MOLECULAR ANALYSIS OF PILUS EXPRESSION IN *NEISSERIA GONORRHOEAE* AND *NEISSERIA MENINGITIDIS*

Thesis submitted in accordance
with the requirements of the
University of Liverpool
for the degree of
Doctor in Philosophy

by

Anthony Charles Farrar Perry, BSc.(Hons.)

Department of Microbiology
University of Liverpool

September 1986

Tris-HCl, 50 mM (pH 8.0); EDTA, 10 mM; Triton X-100, 2 % (v/v).

vii. The lysate was sequentially extracted with phenol-$CHCl_3$ and $CHCl_3$ and the resultant solution supplemented with sterile, distilled water to 3.8 g.

viii. 4.8 g CsCl was dissolved in the lysate mix prior to the addition of 150 µl of a 10 $mg.ml^{-1}$ solution of EB.

ix. The EB-CsCl-lysate mix was adjusted to give a refractive index of 1.392 (sucrose equivalent of 36 % [w/v]) and removed to a polyallomer tube.

x. Further steps were essentially as described in 2.2.2 ix - xii, except that DNA samples were not necessarily pooled.

2.2.4 Maxipreparations of plasmid DNA

Bulk plasmid preparations were performed by a modification of the method of Humphreys et al. (1975). Incubations were on ice unless detailed to the contrary.

i. A starter culture was diluted 1:100 in 250-500 ml fresh nutrient broth containing suitably selective antibiotics.

ii. The diluate was incubated at 37°C until an OD_{660} of 0.8-1.0 had been reached, prior to the addition of chloramphenicol. Incubation was continued at 37°C with shaking for 16-20 h.

iii. Cells were harvested, washed in phosphate buffer, and resuspended in a solution containing 50 mM Tris-HCl (pH 8.0), 25 % (w/v) sucrose.

iv. 5 ml lysozyme solution (0.25 Tris-HCl [pH 8.0], 5 $mg.ml^{-1}$ lysozyme) was added and the mixture briefly vortexed prior to inubation for 5 min.

v. Following the addition of 4.4 ml of a solution of 0.25 M EDTA (pH 8.0) incubation was continued for 5 min.

vi. Cells were lysed by the addition of 18 ml Brij solution and the resultant lysate cleared by centrifugation at 40 000g (4°C) for approximately 50 min. Brij solution is: Tris-HCl, 50 mM (pH 8.0); EDTA, 62.5 mM; Brij 58, 1 % (w/v); sodium deoxycholate, 0.4 % (w/v).

vii. The supernatant was removed and supplemented with NaCl to 0.5 M.

viii. PEG was added to a final concentration of 10 % (w/v). The lysate-NaCl-PEG solution was incubated for 2-5 h.

ix. PEG-precipitated material was collected by centrifugation at 2 600g (4°C) for 5 min, and redissolved in 2 ml sterile TE.

x. The resultant solution was made up to 3.8 g with sterile, distilled water and 4.8 g CsCl added.

xi. PEG was displaced from solution as a plug by centrifugation at 1 000g for 2 min.

xii. To the remaining solution was added 150 µl of a 10 $mg.ml^{-1}$ solution of EB and the refractive index adjusted to 1.392.

xiii. A CsCl isopycnic density gradient was then formed by centrifugation of this solution at 180 000g for 16-20 h at 18°C. Subsequent DNA recovery and purification was as described previously (2.2.3 x.).

2.2.5 Minipreparations of plasmid DNA

Small quantities of plasmid DNA were isolated essentially using a method described elsewhere (Close & Rodriguez, 1982). Plastic 1.5 ml Eppendorf tubes were used throughout. The components of minipreparation solutions used here are described in 2.2.5 vii. All incubations were on ice unless described otherwise.

i. Cells from 1.5 ml of a fresh overnight culture were pelleted and resuspended in 150 µl solution 1. 5 µl RNase A solution (10 $mg.ml^{-1}$) was added.

ii. 350 µl solution 2 was added and the mixture vortexed prior to incubation for 10 min.

iii. Following the addition of 250 µl solution 3, the lysate was mixed well by inversion and centrifuged for 5 min.

iv. 800 µl of the resulting supernatant was transfered to a fresh tube, to which 700 µl propan-2-ol was added.

v. This was mixed by inversion and subjected to centrifugation for 5 min in order to pellet the precipitated DNA.

vi. The DNA pellet was washed with 70 % (v/v) of ethanol at -20°C, dried *in vacuo*, and resuspended in 10-20 µl sterile, double-distilled water. If required for restriction enzyme analysis, DNA was further purified by sequential phenol-$CHCl_3$, $CHCl_3$ and diethyl ether extraction, and precipitated with ethanol.

vii. Solutions used were:

Solution 1;	Tris-HCl (pH 8.0)	50	mM
	EDTA	50	mM
	Sucrose	20 %	(w/v),
Solution 2;	NaOH	200	mM
	SDS	1 %	(w/v),
Solution 3;	Sodium acetate (pH 4.8)	3	M.

2.2.6 Transformation of E.coli

The method of transformation employed here was based on that of Brown et al. (1979).

i. An overnight culture of the strain to be transformed was diluted 1:100 in fresh, prewarmed nutrient broth, and incubated at 37°C with shaking (approximately 140 rpm).

ii. When the culture had reached an OD_{660} of approximately 0.2, cells were harvested and washed once with 1/5th of the original volume of ice-cold, 10 mM $CaCl_2$.

iii. Cells were then resuspended in 1/50th of the original volume of ice-cold, 75 mM $CaCl_2$.

Abbreviations

Ap^r	Ampicillin resistant/resistance
bp	Base pair(s)
Brij 58	Polyoxyethylene 20 cetyl ether
BSA	Bovine serum albumin
CsCl	Caesium chloride
DNase	Deoxyribonuclease
dNTP(s)	Monodeoxynucleoside-5'-triphosphate(s)
ddNTP(s)	Dideoxynucleoside-5'-triphosphate(s)
DTT	DL-dithiothreitol
EB	Ethidium bromide
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N',-tetraacetic acid
g	Force x gravitational
h	Hour(s)
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	kilobase pair(s)
M	Molar
mA	Milliamperes
min	Minute(s)
M_r	Apparent relative molecular mass(es)
OD_{660}	Optical density (wavelength = 660 nm)
P+	Piliated
P-	Non-piliated
PEG	Polethylene glycol 6000

iv. Typically, 200 µl cells were added to 10-500 ng DNA solution and incubated on ice for 50 min.

v. The transformation mix was then heat-pulsed for approximately 2 min at 42°C prior to the addition of 0.5-1 ml of fresh, prewarmed nutrient broth.

vi. Following incubation at 37°C, cells were plated on selective media at a range of concentrations likely to give discrete colonies.

vii. Plates were incubated overnight at 28°C or 37°C as appropriate.

2.2.7 *In vitro* DNA manipulations

Standard DNA manipulations have been described previously (Maniatis *et al.*, 1982). DNA was typically stored frozen at -20 or -70°C. Restriction enzyme digests were terminated either by the addition of 1/10th volume loading buffer, prior to agarose gel electrophoresis, or by phenol-$CHCl_3$ extraction. Digestion with nuclease BAL 31 was terminated by the addition of 1/10th volume 0.2 M EGTA (pH 8.0). The action of calf intestinal alkaline phosphatase was terminated by the addition of a solution containing SDS. Ligation of DNA molecules was in a solution containing 20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10 mM DTT, 1 mM

dATP and was catalysed by T4 DNA ligase at 0.2 U and 1 U μg^{-1} for 'sticky-' and 'blunt-ended' ligations respectively. Ligation reactions proceeded at 16°C for 16-24 h.

Formamide was deionised by stirring for 30 min in the presence of Amberlite MB-1 mixed-bed resin and stored aliquoted at -20°C.

Labelling of DNA probes was with [α-^{32}P]dCTP (approximately 3 000 $Ci.mmol^{-1}$) was by the method of Maniatis et al. (1982) or using the Polymeraid random hexanucleotide DNA labelling kit as directed by the vendor.

A coupled transcription-translation kit (CTT) was used to label plasmid-encoded proteins in a cocktail containing [^{35}S]methionine (> 800 $Ci.mmol^{-1}$). The method employed was that of Pratt (1984). Samples were analysed electrophoretically (see 2.2.13).

2.2.8 Agarose gel electrophoresis

Restriction fragments and uncut plasmid DNA were analysed by electrophoresis through an horizontal 0.7-1.2 % (w/v) agarose gel containing EB (0.1 $\mu g.ml^{-1}$). Gels were buffered with TBE and typically run at a constant amperage of 50 mA. Electrophoresed DNA was visualised over a 302 nm UV light source, and where necessary, photographed.

2.2.9 Isolation of discrete DNA fragments

Purification of DNA fragments was by electro-elution from gels containing 1.0-1.3 % (w/v) low melting-point agarose. Restriction fragments were sufficiently resolved by electrophoresis and a pre-assembled visking-3MM paper matrix inserted into the gel parallel to, and infront of the band to be removed, with the 3MM paper proximal to the band. Electrophoresis was continued so that migration of DNA was towards the visking-3MM barrier for the minimum time to ensure that the desired band had eluted into the matrix. The direction of the electric current in the apparatus was then reversed for 10-15 s to dissociate any DNA that was strongly bound to the visking-3MM barrier. The matrix was then removed to a 400 µl tube that had been punctured at its base, and the liquid component collected in a 1.5 ml Eppendorf tube by centrifugation for 30-60 s. Small amounts (< 10 µg) of DNA recovered in this way were normally purified by phenol-$CHCl_3$ extraction. Large amounts (>25 µg) of DNA were further purified by CsCl-EB density centrifugation. Purity of isolated fragments was checked electrophoretically.

2.2.10 DNA-DNA hybridisation: Southern blotting

Following electrophoresis, DNA was transferred to nitrocellulose filters by the method of Southern (1975) with the modifications of Maniatis et al. (1982). DNA-DNA hybridisations were as

described by Maniatis et al. (1982). All hybridisations were performed under conditions of high stringency in the presence of 25 ng labelled probe and 50 % formamide at 42°C. Excess, unbound label was removed from each filter by washing at 68°C in a total of 2 l of a solution containing 0.1 X SSC, 0.1 % SDS (w/v) over 2h with 4 changes. Autoradiography was at -70°C for 1-72 h using an intensifying screen.

2.2.11 DNA-DNA hybridisation: colony hybridisation

The protocol adopted was as outlined by Hanahan & Meselson (1980) with minor modifications.

i. Cultures to be screened (usually transformations with gene libraries) were plated on selective media to give a density of 200-1000 colonies.plate^{-1}. Incubation was normally overnight at 28°C.

ii. Colonies were replica-plated using sterile nitrocellulose filters keyed to each plate using a pin-prick system. Original plates were stored at 4°C until required.

iii. Filters were then removed, placed with replica colonies uppermost on fresh selective plates and incubated at 37°C for several hours.

iv. Filters were then placed on nutrient agar plates containing chloramphenicol at 500 $\mu g.ml^{-1}$ and incubation continued at 37°C for 16-20 h.

v. Colonies were lysed by sequentially laying filters colony-side-up on 3 sheets of 3MM paper drenched as detailed below. After each 5 min step, excess solution was removed by blotting.

Filter placed on 0.5 M NaOH-soaked 3MM, 5 min.
" " " " " " " "
" " " 1 M Tris-HCl (pH 8.0)-soaked 3MM, 5 min.
" " " " " " " " " "
" " " Neutralising solution-soaked 3MM, 5 min.

vi. Filters were air-dried and baked <u>in vacuo</u> for 2 h at 80°C on a single sheet of 3MM paper.

vii. Filters were not subjected to a prehybridisation step. DNA-DNA hybridisation, subsequent washes and autoradiography of filters was as for Southern hybridisation (2.2.10).

viii. Positive colonies (if any) were identified by alignment of autoradiographs with original plates.

2.2.12 Immunological screening

Immunological screening was by a modification of the method of Helfman *et al*. (1984) using monoclonal antibody SM1.

i. After overnight growth on selective media at 28°C replicas of colonies were made using sterile nitrocellulose filters that had been keyed to each plate using a series of pin-pricks. Original plates were stored at 4°C until required.

ii. Replica filters were placed with colonies uppermost on fresh selective plates and incubated at 37°C for 4-6 h.

iii. Colonies were then lysed by placing filters in a $CHCl_3$-saturated atmosphere for 20-30 min.

iv. Complete lysis and blocking of 'non-specific' binding sites was ensured by placing each filter in a petri-dish containing 10 ml BSA binding solution and incubating for 6 h at 30 rpm.

v. Filters were washed in 10 ml TS for 5 min.

vi. Following the removal of TS, 10 ml of TSA buffer containing antibody was added, and incubation continued for 16-20 h.

vii. Filters were then washed in 5 changes of TS over 3 h.

viii. Each filter was then incubated for 16-20 h in 10 ml TSA buffer containing ^{125}I-labelled Staphylococcus aureus protein A (0.01 $\mu Ci.ml^{-1}$).

ix. Step vii. was repeated prior to drying of filters. Autoradiography of filters was with intesifying screens at -70°C. Positive colonies were identified by reference of autoradiographs to original plates.

2.2.13 Immunological dot blotting

Bacterial suspensions (2 µl of $>10^8$ cells ml^{-1}) were applied to sheets of nitrocellulose, allowed to dry and lysed by exposure to $CHCl_3$ vapour. Excess protein binding was blocked with BSA and the sheets were reacted with antibody, washed and subsequently reacted with ^{125}I-labelled protein A as described by Virji & Heckels (1983). Immunological reactivity was detected following autoradiography for 24-48 h.

2.2.14 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Linear gradient SDS-PAGE (10-17.5 % [w/v]) was performed as described by Laemmli (1970). Estimation of protein in samples was

by the method of Lowry et al. (1951). All samples were boiled for 3 min in the presence of an equal volume of 2X FSB. Gels were run at constant amperages of 10 mA for 60 min and 20 mA until the tracking dye had migrated to the bottom.

Where necessary, gels were sequentially soaked in staining and destaining solutions for 6-24 h in each case, and dried onto 3MM paper using a Bio-Rad vacuum drying apparatus, prior to autoradiography at room temperature for 24-48 h, with an intensifying screen. Staining solution is: methanol, 45 % (v/v); glacial acetic acid, 5 % (v/v); coomassie blue (omitted in destaining solution) 0.1 % (w/v).

2.2.15 Western blotting

Proteins separated by SDS-PAGE were transferred to nitrocellulose filters by the method of Towbin et al. (1979) in a Bio-Rad Transblot Cell containing Western blot buffer, at a constant voltage of 28-30 V for 16-20 h. Filters were removed to BSA binding solution for 6-8 h, washed once with TS (5 min) and soaked in a solution of TSA buffer containing antibody for 16-20 h. Filters were then submerged in 5 changes of TS over 3 h prior to incubation in TSA buffer containing ^{125}I-labelled Staphylococcus aureus protein A (0.01 $\mu Ci.ml^{-1}$). Excess, unbound protein A was removed by 5-fold washing in TS before filters were dried and subjected to autoradiography at -20^{o}C for 24-48 h in

the presence of an intensifying screen.

2.2.16 DNA sequencing

DNA sequences were determined by dideoxy chain termination methods similar to those described elsewhere (Sanger et al., 1977; Messing et al., 1981; Amersham International, Cloning and Sequencing Handbook, 1984; New England Biolabs, M13 Cloning and Sequencing System. A Laboratory Manual 1985).

A. Preparation of RF DNA

Cultures containing M13mp18 and M13mp19 were established as described in the Amersham International Cloning and Sequencing Handbook and used for the isolation of RF plasmid DNA as described in 2.2.4.

B. M13 RF transfection

i. 1 ml of an overnight culture of JM103 was used to inoculate 100 ml fresh, prewarmed 2T3Y broth. Incubation was at 37°C with shaking (approximately 140 rpm).

ii. When the culture had achieved an OD_{660} of 0.2-0.4 cells

were harvested, washed in 1/5th of their original volume of ice-cold, 10 mM $CaCl_2$, reharvested and resuspended in a final volume of 1-4 ml ice-cold 75 mM $CaCl_2$.

iii. 0.3 ml of the competent cell suspension was added to 10-100 ng of DNA, the mixture gently vortexed and incubated on ice for 50 min.

iv. The transformation mixture was heat-pulsed at 42°C for approximately 2 min.

v. To the resultant cell suspension was added 270 µl of a mixture containing: 40 µl IPTG solution (100 mM); 40 µl X-Gal solution (2 % [w/v] in dimethyl formamide); 200 µl of a fresh culture of JM103.

vi. This mixture was then added to 3 ml molten H top agar (that had been held at 50°C), mixed, and poured onto a prewarmed H-plate.

vii. When the agar had set, plates were incubated at 37°C overnight.

C. Preparation of M13 single-stranded (ss) template

i. White plaques were used to seed 1.5 ml aliquots of 2T3Y

RF	Replicative form
RNase	Ribonuclease
rpm	Revolutions per minute
s	Second(s)
SDS	Sodium dodecyl sulphate
SPP1	*subtilis* 'phage P1
Tc^r	Tetracycline resistant
Tc^s	Tetracycline sensitive
U	Unit(s)
V	Volt(s)
v	Volume
w	Weight
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

broth containing a 1:100 dilution of a fresh overnight culture of JM103 in 3 x 0.5", capped glass tubes. Incubation proceeded at 37°C with shaking (900-1 000 rpm) for 5-6 h. Subsequent steps employed microfuges and 1.5 ml Eppendorf tubes.

ii. Cells were pelleted by centrifugation for 10 min and 0.8 ml of supernatant was transferred to a fresh tube containing 200 µl of sterile PEG-NaCl solution (PEG 20 % [w/v]; NaCl, 2.5 M). Homogeneity of this mixture was achieved by vortexing before incubating on ice (30 min).

iii. Precipitated viral particles were collected by centrifugation for 5 min and the supernatant discarded. Following further centrifugation (2 min), residual PEG-NaCl solution was carefully removed using a finely-drawn glass pipette.

iv. The viral pellet was resuspended in 100 µl TE and vortexed for 20 s in the presence of 50 µl TE-equilibrated, triple-distilled phenol.

v. The resultant emulsion was allowed to stand at room temperature for 5 min, revortexed for 15 s and subjected to centrifugation for 5 min.

vi. 80 µl of the upper (aqueous) phase was removed to a fresh tube containing 8 µl 3 M sodium acetate solution (pH 4.8) and 200 µl ethanol at -20°C.

vii. Tubes were incubated at -70°C for 16-20 h, and DNA precipitates harvested by centrifugation for 7 min. The supernatant was decanted and discarded.

viii. Viral DNA pellets were washed with ethanol (held at -70°C) and dried *in vacuo*. Ss preps were resuspended in 18 µl of sterile, double-distilled water and stored at -20°C.

D. DNA sequencing reactions

dNTPs and ddNTPs were dissolved in TE. dNTP:ddNTP ratios are indicated overleaf. Long runs (where over 250 bp were to be read) employed nucleotide mixes at concentrations shown in brackets.

	Nucleotide Reaction Mix			
Components (µl)	A	C	G	T
0.5 mM dCTP	50	2.5	50	50
0.5 mM dGTP	50	50	2.5	50
0.5 mM dTTP	50	50	50	2.5
0.1 mM ddATP	8 (5)			
1.0 mM ddCTP		10 (7)		
1.0 mM ddGTP			20 (14)	
10 mM ddTTP				6 (4)
TE	42 (45)	87 (90)	77 (83)	91 (93)

E. Selective screening by T-tracking

Recombinant 'phages carrying DNA inserts were distinguished from deletants and 'phages carrying undesired DNA inserts by diagnostic T-Tracking. Reactions were in Eppendorf tubes.

i. To 2.2 µl ss template was added 0.8 of a solution containing 0.3 µl Klenow reaction buffer; 0.5 µl M13 universal primer (1.2 ng.μl^{-1}). Klenow reaction buffer is: Tris-HCl (pH 8.0), 10 mM; $MgCl_2$, 5 mM.

ii. The template-primer solution was left to anneal at 58°C for 2 h.

iii. The following prereaction mix was prepared (for 10 clones):

T mix	20 µl,
[α-^{35}S]dATPαS (>600 Ci.mmol^{-1})	1.5 µl,
Klenow enzyme	2 U,
100 mM DTT	1 µl.

iv. 2 µl prereaction cocktail was spotted onto the rim of each tube containing template-primer solution, and the chain elongation reaction initiated by spinning tubes in an Eppendorf 5413 microcentrifuge.

v. Tubes were incubated at 28°C for 20 min followed by the addition of 2 µl chase mix (a solution of all four dNTPs at 0.25 mM). Incubation was continued for 15-20 min.

vi. The reaction was terminated by the addition of 3 µl formamide dye mix and electrophoresed immediately.

F. DNA sequencing reactions

i. The following annealing reaction was established:

Ss template DNA	7 µl,
M13 universal primer	2 µl,
Klenow reaction buffer	1 µl.

ii. Annealing was allowed to proceed at 58°C for 2 h.

iii. The following were added in the amounts shown:

[α-^{35}S]dATPαS (>600 Ci.mmol^{-1})	1 µl,
Klenow enzyme	1 U,
100 mM DTT	1 µl.

iv. 2.5 µl of the resultant cocktail was spotted onto the rims of each of four tubes containing respectively, 2µl A, C, G and T mix. After mixing by centrifugation in an Eppendorf 5413 microfuge, samples were incubated at 28°C for 20 min.

v. 1 µl chase mix (a solution of all four dNTPs at 0.25 mM) was added and incubation continued for 15 min.

vi. Reactions were terminated by the addition of 3 µl formamide dye mix and either electrophoresed immediately, or stored at -20°C (for up to 2 days) until required.

G. Polyacrylamide gel electrophoresis of DNA

This was as per the Amersham International M13 Cloning and Sequencing Handbook (1984) with minor modifications. Samples were boiled for 3 min prior to electrophoresis. Electrophoresis was at a constant amperage of 33 mA for 1.5-8 h.

Autoradiography of gels was for 16-72 h at room temperature without intensifying screens.

H. Analysis of DNA sequences

Computer-assisted analysis of DNA sequences was using the programme of Schwindinger & Warner (1984).

CHAPTER 3

Analysis of Pilin Gene Loci of *Neisseria gonorrhoeae* Strain P9

3.1 Introduction

N.gonorrhoeae strain P9 exhibits antigenic variation of its pili in vitro and in vivo and four well-characterised isogenic P9 variants, designated P9-2, P9-20, P9-35 and P9-37, each express at least one pilus type that is apparently physically and immunologically characteristic for that variant (Lambden et al., 1980, 1981; Virji et al., 1983). Fragments of DNA harbouring pilin expression (pilE) loci that were derived from populations of each P9 pilus variant have been cloned in Escherichia coli using pBR322 (Nicolson et al., 1986). Two such recombinant plasmids, designated pLV260 and pLV270, were derived from the pilus antigenic variant P9-2, although they encode immunologically distinct pilins of 19 and 20 kd respectively (Nicolson et al., 1986).

This chapter includes a description of the gonococcal insert of pLV260. Detailed analysis of the pilE locus of pLV260 enables comparison with other pilin gene loci and the identification of sequences that may play a role in gonococcal pilus gene expression. An investigation of the arrangement of pil-related sequences on pLV260 and the genomes of P9 pilus antigenic variants was initiated.

3.2 Results

3.2.1 Location of the pilE locus of pLV260, a recombinant plasmid that encodes a gonococcal pilin

The restriction enzyme map of pLV260 was determined (Fig. 1). Using this information, a series of restriction endonuclease fragments were sub-cloned into pBR322 (Fig. 1). The physical map of each sub-clone was confirmed by restriction enzyme analysis, allowing alignment of the restriction map of each with that of the parental plasmid pLV260 (Fig. 1). Part of the above analysis was performed in collaboration with I.J. Nicolson.

In independent experiments, each recombinant plasmid sub-clone was used to transform E.coli strain DH1 to Ap^r, prior to immunological screening of representative numbers of colonies with monoclonal antibody SM1. Of the sub-clones analysed, pLV263 and pLV267 alone encoded SM1-reactive pilin(s), indicating that the pilE gene complement of pLV260 lay on a DNA segment of 1.5 kb that possessed PvuII and SmaI sites at its termini (Fig. 1).

To localise further the pilin gene(s) of pLV260, the pilin-encoding sub-clone, pLV267, was linearised at its unique XbaI site and treated with nuclease BAL 31 in conditions conducive to a large range of deletion sizes. Linear molecules treated in this way were self-ligated and used to transform E.coli strain DH1 to

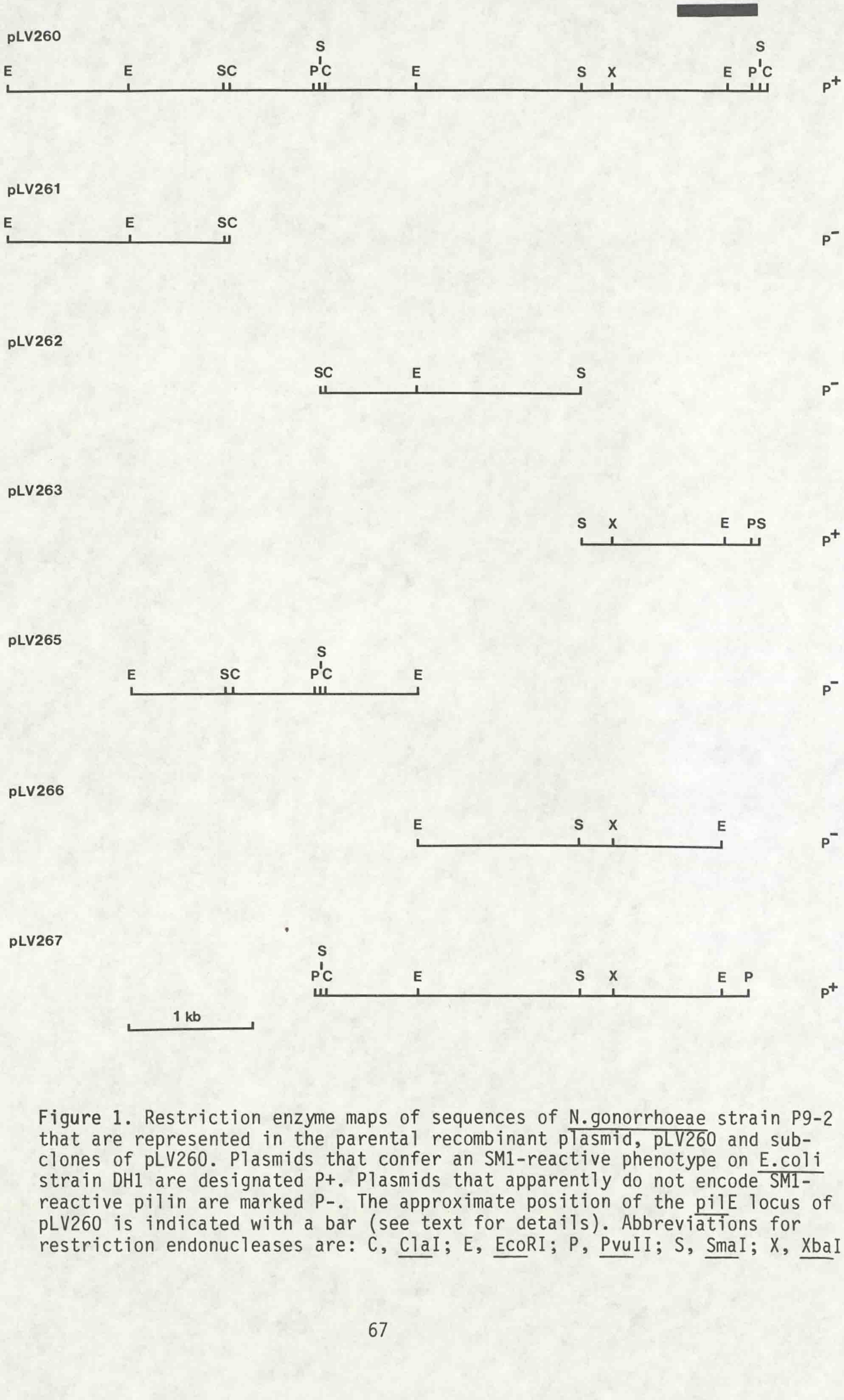

Figure 1. Restriction enzyme maps of sequences of N.gonorrhoeae strain P9-2 that are represented in the parental recombinant plasmid, pLV260 and sub-clones of pLV260. Plasmids that confer an SM1-reactive phenotype on E.coli strain DH1 are designated P+. Plasmids that apparently do not encode SM1-reactive pilin are marked P-. The approximate position of the pilE locus of pLV260 is indicated with a bar (see text for details). Abbreviations for restriction endonucleases are: C, ClaI; E, EcoRI; P, PvuII; S, SmaI; X, XbaI.

