoRI and SalI were used since the arms of the lambda replacement vector EMBL4 do not carry any sites for these enzymes. Except for recombinants complementing an argB gene defect, recombinants complementing a particular arginine gene defect showed identical EcoRI and SalI restriction patterns, suggesting that specific gene defects were largely complemented by a single recombinant bacteriophage. Because of this apparent identity of the clones, one recombinant bacteriophage complementing defects in argA (EFP50), argE (EFP30), argG (EFP80), argIF (EFP40), carA (EFP10) or carB (EFP20) was selected for further characterization. Two different overlapping recombinants (EFP60 and EFP70), complementing an argB gene defect, were selected. Some characteristics of the DNAs from the selected recombinant bacteriophages carrying gonococcal arginine biosynthesis genes are shown in Appendix A (Table A-1). Interestingly, recombinant bacteriophage DNAs complementing argA (EFP50) or argE (EFP30) showed identical EcoRI and SalI restriction patterns. The size of the genomic DNA inserts for the selected recombinants ranged from 11.9 to 16.1 kb (Appendix A, Table A-1). Southern blot hybridizations were performed in order to verify that selected recombinant bacteriophages carried

genomic DNA inserts of gonococcal origin (data not shown). Recombinant DNA molecules from each selected bacteriophage were digested with EcoRI in order to release the inserted DNA from the rest of the bacteriophage. DNA fragments were transferred onto nitrocellulose and then hybridized under conditions of high stringency with a ^{32}P -labelled N. gonorrhoeae total genomic DNA probe prepared from strain CH811 and also with a ^{32}P -labelled E. coli total genomic probe prepared from strain JM240. For all recombinants the DNA insert hybridized only with the N. gonorrhoeae probe thereby suggesting its gonococcal origin. For some recombinant bacteriophages (i.e. clones EFP30 and EFP40), the reverse hybridization using the clone as a probe had to be performed to detect hybridization to gonococcal DNA. The increased sensitivity provided by this strategy for hybridization was apparently required for these two clones. This may be explained by a low G+C content for the gonococcal genomic DNA inserted in these clones.

It is possible that recombination has occurred between the DNA insert from the clones and an homologous region from the E. coli chromosome since the library was both amplified and screened by infection of Rec⁺ E. coli hosts. The fact that no detectable signals were observed for hybridization between

genomic inserts and E. coli genomic DNA may indicate the absence of large segments of E. coli genomic DNA in the selected recombinant bacteriophages. However, this methodology may be inappropriate to detect the presence of small regions from the E. coli genome in the selected clones which may have originated from homologous recombination.

Hybridization with cloned E. coli arginine genes. Clones complementing defects in carA (EFP10), carB (EFP20), argB (EFP70), argE (EFP30) and argIF (EFP40) were further characterized by hybridization, under conditions of various stringencies, with ³²P-labelled recombinant plasmid DNA containing the corresponding E. coli K-12 cloned arginine gene. Prior to hybridization with the E. coli arginine genes, the recombinant bacteriophages were digested with EcoRI or BamHI to release the cloned DNA from both arms of the bacteriophage. This was essential since the probes contained lambda DNA which could hybridize with the left (19.4 kb) and/or the right (9.2 kb) arms of EMBL4. EcoRI digests of EFP10 and EFP20 were hybridized with pMC50 (contains the E. coli carAB operon) (Fig. 1-1), clone EFP70 digested with EcoRI + SmaI was hybridized with a fragment from pMC7 (contains the E. coli argB gene) (Fig. 1-2) and an EcoRI digest of EFP40 was hybridized with pAI1 (contains the E. coli argI gene)

Figure 1-1. Hybridization under conditions of high stringency of the gonococcal clones EFP10 (carAB⁺) and EFP20 (carB⁺) with the corresponding *E. coli* genes. (Ia) Ethidium bromide-stained agarose gel of clones EFP10 (carAB⁺) (lane 1) and EFP20 (carB⁺) (lane 2) digested with EcoRI. (Ib) Southern blot hybridization of the same gel using radiolabelled plasmid pMC50. (IIa) Ethidium bromide-stained agarose gel of clone EFP10 digested with EcoRI. (IIb) Southern blot hybridization of the same gel using radiolabelled EFP20. The bands corresponding to the arms of EMBL4 (19.4 and 9.2 kb) were cut out from the gel to prevent cross-hybridization between lambda sequences. The size of some of the fragments in kilobases is indicated.

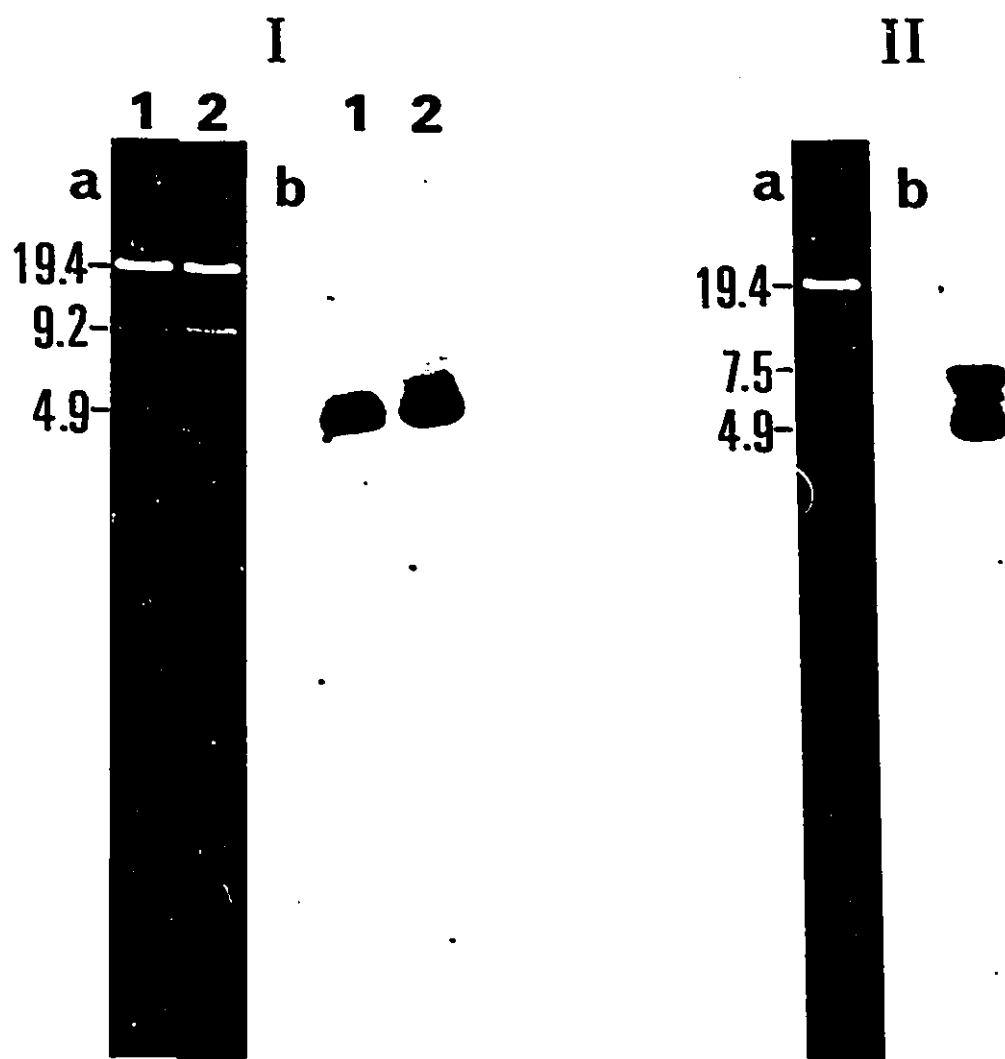
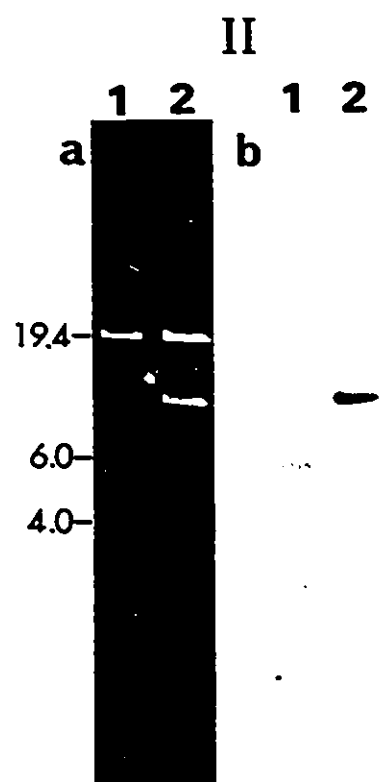
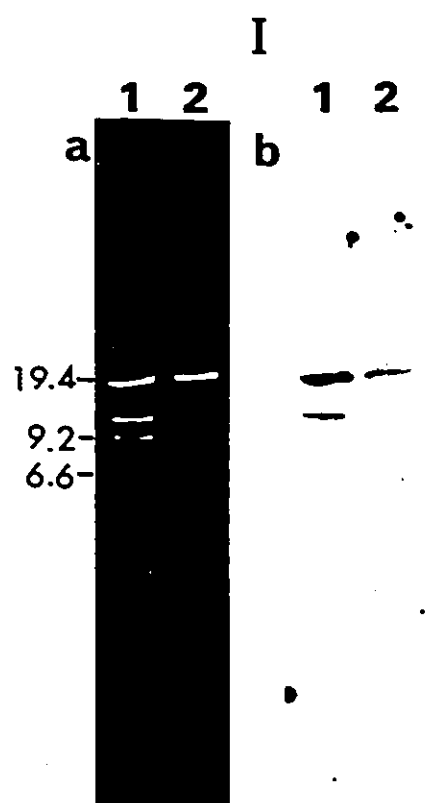


Figure 1-2. Hybridization under conditions of low stringency (T_m -50°C) of the gonococcal clones EFP40 (argF^{*}) and EFP70 (argB^{*}) with the corresponding *E. coli* genes. (Ia) Ethidium bromide-stained agarose gel of clone EFP40 (argF^{*}) digested with BamHI (lane 1) or with EcoRI (lane 2). (Ib) Southern blot hybridization of the same gel using radiolabelled plasmid pAI1 (carries the *E. coli* argI gene). (IIa) Ethidium bromide-stained agarose gel of clone EFP60 (argB^{*}) digested with EcoRI + SmaI (lane 1) or with EcoRI (lane 2). (IIb) Southern blot hybridization of the same gel using a radiolabelled fragment from pMC7 (carries the *E. coli* argB gene). The approximate size for some of the fragments in kilobases is indicated. For more exact fragment sizes, see Table A-1.



(Fig. 1-2). Under conditions of high stringency, which allowed the formation of hybrids between sequences having around at least 80%² similarity (Howley *et al.*, 1979), only clones EFP10 (*carA*⁺) and EFP20 (*carB*⁺) hybridized with the corresponding *E. coli* gene (Fig. 1-1). This suggested that the DNA sequences of *carA* and *carB* genes were more conserved than those of *argIF* or *argB* genes in these two organisms. A 4.9 kb *EcoRI* fragment, which is present in both EFP10 and EFP20 digests, hybridized with pMC50 suggesting that *N. gonorrhoeae* CPSase genes are found on this fragment (Fig. 1-1). Cross-hybridization of clone EFP10 with clone EFP20 confirmed that both 4.9 kb fragments were identical (Fig. 1-1). An additional 7.5 kb genomic DNA fragment from EFP10 hybridized with EFP20 suggesting that the gonococcal inserts from EFP10 and EFP20 are largely overlapping.

Under conditions of low stringency for hybridization, which allowed the formation of hybrids between sequences having around as much as 35%² base mismatch (Howley *et al.*, 1979), plasmid pAI1 (contains the *E. coli argI* gene) hybridized with the gonococcal genomic insert from EFP40 (*argF*⁺) (Fig. 1-2). pAI1 hybridized with one or both

²For these calculations, it was assumed that every 1% base mismatch between two DNAs lowers the T_m of the hybrid molecule by 1.4°C (Howley *et al.*, 1979).

comigrating 6.2 kb EcoRI fragments or with a 10.1 kb BamHI genomic DNA fragment (Fig. 1-2). The left arm of the bacteriophage (19.4 kb) also hybridized with pAI1. Under the same conditions for hybridization, a 5.3 kb EcoRI-HindIII fragment from pMC7 (carries the *E. coli* argB gene) did not hybridize with the genomic insert from EFP70 (argB⁺) (Fig. 1-2). The hybridization signals observed were the result of the hybridization of the pMC7 probe with the right arm of the bacteriophage. These results suggested that, in *N. gonorrhoeae* and *E. coli*, the nucleotide sequence of the ornithine transcarbamoylase (OTCase) gene (argF in *N. gonorrhoeae* and argI in *E. coli*) is more conserved than that of the acetylglutamate 5-phosphotransferase gene (argB).

The genomic insert from clone EFP30 (argE⁺) did not hybridize, under conditions of low stringency, with a probe prepared from pMC7 (1.8 kb HindIII fragment plus 3.0 kb HindIII fragment) which contains the entire *E. coli* argE gene (Crabeel et al., 1979) (data not shown). This finding may suggest that an ornithine deacetylase gene (argE) is not present on clone EFP30.

Co-complementation studies. In order to ascertain linkage between gonococcal arginine genes, the various recombinant

bacteriophages were used to systematically infect 10 different *E. coli* K-12 arginine auxotrophs sensitive to lambda bacteriophage infection. Only clones EFP10 (carA⁺), EFP30 (argE⁺) and EFP50 (argA⁺) were able to complement more than one arginine gene defect (Table 1-3). Clone EFP10 co-complemented a defect in carB suggesting that carA and carB genes are closely linked in *N. gonorrhoeae*. Clones EFP30 and EFP50, originally selected for their ability to complement argE and argA gene defects, respectively, were both able to complement argA and argE gene defects in *E. coli*. Restriction endonuclease analysis of EFP30 and EFP50 and cross-hybridization between these clones confirmed that both were identical (data not shown).

Enzymatic assays. Assays for acetylornithine deacetylase (AODase) and for ornithine acetyltransferase (OATase) were performed with a culture of *E. coli* AB1157 (deficient in AODase) complemented with the recombinant bacteriophage EFP30 (Table 1-4). AODase activity was not found in *E. coli* AB1157 confirming that this strain is defective in argE. This enzymatic activity was also absent in strain AB1157 complemented with the bacteriophage EFP30 indicating that the gonococcal genomic DNA insert of EFP30 does not carry a functional AODase gene. As expected, AODase activity was found

Table 1-3. Complementation of ten different *E. coli* arginine auxotrophs by recombinant bacteriophages carrying gonococcal arginine biosynthesis genes.

Arginine gene defects in <i>E. coli</i>	Complementation by recombinant bacteriophages ^a			
	EFP19 (<u>carA</u> ⁺)	EFP20 (<u>carB</u> ⁺)	EFP30 (<u>argE</u> ⁺)	EFP40 (<u>argF</u> ⁺)
<u>argA</u>	-	-	+	-
<u>argB</u>	-	-	-	-
<u>argC</u>	-	-	-	-
<u>argE</u>	-	-	+	-
<u>argIF</u>	-	-	-	+
<u>argG</u>	-	-	-	-
<u>argH</u>	-	-	-	-
<u>carA</u>	+	-	-	-
<u>carB</u>	+	+	-	-
<u>argECBH</u>	-	-	-	-

	EFP50 (<u>argA</u> ⁺)	EFP60 (<u>argB</u> ⁺)	EFP70 (<u>argB</u> ⁺)	EFP80 (<u>argG</u> ⁺)
<u>argA</u>	+	-	-	-
<u>argB</u>	-	+	+	-
<u>argC</u>	-	-	-	-
<u>argE</u>	+	-	-	-
<u>argIF</u>	-	-	-	-
<u>argG</u>	-	-	-	+
<u>argH</u>	-	-	-	-
<u>carA</u>	-	-	-	-
<u>carB</u>	-	-	-	-
<u>argECBH</u>	-	-	-	-

^a Complementation is indicated as the inability to correct (-) or the ability to complement (+) the defect in the arginine biosynthesis pathway.

Table 1-4. Activities of AODase, OATase and AGSase in cell-free extracts and in cell lysates of *E. coli* mutants complemented with the bacteriophage EFP30.

Cell-free extracts	Specific activity ^a (nmol/min per mg of protein)		
	AODase	OATase	AGSase
<i>E. coli</i> JM240	2640	NT	NT
<i>E. coli</i> AB1157	0	0	0
<i>E. coli</i> AB1157 + EFP30 ^a	0	1670	NT
<i>E. coli</i> NK5992	NT ^c	NT	0
<i>E. coli</i> NK5992 + EFP30 ^a	NT	NT	0
<i>P. aeruginosa</i> 88-301	NT	316	NT

^a Cell-free extracts prepared by lysing the *E. coli* mutants AB1157 and NK5992 by infection with the complementing bacteriophage EFP30.

^b Specific activities for AODase and for OATase are expressed in nmoles of ornithine produced per min per mg of protein. Specific activity for AGSase is expressed in nmole of N-acetylglutamate produced per min per mg of protein.

^c NT, Not tested

in E. coli JM240 (AODase-producing) which was used as positive control. OATase activity was not detected in E. coli AB1157 but was present in the extract of E. coli AB1157 complemented with the bacteriophage EFP30 thereby showing that OATase was synthesized in E. coli. Interestingly, the OATase activity for the complemented E. coli AB1157 was 5 times higher than the activity observed with our positive control P. aeruginosa 88-301. This suggested that the OATase gene was located on the recombinant bacteriophage EFP30. The AODase and OATase assays confirmed that the argE gene defect in E. coli AB1157 was complemented by the gonococcal OATase gene (i.e. argJ) located on EFP30. For this reason, the arginine biosynthesis gene from N. gonorrhoeae present on clone EFP30 is designated argJ rather argE.

An assay for acetylglutamate synthetase (AGSase) was also performed (Table 1-4). This enzyme activity was not detected in a cell-free extract prepared from E. coli NK5992 (AGSase deficient) or in a culture of E. coli NK5992 complemented with the bacteriophage EFP30. The inability to detect AGSase in our positive control E. coli AB1157 (AGSase-producing) may be explained by the low and unstable activities of this enzyme in cell-free extracts from E. coli (Vyas and Mass, 1963). Therefore, as a result of the instability of this enzyme, it

was not possible to establish whether the absence of AGSase activity observed with the complemented strain NK5992 is attributable to the absence of an AGSase gene on clone EFP30 or to the inactivation of the enzyme itself.

DISCUSSION

These results show that a number of arginine biosynthesis genes (argA, argB, argG, argF, argJ, carA and carB) from N. gonorrhoeae can be functionally expressed in E. coli. Others (Clarke and Carbon, 1978; Wood et al., 1983; Mountain et al., 1984; Jeenes et al., 1986) have reported the expression in E. coli of several arginine biosynthesis genes from heterologous hosts. The inability to isolate clones complementing defects in argC or argH is probably explained by the absence of intact genes in the gene bank constructed rather than by their failure to be functionally expressed in E. coli.

Although enough recombinant bacteriophages were obtained to create many N. gonorrhoeae genomic libraries, the yield of recombinants was nearly a thousand times lower than that predicted by the manufacturer of the cloning vector (Promega Biotec). This lower yield may be attributable to the restriction of gonococcal DNA by the Mcr restriction system of E. coli. The Mcr restriction system of E. coli, which is encoded by two genes (mcrA and mcrB), is directed against DNA with methylated cytosine (Marinus, 1987). Since N. gonorrhoeae possesses at least six cytosine methyltransferases (Korch et al., 1983), cloning of gonococcal DNA in E. coli hosts

proficient in the Mcr system may be difficult. The E. coli host NM539 (mcrA⁻mcrB⁺), used in this study to amplify the library, could restrict the gonococcal DNA with methylated cytosine since mutations in both genes are required to eliminate the ability to restrict DNA (Marinus, 1987). Incidentally, Kretz et al. (1989) have compared the cloning efficiency of methylated DNA between mcrA⁻mcrB⁺ and mcrA⁻mcrB⁻ hosts and found a 250 fold decrease in cloning efficiency with the mcrB proficient strain. As a consequence, the genomic library constructed during this study may not be representative of the whole gonococcal genome. This may explain our inability to isolate the gonococcal argC and argH genes. This could also explain why so few different overlapping clones complementing a particular gene defect were obtained. Another possibility is that a higher number of Sau3A restriction sites is found around these genes thereby reducing the yield of 10-20 kb restricted fragments representing those areas of the genome.

The Mcr restriction system may also be responsible for our previous unsuccessful attempts to construct a gonococcal genomic library in plasmid cloning vectors (i.e. pUC12 and pBR322) since Mcr proficient E. coli hosts were used (i.e. HB101, mcrA⁺ mcrB⁻ and C-600, mcrA⁺ mcrB⁺) (Raleigh and Wilson,

1986). These attempts yielded very few clones carrying deleted inserts. These observations have been confirmed by others (P.R. Martin, Dept. of Microbiology and Public Health, Michigan State University, personal communication) who could not obtain a single recombinant plasmid from a N. gonorrhoeae genomic library constructed in pUC12 amplified by using E. coli HB101 (mcrA*mcrB). However, the use of an E. coli host defective in both mcr genes to amplify the pUC12 library yielded several thousands of recombinant plasmids. This shows that the Mcr system seriously hampers the cloning of gonococcal DNA in E. coli and that hosts deficient in that restriction system are more suitable for that purpose.

Acetylornithine deacetylase (AODase) activity (encoded by argE) has not been detected in N. gonorrhoeae (Shinners and Catlin, 1978). Therefore, our ability to isolate a clone of gonococcal genomic DNA able to complement an argE gene defect in E. coli suggested that the ornithine acetyltransferase (OATase) encoded by argJ can replace the AODase activity in E. coli. This was confirmed by enzymatic assays performed with a lysate prepared from the E. coli mutant defective in AODase activity complemented by the recombinant bacteriophage EFP30 showing the presence of a high OATase activity but the absence of OADase activity. This established that N. gonorrhoeae

OATase is being synthesized in E. coli and that the gonococcal OATase gene is located on clone EFP30. This is the first report of this heterologous complementation and of the cloning of an OATase gene.

The isolation of a recombinant bacteriophage complementing E. coli arginine gene defects in both argA and argE initially suggested that argA and argJ genes were closely linked and possibly organized in a single operon. However, in a study in which we determined the arginine gene defects for 319 arginine-requiring clinical isolates of N. gonorrhoeae (Chapter two; Picard and Dillon, 1989b), we found that 174 strains (55%) were defective in argJ but that only one was defective in argA which may suggest that the expression of argA and argJ is not regulated in a single operon in N. gonorrhoeae. OATase (encoded by argJ), which has the ability to transfer an acetyl group from acetylornithine to a glutamate molecule, may be able to complement a defect in AGSase (encoded by argA) which also catalyzes the transfer of an acetyl group onto a glutamate molecule (Fig. LR-1). Thus, according to this hypothesis, substrate ambiguity of the OATase would allow this enzyme to transfer onto glutamate the acetyl group from either acetylornithine or acetyl-CoA. Therefore, a clone carrying the gonococcal argJ gene would be

able to complement both argA and argE gene defects in E. coli. The presence of OATase and the apparent absence of AGSase in methanogenic bacteria (Meile and Leisinger, 1984) may suggest that methanogens possess an OATase able to catalyse the formation of acetylglutamate from glutamate. On the other hand, the fact that argA mutants of P. aeruginosa (defective in AGSase) are arginine-requiring (Haas et al., 1977) indicates that AGSase is probably required for arginine synthesis in an organism recycling the acetyl group by the OATase activity. Consequently, this question remains open. Recently, valuable information on that question has been obtained through a collaboration with the laboratory of Dr. M.H. Mulks (Department of Microbiology and Public Health, Michigan State University, Mich.). Dr. Mulks' group has isolated a recombinant plasmid (pRGE1) carrying a 3.6 kb insert of gonococcal genomic DNA which complements an argE lesion in E. coli (P.R. Martin, D.A. Simpson, and M.H. Mulks. 1988. International Pathogenic Neisseria conference, October 16-21, Atlanta GA. MB6, p. 45, Abstr.) as well as another recombinant plasmid by complementation of an argA mutation in E. coli (M.H. Mulks, personal communication). Co-complementation studies revealed that pRGE1 (argE⁺) also complemented a defect in argA like clone EFP30 and that the argA⁺ plasmid could not co-complement an argE gene defect

(M.H. Mulks, personal communication). Interestingly, the gonococcal inserts from these two recombinant plasmids did not cross-hybridize (M.H. Mulks, personal communication) suggesting that an AGSase gene (argA) is not present on pRGE1 (argE^{*}). These experiments indicate that an AGSase gene (argA) is not responsible for the ability of pRGE1 to complement an argA lesion in E. coli. It also suggests that an AGSase gene is present in N. gonorrhoeae. These observations are supportive of a hypothesis of this thesis whereby the gonococcal OATase gene (argJ) would be able to complement gene defects in both AGSase and AODase.

OATase and AGSase activities are both implicated in recycling the acetyl group from acetylornithine. Morris and Thompson (1975), who found that in Chlorella vulgaris AGSase and OATase activities could not be separated during purification, suggested that both activities were the properties of a single enzyme. By contrast, in Pseudomonas aeruginosa, the two enzymatic activities are separable by gel filtration suggesting the presence of two enzymes (Haas et al., 1977). In N. gonorrhoeae, these enzymatic activities cannot presently be distinguished since AGSase activity has not yet been examined in this micro-organism. The complementation of argA and argE gene defects in E. coli may

be attributable to the production of a single gonococcal enzyme possessing both AGSase and OATase activities. According to the data presented in this study this enzyme would be the product of either a single gene or of two closely linked genes.

