Control of *Neisseria gonorrhoeae* pilin gene expression by environmental factors: involvement of the *pilA/pilB* regulatory genes

Mireille Larribe,¹ Muhamed-Kheir Taha,¹ Andrzej Topilko² and Christian Marchal¹

Author for correspondence: Mireille Larribe. Tel: +33 1 45 68 89 58. Fax: +33 1 45 68 89 53.
e-mail: mlarribe@pasteur.fr

Unité des *Neisseria*¹ and
Station Centrale de
Microscopie Electronique²,
Institut Pasteur, 28 rue du
Dr Roux, 75724 Paris Cedex
15, France

The control of the expression of the pilin gene (*pilE*) in *Neisseria gonorrhoeae* under a wide variety of growth conditions has been studied. The expression of *pilE* was measured using transcriptional fusions between *pilE* and the gene encoding chloramphenicol acetyltransferase (CAT), and the level of pilin production was measured by Western blot analysis. Many of the conditions tested affected both growth rate and pilin gene expression (e.g. isoleucine, high osmolarity, high temperature, anaerobic growth, pH 6, urea and iron depletion). Changes in the level of many other proteins were also observed, depending on the conditions, indicating that gonococci undergo an adaptive response to environmental variations. Moreover, environment-induced changes in the level of many proteins, including pilin, seem to involve the *pilA/pilB* regulatory system, which has been previously proposed to modulate the expression of the gonococcal pilin gene.

Keywords: *Neisseria gonorrhoeae*, pilin expression, environmental factors

INTRODUCTION

Neisseria gonorrhoeae is an exclusively human pathogen which colonizes different mucosal surfaces of the urogenital tract and other sites such as the rectum, the oropharynx or the cornea (Watt & Ward, 1980; Gorby & Schaefer, 1992; Molesh *et al.*, 1994). The bacteria are exposed to a variety of different growth conditions during infection. The ability of the gonococcus to infect its host is conditioned by successive steps of interactions with host cells and by the survival of the bacteria.

Gonococcal pili, which are composed mainly of subunits called pilin, are required for initial attachment of the bacteria to epithelial cells (Swanson *et al.*, 1987), probably in conjunction with outer-membrane proteins such as PilC (Rudel *et al.*, 1995). Opa proteins, which are localized in the outer membrane, are also involved in cell–bacteria interactions (Waldbeser *et al.*, 1994). Pilin and Opa proteins undergo phase and antigenic variation (Segal *et al.*, 1985; Seifert & So, 1988; Robertson & Meyer, 1992). In addition, the pilin gene is transcriptionally regulated by the products of two genes, *pilA/pilB*, which act in a manner analogous to the

family of bacterial two-component regulators (Taha *et al.*, 1988, 1991). The *pilA* gene seems to be essential and its product appears to play a crucial role related to bacterial cell growth. Indeed, when *pilA* is inactivated by transposon insertion, only the *pilA*⁺/*pilA*::mTn3Cm-3 heterodiploids are viable (Taha *et al.*, 1988). In addition, it has been shown that PilA protein is implicated in the gonococcal stress response (Taha *et al.*, 1992) and in the resistance to the bactericidal effect of normal human serum (Taha, 1993). The cellular location of PilA and PilB, the similarities in amino acid sequence with members of the family of two-component regulators and the phosphorylation of PilA (Taha & Giorgini, 1995) suggest that these two proteins may be involved in the modulation of pilin gene expression by environmental factors.