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Ap^r. Transformant colonies were then screened for pilin production with antibody SM1. Six SM1-reactive and SM1-non-reactive clones were chosen at random for restriction analysis of recombinant plasmids using EcoRI (data not shown). Each pilin-encoding plasmid possessed 3 EcoRI fragments, the smallest and largest of which (1.09 kb and 4.62 kb respectively) were common to pLV267. However, the size of the remaining EcoRI fragment of each pilin-eliciting plasmid was variable. This segment, which presumably corresponded to the 2.65 kb EcoRI fragment that harboured the unique XbaI site of pLV267, was reduced in one pilin-encoding plasmid deletant by 1.11 kb, to 1.54 kb. The analogous fragments of pilin-deficient plasmids were at most approximately 1.5 kb, and in some cases deletion of an EcoRI site had occurred. Assuming deletion by nuclease BAL 31 to have been symmetrical about the XbaI site of pLV267, the boundary of the pilin coding sequence therefore lay approximately 0.55 kb to one side of this site. The pilE gene of pLV260 was resident upon the 1.5 kb SmaI-PvuII fragment (vide supra) that contains, approximately 0.26 kb from its SmaI terminus, the unique XbaI site (Fig. 1). Hence, the XbaI-proximal boundary of the pilE gene of pLV260 lay approximately 0.55 kb from the XbaI site on the 1.5 kb SmaI-PvuII fragment (Fig. 1).

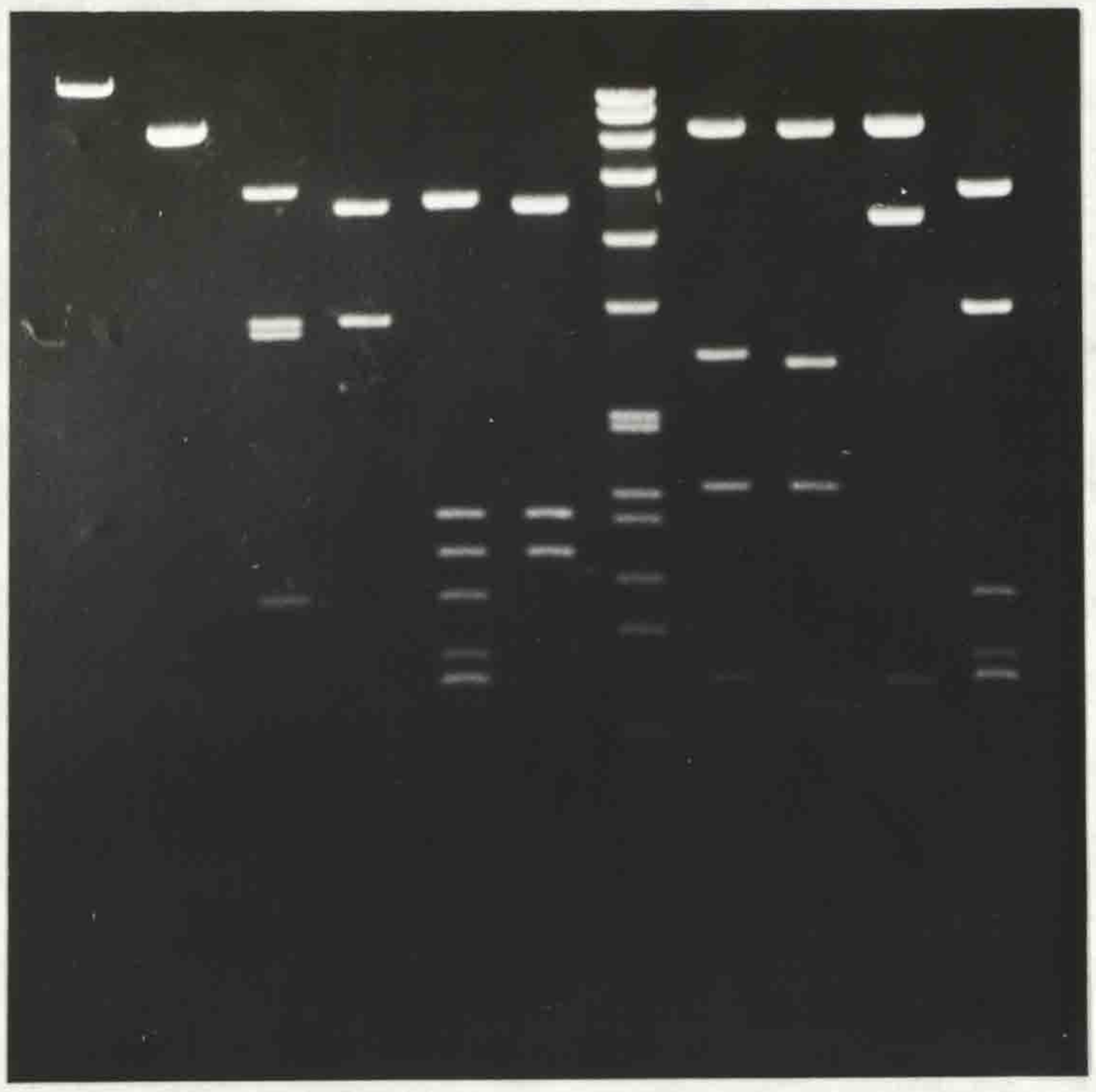
According to these results, the pilE gene of pLV260 required a minimum of approximately 0.6 kb coding capacity. Indeed, the pilin expression loci of N.gonorrhoeae $MS11_{ms}$ are each encoded by DNA tracts of similar size (Meyer et al., 1984). Furthermore, a

DNA segment of 0.6 kb has coding potential for a polypeptide of approximately 20 000, in good agreement with the apparent M_r of the pilin encoded by pLV260 (Nicolson et al., 1986). Assuming that the pilin encoded by pLV260 required a coding sequence of approximately 0.6 kb, these data were not consistent with the possibilty that pLV260 possessed more than one pilE locus.

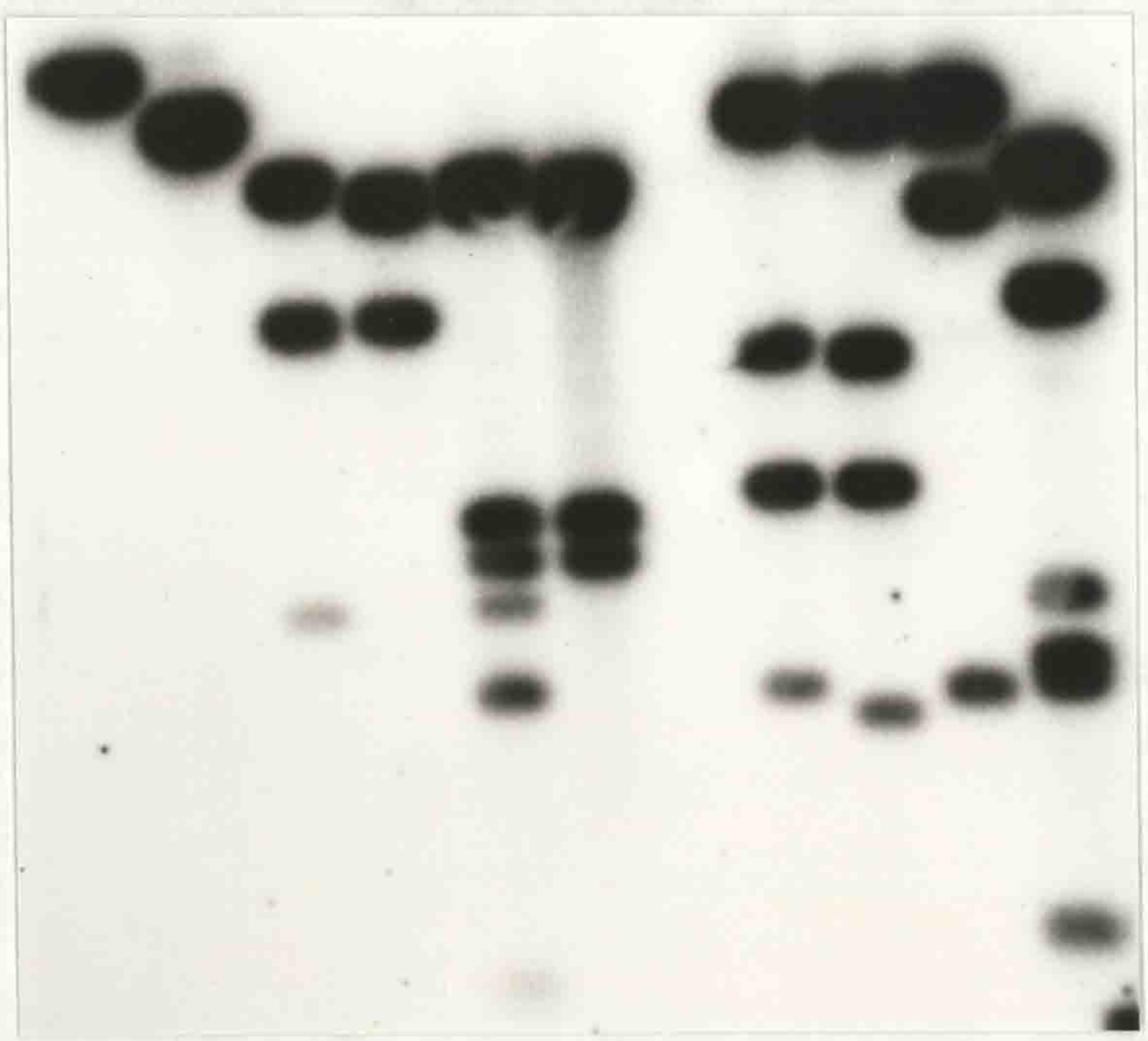
3.2.2 Multiple pilE-related sequences are present on plasmid pLV260 and genomic DNA isolated from P9 pilus antigenic variants

In an attempt to localize any region(s) of homology between plasmids pLV260 and pLV270, pLV270 was used as probe in Southern hybridisation analysis of pLV260 cut with either SmaI, ClaI, EcoRI or combinations of two of these enzymes (Fig. 2). With the exception of an EcoRI-SmaI fragment of 0.82 kb, plasmid pLV270 hybridised to all fragments of pLV260 that were visualised (Fig. 2). The behaviour of sequences present on the 0.82 kb EcoRI-SmaI fragment of plasmid pLV260 is discussed below. Since pLV260 possessed two EcoRI fragments of approximately 2.5 kb that were poorly resolved on this gel, it was possible that only one such fragment hybridised to pLV270. However, almost all fragments of pLV260 generated by digestion with EcoRI and one other enzyme hybridised. Furthermore, in an experiment in which the two EcoRI fragments were independently electrophoresed and blotted, prior to probing with pLV270 as before, both fragments clearly

Figure 2. Agarose gel electrophoresis (A) and Southern hybridisation analysis (B) of restriction fragments of plasmids pLV260 and pLV270. Insets A and B represent the same gel. Tracks are: 1. pLV260 DNA cleaved with HindIII; 2. pLV270 DNA cleaved with SmaI; 3. pLV260 DNA cleaved with EcoRI; 4. pLV270 DNA cleaved with EcoRI; 5. pLV260 DNA cleaved with EcoRI + SmaI; 6. pLV270 DNA cleaved with EcoRI + SmaI; 7. SPP1 DNA cleaved with EcoRI; 8. pLV260 DNA cleaved with SmaI; 9. pLV260 DNA cleaved with SmaI + ClaI; 10. pLV260 DNA cleaved with ClaI; 11. pLV260 DNA cleaved with ClaI + EcoRI. Plasmid pLV270 was used as probe in Southern hybridisation analysis.



A



B

hybridised (data not shown).

Assuming hybridisation to have been exclusively between fragments of pLV260 and the gonococcal insert of pLV270, these results reflect repetition of at least one extensive DNA sequence throughout the 6.1 kb insert of pLV260.

Such sequence repetition may have been due to the presence on pLV260 of multiple pilin gene sequences. Indeed, the chromosomes of other strains of N.gonorrhoeae contain many pilin gene sequences (Meyer et al., 1982; Swanson et al., 1985). To investigate the arrangement of pilE-related loci upon the chromosome of N.gonorrhoeae P9, approximately 1.2 kb of the gonococcal insert of pLV260 was used in Southern hybridisation analysis of genomic DNA that had been derived from P9 pilus variants and digested to completion with endonuclease ClaI (Fig. 3). P9-2, P9-20, P9-35 and P9-37 total DNAs that had been subjected to Southern analysis in this way possessed one of three distinguishable hybridisation patterns comprising at least seven bands. With the exception of a faintly hybridising 2.3 kb ClaI fragment that was generated on cleavage of P9-37 DNA, total DNA preparations from P9-2, P9-35, and P9-37 yielded apparently identical hybridisation patterns. In each case, a 3.8 kb ClaI fragment was present that disappeared on subsequent cleavage with XbaI (Fig. 3). A ClaI fragment of similar size was also represented in the P9-20 hybridisation pattern. This fragment may harbour the intact pilin coding sequence of each strain, since

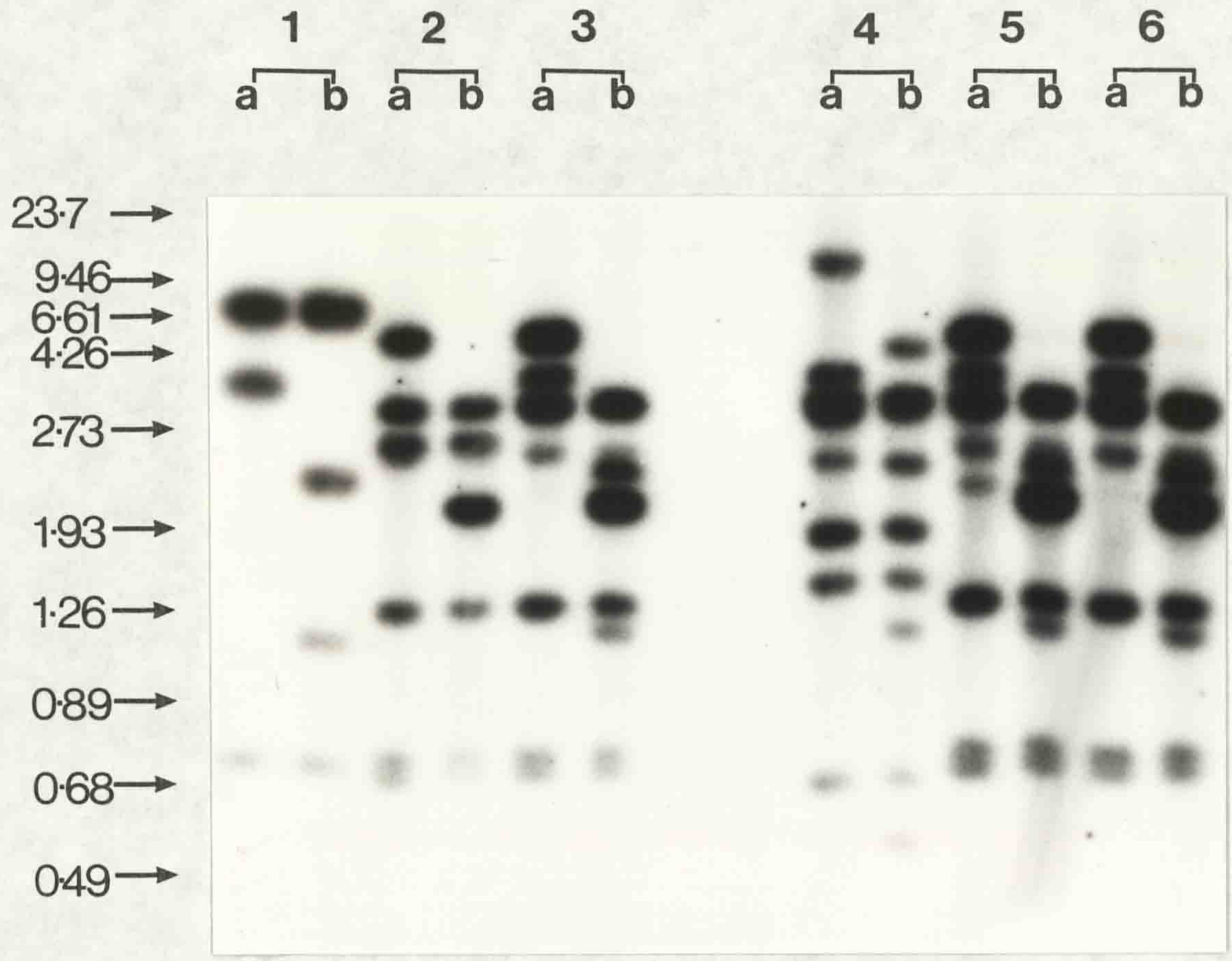


Figure 3. Southern hybridisation analysis of total DNA from P9 pilus variants. DNA was digested with ClaI (a), or ClaI + XbaI (b). The 1 kb EcoRI fragment of plasmid pLV260 (Fig. 5) was used as probe. Probed DNA was: 1. plasmid pLV260; 2. total DNA from strain P9-2(P-); 3. total DNA from strain P9-2(P+); 4. total DNA from strain P9-20; 5. total DNA from strain P9-35; 6. total DNA from strain P9-37. Brackets indicate that DNA had been isolated from the same variant. DNA sizes are in kb.

the (analogous) 3.8 kb ClaI fragment of pLV260 a) contains a pilE locus, and b) is digested by XbaI to give fragments of and 1.3 and 2.5 kb. Fragments of similar size were represented in hybridisation patterns of all P9 total DNA preparations that had been digested with ClaI and XbaI (Fig. 3). The hybridisation pattern of a P- isogenic variant of P9-2 whose total DNA had been completely digested with ClaI, lacked a band corresponding to a fragment of 3.8 kb (Fig. 3). Furthermore, since pilE-homologous ClaI fragments of identical size were apparently shared by pLV260 and the genome of strain P9-2, it is unlikely that the gonococcal insert of pLV260 had undergone rearrangement *in vitro*.

The respective hybridisation patterns obtained for P9-20 DNA digested to completion with EcoRI, PstI, and PvuII (data not shown) and ClaI (Fig. 3) are markedly different from those obtained for similarly-treated DNA that had been isolated from P9-2, P9-35 and P9-37. However, using pilin gene sequences as probes, substantial differences have also been revealed between the Southern blotting patterns of sibling pilus variants of *N.gonorrhoeae* strain MS11 (Swanson *et al.*, 1985).

Apparent discrepancies between the chromosomal arrangements of pilE-related sequences of isogenic P9 pilus variants may be associated with pilus antigenic variation. To investigate this possibility, total genomic DNA was isolated from a series of gonococcal clones, each of which had been derived from a single colony of *N.gonorrhoeae* P9-2 and that expressed pilin(s) of

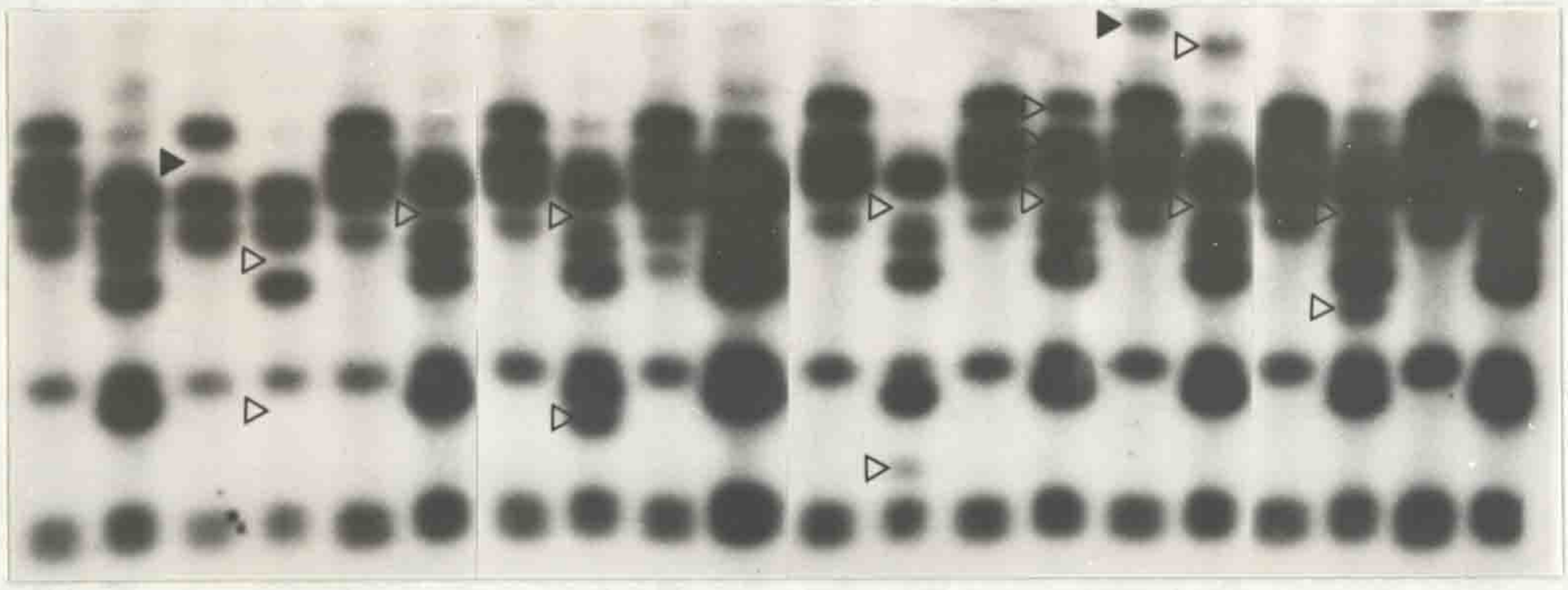


Figure 4. Southern hybridisation analysis of total DNA from P9-2 and P9-2 pilus variants. DNA was digested with ClaI (a), or ClaI + XbaI (b). The 1.2 kb XbaI-PvuII fragment of plasmid pLV260 was used as probe. Brackets indicate that DNA had been isolated from the same variant. Apparent differences between the ClaI (▶), and ClaI + XbaI (▷) hybridisation patterns of DNA from P9-2 and progeny populations are shown.

differing M_r (I.J. Nicolson, personal communication). Total DNA from each P9-2 derivative was digested to completion with either ClaI or XbaI and ClaI, prior to Southern hybridisation analysis using the 1.2 kb XbaI-PvuII fragment of pLV260 as probe (Fig. 4). Several P9-2 derivatives differed from their progenitor strain with respect to hybridisation patterns (Fig. 4). Assuming pilus antigenic variation to have been concomitant with changes in pilin M_r exhibited by P9-2 derivatives (Virji & Heckels, 1983; Nicolson et al., 1986), it is likely that pilus antigenic variation of strain P9-2 involves readily demonstrable rearrangement of pil-related chromosomal sequences. Furthermore, a pilus phase variant of strain P9-2 that did not elaborate SM1-

reactive pilin, had apparently undergone deletion of the 3.8 kb pilE-homologous ClaI fragment that is present on the P+ P9-2 chromosome (Figs 3 & 4).

The relationship between pilin gene sequences present on plasmid pLV260 and strain P9 pilus phase and antigenic variants, was further analysed using a series of probes that had been derived from pLV260 (Fig. 5). With the exception of a 0.75 kb ClaI-EcoRI fragment (probe 4; Fig. 5), probes produced hybridisation patterns that were essentially identical (data not shown). P9-20 total DNA that had been digested with ClaI and XbaI contained two fragments that hybridised to probe 4, of 0.52 and 2.55 kb. Similarly digested DNA from P9-35 and P9-2 produced single probe 4-reactive bands that corresponded to a fragment of approximately 2.5 kb in each case. Indeed, probe 4 is resident upon the 2.55 kb XbaI-ClaI fragment of pLV260 (Fig. 5). A fragment of this size was replaced on the genome of a P- derivative of P9-2, by a probe 4-homologous fragment of 2.84 kb. A chromosomal segment of DNA corresponding to probe 4 may, therefore, undergo a rearrangement that is concomitant with P9-2 pilus phase variation.

3.2.3 Sequence analysis of the pilE gene of pLV260

The primary structure of the pilE region of strain P9-2 was determined by sequencing the 1.4 kb XbaI-ClaI fragment of pLV260 that encoded an SM1-reactive pilin. To this end, a series of

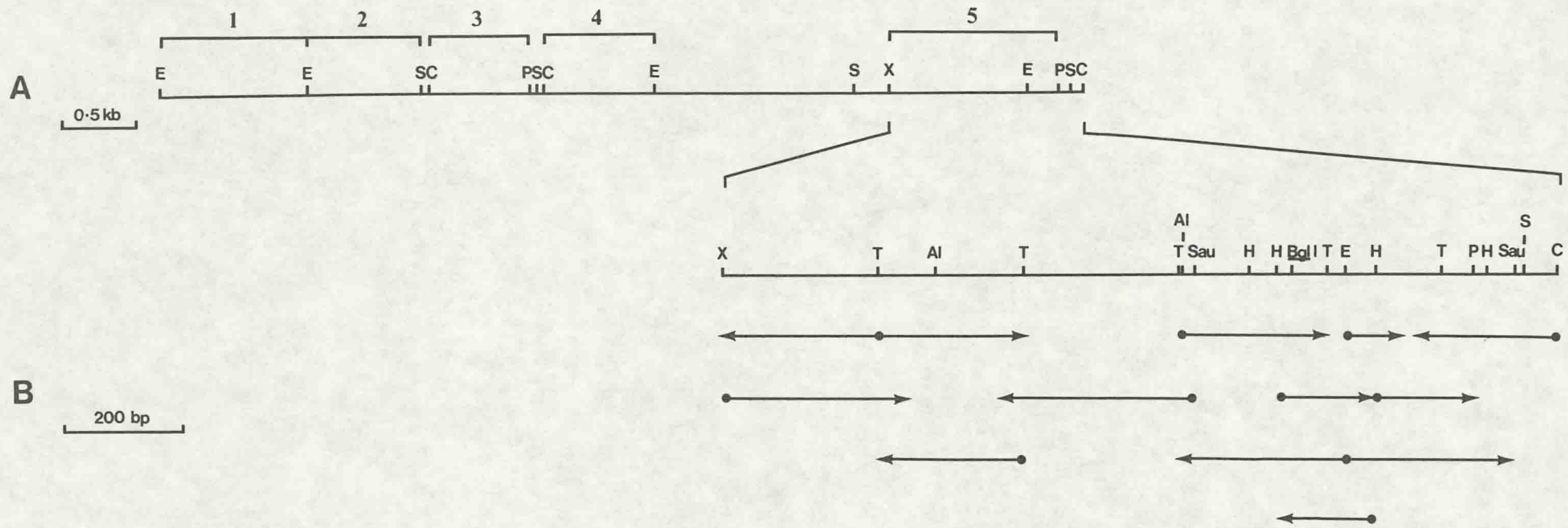


Figure 5. A. Restriction endonuclease map of recombinant pilin-encoding plasmid pLV260. Positions of fragments corresponding to probes derived from pLV260 (probes 1-5) are indicated. B. Detailed restriction analysis of the sequenced region of pLV260. Arrows designate the sequencing strategy employed. Abbreviations for restriction enzymes are as for Fig. 1, with the additions of: Al, AluI; H, HaeIII; Sau, Sau3A1; T, TaqI.