The enzyme carbamoylphosphate synthetase (CPSase) consists of a small and a large subunit encoded by the genes carA and carB, respectively (Cunin, 1983). These two genes are closely linked in N. gonorrhoeae as in all procaryotes investigated to date and may be organized in a single operon, as in E. coli and S. typhimurium (Abdelal and Ingraham, 1975; Haas et al., 1977; Gigot et al., 1980; Mountain et al., 1984; Kilstrup et al., 1988).

Hybridization data indicate that the CPSase genes from N. gonorrhoeae and E. coli are approximately 80% similar. Similarly, Kerppola and Kahn (1988) also found, in hybridization studies, that the CPSase genes from Rhizobium meliloti were highly similar (i.e. around 80% similarity) to those from E. coli. This is consistent with the finding that the nucleotide sequence of the carA gene from the gram-negative bacteria P. aeruginosa exhibits approximately 64% homology with its counterparts from E. coli and S. typhimurium.

(Wong and Abdelal, 1990) showing that carA is well conserved in procaryotes. To our knowledge, the nucleotide sequence of carB has not been determined for procaryotes other than E. coli. Furthermore, the amino acid sequences of CPSases from procaryotic and eucaryotic organisms share some well conserved regions (Nyunoya et al., 1985; Werner et al., 1985). The comparison of the amino acid sequences for the CPSases from Saccharomyces cerevisiae and the rat with E. coli shows approximately 42% of homology which encompasses the entire sequence of both the small and the large subunits of the two eucaryotic enzymes (Nyunoya et al., 1985; Werner et al., 1985).

The ornithine transcarbamoylase (OTCase) gene (argF) from N. gonorrhoeae and E. coli hybridized with each other under conditions of low stringency. Itoh et al. (1988) found that anabolic OTCase genes from P. aeruginosa and E. coli had approximately 46% of identity at the nucleotide level suggesting that procaryotic OTCases are relatively well conserved. Interestingly, the arcB gene encoding the P. aeruginosa catabolic OTCase, which is involved in arginine degradation, exhibits a higher homology (i.e. approximately 63%) with the E. coli anabolic OTCase encoded by argF (Baur et al., 1987). For this reason, a common origin for P. aeruginosa

arcB and E. coli argF has been suggested, despite the fact that the product of these genes have different physiological roles (Itoh et al., 1988). The nucleotide sequence of the N. gonorrhoeae argF gene is also very similar to E. coli argI and argF genes as well as to P. aeruginosa arcB (M.H. Mulks, personal communication). These observations seem to be in agreement with our finding that the gonococcal OTCase gene hybridize to the E. coli argI gene.

The acetylglutamate 5-phosphotransferase genes (argB) from N. gonorrhoeae and E. coli do not appear to share significant homology. Recently, Parsot et al. (1988) found that the amino acid sequence of the E. coli acetylglutamate 5-phosphotransferase showed 24% identity with the sequence of the functionally equivalent enzyme from Saccharomyces cerevisiae. The comparison of the amino acid sequences of the OTCase (encoded by argF) and of the CPSase (encoded by carA and carB) between these same two organisms revealed 31% and 40% of identity of the residues, respectively, with the presence for both enzymes of highly conserved regions (Feller et al., 1983; Werner et al., 1985; Huygen et al., 1987). These observations seem to be in agreement with our findings which suggest that the nucleotide sequence of the argB gene is less conserved than that of carA, carB, and argF.

In procaryotes, arginine biosynthesis genes exhibit a wide variety of arrangements (Cunin, 1983; Haas et al., 1977). They are highly clustered in Bacillus subtilis for which eight arginine genes have been cloned on a 12 kb DNA fragment (Mountain et al., 1984). By contrast, these genes are unlinked and scattered over the chromosome in P. aeruginosa (Haas et al., 1977). The clustering of arginine biosynthesis genes is intermediate in the Enterobacteriaceae with the genes argE, argC, argB and argH closely linked (Cunin, 1983). The data reported in this thesis suggest that gonococcal arginine genes are scattered as in the Pseudomonadaceae. The gene cluster argECBH, typical of Enterobacteriaceae, appears to be absent in N. gonorrhoeae. Since N. gonorrhoeae and P. aeruginosa recycle the acetyl group in the pathway as opposed to enteric bacteria or Bacillaceae, and since arginine biosynthesis genes are scattered in both organisms, N. gonorrhoeae seems to be more closely related to the Pseudomonadaceae than to enteric bacteria or Bacillaceae. Such a relatedness is precisely what modern phylogenetic studies suggest (Woese, 1987). Furthermore, the previous demonstration that gonococci grow anaerobically with nitrite as a terminal electron acceptor (Knapp and Clark, 1984) provides another functional similarity between N. gonorrhoeae and the Pseudomonadaceae.

CHAPTER TWO

BIOCHEMICAL AND GENETIC STUDIES WITH ARGININE AND PROLINE
AUXOTROPHS OF NEISSERIA GONORRHOEAE

INTRODUCTION

Clinical isolates of Neisseria gonorrhoeae have accumulated spontaneous mutations in genes encoding enzymes implicated in a variety of biosynthesis pathways (Carifo and Catlin, 1973; Catlin, 1977). The most frequently encountered auxotrophic defects are found in the arginine and the proline biosynthesis pathways (Carifo and Catlin, 1973; Catlin, 1977; Copley and Egglestone, 1983; Dillon and Pauzé, 1984; Hendry and Stewart, 1979). Arginine and proline are both synthesized from glutamate in eight and three enzymatic steps, respectively (Fig. LR-1). In N. gonorrhoeae, arginine and uracil auxotrophy are frequently associated (Copley and Egglestone, 1983; Dillon and Pauzé, 1984; Hendry and Stewart, 1979). Shinnars and Catlin (1982) proposed that a defect in the carbamoylphosphate synthetase (CPSase) genes (carA and carB) may account for this dual requirement since carbamoylphosphate (CP) is an intermediate in both the arginine and the pyrimidine biosynthesis pathways (Fig. LR-1).

Catlin and Nash (1978) studied the prevalence of arginine biosynthesis gene defects for 212 clinical isolates of N. gonorrhoeae from the United States by using the ability of the strains to utilize compounds known to be precursors of

arginine biosynthesis. An important limitation of their methodology was the inability to distinguish strains having a CPSase gene (carAB) defect from those having an ornithine transcarbamoylase (OTCase) gene (argF) defect. Using a biochemical method which seems to allow such distinctions, we studied the prevalence of argA, argB, argC, argD, argJ, argF, argG, argH and carAB gene defects for a group of 319 arginine-requiring strains of N. gonorrhoeae. Subsequently, some of those strains were transformed with cloned gonococcal arginine biosynthesis genes (Chapter one; Picard and Dillon, 1989a). Clones carrying proA, proB or proC genes from N. gonorrhoeae were used in gonococcal transformation assays with proline-requiring strains in order to identify their proline gene defects.

MATERIALS AND METHODS

Bacterial strains, growth conditions and identification.

A total of 324 strains of N. gonorrhoeae (isolated between 1976 and 1988) defective in arginine and/or proline biosynthesis were selected from the culture collection of the National Laboratory for Sexually Transmitted Diseases (Laboratory Centre for Disease Control, Ottawa, Ontario) for this study (Dillon and Pauzé, 1984; King et al., 1987; Moreno et al., 1987). Most isolates (288) were from Canadian sources while others (36) were from Jamaican (15), Brazilian (13) and Chilean (8) sources. The Canadian isolates were collected over a decade and were submitted by various laboratories across Canada for a number of national surveys and programs. GCMB (Difco Laboratories, Detroit, Michigan) with Kellogg's defined supplement as modified by Dillon (1983) was used for the routine cultivation of N. gonorrhoeae strains in a humid environment in the presence of 5% CO₂ at 35°C. When required, New York City media (Frappier Diagnostic Inc., Laval, Québec, Canada) was used to purify the strains. The Gonogen confirmation test kit (Bio-Mega Diagnostic Inc., Montréal, Québec, Canada) was used to confirm the strains as N. gonorrhoeae. A streptomycin resistant (Str^r) N. gonorrhoeae strain (FS62), derived from strain F62 (Evins and Knapp,

1988), was also used.

E. coli K-12 proline auxotrophs used in this study are listed in Table 2-1. E. coli was grown using tryptic soy medium (Difco), NZY medium or synthetic medium as described earlier (Chapter one). Prior to infection with lambda bacteriophages, E. coli was grown to mid-log phase either in NZY broth or liquid minimal medium supplemented with 0.5% maltose (Chapter one). Ampicillin (100 mg/L) was added for the maintenance of plasmids.

Auxotype determination. The auxotypes of all N. gonorrhoeae strains used in this study were reconfirmed as described previously (Hendry and Stewart, 1979; Dillon, 1983). The ability of N. gonorrhoeae arginine auxotrophs to utilize intermediates of arginine biosynthesis was investigated using a modification of the method used by Catlin and Nash (1978). The various intermediates were added to the synthetic media for N. gonorrhoeae at a final concentration of 100 mg/L. The intermediate carbamoylphosphate (CP), at a concentration of 150 mg/L, combined with L-ornithine was also tested to identify defects in carAB and argF genes. Various E. coli arginine auxotrophs (Chapter one, Table 1-1) were used as growth controls to test the minimal medium containing the

Table 2-1. E. coli strains.

<u>E. coli</u> K-12 strains	Relevant genotype
<u>Proline auxotrophs</u>	
χ^{462}	<u>proA180</u> , <u>leuB6</u> , <u>purE42</u> , <u>trpE38</u> , <u>lysA23</u> , <u>metE70</u> , λ^- , λ^s
χ^{474}	<u>proB91</u> , <u>leuB6</u> , <u>purE42</u> , <u>trpE38</u> , <u>lysA23</u> , <u>metE70</u> , λ^- , λ^s
χ^{478}	<u>proC32</u> , <u>leuB6</u> , <u>purE42</u> , <u>trpE38</u> , <u>lysA23</u> , <u>metE70</u> , λ^- , λ^s

intermediates of arginine biosynthesis. Amino acids and arginine biosynthesis pathway intermediates were purchased from Sigma Chemical Co.

DNA preparation. Isolation and purification of genomic DNA from both *N. gonorrhoeae* and *E. coli* were performed using a modified procedure of Marmur (1961). For the isolation of *N. gonorrhoeae* genomic DNA, cells were collected from confluent overnight growth of non-piliated cells on 2 GCMB plates (15 X 100 mm). Cells were then suspended in 20 mL of TE buffer containing 25% sucrose. For the isolation of *E. coli* genomic DNA, cells collected from a 100 mL late log phase culture by centrifugation (5,000 rpm for 10 min) were suspended in 10 mL of TE supplemented with 25% sucrose. The preparation was extracted with phenol-chloroform and digested with proteinase K (100 µg/mL) for one h at 37°C. A second phenol-chloroform extraction was performed prior to precipitation of the DNA with ethanol.

For large-scale isolation of plasmid DNA, cells were lysed by an alkaline-SDS treatment essentially as described by Ng *et al.* (1987). Bacterial cells were prepared as described in Chapter one. Subsequently, plasmid DNA was further purified by centrifugation to equilibrium in cesium chloride-ethidium

bromide gradients (Chapter one). Minipreparations of plasmid DNA were performed by the method of Birnboim and Doly (1979). Recombinant DNA was isolated from phage lysates by using Lambdasorb phage adsorbent (Chapter one).

Restriction endonuclease digestion and electrophoresis. Restriction endonuclease digestions were performed as described in Chapter one. *N. gonorrhoeae* total genomic DNA was digested overnight at 37°C with an excess of restriction enzymes to ensure complete digestion. Electrophoresis of DNA was achieved using 1.0% agarose gels (Chapter one). For a better resolution of fragments from restricted total genomic DNA, electrophoresis was carried out as described earlier but using a long gel (22 cm) apparatus run at 1 V/cm for approximately 30 h.

Cloning of proline genes and complementation of *E. coli* auxotrophs. The gonococcal genomic library constructed in the lambda cloning vector EMBL4 was screened for recombinant bacteriophages carrying gonococcal proline biosynthesis genes as described earlier (Chapter one). Using a shotgun cloning approach, the gonococcal proC gene was subcloned from the selected recombinant bacteriophage into the plasmid cloning vector pGEM-3Z (Promega Biotec). The vector and the insert

DNA, both digested with the restriction enzyme BamHI, were ligated using T4 DNA ligase (Chapter one). The molar ratio of vector:insert used was approximately 1:3. Recombinant plasmids were introduced into cells of E. coli proline auxotrophs via transformation. Recombinants carrying proline biosynthesis genes were selected on minimal media supplemented with ampicillin but lacking proline. A similar approach was used to subclone several arginine biosynthesis genes from N. gonorrhoeae (i.e. carA, carB and argJ); however, these attempts were unsuccessful. A second N. gonorrhoeae genomic library in pGEM-12 (Clontech Laboratories Inc., Palo Alto, CA) was screened for clones carrying proA and proB genes. Since this library was amplified by transforming E. coli strain HB101 (defective in proA), transformants carrying the gonococcal proA gene were directly selected on synthetic medium lacking proline and containing ampicillin. The ability of all recombinant plasmids to complement an E. coli proline gene defect was confirmed by re-transforming the E. coli proline auxotrophs with plasmid DNA isolated from the purified original transformants. E. coli plasmid transformation was performed as described by Miller (1987).

Probe preparation and blot hybridization. Chromosomal and plasmid DNA from N. gonorrhoeae or E. coli was labelled by

nick translation using [α - 32 P]dATP (Chapter one) or by random priming using digoxigenin-deoxyuridine-triphosphate (dig-dUTP) (Boehringer Mannheim Canada (BMC)). Random priming was performed using the reagents from a kit available from BMC for the non-radioactive detection of hybrid DNAs. The labelling was achieved as recommended by the manufacturer (BMC). Prior to labelling, plasmid DNA was linearized by digestion with a single cutter restriction endonuclease. DNA fragments used as probes were isolated from low-melting-temperature agarose (Chapter one). DNA was transferred to nylon Hybond-N membranes as described earlier (Chapter one). Prior to the transfer of large genomic DNA, the gel was irradiated under U.V. light for 5 min to nick the DNA and thereby facilitate the transfer of large chromosomal DNA fragments onto the membrane. Hybridizations with radioactive probes were carried out at $T_m - 30^\circ\text{C}$ (Chapter one). Hybridizations with the non-radioactive probes (dig-dUTP labelled) were performed under conditions of high stringency ($T_m - 30^\circ\text{C}$) by following the recommendations provided with the non-radioactive detection kit (BMC). Hybrid detection by enzyme-linked immunoassay (ELISA) was carried out by using the reagents from the kit as recommended by the manufacturer.

Gonococcal transformation. Arginine and proline

auxotrophs of N. gonorrhoeae, maintained as type 2 colonies (i.e. colonies of piliated gonococcal cells) (Kellogg et al., 1963), were treated with sterile preparations of purified bacteriophage or plasmid DNA carrying inserts of gonococcal DNA prepared from E. coli as described by Janik and Heym (1976). Piliated cells taken from isolated type 2 colonies were mixed with 0.5 μ g of recombinant DNA (in a volume of 5 μ L or less). For transformations to streptomycin resistance, cells were treated with 2.5 μ g of total genomic DNA from the Str^r N. gonorrhoeae strain FS62. The cell-DNA mixture was initially spread onto a GCMB plate and incubated for approximately 6 h before plating onto appropriate auxotyping medium or GCMB agar containing streptomycin (500 mg/L). Transformant colonies were visualized after 24 or 48 h of incubation. The identification and the selection of the colonies of piliated cells (i.e. type 2 colonies) which have a morphology different from colonies of non-piliated cells was facilitated with the use of a stereoscopic microscope (Zeiss, model LPS-7.5).

RESULTS

Utilization of arginine biosynthesis intermediates by N. gonorrhoeae. A total of 319 arginine-requiring strains of N. gonorrhoeae was examined for the ability of the strains to utilize seven of the nine intermediates of arginine biosynthesis (Table 2-2). L-glutamate did not satisfy the arginine requirement of any strain tested indicating that all strains had at least one genetic defect in their arginine biosynthesis pathway. The arginine requirement of one strain was satisfied by N-acetylglutamate suggesting that this strain was defective in the N-acetylglutamate synthetase gene (argA). None of the strains appear to be defective in the 3 steps involved in the conversion of N-acetylglutamate to N-acetylornithine since N-acetylornithine did not satisfy the arginine requirement for any additional strains (Table 2-2). However, 55% (174/319) of the strains were defective in the fifth step of the pathway (conversion of N-acetylornithine to L-ornithine) suggesting that those ornithine-requiring strains were defective in the ornithine acetyltransferase (OATase) gene (argJ). The arginine requirement of many strains (44% or 141/319) was satisfied by L-citrulline suggesting that these strains were defective in the conversion of L-ornithine + CP to yield L-citrulline. The ability of only 15 of those

Table 2-2. Number of arginine-requiring strains of N. gonorrhoeae capable of utilizing intermediates of arginine biosynthesis for growth.

Arginine intermediate tested	Gene defect(s) ^a possibly implicated	Arginine requirement satisfied (No. of Arg ⁺ strains)
L-glutamate	none	0
N-acetylglutamate	<u>argA</u>	1
N-acetylornithine	<u>argB</u> , <u>argC</u> , <u>argD</u> ^c	1
L-ornithine	<u>argJ</u>	175
L-ornithine + CP ^b	<u>carA</u> , <u>carB</u>	190
L-citrulline	<u>argF</u>	316
L-argininosuccinate	<u>argG</u>	319
L-arginine	<u>argH</u>	319

^a These gene defects may be combined with defect(s) in the genes implicated in the preceding steps of the pathway.

^b CP, carbamoylphosphate.

^c Since the intermediates N-acetylglutamate-phosphate and α -N-acetylglutamate-semialdehyde were not tested because these compounds are not commercially available, we could not distinguish between strains having gene defects in argB, argC or argD.

141 strains (i.e. 11%) to grow on minimal medium supplemented with L-ornithine + CP suggests that they require CP for growth and, consequently, appear to be defective in the CPSase genes (carAB). The other 126 strains (i.e. 89%) required L-citrulline for growth, suggesting that they were defective in the OTCase gene (argF). The arginine requirement for three isolates was satisfied by L-argininosuccinate suggesting a defect in the argininosuccinate synthetase gene (argG). No strains were found to be defective in the last step of the pathway catalysed by the argH encoded enzyme (Table 2-2). Since this biochemical method allows the identification of the latest defective step in arginine biosynthesis, it is possible that hidden gene defect(s) are present in the preceding steps of the pathway.

Association of auxotype with gene defects. The distribution by auxotypes of arginine biosynthesis gene defects, identified by the ability of the strains to utilize arginine biosynthesis intermediates for growth, is presented in Table 2-3. Only strains of auxotypes CUH (14) or CUHL (1) required CP for growth suggesting a carAB gene defect for these strains. Most of the CUH strains (83%) were defective in carAB. All other strains (124) requiring citrulline and uracil, which are mostly of auxotypes PCU and CU, were

Table 2-3. Possible arginine biosynthesis gene defects for 319 isolates of *N. gonorrhoeae* distributed by auxotypes.

Auxotypes ^a	Number of isolates	Arginine gene defects ^b (percentage of isolates)
PCU	86	<u>argF</u> (100)
PO	74	<u>argJ</u> (99), <u>argA</u> (1)
O	56	<u>argJ</u> (100)
OUH	41	<u>argJ</u> (100)
CU	27	<u>argF</u> (100)
CUH/CUHL	18	<u>carAB</u> (83), <u>argF</u> (17)
PAU	3	<u>argG</u> (100)
PCUL/PCUM/ PCUH/C	10	<u>argF</u> (100)
POH/POUH/ OU/OM	4	<u>argJ</u> (100)

^a Requirements used for auxotyping are abbreviated as follows; P: proline; O: ornithine; C: citrulline; A: arginine; U: uracil; H: hypoxanthine; L: leucine; M: methionine.

^b Possible arginine gene defects identified by the ability of the strains to utilize the intermediates of arginine biosynthesis for growth.

apparently defective in the OTCase gene (argF) since they required L-citrulline for growth.

Analysis by hybridization of genomic DNA from CPSase defective N. gonorrhoeae. Since large segments of the gonococcal cryptic plasmid has the ability to insert into the gonococcal chromosome (Fagblom et al., 1986), the possibility that it may represent a mechanism of natural mutagenesis utilized to inactivate biosynthesis genes in N. gonorrhoeae was investigated. To verify this hypothesis, the genomic DNA from six strains defective in their CPSase gene was analyzed by hybridization to a gonococcal cryptic plasmid probe and also to a probe specific to the gonococcal CPSase genes prepared from the 4.9 kb EcoRI fragment of EFP10 (Fig. 1-1).

Genomic DNA from nine N. gonorrhoeae strains (six defective in carAB and three controls not defective in carAB) was digested to completion with the restriction endonucleases AvaII, SmaI or EcoRI (Table 2-4). The gonococcal genomic DNA was digested only partially with the restriction endonucleases PstI and HindIII possibly due to the presence of inhibitors of enzyme activity in the DNA preparations. Approximately 3.0 μ g of digested DNA was electrophoresed in duplicate through

Table 2-4. Sizes of the genomic DNA fragments from 9 N. gonorrhoeae strains which hybridized with the gonococcal cryptic plasmid or with the gonococcal CPSase genes probes.

^a Relevant properties of the strains: (i) Requirements used for auxotyping are abbreviated as in Table 2-3. (ii) The presence (4.2⁺) or the absence (4.2⁻) of extrachromosomal cryptic plasmid is indicated. (iii) The growth requirement for carbamoylphosphate is designated as follows: CP⁻ (requiring strain) or CP⁺ (non-requiring strain).

^b Hybridization signals corresponding to extrachromosomal cryptic plasmid have been excluded.

^c ---, no hybridization signal was observed.

^d NT, the genomic DNA was not tested since it could not be digested to completion with the particular restriction enzyme.

^e NR, no nutritional requirement.

<u>N. gonorrhoeae</u> strain	Size (kb) of the genomic DNA fragment hybridizing with the probe ^b					
	<u>Ava</u> II digests		<u>Sma</u> I digests		<u>Eco</u> RI digests	
	CPSase genes	cryptic plasmid	CPSase genes	cryptic plasmid	CPSase genes	cryptic plasmid
(auxotype, 4.2 kb plasmid content, CP requirement) ^a						
NS-543 (CUH, 4.2 ⁺ , CP ⁻)	11.0	--- ^c	6.5	13.5	NT ^d	NT
C5 (CUH, 4.2 ⁺ , CP ⁻)	12.5	---	6.8	13.5, 38.0	NT	NT
NS-686 (CUH, 4.2 ⁺ , CP ⁻)	8.7	---	9.2, 28.0	13.5, 38.0	23.0, 30.0	7.8, 36.0
NS-1061 (CUH, 4.2 ⁺ , CP ⁻)	9.4	---	9.7, 28.0	7.3, 11.8, 13.5, 38.0	23.0, 30.0	7.8, 36.0
NS-1401 (CUH, 4.2 ⁺ , CP ⁻)	9.0	---	9.4,	13.5 28.0	NT	NT
NS-303 (CUH, 4.2 ⁺ , CP ⁻)	11.0, 11.8	---	6.5, 8.1, 20.0, 38.0	13.5	NT	NT
CH811 (NR ^e , 4.2 ⁻ , CP ⁺)	11.8	---	6.3, 6.5	---	5.8	---
J163A (PO, 4.2 ⁺ , CP ⁺)	11.8	8.7	6.5, 7.8, 22.0	7.2, 11.0, 13.2, 36.0	5.9 14.0	11.0 13.2
I-1403 (PCU, 4.2 ⁻ , CP ⁺)	9.2	---	9.7	---	14.0	---

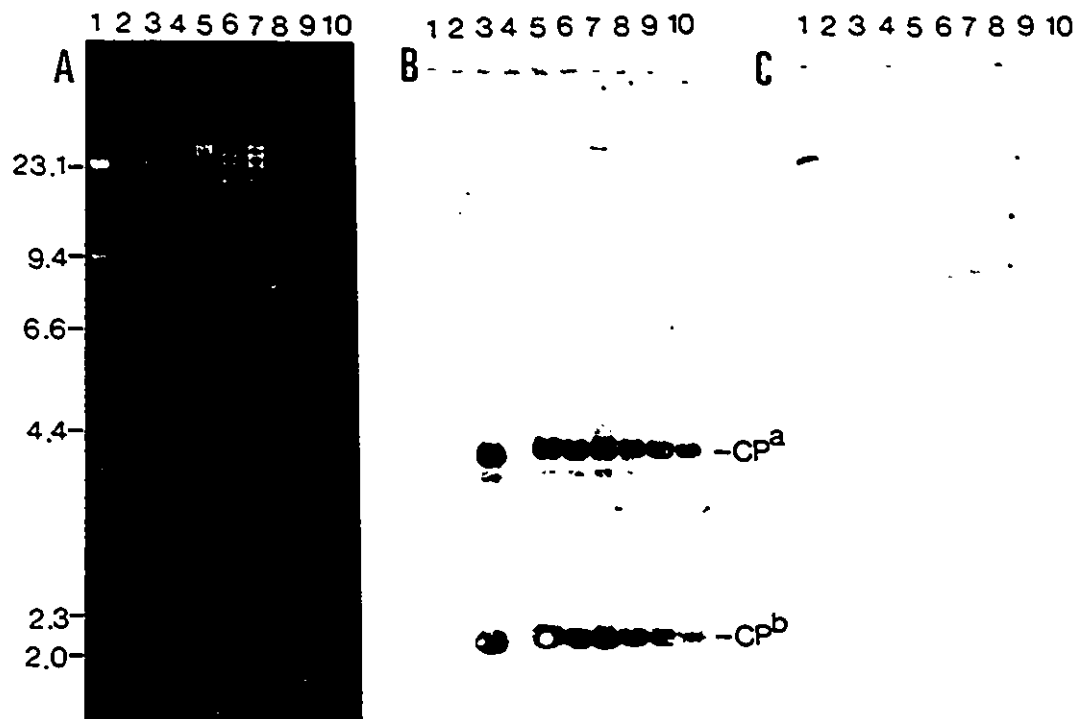
long agarose gels. Southern blot hybridization analysis using non-radioactive probes was performed from two identical gels: One membrane was hybridized with the gonococcal cryptic plasmid while the other membrane was hybridized with the CPSase genes probe. It was found that no single DNA fragment hybridized with both probes for all six CPSase defective strains suggesting that cryptic plasmid DNA sequences were not present on the genomic DNA fragments carrying the defective CPSase gene(s) (Table 2-4 and Fig. 2-1). The sizes of the genomic DNA fragments hybridizing to the cryptic plasmid probe or to the gonococcal CPSase genes probe are given in Table 2-4. Some of those bands may be difficult to visualize or not seen on Fig. 2-1 (panels b and c) since their hybridization signal with the probe was very low. The expected loss of resolution attributable to two subsequent photography of the blots necessary to obtain Fig. 2-1 would be responsible for the inability to visualize those bands which were already very faint on the original blot.