In the present study, we investigated the effects of numerous growth conditions on the control of expression of the pilin gene, some of which reflect the habitat of *N. gonorrhoeae* (high osmolarity, high urea concentration, anaerobiosis). The level of regulation of pilin gene expression was analysed using a transcriptional fusion between the *cat* gene [encoding chloramphenicol acetyltransferase (CAT)] and the promoter of the pilin gene (*pilE*) in *N. gonorrhoeae* strain MS11. The influence of growth conditions on *pilE* transcription

Abbreviation: CAT, chloramphenicol acetyltransferase.

and on the amount of pilin synthesized is reported. The role of the *pilA/pilB* system in the response to these external signals was also analysed using *pilA* and *pilB* mutants. Variations in the amounts of several proteins, such as the heat-shock protein Gsp63, under these different conditions were also tested. We discuss the role of PilA and PilB in gonococcal adaptation to the environment.

METHODS

Bacterial strains and growth conditions. The variants of the gonococcal strain MS11 used in this study are listed in Table 1. The transcriptional fusions between the *pilE* promoter and the *cat* gene at the *pilE1* site (CAT-1) or *pilE2* site (CAT-2) of the strain MS11 have been previously described (Taha *et al.*, 1988). The strains harbouring the *pilE-cat* fusions produce CAT as well as the pilin protein. The mutations in *pilA*, *pilB* and *opaE1* obtained by insertions of the mTn3Cm-3 transposon were previously described by Taha *et al.* (1988). The *pilA* mutants are always heterodiploid for the *pilA* gene and homodiploid for the neighbouring gene, *pilB*. The mutation *pilAk* results from the insertion of a *Bam*HI fragment carrying the *aph-3'* gene of Tn5 (encoding the aminophosphotransferase and conferring kanamycin resistance) into the *Not*I site in *pilA* (Taha & Giorgini, 1995). Bacteria were grown at 37 °C under 5% (v/v) CO₂ on G medium with G supplements (Sanofi-Diagnostics Pasteur) or on the minimal medium as previously described by Larribe *et al.* (1991). These conditions are indicated as standard growth conditions. When needed, chloramphenicol and kanamycin were added at final concentrations of 10 µg ml⁻¹ and 40 µg ml⁻¹, respectively. The effect of amino acid supplements was studied on solid minimal or G medium. For anaerobic cultures, the Petri dishes containing sodium nitrite discs (40 mM) were incubated in an anaerobic chamber under CO₂/H₂ (5:95, v/v). The studies of increased osmolarity were performed in liquid minimal or G medium. Iron starvation was achieved in a solid minimal medium in which iron had been chelated by the siderophore desferrioxamine (Desferal; 15 mM). Growth at 41 °C was performed on solid G medium.

CAT assay. The cells were centrifuged, washed, resuspended in Tris/HCl (50 mM, pH 7.8, containing 30 µM DTT) and sonicated. The CAT assay was performed as previously described by Shaw (1975). CAT activity is expressed as U (mg protein)⁻¹ in total cell extracts (1 CAT unit corresponding to 1 nmol chloramphenicol acetylated min⁻¹). The results obtained under a given growth condition were expressed as

the percentage of the value measured under standard growth conditions. Experiments were repeated at least twice and usually about five times.

The CAT assay cannot be used to monitor *pilE* expression in the *pilA/pilB* mutants since their mutations were obtained by the insertion of the transposon mTn3Cm-3, which confers chloramphenicol resistance. In order to study the role of PilA and PilB in the modulation of *pilE* gene expression under different environmental conditions, the amounts of pilin were measured by Western blot analysis. In each case, the results are expressed as the percentage of the pilin level in the wild-type strain MS11-A.

Pilin detection by Western blot analysis. Western blotting was carried out using ³⁵S-labelled protein A (Amersham) as previously described by Dupuy *et al.* (1991). The anti-pilin serum was prepared as previously described by Dupuy *et al.* (1991). After exposure, the bands were cut out of the membrane and radioactivity was measured in a liquid scintillation counter. Results were expressed as the percentage of c.p.m. (mg total protein)⁻¹ for a given growth condition compared to the standard growth conditions. Experiments were performed at least twice and generally five times. SDS-PAGE analyses were performed for protein profiles; the gels were stained with Coomassie Brilliant Blue or with silver nitrate.