CHAPTER 1

General Introduction

overlapping restriction fragments were sub-cloned into phages M13mp18 or M13mp19 using the strategy detailed in Fig. 5, prior to DNA sequencing. The complete nucleotide sequence of the 1.4 kb XbaI-ClaI fragment of pLV260 is shown in Fig. 6. Fig. 7 shows a sequencing ladder corresponding to part of the pilE locus of P9-2.

An open reading frame (orf) present on this sequence between nucleotide positions 735 and 1236 was inferred to correspond to the pilE gene because a) it contained an EcoRI site (bp 1028-1033) that was shown by sub-cloning analysis to lie within the pilE gene, b) it encoded a protein of predicted M_r 17 000, in good agreement with the observed size of pilin elaborated by E.coli harbouring pLV260 (Nicolson et al., 1986) and c) it could be aligned with the pilE gene of N.gonorrhoeae strain $MS11_{ms}$ (Meyer et al., 1984). Furthermore, alignment of the pilE loci of pLV260 and strain $MS11_{ms}$ (Meyer et al., 1984) indicated that the ribosome binding site and promoter sequences of the pilin gene pLV260 were at nucleotide positions 723-727 and 632-638 respectively (Figs 6 & 8). However, use of these sequences has not been demonstrated for pLV260.

Comparison of the predicted N-terminal amino acid sequence of the putative pLV260 pilE gene with the N-terminal amino acid sequence of purified gonococcal pilin indicated that the pilE gene product of pLV260 possessed a 'leader' peptide of 7 amino acids (Fig. 8). Indeed, nucleotides 1 to 332 of the putative pilE

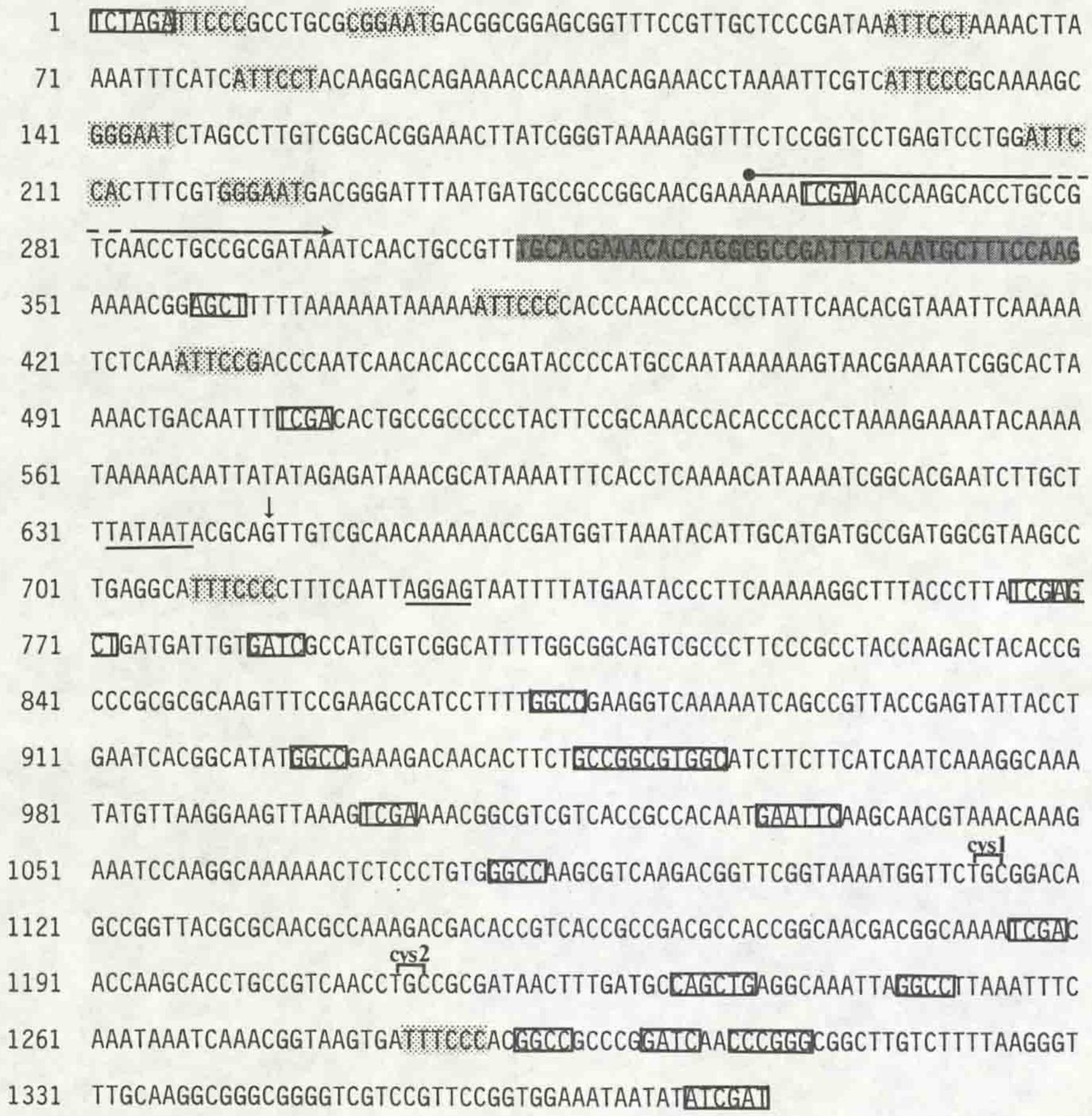


Figure 6. DNA sequence analysis of the 1.4 XbaI-ClaI fragment of plasmid pLV260, harbouring an intact pilE gene. The -10 sequence and ribosome binding site are underlined. The arrow at postion 643 indicates the putative start of pilin mRNA synthesis. Restriction enzyme sites are boxed. The positions RS1- (▓) and RS3- (▒) like elements are indicated. Positions of expressed cys1 and cys2 codons are shown. A region upstream of the expressed pilin gene that is homologous with the expressed cys2 region is designated with an arrow.

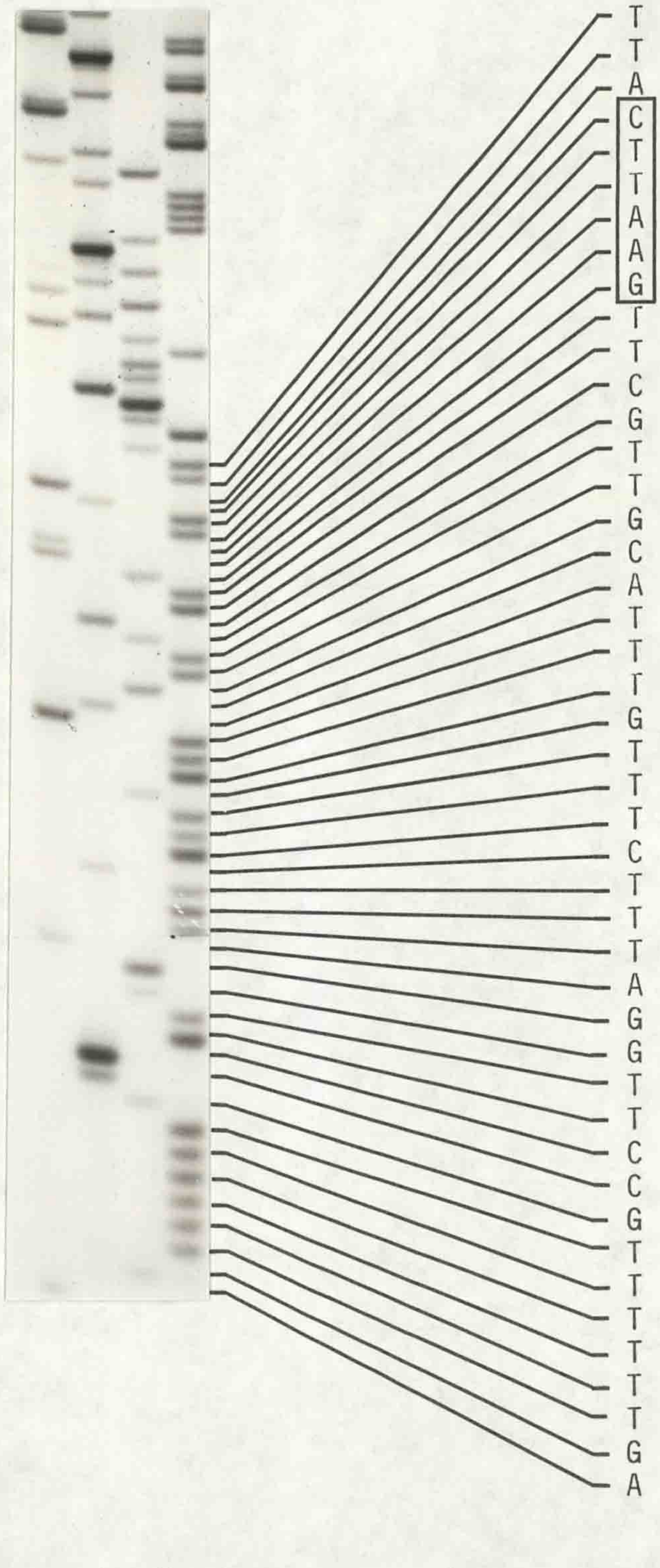


Figure 7. Sequencing ladder corresponding to the negative sense strand of part of the pilE gene of plasmid pLV260 (bp 985-1069; Fig. 6). Interpretation of a section of this ladder is shown. The EcoRI site represented in this sequence is boxed.

Figure 8. Nucleotide sequence comparison between the pilE locus of pLV260 (bp 469-1378; Fig. 6) and an analogous locus of N.gonorrhoeae strain $MS11_{ms}$ (Meyer et al., 1984). Deduced amino acid sequences appear below the coding sequence of each structural pilin gene. Dashes indicate no sequence differences observed in these postions. Parentheses indicate deletions of nucleotides. Nucleotide insertions, and where appropriate, predicted amino acid insertions, are also recorded. If the nucleotide sequence change is not accompanied by a change in amino acid, only the codon change is shown. The -10 sequence and ribosome binding site are underlined. Selected restriction enzyme sites are boxed.

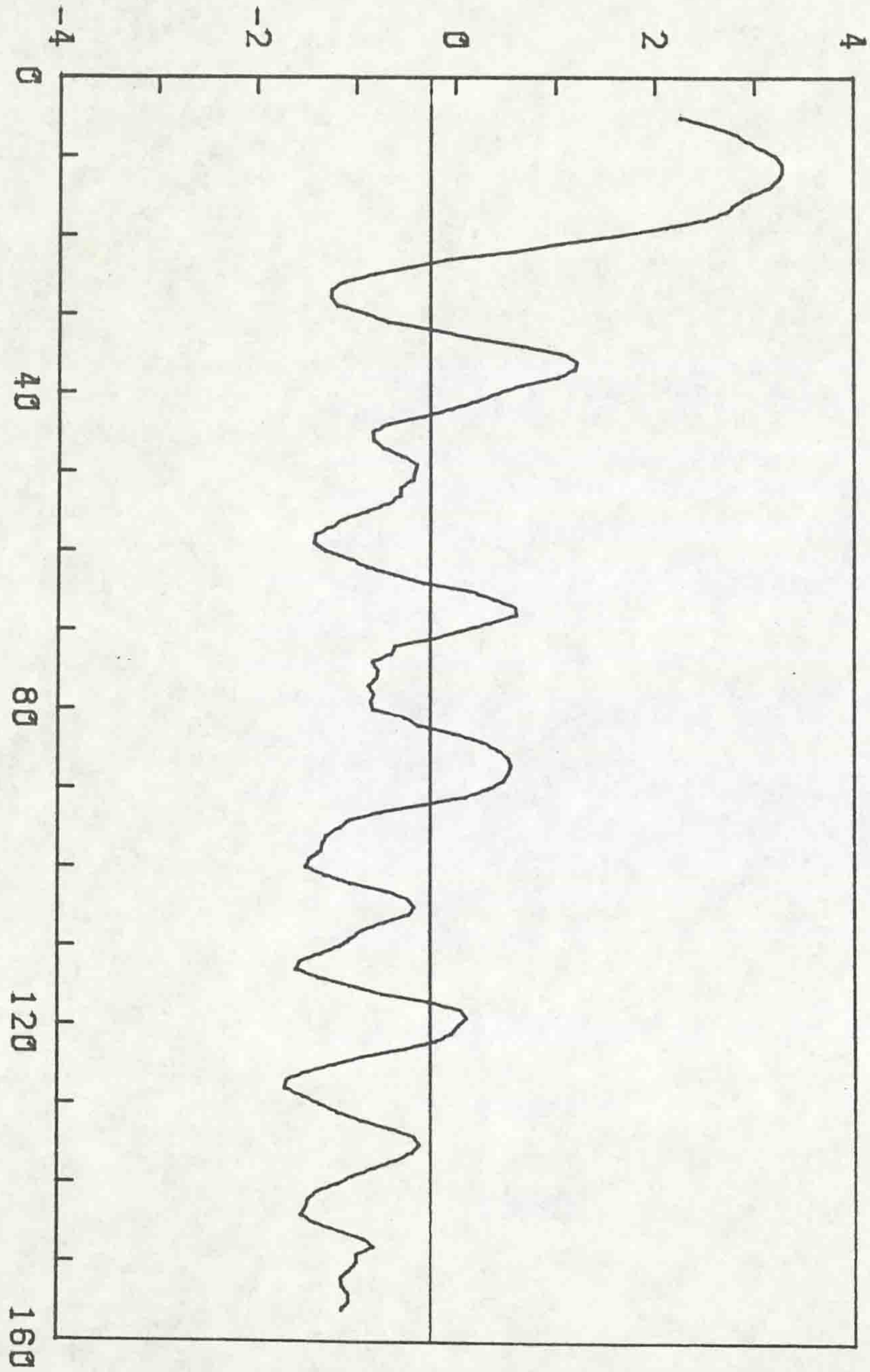
```
pLV260     AAAGTAACGAAAATCGGCACTAAAACTGACAATTTTCGACACTGCCGCCC()CCTACTTCCGCAAACC
MS11ms     ----------------------------------------------------C----------------

pLV260     ACACCCACCTAAAAGAAAATACAAAATAAAAACAATTATATAGAGATAAACGCATAAAATTTCACCT
MS11ms     ------------------------------------------------------------------

pLV260     CAAAACATAAAATCGGCACGAATCTTGCTTTATAATACGCAGTTGTCGCAACAAAAAACCGATGGTT
MS11ms     ------------------------------------------------------------------

pLV260     AAATACATTGCATGATGCCGATGGCGTAAG()CCTGAGGCATTTCCCCTTTCAATTAGGAGTAATTTT
MS11ms     -------------------------()----C--------()-------------------------

pLV260     ATG AAT ACC CTT CAA AAA GGC TTT ACC CTT ATC GAG CTG ATG ATT GTG ATC
           Met Asn Thr Leu Gln Lys Gly Phe Thr Leu Ile Glu Leu Met Ile Val Ile
MS11ms     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

pLV260     GCC ATC GTC GGC ATT TTG GCG GCA GTC GCC CTT CCC GCC TAC CAA GAC TAC
           Ala Ile Val Gly Ile Leu Ala Ala Val Ala Leu Pro Ala Tyr Gln Asp Tyr
MS11ms     --T --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

pLV260     ACC GCC CGC GCG CAA GTT TCC GAA GCC ATC CTT TTG GCC GAA GGT CAA AAA
           Thr Ala Arg Ala Gln Val Ser Glu Ala Ile Leu Leu Ala Glu Gly Gln Lys
MS11ms     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

pLV260     TCA GCC GTT ACC GAG TAT TAC CTG AAT CAC GGC ATA TGG CCG AAA GAC AAC
           Ser Ala Val Thr Glu Tyr Tyr Leu Asn His Gly Ile Trp Pro Lys Asp Asn
MS11ms     --- --- --C --- --- --- --- --- --- --- --- -A- --- --- G-- A-- ---
                                                       Lys         Glu Asn

pLV260     ACT TCT GCC GGC GTG GCA TCT TCT TCA TCA (  ) ATC AAA GGC AAA TAT GTT
           Thr Ser Ala Gly Val Ala Ser Ser Ser Ser     Ile Lys Gly Lys Tyr Val
MS11ms     --- --- --- --- --- --- --C C-C C-C --- GAC --- --- --- --- --- ---
                                       Pro Pro     Asp

pLV260     AAG GAA GTT AAA GTC GAA AAC GGC GTC GTC ACC GCC ACA ATG AAT TCA AGC
           Lys Glu Val Lys Val Glu Asn Gly Val Val Thr Ala Thr Met Asn Ser Ser
MS11ms     --A --G --- G-- --T A-- --- --- --- --T --- --- --- --- CT- --- ---
           Lys         Glu     Lys                                 Leu

pLV260     AAC GTA AAC AAA GAA ATC CAA GGC AAA AAA CTC TCC CTG TGG GCC AAG CGT
           Asn Val Asn Lys Glu Ile Gln Gly Lys Lys Leu Ser Leu Trp Ala Lys Arg
MS11ms     GG- --- --- --T --- --- A-- --- --- --- --- --- --- --- --- -G- ---
           Gly         Asn         Lys                                 Arg

pLV260     CAA GAC GGT TCG GTA AAA TGG TTC TGC GGA CAG CCG GTT ACG CGC AAC GCC
           Gln Asp Gly Ser Val Lys Trp Phe Cys Gly Gln Pro Val Thr Arg Asn Ala
MS11ms     G-- A-- --- --- --- --- --- --- --- --- --- --- --- --- --- -C- -A-
           Glu Asn                                                     Thr Asp

pLV260     AAA GAC GAC ACC GTC ACC GCC GAC GCC ACC GGC AAC GAC GGC AAA (  ) ATC
           Lys Asp Asp Thr Val Thr Ala Asp Ala Thr Gly Asn Asp Gly Lys     Ile
MS11ms     (  ) --- --- --- --T G-- -A- -C- (          ) --A --- --- --- GAA ---
                       Ala Asp Ala             Lys                     Glu

pLV260     GAC ACC AAG CAC CTG CCG TCA ACC TGC CGC GAT AAC(   )TTT GAT GCC AGC
           Asp Thr Lys His Leu Pro Ser Thr Cys Arg Asp Asn     Phe Asp Ala Ser
MS11ms     --- --- --- --- --- --- --- --- --- --- --- --G GCA -C- --- --- -AA
                                                       Lys Ala Ser         Lys

pLV260     TGA GGCAAATTAGGCCTTAAATTTCAAATAAATCAAACGGTAAGTGATTTCCCACGGGCGGCCGGAT
           TER
MS11ms     --- ----------------------T----------G-------------T----( )---------

pLV260     CAACCCGGGCGGCTTGTCTTTTAAGGGTTTGCAAGGCGGGCGGGGTCGTCCGTTCCGGTGGAAATAA
MS11ms     ------------------------------------------------------------------

pLV260     TATATCGAT
MS11ms     ---------
```

genes of pLV260 and pLV270 were identical, and the pilin polypeptide encoded by pLV270 is apparently processed in E.coli (I.J. Nicolson, personal communication). The predicted amino acid sequence of the prepilin 'leader' peptide of N.gonorrhoeae strain P9-2 is similar in each case to that of P.aeruginosa (Sastry et al., 1985) and B.nodosus (Elleman et al., 1986). Alignment of the pilE gene sequences of pLV260 and $MS11_{ms}$ indicated that a single, silent nucleotide difference existed between the C regions (bp 1-159) of each gene (Fig. 8). However, many amino acid differences existed between the SV and HV sections of the respective pilE loci (Fig. 8). Sequence disparities between $MS11_{ms}$ and pLV260 pilE loci are presumably responsible for antigenic differences that exist between their gene products (J.E. Heckels, personal communication).

A hydropathicity plot based on the predicted amino acid sequence of the pLV260 pilE gene product, excluding its putative leader sequence (Fig. 9) indicated a protein with a hydrophobic N-terminus. Presumptive hydrophilic C-terminal regions may correspond to pilus epitopes (Nicolson & Perry, manuscript in preparation).

The pilE locus of pLV260 contained several classes of DNA segments that were not related to pilin coding sequences (Fig. 6). DNA segments RS1 and RS3 have previously been described and are present on the major silent pilus gene locus of strain $MS11_{ms}$, pilS1 (Meyer et al., 1984; Haas & Meyer, 1986). The RS3-

Figure 9. Hydropathy plot of the predicted amino acid sequence of the mature pilE gene product of plasmid pLV260. Hydrophobicity indeces (y axis) were calculated according to the method of Eisenberg et al. (1982). Areas above the mid-point line (i.e. with positive values) are hydrophobic relative to those below. Values were averaged with a periodicity of 4 amino acids.


Hydropathy Index
-4
-2
0
2
4
0
40
80
120
160
Sequence number

related family of repeated segments of pLV260 shared a 6 bp core sequence, 5'-ATTCCC-3' or, less frequently, 5'-GGGAAT-3' that exhibited 'wobble' at positions 1 or 6 of each hexanucleotide (Fig. 6). RS3-like elements were clustered upstream of the pilE reading frame, usually in groups of 2 or 3 that were interspersed within each group by 3-8 bp (Fig. 6). Several RS3-related elements, located at nucleotide positions 19-24, 140-145 and 220-225 were each present on DNA segments that were reminiscent of the sequence, 5'-AGCGAATG-3' (Fig. 6). This sequence mediates deletionogenesis involving the gonococcal cryptic plasmid, pJD1 (Hagblom et al., 1986). It is as yet unclear whether pJD1 deletionogenic, and RS3 sequences are functionally related.

The XbaI-ClaI fragment of pLV260 harboured a single RS1-like sequence that extended from bp 311 to 350 (Fig. 6; Haas & Meyer, 1986). The RS1-like sequence was resident on a DNA segment of 96 bp that differed at only 5 nucleotide positions from a portion of the pilS1 locus of $MS11_{ms}$ (Haas & Meyer, 1986). Part of the 96 bp tract possessed a copy of a cys2 coding region that is conserved on pilin expression loci.

3.3 Discussion

The gonococcal insert of pLV260 contains, in addition to a single expressed pilin gene, multiple unexpressed copies of pilE-related sequences. Using DNA probes derived from pLV260, antigenic pilus variants of N.gonorrhoeae strain P9 have each been shown to harbour many pilin gene loci, in common with other gonococcal strains (Meyer et al., 1982; Swanson et al., 1985). A better understanding of the chromosomal arrangement of silent P9 pilin loci will be achieved by analysis of a series of large, overlapping gonococcal DNA fragments. However, the genomic arrangement of pilS loci is apparently not static, but varies between some P9 pilus antigenic variants (Figs 3 & 4). Such chromosomal rearrangements probably reflect intragenic recombination involving one or more pilS loci and the pilE locus of strain P9.

Chromosomal rearrangement of pilE-related sequences had also occured in a P- pilus phase variant of sub-strain P9-2 (Figs 3 & 4). The 3.8 kb chromosomal ClaI fragment that presumably harbours the pilE gene of sub-strain P9-2 has been replaced in the P- variant by a ClaI fragment of 2.8 kb (Fig. 3). Furthermore, this fragment is apparently refractory to cleavage by XbaI and contains sequences that are homologous to probe 4, whereas P9-2 DNA that had been digested with ClaI and XbaI contained a single probe 4-homologous fragment of 2.5 kb (data not shown). One

1.1 Bacterial fimbriae: a subset of adhesion determinants in pathogenesis

Successful infectious agents can interact with, and subsequently colonise host tissue. Such colonisation may be the result of a specific interaction between host and microbial cell surface components. The biochemical nature of these components determines the tissue tropism exhibited by potentially infectious agents. Hence, Acquired Immune Deficiency Syndrome (A.I.D.S.) -linked virus HIV-I, possesses one or more surface glycoproteins that engender it with a tropism for the OK $T4^+$ subset of T-cells (Klatzmann et al., 1984; Allan et al., 1985), whereas exposed protein M of Streptococcus pyogenes mediates attachment to pharyngeal epithelial cells in a specific manner (Lancefield, 1962; Ellen & Gibbons, 1972). Microbial components that promote attachment to host tissue (adhesins) can be involved in the establishment of a pathogenic infection, as in the cases of HIV-I and S.pyogenes. The role played by adhesins in the disease process defines their importance as pathogenicty determinants. Expression by Haemophilus influenzae type b of fimrial appendages is apparently not important for colonisation of rat nasopharyngeal cells (Stull et al., 1984). In contrast, the presence of analogous fimbriae (pili) on Pseudomonas aeruginosa is important for adhesion of non-mucoid, but not mucoid strains to the mouse tracheal epithelium (Ramphal et al., 1984). Indeed, it is likely that fimbrial adhesins constitute a major subset of pathogenicity determinants in many bacterial species.

interpretation of these results, is that the pilE-harbouring ClaI fragment of P9-2 can undergo a deletion of 1 kb in vivo that results in removal the XbaI site and sequences at the 5' end of pilE (Figs 5 & 6). Such a deletion would result in an irreversible P+ to P- phase transition, assuming that pilE promoter and N-terminal coding sequences were absent from other loci on the genome of P9-2(P-). It is not known whether the P- P9-2 phase variant studied here can revert to a P+ state.

Pilus phase variation of N.gonorrhoeae strain $MS11_{ms}$ is associated with chromosomal deletions that seemingly have a fixed end-point at the 3' end of one, or both of its pilE loci (Segal et al., 1985). Sequence analysis revealed that a 41 bp sequence comprising the cys2 region of pilE was present as a direct repeat approximately 1 kb upstream on the gonococcal insert of pLV260 (Fig. 6). Associated with the silent cys2 region is an RS1-like element (Fig. 6; Haas & Meyer, 1986). It remains to be seen whether or not pilus phase variation in P9-2 can occur as a result of deletion across these two cys2 loci, although the analogous intervening DNA segment in pLV270 is excised efficiently in E.coli JM103 (I.J. Nicolson, personal communication). Since JM103 is $recA^+$, the analogous 'RecA' protein of N.gonorrhoeae strain P9 may play a role in P+ to P- pilus phase variation.

Alternatively, pilus phase and antigenic variation may occur via site-specific recombination mechanisms that are RecA-independent.

Indeed, nucleotides 1-450 of the pilE region of P9-2 harbour approximately 10 RS3-related elements that may act either directly as recombinase targets, or indirectly, by altering the local secondary DNA structure to produce a recombination signal. The importance of RS3 (and RS1) elements in gonococcal pilus expression is indicated by their conservation on pil loci on N.gonorrhoeae strain $MS11_{ms}$ (Haas & Meyer, 1986).

Pilus expression in strain $MS11_{ms}$ is believed to involve a trans-acting regulator that is, in turn, regulated by the pilus phase switch (Segal et al., 1985). Should an analogous regulator exist in strain P9-2, it is unlikely to be encoded in its entirety by the XbaI-ClaI fragment that harbours the P9-2 pilE gene, due to the absence of a suitable candidate orf or promoter/ribosome-binding-site. Neither does this fragment contain significant sequence homology with the recombination sites of the Cin, Gin, or Pin invertases (Hin is discussed in Chapter 5) that mediate gene expression via chromosomal rearrangement in other organisms (Plasterk & Van de Putte, 1984).

However, pLV260 contains a SmaI-ClaI repeat that is identical to that of pNG1100, a recombinant pilin-encoding plasmid whose gonococcal insert was derived from strain $MS11_{ms}$ (Meyer et al., 1982). Present at positions 1356-1378 upon the SmaI-ClaI fragment is a sequence that is conserved with recognition sequences of several DNA binding proteins (Fig. 10).