Hagblom et al. (1986) have shown, by hybridization of N. gonorrhoeae genomic to the gonococcal cryptic plasmid, that segments of the cryptic plasmid were found integrated into the gonococcal chromosome. The faint bands seen on Fig. 2-1 (panel b) are probably also the result of hybridization of the

Figure 2-1. Hybridization of genomic DNA from six CPSase defective strains of *N. gonorrhoeae* with the cryptic plasmid and with the gonococcal *carAB* probes. (a) Ethidium bromide-stained agarose gel of genomic DNA from 9 *N. gonorrhoeae* strains digested with *Sma*I. 1) lane 1; *Hind*III digested lambda DNA. 2) lanes 2 to 4; DNA from strains non CP-requiring: 1-1403 (lane 2), J163A (lane 3), CH811 (lane 4). 3) lanes 5 to 10; DNA from strains of auxotype CUH and CP-requiring: NS-303 (lane 5), NS-1401 (lane 6), NS-1061 (lane 7), NS-686 (lane 8), C5 (lane 9), NS-543 (lane 10). (b) Southern blot hybridization of the same gel with dig-dUTP labelled cryptic plasmid. (c) Southern blot hybridization of the same gel with dig-dUTP labelled CPSase genes probe. Bands corresponding to extrachromosomal cryptic plasmid are indicated.

^a Hybridization signal corresponding to the band of open-circular extrachromosomal cryptic plasmid (CP).

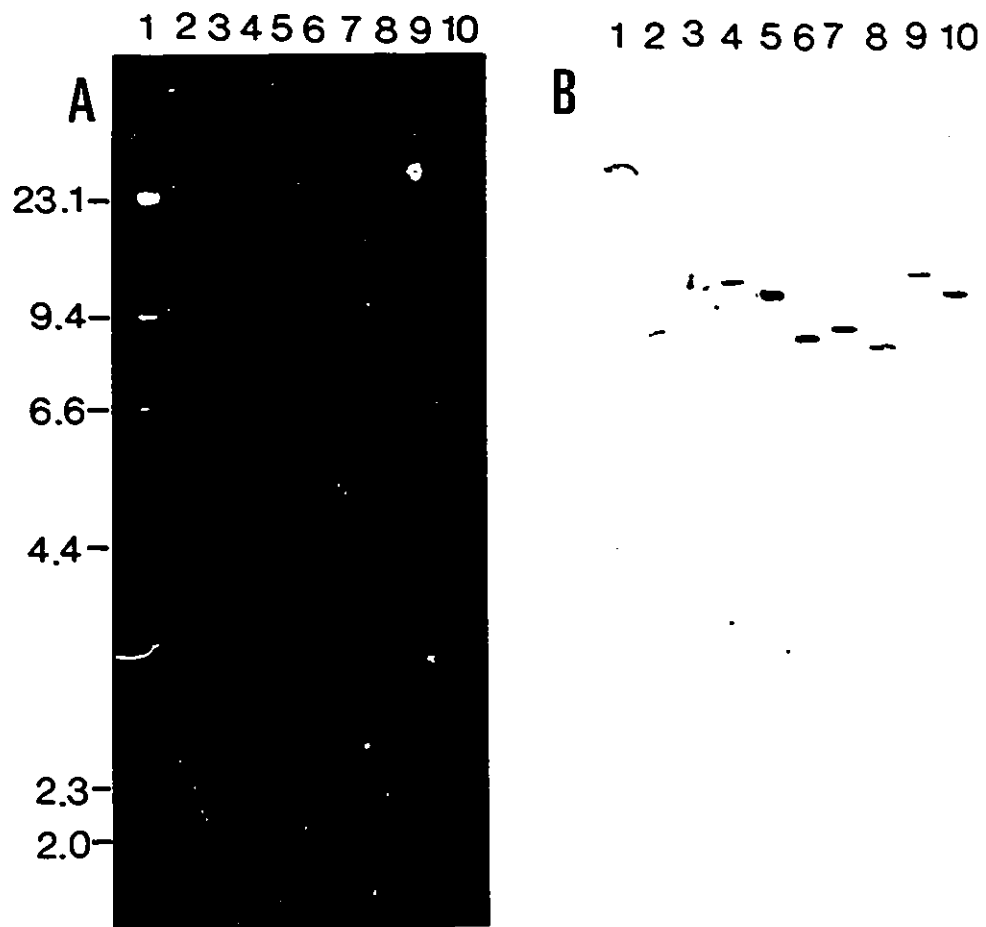
^b Hybridization signal corresponding to the band of closed-circular extrachromosomal cryptic plasmid (CP).



cryptic plasmid probe at cryptic plasmid segments integrated into the gonococcal chromosome. The low hybridization strength observed (Fig. 2-1, panel b) may be explained by the integration of small segments from the cryptic plasmid into the chromosome of the strains tested. Massive binding of the cryptic plasmid probe to extrachromosomal cryptic plasmid (e.g. bands of CCC and OC plasmid DNA) could also be responsible for the low hybridization strength at integrated cryptic plasmid segments. The lower hybridization strength with the gonococcal carAB probe observed with genomic DNA digested with SmaI (Fig.2-1, panel c) as compared to genomic DNA digested with AvaII (Fig. 2-2, panel b) could be explained by the presence of many SmaI fragments hybridizing with that probe. Furthermore, SmaI fragments smaller than 2 kb, which may have hybridized with the carAB probe, ran off the gel (Fig. 2-1, panel c).

Genomic DNA from the two plasmid-free strains (CH811 and 1-1403) did not hybridize with the cryptic plasmid probe (Table 2-4; Fig.2-1). Interestingly, the pattern of hybridization observed with the carAB probe for the AvaII digests from the six CPSase defective strains (which are all of same auxotype and serotype) was different for each strain (Table 2-4 and Fig. 2-2). This high polymorphism is also

Figure 2-2. Hybridization of genomic DNA from six CPSase defective strains of *N. gonorrhoeae* with the gonococcal carAB probe. (a) Ethidium bromide-stained agarose gel of genomic DNA from 9 *N. gonorrhoeae* strains digested with AvaII. 1) lane 1; HindIII digested lambda DNA. 2) lanes 2 to 4; DNA from strains non CP-requiring: 1-1403 (lane 2), J163A (lane 3), CH811 (lane 4). 3) lanes 5 to 10; DNA from strains of auxotype CUH and CP-requiring: NS-303 (lane 5), NS-1401 (lane 6), NS-1061 (lane 7), NS-686 (lane 8), C5 (lane 9), NS-543 (lane 10). (b) Southern blot hybridization of the same gel with dig-dUTP labelled CPSase genes probe.



observed with the SmaI digests (Table 2-4 and Fig. 2-1).

Isolation and characterization of Pro⁺ recombinant bacteriophages and plasmids. In order to complement gene defects in the proline biosynthesis pathway, three recombinant bacteriophages (EPL10, EPL20 and EPL40), which complemented E. coli gene defects in proA, proB or proC (Table 2-5), were selected from the N. gonorrhoeae lambda genomic library by lytic complementation (Chapter one). For all recombinants, the gonococcal origin of the DNA inserts was verified by Southern blot hybridization using total genomic DNA probes from N. gonorrhoeae or E. coli as described in Chapter one. Further information on these recombinant bacteriophages is given in Appendix A (Table A-1).

In order to determine if proline and arginine biosynthesis genes were closely linked in N. gonorrhoeae, the three recombinant bacteriophages carrying proline genes were tested for their ability to complement arginine biosynthesis gene defects. Nine E. coli arginine auxotrophs defective in the different genes implicated in arginine biosynthesis (Chapter one, Table 1-1) were systematically infected with clones EPL10, EPL20 and EPL40 (Chapter one). None of the three recombinant bacteriophages could complement any of the

Table 2-5. Some properties of clones carrying proline biosynthesis genes from *N. gonorrhoeae*.

Clones	<i>E. coli</i> gene defect(s) complemented	Size of the gonococcal genomic DNA insert (kb)
Bacteriophages		
EPL10 ^a	<u>proA</u> , <u>proB</u>	13.1
EPL20 ^a	<u>proA</u>	12.6
EPL40 ^a	<u>proC</u>	19.1
Plasmids		
pGFP1	<u>proA</u> , <u>proB</u>	3.0
pGFP2	<u>proC</u>	3.6

^a These three recombinant bacteriophages were selected from the library by Pierre Ledoux who did a four credit honours project in our laboratory during the semester of winter 1988.

arginine biosynthesis gene defects suggesting that arginine and proline biosynthesis genes are not closely linked in N. gonorrhoeae.

The recombinant plasmid pGFP2, complementing a proC gene defect in E. coli, was obtained by subcloning a 3.6 kb BamHI fragment of gonococcal genomic DNA from recombinant bacteriophage EPL40 (proC*) into the BamHI site of pGEM-3Z (Table 2-5). Southern blot of EPL40 BamHI digest probed with pGFP2 showed that only a 3.6 kb BamHI fragment from EPL40 hybridized with the plasmid thereby confirming that the 3.6 kb DNA fragment cloned into pGEM-3Z originated from EPL40. Plasmid pGFP1, able to complement both proA and proB gene defects in E. coli, was isolated from the gonococcal genomic library constructed in pGEM-1Z (Table 2-5).

Transformation of N. gonorrhoeae arginine and proline auxotrophs. Transformation assays were performed with 23 arginine-requiring strains of N. gonorrhoeae using DNA from recombinant bacteriophages carrying characterized gonococcal arginine biosynthesis genes (Chapter one). The arginine gene defect of strains requiring L-ornithine + CP (3 strains), L-ornithine (5 strains), L-citrulline (4 strains) or L-argininosuccinate (1 strain) was apparently corrected by

chromosomal marker rescue with the transforming DNA suggesting that these strains have a single gene defect in the arginine biosynthesis pathway (Table 2-6). The three strains requiring CP were transformed with DNA isolated from bacteriophages EFP10 (complements carA and carB gene defects in E. coli) and EFP20 (complements carB gene defect in E. coli) in order to identify their CPSase gene defect (Table 2-6). Strains NS-303 and NS-716 were complemented by DNA from EFP10 (carAB⁺) but not by DNA from EFP20 (carB⁺), suggesting that these strains were defective in carA gene. Strain NS-1426 was complemented by both EFP10 (carAB⁺) and EFP20 (carB⁺) suggesting that this strain was defective in carB. Ten out of the 23 arginine-requiring strains tested could not be transformed to arginine prototrophy. In order to verify that the ten strains were competent, transformation assays with genomic DNA from N. gonorrhoeae strain FS62 (Str^r) were performed. Only five of the ten strains could be transformed to streptomycin resistance. Non-transformable strains were of auxotypes CU (3), CUH (1) and PCU (1).

Transformation assays were also performed with 12 proline-requiring strains of N. gonorrhoeae by systematically transforming them with DNA from the recombinant bacteriophages EPL10 (proAB⁺), EPL20 (proA⁺) and EPL40 (proC⁺) (Table 2-7).

Table 2-6. Transformable strains of N. gonorrhoeae having different arginine biosynthesis gene defects with recombinant bacteriophage DNA isolated from E. coli and carrying gonococcal arginine biosynthesis genes.

^a Abbreviations of the requirements used for auxotyping are given in Table 2-3.

^b Transformation assay is indicated as the ability (+) or the inability (-) of transforming DNA to correct the defect in the arginine biosynthesis pathway.

^c CP, carbamoylphosphate.

Recipient strains of <i>N. gonorrhoeae</i> (Auxotype) ^a		Arginine intermediates required for growth	Transforming DNA	Transformation assay ^b
NS-303	(CUH)	CP ^c + L-ornithine	1) EFP10 (<u>carAB</u> ⁺) 2) EFP20 (<u>carB</u> ⁺)	+ -
NS-716	(CUH)	CP + L-ornithine	1) EFP10 (<u>carAB</u> ⁺) 2) EFP20 (<u>carB</u> ⁺)	+ -
NS-1426	(CUH)	CP + L-ornithine	1) EFP10 (<u>carAB</u> ⁺) 2) EFP20 (<u>carB</u> ⁺)	+ +
NS-718	(OUH)	L-ornithine	EFP30 (<u>argJ</u> ⁺)	+
NS-933	(OUH)	L-ornithine	EFP30 (<u>argJ</u> ⁺)	+
NS-888	(O)	L-ornithine	EFP30 (<u>argJ</u> ⁺)	+
NS-26	(POH)	L-ornithine	EFP30 (<u>argJ</u> ⁺)	+
NS-951	(POUH)	L-ornithine	EFP30 (<u>argJ</u> ⁺)	+
NS-506	(PCUH)	L-citrulline	EFP40 (<u>argF</u> ⁺)	+
NS-1028	(PCU)	L-citrulline	EFP40 (<u>argF</u> ⁺)	+
NS-1634	(PCU)	L-citrulline	EFP40 (<u>argF</u> ⁺)	+
NS-1190	(PCU)	L-citrulline	EFP40 (<u>argF</u> ⁺)	+
NS-1459	(PAU)	L-argininosuccinate	EFP80 (<u>argG</u> ⁺)	+

Table 2-7. Transformation of proline-requiring strains of N. gonorrhoeae with recombinant bacteriophage DNA isolated from E. coli and carrying gonococcal proline biosynthesis genes.

^a Abbreviations of the requirements used for auxotyping are given in Table 2-3.

^b Transformation is indicated as the ability (+) or the inability (-) of transforming DNA to correct the defect in the proline biosynthesis pathway.

^c ND, not determined.

Recipient strains of <u>N. gonorrhoeae</u> (Auxotype) ^a	Transformation by Bacteriophage DNA ^b			Proline biosynthesis gene defect of the strain
	EPL10 (<u>proAB</u> ⁺)	EPL20 (<u>proA</u> ⁺)	EPL40 (<u>proC</u> ⁺)	
F62 (P)	+	+	-	<u>proA</u>
NS-868 (P)	+	+	-	<u>proA</u>
NS-898 (P)	+	+	-	<u>proA</u>
NS-983 (P)	+	+	-	<u>proA</u>
NS-1190 (PCU)	+	-	-	<u>proB</u>
NS-1634 (PCU)	+	-	-	<u>proB</u>
2152 (PCU)	+	-	-	<u>proB</u>
NS-506 (PCUH)	+	-	-	<u>proB</u>
NS-26 (POH)	+	-	-	<u>proB</u>
NS-951 (POUH)	+	+	-	<u>proA</u>
NS-1459 (PAU)	+	-	-	<u>proB</u>
NS-829 (P)	-	-	-	ND ^c

The proline gene defect of 11 of the 12 strains was apparently corrected by transforming DNA from EPL10 (proAB⁺) but not from EPL40 (proC⁺) suggesting that all strains were defective in proA and/or proB. The proline gene defect of 5 of these strains was complemented by EPL20 (proA⁺) DNA suggesting that they were defective in proA gene. However, the possibility that the proline gene defect of those strains was corrected by recombination with a portion of the inactivated proB gene, possibly present on EPL20 (proA⁺), cannot be excluded. The six other strains which were complemented only by EPL10 (proAB⁺) were probably defective in proB. Strains of auxotype P were all defective in proA and those of auxotype PCU or PCUH were all defective in proB (Table 2-7). The proline gene defect of one strain (Table 2-7) could not be complemented by any of the proline biosynthesis genes. Furthermore, this strain could not be transformed to streptomycin resistance with the Str^r genomic DNA from strain FS62 suggesting that this strain was not competent for genetic transformation.

The 11 transformable proline-requiring strains were also transformed with recombinant plasmids pGFP1 (proAB⁺) and pGFP2 (proC⁺) either in linearized (i.e. digested with EcoRI) or in closed-circular (CCC) form. The proline biosynthesis gene defect of all strains was complemented by linearized or CCC

plasmid pGFP1 (proAB⁺) but not by plasmid pGFP2 (proC⁺), in accordance with our findings using recombinant bacteriophage genomes as transforming DNA (Table 2-7). The yield of transformants appeared to be similar with transformation assays using equimolar quantities of either bacteriophage DNA from EPL10 (proAB⁺), linearized pGFP1 (proAB⁺) or CCC form of pGFP1 (proAB⁺).

DISCUSSION

Most of the 319 arginine auxotrophs of N. gonorrhoeae tested in the present study, appear to be defective either in the conversion of N-acetylornithine to L-ornithine (55%) or in the conversion of L-ornithine plus CP to L-citrulline (44%). The other strains (1%) were apparently defective in the N-acetylglutamate synthetase gene (argA) or in the argininosuccinate synthetase gene (argG). These results are similar to findings by Catlin and Nash (1978) who showed that the arginine biosynthesis gene defects for 212 arginine-requiring isolates of N. gonorrhoeae from the United States were predominantly defective in the fifth (69%) or in the sixth (30%) step of the pathway.

We have found that only 11% of isolates defective in the conversion of L-ornithine to L-citrulline required CP for growth suggesting that most of these strains had an OTCase gene (argF) defect. Therefore, those OTCase defective strains, which require both uracil and citrulline, must have a gene defect other than carAB in the pyrimidine biosynthesis pathway accounting for their uracil requirement. Shinnars and Catlin (1982) showed that all eleven citrulline and uracil requiring strains of N. gonorrhoeae examined (six of auxotype CU and

five of auxotype CUH) had normal OTCase activity but lacked CPSase activity suggesting that a CPSase gene defect was attributable to their dual requirement for citrulline and uracil. These findings appear to differ from this study, since we have found that relatively few strains requiring both citrulline and uracil required CP for growth. This difference could be partly attributable to the large proportion of strains of auxotype PCU in our collection (accounting for 68% of strains having a dual requirement for citrulline and uracil in this study) and their absence in the group of eleven strains investigated by Shinnars and Catlin (1982). However, enzymatic assays recently performed (M.H. Mulks, unpublished data) with several *N. gonorrhoeae* strains of auxotype PCU indicate that these strains possess normal OTCase activity but lack CPSase activity. Although none of these strains was used in our study, this finding may indicate that a CP requirement for growth to identify a car gene defect in *N. gonorrhoeae* is not valid. On the other hand, transformation assays with PCU strains (Table 2-6), which suggest that these strains are defective in argF, support our finding that these strains do not appear to require CP for growth. Clearly, enzymatic assays for OTCase and CPSase with some of the *N. gonorrhoeae* strains requiring citrulline and uracil used in this study will help to resolve this question. This apparent discrepancy may be

explained by possible problems associated with CP transport. Although published data on CP transport are apparently inexistant, the fact that many phosphorylated compounds (e.g. nucleotides) are excluded from bacteria (Hays, 1978; Leive and Davis, 1980) may indicate that transport systems for these compounds may be inefficient or absent. On the other hand, efficient bacterial transport systems exist for glucose-P, glycerol-P and phosphate (Silver, 1978; Leive and Davis, 1980). This study showed that E. coli mutants defective in carA (i.e. strain MI178) and carB (i.e. strain JEF8) as well as N. gonorrhoeae strains of auxotype CUH (14) or CUHL (1) were able to grow on minimal media supplemented with CP + ornithine. These findings indicate that CP can be utilized for growth by E. coli and N. gonorrhoeae. The E. coli positive growth controls may not be appropriate since transport systems for such compounds may vary between bacterial species. Most N. gonorrhoeae strains, of auxotype CUH, are apparently able to utilize CP for growth. However, PCU strains may be unable to utilize CP for growth. A porin possibly implicated in CP transport which is present on CUH cells but absent on PCU cells may explain this apparent differential uptake of CP in N. gonorrhoeae.

Only strains of auxotype CUH appear to require CP for

growth suggesting a gene defect in carAB. This finding agrees with that reported by Shinnars and Catlin (1982) who found that strains of auxotype CUH lacked CPSase activity. The requirement for CP was not universal in CUH strains as 17% were unable to utilize CP + ornithine for growth suggesting that they were defective in argF. Thus, N. gonorrhoeae strains of auxotype CUH appear to be heterogeneous at the genetic level. Interestingly, CUH strains apparently defective in carAB, which were all of same serovar (1A-1/2), had a serovar different from the CUH strains apparently defective in argF (serovar 1B-3), indicating a possible relationship between the arginine gene defect and serovar. According to previous findings (Shinnars and Catlin, 1982), the CUH strains requiring CP for growth are probably also defective in argJ. The citrulline requirement for 27 isolates of auxotype CU was not satisfied by ornithine and CP suggesting that these strains were defective in the OTCase gene, like the PCU strains. These results are different from those obtained by Shinnars and Catlin (1982) who showed that all six CU strains examined had normal OTCase activity but lacked CPSase activity. These observations may suggest that N. gonorrhoeae arginine auxotroph populations are heterogeneous. In this regard, the identification of the arginine gene defect of N. gonorrhoeae strains by this biochemical method may be useful

for epidemiological purposes since it may allow the differentiation of strains with the same auxotype. On the other hand, this may raise questions about the validity of CP requirement for growth to identify car gene defects. Enzymatic assays for OTCase and CPSase are required to verify this.

The selection of most arginine-requiring strains of N. gonorrhoeae used in this study (75%) was based on their ornithine or citrulline requirements. As a result, strains defective in the two biosynthesis steps involved in the conversion of citrulline to yield arginine could not be identified initially. More recently, arginine was used for auxotyping in a national study (Dillon et al., 1990) allowing the isolation of three argG defective strains (selected for this study) among 696 arginine-requiring strains. This suggests a low incidence of this gene defect in N. gonorrhoeae. Strains of N. gonorrhoeae defective in argG have been screened for (Catlin and Nash, 1978), but not previously identified.

According to our data, the use of ornithine for auxotyping appears to be a good screening procedure for strains with an argJ gene defect since, except for one strain, all ornithine-requiring strains were apparently defective in

argJ. However, it is clear that the argJ mutations in ornithine-requiring strains may be combined with other mutations in any of the first four steps of the arginine biosynthesis pathway that would be hidden by the argJ gene defect. Arginine, when used in combination with citrulline for auxotyping, allows the identification of strains with an argG gene defect since all arginine-requiring strains isolated in this study were apparently defective in argG.

Hagblom et al. (1986) have shown that both the entire cryptic plasmid from *N. gonorrhoeae* or portions of it were integrated into the gonococcal chromosome at several locations. Our study also suggests that sequences from the cryptic plasmid were integrated into the gonococcal chromosome. Genomic DNA from the two plasmid-free strains did not hybridize with the cryptic plasmid probe suggesting that the integration of the gonococcal cryptic plasmid into the chromosome is not universal. This finding differs from that by Hagblom et al. (1986) who showed that the single plasmid-free strain tested hybridized with the cryptic plasmid, although the hybridization signal with that strain was weaker and limited to fewer genomic DNA fragments than with strains carrying the cryptic plasmid. According to the hybridization data with genomic DNA from several CPSase defective strains of

N. gonorrhoeae, the cryptic plasmid does not appear to play a role in the inactivation of the CPSase genes. Clearly, we cannot exclude the possibility that insertional mutagenesis by the cryptic plasmid is utilized to inactivate other gonococcal genes. Such an elegant mechanism of mutagenesis may play a role in the creation of the numerous naturally-occurring N. gonorrhoeae strains with multiple biosynthesis gene defects.

Interestingly, hybridization patterns of restricted genomic DNA from six CPSase defective strains of the same auxotype and serotype with a gonococcal CPSase gene probe was different for each strain. Abundant restriction site polymorphism has also been observed for the gonococcal iga and proAB genes (Mulks and Knapp, 1985; M.H. Mulks, personal communication). The gonococcal carAB probe used in this study may be useful for epidemiological purposes to distinguish between gonococcal strains which appear identical on the basis of routinely used epidemiological markers. The usefulness of this probe as an epidemiological tool for N. gonorrhoeae is presently being further investigated in our laboratory with a large number of strains representative of the different gonococcal auxotypes. Preliminary data indicate that PCU strains cannot be differentiated on the basis of hybridization patterns of genomic DNA with this probe. Therefore, the

usefulness of this probe as an epidemiological tool seems to be limited. The high polymorphism in hybridization patterns of genomic DNA with that probe observed with strains of auxotype CUH defective in their CPSase gene(s) may be attributable to heterogeneity of these mutations. However, the observed restriction site polymorphism may not be within carAB but rather in the flanking sequences of those genes since the size of the genomic DNA fragments (e.g. 8.7 to 11.8 kb for AvaII) hybridizing with the carAB probe are larger than the gonococcal CPSase genes.