Electron microscopy. Gonococci from solid or liquid cultures were gently dissociated and resuspended in phosphate buffer. Copper grids (400 mesh) coated with collodion-carbon support film were floated for 3 min on the bacterial suspension. For rotary shadowing, the grids with attached gonococci were mounted on a rotary holder. The stage was positioned 10 cm from a platinum-carbon source and adjusted to an angle of 6 ° with respect to the electron gun. Samples were rotary-shadowed (100 r.p.m.). Metal shadowing was sufficient after 20 s evaporation. All specimens were examined using a JEOL JEM100CXII electron microscope.

RESULTS

Pilin gene expression is related to growth rate in *N. gonorrhoeae*

We first tested the effect of environmental factors on growth of *N. gonorrhoeae* on standard G and minimal media. Growth was evaluated by assaying the protein content of cells from solid or liquid medium cultures after the same incubation time. Several factors reduced growth moderately (50% reduction of growth under

Table 1. Bacterial strains

Strain	Genotype or description	Reference
MS11-A	<i>pilE1</i> , <i>pilE2</i>	Segal <i>et al.</i> (1985)
4.1C	<i>pilE1</i> , Δ <i>pilE2</i> , derived from MS11-A	Segal <i>et al.</i> (1985)
MS11. <i>opaE1a</i>	<i>opaE1::mTn3Cm-3</i>	Taha <i>et al.</i> (1988)
MS11.CAT-1	<i>pilE1-cat</i> (transcriptional fusion)	Taha <i>et al.</i> (1988)
MS11.CAT-2	<i>pilE2-cat</i> (transcriptional fusion)	Taha <i>et al.</i> (1988)
MS11. <i>pilBa</i>	<i>pilB::mTn3Cm-3</i>	Taha <i>et al.</i> (1988)
MS11. <i>pilA</i> ⁺ / <i>Aa</i>	<i>pilA::mTn3Cm-3</i>	Taha <i>et al.</i> (1988)
MS11. <i>pilA</i> ⁺ / <i>Ak</i>	<i>pilA::aph-3'</i>	This work
MS11. <i>pilA</i> ⁺ / <i>Ak.pilBa</i>	<i>pilA::aph-3'</i> ; <i>pilB::mTn3Cm-3</i>	This work

Table 2. CAT activity and level of pilin of the different strains under standard growth conditions

The standard minimal medium contained 30 mM dextrose, 4 mM NH_4Cl , 0.35 mM cysteine, 0.15 mM cystine, 39 mM P_i , 0.25 mM CaCl_2 and 2 mM MgCl_2 and was incubated at 37 °C under 5% CO_2 .

Strain	CAT activity*	Pilin†
MS11.CAT-1	100 (430 ± 50)	92 ± 4
MS11.CAT-2	100 (310 ± 37)	78 ± 3
MS11-A		100
MS11.pilBa		125 ± 33
MS11.pilA ⁺ /pilAa		40 ± 8
MS11.pilA ⁺ /pilAk		140 ± 2
MS11.pilA ⁺ /pilAk.pilBa		140 ± 2

*For MS11.CAT-1 and MS11.CAT-2, CAT activity under standard conditions was taken as 100%. The absolute values of CAT activity for each fusion are indicated in parentheses and expressed as U (mg protein)⁻¹ ± SD. One CAT unit = 1 nmol chloramphenicol acetylated min⁻¹ at room temperature.

†For each strain under standard growth conditions, the level of pilin is expressed as the percentage ± SD of values obtained with MS11-A wild-type.

anaerobic conditions and temperature between 30 and 37 °C). Other factors reduced growth severely (90% reduction of growth) [high osmolarity (300 mM NaCl or KCl, or 600 mM sucrose), iron depletion, 400 mM urea, increased temperature from 37 to 41 °C and pH 6]. These results revealed a very narrow range of favourable growth conditions, especially for temperature, pH, saline concentration and oxygenation.