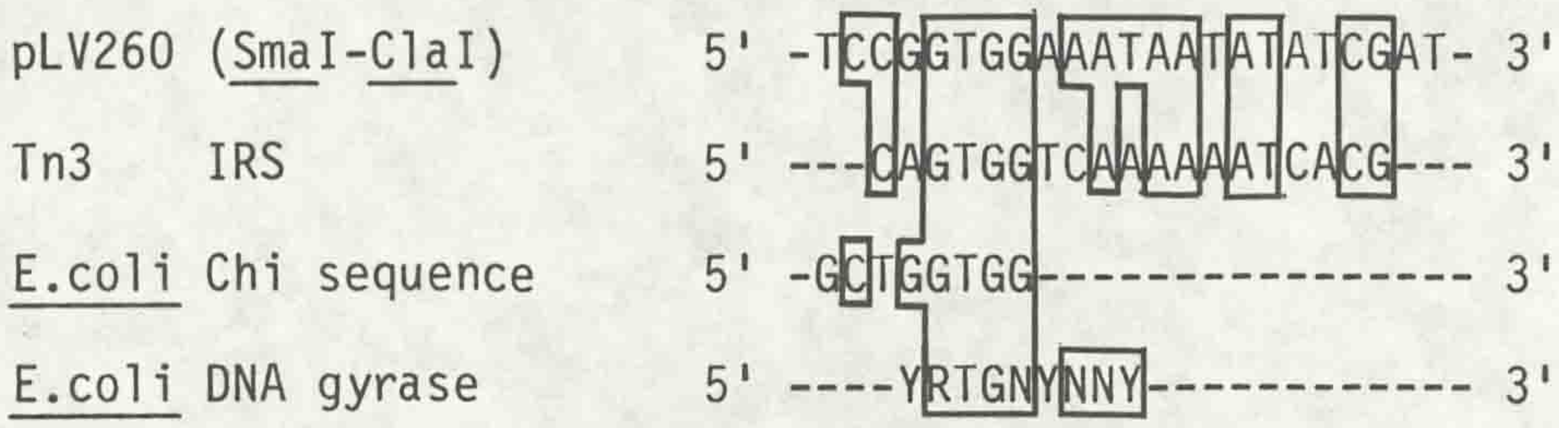


Figure 10. Comparison of the nucleotide sequence of part of the SmaI-ClaI fragment of the pilE locus of N.gonorrhoeae strain P9-2 with recombinase recognition sequences (Heffron et al., 1979; Smith et al., 1980; Naito et al., 1984). Consensus sequences are boxed.

The significance of these sequence similarities, if any, is unknown. However, the degree of homology between SmaI-ClaI and Tn3 IRS sequences may indicate that gonococcal pilus expression occurs via one or more duplicative transposition events that involve pilin gene segments (Segal et al., 1985, 1986). Such transposition, should it occur, may be mediated by a transposase acting at the the pseudo-IRS sequence present on the SmaI-ClaI fragment. Moreover, the SmaI-ClaI segment is located at probably all gonococcal pilin gene loci (Segal et al., 1985) and is apparently present in 3 copies on pLV260. The role of the SmaI-ClaI repeats in pilus phase and antigenic variation in P9 awaits elucidation.

CHAPTER 4

Inter-Strain Homology of Pilin Gene Sequences in *Neisseria meningitidis* Isolates that Express Markedly Different Antigenic Pilus Types

4.1 Introduction

Neisseria meningitidis is an important pathogen whose only natural host is man. Infections caused by the meningococcus can have high morbidity and mortality and include meningitis, arthritis and acute fulminant septicaemia. N.meningitidis infections may occur sporadically or in epidemics (Bovre & Gedde-Dahl, 1980) and during non-epidemic periods the meningococcus is carried asymptomatically in the nasopharynx of 5-10% of the population (Greenfield et al., 1971).

Little is known of the involvement of pili in meningococcal disease. However, pili are present on N.meningitidis isolated from mucosal surfaces (Craven et al., 1983; Kristiansen et al., 1984) and following tissue invasion (Stephens & McGee, 1981). Indeed, antibodies to pili have been detected subsequent to fulminant meningococcal infection (Poolman et al., 1983) and pili may mediate attachment of N.meningitidis to human buccal epithelial cells and erythrocytes (Trust et al., 1983).

Meningococcal pili are homopolymers of a pilin polypeptide subunit. Pilin molecules from some N.meningitidis isolates share biochemical, physical and immunological properties with pilins of N.gonorrhoeae (Virji & Heckels, 1983; Stephens et al., 1985). Monoclonal antibodies have been isolated that cross-react with meningococcal and gonococcal pilins (Virji & Heckels, 1983; Olafson et al., 1985). Pilin species from different clinical

isolates of N.meningitidis vary in size from 13-22 kd (Virji & Heckels, 1983; Diaz et al., 1984; Olafson et al., 1985). Pilin size variation is also exhibited by gonococci and is associated with changes in the immunological properties of pilin that account for gonococcal pilus antigenic variation (Virji et al., 1983). Moreover, like gonococci, meningococci can undergo pilus phase variation that does not necessarily result in loss of pilin production (Stephens et al., 1985; J.E. Heckels, personal communication). The genetic mechanisms of pilus phase and antigenic variation in N.gonorrhoeae and N.meningitidis may therefore be related.

In this chapter, physical and immunological properties of N.gonorrhoeae and N.meningitidis pilins are compared, and an investigation of meningococcal pilin gene loci described.

4.2 Results

4.2.1 Clinical isolates of N.meningitidis contain sequences that are homologous to the pilE locus of N.gonorrhoeae

Samples of genomic DNA from 14 clinical isolates of N.meningitidis were independently subjected to Southern blot analysis using a DNA probe corresponding to the pilE locus of N.gonorrhoeae strain P9-2 (Chapter 3; Figs 11 & 12). In each case, and in a further 5 examples (data not shown), strong hybridisation signals were obtained under conditions of high stringency, indicating extensive DNA homology. Two non-pathogenic isolates of the genus Neisseria (N.sicca and N.pharyngis) did not contain pilE-homologous sequences (W.N. Spencer, personal communication). Plasmids have not been detected in meningococcal DNA preparations. It is therefore likely that genomic sequences of N.meningitidis isolates that are homologous with the pilE locus of N.gonorrhoeae are located chromosomally.

Southern analysis of N.meningitidis DNA digested with ClaI and XbaI and probed with the P9-2 pilE gene revealed at least 5 different hybridisation patterns, each with 2 or more strongly hybridising fragments, representing a total of between 2.6 kb (C114) and approximately 24 kb (for example, C311) of each genome (Figs 11 & 12). Digestion of total DNA from 5 more meningococcal strains with ClaI and XbaI resulted in a further 4 unique

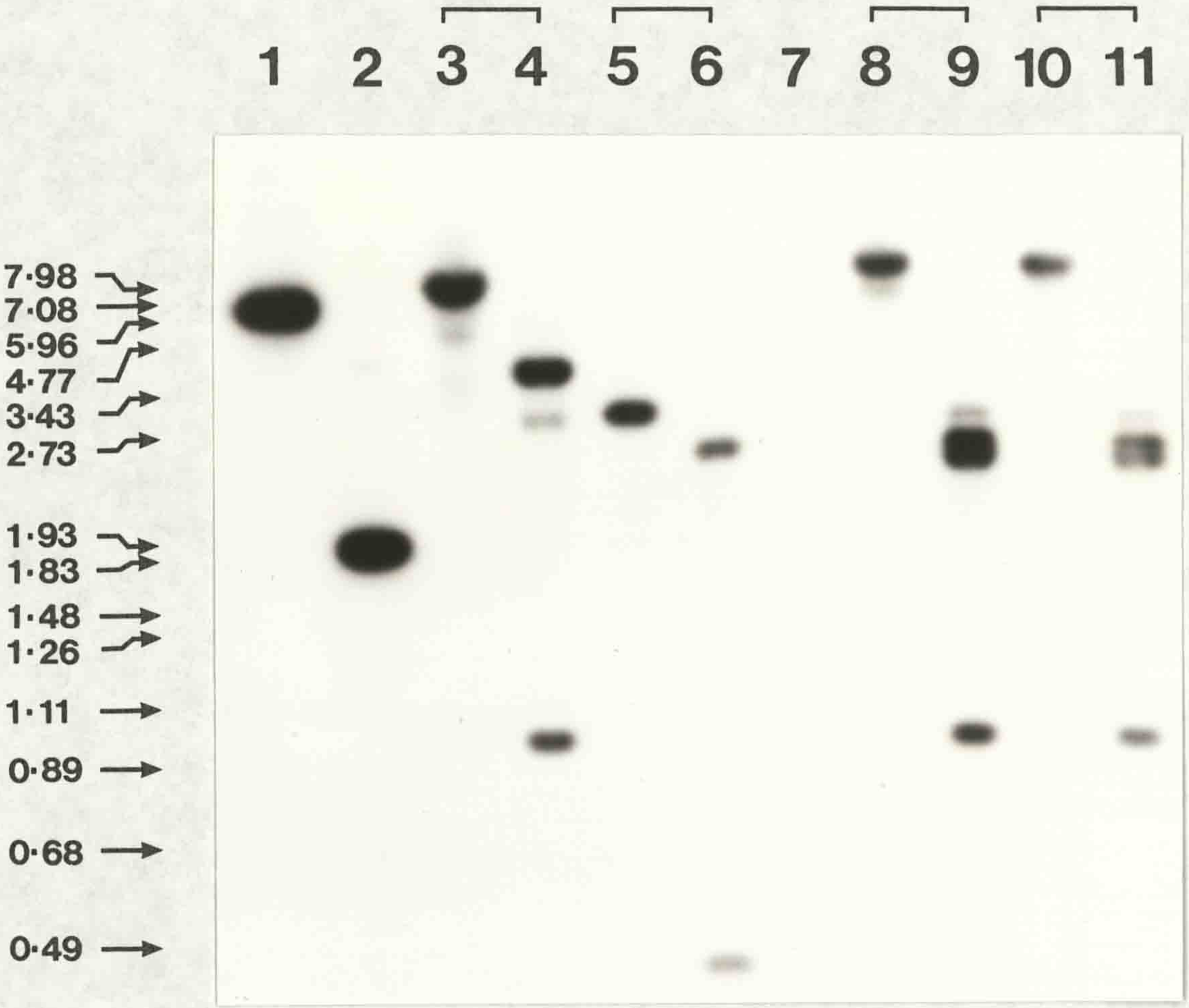


Figure 11. Southern hybridisation analysis of total DNA from 4 N.meningitidis isolates using a 1.2 kb DNA fragment containing the cloned pilE locus of N.gonorrhoeae strain P9-2 as probe. Lanes: 1. & 2. pilE-homologous DNA size markers of 6.64 and 1.95 kb respectively; 3. strain C111 DNA digested with ClaI; 4. strain C111 DNA digested with ClaI + XbaI; 5. strain C114 DNA digested with ClaI; 6. strain C114 DNA digested with ClaI + XbaI; 7. SPP1 DNA digested with EcoRI; 8. strain C115 DNA digested with ClaI; 9. strain C115 DNA digested with ClaI + XbaI; 10. strain C151 DNA digested with ClaI; 11. strain C151 DNA digested with ClaI + XbaI. Bracketed numbers refer to DNA from the same isolate. Sizes of DNA markers are in kb.

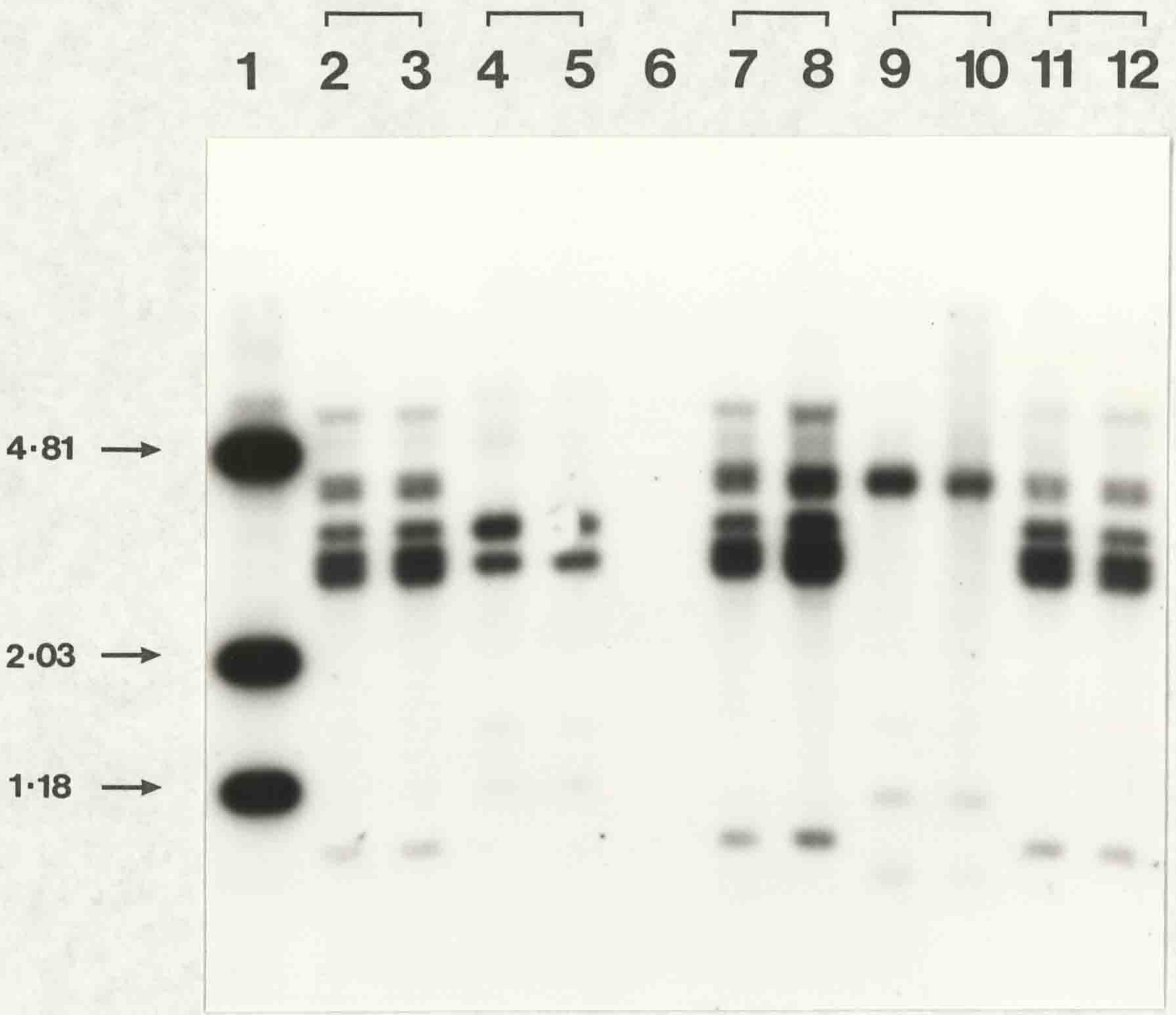


Figure 12. Southern hybridisation analysis of total DNA from 10 N.meningitidis isolates using the pilE probe as described for Fig. 11. Bracketed numbers refer to DNA from meningococcal strains that had been isolated from either the CSF or blood of the same patient (Table 1). All meningococcal DNA preparations were digested to completion with ClaI + XbaI. Lanes: 1. pilE-homologous DNA size markers; 2. strain C3II DNA; 3. strain C312 DNA; 4. strain C318 DNA; 5. strain C319 DNA; 6. SPP1 DNA digested with EcoRI; 7. strain C321 DNA; 8. strain C322 DNA; 9. strain C341 DNA; 10. strain C342 DNA; 11. strain C359 DNA; 12. strain C360 DNA. Fragment sizes are in kb.

Southern hybridisation patterns (data not shown). Similar analysis of meningococcal isolates, but using a probe containing silent pil DNA sequences derived from a portion of the P9-2 genome upstream of its pilE locus (probe 1; Chapter 3) produced hybridisation patterns indistinguishable from those obtained using the pilE probe (data not shown). Cleavage of N.meningitidis total DNA with ClaI and XbaI produced one Southern hybridisation pattern, exemplified by that of strain C311, that is apparently shared by at least 5 other N.meningitidis isolates (Figs 11 & 12).

Total DNA was extracted from N.meningitidis strains that had been isolated from both the blood and CSF of each of five patients during the course of meningococcal infection. Southern analysis of total genomic DNA using the P9 pilE probe revealed that meningococcal isolates from the blood and CSF of the same patient possessed apparently identical hybridisation patterns (Fig. 12). Where N.meningitidis isolates were from the same patient, their genomic DNA fingerprinting profiles were identical (data not shown), indicating that they are closely related (Kristiansen et al., 1984).

Comparison of Southern analyses of DNA isolated from strains C311, C312 and their respective P- derivatives and that was subsequently digested with ClaI and XbaI, revealed a chromosomal rearrangement involving pilE-homologous DNA sequences, including two strongly hybridising fragments present in each strain, of

1.2 Fimbrial adhesins as pathogenicity determinants

Fimbriae may be obligatory for the establishment of infections by certain pathogenic Escherichia coli. Type 1 (common type) fimbriae (Duguid et al., 1955) have been implicated in adhesion of E.coli in vitro and in vivo (Knutton et al., 1984; Jayappa et al., 1985). Furthermore, the capacity of type 1-fimbriate E.coli to colonise the bladder uroepithelium of BALB/c mice is enhanced compared to that of isogenic derivatives that lack type 1 fimbriae (Hultgren et al., 1985). The adhesion properties of type 1 fimriae, including haemagglutination of guinea pig erythrocytes, are susceptible to complete inhibition by D-mannose (Old, 1972; Duguid & Old, 1980) and protection against E.coli-induced urinary tract infections can be achieved using hybridoma antibodies directed against D-mannose receptors (Abraham et al., 1985). A similar protective effect is achieved using antibodies against the supramolecular structure of type 1 fimbriae, but not with hybridoma antibodies specifically against their dimeric protein subunit (Abraham et al., 1985). The gene encoding the subunit of type 1 fimbriae (pilA) is distinct from the determinant(s) of guinea pig erythrocyte haemagglutination (Orndorff & Falkow, 1985; Minion et al., 1986). The presence on E.coli of type 1 fimbriae alone may therefore not be sufficient to mediate such haemagglutination. The role of type 1 fimbriae in promoting uroepithelial adhesion of E.coli has also been questioned (Svanborg-Eden et al., 1977; Korhonen et al., 1981) and the ubiquity of type 1 fimbriae amongst both clinical and

2.65 and 2.9 kb (Fig. 13). Two types of rearrangement were evident. P+ to P- phase variation of strain C311 was accompanied by deletion of both strongly hybridising pilE-homologous fragments, whereas loss of piliation in C312 involved replacement of analogous fragments by a single fragment of intermediate size. No pilus phase variation was detected by electron microscopy during 40 serial, daily subcultures in vitro of meningococcal isolates C318 and C319. This is consistent with a previous observation that the P+ phenotype can be stably maintained by some strains of N.meningitidis (Trust et al., 1983). Furthermore, DNA from subcultures of strains C318 and C319 produced hybridisation patterns that were identical to those exhibited by their progenitors (data not shown).

Cleavage of total DNA from strains C311 and C312 with a combination of ClaI and XbaI and subsequent hybridisation analysis with probe 4 (Chapter 3) indicated that each strain possessed one probe 4-homologous fragment (data not shown). Probe 4-homologous fragments were of 0.87 kb and were apparently conserved on the genomes of isogenic P- phase variants. Digestion of strain C114 total DNA with XbaI and ClaI produced a single probe 4-homologous segment of 0.95 kb (data not shown). Probe 4-homologous genomic fragments did not hybridise with the 1.4 kb XbaI-ClaI fragment of pLV260 that contains an intact gonococcal pilE locus (Chapter 3; data not shown). The importance of sequences corresponding to probe 4 is indicated by their inter-specific conservation, although their function (if any) awaits

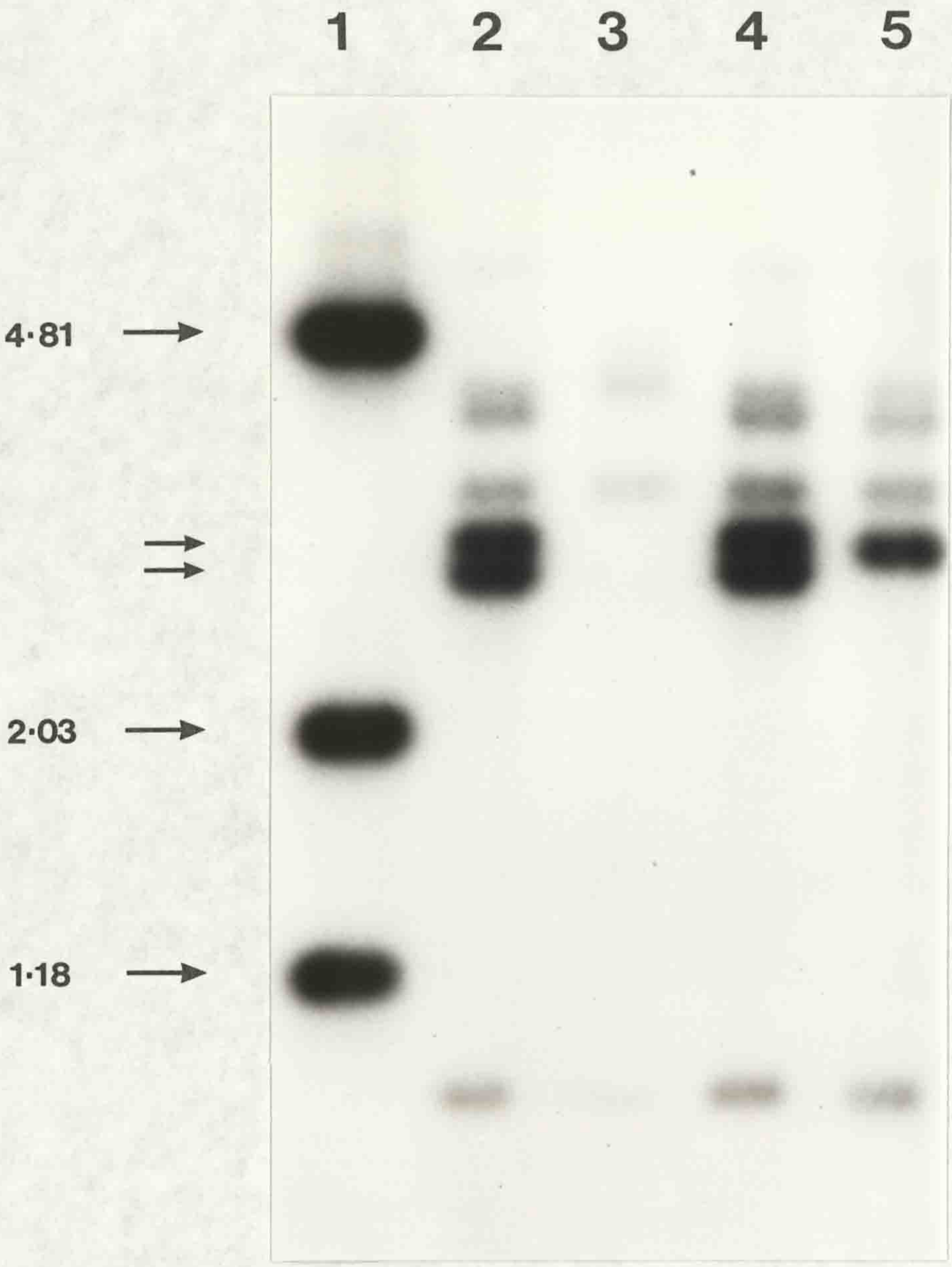


Figure 13. Southern hybridisation analysis of total DNA from 2 P+ meningococcal isolates and their P- derivatives. The probe was as described in Fig. 11. N.meningitidis DNA preparations were digested to completion with ClaI + XbaI. Lanes: 1. pilE-homologous DNA size markers; 2. strain C311 (P+) DNA; 3. strain C311 (P-) DNA; 4. strain C312 (P+) DNA; 5. strain C312 (P-) DNA. The approximate postions of two strongly hybridising DNA fragments involved in rearrangement (see text) are indicated by arrows. Fragment sizes are in kb.

elucidation.

4.2.2 Marked immunological disparity exists among meningococcal pilin proteins

Total protein from 15 N.meninigitidis isolates was subjected to Western blotting analysis using monoclonal antibody SM1 (Fig. 14). Twelve isolates cross-reacted with SM1 and all labelled polypeptides were in the size range 17.5-21.1 kd (Fig. 14). The sizes of these proteins are in good agreement with those described previously for meningococcal pilin molecules (Virji & Heckels, 1983; Diaz et al., 1984; Olafson et al., 1985).