By genetic transformation with gonococcal genomic DNA cloned into an *E. coli* lambda cloning vector, it was apparently possible to correct the arginine biosynthesis gene defects for 13 of 23 *N. gonorrhoeae* arginine auxotrophs which appear to be defective in carAB, argJ, argF, or argG on the basis of their growth requirement for arginine biosynthesis intermediates. The ability to transform to arginine prototrophy some of the strains by transformation with a single recombinant bacteriophage suggest that these strains have a single gene defect in the arginine biosynthesis pathway. However, findings by Shinnars and Catlin (1982) indicate that strains defective in both GATase and CPSase activities are common. The large gonococcal genomic DNA insert

(11.9 to 15.1 kb) in the clones may contain a closely linked portions of a gene or an inactivated gene (possibly during cloning in the *E. coli* *Rec*⁺ host) which is implicated in arginine biosynthesis (including a possible regulatory gene) or in arginine transport. Homologous recombination with these clones may be able to transform to arginine prototrophy strains with more than one gene defect in the arginine biosynthesis pathway. Recently, M.H. Mulks *et al.* (P.R. Martin, D.A. Simpson and M.H. Mulks, Abstr. Int. Pathogenic Neisseria Conf. 1988, MB6, p. 45) have also succeeded in correcting an arginine gene defect of a *N. gonorrhoeae* strain with the gonococcal *argJ* gene cloned into an *E. coli* plasmid cloning vector. Since the gonococcal arginine biosynthesis genes were cloned into a lambda cloning vector, which does not replicate in *N. gonorrhoeae*, the transformants were presumably produced by recombination between the recombinant bacteriophage and the corresponding defective gene on the gonococcal chromosome (Stein, 1989). This chromosomal marker rescue approach occurs even in the presence of host-mediated restriction (Stein, 1989). Our failure to correct the arginine gene defect of 10 strains by genetic transformation may be caused by multiple genetic defects in their arginine biosynthesis pathway. In fact, findings by Shinnars and Catlin (1982) indicate that *N. gonorrhoeae* arginine auxotrophs

defective in both argJ and carAB are common. Our inability to transform 5 of the 10 strains to streptomycin resistance suggests that those 5 strains were not competent for genetic transformation. Recently, Biswas et al. (1989a) have isolated transformation-deficient mutants of piliated N. gonorrhoeae after chemical mutagenesis. The natural occurrence of such N. gonorrhoeae mutants may possibly explain why some of the gonococcal strains in this study were non-transformable.

Clones of gonococcal genomic DNA complementing E. coli gene defects in proA, proB and proC were isolated. The gonococcal proC gene had not been previously cloned. As previously noted by Stein et al. (1984), we also found that proA and proB proline biosynthesis genes were closely linked in N. gonorrhoeae; the gonococcal proC gene appears to be unlinked to the proAB genes, as in E. coli K-12 (Bachmann, 1990). Furthermore, no evidence of linkage between proline and arginine biosynthesis genes was found. This suggests that the dual requirement for proline and arginine observed in 54% of the N. gonorrhoeae strains used in this study is attributable to at least one genetic defect in each pathway.

The precursors of proline biosynthesis are not commercially available due to their high instability (Csonka

and Baich, 1983). Consequently, the enzymes implicated in this pathway have been difficult to assay (Csonka and Baich, 1983). For *N. gonorrhoeae*, none of the proline biosynthesis enzymes has been assayed. By using genetic complementation of proline-requiring strains with gonococcal proline genes cloned in *E. coli* cloning vectors which do not replicate in *N. gonorrhoeae*, these problems were overcome and specific proline biosynthesis gene defects in *N. gonorrhoeae* were apparently corrected. This approach allowed the identification of possible proline gene defects for eleven *N. gonorrhoeae* proline auxotrophs. Since none of the 11 strains investigated appear to have a proC gene defect, these data suggest that strains defective in proA or proB are more prevalent than those defective in proC. Strains defective in proA or proB appear to have a similar incidence. Interestingly, strains of auxotype P appear to be defective in proA and those of auxotype PCU were apparently defective in proB indicating that auxotyping may prove to be a useful method to identify proline gene defects. Future studies with a larger number of strains will elucidate the validity of these hypotheses. The finding that strain FS62 (a Str^r derivative from strain F62) was defective in proA agrees with the study by Stein *et al.* (1984) in which genetic complementation in trans with recombinant plasmids stable in that strain was used. However, because the

plasmid cloning vector (i.e. the shuttle vector pLES2) used by Stein et al., (1984) is unstable in most N. gonorrhoeae strains (V.L. Clark, personal communication), the chromosome marker rescue approach, which can be used with most strains, seems to be more appropriate than genetic complementation in trans to identify biosynthesis gene defects in the gonococcus.

The finding that gonococcal proline biosynthesis genes cloned into a lambda replacement vector (vector size: 28.6 kb and insert size: 10-20 kb) transformed N. gonorrhoeae apparently as well as genes cloned into a plasmid cloning vector (vector size: 2.8 kb and insert size: 3.0 kb) suggests that the size and the type of vector DNA as well as the size of genomic inserts do not affect significantly the efficiency of marker rescue. Furthermore, the recombinant plasmid pGFP1 (proAB⁺), either linearized by digestion with a restriction endonuclease or in its covalently closed circular (CCC) form, was apparently able to transform N. gonorrhoeae with similar efficiencies suggesting that the conformation of the plasmid is not critical for chromosomal marker rescue.

CHAPTER THREE

MOLECULAR CHARACTERIZATION
OF THE GONOCOCCAL CPSase GENES

INTRODUCTION

Carbamoyl phosphate (CP) is a precursor of both arginine and pyrimidine biosynthesis (Fig. LR-1). In most bacteria, CP is synthesized from glutamine, ATP and CO₂ by a single enzyme, the glutamine-dependent CPSase which provides CP for both the arginine and the pyrimidine biosynthesis pathways (Cunin, 1983). In *E. coli*, the CPSase enzyme consists of two subunits which are the products of two adjacent genes carA and carB constituting an operon oriented from A to B (Mergeay *et al.*, 1974; Gigot *et al.*, 1980; Cunin, 1983). The *E. coli* carAB operon is regulated by two tandem promoters which are regulated differently (Fig. LR-2); the upstream promotor (P1) is controlled by the pyrimidines while the second promotor (P2) is repressed by arginine (Piette *et al.*, 1984). The *S. typhimurium* carAB operon is also regulated by two tandem promoters in an apparently similar fashion (Kilstrup *et al.*, 1988). In *E. coli*, carA encodes the small subunit (MW 42 kDa) of the enzyme which carries the glutamine-binding site (Cunin, 1983). carB encodes the large subunit (MW 130 kDa) which catalyzes the synthesis of CP from ammonia (Cunin, 1983). The large subunit carries the targets for all known allosteric effectors of the CPSase (Trotta *et al.*, 1971). The carB gene, which is more than 3 kb in size, arose from an internal

duplication of a smaller ancestral gene (Nyunoya and Lusty, 1983).

The E. coli carAB operon has been entirely sequenced (Nyunoya and Lusty, 1983; Piette et al., 1984). carA has also been sequenced for the procaryotes S. typhimurium and P. aeruginosa (Kilstrup et al., 1988; Wong and Abdelal, 1990). The nucleotide sequence of the carA gene from P. aeruginosa exhibits approximately 64% homology with its counterparts from the enterics E. coli and S. typhimurium indicating that carA is well conserved in procaryotes (Wong and Abdelal, 1990).

In the procaryote Bacillus subtilis as well as in the eucaryotes, two different CPSase enzymes (one arginine-specific and the other pyrimidine-specific) are present (Davis, 1983; Cunin et al., 1986). In yeast and mammals, the arginine-specific CPSase is an independent enzyme but the pyrimidine-specific CPSase is part of a multifunctional protein (Nyunoya et al., 1985; Werner et al., 1985; Maley and Davidson, 1988). DNA sequence analysis of the arginine-specific yeast (Werner et al., 1985) and rat (Nyunoya et al., 1985) CPSases revealed that both enzymes were evolutionarily related to the E. coli CPSase (Nyunoya and Lusty, 1983; Piette et al., 1984). The comparison of the amino acid sequences for

the eucaryotic CPSases from rat and yeast with that from E. coli shows 42% and 40% of homology, respectively. The homology encompasses the entire sequence of both the small and the large subunits of the enzyme (Nyunoya et al., 1985; Werner et al., 1985).

Hybridization studies indicated that the N. gonorrhoeae CPSase genes were very similar to those of E. coli (Chapter one; Picard and Dillon, 1989a). In this study, the gonococcal CPSase genes are further characterized by PCR amplification of several portions of the gonococcal carA and carB genes using E. coli-specific primers.

MATERIALS AND METHODS

DNA preparation. Recombinant DNA was isolated from phage lysates (Chapter one). Large scale isolations and minipreparations of plasmid pMC50 (carries the *E. coli* carAB operon) were as described in Chapter two. Isolation and purification of genomic DNA from *N. gonorrhoeae* strain CH811 were as described in Chapter two.

Restriction endonuclease digestions and electrophoresis. Restriction endonuclease digestions were performed as described in Chapter one. Electrophoresis of DNA was performed using 1.0 or 1.5% agarose gels (Chapter one).

Probe preparation and blot hybridization. Plasmids or DNA fragments were labelled with dig-dUTP by random priming (Chapter two). DNA fragments used as probes were isolated from low-melting-temperature agarose (Chapter one). DNA was transferred from agarose gels to nylon membranes as described in Chapter one. Hybridizations with the non-radioactive probes and hybrid detection were performed as described earlier (Chapter two).

PCR amplification. Amplifications were performed in

reaction volumes of 50 μ L or 100 μ L containing 10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5-3.0 mM MgCl₂, 0.01% (w/v) gelatin, each primer at 0.05-1.0 μ M, each dNTP (dATP, dCTP, dTTP, dGTP) at 100-200 μ M and 1 unit/50 μ L of Taq DNA polymerase. DNA concentrations used for PCR amplifications were as follows: 100 ng /50 μ L for genomic DNA amplifications; 1 ng/50 μ L for amplifications from linearized plasmid DNA, recombinant bacteriophage genomes and DNA fragments purified from agarose gels. To reduce evaporation, the reaction mixture was overlaid with 50 or 100 μ L of mineral oil (Perkin-Elmer Cetus). Amplifications were carried out for 30-40 cycles using a thermocycler (Model PCR1000, Perkin-Elmer Cetus, Norwalk, CT). The DNA was denatured at 94°C for 1 min, annealed at 40-55°C for 1-2 min and extended for 1-2 min at 72°C. After the last cycle, the template was extended at 72°C for an additional 7 min and then chilled slowly to 4°C. A control amplification which contains all the components of the polymerase chain reaction except the template DNA was included to ensure that DNA amplification did not result from contaminants in the reaction mixture. The amplification products were analysed by agarose gel electrophoresis using 8 μ L from each reaction. Some amplified DNA fragments were purified from agarose gels by using the Gene Clean kit (BIO/CAN Scientific Inc., Mississauga, Ontario) according to

the instructions of the manufacturer (BIO 101 Inc., La Jolla, CA). The use of these purified DNA fragments in PCR reactions significantly reduced PCR artifacts and yielded homogeneous amplification products. The nucleotides and the 10X PCR reaction buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM MgCl₂, 0.1% (w/v) gelatin) were purchased from Perkin-Elmer Cetus. When required, the PCR reaction buffer was supplemented with more MgCl₂ to obtain final concentrations of 2.0-3.0 mM. The Tag polymerase was obtained from Perkin-Elmer Cetus or Bethesda Research Laboratories (Burlington, Ontario).

Four primers (A1, B1, B2 and B3) of 24 to 36 nucleotides in length were used (Table 3-1). These primers were synthesized and purified by HPLC (high pressure liquid chromatography) at the University of Ottawa Biotechnology Research Institute (Ottawa, Ontario). The nucleotide sequence of the oligonucleotides was selected from the E. coli carAB operon sequence. The rationale for the selection of these four oligonucleotides was primarily based on the fact that these sequences possess a higher degree of homology to their counterparts in the two eucaryotic CPSase genes than the rest of the E. coli carAB operon (Lusty et al., 1983; Nyunoya et al. 1985; Werner et al., 1985). The selected oligonucleotide sequences are dispersed over most of the E. coli carAB

Table 3-1. Primers used for PCR amplifications of the gonococcal CPSase genes.

Primer	Sequence ^a
A1	5'-GGGGAAGTCGTTTTCAATACTTCA-3'
B1	5'-TATTTTCGAA/GTAGTCAAATTCACACGCCTGACCGAT-3'
B2	5'-ATACTGCAG/GTTGGCGAAGTTATGGCCATTGGT-3'
B3	5'-GACTTCCCCGGTAGAGCGCATTTC-3'

^a The nucleotides to the left of the slash are absent in the gene sequence and have been introduced into the primer to create a restriction site at the 5' end: HindIII for B1 and PstI for B2. In addition, an overhang of three nucleotides (TAT for B1 and ATA for B2) was placed at the 5' end of the restriction site to facilitate enzyme binding and restriction.

operon (Fig. 3-2). The alignment of the four selected oligonucleotide sequences from the *E. coli carAB* operon (Nyunoya and Lusty, 1983; Piette *et al.*, 1984) with the corresponding regions of the eucaryotic CPSase genes from *S. cerevisiae* (Lusty *et al.*, 1983; Werner *et al.*, 1985) and the rat (Nyunoya *et al.*, 1985) is presented in Fig. 3-1. The primer sequences were taken from the *E. coli carAB* operon sequence (Nyunoya and Lusty, 1983; Piette *et al.*, 1984) since these oligonucleotides were designed to amplify portions of the procaryotic CPSase genes from *N. gonorrhoeae*. Differences in the codon usage for *N. gonorrhoeae* (West and Clarke, 1989) as compared to *E. coli* (Nyunoya and Lusty, 1983; Piette *et al.*, 1984) were also considered for the determination of the primer sequences (Fig. 3-1). Based on the nucleotide sequence of the *E. coli carAB* operon (Nyunoya and Lusty, 1983; Piette *et al.*, 1984), the predicted sizes for the amplification products using different pair combinations of these primers (Fig. 3-2) were as follows: (i) A1-B1; 1169 nucleotides, (ii) B2-B1; 560 nucleotides, (iii) B2-B3, 1571 nucleotides and (iv) A1-B3; 3836 nucleotides.

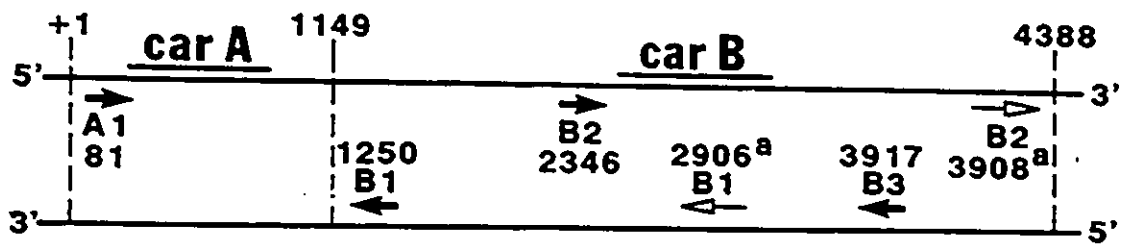
Figure 3-1. Comparison of the nucleotide sequence of the four primer sequences (A1, B1, B2 and B3) used for PCR amplifications with the corresponding regions of the *E. coli*, the yeast and the rat CPSase genes (Lusty *et al.*, 1983; Nyunoya and Lusty, 1983; Nyunoya *et al.*, 1985). The nucleotides from the primer sequence conserved in three (*), two (+) or in only one (-) CPSase genes are indicated.

^a Indicates the nucleotide sequence position from the 5' to the 3' end of the corresponding sequence in the *E. coli carAB* operon (Nyunoya and Lusty, 1983).

		***	***	++-	***	***	***	***	++-
A1	5'-GGG	GAA	GTC	GTT	TTC	AAT	ACT	TCA	-3'
<u>E. coli</u>	81-GGG	GAA	GTC	GTT	TTC	AAT	ACT	TCA	-105 ^a
Yeast	GGT	GAA	ACA	GTT	TTC	ACT	ACT	TCT	
Rat	GGC	GAA	GTG	GTT	TTT	AAT	ACT	GGC	
		***	-**	+++	+++	***	-**	+++	***
B1	5'-GTA	GTC	AAA	TTC	ACA	CGC	CTG	ACC	GAT-3'
<u>E. coli</u>	1250-GTA	GTC	AAA	CTC	ACA	CGC	CTG	ACC	GAT-1223 ^a
Yeast	GTA	ATC	AA A	TTC	ACC	TGC	CTG	ACC	AAT
Rat	GTA	ATC	GAA	TTC	ACC	AGC	TTG	ACC	AAT
		***	***	***	***	***	***	***	***
B2	5'-GTT	GGC	GAA	GTT	ATG	GCC	ATT	GGT	-3'
<u>E. coli</u>	2346-GTT	GGC	GAA	GTG	ATG	GCG	ATT	GGT	-2370 ^a
Yeast	GTA	GGC	GAG	GTC	ATG	GCC	ATT	GGT	
Rat	GTT	GGA	GAA	GTT	ATG	GCT	ATT	GGT	
		+++	+++	-**	-**	+++	---	***	+++
B3	5'-GAC	TTC	CCC	GGT	AGA	GCG	CAT	TTC	-3'
<u>E. coli</u>	3919-GAC	TTC	CCC	GGT	AGA	GCG	CAT	TTC	-3893 ^a
Yeast	AAC	TTC	ACC	AGT	TGA	TGC	CAT	TTC	
Rat	GAC	CTC	TCC	AGT	AGA	AGC	CAT	CTC	

Figure 3-2. Nucleotide positions of the four primer sequences on the E. coli carAB operon used for PCR amplifications. These primer sequences are designated with a black arrowhead. The position of the second copy of oligonucleotides B1 and B2 in the internally duplicated carB gene is designated with a white arrowhead.

^a Oligonucleotides B1 and B2 are found at two different locations in the E. coli carB gene since it is duplicated (Nyunoya and Lusty, 1983).



RESULTS

Subcloning of carA and carB. Attempts to subclone into the plasmid vectors pGEM-3Z and pGEM-7Z the gonococcal carA and carB genes from recombinant bacteriophages EFP10 (carries the gonococcal carA and carB genes) and EFP20 (carries the gonococcal carB gene) were unsuccessful. Many attempts were performed by using a shotgun cloning approach. The cloning vector and the recombinant bacteriophage were both digested with the same restriction endonuclease (EcoRI, SalI, HindIII and SmaI were tried out) and then ligated with T4 DNA ligase using a molar ratio vector:insert of approximately 1:3. The ligation mix was used to transform E. coli K-12 strains MI178 (carA mutant) and JEF8 (carB mutant). Selection for transformants was carried out in minimal medium lacking arginine and containing ampicillin. The failure of shotgun cloning could be attributable to the presence, in the gonococcal CPSase genes, of a recognition site for the restriction endonucleases tested. The large size of carB (around 3 kb in E. coli) could make the cloning of that gene more difficult.

Attempts to clone the 4.9 kb-EcoRI fragment from recombinant bacteriophage EFP10 (extracted from low melting

temperature agarose), which hybridizes with the *E. coli* carAB genes (Chapter One, Fig. 1-1), by complementation of car gene defects in *E. coli* were also unsuccessful. Recently, N. Bigelow (this laboratory) has cloned this 4.9 kb DNA fragment in pBR322 by selection for antibiotic resistance markers. This recombinant plasmid has not been tested for its ability to complement car gene defects in *E. coli*. My inability to isolate this clone may be explained by the fact that this fragment does not carry the entire carA or carB genes since the screening system for transformants was based on the ability of the clones to complement car gene defects in *E. coli*.

The failure of the strategies to subclone the gonococcal carA and carB genes described above could also be explained by the low frequencies of transformation (i.e. approximately $1-5 \times 10^3$ transformants/ μ g of pGEM-3Z) with *E. coli* strains MI178 and JEF8. Another technical problem encountered was the presence of "inhibitors" in some batches of minimal medium which inhibited the growth of most cells from the *E. coli* arginine auxotrophs.

Alternatively, a *N. gonorrhoeae* genomic library in pGEM-1Z (Chapter Two) was screened by complementation of carA and

carB gene defects in E. coli. The E. coli mutants were transformed either by the calcium chloride procedure or by electroporation (yielded frequencies of up to approximately 1×10^{10} transformants/ μ g of pGEM-32). These strategies were also unsuccessful possibly because of the non-representativity of the library since it was amplified in E. coli HB101 which possesses the MCR restriction system.

PCR amplifications of the gonococcal CPSase genes. Initially, the correctness and the specificity of the four primers used in this study (Table 3-1) were verified by PCR amplifications carried out with pMC50 linearized with HindIII as template DNA (the plasmid pMC50 carries the entire E. coli carAB operon on a 5.6 kb HindIII fragment (Piette et al., 1984)). For all primer combinations, a homogeneous amplification product of the predicted size (Nyunoya and Lusty, 1983; Piette et al., 1984) was obtained (i.e. A1-B1, 1.2 kb; B2-B1, 0.6 kb; B2-B3, 1.6 kb; and A1-B3, 3.8 kb) (Fig. 3-2, 3-3A and 3-4A). The size of the amplified DNA ranged from 0.6 kb (primers B2-B1; Fig. 3-3A, lane 1) to 3.8 kb (primers A1-B3; Fig. 3-3A, lane 12). Primer combinations A1-B1 and B2-B3 each yielded an amplification product of 1.2 kb and 1.6 kb, respectively (Fig. 3-3A, lanes 7 and 18). PCR amplification of the 3.8 kb fragment with A1-B3 was particularly efficient

Figure 3-3. Amplification by PCR^b of recombinant bacteriophage DNA from EFP10 (carAB⁺), recombinant plasmid pMC50 (carries the entire *E. coli carAB* operon) and *N. gonorrhoeae* chromosomal DNA from the prototrophic strain CH811. (A) Amplifications with primers B1-B2 (lanes 1 to 4), A1-B1 (lanes 7 to 9), A1-B3 (lanes 12 to 15) and B2-B3 (lanes 18 to 21). Template DNA used for PCR amplifications was EFP10 (lanes 4, 9, 15, 20 and 21), *N. gonorrhoeae* genomic DNA (lanes 2, 3, 8, 13, 14 and 19) and pMC50 (lanes 1, 7, 12 and 18). Lanes 5, 11 and 17 contain HindIII-digested lambda DNA^a and lanes 6, 10 and 16 are empty. (B) Southern blot hybridization of the same gel for dig-dUTP-labelled gonococcal CPSase genes probe (prepared from the 4.9 kb EcoRI fragment from clone EFP10). The size of the fragments in kilobases is indicated.

^a The probe hybridized weakly with lambda DNA since it contains broken lambda DNA taken up during the purification by extraction from low-melting-temperature agarose of the 4.9 kb EcoRI fragment from recombinant bacteriophage EFP10 used as a probe.

^b Control amplifications which contain all the components of the polymerase chain reaction except the template DNA were performed. These reactions were analyzed by electrophoresis in another agarose gel.