The virulence and the piliation of the gonococcus have been previously reported to be modified in the presence of amino acids (Saikh *et al.*, 1989), during anaerobiosis or under limitation for glucose, cystine or iron (Keevil *et al.*, 1986, 1989). We analysed the effects of environmental factors on the transcription of the *pilE1* and *pilE2* genes by using a transcriptional fusion between the pilin gene promoter and the promoter-less *cat* gene encoding CAT (Taha *et al.*, 1988). The *pilE*-*cat* fusion was integrated by homologous recombination into the wild-type strain MS11-A at the *pilE1* site (CAT-1) or at the *pilE2* site (CAT-2). The pilin level was also evaluated by Western blot analysis using anti-pilin antibodies. The CAT activities obtained with the two fusions CAT-1 and CAT-2 (Table 2) showed expression at both sites. However CAT-2 showed a reproducibly lower level of expression than CAT-1. Pilin level was not affected by the insertion of the *cat* gene (Table 2). As indicated in Table 3, all conditions which reduced the gonococcal growth rate in the wild-type MS11-A strain were also shown to reduce pilin gene transcription and pilin level, with the exception of iron depletion. Indeed, the latter condition, which severely reduced the growth rate, seemed to have no effect on pilin gene transcription or on the pilin level (Table 3).

The presence of some amino acids seemed to affect growth rate and pilin gene expression in the same manner. When isoleucine was added at 3 mM, it induced a fourfold and twofold increase in the expression of the *pilE*-*cat* fusion and the pilin level, respectively (Table 3). It also provoked a threefold increase in growth rate.

We next examined the piliation of wild-type strain MS11-A under standard and high osmolarity growth conditions using electron microscopy. We observed that

Table 3. CAT activity and level of pilin under different environmental conditions

CAT activity and level of pilin are expressed as the percentage ± SD of the values observed in standard G medium.

Strain	pH 6	Anaerobiosis	41 °C	High osmolarity*		Urea (400 mM)	Isoleucine (3 mM)	Fe starvation
				KCl	NaCl			
MS11.CAT-1								
CAT activity	52 ± 9	28 ± 7	65 ± 7	41 ± 8	39 ± 7	22 ± 11	558 ± 180	162 ± 23
Pilin	48 ± 28	34 ± 8	51 ± 3	51 ± 2	56 ± 2	51 ± 2	242 ± 3	124 ± 8
MS11.CAT-2								
CAT activity	37 ± 20	37 ± 7	49 ± 7	39 ± 7	34 ± 11	19 ± 8	640 ± 250	128 ± 15
Pilin	52 ± 12	30 ± 6	58 ± 17	77 ± 16	69 ± 7	63 ± 6	309 ± 137	145 ± 7
MS11								
Pilin	43 ± 6	47 ± 8	39 ± 4	83 ± 10	69 ± 15	85 ± 15	170 ± 6	148 ± 13
MS11.pilBa								
Pilin	156 ± 40	85 ± 31	73 ± 50	168 ± 17	270 ± 40	159 ± 17	77 ± 3	84 ± 10
MS11.pilA ⁺ /pilAa								
Pilin	76 ± 11	12 ± 5	47 ± 14			97 ± 18	42 ± 3	185 ± 35
MS11.pilA ⁺ /pilAk								
Pilin	75 ± 13	82 ± 15	96 ± 13			102 ± 6	156 ± 50	120 ± 10

* High osmolarity was obtained by addition of 300 mM KCl or NaCl.

Fig. 1. Piliation status of wild-type gonococci and mutants observed by electron microscopy using the rotative shadowing method. Two growth conditions were tested: minimal medium (a, c, e) and minimal medium supplemented with 300 mM KCl (b, d, f). (a, b) MS11-A wild-type strain; (c, d) 4.1C wild-type strain; (e, f) mutant MS11.*pilBa*. Bars, 1 μ m.

the bacteria grown under high osmolarity were almost devoid of pili, although the pilin level was only reduced by 50% in strain MS11-A (Fig. 1).