Where derived from the same patient, the size of SM1-reactive pilin(s) from each isolate was apparently identical. This presumably reflects shared parentage of paired isolates. At least two meningococcal strains, paired isolates C341 and C342, appeared to express two size classes of SM1-reactive pilin each (Fig. 14). The size difference between the two pilin species of C341 (and of C342) is 1.2 kd, accounting for a difference in length of approximately 12 amino acids. Similar intra-strain pilin size differences have been revealed by immunoblotting of N.gonorrhoeae strain P9 (I.J.Nicolson, unpublished observation). Three N.meningitidis isolates, C114, C318 and C319, failed to react with monoclonal antibody SM1 when total protein was subjected to Western blotting (Fig. 14). Furthermore, C114, C318

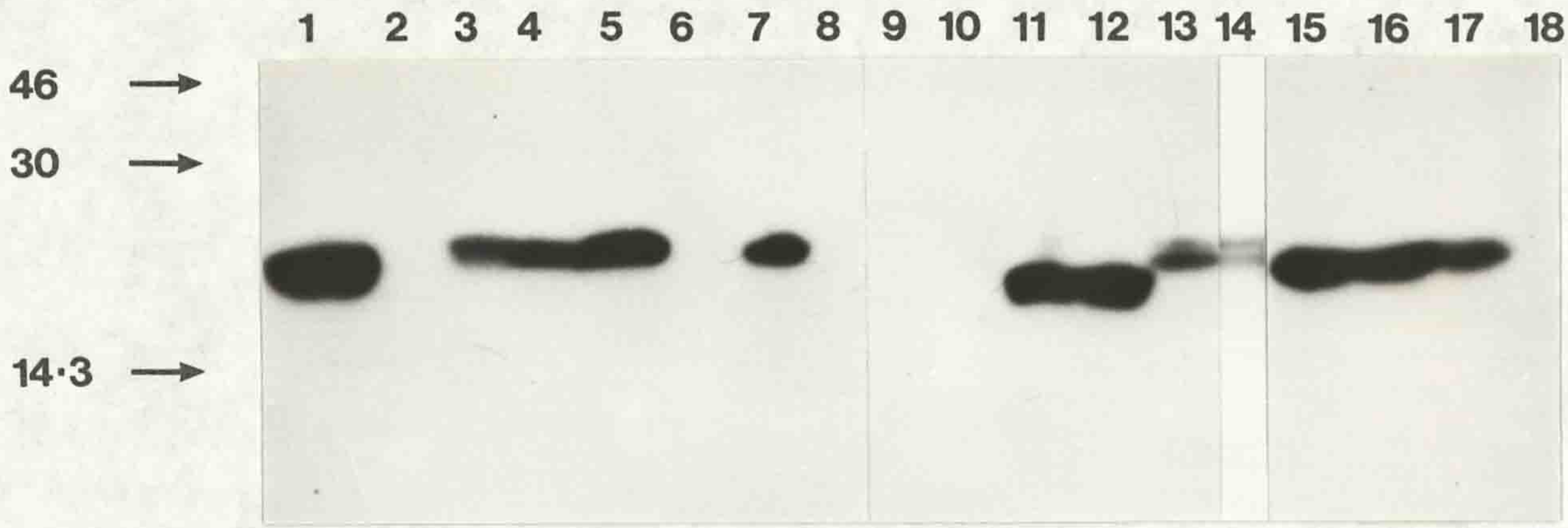


Figure 14. Western blot analysis of total protein from each of 17 meningococcal strains using monoclonal antibody SM1. Lanes: 1. strain C111; 2. strain C114; 3. strain C115; 4. strain C151; 5. strain C311 (P+); 6. strain C311 (P-); 7. strain C312 (P+); 8. strain C312 (P-); 9. strain C318; 10. strain C319; 11. strain C321; 12. strain C322; 13. strain C341; 14. strain C342; 15. strain C359; 16. strain C360; 17. strain C361; 18. total protein from E.coli DH1. Sizes are in kd.

and C319 did not react with SM1 on analysis by dot blotting (data not shown). To investigate further the nature of pilins elaborated by the SM1 non-reactive strains, rabbit antiserum raised against purified pili from N.meningitidis strain C114 was used in Western blot analysis of total protein from meningococcal isolates (Fig. 15). A subset of proteins from strains C114, C318 and C319 reacted strongly with anti-C114 pilus polyclonal antibody (Fig. 15). Western analysis of the C114 pilus preparation used to generate polyclonal rabbit antiserum revealed several bands (data not shown) indicating the presence in the pilus preparation, of meningococcal components other than pili. Such impurities would probably have given rise to rabbit anti-C114 antibodies, not directed against C114 pili, that would recognise non-pilin epitopes on Western blotting. However, apart from a polypeptide of approximately 17.1 kd, Western blotting analysis revealed that the smallest protein to react with anti-C114 pilus polyclonal antibody in total C114 protein (Fig. 15) that was also present in the C114 pilus preparation (data not shown) had an apparent molecular weight of 26 200 (Fig. 15). Since bacterial pilins are commonly in the size range 12-22 kd (for example Every, 1979; Sastry et al., 1983; Diaz et al., 1984), it is likely that N.meningitidis strain C114 produces pilin(s) of approximately 17.1 kd and that the pilins for strains C318 and C319 are each of approximately 15.7 kd (Fig. 15). Total protein from SM1-reactive meningococcal isolates reacted no more strongly with anti-C114 pilus polyclonal antibody than did the negative controls (total protein from P- meningococci and

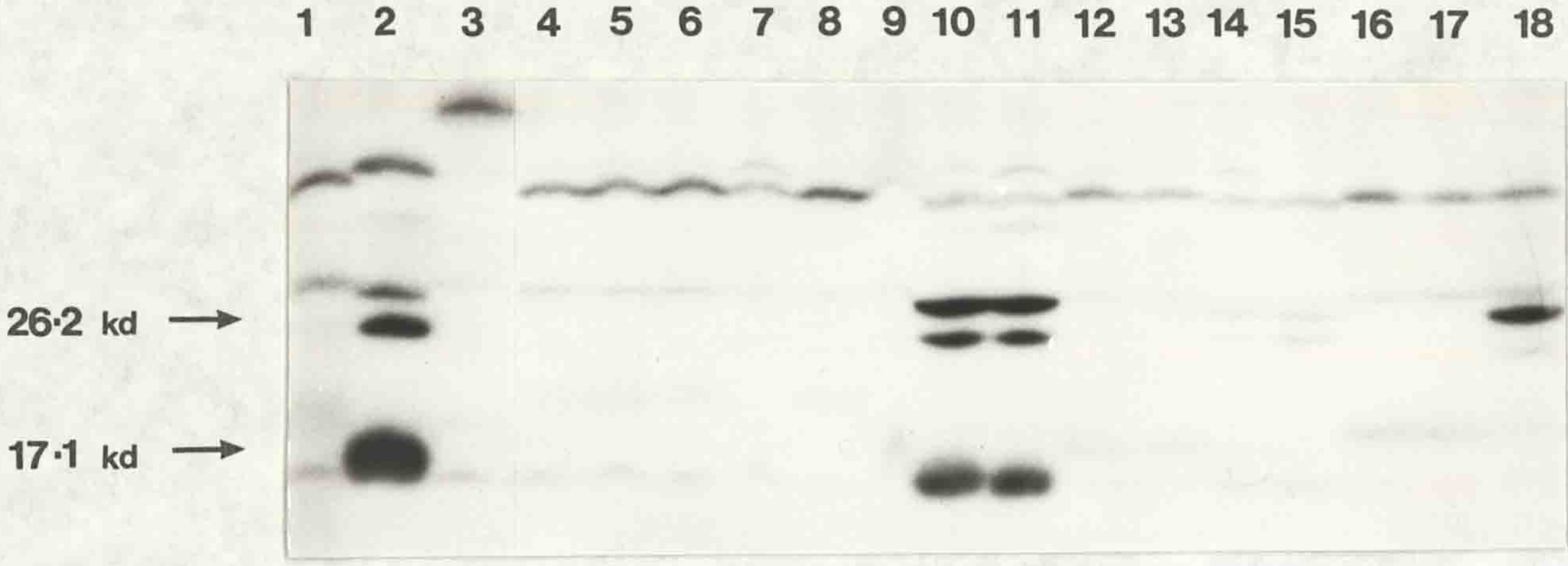


Figure 15. Western blot analysis of total protein from each of 17 meningococcal strains using polyclonal antibody raised against whole pili from N.meningitidis strain C114. Lanes: 1. strain C111; 2. strain C114; 3. total protein from E.coli DH1; 4. strain C115; 5. strain C151; 6. strain C311 (P+); 7. strain C311 (P-); 8. strain C312 (P+); 9. strain C312 (P-); 10. strain C318; 11. strain C319; 12. strain C321; 13. strain C322; 14. strain C341; 15. strain C342; 16. strain C359; 17. strain C360; 18. strain C361.

E.coli)(Fig. 15). This suggests that N.meningitidis can express at least two distinct pilus types that are markedly dissimilar immunologically. Furthermore, it is unlikely that both antigenic pilus types were simultaneously expressed in source populations of meningococci, since each N.meningitidis isolate, with the exception of strain C361, reacted with SM1 monoclonal, or anti-C114 pilus polyclonal antibodies, but not both. Strain C361 contained, in addition to an SM1-reactive protein, a polypeptide of approximately 26 kd that reacted with the anti-C114 pilus polyclonal antibody preparation (Fig. 15). Variation in components of approximately 26 kd between strains of N.meningitidis has been observed previously (Poolman et al., 1980a).

4.3 Discussion

It has been shown previously that some N.meningitidis isolates express pili that are immunologically related to those of N.gonorrhoeae (Virji & Heckels, 1983; Olafson et al., 1985). The pili of many meningococcal isolates possess an epitope, present in all piliated gonococci so far tested, that is recognized by the monoclonal antibody SM1 (Virji & Heckels, 1983). This study shows that meningococcal pili that do not react with SM1 have few, if any, epitopes in common with SM1 reactive meningococcal pili. All strains expressing SM1 non-reactive pili that we have tested belong to serogroup C (Table 1). It is not yet clear whether there is a correlation between capsular serogroup and pilus type, although SM1 non-reactive pili have previously been isolated from N.meningitidis strains of serogroups A,B and C (Diaz et al., 1984).

The genomes of all SM1 non-reactive isolates of N.meningitidis analysed possess at least one region that is homologous to the pilE gene of N.gonorrhoeae strain P9. Such pilE-homologous sequences do not however encode an SM1 reactive epitope on pili expressed by N.meningitidis strains C114, C318, or C319. Furthermore, it is unlikely that the homologies reflect the presence of expressed sequences analogous to any immunogenic region of the pilE locus in pLV260, since anti-C114 pilus polyclonal antiserum fails to react significantly with total protein from the gonococcal antigenic pilus variant P9-2 on

Western blotting (unpublished observations). The N-terminal region of gonococcal pilin is immunorecessive (Schoolnik, et al., 1984) and the first 30 or so amino acids are strongly conserved in the N-terminal regions of pilins from gonococci and several other Gram negative bacteria, including meningococci (Hermondson et al., 1978; Olafson et al., 1985), Pseudomonas aeruginosa (Sastry et al., 1983), Bacteroides nodosus (McKern et al., 1983) and Moraxella nonliquefaciens (Froholm et al., 1977). It is possible that SM1 non-reactive and SM1 reactive pilins share extensive homology between their N-terminal (conserved) regions but that the former lacks the SM1 reactive epitope.

SM1-non-reactive pili may share antigenic determinants with gonococcal pili although such determinants are not immunoreactive following immunoblotting (Diaz et al., 1984). However, N.meningitidis strains C114, C318 and C319 were not labelled on dot blot analysis of total protein from each, using polyclonal antisera raised against N.gonorrhoeae strain P9-20 (unpublished observations). Nevertheless, this possibilty requires further investigation.

The N-terminal, conserved region of gonococcal pilin is encoded by a DNA tract of approximately 0.15 kb (Meyer et al., 1984). Digestion of DNA from strains C318 and C319 with restriction endonucleases ClaI and XbaI produced three fragments (each >0.45 kb) that are homologous to the P9-2 pilE gene (ca. 0.5 kb; Chapter 3). Thus, if each pilE-homologous DNA fragment harbours

coding information for the N-terminal portion of its respective pilin, at least two such non-contiguous DNA sections would have to exist.

It is possible that meningococcal sequences that are homologous to the pilE locus of N.gonorrhoeae are not expressed in SM1 non-reactive strains. Such putatively silent loci may potentiate pilus antigenic variation, possibly by a mechanism of intragenic recombination similar to that exhibited by N.gonorrhoeae (Hagblom et al., 1985). If this were the case, the SM1 non-reactive pilus would have to be encoded by a separate, non-homologous pilE gene or genes. Detailed analysis of pil loci in SM1 non-reactive meningococci will show whether or not this is so. It also remains to be seen whether switching of the immunological type of pilus between SM1-reactive and non-reactive can occur.

Pilus phase variation in N.gonorrhoeae strain $MS11_{ms}$ can involve deletion of one, or both pilE loci (Segal et al., 1985). Where both pilE loci are deleted, pilus phase transition is irreversible, although the loss of a single pilE locus can be restored by duplication of the remaining intact pilin gene resulting in a P- to P+ switch (Segal et al., 1985). The SM1-reactive N.meningitidis strains C311 and C312 exhibit distinct chromosomal rearrangements, also involving deletion of pilE-homologous sequences, which are in each case associated with pilus phase variation. It is possible that strains C311 and C312 also each possess two (or more) pilus expression loci, analogous

environmental isolates (Deneke et al., 1979; Duguid et al., 1979) implies that they are not, by themselves, virulence determinants. Alternatively, one or more additional factors may act in concert with the pilA gene product to confer the haemagglutination phenotype and other adherence properties upon type 1 fimbriae. Indeed, the function of at least one gene, pilD, that is closely linked to pilA is unknown, although it encodes a polypeptide of 14 kd that is not sufficient of itself to restore adhesive function to haemagglutination-deficient type 1 fimbriae (Orndorff & Falkow, 1984a). Other genes involved in expression of type 1 fimbriae include hyp, deletion of which results in a 40-fold increase in expression of the pilA gene product (Orndorff & Falkow, 1984a, 1984b; Orndorff et al., 1985), and at least one factor that modulates phase variation (Brinton, 1959).

Phase variation of type 1 fimbriae, whereby individual cells alternate between fimbriate and non-fimbriate states (Brinton, 1959) may be the result of metastable transcriptional regulation of pilA by an element closely linked to it (Orndorff et al., 1985). This element possibly acts in cis, although one or more additional, trans-active factors may also be required (Freitag et al., 1985).

E.coli is capable of expressing fimriae whose phenotypic and genetic properties are distinct from, but related to those of type 1 fimbriae. The gene cluster responsible for expression of type 1 fimbriae resembles the genetic region encoding production

to pilE1 and pilE2 of N.gonorrhoeae strain $MS11_{ms}$ (Meyer et al., 1982; Segal et al., 1985), that are involved in pilus phase variation. Furthermore, the extent of deletion of pilE-homologous sequences in P- derivatives of C311 and C312 may determine whether or not the P+ phenotype can be restored in each case. P- pilus variants of N.meningitidis strains C311 and C312 are deficient in the production of immunoreactive pilin, although P+ to P- phase transition events in meningococci do not necessarily result in loss of pilin expression (Stephens et al., 1984). Similar phenomena occur in gonococci (Segal et al., 1985; Swanson et al., 1986) and pilus phase and antigenic variation in N.gonorrhoeae and N.meningitidis may therefore occur by a related mechanism.

Pilus antigenic variation in N.gonorrhoeae involves reassortment of silent pilin gene sequences (Hagblom et al., 1985; Haas & Meyer, 1986) and can occur in vivo (Lambden et al., 1981). Pilus antigenic variation of N.meningitidis can occur in vitro (J.E. Heckels, personal communication) and might also occur during the course of infection. Pilus phase or antigenic variation between N.meningitidis isolates from the blood and CSF of the same patient has not been demonstrated. However, the possibilty that such variation occurs when the organism makes the transition from the naso-pharyngeal tract to the blood or CSF will be the subject of future studies.

CHAPTER 5

A Silent Pilin Locus of *Neisseria meningitidis* Contains Truncated, Tandemly-Repeated Pilin Gene Segments

5.1 Introduction

A subset of N.meningitidis isolates are capable of elaborating pili whose immunological and physical characteristics are different from those of gonococcal pili (Virji & Heckels, 1983; Diaz et al., 1984; Chapter 4). However, three such isolates, C114, C318 and C319 harbour genomic sequences that are homologous with the pilE locus of N.gonorrhoeae strain P9 (Chapters 3 & 4). To investigate this apparent contradiction, a detailed analysis of the pilE-homologous chromosomal region of N.meningitidis strain C114 was undertaken.

5.2 Results

5.2.1 Cloning and preliminary analysis of a pil locus of N.meningitidis strain C114

Total genomic DNA from N.meningitidis strain C114 was partially digested with ClaI. The resulting fragments were were ligated to ClaI-linearised pBR322 DNA that had subsequently been treated with alkaline phosphatase. Ligated DNA molecules were used to transform E.coli strain GC1 to Ap^r. E.coli strain GC1 was chosen as host in this experiment because it has previously been shown to be proficient in expression of gonococcal pilin genes on primary passage (Meyer et al., 1982; Nicolson et al., 1986). E.coli strains DH1, RR1 and C600 do not appear to express gonococcal genes that have not previously been passaged in E.coli strain GC1 (M. So, personal communication). Similar constraints may exist on the expression of meningococcal genes in E.coli given the genealogical relatedness of N.meningitidis and N.gonorrhoeae (Chun et al., 1985).

Approximately 14 000 Ap^r, Tc^s transformant colonies were screened for possession of DNA sequences homologous to the pilE locus of N.gonorrhoeae strain P9-2, using the 1.2 kb XbaI-PvuII fragment of pLV260 as probe (Chapter 3). Confirmation of a positive result was by secondary screening. Ten positive E.coli clones were obtained in this way and each possessed a pilE-homologous

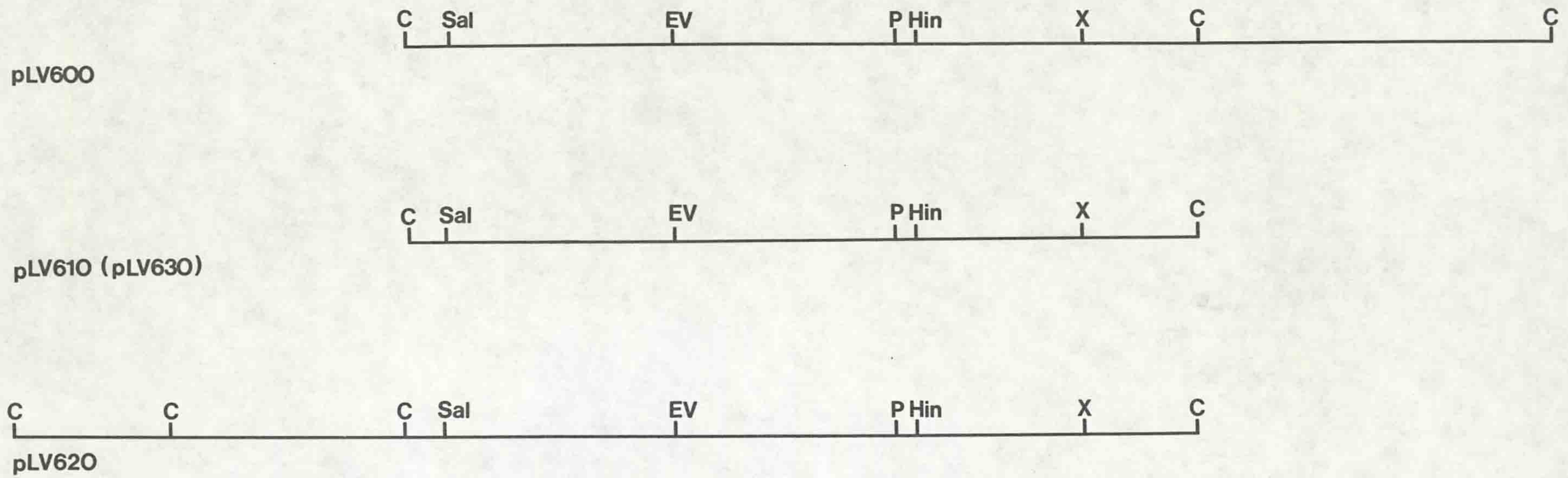


Figure 16. Restriction enzyme maps of sequences derived from N.meningitidis strain C114 that are represented in recombinant pilE-homologous plasmids (see text for details). Abbreviations for restriction enzymes are as for Fig. 1, with the additions of: Hin, HindIII; RV, EcoRV; Sal, SalGI.

recombinant plasmid that contained a DNA insert of one of 3 sizes (data not shown). Four such recombinant plasmids (designated pLV600, pLV610, pLV620 and pLV630), included representatives of each size class and were chosen for analysis by restriction enzyme mapping (Fig. 16). Plasmids pLV610 and pLV630 possessed apparently identical restriction maps. All pilE-homologous recombinant plasmids analysed by restriction mapping possessed a consensus ClaI fragment of approximately 3.1 kb (Fig. 16). The orientation of this fragment with respect to pBR322 sequences was different in pLV620 compared to plasmids pLV600 and pLV610 (data not shown). The relative positions of the 3 ClaI fragments on the meningococcal insert of pLV620 were determined by BAL 31 nuclease deletion analysis of XbaI-linearised pLV620 DNA (data not shown).

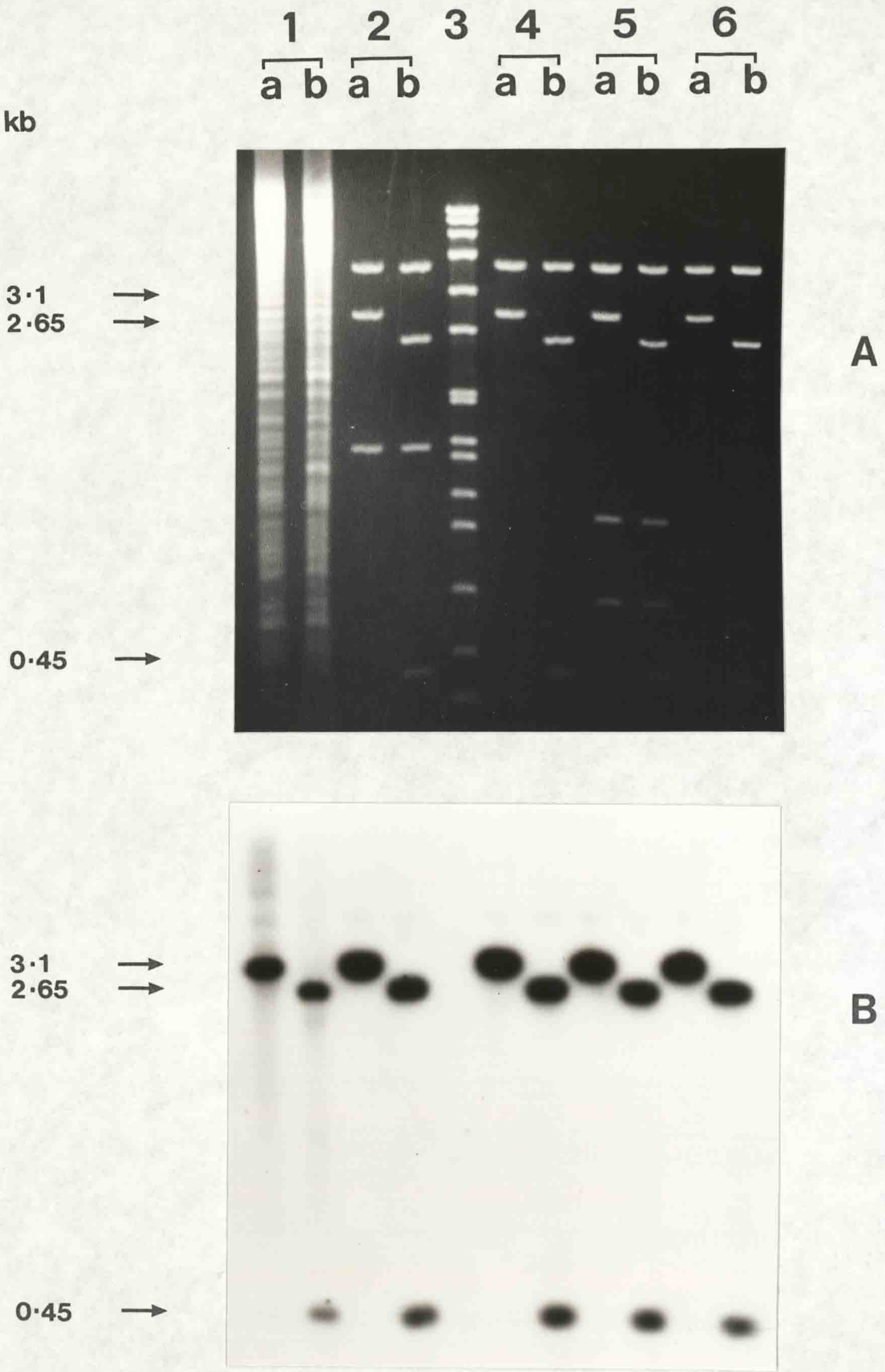
Southern hybridisation analysis of total DNA that had been respectively digested with several combinations of restriction endonucleases indicated that the chromosome of strain C114 possessed a single pilE-homologous locus (data not shown). The same analyses showed that ClaI fragments of meningococcal origin that were represented in recombinant plasmid pLV600 were not contiguous on the chromosome of strain C114. The arrangement of these ClaI segments in pLV600 presumably reflects their juxtaposition *in vitro*. However, Southern hybridisation data were not inconsistent with the observed arrangement of restriction fragments on the meningococcal inserts of plasmids pLV610 or pLV620 (data not shown).

Southern hybridisation analysis indicated that the unique pilE-homologous ClaI fragment of N.meningitidis strain C114 was identical, or very similar to the consensus 3.1 kb ClaI fragment of plasmids pLV600, pLV610, pLV620 and pLV630 (Fig. 17). This implied that pilE-homologous plasmids had not undergone gross, aberrant recombination event(s), either in vitro or in E.coli, during the course of isolation and analysis.

Western and dot blot analysis using polyclonal antisera raised against purified pili from N.meningitidis strain C114 indicated that plasmid pLV610 did not encode immunoreactive pilins that are expressed in E.coli strains GC1 (data not shown) or DH1 (Fig. 18). Similar results were obtained with plasmids pLV600 and pLV620 (data not shown). However, in vitro analysis of proteins encoded by plasmids pLV600, pLV610 and pLV620 revealed that the transcriptional-translational machinery of E.coli could function using meningococcal genes as substrates (Fig. 19). These findings suggested that pilE-homologous sequences were not expressed in N.meningitidis strain C114.

All recombinant plasmids encoded proteins of 13.6, 33.3 and 34.7 kd (Fig. 19). These proteins were produced in relatively large amounts and were apparently not encoded by pBR322 (Fig. 19). It is therefore likely that the structural gene of each protein is located on the consensus 3.1 kb ClaI fragment of pilE-homologous plasmids. No protein corresponding in apparent M_r to that of N.meningitidis strain C114 pilin (15 700) was present (Fig. 19).