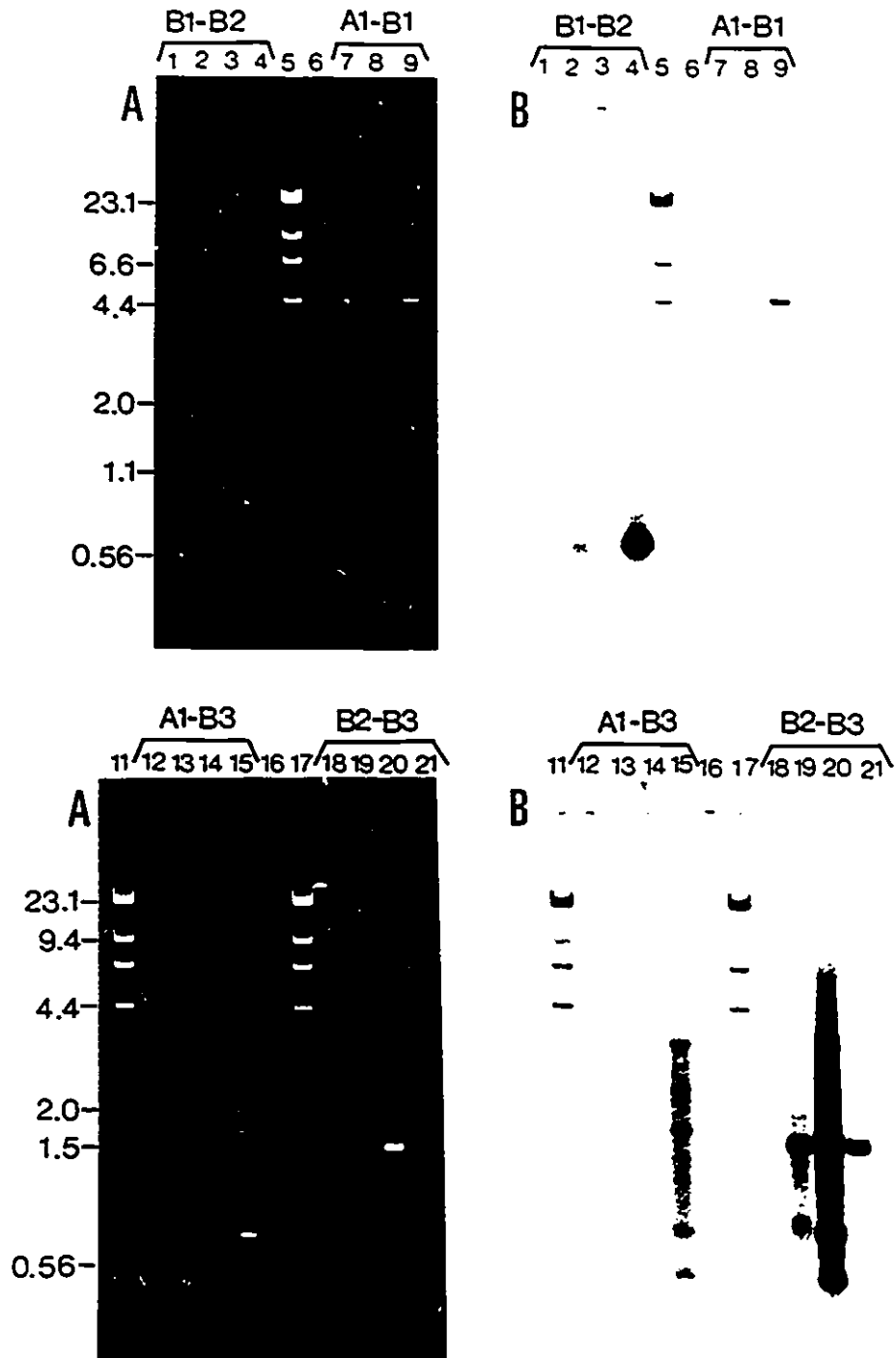
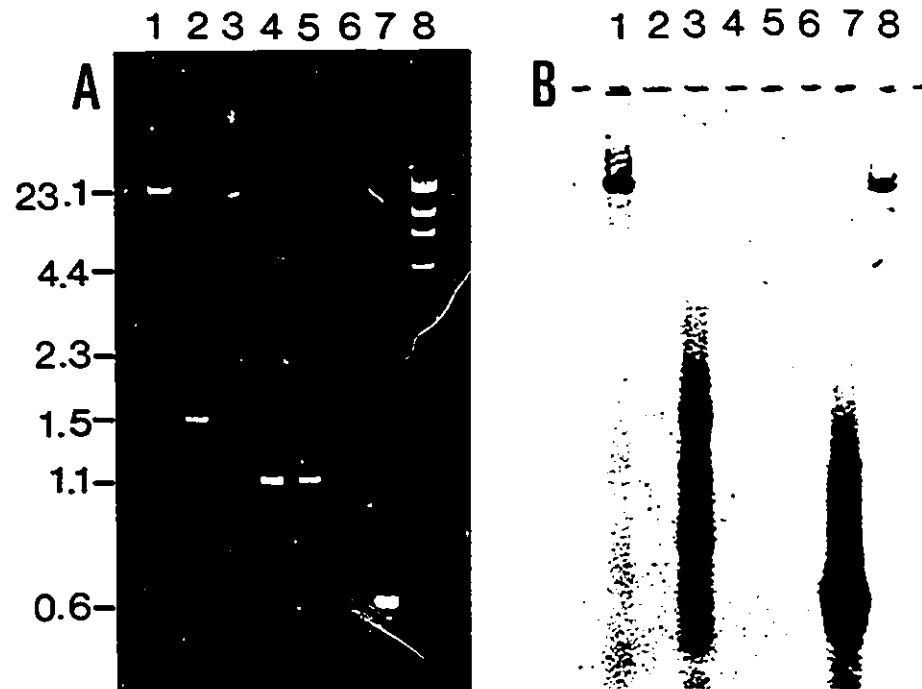


Figure 3-4. Amplification by PCR^b of recombinant bacteriophage DNA from EFP10 (carAB⁺) and recombinant plasmid pMC50 (carries the entire *E. coli carAB* operon). (A) Amplifications with primers B2-B3 (lanes 2 and 3), A1-B1 (lanes 4 and 5) and B1-B2 (lanes 6 and 7) . The template DNA used as template for PCR amplifications was EFP10 (lanes 3, 5 and 7) and pMC50 (lanes 2, 4 and 6). Lanes 1 and 8 contain undigested EFP10 DNA and HindIII digested lambda DNA^a, respectively. (B) Southern blot hybridization of the same gel for dig-dUTP-labelled gonococcal CPSase gene probe (prepared from the 4.9 kb EcoRI fragment from clone EFP10). The size of the fragments in kilobases is indicated.

^a The probe hybridized weakly with lambda DNA since it contains broken lambda DNA taken up during the purification by extraction from low-melting-temperature agarose of the 4.9 kb EcoRI fragment from recombinant bacteriophage EFP10 used as a probe.

^b Control amplifications which contain all the components of the polymerase chain reaction except the template DNA were performed. These reactions were analyzed by electrophoresis in another agarose gel.



with extension periods of 2 min at 72°C for both pMC50 and E. coli genomic DNA from a prototrophic K-12 strain (data not shown).

PCR amplifications of the gonococcal carA and carB genes were then carried out under various conditions using bacteriophage EFP10 (carAB*) DNA and N. gonorrhoeae genomic DNA from a prototrophic strain (CH811) as template (Figure 3-3A). The amplification products were generally more heterogeneous for PCR reactions performed with gonococcal DNA (EFP10 and total genomic DNA) than for reactions carried out with E. coli DNA (pMC50 and total genomic DNA) (data not shown). By using primer concentrations of 0.05 to 0.1 μM instead of 1.0 μM typically used in PCR reactions (Saiki et al., 1988), a more homogeneous amplification product was obtained with gonococcal DNA (Fig. 3-3 and 3-4). The comparison of the amplification products obtained with primer (B2-B3) concentrations of 0.2 μM (lane 20) and 0.1 μM (lane 21) is shown on Fig. 3-3. Kreitman and Landweber (1989) have also found that the use of lower primer concentration (i.e. 0.05 to 0.1 μM) yielded a more homogeneous amplification product. Since the primer sequence was based on the E. coli carAB operon sequence, amplifications of the gonococcal CPSase genes were initiated at a low annealing temperature (i.e.

40°C) and then gradually increased to 55°C in order to reduce unspecific priming. It was found that annealing temperatures above 50°C were not suitable for all primer combinations. This may be attributable to mismatches between the sequences of the primers and that of the *N. gonorrhoeae* CPSase genes since the primers were selected from the *E. coli carAB* operon sequence. Other factors such as the length of the annealing and of the extension periods as well as the concentration of Mg⁺⁺ ions were also controlled to increase the homogeneity of the amplification product.

The amplifications with the gonococcal genomic DNA and with EFP10 DNA apparently yielded several common fragments as revealed by the pattern of hybridization of the amplification products with the gonococcal *carAB* probe (Fig. 3-3). Interestingly, the amplifications of gonococcal DNA with primers B2-B1, A1-B1 and B2-B3 all yielded an amplification product (0.6, 1.1 and 1.5 kb, respectively; Fig. 3-3, lanes 4, 9 and 21) of size similar to that obtained from the amplification of pMC50 using the same primers (Fig. 3-3, lanes 1, 7 and 18). Southern blot hybridization showed that gonococcal DNA amplified with primers B2-B3 and B2-B1 hybridized with the gonococcal probe (Fig. 3-3, lanes 2, 3, 4, 19, 20 and 21; Fig. 3-4, lanes 3 and 7). The amplification

product from pMC50, which is of similar size, gave a much weaker hybridization signal than the gonococcal amplification product (Fig. 3-3, lanes 1 and 18; Fig. 3-4, lanes 2 and 6) indicating that these DNA fragments are different from the gonococcal amplification product. This is an important fact when one considers the potential risk of DNA contamination associated with the use of PCR. The amplification with primers A1-B3 yielded a product of 3.3 kb which hybridized well with the gonococcal probe (Fig. 3-3, lane 15). Since the *E. coli* amplification product with these primers is 3.85 kb (Fig. 3-3, lane 12), these results suggest that the *N. gonorrhoeae* CPSase genes are slightly shorter than the *E. coli* carAB operon.

The 1.1 kb fragment produced with *N. gonorrhoeae* amplifications using primers A1-B1 hybridized very weakly with the gonococcal probe (Fig. 3-3, lane 9; Fig. 3-4, lane 5; and other data not shown). An even weaker hybridization signal was observed with the 1.1 kb fragment produced with *E. coli* amplification (Fig. 3-3, lane 7; Fig. 3-4, lane 4; and other data not shown). A possible explanation would be the absence or a deletion of the carA gene on the gonococcal probe. In fact, the 4.9 kb EcoRI fragment (purified from the recombinant bacteriophage EFP10) from which the probe was prepared is found in both EFP10 (carA⁺) and EFP20 (carB⁺) recombinant

bacteriophages (Chapter one) suggesting that this fragment does not carry the entire gonococcal carA gene. PCR amplifications using the 4.9 kb EcoRI fragment from bacteriophage EFP10 as template did not yield any amplification product with the A1-B1 primer combination, thereby also suggesting that this fragment does not carry the entire carA gene (data not shown). Amplifications of this template using primers B2-B1 and B2-B3 produced DNA fragments of size identical to that observed with amplification from EFP10 and the gonococcal chromosome (data not shown). The A1-B1 amplification product was probed with EFP10 DNA (carries the entire gonococcal carA and carB genes). This hybridization did not show either an hybridization signal suggesting that the amplified DNA fragment was not of gonococcal origin. The ability of the 5.6 kb HindIII fragment from pMC50 (carries the E. coli carAB operon) to hybridize preferably with the 1.1 kb fragment (data not shown) suggests that this DNA originated from E. coli. This hypothesis was confirmed by M13 sequencing which showed that 376 and 408 nucleotides from each end of the fragment matched perfectly with the corresponding region of the E. coli CPSase gene sequence. This situation may be explained by a contamination of the PCR reaction with E. coli DNA carrying the CPSase genes (i.e. pMC50 or E. coli genomic DNA).

The three amplification products from EFP10 which were of size similar to that from pMC50 amplifications were purified from agarose gels and used as template for subsequent PCR amplifications. This strategy yielded more homogeneous amplification products (Fig. 3-4A, lanes 3, 5 and 7). The 0.6 kb amplification product from EFP10 with primers B2-B1 has been cloned in M13 and partially sequenced. The preliminary sequence for this portion of the gonococcal carB gene is presented in Appendix B.

DISCUSSION

Attempts to subclone the gonococcal carA and carB genes from the recombinant bacteriophage EFP10 (carAB⁺) in plasmid vectors were unsuccessful³. Other attempts to select a recombinant plasmid carrying those genes from a purchased N. gonorrhoeae genomic library constructed in the plasmid pGEM-12 (Chapter two) by transformation and electroporation of E. coli arginine auxotrophs were also unsuccessful³. Amplification by PCR of the gonococcal CPSase genes represented an attractive alternative strategy since N. gonorrhoeae and E. coli CPSase genes are well conserved (Chapter one). Therefore, primer sequences for PCR amplification were chosen among regions of the E. coli carAB operon which are highly conserved in the eucaryotic CPSase genes from the yeast and the rat. PCR with these primers yielded amplified copies of various portions of the N. gonorrhoeae CPSase genes. Similarly, Shyamala and Ames (1989) took advantage of the extensive homology between the genome of Salmonella typhimurium and E. coli by using S. typhimurium sequences to amplify regions of the E. coli histidine permease genes.

³ More details about the strategies used to subclone the gonococcal carA and carB as well as possible explanations for their failure are given in the Result section (Chapter 3).

Since the four PCR primers used in this study are dispersed over most of the *E. coli* carAB operon, the comparison of the *N. gonorrhoeae* with the *E. coli* amplification products using these primers has given some information about the size and the structure of the gonococcal CPSase genes. For example, the size of the gonococcal amplification products for primers B2-B1 and B2-B3, which are slightly shorter than the *E. coli* amplification product, suggests that the *N. gonorrhoeae* carAB genes may be slightly smaller than the *E. coli* carAB operon.

Primers B2 and B1 were originally designed to amplify fragments with primers B3 and A1, respectively (Fig. 3-2). As a consequence, PCR amplification of the CPSase genes using primers B1-B2 should not yield an amplification product since DNA synthesis from these primers is achieved in opposite directions. Therefore, the ability of primers B2-B1 to amplify a portion of the *N. gonorrhoeae* carB gene suggests that sequences similar to those of primers B1 and B2 are present elsewhere in the gonococcal CPSase genes which allow the annealing of the primers. In *E. coli*, a portion of the carB gene can be amplified with the primer combination B2-B1 since this gene is internally duplicated (Nyunoya and Lusty, 1983). These results suggest that the *N. gonorrhoeae* carB gene may be

internally duplicated as its E. coli counterpart. The fact that the gonococcal amplification product with primers B2-B1 matches well in size with the E. coli carB amplification product indicates that the gonococcal carB gene duplication is similar to that of E. coli. This is supported by preliminary sequence data (Appendix B, Fig. B-2) of the B2-B1 gonococcal amplification product which suggests that its nucleotide sequence matches well (i.e. approximately 70% similarity) with the corresponding region of the E. coli carB gene. This carB internal duplication is also found in the yeast and in the rat CPSase genes suggesting that this duplication occurred before the divergence of procaryotes and eucaryotes (Lusty et al., 1983; Nyunoya et al., 1985). Thus, this simple PCR approach can be used to demonstrate the presence of a gene duplication.

Future research should be oriented toward sequencing the entire gonococcal CPSase genes including the regulatory region(s) in order to obtain the complete sequence of those genes for comparisons with procaryotic and eucaryotic CPSase genes. The study of the regulatory region(s) for the gonococcal CPSase genes is of particular interest since CP is an intermediate in arginine and pyrimidine biosynthesis. This will address the following questions: Are the gonococcal CPSase genes organized in an operon? Are tandem promoters

responsible for the regulation of these genes as in E. coli and S. typhimurium? Does repression or attenuation play a role in the regulation of the gonococcal CPSase genes?

CONCLUSIONS

The complementation of various E. coli mutants defective in arginine biosynthesis genes with clones of N. gonorrhoeae genomic DNA combined with enzymatic assays for several arginine biosynthesis enzymes has provided the following fundamental knowledge about the arginine biosynthesis pathway:

- (i) Arginine biosynthesis genes from N. gonorrhoeae can be functionally expressed in E. coli.
- (ii) The OATase enzyme (encoded by argJ) activity can functionally replace the activity of the enzyme AODase (encoded by argE).
- (iii) OATase can be synthesized in E. coli.
- (iv) The OATase (encoded by argJ) may also be able to functionally replace AGSase (encoded by argA) activity. This hypothesis appears to be valid since a clone of gonococcal DNA which apparently carries only the OATase gene complements E. coli gene defects in both argA and argE (M.H. Mulks, personal communication).

Co-complementation studies with the various recombinant bacteriophages isolated to study the organization of arginine and proline biosynthesis genes indicate that:

- (i) Most gonococcal arginine biosynthesis genes are unlinked as in the Pseudomonadaceae suggesting that N. gonorrhoeae is

more closely related to the Pseudomonadaceae than to enteric bacteria or Bacillaceae in which arginine biosynthesis genes are clustered.

(ii) The gonococcal carA and carB genes are closely linked suggesting that they are organized in an operon as in E. coli and S. typhimurium.

(iii) The gonococcal proC gene is not closely linked with the proBA gene cluster reflecting the organization of proline biosynthesis genes encountered in other bacterial species.

(iv) Arginine and proline biosynthesis genes are unlinked in N. gonorrhoeae.

The studies on the organization of arginine and proline biosynthesis genes have provided additional information for improving the knowledge of the genetic map for the N. gonorrhoeae chromosome (West and Clark, 1989).

Hybridization studies of several gonococcal arginine biosynthesis genes with the corresponding E. coli genes indicate that:

(i) The CPSase genes (carA and carB) from N. gonorrhoeae and E. coli are very similar. By taking advantage of this apparently high similarity, it was possible to use primers homologous to the E. coli carAB operon sequence to amplify by

PCR several portions of the gonococcal CPSase genes.

(ii) The nucleotide sequence of carAB genes is more conserved than that of argF and argB genes in these two organisms and that the nucleotide sequence of argB is less conserved than that of argF.

The characterization of arginine and proline biosynthesis gene defects in naturally-occurring auxotrophs of N. gonorrhoeae uncovered heterogeneity at the genetic level:

(i) Strains having a dual requirement for citrulline and uracil appear to be defective either in the CPSase genes or in the OTCase gene. These gene defects may be combined with other arginine biosynthesis gene defect(s) in the preceding steps of the pathway.

(ii) The inability of strains of auxotypes CUH to convert ornithine to citrulline appears to be attributable to a defect either in the CPSase genes or in the OTCase gene.

(iii) Based on hybridization studies with the gonococcal carAB probe, the CPSase gene mutations encountered in the gonococcus may be heterogeneous. Hybridization data suggest that those mutations are not mediated by the insertion of the gonococcal cryptic plasmid into the chromosome.

(vi) Arginine and proline auxotrophy in N. gonorrhoeae appears to be attributable to defects into a variety of biosynthesis

genes: Defects in genes argJ, argF, carAB, argG, argA, proA and proB were apparently identified.

Genetic studies on arginine and proline auxotrophs have pointed out several observations with potential epidemiological applications:

- (i) There is possibly a relationship between the proline gene defect of a strain and its auxotype (i.e. proB versus auxotype PCU).
- (ii) Auxotyping of N. gonorrhoeae using ornithine and arginine appears to be a good procedure to identify strains with gene defects in argJ and argG, respectively.
- (iii) The use of the gonococcal CPSase probe for screening for restriction fragments length polymorphism may provide a useful epidemiological marker for N. gonorrhoeae.

The chromosome marker rescue approach used in this study seems to represent a useful approach to identify and study the prevalence of biosynthesis gene defects in the gonococcus and offers distinct advantages as compared with genetic complementation in trans. This method may be particularly useful to identify gene defects in biosynthesis for which the precursors are not commercially available, such as for proline biosynthesis.

PCR amplifications of the gonococcal CPSase genes using various combinations of E.coli-specific primers indicated that:

- (i) The gonococcal carB gene is duplicated. PCR amplifications and DNA sequencing suggest that this duplication is similar to that encountered in E. coli.
- (ii) The size of the gonococcal CPSase genes is slightly reduced as compared with E. coli.

REFERENCES

- Abdelal, A.T.H., and J.L. Ingraham. 1975. Carbamoylphosphate synthetase from Salmonella typhimurium. Regulation, subunit composition and function of the subunits. J. Biol. Chem. 250:4410-4417.
- Abdelal, A.T. 1979. Arginine catabolism by microorganisms. Annu. Rev. Microbiol. 33:139-168.
- Adams, E., and L. Frank. 1980. Metabolism of proline and the hydroxyprolines. Annu. Rev. Biochem. 49:1005-1061.
- Ashford, W., R. Golash, and V. Hemming. 1976. Penicillin-producing Neisseria gonorrhoeae. Lancet ii:657-658.
- Dachmann, B.J. 1990. Linkage map of Escherichia coli K-12, Edition 8. Microbiol. Rev. 54:130-197.
- Baur, H., V. Stalon, P. Falmagne, E. Luethi, and D. Haas. 1987. Primary and quaternary structure of the catabolic ornithine carbamoyltransferase from Pseudomonas aeruginosa. Eur. J. Biochem. 166:111-117.
- Bencini, D.A., J.E. Houghton, T.A. Hoover, K.F. Foltermann, J.R. Wild, and G.A. O'Donovan. 1983. The DNA sequence of argI from Escherichia coli K-12. Nucleic Acid Res. 11:8509-8518.
- Birnboim, H.C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Res. 7:1513-1523.
- Biswas, G.D., T. Sox, E. Blackman, and P.F. Sparling. 1977. Factors affecting genetic transformation in Neisseria gonorrhoeae. J. Bacteriol. 129:983-992.
- Biswas, G.D., and P.F. Sparling. 1981. Entry of double-stranded deoxyribonucleic acid during transformation of Neisseria gonorrhoeae. J. Bacteriol. 145:638-640.
- Biswas, G.D., J.B. Graves, T.F. Sox, F.C. Tenover, and P.F. Sparling. 1982. Marker rescue by homologous recipient plasmid during transformation of gonococci by a hybrid Pc^r plasmid. J. Bacteriol. 151:77-82.
- Biswas, G.D., J.B. Graves, R. Schwalbe, and P.F. Sparling.

1986. Construction of isogenic gonococcal strains varying in the presence of a 4.2 kilobase cryptic plasmid. *J. Bacteriol.* 167:685-694.

Biswas, G.D., S.A. Lacks, and P.F. Sparling. 1989a. Transformation-deficient mutants of piliated Neisseria gonorrhoeae. *J. Bacteriol.* 171:657-664.

Biswas, G.D., S.A. Thompson, and P.F. Sparling. 1989b. Gene transfer in Neisseria gonorrhoeae. *Clin. Microbiol. Rev.* 2:S24-S28.

Bowmer, E.J. 1976. Penicillinase-producing Neisseria gonorrhoeae. *Can. Dis. Weekly Rep.* 2-48:1.

Brown, K., P.W. Finch, I.D. Hickson, and P.T. Emmerson. 1987. Complete nucleotide sequence of the Escherichia coli *argA* gene. *Nucleic Acid Res.* 15:10586.

Burnstein, K.L., D. Dyer, and P.F. Sparling. 1988. Preferential uptake of restriction fragments from the gonococcal cryptic plasmid by competent Neisseria gonorrhoeae. *J. Gen. Microbiol.* 134:547-557.

Campbell, L.A., and R.E. Yasbin. 1984. Mutagenesis of Neisseria gonorrhoeae: Absence of error-prone repair. *J. Bacteriol.* 160:288-293.

Cannon, J.G., and P.F. Sparling. 1984. Genetics of the gonococcus. *Annu. Rev. Microbiol.* 38:111-113.

Carifo, K., and B.W. Catlin. 1973. Neisseria gonorrhoeae auxotyping: differentiation of clinical isolates based on growth responses on chemically defined media. *Appl. Microbiol.* 26 :223-230.

Catlin, B.W. 1973. Nutritional profiles of Neisseria gonorrhoeae, Neisseria meningitidis and Neisseria lactamica in chemically defined media and the use of growth requirements for gonococcal typing. *J. Infect. Dis.* 128:178-194.

Catlin, B.W. 1975. Evolution of Neisseria gonorrhoeae-1975, p. 453-466. In D. Schleissinger (ed.), *Microbiology-1976*. American Society for Microbiology, Washington, D.C.

Catlin, B.W. 1977. Nutritional requirements and auxotyping. p. 91-109. In R.B. Roberts (ed.). *The gonococcus*. John Wiley and Sons, Inc., New York.

- Catlin, B.W., and E.H. Nash. 1978. Arginine biosynthesis in gonococci isolated from patients, p.1-7. In G.F. Brooks, E.C. Gotschlich, K.K. Holmes, W.D. Sawyer and F.E. Young (ed.), Immunobiology of *Neisseria gonorrhoeae*. American Society for Microbiology, Washington, D.C.
- Charlier, D., G. Weyens, M. Roovers, J. Piette, C. Bocquet, A. Piérard and N. Glansdorff. 1988. Molecular interactions in the control region of the carAB operon encoding *Escherichia coli* carbamoylphosphate synthetase. J. Mol. Biol. 204:867-877.
- Clarke, L., and J. Carbon. 1978. Functional expression of cloned yeast DNA in *Escherichia coli*: specific complementation of arginosuccinate lyase (argH) mutations. J. Mol. Biol. 120:517-532.
- Copley, C.G., and S.I. Egglestone. 1980. Auxotyping of *Neisseria gonorrhoeae* isolated in the United Kingdom. J. Med. Microbiol. 16:295-302.
- Crabeel, M., D. Charlier, R. Cunin, and N. Glansdorff. 1979. Cloning and endonuclease restriction analysis of argE and of the control region of the argECBH bipolar operon in *Escherichia coli*. Gene 5:207-231.
- Crawford, I.P. 1975. Gene rearrangements in the evolution of the tryptophan synthetic pathway. Bacteriol. Rev. 39:87-120.
- Csonka, L.N., and A. Baich. 1983. Proline biosynthesis, p. 35-51. In K.M. Herrmann and R.L. Somerville (ed.), Amino acids: Biosynthesis and genetic regulation. Addison-Wesley Publishing Company Inc., Boston, Mass.
- Cunin, R. 1983. Regulation of arginine biosynthesis in prokaryotes, p.53-78. In K.M. Herrmann and R.L. Somerville (ed.), Amino acids: Biosynthesis and genetic regulation. Addison-Wesley Publishing Company Inc., Boston, Mass.
- Cunin, R., T. Eckhardt, J. Piette, A. Boyen, A. Piérard, and N. Glansdorff. 1983. Molecular basis of modulated regulation of gene expression in the regulon of *Escherichia coli* K-12. Nucleic Acids Res. 11:5007-5019.
- Cunin, R., N. Glansdorff, A. Piérard, and V. Stalon. 1986. Biosynthesis and metabolism of arginine in bacteria. Microbiol. Rev. 50:314-352.
- Datta, A.R., R. Ostroff, and A.M. MacQuillan. 1987. Genetic

and physical characterization of proBA genes of the marine bacterium Vibrio parahaemolyticus. Appl. Environ. Microbiol. 53:2733-2738.

Davies, J.K. 1989. DNA restriction and modifications systems in Neisseria gonorrhoeae. Clin. Microbiol. Rev. 2:S78-S82.

Davis, R.H. 1983. Arginine synthesis in eucaryotes, p. 81-102. In K.M. Herrmann and R.L. Somerville (ed.), Amino acids: Biosynthesis and genetic regulation. Addison-Wesley Publishing Company Inc., Boston, Mass.

Davis, R.W., D. Botstein, and J.R. Roth. 1980. A manual for genetic engineering: Advanced bacterial genetics, p. 49-53, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Dénes, G. 1970. Ornithine acetyltransferase (Chlamydomonas reinhardtii). Methods Enzymol. 17A:273-277.

Deutch, A.H., C.J. Smith, K.E. Rushlow, and P.J. Kretschmer. 1982. Escherichia coli γ -pyrroline-5-carboxylate reductase: gene sequence, protein overproduction and purification. Nucleic Acid Res. 10:7701-7714.

Deutch, A.H., K.E. Rushlow, and C.J. Smith. 1984. Analysis of the Escherichia coli proBA locus by DNA and protein sequencing. Nucleic Acid Res. 12:6337-6355.

Dillon, J.R., and M. Pauzé. 1981. Relationship between plasmid content and auxotype in Neisseria gonorrhoeae isolates. Infect. Immun. 33:625-628.

Dillon, J.R. 1983. Laboratory methods for Neisseria gonorrhoeae. Publication H47-58/1983E, Health and Welfare Canada, Ottawa, Ontario.