Taken together, these data indicate that pilin gene expression and growth rate are clearly related and that *N. gonorrhoeae* modulates the expression of the pilin gene in response to external signals.

Analysis of *pilA* and *pilB* mutants

We next analysed the behaviour of *pilA* and *pilB* mutants under different growth conditions described in this study. We tested the previously described *pilA*⁺/*pilAa* heterodiploid and *pilBa* mutant (Taha *et al.*, 1988). Moreover, we constructed a new *pilA* heterodiploid (*pilA*⁺/*pilAk*). The *pilAk* allele was obtained by the insertion of the *aph-3'* gene (which confers resistance to kanamycin) into the *NotI* site in the *pilA* gene (Fig. 2). The new recombinant plasmid, pNG17K, was then used to transform the wild-type strain MS11-A and the

mutant MS11.*pilBa*. All *pilA* mutants were heterodiploid *pilA*⁺/*pilA*::mTn3Cm-3 or *pilA*⁺/*pilA*::*aph-3'* (data not shown). In the gonococcus, it has been previously shown that *pilA* is an essential gene, as its inactivation by transposon insertion is lethal, and only *pilA*⁺/*pilA*::mTn3Cm-3 heterodiploids can be obtained (Taha *et al.*, 1988). In the *pilA*⁺/*pilAa* heterodiploid, a reduction in pilin gene transcription is observed. This phenotype results from the negative transdominant effect of the product of the *pilAa* allele on that of the wild-type allele (Taha *et al.*, 1988). Moreover, the phenotype of *pilA* heterodiploids depends on the site of the insertional inactivation on the *pilA* gene (Taha *et al.*, 1991).

Under standard growth conditions (minimal medium), the amount of pilin was higher in the mutants *pilBa*, *pilA*⁺/*pilAk* and *pilA*⁺/*pilAk.pilBa* when compared to the strains MS11-A, MS11.CAT-1 and MS11.CAT-2. The pilin level was lower in the mutant *pilA*⁺/*pilAa* (Table 2; Fig. 3, lanes 6–10).

The external conditions tested in this study which modified the amount of pilin had few or no effects in the *pilBa* mutant (Table 3; Fig. 3, lanes 3 and 8), in particular pH 6, urea (which reduced the pilin level in the wild-type strain) and isoleucine (which increased the pilin level in the wild-type strain). However, the piliation state of the *pilBa* mutant is severely reduced under high osmolarity conditions (Fig. 1e, f). These data indicate that factors other than the pilin level are also involved in piliation in the gonococci. The *pilA*⁺/*pilAk* heterodiploid showed the same behaviour as the *pilBa* mutant (Table 3; Fig. 3, lanes 4 and 9). A different situation was observed for the heterodiploid *pilA*⁺/*pilAa*. Indeed, the effect of growth at pH 6 or in the presence of 400 mM urea on pilin level was abolished in this heterodiploid (Table 3). The presence of 3 mM isoleucine reduced the pilin level in the *pilA*⁺/*pilAa* heterodiploid while it increased this level in the wild-type strain (Table 3). However, other conditions, growth under anaerobiosis or at 41 °C (Fig. 3, compare lanes 1 and 2 to 6 and 7), had the same effect on both the wild-type strain and the *pilA*⁺/*pilAa* heterodiploid. The modulation of pilin synthesis seemed to be more pronounced in the *pilA*⁺/*pilAa* heterodiploid for the growth under anaerobic conditions (Table 3). These data indicate that the response of *pilE* gene expression in *N. gonorrhoeae* to several external signals passes through the *pilA/pilB* regulatory system.