Figure 17. Agarose gel electrophoresis (A) and Southern hybridisation analysis (B) of recombinant pilE-homologous plasmid, and N.meningitidis strain C114 total DNAs. Insets A. and B. represent the same gel. The 1.2 kb XbaI-PvuII fragment of plasmid pLV260 was used as probe in Southern hybridisation analysis. Meningococcal and recombinant plasmid DNAs were digested with ClaI (a) or ClaI + XbaI (b). Lanes are: 1. strain C114 DNA; 2. pLV600 DNA; 3. SPP1 DNA digested with EcoRI; 4. pLV610 DNA; 5. pLV620 DNA; 6. pLV630 DNA.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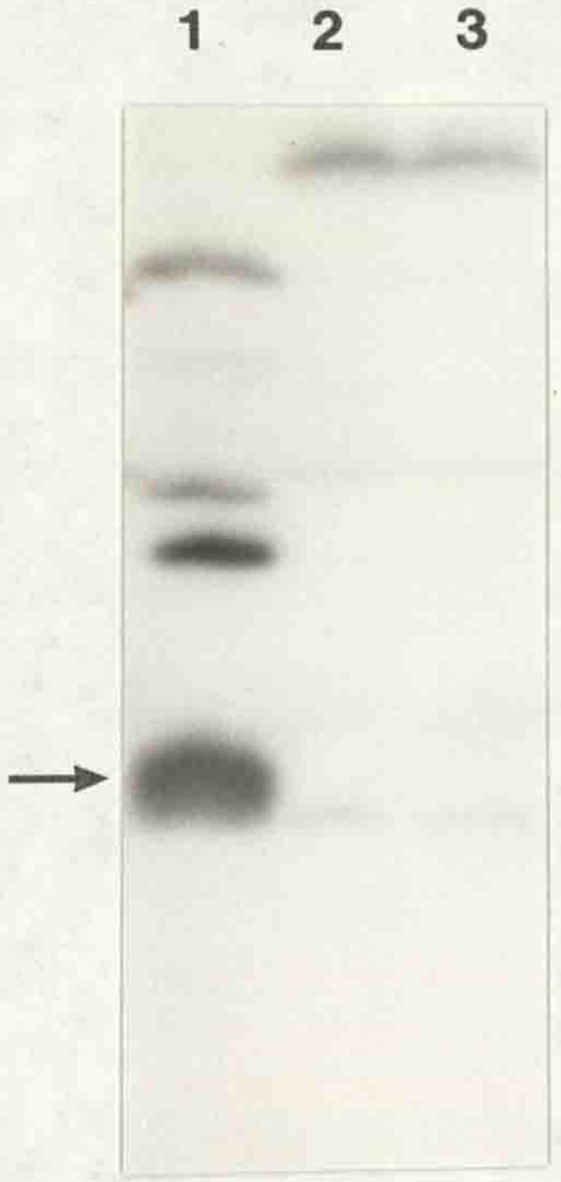


Figure 18. Western blot analysis of total protein of: 1. N.meningitidis strain C114; 2. E.coli strain DH1(pLV610); 3. E.coli strain DH1(pBR322). The position of pilin elaborated by N.meningitidis strain C114 is marked with an arrow.

of K88 antigen (Kehoe et al., 1981; Mooi et al., 1982; Orndorff & Falkow, 1984a), a fimbrial adhesin that is present on many enterotoxigenic E.coli isolated from neonatal piglets with diarrhoea (Dougan & Morrissey, 1985). The K88 determinant, which is normally located on plasmids of at least 70 kb (Shipley et al., 1978), promotes adhesion and subsequent colonisation of the small bowel of piglets by E.coli (Dougan et al., 1986). Such colonisation involves interaction between K88 fimbriae and piglet receptor molecules that can vary intraspecifically (Sellwood, et al., 1975; Gaastra & de Graaf, 1982). Indeed, the existence of more than one porcine K88 antigen receptor phenotype may have placed upon E.coli expressing K88 antigen a selective pressure that has resulted in K88 antigenic variation. At least three major antigenic variants of K88 exist, designated K88ab, K88ac (Orskov, et al., 1964) and K88ad (Guinee & Jansen, 1979), each comprising serologically conserved and variable components, termed a and b, c or d respectively. The antigenic identity of K88 fimbrial variants is endowed by their homopolymerically arranged protein subunits. Irrespective of immunological properties, K88 antigen subunits comprise 264 amino acids and have conserved N- and C-terminal amino acid sequences that may be important for stabilisation of fimbrial quaternary structure (Klemm, 1981; Gaastra, et al., 1981, 1983; Dykes et al., 1985). Alterations at the DNA level of sequences encoding the variable portion of the K88 fimbrial subunit have resulted in K88ab, K88ac, K88ad and additional related antigenic variants (Gaastra et al., 1983; Dykes et al., 1985). The mechanism by which these

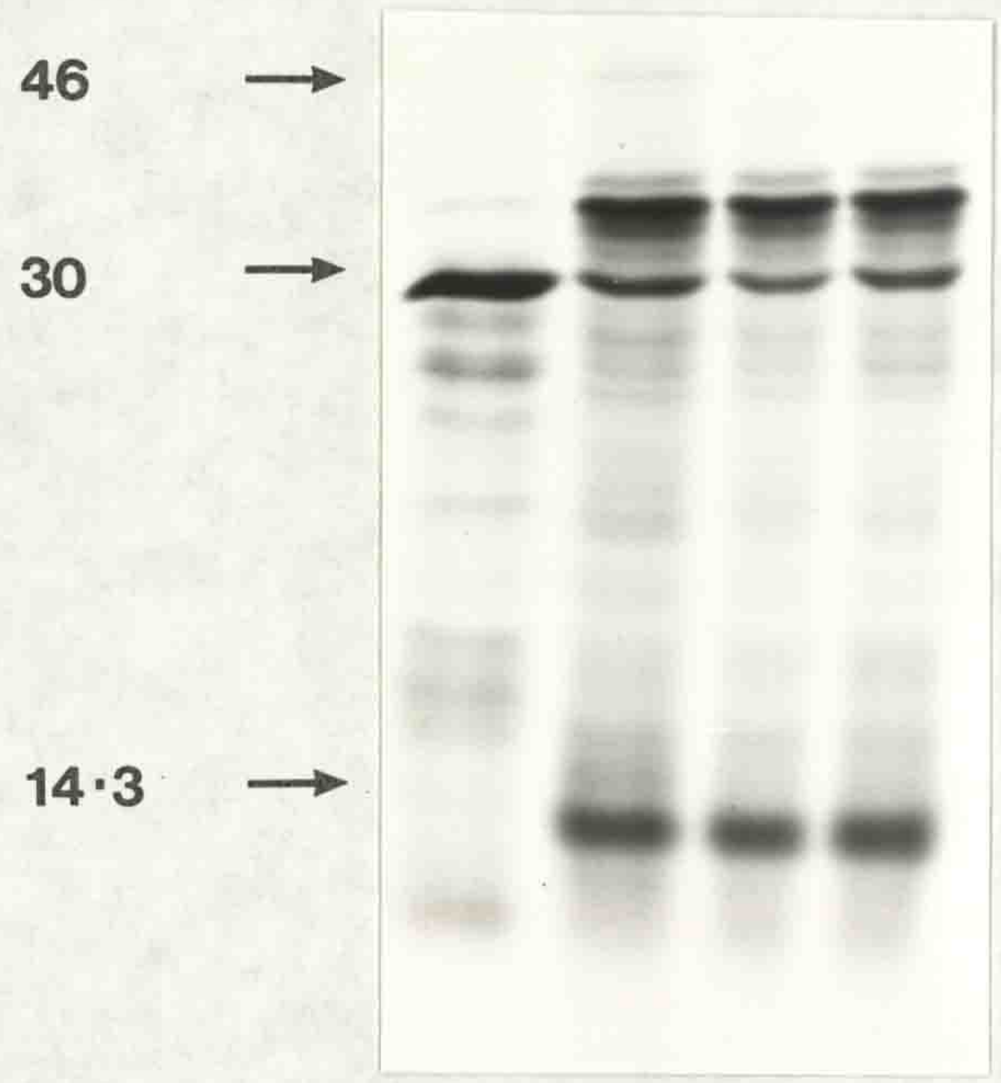
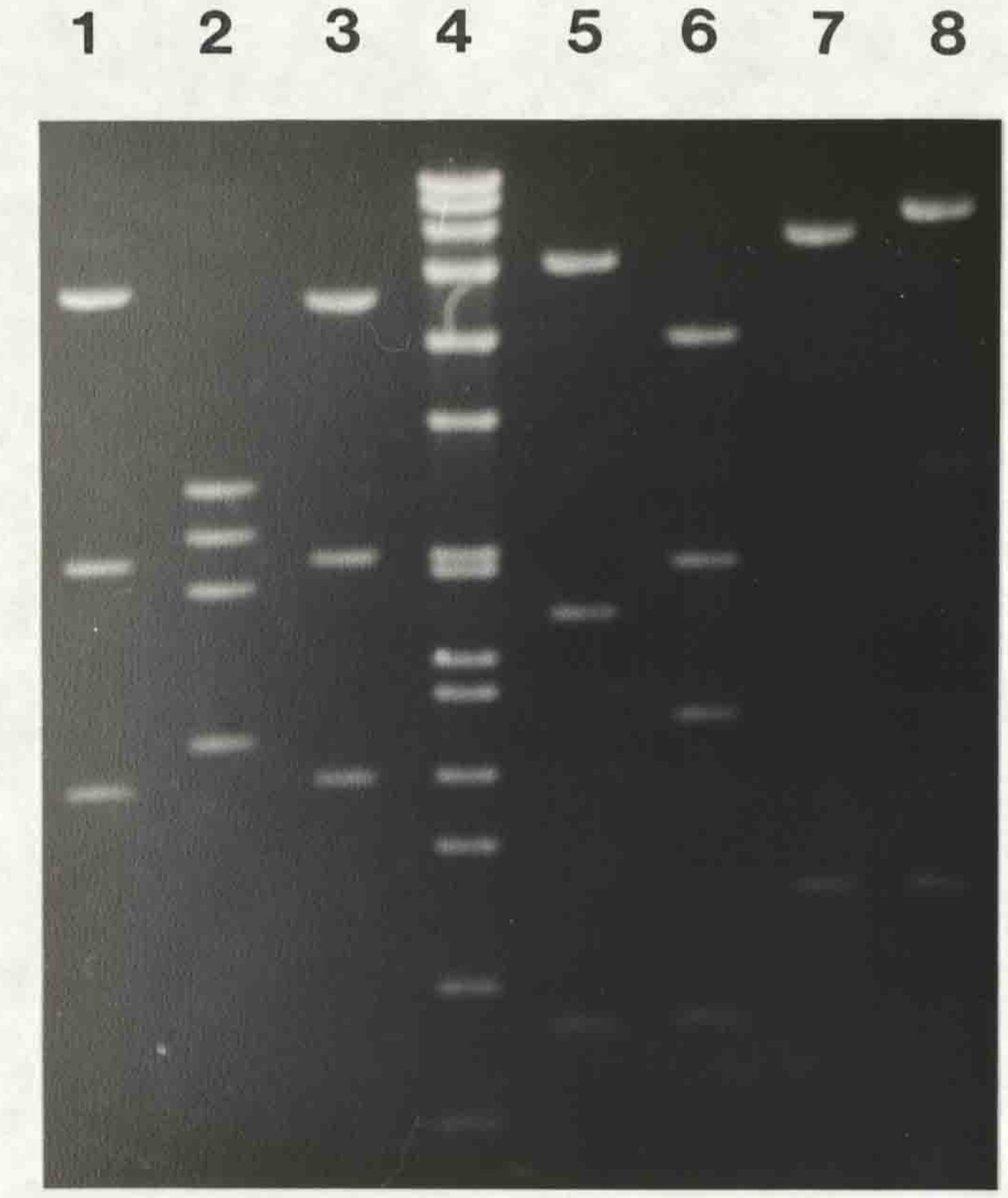


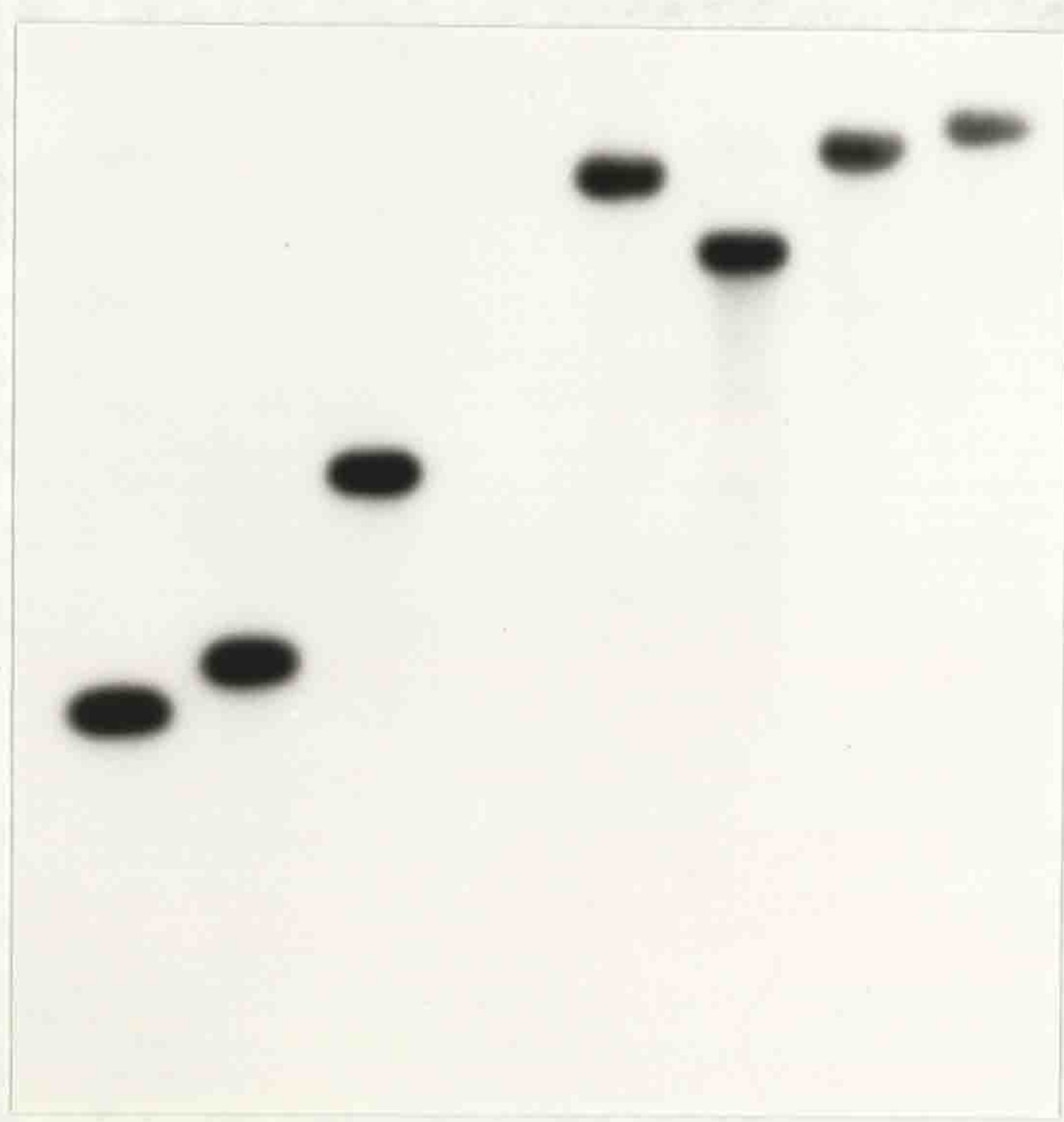
Figure 19. Identification of [^{35}S]methionine-labelled cloned gene products synthesised *in vitro*. DNA substrates were: 1. plasmid pBR322; 2. plasmid pLV600; 3. plasmid pLV610; 4. plasmid pLV620. Sizes of markers are in kd.

However, cloned proteins of 33.3 or 34.7 may have represented pilin dimers that were not immunoreactive. Proteins encoded by the meningococcal ClaI insert of plasmid pLV610 await detailed characterisation.

Plasmid pLV610 DNA that had been independently digested with different combinations of restriction endonucleases was subjected to Southern hybridisation analysis using the pilE locus of pLV260 as probe (Fig. 20). In this way, pilS sequences were localised to a 1.15 kb HindIII-ClaI fragment that is resident upon the meningococcal insert of pLV610 (Fig. 20). Southern hybridisation patterns generated by probing restriction endonuclease fragments of total DNA from N.gonorrhoeae or N.meningitidis with either the 1.2 kb XbaI-PvuII fragment of pLV260 (Chapter 3; Fig. 5) or the 1.15 HindIII-ClaI fragment of pLV610 were indistinguishable (data not shown). Since the chromosome of N.gonorrhoeae contains pilS loci that lack N-terminal pilin coding sequences (Haas & Meyer, 1986), this finding suggested that sequences corresponding to the variable region of the gonococcal pilE gene might be present on the 1.15 kb HindIII-ClaI fragment of pLV610. This possibility was investigated by DNA sequence analysis.

Figure 20. Agarose gel electrophoresis (A) and Southern hybridisation analysis (B) of restriction fragments of plasmid pLV610. Panels A and B represent the same gel. The 1.2 kb XbaI-PvuII fragment of pLV260 was used as probe in Southern hybridisation analysis. Tracks are plasmid pLV610 DNA digested with: 1. HindIII + ClaI; 2. PvuII + ClaI; 3. EcoRV + ClaI; 4. SPP1 DNA digested with EcoRI; 5. HindIII + SalGI; 6. PvuII + EcoRI; 7. SalGI + EcoRV; 8. SalGI.


1 2 3 4 5 6 7 8
1·15 kb
A


1·15 kb
B

5.2.2 Pilin gene sequences that are homologous with the pilE locus of N.gonorrhoeae strain P9 exist as tandem repeats in N.meningitidis strain C114

The DNA sequence of the 1.3 kb PvuII-ClaI fragment of plasmid pLV610, harbouring a cloned pilS locus of N.meningitidis strain C114, was determined. To this end, a series of overlapping restriction fragments were cloned into M13 'phages mp18 and mp19 according to the strategy indicated in Fig. 21. The nucleotide sequence of the 1.3 kb PvuII-ClaI fragment of plasmid pLV610 is presented in Fig. 22.

Comparison of the meningococcal pilS locus with the sequence of the pilE gene of N.gonorrhoeae strain P9-2 enabled the identification of two sections (copies 1 and 2) that were strongly reminiscent of the gonococcal pilus structural gene (Figs 22 & 23). Each copy included a coherent open reading frame that comprised a variant pilin gene copy that was truncated at its 5' terminus and lacked coding information corresponding to a putative SM1-reactive epitope (Virji et al., 1983; Schoolnik et al., 1984). Sequencing ladders representing comparable portions of each truncated pilin gene copy are shown in Fig. 24.

Copies 1 (bp 436-639) and 2 (bp 869-1138) of the pilS locus of N.meningitidis strain C114 each encoded a portion of a variant pilin gene (Figs 22 & 23). Both copies exhibited nucleotide exchanges as well as insertions and deletions of whole triplet

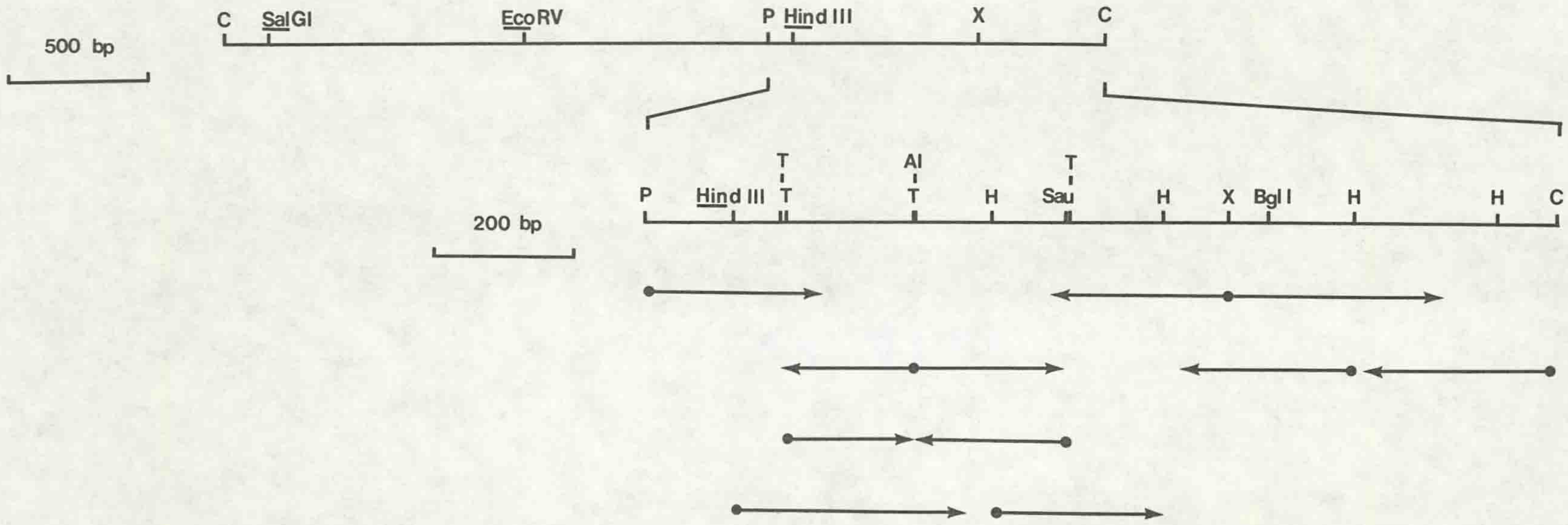


Figure 21. Restriction endonuclease analysis of plasmid pLV610 showing a detailed restriction map of the sequenced region. Arrows designate the sequencing strategy employed. Abbreviations are as for Fig. 5.

Figure 22. DNA sequence analysis of the 1.3 kb PvuII-ClaI fragment of plasmid pLV610. Two pilus gene segments (copies 1 and 2) have been identified and their putative start positions (marked 1 and 2 respectively) are shown. The 3' terminal nucleotide of copy 1 is designated by an asterisk. The stop codon terminating copy 2 is underlined. Circles mark the central nucleotide of cys codons present on copies 1 and 2. Computer-assisted analysis has located RS1- (▓) and RS3- (▒) like elements (Haas & Meyer, 1986). Selected restriction sites are boxed. Numbers refer to positions of nucleotides in the left-hand column. A near-perfect palindrome (bp 1192-1215) is located with a bar. The sequence shown here was derived using the strategy indicated in Fig. 21.

```
   1  CAGCTGACGTTTGAGCGGCATTTGACTTCGCCGGACGGTATGTTACCCTT
  51  GCCGTTTTAACCAACAAGCAACGAAAGGACAAATATGGGCAGCCTGATTA
 101  TTGAAGATTTGCAGGAAAGCTTCGGAAAAGAAGCAGTTAAAGGCAAAGAG
 151  ATTACCGTGCATTACACAGGTTGGCTGGAAGACGGCACCAAATTCGACTC
 201  CAGCCTCGACCGCCGCCAGCCGCTGACCATCACGCTCGGCGTCGGACAAG
 251  TCATCAAAGGCTGGGACGAAGGCTTCGGCGGAATGAAGGAGGGCGGCAAA
 301  CGCAAGCTGACCATCCCTTCGGAAATGGGCTACGGCGCACACGGCGCGGC
 351  GGCGTGATTCCCCCGCACGCCACTTTGATATTTGAAGTCGAGCTTGCTGAA
                          1⇨
 401  AGTGTACGAATAAAGCTGCCTGGGCAATACCGTCTGACATAATGGCTTCA
 451  AGCAACGTAAACAAAGAAATCAAAGACAAAAAACTCTCCCTGTGGGCCAA
                          •
 501  GCGTCAAGACGGTTCGGTAAAATGGTTCTGCGGACAGCCGGTTACGCGCA
 551  ACGACACCGCCAAAGACGACACCGTCGCCGCCGACACCGACACCGCCAAG
                          •        *
 601  AAGATCGACACCAAGCACCTGCCGTCAACCTGCCGCGATGATTCATCTAC
 651  CGGTTGCATAGAAACACCACGGCGCCGACTTCAAACACTTCCAAAAAATCA
 701  GCCGTTACCGAGTATTGCCCGAATCACGGCACATGGCCGAAAAACTTCGT
 751  CATTCCCGCGCAGGCGGGAATCTAGGTCTGTCGGCACGGAAACTTATCGG
 801  GTAAAACGGTTTCTTGAGATTTTGCGTTCTAGATTCCCACTTTCGTGGGA
                     2⇨
 851  ATGACGGGATTTAATGATGCCGCCGGCGTGGCATCCGCCTCCGACATCAA
 901  AGGCAAATATGTTGAGAAAGTTGAAGTCAAAAACGGCGTCGTTACCGCCG
 951  AAATGAAATCAAGCGGCGTAAACAAAGAAATCCAAGGCAAAAAACTCTCC
                                   •
1001  CTGTGGGCCAAGCGTCAAGACGGTTCGGTAAAATGGTTCTGCGGACAGCC
1051  GGTTGCGCGCAACGACAAAGCCGACACCGACAAAATCGACACCAAGCACC
                •
1101  TGCCGTCAACCTGCCGCGACGCAGCATCTGCCGATTAAGGCAAATTATAC
                                                  ----
1151  CATAAATTTTAAATAAATCAAGCGGTAAATGATTTTCCACGGCCACTCGG
      ----------
1201  ATTAATCCGGGTGGCTTCCTTTTTAAAGGTTTGCAAGGCAAGCGGGGTCG
1251  TCCGTTTTGGTGGAAATAATATATCGAT
```

Figure 23. The DNA and predicted amino acid sequences of copy 1 and copy 2 of the pilS locus of recombinant plasmid pLV610, compared with nucleotide, and deduced amino acid sequences of part of the gonococcal pilE gene resident upon plasmid pLV260 (bp 924-1378; Fig. 6). Dashes indicate no sequence differences observed in these positions. Codon and amino acid changes are indicated where found. Parentheses indicate codon deletions. If the DNA sequence change is not accompanied by a change in amino acid at that postion, only the codon change is shown. cys codons present on each pilin gene segment are underlined. DNA sequences 3' to copy 2, including a pseudo-SmaI-ClaI, repeat are also shown.

```
pilE    TGG CCG AAA GAC AAC ACT TCT GCC GGC GTG GCA TCT TCT TCA TCA ATC AAA GGC AAA
        Trp Pro Lys Asp Asn Thr Ser Ala Gly Val Ala Ser Ser Ser Ser Ile Lys Gly Lys

copy 2                                 --- --- --- --C G-C --C GAC --- --- --- --- ---
                                                       Ala Ala     Asp

copy 1                                                                -A- -T- --- GC- --- ---
                                                                      Asp Ile     Ala

pilE    TAT GTT AAG GAA GTT AAA GTC GAA AAC GGC GTC GTC ACC GCC ACA ATG AAT TCA AGC
        Tyr Val Lys Glu Val Lys Val Glu Asn Gly Val Val Thr Ala Thr Met Asn Ser Ser

copy 2  --- --- G-- A-- --- G-- --- A-- --- --- --- --T --- --- GA- --- --A --- ---
                Glu Lys     Glu     Lys                         Glu     Lys

copy 1  --- --- --- --- --- --- A-- A-- --- --- --- --- --- --- --- --- --- --- ---
                                Lys Ser

pilE    AAC GTA AAC AAA GAA ATC CAA GGC AAA AAA CTC TCC CTG TGG GCC AAG CGT CAA GAC
        Asn Val Asn Lys Glu Ile Gln Gly Lys Lys Leu Ser Leu Trp Ala Lys Arg Gln Asp

copy 2  GG- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
        Gly

copy 1  --- --- --- --- --- --- --- --- --- --- --- --- --- --- -A- --- --- --- ---
                                                                Asp

pilE    GGT TCG GTA AAA TGG TTC TGC GGA CAG CCG GTT ACG CGC AAC GCC AAA GAC GAC ACC
        Gly Ser Val Lys Trp Phe Cys Gly Gln Pro Val Thr Arg Asn Ala Lys Asp Asp Thr

copy 2  --- --- --- --- --- --- --- --- --- --- --- G-- --- --- -A- --- --- --- ---
                                                    Ala         Asp

copy 1  --- (           ) --- G-- -A- -C- --- AC- GCC AAG --G --- --- --- --- --- ---
                              Ala Asp Thr     Thr Ala Lys Lys

pilE    GTC ACC GCC GAC GCC ACC GGC AAC GAC GGC(       )AAA ATC GAC ACC AAG CAC CTG
        Val Thr Ala Asp Ala Thr Gly Asn Asp Gly         Lys Ile Asp Thr Lys His Leu

copy 2  (                                   )-A-(       )--- --- --- --- --- --- ---
                                              Asp

copy 1  --- --- --- --- --- ---]

pilE    CCG TCA ACC TGC CGC GAT(   )AAC TTT GAT GCC AGC TGA GGCAAATTAGGCCTTAAATTTCA
        Pro Ser Thr Cys Arg Asp     Asn Phe Asp Ala Ser TER

copy 2  --- --- --- --- --- --C GCA GCA -C- (   ) --- GAT -A- ---------TA-()A-------T-
                              Asp Ala Ala Ser         Asp

pilE    AATAAATCAAACGTAAGTGATTTCCCACGGCCGCCGGATCAACCCGGGCGGCTTGTCTTTT()AAGGGTTTGCAAG

copy 2  ----------G-----A------T--------A-T----T--T-----T-----C()-----T--A----------

pilE    GCGGGCGGGGTCGTCCGTTCCGGTGGAAATAATATATCGAT

copy 2  --AA---------------TT-------------------]
```

alterations have been achieved is poorly understood. However, antigenic variation of P-fimbriae, a class of adhesins associated with pyelonephritogenic E.coli (Korhonen et al., 1982) is probably a complex phenomenon given their antigenic heterogeneity (Orskov & Orskov, 1983; Rhen et al., 1983a; Pere et al., 1986). P-fimbriae are also associated with mannose resistant haemagglutination of human erythrocytes (Vaisanen et al., 1981) and promote attachment of E.coli to uroepithelial and periepithelial cells (Svanborg-Eden et al., 1977) via interactions between a glycosphyngolipid digalactoside receptor moiety and a fimbrial receptor binding domain that is immunorecessive and presumably conserved (Kallenius et al., 1981; Hanley et al., 1985). Indeed, N-terminal amino acid sequences of antigenically distinct P-fimbrial subunits are nearly identical (Hanley et al., 1985; Klemm, 1985). However, genetic determinants for the P-fimbrial structural subunit and haemagglutination may be distinct. Deletion of the KS71B structural subunit gene of recombinant plasmids encoding the P-fimbrial antigen KS71B (Rhen et al., 1983b) results in a haemagglutination-proficient, non-fimbriate phenotype (Rhen et al., 1986). Moreover, the genetic determinant of haemagglutination mediated by the P-fimbrial antigen, Pap, is distinct from the gene papA which encodes the Pap structural subunit (Normark et al., 1983). The genetic determinant of KS71B-mediated haemagglutination is separated from the KS71B structural subunit gene on the chromosome of KS71 by approximately 6 kb (Rhen et al., 1986). Since type 1 fimbriation is also distinct from haemagglutination function, the