Dillon, J.R., and M. Pauzé. 1984. Introductory address: Resistance to antimicrobial agents. What next for Neisseria gonorrhoeae. Sex. Transm. Dis. 11:353-359.

Dillon, J.R., G.S. Bezanson, and K.-H. Yeung. 1985. Basic techniques, p. 1-126. In Dillon, J.R., A. Nasim, and E.R. Nestmann (ed). Recombinant DNA methodology. John Wiley and Sons, New York.

Dillon, J.R., and K.-H. Yeung. 1989. β -lactamase plasmids and chromosomally mediated antibiotic resistance in pathogenic Neisseria species. Clin. Microbiol. Rev. 2:5125-5133.

Dillon, J.R., S. Hickey, K.-H. Yeung, C. Druxler, M. Pauzé, M. Carballo, N. Bigelow, and the National Co-operative Study Group. 1990. National study to determine clinical and biological factors influencing Neisseria gonorrhoeae infections and treatment in children and adults. Final Report. The National Laboratory for Sexually transmitted Diseases. Laboratory Centre for disease control, Health and Welfare Canada, Ottawa, Ontario, Canada.

Dougherty, T.F., A. Asmus, and A. Tomasz. 1979. Specificity of DNA uptake in genetic transformation of gonococci. *Biochem. Biophys. Res. Commun.* 86:97-104.

Duff, M.K., and J.K. Davies. 1988. Multiple restriction-modification systems in Neisseria gonorrhoeae. *Gene* 74:227-228.

Eisenstein, B.I., T.J. Lee, and P.F. Sparling. 1977. Penicillin sensitivity and serum resistance are independent attributes of strains of Neisseria gonorrhoeae causing disseminated gonococcal infection. *Infect. Immun.* 15:834-841.

Elmros, T., L.G. Buiman, and G.D. Bloom. 1975. Autolysis of Neisseria gonorrhoeae. *Proc. Soc. Exp. Biol. Med.* 145:1418-1421.

Evins, G.M., and J.S. Knapp. 1988. Characterization of Neisseria gonorrhoeae reference strains used in development of serologic classification systems. *J. Clin. Microbiol.* 26:358-363.

Feller, A., M. Werner, and A. Piérard. 1983. Sequence homologies between the small subunit of carbamoylphosphate synthetase of Saccharomyces cerevisiae and other glutamine amidotransferases. *Arch. Int. Physio. Biochim.* 91:B100-B101.

Frischauf, A.M., H. Lehrach, A. Poustka, and N. Murray. 1983. Lambda replacement vectors carrying polylinker sequences. *J. Molec. Biol.* 170:827-842.

Gallant, J.A. 1979. Stringent control in Escherichia coli. *Annu. Rev. Genet.* 13:393-415.

Gardner, M.M., D.O. Hennig, and R.A. Kelln. 1983. Control of *arg* gene expression in Salmonella typhimurium by the arginine repressor from Escherichia coli K-12. *Mol. Gen. Genet.* 189:458-462.

Gascoyne, D.M., J. Heritage, and P.M. Hawkey. 1990. The 25.2 MDa tetracycline-resistance plasmid is not derived from the 24.5 MDa conjugative plasmid of Neisseria gonorrhoeae. J. Antimicrobials Chemotherapy 25:39-47.

Gigot, D., M. Crabeel, A. Feller, D. Charlier, W. Lissens, N. Glansdorff, and A. Piérard. 1980. Patterns of polarity in the Escherichia coli carAB gene cluster. J. Bacteriol. 127:302-308.

Glansdorff, N. 1965. Topography of cotransducible arginine mutations in Escherichia coli K-12. Genetics 51:167-179.

Glansdorff, N. 1987. Biosynthesis of arginine and polyamines, p. 321-344. In F.C. Neidhardt (ed.), Escherichia coli and Salmonella typhimurium: Cellular and molecular biology. American Society for Microbiology, Washington, D.C.

Goodman, S.D., and J.J. Scocca. 1988. Identification and arrangement of the DNA sequence recognized in specific transformation of Neisseria gonorrhoeae. Proc. Natl. Acad. Sci. USA 85:6982-6986.

Graves, J.G., G.D. Biswas, and P.F. Sparling. 1982. Sequence-specific DNA uptake in transformation of Neisseria gonorrhoeae. J. Bacteriol. 152:1071-1077.

Gyllensten, U.B., and H.A. Erlich. 1988. Generation of single stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA-DQ alpha locus. Proc. Natl. Acad. Sci. USA 85:7652-7656.

Gyllensten, U.B. 1989. PCR and DNA sequencing. Biotechniques 7:700-708.

Haas, D., V. Kurer, and T. Leisinger. 1972. N-acetylglutamate synthetase of Pseudomonas aeruginosa. An assay in vitro and feedback inhibition by arginine. Eur. J. Biochem. 31:290-295.

Haas, D., B.W. Holloway, A. Schambock, and T. Leisinger. 1977. The genetic organization of arginine biosynthesis in Pseudomonas aeruginosa. Molec. Gen. Genet. 154:7-22.

Hagblom, P., C. Korch, A.-B. Jonsson, and S. Normark. 1986. Intragenic variation by site-specific recombination in the cryptic plasmid of Neisseria gonorrhoeae. J. Bacteriol. 167:231-237.

- Hall, B.G., S. Yokoyama, D.H. Calhoun. 1983. Role of cryptic genes in microbial evolution. *Mol. Biol. Evol.* 1:109-124.
- Hays, J.B. 1978. Group translocation transport systems, p.43-102. *In* B.P. Rosen (ed.), *Bacterial transport*. Marcel Dekker Inc., New-York.
- Hendry, A.T., and I.O. Stewart. 1979. Auxanographic grouping and typing of Neisseria gonorrhoeae. *Can. J. Microbiol.* 25:512-521.
- Hoare, D.S., and S.L. Hoare. 1966. Feedback regulation of arginine biosynthesis in blue-green algae and photosynthetic bacteria. *J. Bacteriol.* 92:375-379.
- Holloway, B.W., V. Krishnapilla, and F. Morgan. 1979. Chromosomal genetics of Pseudomonas. *Microbiol. Rev.* 43:73-102.
- Howley, P.M., M.A. Israel, M.-F. Law, and M.A. Martin. 1979. A rapid method for detecting and mapping homology between heterologous DNAs. *J. Biol. Chem.* 254:4876-4883.
- Hutson, J.Y., and M. Downing. 1968. Pyrimidine biosynthesis in Lactobacillus leichmannii. *J. Bacteriol.* 96:1249-1254.
- Huygen, R., M. Crabeel, and N. Glansdorff. 1987. Nucleotide sequence of the ARG3 gene of the yeast Saccharomyces cerevisiae encoding ornithine carbamoyltransferase. *Eur. J. Biochem.* 166:371-377.
- Itikawa, H., S. Baumberg, and H.J. Vogel. 1968. Enzyme basis for a genetic suppression: Accumulation and deacetylation of N-acetylglutamate- γ -semialdehyde in enterobacterial mutants. *Biochem. Biophys. Acta.* 159:547-550.
- Itoh, Y., L. Soldati, V. Stalon, P. Folmagne, Y. Terawaki, T. Leisinger, and D. Haas. 1988. Anabolic ornithine carbamoyltransferase of Pseudomonas aeruginosa: Nucleotide sequence and transcriptional control of the argF structural gene. *J. Bacteriol.* 170:2725-2734.
- Jacob, F., and J. Monod. 1961. Genetic regulatory mechanism in the synthesis of proteins. *J. Mol. Biol.* 3:318-356.
- Janik, A., and G.A. Heym. 1976. Genetic transformation as a tool for detection of Neisseria gonorrhoeae. *J. Clin. Microbiol.* 4:71-81.

- Jeenes, D.J., L. Soldati, H. Baur, J.M. Watson, A. Mercenier, C. Reimann, T. Leisinger, and D. Haas. 1986. Expression of biosynthetic genes from Pseudomonas aeruginosa and Escherichia coli in the heterologous host. *Mol. Gen. Genet.* 203:421-429.
- Kellogg, D.S., Jr., W.L. Peacock, Jr., W.E. Deacon, L. Brown, and C.I. Pirkle. 1963. Neisseria gonorrhoeae. I. Virulence is genetically linked to colony variation. *J. Bacteriol.* 85:1274-1279.
- Kenyon, J.E. 1978. Neisseria gonorrhoeae: Acquisition of auxotrophy in the mouse. *Current Microbiol.* 1:253-256.
- Kerppola, T.K., and M.L. Kahn. 1988. Genetic analysis of carbamoylphosphate synthesis in Rhizobium meliloti 104A14. *J. Gen. Microbiol.* 134:921-929.
- Kilstrup, M., C. Lu, A. Abdelal, and J. Neuhard. 1988. Nucleotide sequence of the carA gene and regulation of the carAB operon in Salmonella typhimurium. *Eur. J. Biochem.* 176:421-429.
- King, S.D., A.R. Brathwaite, and J.R. Dillon. 1987. Treatment of gonorrhea and susceptibility to antimicrobials of PPNG and non-PPNG strains in Jamaica. *Genitourin Med.* 63:365-370.
- Kingsbury, D.T. 1969. Estimate of the genome size of various microorganisms. *J. Bacteriol.* 98:1400-1401.
- Knapp, J.S., and V.L. Clark. 1984. Anaerobic growth of Neisseria gonorrhoeae coupled to nitrite reduction. *Infect. Immun.* 46:176-181.
- Knapp, J.S., and K.K. Holmes. 1975. Disseminated gonococcal infections caused by Neisseria gonorrhoeae with unique growth requirements. *J. Infect. Dis.* 132:204-208.
- Knapp, J.S., C. Thornsberry, G.A. Schoolnik, P.J. Wiesner, K.K. Holmes, and the Cooperative Study Group. 1978. Phenotypic and epidemiological correlates of auxotype in Neisseria gonorrhoeae. *J. Infect. Dis.* 138:160-165.
- Knapp, J.S., M.H. Mulks, I. Lind, H.B. Short, and V.L. Clark. 1985a. Evolution of gonococcal populations in Copenhagen, 1928-1979, p. 82-88. In G.K. Schoolnik, G.F. Brooks, S. Falkow, C.E. Frasch, J.S. Knapp, J.A. McCutchan and S.A. Morse (ed.), The pathogenic Neisseria. American Society for Microbiology, Washington, D.C.

- Knapp, J.S., E.G. Sandstrom, and K.K. Holmes. 1985b. Overview of epidemiological and clinical applications of auxotype/serovar classification of Neisseria gonorrhoeae, p. 6-12. In G.K. Schoolnik, G.F. Brooks, S. Falkow, C.E. Frasch, J.S. Knapp, J.A. McCutchan and S.A. Morse (ed.), The pathogenic Neisseria. American Society for Microbiology, Washington, D.C.
- Knapp, J.S., K.K. Holmes, P. Bonin, and E.W. Hook III. 1987. Epidemiology of gonorrhea: distribution and temporal changes in auxotype/serovar classes of Neisseria gonorrhoeae. Sex. Transm. Dis. 14:26-32.
- Koomey, M.J., R.E. Gill, and S. Falkow. 1982. Genetic and biochemical analysis of gonococcal IgA1 protease; cloning in Escherichia coli and construction of mutants of gonococci that fail to produce the activity. Proc. Natl. Acad. Sci. USA 79:7881-7885.
- Koomey, M.J., and S. Falkow. 1987. Cloning of the recA gene of Neisseria gonorrhoeae and construction of gonococcal recA mutants. J. Bacteriol. 169:790-795.
- Korch, C., P. Hagblom, and S. Normark. 1983. Sequence-specific DNA modification in Neisseria gonorrhoeae. J. Bacteriol. 155:11324-11332.
- Kreitman, M., and L.F. Landweber. 1989. A strategy for producing single-stranded DNA in the polymerase chain reaction. A direct method for genomic sequencing. Gene Anal. Techn. 6:84-88.
- Kretz, P.L., C.H. Reid, A. Greener, and J.M. Short. 1989. Effect of lambda packaging extract mcr restriction activity on DNA cloning. Nucleic Acid Res. 17:5409.
- Lacroute, F., A. Piérard, M. Grenson, and J.M. Wiame. 1965. The biosynthesis of carbamoylphosphate in Saccharomyces cerevisiae. J. Gen. Microbiol. 40:127-142.
- Lederberg, E.M., and J. Lederberg. 1952. Genetic studies of lysogenicity in Escherichia coli. Genetics 38:51-64.
- Lee, E.C., R.I. Walker, and P. Guerry. 1985. Expression of Campylobacter genes for proline biosynthesis in Escherichia coli. Can. J. Microbiol. 31:1064-1067.
- Leisenger, T. 1987. Biosynthesis of proline, p. 345-351. In

F.C. Neidhardt (ed.), Escherichia coli and Salmonella typhimurium: Cellular and molecular biology. American Society for Microbiology, Washington, D.C.

Leive, L.L., and B.D. Davis. 1980. Cell envelope; spores, p. 71-110. In B.D. Davis, R. Dulbecco, H.N. Eisen and H.S. Ginsberg (eds.), Microbiology, 3rd edition. Harper and Row Publishers, New-York.

Lusty, C.J., E.E. Widgrew, K.E. Broglie, and H. Nyunoya. 1983. Yeast carbamoyl phosphate synthetase. J. Biol. Chem. 258:14466-14472.

Maas, W.K., and A.J. Clark. 1964. Studies on the mechanism of repression of arginine biosynthesis in Escherichia coli. II. Dominance of repressibility in diploids. J. Mol. Biol. 8:365-370.

Maham, M.J., and L.N. Csonka. 1983. Genetic analysis of the proBA genes of Salmonella typhimurium. J. Bacteriol. 156:1249-1262.

Maley, J.A., and J.N. Davidson. 1988. Identification of the junction between the glutamine amidotransferase and carbamyl phosphate synthetase domains of the mammalian CAD protein. Biochem. Biophys. Res. Commun. 154:1047-1053.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1982. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Manson, M.D., and C. Yanofsky. 1976. Naturally occurring sites within the Shigella dysenteriae tryptophan operon severely limit tryptophan biosynthesis. J. Bacteriol. 126:668-678.

Marinus, M.G. 1987. DNA methylation in Escherichia coli. Ann. Rev. Genet. 21:113-131.

Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. 3:208-218.

Mathis, L., and J.J. Scoocca. 1984. On the role of pili in transformation of Neisseria gonorrhoeae. J. Gen. Microbiol. 130:3165-3173.

Matsumoto, H., S. Hosogaya, K. Suzuki, and T. Tazaki. 1975. Arginine gene cluster of Serratia marcescens. Jpn. J.

Microbiol. 19:35-44.

McDonald, I.J., and K.G. Johnson. 1975. Nutritional requirements of some nonpathogenic Neisseria grown in simple synthetic media. Can. J. Microbiol. 21:1198-1204.

Meile, L., and T. Leisinger. 1984. Enzymes of arginine biosynthesis in methanogenic bacteria. Experientia 40:899-900.

Mergeay, M., D. Gigot, J. Beckmann, N. Glansdorff, and A. Piérard. 1974. Physiology and genetics of carbamoylphosphate synthesis in Escherichia coli K-12. Mol. Gen. Genet. 133:299-316.

Meyer, T.F. 1987. Molecular basis of surface antigen variation in Neisseria. Trends Genet. 3:319-324.

Miller, H. 1987. Practical aspects of preparing phage and plasmid DNA: Growth, maintenance, and storage of bacteria and bacteriophage, p. 145-170. In S.L. Berger and A.R. Kimmel (ed.), Guide to molecular cloning techniques. Methods Enzymol., vol. 152. Academic Press, San Diego.

Mitchell, L.G., and C.R. Merril. 1989. Affinity generation of single-stranded DNA for dideoxy sequencing following the polymerase chain reaction. Anal. Biochem. 178:239-242.

Morello, J.A., S.A. Lerner, and M. Bohnhoff. 1976. Characteristics of atypical Neisseria gonorrhoeae from disseminated and localized infections. Infect. Immun. 13:1510-1516.

Moreno, J.G., J.R. Dillon, R. Arroyave, A. Maldonado, F. Fich, A. Salvo, D. Villalobos, P. Vincent, and M. Pauzé. 1987. Identification of penicillinase producing Neisseria gonorrhoeae in Chile during clinical and microbiological study of gonococcal susceptibility to antimicrobial agents. Genitourin Med. 63:6-12.

Morris, C.J., and J.F. Thompson. 1975. Acetyl coenzyme A-glutamate acetyltransferase and N-acetylornithine-glutamate acetyltransferase of Chlorella. Plant Physiol. 55:960-967.

Morse, S.A., and L. Barteinstein. 1974. Factors affecting autolysis of Neisseria gonorrhoeae. Proc. Soc. Exp. Biol. Med. 145:1418-1421.

Morse, S.A., S.R. Johnson, J.W. Biddle, and M.C. Roberts.

1986. High-level tetracycline resistance in Neisseria gonorrhoeae is the result of acquisition of streptococcal tetM determinant. *Antimicrob. Agents Chemother.* 30:664-670.

Mountain, A., N.H. Mann, R.N. Munton, and S. Baumberg. 1984. Cloning of a Bacillus subtilis restriction fragment complementing auxotrophic mutants of eight Escherichia coli genes of arginine biosynthesis. *Mol. Gen. Genet.* 197:82-89.

Mulks, M.H., and J.S. Knapp. 1985. Immunoglobulin A1 protease types of Neisseria gonorrhoeae, p. 51-56. In G.K. Schoolnick, G.F. Brooks, S. Falkow, C.E. Frasch, J.S. Knapp, J.A. McCutchan and S.A. Morse (ed.), *The pathogenic Neisseriae*. Proceedings of the Fourth International Symposium. American Society for Microbiology, Washington DC.

Ng, L., M.E. Stiles, and D.E. Taylor. 1987. DNA probes for identification of tetracycline resistance genes in Campylobacter species isolated from swine and cattle. *Antimicrob. Agents Chemother.* 31:1669-1674.

North, A.K., M.C.M. Smith, and S. Baumberg. 1989. Nucleotide sequence of a Bacillus subtilis arginine regulatory gene and homology of its product to the Escherichia coli arginine repressor. *Gene* 80:29-38.

Nyunoya, H., and C.J. Lusty. 1983. The carB gene of Escherichia coli: a duplicated gene coding for the large subunit of carbamoyl-phosphate synthetase. *Proc. Natl. Acad. Sci. USA* 80:4629-4633.

Nyunoya, H., K.E. Brohlie, E.E. Widgrew, and C.J. Lusty. 1985. Characterization and derivation of the gene coding for mitochondrial carbamyl phosphate synthetase I of rat. *J. Biol. Chem.* 260:9346-9356.

Paik, G. 1980. Reagents, strains and miscellaneous tests procedures, p. 1000-1024. In E.H. Lennette, A. Balows, W.J. Hausler and J.P. Truant (ed.), *Manual of clinical microbiology*, 3rd ed. American Society for Microbiology, Washington, D.C.

Paranchych, W. 1966. Stages in phage R17 infection: The role of divalent cations. *Virology* 28:90-99.

Parsot, C., A. Boyen, G.N. Cohen, and N. Glansdorff. 1988. Nucleotide sequence of Escherichia coli argB and argC genes: Comparison of N-acetylglutamate kinase and N-acetyl-

glutamate- γ -semialdehyde dehydrogenase with homologous and analogous enzymes. *Gene* 68:275-283.

Percival, A., J. Rowlands, J.E. Crockill, C.D. Alergant, O.P. Arya, E. Rees, and E.H. Annels. 1976. Penicillinase-producing gonococci in Liverpool. *Lancet* ii:1379-1382.

Phillips, I. 1976. Beta-lactamase producing penicillin-resistant gonococcus. *Lancet* ii:656-657.

Picard, F.J., and J.R. Dillon. 1989a. Cloning and organization of seven arginine biosynthesis genes from Neisseria gonorrhoeae. *J. Bacteriol.* 171:1644-1651.

Picard, F.J., and J.R. Dillon. 1989b. Biochemical and genetic studies with arginine and proline auxotrophs of Neisseria gonorrhoeae. *Can. J. Microbiol.* 35:1069-1075.

Piérard, A., N. Glansdorff, M. Mergeay, and J.M. Wiame. 1965. Control of the biosynthesis of carbamoylphosphate in Escherichia coli. *J. Mol. Biol.* 14:23-46.

Piérard, A. 1966. Control of Escherichia coli carbamoylphosphate synthetase by antagonistic allosteric effectors. *Science* 154:1572-1573.

Piérard, A., N. Glansdorff, D. Gigot, M. Crabeel, P. Halleux, and L. Thiry. 1976. Repression of Escherichia coli carbamoylphosphate synthetase: relationship with enzyme synthesis in the arginine and pyrimidine pathways. *J. Bacteriol.* 127:291-301.

Piette, J., R. Cunin, F. Van Vliet, D. Charlier, M. Crabeel, Y. Ota, and N. Glansdorff. 1982. Homologous control sites and DNA transcription starts in the related argF and argI sites of Escherichia coli K12. *EMBO J.* 1:853-857.

Piette, J., H. Nyunoya, C.J. Lusty, R. Cunin, G. Weyens, M. Crabeel, D. Charlier, N. Glansdorff, and A. Piérard. 1984. DNA sequence of the carA gene and the control region of carAB: Tandem promoters, respectively controlled by arginine and the pyrimidines, regulate the synthesis of carbamoylphosphate synthetase in Escherichia coli K12. *Proc. Natl. Acad. Sci. USA* 81:4134-4138.

Piffaretti, J.C., A. Arini, and J. Frey. 1988. pUB307 mobilizes resistance plasmids from Escherichia coli into Neisseria gonorrhoeae. *Mol. Gen. Genet.* 212:215-218.

Potvin, B., and H. Gooder. 1975. Carbamoylphosphate synthesis in Bacillus subtilis. Biochem. Genet. 13:125-143.

Prozesky, O.W., W.O.K. Grabow, S. van der Merwe, and J.N. Coetzee. 1973. Arginine cluster in Proteus providence group. J. Gen. Microbiol. 77:237-240.

Rabussay, D., L. Uher, G. Bates, and W. Piastrich. 1987. Electroporation of mammalian and plant cells. Focus 9:1-5.

Raleigh, A.R., and G. Wilson. 1986. Escherichia coli K-12 restricts DNA containing 5-methylcytosine. Proc. Natl. Acad. Sci. USA 83:9070-9074.

Rigby, T.M.J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labelling deoxyribonucleic acid to high speed activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. 113:237-251.

Roberts, M., L.P. Elwell, and S. Falkow. 1977. Molecular characterization of two beta-lactamase-specifying plasmids isolated from Neisseria gonorrhoeae. J. Bacteriol. 131:557-563.

Roberts M., and S. Falkow. 1977. Conjugal transfer of R plasmids in Neisseria gonorrhoeae. Nature (London) 266:630-631.

Roberts, M., P. Piot, and S. Falkow. 1979. The ecology of gonococcal plasmids. J. Gen. Microbiol. 114:491-494.

Roberts, M. 1989. Plasmids of Neisseria gonorrhoeae and other Neisseria species. Clin. Microbiol. Rev. 2:S18-S23.

Rodriguez, R.L., and R.C. Tait. 1983. Recombinant DNA techniques: Introduction. Benjamin/Cummings Publishing Company, Minlo Park, California.

Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.E. Mullis, and H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual. 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanderson, K.E., and P.E. Hartman. 1978. Linkage map of

Salmonella typhimurium, edition V. Microbiol. Rev. 42:471-519.

Sanger, F., S. Nicklen, and A. Coulson. 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

Sarafian, S.K., and J.S. Knapp. 1989. Molecular epidemiology of gonorrhea. Clin. Microbiol. Rev. 2:S49-S55.

Seifert, H.S., R.S. Ajioka, C. Marchal, P.F. Sparling, and M. So. 1988. DNA transformation leads to pilin antigenic variation in Neisseria gonorrhoeae. Nature (London) 336:392-395.

Seifert, H.S., R.S. Ajioka, D. Paruchuri, F. Heffron, and M. So. 1989. Shuttle mutagenesis of Neisseria gonorrhoeae: Pilin null mutations lower DNA transformation competence. J. Bacteriol. 172:40-46.

Shinners, E.N., and B.W. Catlin. 1978. Arginine biosynthesis in Neisseria gonorrhoeae: Enzymes catalyzing the formation of ornithine and citrulline. J. Bacteriol. 136:131-135.

Shinners, E.N., and B.W. Catlin. 1982. Arginine and pyrimidine biosynthetic defects in Neisseria gonorrhoeae strains isolated from patients. J. Bacteriol. 151:295-302.

Shyamala, V., and G.F. Ames. 1989. Amplification of bacterial genomic DNA by the polymerase chain reaction and direct sequencing after asymmetric amplification: Application to the study of periplasmic permeases. J. Bacteriol. 171:1602-1608.

Silver, S. 1978. Transport of cations and anions, p.221-324. In B.P. Rosen (ed.), Bacterial transport. Marcel Dekker Inc., New-York.

Sisco, K.L., and G.O. Smith. 1979. Sequence specific DNA uptake in Haemophilus transformation. Proc. Natl. Acad. Sci. USA 76:972-976.

Smith, M.C.M., L. Czaplewski, A.K. North, S. Baumberg, and P.G. Stockley. 1989. Sequences required for regulation of arginine biosynthesis promoters are conserved between Bacillus subtilis and Escherichia coli. Mol. Microbiol. 3:23-28.

Smith, H.O., D.B. Dannen, and R.A. Deich. 1981. Genetic transformation. Annu. Rev. Biochem. 50:41-68.

Somerville, R.L. 1983. Tryptophan: Biosynthesis, regulation and large-scale production, p. 351-374. In K.M. Herrmann and R.L. Somerville (ed.), Amino acids: Biosynthesis and genetic regulation. Addison-Wesley Publishing Company Inc., Boston, Mass.