Analysis of the protein profile in *pilA/pilB* mutants compared to the wild-type strain

It has been suggested previously that *pilA/pilB* genes constitute a pleiotropic regulatory system which could be involved in the regulation of several aspects of the gonococcal virulence (Taha *et al.*, 1992; Taha, 1993). In view of this, we examined the pleiotropic effects of *pilA/pilB* mutations on the level of other proteins produced by *N. gonorrhoeae*.

We first used SDS-PAGE analysis of total cell proteins to compare the protein profile of the wild-type strain

Fig. 2. Schematic representation of the *pilA/pilB* region of *N. gonorrhoeae*. Black arrowheads indicate the position of the mutational insertions of mTn3Cm-3 or *aph-3'*, with numbers in parentheses corresponding to the amino acid deduced from the nucleotide sequence.

Fig. 3. Western blot analysis of whole-cell extracts of *N. gonorrhoeae* using anti-pilin antibody. Bacteria were grown at 41 °C (lanes 1–5) or at 37 °C (lanes 6–10). The strains analysed were MS11-A (lanes 1 and 6), MS11.*pilA*⁺/*pilAa* (lanes 2 and 7), MS11.*pilBa* (lanes 3 and 8), MS11.*pilA*⁺/*pilAk* (lanes 4 and 9) and MS11.*pilA*⁺/*pilAk.pilBa* (lanes 5 and 10).

MS11-A under the standard growth conditions to those under anaerobic, high temperature or high osmolarity growth conditions. The levels of several proteins were observed to be modified in the wild-type strain grown under the conditions tested compared to the standard growth conditions. These data are summarized in Table 4. Variations in the amounts of some proteins were specific for one condition. Some other proteins were modulated in the same way under the three conditions tested (e.g. the 66 kDa protein).

We observed differences between *pilA* or *pilB* mutants and the wild-type strain MS11-A grown under standard conditions. As shown in Table 4, each mutant (*pilA*⁺/*pilAa*, *pilA*⁺/*pilAk* and *pilBa*) has a distinct protein profile. Indeed, the increase in the 66 kDa and the 41 kDa proteins observed in the wild-type strain under anaerobic, high temperature or high osmolarity growth conditions is also observed in the *pilA*⁺/*pilAa* mutant grown under standard growth conditions. In the same manner, the increase of proteins of 170, 66 and 53 kDa observed under high osmolarity or under anaerobic growth conditions in the wild-type strain is also

present in the *pilBa* mutant grown under standard conditions. These data indicate that the modulation observed in the level of some proteins in the mutants mimics that observed in the wild-type strain under these growth conditions.

We also compared the ability of the wild-type strain and the different mutants to adapt their protein level when shifted to different growth conditions. The *pilAk* mutation abolishes or reduces the ability to modulate production of various proteins according to environmental conditions, e.g. under anaerobiosis (Fig. 4, compare lane 3 to lane 7) or at 41 °C or under high osmolarity (data not shown). The mutation *pilBa* affects the changes in protein profile at least under high osmolarity (Larribe *et al.*, 1991) and acidic conditions (data not shown). The *pilA*⁺/*pilAa* mutant conserved the ability to change the relative amount of different proteins at least after the switch to anaerobiosis (Fig. 4, compare lane 1 to lane 10).

As the growth conditions tested in this study may be viewed as stress conditions, we next analysed the level of the gonococcal protein Gsp63, which is related to the GroEL protein of *Escherichia coli*. Gsp63 has been reported to be increased in gonococci grown under stress conditions (Pannekoek *et al.*, 1992). We used monoclonal antibodies directed against epitope G6 of the Gsp63 protein to measure the level of this protein (Pannekoek *et al.*, 1992). When compared to the wild-type strain, the level of Gsp63 was increased under standard growth conditions in the *pilA*⁺/*pilAa* heterodiploid, confirming previous results (Fig. 5) (Taha *et al.*, 1992). Under growth at 41 °C, the *pilBa* mutant and the *pilA*⁺/*pilAk* heterodiploid showed an increase in the level of Gsp63, while the *pilA*⁺/*pilAa* heterodiploid showed a decrease in the level of this protein (Fig. 5). Surprisingly, the level of Gsp63 decreased in the wild-type strain and in the *pilA*⁺/*pilAa* heterodiploid mutant under anaerobic growth conditions while it increased in *pilA*⁺/*pilAk* and *pilA*⁺/*pilAk.pilBa* mutants. These data indicate that gonococci undergo an adaptive response upon exposure to stress conditions and that the regulatory system *pilA/pilB* seems to be involved in the induction of this response.