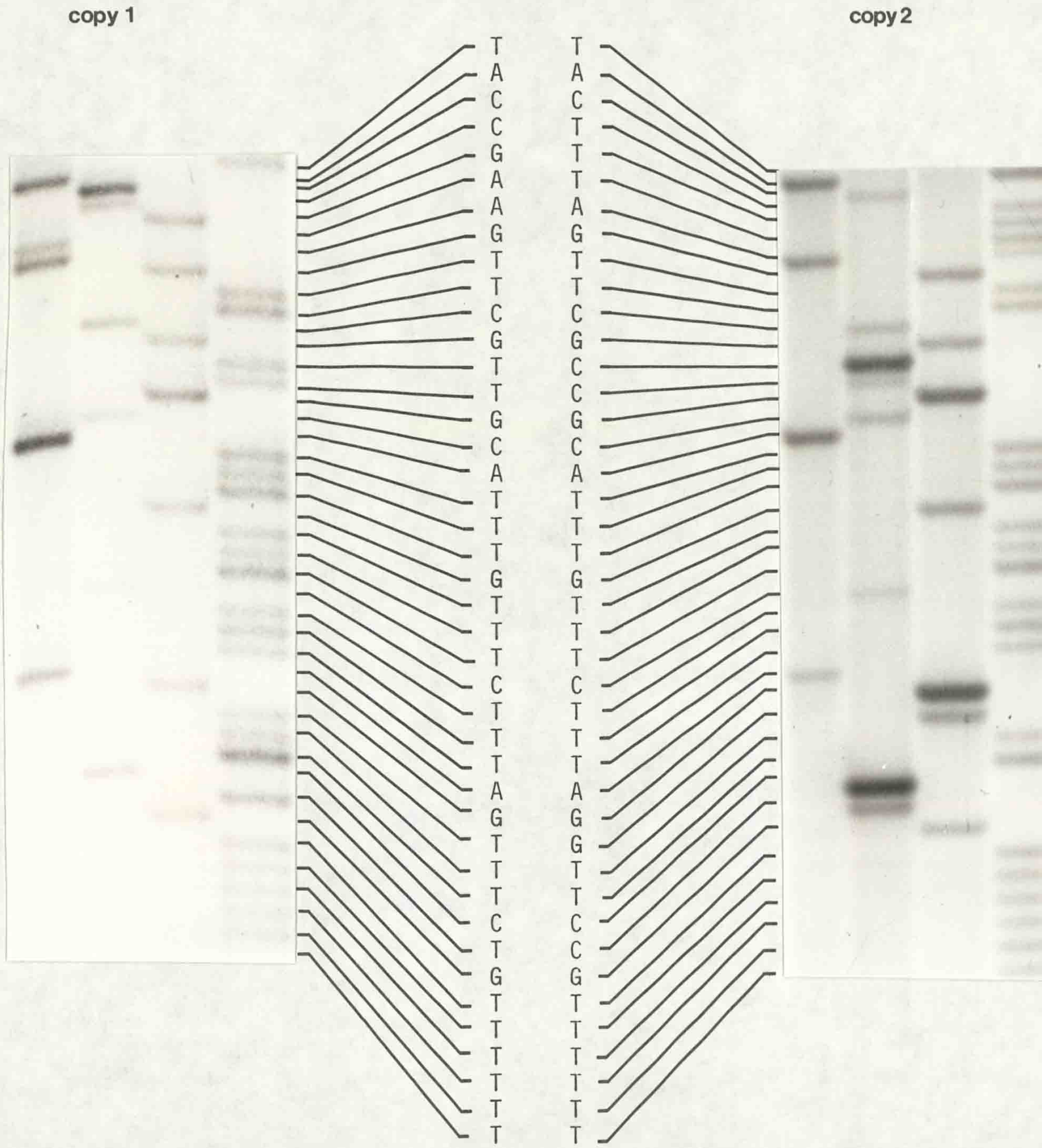


Figure 24. DNA sequence analysis of copies 1 and 2 of the pilS locus of recombinant plasmid pLV610. Sequencing ladders represent the same strand of analogous segments of each copy. Where difficult to interpret, sequences were determined by analysis of the opposite strand of the same segment.

codons. In addition, each copy harboured two regions that were highly conserved with respect to both expressed cys regions of the pilE locus of N.gonorrhoeae strain P9 (Figs 22 & 23). Sequences that lay between the two cys regions of each copy exhibited considerable differences compared to the analogous segment of the pilE gene of N.gonorrhoeae strain P9 (Fig. 23). Unlike copy 1, the 3' terminus of copy 2 possessed a stop codon (TAA) in the correct frame (Fig. 23). Copies 1 and 2 were separated by a tract of DNA that contained a 40 bp sequence identical at all but 3 nucleotide positions with an RS1 element resident at the pilS1 locus of N.gonorrhoeae strain $MS11_{ms}$ (Fig. 22; Haas & Meyer, 1986). In addition, the DNA segment between copies 1 and 2 contained two sets of invertedly-repeated, RS3-like elements (Fig. 22; Haas & Meyer, 1986). Resident on the pilS locus of N.meningitidis strain C114 was a DNA segment that was bounded by a pseudo-SmaI site (5'-TCCGGG-3') at its 5' terminus (nucleotides 1206-1211) and a ClaI site at its 3' terminus (nucleotides 1273-1278). This segment shares extensive homology with the SmaI-ClaI repeat of N.gonorrhoeae (Meyer et al., 1984; Fig. 23). A short sequence of the meningococcal pilS locus (Fig. 22; bp 387-410) resembled codons 11-18 of the pilE gene of N.gonorrhoeae (Fig. 8; Chapter 3):

```
pLV260 (pilE)  ATC GAG CTG ATG ATT GTG ATC GCC
                  ** *** ***  ** *   ***   * *
pLV610 (pilS)  GTC GAG CTG CTG AAA GTG TAC GAA.
```

The pilS locus of N.meningitidis strain C114 apparently did not contain an arrangement of sequences that was likely to direct transcription and/or translation. Promoter sequences of several genes that function in E.coli, including Klebsiella pneumoniae ntrA and ntrC nif genes, and the expressed pilin genes of N.gonorrhoeae, Moraxella bovis, and Pseudomonas aeruginosa are structurally related but do not contain classical -10 and -35 conserved regions (M. So, personal communication). Such nif-like promoter sequences are apparently not present immediately upstream of pilin gene copies 1 and 2 of the pilS locus of N.meningitidis strain C114. The function of a sequence that is reminiscent of the -10 sequence of the M.bovis pilE analogue and is present at base positions 175-187 of strain C114 pilS (Fig. 22) awaits investigation.

5.3 Discussion

The chromosome of N.meningitidis strain C114 harbours an apparently silent locus, pilS, comprising two truncated, tandemly-arranged variant pilin genes. Each pilin gene copy lacks an extensive fraction of 5' sequence information corresponding to coding information for the N-terminal portion of pilin. The pilS locus is the only region of the chromosome of N.meningitidis strain C114 that possesses sequences that are extensively homologous to the pilE locus of N.gonorrhoeae strain P9. The N-terminal of pilin produced by strain C114 is therefore encoded by a tract of DNA that shares little, or no homology with sequences encoding the N-terminal region of gonococcal pilin. Presumably, such differences are also reflected in the primary structures of pilins elaborated by N.meningitidis strain C114 and N.gonorrhoeae. Indeed, amino-terminal amino acid sequences of several bacterial pilins are markedly dissimilar from analogous sequences in gonococcal pilin (Yoshimura et al., 1985). It is as yet unknown whether any of the pilin genes of such species are homologous with genomic sequences present in N.meningitidis strain C114. Answers to questions concerning the primary structure of N.meningitidis strain C114 pilin may ultimately involve sequence analysis of pilus expression site(s).

Copies 1 and 2 of the meningococcal pilS locus do not contain out-of-frame DNA sequence alterations compared with the pilE locus of N.gonorrhoeae strain P9. Assuming such changes would

have occurred had either pilin gene copy become vestigial, copies 1 and 2 presumably play a role in pilus expression. Each copy may therefore compromise one or more cartridges of pilin gene information that can participate in intragenic recombination, resulting in a gene conversion event. Pilin gene conversion apparently occurs in N.gonorrhoeae strain $MS11_{ms}$ and may involve repeated elements resident at pilus gene loci (Segal et al., 1986; Haas & Meyer, 1986). Indeed, the arrangement of copies 1 and 2 on the pilS locus of N.meningitidis strain C114 is similar to that of copies 1 and 2 of the pilS1 locus of N.gonorrhoeae strain $MS11_{ms}$ (Haas & Meyer, 1986).

Several families of repeats, notably RS1-, RS3- and SmaI-ClaI-like elements are present at the pilS locus of N.meningitidis strain C114. Inter-specific conservation of such elements at pilin gene loci is possibly an indication of their importance in pilus expression. Multiple copies of repeated families of elements have not been detected on the genome of strain C114 at loci other than pilS in Southern hybridisation analyses using pilin gene sequences as probes. Should they exist at sites other than pilS, repeated elements would therefore be interspersed by extensive segments of DNA that are heterologous to the pil probes used.

The pseudo-SmaI-ClaI repeat of N.meningitidis strain C114 harbours a sequence (Fig. 22; bp 1216-1236) that shares considerable homology with part of the Hin (inverted repeat

right, IRR) recombination site (Zieg & Simon, 1980):

Hin recognition site 5'- TTCCTTTTGGAAGGTTTTTGA -3'
******** ******* *

pLV610 (p-SmaI-ClaI) 5'- TTCCTTTTTAAAGGTTTGCAA -3'.

This sequence is present at the 3' terminus of a 24 bp near-perfect palindrome that is flanked at its 5' end by part of a HaeIII site (Fig. 22). Such an arrangement of sequences is absent from the termini of the invertible H-segment of S.typhimurium (Zieg & Simon, 1980) but is conserved (in a modified form) on the corresponding region of the gonococcal SmaI-ClaI repeat (Fig. 23). It is possible that sequences resident upon the genomic pseudo-SmaI-ClaI repeat of N.meningitidis are involved in recombination events that are mediated by a meningococcal Hin analogue. This possibilty awaits investigation.

The boundaries of pilin gene copies 1 and 2 of the pilS locus of N.meningitidis strain C114 have been assigned by comparison with the sequence of the pilE locus of pLV260 (Fig. 23). Copies 1 and 2 are separated by an insertion of DNA, including an RS1-like sequence (Haas & Meyer, 1986), which might infact comprise part of either copy 1 or 2 or both (Fig. 22). However, stop codons in all three translational reading frames are distributed throughout this tract (Fig. 22).

The pilS1 locus of N.gonorrhoeae strain $MS11_{ms}$ is separated by a

chromosomal segment of approximately 15 kb from pilE1 (Meyer et al., 1984). 'Chromosome walking' analyses using sequences specific to the pilS locus may therefore locate the pilus expression gene(s) of N.meningitidis strain C114, assuming both meningococcal loci are linked.

CHAPTER 6

Epilogue

Meningococcal pili can be divided into two groups on the basis of immunological reactivity. Members of one group react with the monoclonal antibody SM1 and may be similar to gonococcal pili. In contrast, members of the second group do not possess an SM1-reactive epitope. Findings presented in this work suggest that some (or all) meningococcal pili that do not react with SM1 are markedly different from gonococcal pili with respect to their serological properties. However, pili from some SM1-non-reactive N.meningitidis isolates are immunoprecipitable using polyclonal antisera directed against pili from N.gonorrhoeae strain P9-2, suggesting that there may be close immunological relatedness between such pilus types (Diaz et al., 1984). This apparent contradiction may reflect immunological diversity among meningococcal pili that do not possess an SM1-reactive epitope. Further analysis of such pili from N.meningitidis isolates that apparently do not share close genealogical relatedness will shed light on this possibility.

N.meningitidis and N.gonorrhoeae possess pilin gene loci that share considerable homology. The genome of at least one N.meningitidis isolate, strain C114, apparently does not possess pilin gene sequences that encode an SM1-reactive epitope or N-terminal region. Furthermore, the N-terminal portion of gonococcal pilin contains a receptor binding domain that may reside near to the SM1-reactive epitope (Virji et al., 1983; Schoolnik et al., 1984). Thus, it is possible that the pili of N.meningitidis strain C114 and N.gonorrhoeae exhibit different

receptor binding properties. The ability of meningococci to exhibit different receptor binding specificities might enhance sequential colonisation of different tissue types during the course of infection (Trust et al., 1983; Heckels, 1986). It remains to be seen whether or not meningococci can produce daughters whose pili possess receptor binding qualities that are distinct from those of their progenitor. Moreover, individual meningococcal (and gonococcal) cells may possess machinery that enables them to elaborate more than one pilus type simultaneously.

Alterations in meningococcal piliation, including pilus phase and antigenic variation, might involve recombination between genomic segments of pilin coding information. The chromosomes of several strains of N.meningitidis have been shown to harbour multiple pilin gene loci. Such loci presumably contain sequences corresponding to either the SV, HV, or Sma-Cla repeat regions (or a combination of these) of the gonococcal pilE gene. With the exception of N.meningitidis strain C114, it is not known whether native pilE-homologous meningococcal sequences are expressed. However, it is likely that genomic sequences of N.meningitidis that encode an SM1-reactive domain would be homologous to analogous gonococcal sequences. Attempts to clone out the expressed pilin genes of 5 SM1-reactive meningococcal isolates in E.coli, using the plasmid vector pBR322 have hitherto proven unsuccessful (unpublished observations). The reason(s) for this is unclear and may be a function of the cloning strategy

organisation of the cistron encoding KS71B fimbriae may resemble that of analogous loci required for type 1 fimbriation (Orndorff & Falkow, 1984a; Minion *et al.*, 1986). Indeed, some (or all) of the genes required for fimbrial biogenesis are clustered on the chromosomes of several P-fimbriate *E.coli*, including those elaborating $F7_1$ (Van Die *et al.*, 1985), $F7_2$ (Van Die *et al.*, 1984) and Pap (Norgren *et al.*, 1984) fimbriae. The chromosomes of some *E.coli* isolates from urinary tract infections harbour unlinked, duplicate copies of the gene cluster (*pap*) responsible for the biosythesis of Pap fimbriae (Hull *et al.*, 1985, 1986). Although both copies of *pap* express P-related antigen, only one expresses an adhesin capable of promoting D-mannose resistant haemagglutination (Hull *et al.*, 1986).

D-mannose resistant haemagglutination is mediated almost exclusively by *E.coli* (Hull *et al.*, 1984). Extraintestinal pathogenic isolates of the family *Enterobacteriaceae*, with the exception of some *Morganella* sp. do not mediate D-mannose resistant haemagglutination, and none possess *pap*-related DNA sequences (Hull *et al.*, 1984). In contrast, type 1 fimbriae, characterised by mannose inhibitable haemagglutination, are expressed by representatives of at least nine genera of the family *Enterobacteriaceae* and the chromosomes of two *Shigella* sp. isolates contain sequences related to the *pil* region of *E.coli* Duguid & Gillies, 1958; Duguid & Campbell, 1969; Buchanan *et al.*, 1985). However, members of the *Enterobacteriaceae* exhibit inter-generic size and immunological variation of the structural

employed. Alternatively, DNA fragments harbouring an intact N.meningitidis pilE locus may be lethal to E.coli, either due to the action of cloned meningococcal pilin, the gene product of a gene closely linked to pilE, or some other factor(s). These potential problems could be circumvented by employing an alternative cloning strategy and using a vector that minimised expression of cloned genes. Detection of cloned pilE genes in this regime would be by DNA hybridisation techniques.

Cloned meningococcal pilE loci whose encoded pilins do not react with SM1 would be detected in E.coli using either DNA hybridisation, or immunological techniques, possibly subject to constraints operating for genes encoding SM1-reactive pilins. However, the need to clone expressed meningococcal pilin gene loci might be obviated if pilE transcripts were sequenced by primer extension methods. Such techniques have recently yielded the sequences of expressed variant pilin genes of N.gonorrhoeae (Hagblom et al., 1985).

Determination of the precise nature of silent pilin gene loci on the gonococcal insert of plasmid pLV260 by DNA sequence analysis may shed light (particularly if interpreted in conjunction with analogous data from recombinant plasmids that encode distinct pilins) on the mechanism of pilin gene conversion. Plasmid pLV260 harbours an additional region, corresponding to probe 4, that does not harbour extensive pilin coding information (Chapter 3). The proximity of this segment with respect to the pilE locus of

N.gonorrhoeae strain P9 suggests that it may be involved in pilus expression. Furthermore, hybridisation data indicate that probe 4-homologous segments are present at only one or two loci on the genomes of N.gonorrhoeae and N.meningitidis and are therefore unlikely to contain opa sequences (Stern et al., 1984). Sequences corresponding to probe 4 may therefore encode one or more proteins that are involved in pilus assembly. Indeed, clusters of genes involved in pilus expression have been reported for E.coli (for example Mooi et al., 1982; Normark et al., 1983; Orndorff & Falkow, 1984a). Although sequences corresponding to probe 4 are closely linked to the pilE locus of N.gonorrhoeae strain P9-2, it is not known whether such sequences are resident in the neighbourhood of pilin loci on the genome of N.meningitidis. In addition, preparations of gonococcal and meningococcal pili may not be homogeneous, but could contain components other than pilin (J.K. Davies, personal communication). The relationship (if any) between such putative pilin-associated components and probe 4-analogous sequences in N.gonorrhoeae and N.meningitidis awaits elucidation by nucleotide sequencing and analysis of encoded proteins.

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Abstract

The recombinant plasmid pLV260 contains a DNA segment derived from N.gonorrhoeae strain P9-2. The gonococcal insert of plasmid pLV260 harboured several pilin gene loci, one of which (pilE) was expressed in E.coli. The DNA sequence of the pilE locus of pLV260 shared several features with primary structures of other pilin gene loci (Meyer et al., 1984; Haas & Meyer, 1986). Shared characteristics included a SmaI-ClaI segment, RS1- and RS3-like elements and an open reading frame that corresponded to a structural pilin gene (Meyer et al., 1984; Haas & Meyer, 1986). Hitherto undescribed characteristics were also identified.

Probes derived from plasmid pLV260 have been used to demonstrate the presence of several genomic pil loci in pilus antigenic variants of N.gonorrhoeae strain P9. Similar analyses have demonstrated pilE-homologous sequences on the chromosomes of N.meningitidis isolates. pilE-homologous sequences had undergone demonstrable rearrangement in two P- meningococcal phase variants.

N.meningitidis isolates could be classified according to whether or not they reacted with a monoclonal antibody that recognises an epitope common to probably all gonococcal pili. The genomes of members of both immunological classes of N.meningitidis isolate harboured pilE-homologous segments.

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subunits of their type 1 fimbriae (Duguid & Campbell, 1969; Clegg et al., 1985a). Furthermore, lesions in the cloned pil-analogous regions of Enterobacter cloacae, Klebsiella pneumoniae, Salmonella typhimurium and Serratia marcescens are complemented intra-, but not inter-specifically (Clegg et al., 1985b) indicating the requirement of at least one trans-acting function for the expression of type 1 fimbriae by these organisms.

Irrespective of genetic and antigenic properties, type 1 fimbriae from a variety of isolates are morphologically indistinguishable (Duguid & Campbell, 1969). However, some enteropathogenic E.coli express adhesins that are apparently identical to type 1 fimbriae but mediate mannose resistant haemagglutination of bovine erythrocytes (Craviotto et al., 1982). Unlike type 1 antigens, the members of this distinct class of fimbriae, colonisation factor antigens I and II (CFA/I and CFA/II respectively), promote adhesion of enterotoxigenic E.coli strains to brush border duodenal enterocytes in vitro (Knutton et al., 1984). In addition, they are associated with plasmids that possess the genes for heat-stable, or heat-stable and heat-labile enterotoxins (Penaranda et al., 1980; Mullany et al., 1983; et al., 1983; Echeverria et al., 1986). Combinations of immunologically distinct subsets of CFA/II antigens designated CS (coli surface antigen) 1, CS2 and CS3 can be concomitantly expressed (Smyth, 1982; Mullany et al., 1983). Indeed, populations of enteropathogenic and uropathogenic E.coli can simultaneously express additional fimbrial types, including K99

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and F41 (Morris et al., 1980), type 1, KS71C and P-fimbriae (Norwicki et al., 1984) and others (Karch et al., 1985; Norwicki et al., 1985).

The possession by individual E.coli cells of genetic information for several fimbrial types may potentiate recombination events generating variant genes that encode antigenically, and perhaps functionally novel fimbriae. Such recombination may be evidenced by the considerable amino acid conservation between subunits of several fimbrial types (Klemm, 1985) and the presence on the genomes of some fimbriate E.coli strains of multiple copies of fimbrial gene clusters (Hull et al., 1985; Rhen, 1985). The genome of N.gonorrhoeae harbours many copies of analogous sequences (Meyer et al., 1982). It is therefore possble that parallels exist between the mechanisms of genetic modulation of fimbriation in E.coli and N.gonorrhoeae.

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1.3 Pili of the pathogenic Neisseria: properties and molecular environment

Proteinaceous appendages that resemble E.coli somatic fimbriae are present on many pathogenic isolates of the genus Neisseria (Kellogg et al., 1963; Stephens & McGee, 1981). Pili may be important determinants of pathogenicty in the case of N.gonorrhoeae, although little is known of their involvement, if any, in meningococcal disease. Damage by N.meningitidis to human ciliated nasoepithelial cells is mediated in vitro equally well by P+ and P- variants (Stephens et al., 1986), and the ability of meningococci to colonise the mouse nasopharyngeal epithelium in vivo correlates with capsule production (Salit & Tomalty, 1986). Indeed, N.meningitidis is capable of eliciting at least 8 serologically distinct classes of capsular polysaccharide, designated A, B, C, X, Y, Z, W135 and 29E (Branham, 1953; Slaterus, 1961; Evans et al., 1968). Additionally, physical and immunological heterogeneity exists between the major outer membrane protein profiles of different meningococcal isolates (Frasch & Chapman, 1972; Frasch et al., 1976; Poolman et al., 1980a, 1980b). Such outer membrane proteins can be placed, on the basis of their physical properties into classes, designated 1 to 5 in descending order of their M_r (Tsai et al., 1981).

Class 2 and 3 principal outer membrane proteins are functionally equivalent, have M_r of approximately 41 and 38 kd respectively and one or the other (but not both) is probably represented on

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all meningococcal strains (Tsai et al., 1981; Zollinger et al., 1984). N.gonorrhoeae possesses an analogous outer membrane protein type, protein I (P.I), a trans-membrane polypeptide of between approximately 32 and 40 kd which functions as a porin that is apparently necessary for the translocation of essential nutrients across the outer membrane (Johnston et al., 1976; Heckels, 1979; Douglas et al., 1981). P.I molecules exist in one of two forms (P.IA and P.IB) and are present in their outer membrane environment as trimers closely associated with a 31 kd envelope component, protein III (P.III) (Newhall et al., 1980; McDade & Johnson, 1980; Judd, 1982). P.IA and P.IB each exhibit different surface exposures and orientations in the gonococcal outer membrane (Heckels, 1978; Barrera & Swanson, 1984) and like N.meningitidis class 2 and 3 outer membrane proteins, are mutually exclusive and serotype specific (Sandstrom et al., 1982a, 1982b). Such serotype specificity reflects immunological differences of P.I between strains (Sandstrom et al., 1982a), although serologically distinct P.IB molecules possesses a conserved epitope, recognised by SM24, a monoclonal antibody that mediates complement-dependent killing of P.IB-expressing strains (Fletcher et al., 1986; Virji et al., 1986).

P.I exhibits antigenic drift that may involve exhange of genetic material between strains (Sandstrom et al., 1985; Danielsson, et al., 1986) although intra-strain P.I antigenic variation does not usually occur either in vitro or in vivo (Sandstrom & Danielsson, 1980). In contrast, N.gonorrhoeae displays considerable

their use in serotyping. Infection and Immunity 46, 260-266.

immunological differences, within and between strains, of a class of heat-modifiable outer membrane proteins, protein II (Walstad, et al., 1977; Heckels, 1977; Diaz & Heckels, 1982; Newhall et al., 1985). Production by N.gonorrhoeae of protein II (P.II) in vitro results in an opaque colonial phenotype, whereas colonies of P.II-lacking gonococci are transparent (Lambden & Heckels, 1979). Opaque cell lines may express one or more P.II species in vitro (P.IIa, P.IIb, etc.) with distinct immunological profiles and varying between approximately 24 and 30 kd (Swanson, 1978; Lambden et al., 1979; Black, et al., 1984). Antigenic variation of P.II can also take place in the course of experimental or natural gonococcal infection (McBride, et al., 1981; Schwalbe et al., 1985a) and may involve reassortment of DNA sequences related to the expressed P.II structural gene, opaE (Schwalbe et al., 1985b). Indeed, the chromosome of N.gonorrhoeae possesses many, widely distributed opaE-related sequences, the conformation of which can differ between isogenic P.II antigenic variants (Stern et al., 1984; Schwalbe et al., 1985b). Furthermore, immunologically distinct species of P.II may be the products of different genes (Schwalbe & Cannon, 1986) and the gonococcal genome harbours at least two intact, distinguishable copies of opaE (Stern et al., 1985; Schwalbe et al., 1985b). Expression of P.II can be switched on and off at high frequencies (phase variation) in a non-random fashion (Lambden & Heckels, 1979; Mayer, 1982; Black et al., 1984) and expressed and non-expressed forms of the P.II structural gene are apparently different (Schwalbe & Cannon, 1986). However, variant segments of

opaE loci also possess sequences that are conserved with respect to one another (Stern et al., 1984). Such sequence conservation may reflect the structural relationships that exist between immunologically and physically distinct P.II species (Swanson, 1980) whereas sequence discrepancies that exist between different between opaE loci are presumably correlated to the primary structure and surface exposure of P.II molecules that determine their immunological identity (Judd, 1985).

protein II

Variation of P.II expression in N.gonorrhoeae apparently plays an important role in disease. Gonococci isolated from infections localised to the surface mucosa generally express P.II, whereas P.II is often lacking in organisms recovered from disseminated infection (James & Swanson, 1978; Draper et al., 1980). Close interactions between N.gonorrhoeae and blood and epithelial tissue occurs during the course of natural infection and may be mediated, in part, by P.II (King & Swanson, 1978; Lambden et al., 1979). P.II-deficient N.gonorrhoeae interact minimally with, and are resistant to, phagocytic killing by human polymorphonuclear leukocytes in vitro compared to isogenic P.II-producing gonococci (Virji & Heckels, 1986). In addition, P.II has been implicated as a determinant of gonococcal pathogenesis by confering altered resistance to serum, certain antibiotics, steroid hormones and serum proteases (Lambden et al., 1979; Blake et al., 1981; James et al., 1982; Salit, 1982).

The outer membranes of some N.meningitidis strains possess a

group of heat-modifiable polypeptides, class 5 proteins, that are equivalent to gonococcal P.II but whose role in the disease process is unknown (Poolman et al., 1980a; Tsai et al., 1981). Class 5 outer membrane proteins are of approximately 26 kd and like P.II of N.gonorrhoeae, exhibit considerable antigenic variety (Poolman et al., 1980a, 1982) with single meningococcal strains capable of simultaneously elaborating more than one class 5 protein variant (Tsai et al., 1981). A second type of immunovariable meningococcal outer membrane component, class 1 proteins, typically have a M_r of 46 000 and in common with class 5 proteins, are not expressed by all N.meningitidis strains (Tsai et al., 1981). Both class 1 and class 5 proteins are potent immunogens (Wedege & Froholm, 1986) and possess regions that are structurally conserved within each class (Tsai & Frasch, 1980). Conservation of structure is a property that is also common to physically and immunologically heterogeneous species of N.meningitidis and N.gonorrhoeae lipooligosaccharide (Schneider et al., 1984). A component of lipooligosaccharide that is antigenically variable apparently confers serum resistance to certain gonococcal strains (Schneider et al., 1982, 1985).