Southern, E.M. 1975. Selection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Sox, T.E., W. Mohammed, and P.F. Sparling. 1979. Transformation-derived Neisseria gonorrhoeae plasmids with altered structure and function. J. Bacteriol. 138:510-518.

Sparling, P.F. 1966. Genetic transformation of Neisseria gonorrhoeae to streptomycin resistance. J. Bacteriol. 92:1364-1371.

Spratt, S.K., F. Jones, T.E. Shockley, and J.H. Jackson. 1980. Co-transformation of a serum resistance phenotype with genes for arginine biosynthesis in Neisseria gonorrhoeae. Infect. Immun. 29:287-289.

Stein, D.C. 1989. Introduction of cloned genes into Neisseria gonorrhoeae. Clin. Microbiol. Rev. 2:S146-S149.

Stein, D.C., F.E. Young, F.C. Tenover, and V.L. Clark. 1983. Characterization of a chimeric β -lactamase plasmid of Neisseria gonorrhoeae which can function in Escherichia coli. Mol. Gen. Genet. 189:77-84.

Stein, D.C., L.E. Silver, V.L. Clark, and F.E. Young. 1984. Cloning genes for proline biosynthesis from Neisseria gonorrhoeae: Identification by interspecific complementation of Escherichia coli mutants. J. Bacteriol. 158:696-700.

Stein, D.C., S. Gregorie, and A. Piekarowicz. 1988. Restriction of plasmid DNA during transformation but not conjugation in Neisseria gonorrhoeae. Infect. Immun. 56:112-116.

Steinberg, V.I., A.C. Fenton, E.H. Baker, and I.D. Goldberg. 1979. Co-transformation of temperature sensitivity and nutritional markers in Neisseria gonorrhoeae. J. Bacteriol. 140:206-212.

Sugar, I.P., and E. Neumann. 1984. Stochastic model for electric field-induced membrane pores by electroporation.

Biophys. Chem. 19:211-225.

Tenover, F.C., D.C. Stein, F.E. Young, and V.L. Clark. 1985. Construction and characterization of chimeric β -lactamase plasmids of Neisseria gonorrhoeae with altered ability to be mobilized during conjugation. Sex. Transm. Dis. 12:76-82.

Tesh, M.J., and R.K. Miller. 1983. Arginine biosynthesis in Legionella pneumophila: absence of N-acetylglutamate synthetase. Can. J. Microbiol. 29:1230-1233.

Trotta, P., M.E. Burt, R.H. Haschemeyer, and A. Meister. 1971. Reversible dissociation of carbamoylphosphate synthetase into a regulated synthesis subunit and a subunit required for glutamine utilization. Proc. Natl. Acad. Sci. USA 68:2599-2603.

Udaka, S. 1966. Pathway-specific pattern of control of arginine biosynthesis in bacteria. J. Bacteriol. 91:617-621.

Van Vliet, F., R. Cunin, A. Jacobs, J. Piette, D. Gigot, M. Lauwereys, A. Piérard, and N. Glansdorff. 1984. Evolutionary divergence of genes for ornithine and aspartate carbamoyltransferases-Complete sequence and mode of regulation of the Escherichia coli argF gene: Comparison of argF with argI and pyrB. Nucleic Acid Res. 12:6277-6289.

Vogel, H.J. 1970. Arginine biosynthetic system in Escherichia coli. Methods Enzymol. 17A:249-251.

Vogel, H.J., and D.M. Bonner. 1956. Acetylornithinase of Escherichia coli: Partial purification and some properties. J. Biol. Chem. 218:97-106.

Vogel, H.J., and W.L. McLellan. 1970. Acetylornithinase (Escherichia coli). Methods Enzymol. 17A:251-255.

Vyas, S., and W.K. Maas. 1963. Feedback inhibition of acetylglutamate synthetase by arginine in Escherichia coli. Arch. Biochem. Biophys. 100:542-546.

Weislander, L. 1979. A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. Anal. Biochem. 98:305.

Werner, M., A. Feller, and A. Piérard. 1985. Nucleotide sequence of yeast gene CPAI encoding the small subunit of

arginine-pathway carbamoylphosphate synthetase. Homology of the deduced amino acid sequence to other glutamine amidotransferases. *Eur. J. Biochem.* 146:371-381.

West, S.E.H., and V.L. Clark. 1989. Genetic loci and linkage associations in Neisseria gonorrhoeae and Neisseria meningitidis. *Clin. Microbiol. Rev.* 2:S92-S103.

Woese, C.R. 1987. Bacterial evolution. *Microbiol. Rev.* 51:221-271.

Wong, S.C., and A.T. Abdelal. 1990. Unorthodox expression of an enzyme: Evidence for an untranslated region within carA from Pseudomonas aeruginosa. *J. Bacteriol.* 172:630-642.

Wood, A.G., A.H. Redborg, D.R. Cue, W.B. Whitman, and J. Konisky. 1983. Complementation of argG and hisA mutations of Escherichia coli by cloned DNA from the archaeobacterium Methanococcus voltae. *J. Bacteriol.* 156:19-29.

Yanofsky, C., and R. Kolter. 1982. Attenuation in amino acid biosynthetic operons. *Annu. Rev. Genet.* 16:113-134.

Young, F.E., B.H. Hebel, and W. Wong. 1976. Mechanism of action of autolysin(s) of Neisseria gonorrhoeae, p.197-212. In R.B. Roberts (ed.), *The gonococcus*. John Wiley and Sons, New York.

York, M.K., and M. Stodolsky. 1981. Characterization of P1 argF derivatives from Escherichia coli K-12 transduction. I. IS1 elements flank the argF gene segment. *Mol. Gen. Genet.* 186:230-240.

APPENDIX A

Some characteristics of the DNAs from the recombinant
bacteriophages carrying gonococcal arginine
and proline biosynthesis genes.

Table A-1. Some characteristics of the DNAs from the recombinant bacteriophages carrying gonococcal arginine and proline biosynthesis genes.

Clone	Selected marker in <u>E. coli</u>	Gonococcal genomic DNA insert ^a	
		Size of the <u>EcoRI</u> ^b fragments (kb)	Total size (kb)
EFP10	<u>carA</u> ⁺	7.5, 4.9, 1.8	14.2
EFP20	<u>carB</u> ⁺	5.4, 4.8, 1.7	11.9
EFP30	<u>argE</u> ⁺	4.4, 3.9, 2.6, 2.5, 1.7	15.1
EFP40	<u>argIF</u> ⁺	6.8, 2.4, 1.5, 1.3	12.0
EFP50	<u>argA</u> ⁺	4.3, 3.8, 2.6, 2.5, 1.7	14.9
EFP60	<u>argB</u> ⁺	9.5, 3.7	13.2
EFP70	<u>argB</u> ⁺	6.3, 9.5	16.1
EFP80	<u>argG</u> ⁺	10.3, 3.4	13.7
EPL10	<u>proA</u> ⁺	9.2, 3.9	13.1
EPL20	<u>proB</u> ⁺	5.7, 4.6, 2.3	12.6
EPL30	<u>proB</u> ⁺	9.3, 3.9	13.2
EPL40	<u>proC</u> ⁺	7.0, 5.4, 2.7, 2.1, 1.9	19.1

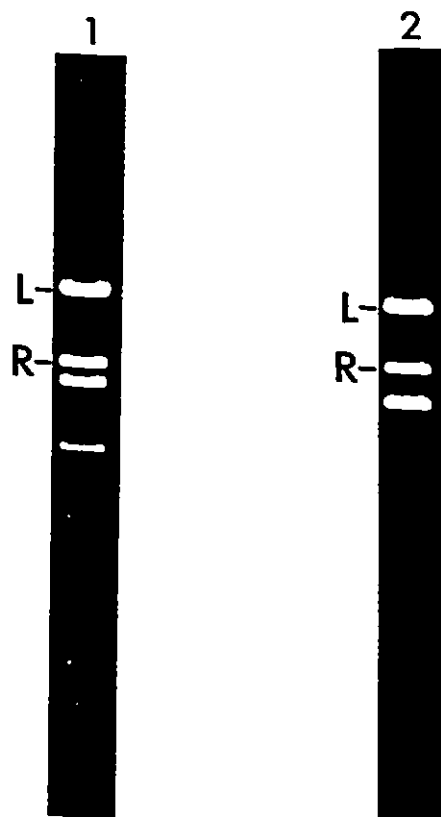
^a The level of uncertainty for fragment sizes is around ± 0.1 kb.

^b EcoRI digests for some of these clones are shown on Fig. A-1.

Figure A-1. EcoRI digested DNA from some of the recombinant bacteriophages carrying gonococcal arginine biosynthesis genes. Ethidium bromide agarose gels^a of clones EFP10 (lane 1) and EFP40 (lane 2) digested with EcoRI. Bands of the right (R; 9.2 kb) and the left (L;19.4 kb) arms of the lambda bacteriophage EMBL4 are indicated.

^a These two EcoRI digests are from two different agarose gels.

Note: This figure shows the bands of DNA fragments not visible upon rephotography on Fig. 1-1 and Fig. 1-2.



APPENDIX B

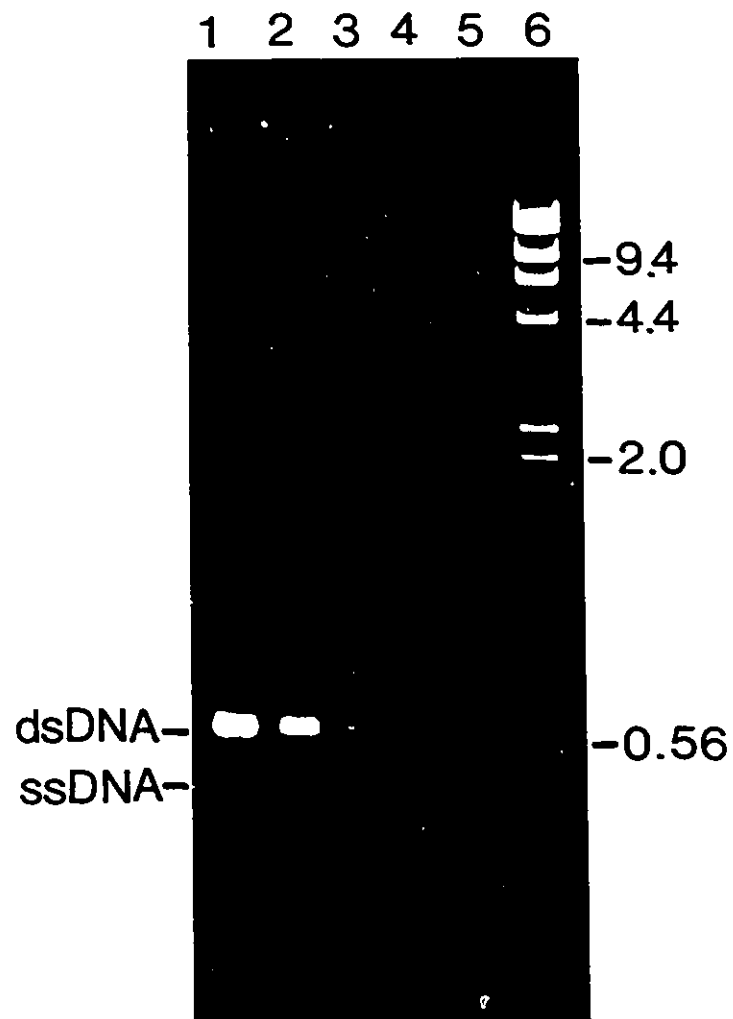
DNA sequence from a portion of the
N. gonorrhoeae carB gene

MATERIALS AND METHODS

DNA preparation. Small-scale preparation of the replicative form of bacteriophage M13 DNA was as described by Sambrook *et al.* (1989). Small-scale preparation of single-stranded (ss) bacteriophage M13 DNA for sequencing was as described in the sequenase protocol (5th edition, U.S. Biochemical Corporation, Cleveland, Ohio). DNA was isolated from 1.2 mL of bacteriophage-containing supernatant. The pellet of ssDNA was washed once with 70% ethanol and then once with 90% ethanol, air dried and resuspended in 20 μ L of TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

Generation of ssDNA by PCR. Asymmetric amplification of ssDNA for direct sequencing was achieved by using a limiting concentration of one of the two primers in order to preferentially amplify one strand (Gyllensten and Erlich, 1988). The concentration of the non-limiting primer was 200 pM and that of the limiting primer was 6.7 pM. The optimal primer ratio to yield ssDNA was estimated by performing several PCR amplifications using different amounts of the limiting primer (Fig. B-1). Besides the asymmetric molar ratio of the primers, the PCR reaction was achieved as described above for the standard PCR. The reaction mixture was subjected

Figure B-1. Asymmetric amplification by PCR: Titration of the limiting primer against the non-limiting primer to determine the optimal primer ratio to yield ssDNA. Amplifications were performed using EFP10 DNA as template with primers B1 (non-limiting at a concentration of 0.2 μ M) and B2 (limiting). The B2/B1 primer ratio were as follows; 1/1 (lane 1), 1/10 (lane 2), 1/20 (lane 3), 1/30 (lane 4) and 1/40 (lane 5). Lane 6 contains HindIII digested DNA. Bands of double-stranded DNA (dsDNA) and of single-stranded DNA (ssDNA) are indicated.



to 35 successive cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. Following asymmetric PCR, the amplification products were analysed by agarose gel electrophoresis. The diffused band of ssDNA was visualized on ethidium bromide-stained gels (Fig. B-1).

Cloning of amplified DNA into M13 vectors. The PCR-amplified DNA was isolated from low-melting-temperature agarose (Chapter one). The DNA was further purified by passage of the sample through prepac chromatography columns (i.e. NACS prepac columns purchased from Bethesda Research Laboratory) used according to the instruction of the manufacturer. The ends of the purified DNA were then repaired by a filling-in reaction with the Klenow DNA polymerase (Boehringer Mannheim). A reaction mixture of 20 μ L containing approximately 500 ng of DNA, 20 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$ and 1 unit of Klenow DNA polymerase was incubated for 3 min at 37°C. Subsequently, 2 μ L of dNTP mix (0.125 mM of each dATP, dCTP, dGTP and dTTP) was added and the reaction mixture was incubated at 37°C for an additional 10 min. The Klenow enzyme was heat-inactivated by a treatment at 65°C for 10 min. At the same time, the replicative form of the cloning vector M13mp19 was digested with SmaI followed by heat inactivation of the restriction endonuclease by two successive incubations

at 65°C for 15 min. Both DNA preparations (i.e. the repaired insert and the digested vector) were precipitated with ethanol. Prior to ethanol precipitation of the insert DNA (range in size from 0.6 to 1.5 kb), transfer RNA (Boehringer Mannheim) was added to the DNA preparation at a concentration of 1 µg/10 µL to improve the efficiency of the precipitation. After two washes with 70% ethanol, the DNA was suspended in TE buffer.

The vector was blunt-end ligated with the insert in a reaction mixture containing 50 mM Tris-HCl [pH 7.6], 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, 5% (w/v) PEG and 4 units/10 µL of T4 DNA ligase (Boehringer Mannheim). For each 10 µL of ligation reaction, 30 ng of vector DNA was combined with a ten-fold molar excess of insert DNA. Ligation was carried out at room temperature for 2 h. The extent of the ligation was verified by agarose gel electrophoresis as described in Chapter one. Reagents used for the filling-in reaction and for the blunt-end ligation were taken from the Erase-a-Base System kit (Promega).

Competent E. coli cells were transfected with 5 µL from the ligation mix as described by Sambrook et al. (1989). Competent and plating cells carrying sex pili were prepared

from strain JM109 carrying an F' episome (F'traD36 proAB⁺ lacI^q lacZ M15) (Sambrook et al., 1989). Competent and plating cells of strains DH5 α F'IQ purchased from Bethesda Research Laboratory were also used. Cell competence was verified with control M13 DNA (Sambrook et al., 1989).

The orientation of DNA cloned in M13 was determined by the ability of the different recombinant bacteriophages to hybridize to each other (Sambrook et al., 1989). Recombinant bacteriophages were tested pairwise by hybridization and then analyzed by agarose gel electrophoresis (Sambrook et al., 1989). Pairs of recombinants carrying complementary sequences hybridized to form structures which migrate more slowly through the gel than single-stranded viral DNA.

DNA sequencing. ssDNA prepared from M13 recombinant bacteriophages or by asymmetric PCR was used as template for dideoxy-mediated chain termination sequencing (Sanger et al., 1977). Prior to its use for sequencing, PCR-amplified ssDNA was selectively precipitated with ethanol, as described by Kreitman and Landweber (1989), in order to remove the oligonucleotides. The DNA pellet from a 100 μ L PCR reaction was washed twice in 70% ethanol, dried under vacuum and suspended in 10 μ L of TE buffer.

The M13 ssDNA was sequenced by using the sequenase sequencing kit (U.S. Biochemical Corp.) with [α - 35 S]dATP according to the instructions provided by the manufacturer using the M13 universal primer. PCR-amplified ssDNA was sequenced using the sequenase sequencing kit or by using the Genesis™ 2000 sequencing kit (Du Pont Canada, Mississauga, Ontario) for automated DNA sequencing. For both protocols the sequencing reaction was performed using the limiting primer in the PCR reaction. For manual sequencing, 3 to 7 μ L of the DNA preparation (each 10 μ L was prepared from a 100 μ L PCR reaction) was used for each sequencing reaction. The dGTP mix was diluted 1:15 instead of 1:5 and the labelling time was about 2 min. Fluorescent dideoxy sequencing using sequenase was performed using 10 to 15 μ L of the DNA preparation according to instructions provided by the manufacturer (Du Pont).

For "manual" sequencing, electrophoresis was carried out by using the Sequi-Gen™ nucleic acid sequencing cell (Bio-Rad). Gel preparation and electrophoresis were performed according to the instructions provided with the apparatus (Bio-Rad). The device which was 21 cm wide and 50 cm long was used with 0.4 mm spacers. Electrophoresis was carried out in 5% polyacrylamide gels ran at 2000 V in Tris-Borate buffer (89

mM Tris [pH 8.3], 89 mM boric acid, 1 mM EDTA) for 2 to 4 h. Generally, double loadings of the samples were made to increase the amount of readable sequence from a single run. After electrophoresis, the sequencing gel was peeled off from the glass plate using 3MM paper, covered with Saran Wrap and dried at 80°C under vacuum using a Bio-Rad gel dryer (Model 583). Autoradiographic exposures of 18 to 72 h were carried out as described earlier (Chapter one). The DNA sequence was read by two individuals (myself and a second person) using a Gelmate™ 1000 Sonic digitizer unit from Beckman.

For automated DNA sequencing electrophoresis was performed by B. Mah (Department of Microbiology and Immunology, University of Ottawa) using the Du Pont Genesis™ 2000 automated sequencer according to instructions provided by the manufacturer. The sequence was read and analyzed by B. Mah using the Macintosh^R program provided with the sequencing apparatus.

DNA sequences were analyzed using the Microgenie™ (Beckman) and the PC Gene (Intelligenetics) sequence analysis programs.

RESULTS

Cloning of amplified DNA from the gonococcal CPSase genes into M13. Following cloning of amplified DNA by the Klenow fill-in method, approximately 20 recombinant M13 bacteriophages carrying the 0.6 kb amplification product with B1-B2 were obtained. The presence of the insert DNA was verified by digesting the replicative form of the recombinants with the restriction endonucleases EcoRI and SalI (data not shown). Four recombinants carrying the 1.1 kb amplification product with A1-B1 were also obtained. However, no recombinant carrying the 1.5 kb fragment amplified with B2-B3 was obtained. Hybridization between ssDNA from the M13 clones indicated that both the 0.6 kb and the 1.1 kb DNA fragments had been cloned in both orientations (data not shown). Attempts to clone the 0.6 kb B1-B2 amplification product into M13 with a directional cloning strategy using the restriction sites introduced at the 5' end of the primers B1 and B2 (Table 3-1) were unsuccessful.

DNA sequence of amplified DNA from the gonococcal CPSase genes. Four M13 clones (two in each orientation) carrying the 0.6 kb fragment amplified with B1-B2 primers were sequenced. The DNA sequence of the 0.6 kb fragment determined by M13

sequencing is presented in Fig. B-2. The first 24 nucleotides and the last 27 nucleotides of the fragment represent the sequence of the E. coli-specific PCR primers B2 and B1, respectively. The sequence was determined for only one strand starting from each end (an overlap of approximately 50 nucleotides was reached in the middle of the fragment). As a consequence, the nucleotide sequence contains some uncertainties particularly in the middle third of the fragment. These ambiguities, which are designated with the letter "N" in the nucleotide sequence, are attributable to band compressions as well as reading difficulties at the top of the sequencing gels. Additional errors may have arise from base misincorporations by the Taq DNA polymerase since the strategy involves the cloning into M13 which singles out individual amplification products. The frequency of misincorporation errors by the Taq DNA polymerase is approximately one error per 400 bp sequenced (Saiki et al., 1988). A semi-graphical representation of the sites for several restriction endonucleases, obtained by analysis of this preliminary sequence, is presented in Fig. B-3.

Analysis of the nucleotide sequence of the three reading frames revealed a single open reading frame without a start codon. The corresponding region of the E. coli CPSase gene is

Figure B-2. Nucleotide sequence for a portion of the gonococcal carB gene amplified from clone EFP10 (carries the gonococcal CPSase genes) using primers B2-B1. Sequencing strategy is indicated with dashed arrows.

^a Nucleotide sequence for the PCR primers B1 and B2.

^b "N" indicates uncertain nucleotide positions.

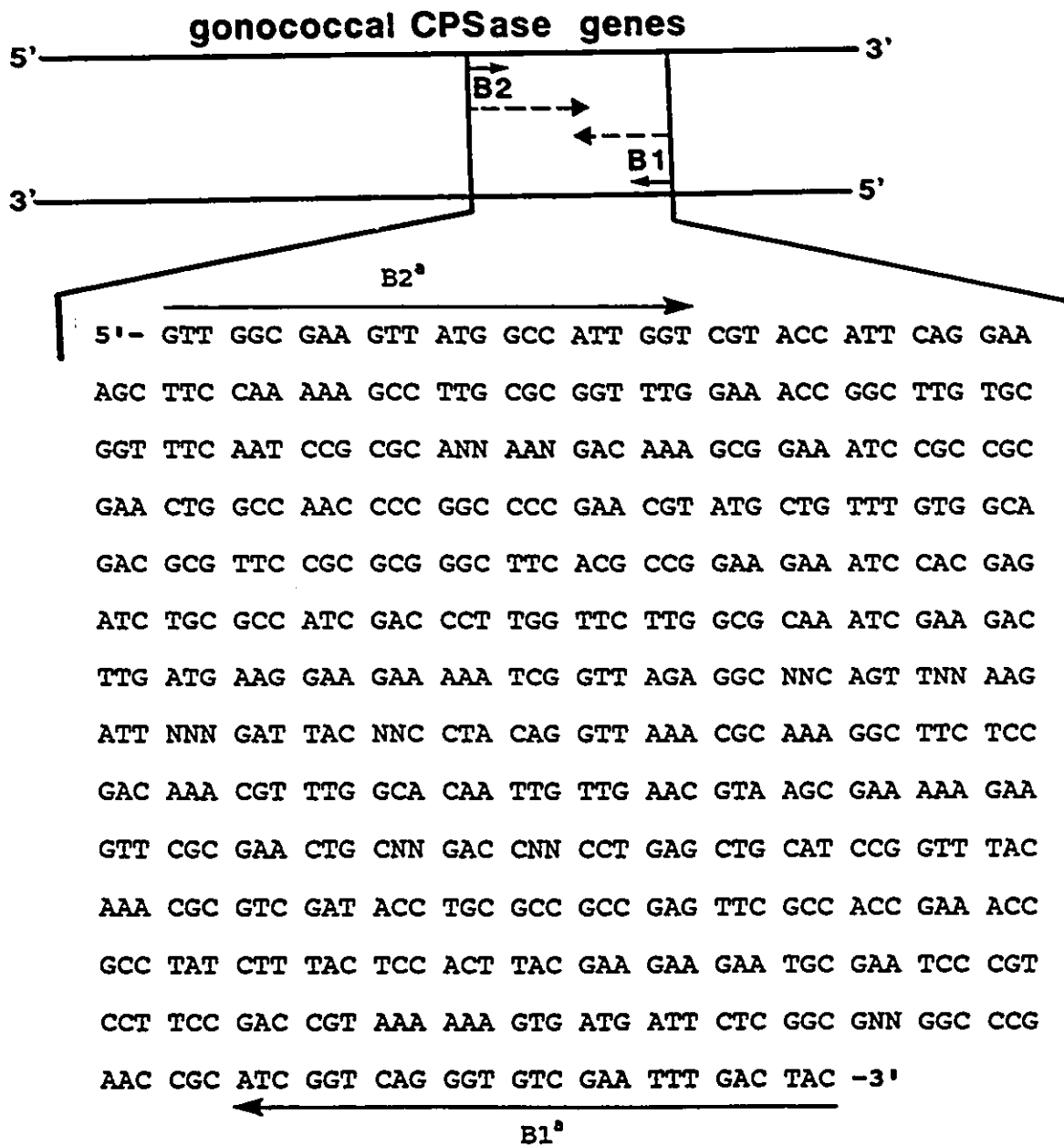
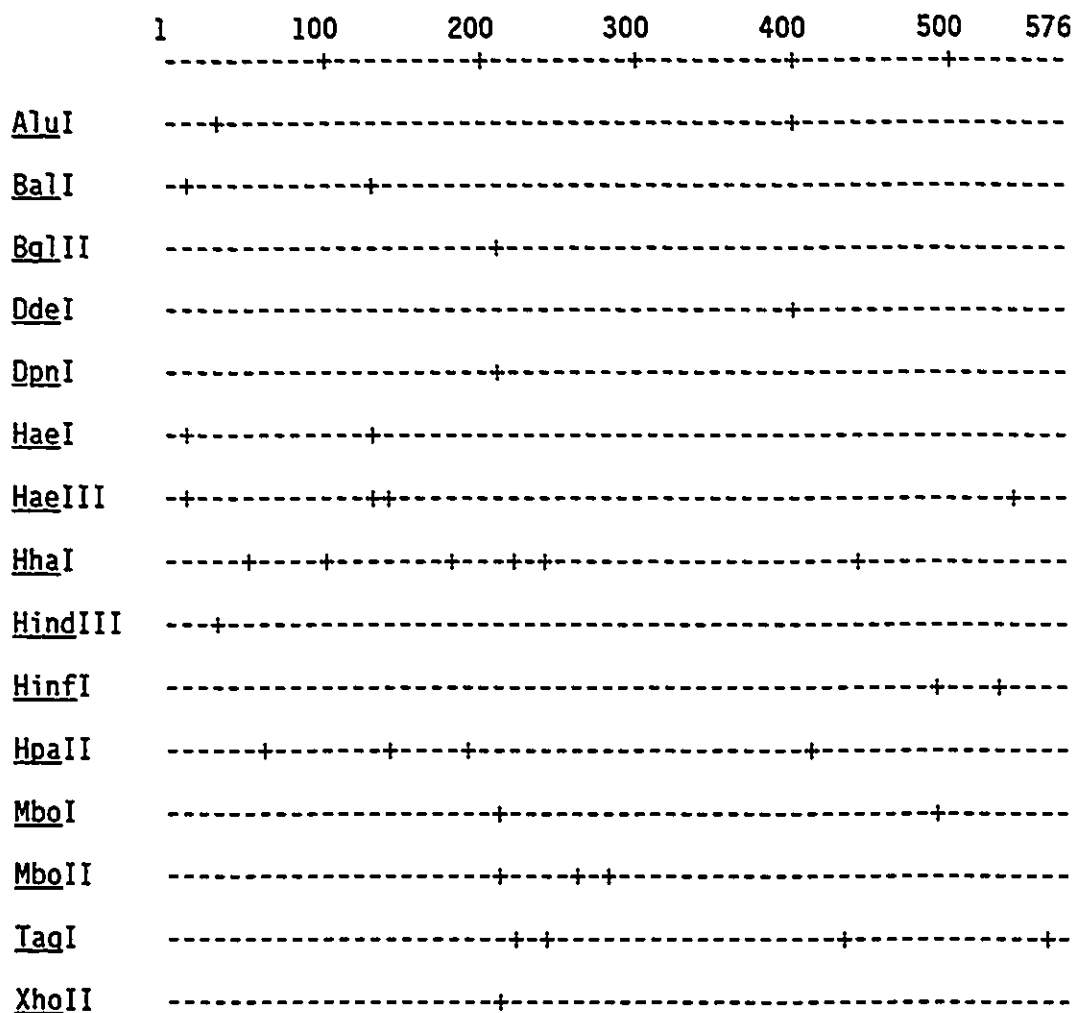


Figure B-1. Semi-graphical representation of the restriction sites on the 576 nucleotide sequence from the gonococcal carB gene for fifteen restriction endonucleases.



also within the carB gene. The alignment of the deduced amino acid sequence with the corresponding region of the E. coli CPSase revealed 60% identity (Fig. B-4). This alignment also showed the presence of five deletions (one of four codons and four of one codon) in the amino acid sequence of the gonococcal CPSase. When the matches between similar amino acids (Microgenie™ sequence analysis manual, Beckman) are considered, the percentage of matches between these amino acid sequences is 71%. For these calculations, each deletion in the gonococcal CPSase amino acids sequence was considered as one mismatch. PCR primer sequences and "X" amino acids (unknown due to sequence uncertainties) were excluded.

At the nucleotide level, the gonococcal CPSase gene and the E. coli carB gene showed 69% identity. For this calculation, "N" nucleotides (uncertain nucleotide positions) and PCR primer sequences were excluded. Each deletion in the gonococcal CPSase gene sequence was considered as one mismatch.

The deduced amino acid sequence for the gonococcal CPSase was compared with the corresponding regions of the three CPSases characterized so far (i.e. CPSases from E. coli, S. cerevisiae and the rat) (Fig. B-4). This sequence comparison

Figure B-4. Alignments of the CPSase amino acid sequences from N. gonorrhoeae, E. coli, S. cerevisiae and the rat. The alignment of the E. coli amino acid sequence with that from the yeast and the rat have been previously proposed (Lusty et al., 1983; Nyunoya et al., 1985). The amino acid positions of the N. gonorrhoeae CPSase matching with (i) three other enzymes (*), (ii) two other enzymes (+) or (iii) only one other enzyme (-) are indicated. The sequence of the PCR primers is shown. The one letter code for the amino acids has been used. Uncertain amino acids are designated with an "x".

*****+ -+*****--- -- **
 1. N. gonorrhoeae carB VGEVMAIGRTIQESFQKALRGLETGLCGFN
 2. E. coli carB 379- VGEVMAIGRTQQESLQKALRGLEV GATGFD
 3. S. cerevisiae CPA2 397- VGEVMAIGRNYEEAFQKALRQVDPSLLGFQ
 4. Rat CPSI 795- VGEVMAIGRTFEESFQKALRMCHPSVDGFT

→
B2

+ -- * - *++ -+ + +-+----- ---- - ** *
 1. P-RXXDKA--E-----I--RRELANPGPERMLFVADAFRAGFTPEEIHCAIDPW
 2. PKVSLDDP--E--ALTKI--RRELKDAGADRIWYIADAFRAGLSVDGVFNLTNIDRW
 3. GSTEFGDQLDE--ALRTPDTRRVL-AIGQALIHENYT-----VERVNELSKIDKW
 4. PRLPMNK---EWPANLDL--RKELSEPSSTRIYAIKALENNMSLDEIVKLTSIDKW

** ----- - - - - - * + - + *++*+ - + * - - - *
 1. FLAQIEDL-MKEEKSVR-GXSXKIXDYXLQVKRK-GFSDKRLA----QLLNVS--EK
 2. FLVQIEELVRLEEKVAEVGITGLNADFLRQLKRK-GFADARLA----KLAGVR--EA
 3. FLYKCMNIVNIYKELESVKSLSDLSKDLLQRAKKGFSQKQIAVTINKHASTNINEL
 4. FLYKMRDILNMDKTLKGLNSESVEETLRQAKEI-GFSDKQLA----KCLGLT--EA

+ *- - -+*--*+---*++--- *-+*+---*--- - - - - +---* *
 1. EVRELXDXPELHPVYKRVDTCAAEFATETAYLYSTYEEECESRPS-DRKKVMILGXG
 2. EIRKLRDQYDLHPVYKRVDTCAAEFATDTAYMYSTYEEECANPSTDREKIMVLGGG
 3. EIRSLRKTGIIIPFVKRIDTLAAEFPAQTNLYTTYNATKNDVEFNENGML-VLGS
 4. QTRRLRLKKNHPWVKQIDTLAAEYPSVTNYLYVTYNGQEHDIKFDEHG-IMILGCG

+-+***--+***-
 1. PNRIGQGVEFDY
 2. PNRIGQGLEFDY -579
 3. VYRIGSSVEFDW -598
 4. PYHIGSSVEFDW -994

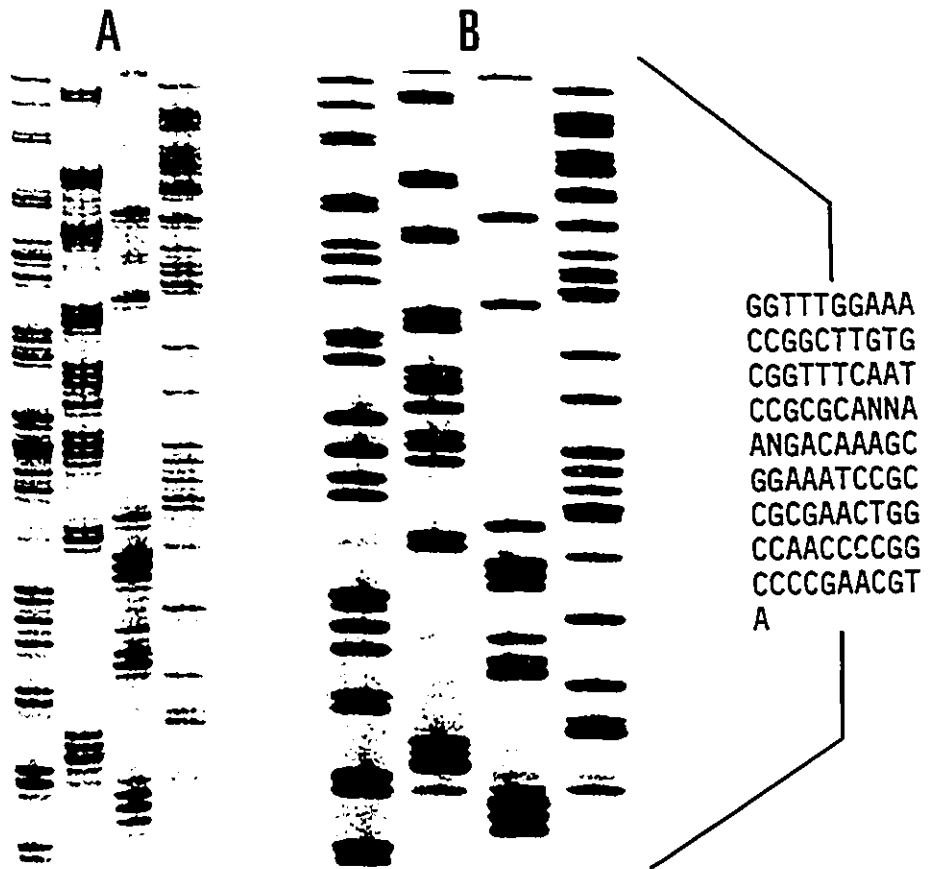
←
B1

shows that 24% of amino acids are conserved in the four enzymes. The comparison of the sequence for the gonococcal CPSase with each enzyme showed identities of 60% with E. coli CPSase, 37% with rat CPSase and 32% with S. cerevisiae CPSase. A number of amino acids in the gonococcal CPSase protein are present in only two other CPSases (14%) or in only one CPSase (35%). The region of the CPSase corresponding to the amino acids 521-546 of the E. coli sequence is well conserved in all enzymes (Fig. B-4). This region, which may correspond to a functional domain of the enzyme, showed 64% of conserved positions in the amino acid sequences of at least three CPSases including the gonococcal CPSase. Of the 43 common residues among E. coli, S. cerevisiae, and rat CPSases, 40 also matched with the gonococcal CPSase (Fig. B-4). Again, for these calculations, the "X" amino acids in the deduced amino acid gonococcal sequence and the PCR primer sequences were excluded. Deletions in the gonococcal CPSase were not considered.

The sequence from a portion of the 0.6 kb fragment amplified with primers B2-B1 was also determined by using ssDNA prepared by asymmetric PCR. The sequencing was performed using two methods: (i) "manual" (traditional) sequencing and (ii) automated DNA sequencing. The resulting sequences were

compared with that obtained with M13 sequencing. The 193 nucleotide sequence determined by manual sequencing matched with 96% of the corresponding sequence determined by M13 sequencing. Seven of the 8 mismatches observed were additions or deletions of one nucleotide which were probably attributable to reading errors. In fact, the background signal, which may be caused by the priming of PCR artifacts, made the reading difficult, especially in nucleotide repetitions (Fig. B-5). The 332 nucleotide sequence obtained by automated DNA sequencing matched only 85% of the nucleotide sequence obtained by M13 sequencing. This important discrepancy is attributable to the high level of background fluorescence noise caused by priming of PCR artifacts. As a consequence, nucleotide assignment was either difficult, or not possible, in many instances (B. Mah, personal communication).

Figure B-5. Comparison of the sequencing ladders obtained with PCR-amplified ssDNA (A) and with ssDNA prepared from M13 recombinant bacteriophages (B).



DISCUSSION

Alignments of preliminary nucleotide and deduced amino acid sequences for the gonococcal and the E. coli CPSases revealed identities of approximately 69% and 60%, respectively, indicating that this area of the CPSase is well conserved. As no procaryotic carB gene other than that from E. coli has been sequenced (Nyunoya and Lusty, 1983), it is difficult to evaluate the significance of this similarity from an evolutionary point of view.

Hybridization data in Chapter One suggest that N. gonorrhoeae and E. coli CPSase genes share regions of at least 80% similarity. However, the preliminary sequencing data in this appendix indicates a lower level of similarity (i.e. approximately 69%) for a portion of the gonococcal carB gene. The comparisons of the amino acid sequence for the entire E. coli CPSase (Nyunoya and Lusty, 1983; Piette et al., 1984) with the sequence for the eucaryotic CPSases from the yeast and the rat revealed overall identities of 40% and 42%, respectively (Lusty et al., 1983; Nyunoya et al., 1985). When the same comparisons are limited to the CPSase region corresponding to the gonococcal sequence (i.e. region 379-579 of the E. coli CPSase amino acid sequence) determined in this

study, these identities in amino acid sequences are 33% for the yeast and 35% for the rat (Lusty et al., 1983; Nyunoya et al., 1985). This suggests that this area of the CPSase may be less conserved than other regions of the enzyme. Furthermore, no functional domain has been assigned to this area of the large subunit of the CPSase (Lusty et al., 1983; Nyunoya et al., 1985). These facts may imply that other regions of the gonococcal CPSase genes are more conserved than the area sequenced. These more conserved regions may account for the ability of E. coli and N. gonorrhoeae CPSase genes to cross-hybridize under hybridization conditions of high stringency (Chapter one; Picard and Dillon, 1989a).

Several strategies using PCR technology have been used for the preparation of template for DNA sequencing (Gyllensten, 1989). One of these strategies consists of generating ssDNA by PCR using an asymmetric molar ratio of the two oligonucleotide primers (Gyllensten and Erlich, 1988); ssDNA generated by this method has been purified and sequenced using several different approaches in order to reduce the sequencing of PCR artifacts (Gyllensten and Erlich, 1988; Gyllensten, 1989; Kreitman and LandWeber, 1989; Mitchell and Merril, 1989; Shyamala and Ames, 1989). In this study, "manual" sequencing performed with ssDNA prepared by

asymmetric PCR yielded DNA sequences with additional uncertainties as compared with the sequence determined by M13 sequencing. This was due to reading difficulties which may be attributable to the priming of PCR artifacts which produced a high background signal. Several approaches have been used to reduce the negative effects of PCR artifacts in DNA sequencing. Recently, Kreitman and Landweber (1989) have shown that the use of primer sequences within the amplified ssDNA (i.e. nested primer) for sequencing yielded ladders with much less background signal. The use of biotinylated primer in the asymmetric PCR reaction to purify the ssDNA on a streptavidin column has also been made to increase the homogeneity of the template for "manual" sequencing (Mitchell and Merril, 1989; Gyllensten, 1989). Therefore, the use of non-purified ssDNA from asymmetric PCR sequenced with the limiting primer in the PCR reaction may not be the optimal method.

The use of automated DNA sequencers to sequence PCR amplified DNA is in its infancy. The combination of these two state of the art technologies which would make DNA sequencing much less labour-intensive, may represent the sequencing method of the future. In this study, the potential use of the Genesis™ 2000 automated DNA sequencer (Du Pont) to directly sequence ssDNA generated by asymmetric PCR using the limiting

primer in the PCR reaction was examined. In our hands, this approach proved unsatisfactory since fluorescence signals showed a high level of background noise which made the reading extremely difficult. In fact, comparison of the sequence determined by automated DNA sequencing with that from the same region of the gonococcal carB gene determined by the conventional M13 sequencing method revealed approximately 15% mismatches between the two sequences. Researchers at Du Pont (Dr. D. Amorese, Du Pont Molecular Genetics Application Laboratory, Boston, Mass., personal communication) have also encountered similar difficulties and are still in the process of optimizing the conditions for the use of the automated DNA sequencer with PCR amplified DNA. Preliminary data from Du Pont laboratories indicate that the use of ssDNA from asymmetric PCR purified on a streptavidin column as well as the use of a nested primer for sequencing may be the method of choice for sequencing PCR amplified DNA with the Du Pont automated sequencer (D. Amorese, personal communication).

APPENDIX C

Transformation of Neisseria gonorrhoeae
by electroporation

INTRODUCTION

In recent years, electroporation has become a widely used technique for the genetic transformation of plant protoplasts and animal cells (Rabussay et al., 1987). The process of electroporation involves the application of a high field strength electric pulse which reversibly permeabilizes the cell membrane thereby allowing the entry of macromolecules such as DNA into the cells (Sugar and Neumann, 1984). This technique has also been used to introduce plasmid DNA into intact cells of various bacterial species (Bio-Rad, Bacterial electroporation manual).

Since electroporation depends on fundamental properties of biological membranes (Sugar and Neumann, 1984), this technique should, in principle, be utilisable with any type of cell. The introduction of DNA into N. gonorrhoeae by electroporation may offer several advantages over natural transformation. It may eliminate the need for DNA uptake sequences on the transforming DNA and also for pili on the competent cells which are both required in natural gonococcal transformation (Cannon and Sparling, 1984; Biswas et al., 1989b).

Our attempts to transform N. gonorrhoeae by electroporation under a wide variety of conditions were unsuccessful. However, transformation of the gonococcus by electroporation with plamid and chromosomal DNA has recently been reported (D.R. Kapczynski and C.A. Genco, Abstr. Int, Pathogenic Neisseria Conf. 1990, p. 95). A brief presentation of some of our unsuccessful strategies as well as possible explanations for their failure are given in this appendix.

MATERIALS AND METHODS

Bacterial strains. *N. gonorrhoeae* strains used for electroporation are listed in Table C-1.

Plasmids and genomic DNA. The 7.2 kb β -lactamase-producing gonococcal plasmid pJD4 and the gonococcal shuttle vector pLES2 (American Type Culture Collection, Rockville, Maryland) were both used as transforming DNA. Plasmid pJD4 was isolated from *N. gonorrhoeae* BG10 or from *E. coli* C-600 (pJD4). Plasmid pLES2 was isolated from *N. gonorrhoeae* BG20. Genomic DNA from a streptomycin-resistant (*str*^r) derivative of *N. gonorrhoeae* 2152 was also used as transforming DNA.

DNA transformation. Transformation by electroporation was carried out by using a Bio-Rad Gene Pulser™ apparatus. The protocols, used for *N. gonorrhoeae* electroporation, were based on the existing methods for electroporation with other bacterial species. The electroporation buffer was modified to reduce the autolysis of gonococci (Young *et al.*, 1976).

Gonococcal cells grown overnight in GC broth were harvested by centrifugation (8,000 rpm for 5 min at 4°C), washed once in cold gonococcal electroporation buffer (GEB)

Table C-1. N. gonorrhoeae strains and plasmids used for electroporation.

<u>N. gonorrhoeae</u> strains (auxotype)	plasmid content
F62 (P) ^a	4.2 kb cryptic
C-5 (CUH) ^a	4.2 kb cryptic
NS757 (PO) ^a	4.2 kb cryptic
2152 (PCU) ^a	plasmid-free
BG10 (PCU) ^b	7.2 kb β -lactamase (pJD4)
BG20 (PCU) ^b	pLES2

^a These strains were selected from the N. gonorrhoeae culture collection of the National Laboratory for Sexually Transmitted Disease (Laboratory Centre for Disease Control, Ottawa, Ontario).

^b Derivatives from N. gonorrhoeae strain 2152 carrying a β -lactamase plasmid introduced by conjugation. Plasmid DNA isolated from these strains was used for N. gonorrhoeae electroporations.

and resuspended in electroporation buffer at 1/20 of the original volume. For gonococcal cells (non-piliated or piliated) grown on GCMB agar, 18 h growth was collected from the surface of the agar plate and suspended in cold electroporation buffer to a turbidity similar to that of a McFarland standard #1 (equivalent to approximately 3×10^8 cells/mL) (Paik, 1980). Cells were harvested by centrifugation and resuspended in electroporation buffer at 1/20 of the original volume.

The dense cell suspension in electroporation buffer (0.8 mL) was added to a pre-chilled electroporation cuvette (inter-electrode distance of 0.4 cm) purchased from Bio-Rad. Subsequently, 0.5 to 5.0 μg of DNA suspended in ddH₂O was added to the cell suspension and mixed by gently tapping the tube. The cell-DNA mixture was left in ice for 0 to 10 min, subjected to a single electrical pulse (capacitance 25 μF , set voltage 1.0-2.5 kV, pulse length 2-20 ms) and returned to ice for 0 to 10 min. GC broth (9 mL) was added to the cells and incubated at 35°C in the presence of 5% CO₂ with periodical gentle agitation for 2 to 8 h to allow the expression of antibiotic resistance. Cells were plated onto GCMB plates containing ampicillin (1 or 5 $\mu\text{g/mL}$) and incubated for up to 72 h.

Gonococcal natural transformation of piliated cells with plasmid DNA was performed essentially as described by Stein et al. (1983).

RESULTS AND DISCUSSION

Initial experiments were performed by using the electroporation buffers recommended by Bio-Rad for E. coli electro-transformations. However, the use of these buffers with N. gonorrhoeae was not appropriate since most gonococcal cells autolysed rapidly in these media. N. gonorrhoeae shows a pronounced tendency to lyse spontaneously when transferred from a growth medium to a variety of liquid environments (Elmros et al., 1975). Therefore, electroporation buffers (30 mM glucose, 0.5 mM spermine, 300 mM sucrose, 0-16 mM MgCl₂, 50 mM HEPES, pH 6.4) in which the autolysis of N. gonorrhoeae is limited were developed for this study. The composition of the gonococcal electroporation buffer (GEB) was based upon available data on biochemical conditions which reduced N. gonorrhoeae autolysis (Morse and Barteinstein, 1974; Elmros et al., 1975; Young et al., 1976). In the recent report of N. gonorrhoeae electroporation (D.R. Kapczynski and C.A. Genco, Abstr. Int. Pathogenic Neisseria Conf. 1990, p. 95) cell autolysis has been minimized by modifying the composition of the electroporation buffer as well as by reducing to a minimum the contact time of cells with electroporation buffer.

β -lactamase plasmids (pJD4 and pLES2) used for N.

gonorrhoeae electroporations with non-piliated or piliated cells were isolated either from an isogenic gonococcal strain or from E. coli. These DNA preparations could efficiently transform by electroporation E. coli strain HB101 to ampicillin resistance (i.e. frequencies of up to 2×10^6 transformants/ μ g of DNA were obtained). Plasmid DNA isolated from E. coli may not be appropriate to transform N. gonorrhoeae by electroporation since it could be restricted in the recipient cell. However, electroporations performed with plasmids pJD4 or pLES2 isolated from an isogenic gonococcal strain did not yield transformants either. Recently, Kapczynski and Genco (D.R. Kapczynski and C.A. Genco, Abstr. Int, Pathogenic Neisseria Conf. 1990, p. 95) have reported the transformation of non-piliated N. gonorrhoeae with the gonococcal 5.1 kb β -lactamase plasmid with efficiencies of up to 1.7×10^4 transformants/ μ g of DNA. Important differences between the two protocols which could explain our failure are presented in Table C-2. The field strength that we used may have been too low (Table C-2). An incubation period of at least 5 h at 37°C in non-selective medium after the electrical pulse is essential to obtain penicillin-resistant (pen^r) transformants (C. Genco, Dept. of Oral Biology, Emory University, Atlanta, personal communication). Therefore, the incubation period that we used may have been too short

Table C-2. Major differences between the protocols for plasmid transformation of *N. gonorrhoeae* by electroporation used by us (protocol A) or by Kapczynski and Genco (protocol B).

Factors of electroporation	Protocols	
	A	B
1. Field strength	4.0- 6.25 kV/cm ^a	12.5 kV/cm
2. Incubation period in non-selective medium after pulse	3-4 h	5 h or more
3. Antibiotic used and its concentration in selective medium	Ampicillin (1 or 5 µg/mL)	Penicillin G (0.02 µg/mL)

^a The electroporation system that we used generated maximum field strength of 6.25 kV/cm

(Table C-2). Our failure to obtain pen^r transformants may be also explained by the use of too high antibiotic concentrations in the selective medium (Table C-2). The lowest antibiotic concentration which inhibits the growth of the recipient cell is usually recommended to recover pen^r transformants of N. gonorrhoeae (Biswas *et al.*, 1982). The possible toxicity of ampicillin to gonococci may represent an additional problem. Our inability to transform N. gonorrhoeae strain 2152 to amp^r by natural transformation with plasmids pJD4 and pLES2 isolated from isogenic strains may suggest that the screening system for transformants was inadequate. On the other hand, plasmid transformation is very inefficient with N. gonorrhoeae when the recipient does not carry an homologous plasmid (transformation frequencies of approximately 1×10^{-7} of pen^r transformants/ μ g of DNA are obtained with the naturally-occurring 7.2 kb β -lactamase-producing plasmid) (Biswas *et al.*, 1982; Cannon and Sparling, 1984). Since the recipient strain used in this study was plasmid-free, the efficiency of transformation may have been too low to obtain amp^r transformants. Therefore, there was no N. gonorrhoeae positive control to verify the validity of the screening system for amp^r transformants.

Attempts to transform N. gonorrhoeae with genomic DNA

from a str^r derivative of strain 2152 were also unsuccessful. Our ability to transform by natural transformation all recipient strains tested to str^r indicates that the screening system for str^r transformants was adequate. Kapczynski and Genco were apparently able to transform the gonococcus with chromosomal DNA under conditions similar to those used for plasmid electroporation (D.R. Kapczynski and C.A. Genco, Abstr. Int, Pathogenic Neisseria Conf. 1990, p. 95). Again, their ability to obtain transformants may be attributable to the use of higher field strength and/or longer incubation periods in non-selective medium after the electrical pulse.