Table 4. SDS-PAGE analysis of total proteins of cells grown in different modified media compared to standard medium

Mol. mass (kDa)	Wild-type MS11*			MS11 mutants under standard growth conditions†		
	Osmolarity	41 °C	Anaerobiosis	<i>pilA</i> ⁺ / <i>Aa</i>	<i>pilA</i> ⁺ / <i>Ak</i>	<i>pilBa</i>
180		+		+		
170	+		+		—	+
150			—			
120	+		+			
100	+					+
97		—	—			
95		—				
90	—	—	—			
83	+		+			
80		—				
73	—	—	—		+	—
66	+	+	+	+	—	+
63	—	—	—	+	+	—
57	—	—	+	—		—
53	+		+			+
45	—	—	—	—		—
43	+	—	—	+	+	
41	+	+	+	+		
39		—	—			
38			—			
35	—	+	—		+	—
33	—	—	—	+	+	
31		+	—	+	+	
27					+	
25	+		+		—	
22			—			
20	—	—	—	—	+	+
19	+	—	—	+	—	
15.5			—			

* High osmolarity was obtained by addition of NaCl, KCl, sucrose or urea. + or — indicates an increase or a decrease compared to the standard conditions of growth for strain MS11.

† Bacteria were grown under standard conditions. + or — represents proteins which increase or decrease in amount compared to the wild-type strain observed under standard conditions.

DISCUSSION

In vitro, *N. gonorrhoeae* can only grow under a limited range of environmental conditions. Changes in temperature, pH, salts or amino acid concentrations between values that do not affect growth of enterobacteria are very unfavourable for the growth of *N. gonorrhoeae*. However, the gonococcus is able to adapt to several different infection sites where it will be under nutrient limitation or stress conditions. This adaptation is the paradigm of this strictly human pathogen. In the present study, we attempted to analyse the effects of environmental factors on the growth and on the level of pilin, one of the major virulence factors in *N. gonorrhoeae*. We have shown that growth is highly sensitive to, and pilin gene expression moderately affected by, a number of environmental factors. We describe a direct cor-

relation between growth rate and pilin expression under the majority of the conditions tested (high osmolarity, anaerobiosis, high temperature). Taken together, our results suggest that growth limitation represses the expression of the pilin gene. Our data are in favour of an adaptation behaviour of gonococci to multiple stress signals, as suggested by the numerous conditions that reduce growth and pilin expression simultaneously. The exception of iron starvation, which stimulates pilin gene expression, may be irrelevant since the gonococcus is able to use the lactoferrin and transferrin of the host as an iron source *in vivo* (Biswas & Sparling, 1995; Cornelissen & Sparling, 1996).

It may be advantageous for the gonococcus to vary the level of pilin gene expression according to growth conditions. For instance, a transient reduction in the

Fig. 4. Silver-nitrate-stained SDS-PAGE gel of total cell extracts of *N. gonorrhoeae*. Bacteria were from anaerobic (lanes 1–5) or aerobic (lanes 6–10) cultures. The strains analysed were MS11.pilA⁺/pilAa (lanes 1 and 10), MS11.CAT-2 (lanes 2 and 6), MS11.pilA⁺/pilAk (lanes 3 and 7), MS11.pilA⁺/pilAk.pilBa (lanes 4 and 8) and MS11-A (lanes 5 and 9). Molecular mass markers are shown to the right. Arrowheads on the left indicate the proteins which show variable levels.

pilin level under unfavourable growth conditions might allow the dissemination of bacteria to other sites more favourable for growth, piliation and colonization. Moreover, this modulation of piliation could be useful for the invasion step, as non-piliated gonococci are more efficiently internalized (Makino *et al.*, 1991).

In other pathogenic bacteria, different variations of the growth conditions modulate regulatory genes for virulence factors [*bvgAS* in *Bordetella pertussis* (Manetti *et al.*, 1994; Graeff-Wohlleben *et al.*, 1995; Prugnola *et al.*, 1995), *regA* in *Pseudomonas aeruginosa* (Franck *et al.*, 1989; Prince *et al.*, 1991) or *toxR* in *Vibrio cholerae*

(Di Rita, 1992)]. This modulation is generally more important for the toxin-encoding genes (10–100-fold) than for the genes implicated in pilus or flagellum biogenesis (2–5-fold). In *N. gonorrhoeae*, our studies on the variations in the levels of pilin and other proteins that are affected by environmental changes indicate that the products of *pilA* and *pilB* participate in this control. The modification of *pilE* expression observed in the presence of isoleucine, urea, under anaerobic growth, high osmolarity (NaCl or KCl addition) or at pH 6 are lost in the *pilBa* mutant. These results support the hypothesis that PilB helps transduce different signals to modify *pilE* expression. In addition, the *pilBa* mutant showed an increase in the level of several proteins, some of which are related to stress response, such as Gsp63.

Analysis of *pilA* mutants confirms that their phenotypes depend on the site of insertional inactivation of *pilA*. The *pilAk* mutation provokes the loss of *pilE* modulation in response to environmental changes. These effects may be explained by the absence of interaction between the PilAk and PilB proteins. Indeed, it has been previously shown that PilA and PilB interact to regulate *pilE* expression (Taha *et al.*, 1991; Taha & Giorgini, 1995).

The *pilA*⁺/*pilAa* heterodiploid has a reduced level of pilin, which is nevertheless modulated by a majority of the tested conditions in the same manner as the wild-type strain (exceptions are high osmolarity and addition of isoleucine). Moreover, the variations in the protein profiles observed in the wild-type strain are also found in this mutant (data not shown). These results could be explained by the conservation of the interaction between PilAa and PilB but an impaired transcriptional activation of the *pilE* gene. The two mutants of the *pilA* gene (*pilAa* and *pilAk*) thus show different responses to environmental stress and could be representative of mutations affecting different functional domains of PilA. Indeed, we have previously shown that PilA has different functional domains (Taha & Giorgini, 1995) and that the observed phenotypes in the heterodiploids are not due to the locations of the insertions of the inactivated *pilA* but rather to the impaired function of PilA (Taha *et al.*, 1988, 1996; Taha, 1993).

PilA/PilB regulatory proteins seem to have pleiotropic effects on the level of several gonococcal proteins. Moreover, this system is involved in the response of gonococci to environmental variations. The identification of the genes encoding these proteins and the analysis of their expression should help to understand

Fig. 5. Western blot analysis of whole-cell extracts of *N. gonorrhoeae* using anti-Gsp63 antibody. Bacteria were grown at 41 °C (lanes 1–5) or 37 °C (lanes 6–10) or under anaerobiosis (lanes 11–14). The strains analysed were MS11-A (lanes 1, 6 and 11), MS11.pilA⁺/pilAa (lanes 2, 7 and 12), MS11.pilBa (lanes 3 and 8), MS11.pilA⁺/pilAk (lanes 4, 9 and 13) and MS11.pilA⁺/pilAk.pilBa (lanes 5, 10 and 14).

the adaptation to environmental variations developed by *N. gonorrhoeae*.

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