Additional outer membrane components that are closely related between N.meningitidis and N.gonorrhoeae and which may play a role in disease include a 70 kd antigen and a surface-exposed, heat-modifiable protein of 20 kd, designated H.8, that exhibits inter- and intra-strain variation (Hitchcock et al., 1985; Martin et al., 1986). The importance of H.8 as a pathogenicity

determinant is indicated by its presence on pathogenic, but not commensal members of the genus Neisseria (Cannon et al., 1984; Black & Cannon, 1985).

Like H.8 antigen, pili may be important determinants of gonococcal and meningococcal pathogenicity. Using negative stain electron microscopy, pili have been demonstrated on N.meningitidis that had been isolated from the nasopharynx, blood and cerebrospinal fluid (CSF) of patients with meningococcal infection (DeVoe & Gilchrist, 1975; Stephens & McGee, 1981). Indeed, N.meningitidis pili promote adhesion in vitro to human erythrocytes and oropharyngeal, but not buccal epithelial cells (Salit & Morton, 1981; Trust et al., 1983). Furthermore, isogenic, non-piliated meningococci and meningococci that had been reversibly de-piliated by the action of mechanical shear forces, trypsin, or antibiotics, all exhibit a markedly decreased ability to attach to human cells compared to their piliated counterparts (Salit & Morton, 1981; Stephens et al., 1984).

The pilus of N.gonorrhoeae, like that of N.meningitidis, is a fimbriate, homopolymeric structure composed of pilin polypeptide molecules. Pilin molecules from some N.meningitidis isolates share biochemical, physical and immunological properties with pilins of N.gonorrhoeae (Virji & Heckels, 1983; Stephens et al., 1985). Indeed, monoclonal antibodies have been isolated that cross-react with meningococcal and gonococcal pilins (Virji & Heckels, 1983; Olafson et al., 1985). One such monoclonal

antibody, SM1, recognises an epitope present on probably all gonococcal pili, but that is absent from the pili of some meningococcal isolates (Virji & Heckels, 1983; Diaz et al., 1984). Since the SM1-reactive epitope of N.gonorrhoeae resides on a conserved region of gonococcal pili that contains the site of interaction with host cell receptor molecules (Gubish et al., 1982), piliated meningococci that lack this epitope may exhibit a different cell receptor specificity (Trust et al., 1983).

Pilin species from different clinical isolates of N.meningitidis vary in size from 13-22 kd (Virji & Heckels, 1983; Diaz et al., 1984; Olafson et al., 1985). Pilin size variation is also exhibited by N.gonorrhoeae, and is associated with changes in the immunological properties of pilin that account for gonococcal pilus antigenic variation (Virji et al., 1983; Heckels, 1986). N,gonorrhoeae strain P9 has been adopted as a paradigm for the study of such variation, and four distinct pilus serotypes, each with a differing subunit size, have been characterised from isogenic P9 variants (Robertson et al., 1977; Lambden, 1981; Virji et al., 1983).

Physical and immunological variation of gonococcal pili can occur both in vitro (Lambden et al., 1980; Nicolson & Perry, unpublished observations) and in vivo (Lambden et al., 1981; Zak et al., 1984). Serological disparities between pili primarily reflect differences in their respective C-terminal amino acid sequences (Hagblom et al., 1985; Nicolson & Perry, manuscript in

preparation). However, antigenically distinct gonococcal pilins share a common primary structure comprising three regions (Rothbard et al., 1984; Schoolnik et al., 1984; Hagblom et al., 1985). The first 53 N-terminal amino acids constitute the constant (C) region and are conserved between immunologically diverse pilin species (Hagblom et al., 1985). Indeed, antisera raised against pilin amino acid residues 41-50 inhibit binding of heterologous pilus variants of N.gonorrhoeae to human endometrial carcinoma cells (Rothbard et al., 1985). Furthermore, the first 30 or so acids are strongly conserved in the N-terminal portion of pilins from N.gonorrhoeae and several other Gram negative bacteria, including meningococci (Hermondson et al., 1978; Olafson et al., 1985), Pseudomonas aeruginosa (Sastry et al., 1983), Bacteroides nodosus (McKern et al., 1983) and Moraxella nonliquefaciens (Froholm & Sletten, 1977). Such amino acid homology presumably reflects conservation of function between the pilins of different species.

A second, semi-variable (SV) region of N.gonorrhoeae pilin molecules comprises amino acid residues 54-114 and contains at least one epitope that is apparently conserved on immunologically distinct gonococcal pili (Hagblom et al., 1985; Virji & Heckels, 1985). The SV region of gonococcal pilin contains changes that usually involve isolated substitutions of charged amino acids, the introduction or removal of an amino acid being balanced by a compensatory alteration nearby involving an oppositely charged one (Hagblom, et al., 1985). In addition to similar

Cloning and detailed analysis of a silent chromosomal pilin gene locus, pilS of N.meningitidis strain C114 revealed sequences corresponding to variable and conserved regions of the pilE locus of N.gonorrhoeae strain P9-2.

substitutions, the hypervariable (HV) domain of gonococcal pilins (amino acid residues 115-C-terminus) can undergo insertions and/or deletions of one to four amino acids (Hagblom, et al., 1985; Nicolson & Perry, manuscript in preparation). Most amino acid changes in the HV region occur within a highly immunogenic section located between two conserved cysteine residues of the pilin polypeptide (Rothbard et al., 1984; Hagblom et al., 1985). The HV portion of gonococcal pilin is immunodominant, and rabbit antisera raised against any one P9 antigenic pilus variant show less than 20% cross-reactivity with isogenic variants expressing heterologous pilus types (Rothbard et al., 1984; Virji et al., 1982).

The molecular mechanism of pilus antigenic variation in N.gonorrhoeae involves rearrangment of mini-cassettes of unlinked, silent DNA segments to yield a fully assembled pilin gene (Hagblom et al., 1985; Segal et al., 1986). Unexpressed pilin gene sequences are distributed throughout the chromosome of N.gonorrhoeae strain $MS11_{ms}$ (Meyer et al., 1984; Haas & Meyer, 1986). The major silent pilus gene locus (pilS1) is closely linked on the genome of $MS11_{ms}$ to a pilin expression site, pilE1 (Meyer et al., 1984). pilS1 harbours six tandemly-arranged, truncated pilin genes, each lacking sequences encoding the N-terminal region of pilin, but containing coding potential for variant SV and HV domains (Haas & Meyer, 1986). Unequally interspersed between the pilin gene segments of pilS1 are three families of short, repeated sequences (RS1, RS2 and RS3) that are

not related to pilus structural genes (Haas & Meyer, 1986). RS sequences may act as recombinogens in the process of pilin gene conversion, mediating genetic exchange between silent sequences, or between silent and expression loci (Hagblom et al., 1985; Haas & Meyer, 1986). Homogenotisation of N.gonorrhoeae strain MS11 with a defective recA gene results in markedly reduced pilin antigenic variation, implying a role for homologous recombination in pilus expression (Koomey & Falkow, 1985).

In addition to an involvement in pilus antigenic variation, pilin gene conversion may give rise to defective pilin polypeptides that are not assembled into mature pili (Swanson et al., 1985). It is possible, therefore, that the mechanisms of gonococcal pilus antigenic and phase variation are related. Pilus phase variation, whereby P+ N.gonorrhoeae can produce P- variants and vice versa, correlates with differences in colonial morphology and gonococcal virulence; P- cells are less infectious than their isogenic P+ variants (Kellogg et al., 1963; Jephcott & Reyn, 1971; Swanson et al., 1971). Like antigenic variation, P+ to P- phase variation occurs as a result of simple or multiple intragenic recombination events, and can involve one or more pilE loci (Meyer et al,. 1982; Segal et al., 1985). In piliated variants of N.gonorrhoeae strain $MS11_{ms}$ deletions of variable size at one or both intact pilE loci, result in loss of piliation (Segal et al., 1985). Where a full genomic complement of pilE loci is deleted, the P+ to P- phase transition is irreversible, although lesions at one pilE locus can be restored by

recombination involving silent and intact pilE loci that co-exist on the same chromosome, resulting in a P- to P+ reversion (Segal et al., 1985). Presumably, $MS11_{ms}$ cells that harbour a single, intact pilE locus remain P- due to the action of a regulator that acts at that locus and which is itself regulated by the P+ to P- switching event (Segal et al., 1985). Indeed, some P- pilus phase variants of N.gonorrhoeae strain $MS11_{ms}$ possess a deleted pilE locus that is not restored on reversion to P+ (Segal et al., 1986). In strains of N.gonorrhoeae that normally possess a single pilE locus in their P+ state (e.g. strains $MS11_{mk}$ and P9), the need for such a regulatory function is obviated (Swanson et al., 1985; Bergstrom et al., 1985; Nicolson et al., 1986).

Regulation of pilus expression in N.gonorrhoeae may have similarities with other piliation control mechanisms. Both N.meningitidis and Haemophilus influenzae can undergo P+ to P- pilus phase variation, the latter at a frequency similar to that of N.gonorrhoeae (Trust et al., 1983; Sable et al., 1985). Furthermore, several Gram negative bacteria exhibit size and/or antigenic variation of their pilin subunits, including Pseudomonas aeruginosa (Sastry et al., 1985), Bacteroides nodosus (Anderson et al., 1986), Bordetella bronchiseptica (Lee et al., 1986) and E.coli (vide supra). In addition, the molecular mechanism(s) of piliation control in N.gonorrhoeae may have parallels with genetic modi operandi in other systems. Studies of prokaryotic and eukaryotic genetic switching systems may shed more light on the regulation of gonococcal pilus expression.

1.4 Several genetic systems are plausibly related to the mechanism(s) of gonococcal piliation control

The characteristics of some P- variants of N.gonorrhoeae strain $MS11_{ms}$ imply an activity involving one or more hitherto unidentified regulators of pilus phase variation (Segal et al., 1985, 1986). Phase switches are also exhibited by Salmonella typhimurium and coliphage Mu, and the genetic modulators of these changes, hin and gin respectively, have been identified and characterised (Lederberg & Iino, 1956; Kamp et al., 1978; Symmonds & Coelho, 1978). The S.typhimurium hin gene product determines the orientation of a chromosomal segment, designated the H-segment (Zieg et al., 1977; Silverman & Simon, 1980). In turn, the orientation of this segment relative to adjacent sequences determines whether flagellin gene H1 or H2 is transcribed, and consequently which of the two flagellar types, H1 or H2, is expressed (Zieg et al., 1977; Silverman & Simon, 1980). Phage Mu also posseses an invertible tract of DNA, the G-segment, whose orientation is determined by the gin gene product, Gin (Daniell et al., 1973; Plasterk et al., 1983). The G-segment (partially) contains two sets of genes, S_V, U and S_V', U' that each encode tail fibre proteins that engender Mu with mutually distinct host specificities (Van de Putte et al., 1980; F. Grundy & M. Howe, cited in Plasterk & Van de Putte, 1984). Phage Mu S_V, U and S_V', U' genes are alternatively located downstream of a promoter that is resident on non-inverting DNA. The orientation of the G-segment determines which of the two sets of genes are

juxtaposed to this promoter and hence, which will be expressed (Plasterk et al., 1983). In this respect, Gin- and Hin-mediated switches are different, since the promoter that alternately directs transcription of S.typhimurium H2 and (indirectly) H1 genes is located upon the invertible H-segment (Zieg et al., 1977; Silverman & Simon, 1980).

Pilus antigenic variation in N.gonorrhoeae is reminiscent of several eukaryotic systems, including the mating-type system in the yeast Saccharomyces cerevisiae (Hicks et al., 1979). The mating-type switch of S.cerevisiae involves site-specific recombination, as a result of which an intact a or α gene is placed at the expression locus, MAT, respectively conferring an a or α mating phenotype (Hicks et al., 1979; Klar & Strathern, 1984). Gene conversion involving silent cassettes of intact genes also occurs in the African trypanosome, Trypanosoma brucei, resulting in variable expression of the surface glycoprotein, VSG (Cross, 1975). The T.brucei genome probably contains 100 or more inactive, complete basic copy (BC) VSG genes (Borst & Cross, 1982; Laurent et al., 1983) and expression of any one of these follows transposition to an active telomeric site, thereby generating a new, expression-linked copy (ELC) (Williams et al., 1979; Hoeijmakers et al., 1980; Pays et al., 1981). Unlike gonococcal pilus antigenic variation, the S.cerevisiae mating-type and T.brucei VSG switches respectively involve transposition of intact, as opposed to fragmented, genes to an expression site. Transposition of structural genes to an expression site also

causes antigenic variation in the bacterium Borrelia hermsii (Plasterk et al., 1985). Serotype switching in B.hermsii occurs when the gene for one variable major protein (VMP) supplants the gene for an antigenically distinct VMP at an expression locus (Plasterk et al., 1985). Transposition of VMP genes involves fusion of variable VMP sequences with constant, 5'sequences (Plasterk et al., 1985) and may therefore resemble assembly of pilin gene segments at an expression locus on the chromosome of N.gonorrhoeae (Hagblom et al., 1985; Segal et al., 1985). Furthermore, assembly of Ig gene segments occurs during human B-cell maturation, and heterocyst differentiation in the cyanobacterium Anabaena involves juxtaposition of scattered isogenomic nitrogen fixation genes (Joho et al., 1983; Golden et al., 1985).

Gonococcal pilus expression may also be regulated by DNA modification, and at least 8 cryptic sequence-specific DNA methylases have been identified in N.gonorrhoeae (Korch et al., 1983, 1985). DNA methylation may regulate expression of the mom gene of coliphage Mu and transposition of IS10 (Toussaint, 1976; Roberts et al., 1985). A role for DNA methylation in the transposition process may, therefore, prove germane to models of gonococcal pilus expression.

1.5 Objectives of this work

To date, genetic studies on pilus phase and antigenic variation in N.gonorrhoeae have focussed on strain MS11 (for example, Meyer et al., 1982; Swanson et al., 1985; Hagblom et al., 1985). Hence, genetic analysis of pilin gene loci from another gonococcal strain may prove illuminating with respect to mechanisms of pilus expression. Such a study was therefore undertaken.

In addition, the aims of this work were extended to initiate an investigation into meningococcal pilus genetics.

CHAPTER 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals were of analytical or reagent grade and supplied by either BDH Ltd. or Sigma Chemical Company unless stated otherwise.

2.1.2 Antibiotics

Ampicillin, tetracycline hydrochloride and chloramphenicol were obtained from Sigma Chemical Company Ltd.

2.1.3 Enzymes

Restriction endonucleases, T4 DNA ligase, calf intestinal alkaline phosphatase, nuclease BAL 31, Escherichia coli DNA-polymerase I and Escherichia coli DNA-polymerase I large fragment ('Klenow enzyme') were obtained from Boerrhinger Mannheim GmbH. Coupled transcription-translation, and random hexanucleotide DNA labelling kits were supplied by P & S Biochemicals Ltd. Lysozyme, DNase I, RNase A and proteinase K were obtained from Sigma Chemical Company Ltd.

2.1.4 Antibodies

Monoclonal antibody SM1 was kindly supplied by Dr J.E. Heckels. The production and properties of monoclonal antibody SM1 have been described elsewhere (Virji & Heckels, 1983).

Rabbit polyclonal antisera raised against purified pili from N.meningitidis strain C114 was a gift from Professor C.A. Hart.

Anti-N.meningitidis strain C114 pilus, and SM1 antibodies were diluted in TSA buffer (see below) 1:50 and 1:2 000 respectively.

2.1.5 Miscellaneous

Polyallomer centifuge tubes were from MSE Scientific Instruments Ltd.

Syringes and S luer needles were supplied by Gillette UK Ltd.

Visking dialysis membranes (18/32") were obtained from Gallenkamp Ltd.

Agarose (medium electroendosmosis grade) and low melting point agarose were supplied by FMC Corporation and Bethesda Research Laboratories respectively. Nitrocellulose membrane filters (BA85) for immuno- and Southern blotting were obtained from Schleicher &

Acknowledgements

Of the several people to whom I am grateful, I extend special thanks to Dr. Jon Saunders, in particular for his inimitable tolerance. I also thank Professor Tony Hart for his friendly assistance, and the SERC for a post-graduate training award. I am indebted to Dr. Dave Greenway and Messrs Chris Wrighton and Kevin Sullivan for their much-appreciated support. In particular, I record a small measure of my ineffable gratitude to Mr Iain Nicolson.

Schull GmbH.

Standard chromatography and 3MM papers were supplied by Whatman Ltd.

Polaroid type 665 positive/negative films were used for photography of agarose gels. X-Omat S X-ray film (Kodak) was used for autoradiography of radioactive filters and gels. X-ray film developer (PQ Universal Developer) and fixer (Hypam Fixer) were obtained from Ilford Ltd.

Bacteriophage SPP1 and lambda DNAs were obtained from P & S Biochemicals Ltd. and Boerhinger Mannheim GmbH respectively.

Radiolabelled compounds and M13 universal sequencing primer (17mer: 5'[-GTAAAACGACGGCCAGT-]3') were supplied by Amersham International Plc.

2.1.6 Media

Media and GC supplement were made using distilled water and unless stated otherwise, were sterilised by autoclaving at 121°C for 15 min. Where appropriate, media contained antibiotics at the following final concentrations: ampicillin, 100 $\mu g.ml^{-1}$; tetracycline hydrochloride, 10 $\mu g.ml^{-1}$; chloramphenicol, 170 $\mu g.ml^{-1}$.

A. Nutrient broth

Lab M nutrient broth No. 2	25	$g.l^{-1}$

B. Glycerol broth

Lab M nutrient broth No. 2	25	$g.l^{-1}$
Glycerol	20	% (w/v)

C. 2T3Y broth

Difco bacto tryptone	16	$g.l^{-1}$
Difco yeast extract	10	$g.l^{-1}$
NaCl	5	$g.l^{-1}$

D. Nutrient agar

Lab M nutrient broth No. 2	25	$g.l^{-1}$
Lab M agar No. 2	12	$g.l^{-1}$

E. H agar

Difco bacto tryptone	10	$g.l^{-1}$
Difco bacto agar	12	$g.l^{-1}$
NaCl	8	$g.l^{-1}$

F. H top agar

Difco bacto tryptone	10	$g.l^{-1}$
Difco bacto agar	8	$g.l^{-1}$
NaCl	8	$g.l^{-1}$

G. Clear typing agar (CTA)

Difco proteose peptone No. 3	11.25	$g.l^{-1}$
KH_2PO_4	1	$g.l^{-1}$
K_2HPO_4	4	$g.l^{-1}$
NaCl	5	$g.l^{-1}$
Soluble starch	1	$g.l^{-1}$
Difco bacto agar	12	$g.l^{-1}$

This mixture was autoclaved and cooled to approximately 50°C prior to the addition of 10 ml of filter-sterilised GC supplement.

GC supplement

D-Glucose	400	$g.l^{-1}$
L-glutamine	5	$g.l^{-1}$
$Fe(NO_3)_3$	0.5	$g.l^{-1}$
Thiamine pyrophosphate (0.2% [w/v])	10	ml

2.1.7 Buffers and solutions

Where necessary, buffers and solutions were sterilised by autoclaving at 121°C for 15 min.

A. BSA binding solution

Tris-HCl (pH 7.5)	0.05	M
NaCl	0.15	M
$MgCl_2$	0.005	M
BSA	3	% (w/v)
Lysozyme	40	$\mu g.ml^{-1}$
DNase I	1	$\mu g.ml^{-1}$

(BSA binding solution used in Western blotting does not contain lysozyme or DNase I.)

B. Denaturation solution

NaOH	0.5	M
NaCl	1.5	M

C. Formamide dye mix

EDTA	0.01	M
Ficoll	10	% (w/v)
Xylene cyanol	0.1	% (w/v)
Bromophenol blue	0.1	% (w/v)
Deionised formamide to	100	ml

D. Final sample buffer

Glycerol	10	% (v/v)
2-Mercaptoethanol	5	% (v/v)
SDS	3	% (w/v)
Bromophenol blue	0.01	% (w/v)
Tris-HCl (pH6.8)	0.0625	M

E. GC TES

Tris-HCl	20	mM
EDTA	1	mM
NaCl	100	mM
	(pH 7.5)	

F. Loading buffer (agarose gels)

EDTA	0.05	M
Urea	4	M
Ficoll	8	% (w/v)
Bromophenol blue	0.1	% (w/v)

G. Neutralising solution

Tris-HCl	1	M
NaCl	1.5	M
	(pH 8.0)	

H. Phosphate buffer

K_2HPO_4	6.7	mM
Na_2HPO_4	24.2	mM

I. 20X SSC solution

Tri-sodium citrate	0.3	M
NaCl	3	M
	(pH 7.0)	

J. 10X TE buffer

Tris-HCl	0.1	M
EDTA	0.01	M
	(pH 8.0)	

K. 10X Tris-borate buffer

Trisma base	0.89	M
Boric acid	0.89	M
EDTA	0.025	M

L. Tris-saline

Tris-HCl	0.05	M
NaCl	1.5	M
	(pH 7.5)	

M. TSA buffer

Tris-HCl	0.02	M
NaCl	0.87	% (w/v)
BSA	5	$mg.ml^{-1}$
NaN_3	7.7	mM
	(pH 7.6)	

N. Western blot buffer

Trisma base	0.02	M
Glycine	0.15	M
Methanol	20	% (v/v)

2.2 Methods

Growth of bacteria in liquid culture was at 37°C with shaking at approximately 140 rpm. Microcentrifugation was at 13 500g in 1.5 ml plastic Eppendorf tubes.

2.2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Tables 1 and 2 respectively. N.meningitidis strains were recent clinical isolates made by Professor C.A. Hart from children at Alder Hey Children's Hospital, Liverpool. Each meningococcal isolate had been subcultured at most twice prior to use. The predominant pilus phase status (P+ or P-) of each of the meningococcal strains used in this study was determined electron-microscopically (Prof. C.A. Hart, personal communication). Strains of N.gonorrhoeae used in this work have been described in detail elsewhere (Lambden et al., 1981).

Bacterial strains were routinely stored as frozen pellets or in glycerol broth at -70°C.

To Mum and Dad

Table 1. Bacterial strains used in this work.

Strain	Relevant phenotype	Source*/reference
N.meningitidis		
C105	serogroup+ C, serotype+ nt.	CSF. This work.
C111	" B " nt.	CSF. "
C114	" C " 15/16.	Blood. "
C115	" B " nt.	CSF. "
C151	" B " 8/5.	CSF. "
C156	" B " 16.	CSF "
C311	" B " nt.	CSF. "
C312	" B " nt.	Blood. "
C318	" C " nt.	Blood. "
C319	" C " nt.	CSF. "
C321	" B " 15/16.	Blood "
C322	" B " 15/16.	CSF. "
C341	" B " 15.	CSF. "
C342	" B " 15.	Blood. "
C359	" B " 15/16.	CSF. "
C360	" B " 15/16.	Blood. "
C361	" B " 15/16.	Blood. "
C368	" Y " 15/16.	Throat "
C369	" nt " nd.	Throat "
C370	" nt " nd.	Pharynx "
N.gonorrhoeae		
P9-2	P+ pilus variant of P9.	Lambden et al. (1981).
P9-20	" " " " "	" " "
P9-35	" " " " "	" " "
P9-37	" " " " "	" " "
E.coli		
DH1	RecA$^-$, r_k- m_k+.	Hanahan (1983).
JM103	Host for DNA sequencing studies.	Messing et al. (1981).
GC1	r_k- m_k+. Proficient in expression of gonococcal pilin.	Meyer et al. (1982).

*Strains that were isolated from the same patient are bracketed.
+Serotypes and serogroups are according to Frasch et al. (1985).
nt. Not typeable.
nd. No data.

Table 2. Plasmids used in this work.

Plasmid	Relevant properties	Source/reference
pBR322	Ap^r, Tc^r.	Bolivar *et al*. (1977).
pLV260	Ap^r. Encodes gonococcal pilin. Derived from *N.gonorrhoeae* strain P9-2.	Nicolson *et al*. (1986).
pLV261	Subclone of pLV260. Pil^-.	This work.
pLV262	Subclone of pLV260. Pil^-.	"
pLV263	Subclone of pLV260. Pil^+.	"
pLV265	Subclone of pLV260. Pil^-.	"
pLV266	Subclone of pLV260. Pil^-.	"
pLV267	Subclone of pLV260. Pil^+.	"
pLV600	Ap^r. Harbours pilin gene sequences derived from *N.meningitidis* strain C114.	This work.
pLV610	Ap^r. Harbours pilin gene sequences derived from *N.meningitidis* strain C114.	"
pLV620	Ap^r. Harbours pilin gene sequences derived from *N.meningitidis* strain C114.	"
pLV630	Ap^r. Harbours pilin gene sequences derived from *N.meningitidis* strain C114.	"

2.2.2 Isolation of N.gonorrhoeae genomic DNA

The method employed for isolating gonococcal DNA was similar to that of Stern et al. (1984).

i. Three CTA plates were seeded to give confluent gonococcal growth. Plates were incubated for 18-24 h at 37°C in the presence of 5-7 % CO_2.

ii. Before continuing, the predominance in resultant lawns of the desired phase variants (i.e. either P+ or P-) was ascertained by stereo-, or electron-microscopy.

iii. Bacterial lawns were resuspended in a total of 10 ml GC TES.

iv. To this suspension was added 0.5 ml of lysis mix, prior to incubation at 37°C for 30 min with gentle shaking. (20 ml lysis mix contains: 4 ml Triton X-100; 1 mg lysozyme; sterile distilled water to 20 ml.)

v. 100 µl of a 5 $mg.ml^{-1}$ solution of proteinase K was added. Incubation was continued at 37°C for 20 min with gentle shaking.

vi. 10 g CsCl was added to, and gently dissolved in the lysate.

vii. 300 µl of a 10 $mg.ml^{-1}$ solution of EB was added.

viii. The EB-CsCl-lysate mix was adjusted to give a refractive index of 1.392 (sucrose equivalent of 36 % [w/v]) and divided equally between two polyallomer tubes.

ix. Polyallomer tubes containing samples were spun at approximately 180 000g at 18°C for 16-20 h.

x. DNA was removed from the resultant CsCl density gradients using sterile syringes that were each equipped with a sterile S luer needle.

xi. The DNA was then pooled and cleansed of EB by 4-fold extraction with CsCl-saturated propan-2-ol.

xii. The DNA solution was then dialysed against 2 changes of TE for 6-18 h prior to purification by sequential extraction with phenol-$CHCl_3$, $CHCl_3$, and diethyl ether. Purified DNA was then precipitated in the presence of ethanol and resuspended in sterile, double-distilled water.

2.2.3 Isolation of N.meningitidis genomic DNA

The method desribed here for the isolation of meningococcal genomic DNA was based on that of Kristiansen et al. (1984). All incubations were on ice.

i. Three CTA plates were seeded to produce meningococcal lawns. Plates were incubated for 18-24 h at 37°C in the presence of 5-7 % CO_2.

ii. Cells were resuspended in approximately 2 ml of a solution containing 50 mM Tris-HCl (pH 8.0), 25 % (w/v) sucrose.

iii. The resultant suspension was transferred to an Eppendorf tube and cells were pelleted.

iv. Following removal of the supernatant, 150 µl of a solution comprising 50 mM Tris-HCl (pH 8.0), 25 % (w/v) sucrose, 5 $mg.ml^{-1}$ lysozyme was added. This mixture was vortexed prior to incubation for 5 min.

v. 60 µl 0.25 M EDTA was added, the mixture vortexed, and incubation continued for a further 5 min.

vi. 300 µl lysis mix was added, followed by 10 µl of a 5 $mg.ml^{-1}$ solution of proteinase K. This cocktail was vortexed prior to incubation for 60 min. Lysis mix is: