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PROMOTER ANALYSIS OF THE *porA* GENE OF *NEISSERIA MENINGITIDIS*

by

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July, 1998

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

TABLE OF CONTENTS

ABSTRACT

Previous work has shown that some *Neisseria meningitidis* strains do not express the Class 1 Outer Membrane Protein (OMP1; the *porA* gene product) as assessed by SDS-PAGE analysis of OMPs. Northern analysis has identified the level of control of *porA* expression as transcriptional. Promoter regions differed in the number of guanosine residues in a poly(G) track located between the -10 and the -35 regions. Strains that do not express the protein either had an adenosine residue within this poly(G) track or contained nine or less guanosine residues in this track. We investigated the role of the promoter region of *porA* in the regulation of transcription. The promoter regions of the *porA* gene from three strains, two of which do not express the OMP1, were analyzed. Promoters were cloned into a promoter-probe vector (pDN19*lac*Ω) possessing a promoterless *lacZ* gene and expressed in *Escherichia coli.* Transcription activities of cloned promoter regions were measured by β-galactosidase assays. The *porA* promoter of strains that did not express the class 1 OMP contained an adenosine residue in their poly(G) track and had lower transcriptional activities when assayed by β-galactosidase activity than the one without the adenosine residue. To assess the importance of spacing and the role of nucleotides between the -10 and the -35 regions, site-specific mutations in this poly(G) track were performed. From the transcription activities of these modified promoters, spacing between the -10 and the -35 regions influenced promoter activity; reduction of the poly(G) stretch by one and two guanosine residues reduced the activity. Replacement of the adenosine residue within the poly(G) track by a guanosine residue increased promoter activity while replacement of a guanosine residue by an adenosine residue decreased the activity. Both the spacing of the poly(G) track and the identity of the nucleotides in this region therefore affect promoter activity.

ABRÉGÉ

Des études précédentes ont démontré que certaines souches de *Neisseria meningitidis* n'expriment pas la protéine de membrane extérieure de classe 1 (OMP1; le produit du gène *porA*) tel que démontré par l'analyse SDS-PAGE des protéines de membrane extérieure. L'analyse Northern a identifié le niveau de contrôle du gène *porA* comme étant transcriptionnel. Les régions du promoteur avaient un nombre différent de résidus guanosines dans un fragment linéaire poly(G) situé entre les régions -10 et -35. Les souches qui n'exprimaient pas la protéine avaient ou bien un résidu adénosine dans ce fragment linéaire poly(G) ou bien un nombre de résidus guanosines inférieur ou égal à neuf. Nous avons étudié le role de cette région du promoteur de *porA* dans la régulation de la transcription de ce gène. Les régions du promoteur de *porA* dans trois souches, dont deux qui n'expriment pas OMP1, furent analysées. Les promoteurs furent clonés dans un vecteur (pDN19*lac*Ω) qui possède un gène *lacZ* dépourvu de promoteur et furent exprimés dans *Escherichia coli*. Les activités de transcription de ces promoteurs clonés ont été mesurées par l'essai β-galactosidase. Les promoteurs du gène *porA* des souches n'exprimant pas OMP1 possédent un résidu adénosine dans leur fragment linéaire poly(G) et ont eu une activité β-galactosidase plus basse que celle ne possèdant pas d'adénosine. Pour évaluer l'importance de l'espace entre les régions -10 et -35 ainsi que l'importance des résidus dans cette région, nous avons procédé à la mutagénèse dirigée de ce fragment linéaire. Nous avons déduit des activités de transcription obtenues pour les promoteurs modifiés que la réduction du nombre de résidus guanosine par un et deux résidus réduisait l'activité. Le remplacement du résidu adénosine dans le fragment linéaire poly(G) par un résidu guanosine augmentait l'activité du promoteur tandis que le remplacement d'un guanosine par un adénosine la réduisait. L'activité du promoteur de *porA* est donc affectée par l'espace entre les régions -10 et -35 ainsi que par l'identité des nucléotides dans cette région.

ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr. Elaine L. Mills and Dr. Francis F. Arhin for their continuous help, support and guidance throughout my graduate studies. I am also extremely grateful to France Moreau for her technical expertise and all her help in my experimental work.

I would also like to thank Dr. James W. Coulton for his help and advice as well as Dr. Gregory T. Marczynski for his helpful suggestions. I am also grateful to Mme. Manon Lorange for providing us with the *N. meningitidis* strains, to Kirsty Salmon for providing the pDN19*lac*Ω vector and to Dr. Michael Apicella for his generous contribution of the pAErmC'G vector.

This thank you extends to my closest friends and my fellow students from the Department of Microbiology and Immunology. I would also like to thank Thierry Casiez for always being there for me in spite of the distance. I am also grateful to my brother and sister for their support.

Finally, I would like to express my deepest gratitude to my parents, Lamia and Wajih, for their continuous love, support and guidance and for giving me the confidence and the determination to achieve any goal.

The financial support of a scholarship from the Fonds pour la formation de Chercheurs et l'Aide à la Recherche (FCAR) is gratefully acknowledged. This research was supported by a grant from For the Love of Kids Foundation, Montreal, Québec.

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

A	adenosine
C	cytidine
DNA	deoxyribonucleic acid
G	guanosine
g	gram
IS	insertion sequence
kb	kilobase-pair
μg	microgram
μl	microliter
μM	micromolar
mg	milligram
ml	milliliter
mM	millimolar
nt	nucleotide
RNA	ribonucleic acid
T	thymidine
Tn	transposon
v/v	volume per volume
w/v	weight per volume

PART I: ANALYSIS OF PROKARYOTIC PROMOTERS

Many of the fundamental principles of gene regulation were first described by Jacques Monod and François Jacob in the 1940s and 1950s. The relative simplicity of the mechanisms they had discovered led them to propose that regulation by repression, induction and feedback inhibition of enzyme activity provided the basis of gene regulation (Yanofsky, 1992).

Besides the regulation of enzyme activity, powerful molecular genetic strategies have allowed us to more recently identify numerous "control features" in cells. These include structural elements that establish promoter strength, messenger RNA stability and translation initiation efficiency. Gene expression is therefore influenced by a combination of both control features and enzyme regulation (Yanofsky, 1992). The discovery of the first promoter in *Escherichia coli* has established this bacterium as the model system for the study of promoters in other prokaryotes.

1. Classical *Escherichia coli* promoters

The genes on the *E. coli* chromosome are frequently organized into independent units of transcription called operons (Reznikoff and McClure; 1986). Each unit contains a DNA sequence termed a promoter that signals the start of transcription initiation, and another sequence that signals transcription termination. The RNA polymerase (RNAP) core enzyme is responsible for carrying out all the steps of transcription except initiation at the promoter. An additional protein called sigma factor is responsible for the efficient recognition of the promoter by the polymerase. The combination of the RNAP core enzyme and the sigma factor constitutes the RNAP holoenzyme. In *E. coli*, the major sigma factor, σ^{70}, allows transcription initiation in cells undergoing exponential growth under normal conditions. When cells are under environmental stress, alternative

sigma factors become important (Burgess *et al.*, 1969; Travers and Burgess, 1969; Helmann and Chamberlain, 1988; Lonetto *et al.*, 1992).

Nucleotide sequence comparisons in *E. coli* have identified two crucial sequence elements at promoters for RNAP-σ^{70}. These are two conserved six-base-pair sequences: the -35 hexamer, located near position -35 relative to the transcription start site and having the consensus sequence 5'-TTGACA-3', and the -10 hexamer, located near position -10 relative to the transcription site and having the consensus sequence 5'-TATAAT-3' (Gross *et al.*, 1992). The importance of these consensus sequences has been confirmed by genetic analyses and footprinting studies (Youderian *et al.*, 1982; Siebenlist *et al.*, 1980). The length of the spacer region between the two hexamers is critical, with an optimal length of 16 to 18 bp. Analysis of the nucleotides in the spacer region has not revealed a conserved sequence (Harley and Reynolds, 1987).

1.1. Defining promoters

Different approaches have been used to define promoters, such as mutational analysis, determination of the 5'-end of the mRNA and binding experiments. In all cases, a final definition of a promoter requires a combination of *in vivo* and *in vitro* experiments (Reznikoff and McClure, 1986).

1.1.1. Mutational analysis

With the advent of recombinant DNA technology, the construction of promoter cloning vectors became possible. These vectors, which are plasmids or phages, provided new *in vivo* approaches to the identification of promoter-containing DNA sequences (Reznikoff and McClure, 1986). They have unique cloning sites located upstream from a gene encoding a function that is easily assayed or selectable: *galK* (galactokinase), *lacZ* (β-galactosidase), *cat* (chloramphenicol acetyltransferase) or *tetA* (tetracycline resistance) (An and Friesen, 1979; West *et al.*, 1979; Casadaban and Cohen 1980; Mandecki and

Reznikoff 1982; Bertrand *et al.*, 1984; Wertman *et al.*, 1984; Munson *et al.*, 1984).

The shotgun cloning of unmapped fragments into the plasmid's cloning site(s), upstream of the indicator gene, allows the identification of fragments containing a promoter by the expression of this reporter gene. These plasmids are also useful in determining the orientation of the promoter within a fragment and in obtaining a qualitative estimate of its level of activity (Reznikoff and McClure; 1986). Random mutagenic treatments as well as the generation of deletions can define one or the other boundary of a promoter (Yu and Reznikoff; 1984) and site-directed mutagenesis can define important sequences in the promoter region (LeClerk and Istock, 1982; Munson *et al.*, 1984).

1.1.2. Determination of the 5'-end of mRNA

The determination of the 5'-end of mRNA is an important piece of information that allows the structural analysis of promoters by determining the transcription start site (Reznikoff and McClure; 1986). In order to determine the 5'-end of mRNA *in vitro*, total RNA is isolated from the cell of interest *in vivo*. The RNA products are subsequently subjected to primer extension analysis *in vitro* and the transcription start site is determined by the comparison of the DNA product and the sequencing of the original DNA fragment using the same primer used for extension of the RNA (Reznikoff and McClure, 1986). In some cases, such as in the *lacZ* system, the determination of an *in vitro* transcription start site was not as easy to verify *in vivo* (Reznikoff *et al.*, 1982, McClure *et al.*, 1982; Peterson and Reznikoff 1984a). In most systems however, there is a general concurrence of the *in vivo* and *in vitro* start site determinations (Reznikoff and McClure; 1986). This suggests that there is a high degree of fidelity between promoter function and recognition *in vitro* and *in vivo*. *In vitro* transcriptional analysis is postulated to advance our understanding of the *in vivo* control of transcription initiation (Reznikoff and McClure; 1986).

1.1.3. Binding experiments

A variety of complementary protocols have been used to define promoter-like elements. These include binding experiments, which rely on the ability of a particular DNA fragment to form a stable open complex with RNA polymerase (Reznikoff and McClure, 1986). The formation of these complexes implies the presence of a promoter sequence in the DNA fragment. However, one must not oversimplify this assumption. It has been observed that some *bona fide* promoters do not result in high-efficiency binding while some sequences that do not appear to be promoters bind to the RNAP *in vitro*. The latter can be one of two classes. The first, called tight binding sites, are unrelated to known promoters and lie at random locations (Kadesch *et al.*, 1980; Melancon *et al.*, 1982). The second class includes the P2 sequence in the *lac* controlling elements (Peterson and Reznikoff 1984b; Malan and McClure 1984; Spassky *et al.*, 1984).This region can form open complexes with RNAP *in vitro* but it does not contain the *lac* consensus promoter.

1.2. Structural analysis

The structural features of the promoter are considered to be important elements in promoter function. Changes in the DNA sequence, RNA polymerase binding and alterations in DNA conformation and supercoiling often have large and specific effects on promoter function.

1.2.1. Sequence analysis

The most highly conserved bases in the -35 and -10 regions of 112 promoters analyzed in *E. coli* were the TTG in the -35 hexamer TTGACA and the TA---T in the -10 region TATAAT (Hawley and McClure, 1983). It was shown that the consensus sequence conferred the highest promoter activity: promoter mutations that decrease homology to the consensus sequence decrease

promoter strength; base-pair alterations that increase homology to the consensus sequence increase promoter activity.

Another feature of the promoter shown to be important for its proper function is the spacing between the -10 and the -35 regions. *In vivo* and *in vitro* studies have shown that optimal spacing between the -10 and the -35 regions is 17 base pairs +/- 1 base pair. Similarly, the distance between the -10 hexamer and the transcriptional start site is approximately 7 base pairs +/- 1 base pair (Reznikoff and McClure, 1986).

Recently, it has been established that, in addition to the -35 and -10 elements, there is a third important sequence element at some *E. coli* promoters (Newlands *et al.*, 1992; Ross *et al.*, 1993; Rao *et al.*, 1994). The transcription activity of the promoters of the *rrn* genes, which encode ribosomal RNA, is increased by a DNA sequence called the Upstream Activator Region (UAR). This AT-rich sequence of approximately 20 bp is located upstream of the -35 region and can be recognized by the RNAP itself. Its exact location with respect to the transcription start site varies from one gene to another.

Promoter strength is therefore likely to be a function of all three promoter elements, with strong promoters having near-consensus sequences and spacing and UARs, and weak promoters having non-consensus promoter elements (Busby and Ebright, 1994).

1.2.2. Protection and sequence

In general, the protection experiments that have been performed on a large number of promoters have shown that recognition of the promoter by RNAP occurs largely from one side of the helix, although RNAP may bind to the DNA in a "wrap-around fashion" (Siebenlist *et al.*, 1980).

The *E. coli* RNAP holoenzyme protects a region of approximately 65 bp when it binds the promoter. The pattern of protection from DNaseI digestion is approximately the same for many promoters, although some differences may be observed between strong promoters and weak promoters (Von Hippel *et al.*,

1984). It is likely that an electrostatic component (Shaner *et al.*, 1983) as well as specific hydrogen bonding to base pairs (von Hippel *et al.*, 1984) are both involved in the binding of RNAP to the DNA.

1.2.3. DNA conformation

The DNA near the promoter region that extends beyond the consensus sequences plays an important role in promoter function, for the simple reason that the DNA present upstream and downstream of the promoter is required for polymerase binding. Furthermore, changes in DNA structure as well as "actions-at-a-distance" are thought to influence gene expression (Reznikoff and McClure, 1986).

An example of changes in DNA structure is the twisting of the DNA (Stefano and Gralla, 1982a/b), which may relate in turn to the supercoiled state of the helix and have several effects on promoter function. In addition, several lines of evidence have shown that certain DNA sequences can exhibit bends within the linear duplex (Kolb *et al.*, 1983; Wu and Crothers, 1984) or kinks, such as the *Eco*RI binding site when complexed to the endonuclease (Frederick *et al.*, 1984). The DNA molecule can therefore show polymorphism and can assume several different conformations in addition to the B-form. One must bear in mind however that small changes such as twisting and bending may not be significant on a structural basis that relates to promoter function (Reznikoff and McClure, 1986).

The effect of supercoiling on promoter activity is considered an example of action-at-a-distance. DNA gyrase can bind at many sites on the DNA strand and introduce negative supercoils into the double helix. The energy input at any one site can be transmitted at a distance to a promoter site where it may be utilized by the RNAP to unwind the DNA and initiate transcription (Reznikoff and McClure, 1986).

In conclusion, several biochemical studies have allowed scientists to identify promoters with a high degree of confidence and to estimate their activity level.

This fundamental information was used by Soberon *et al.*(1982) to construct a totally synthetic yet functional promoter.

2. Emerging types of promoters

The extensive amount of research poured into the effort to define a universal model of prokaryotic promoters has identified a large number of promoters from numerous systems. As previously stated, the important features of a promoter include the -10 and the -35 hexamers as well as the sequences present both upstream and downstream of the promoter. Initial studies which explored those features showed many similarities to the previously defined *E. coli* consensus promoter, yet more recent studies are revealing many differences in several organisms including *E. coli*. Below are reported a few examples of these newly identified bacterial promoters.

2.1. The -10 and the -35 hexamers

2.1.1. Extended -10 regions

It has been observed that many promoters which are subject to positive control *in vivo* have relatively poor homology to the consensus sequence in the -35 region. The positively regulated λ P_{RE} promoter, which is barely recognizable by the RNAP, is dependent on a transcriptional activator protein encoded by the λ gene *cII* (Shimitake and Rosenberg, 1981). The -35 and the -10 regions of this promoter show poor homology to the consensus sequence in *E. coli* and the cII protein binds to a tetranucleotide repeat sequence, $TTGC(n)_6TTGC$, with $(n)_6$ lying precisely at the -35 position. In 1987, Keilty and Rosenberg constructed a constitutive derivative of the P_{RE} promoter called P_{RE}* which contains a perfect -10 sequence (5'- TATAAT -3') and a perfect -35 region (5'- TTGACA -3'). Mutational analysis showed that an efficient cII-independent initiation of

transcription required additional sequences immediately upstream of the -10 region in the absence of a consensus -35 hexamer. Based on these results and earlier evidence (Backendorf *et al.*, 1983; Inokuchi *et al*, 1984; Grana *et al.*, 1985; Ponnanbalam *et al.*, 1986), it was confirmed that the lack of a perfect consensus -35 region required an "extended -10" sequence 5'- TGnTATAAT -3' (Keilty and Rosenberg, 1987). Harley and Reynolds (1987) identified 41 natural *E. coli* promoters with an "extended -10" motif. It should be noted though that 37 of the 41 have at least three out of six base pairs agreeing with the consensus -35 region. Some of them also depend on accessory proteins for initiation *in vivo*. The four exceptions (*aroH*, *htpR*P1, *Tn51*R and *lacI*) have two out of six nucleotides matching the consensus -35 region. Additional promoters with an extended -10 region identified by Kumar *et al.* (1993) and others (for example, *spcP*, *uvrD*P1, *gyrA*P and *infA*P1) also showed good or moderate matches to the -35 consensus sequence. Therefore, it can be concluded that there are no clear examples of "pure" extended -10 promoters, which lack any match to the -35 consensus sequence or are independent of a protein activator. "Addition" of the extended -10 region to normal *E. coli* consensus promoters was shown to increase promoter activity (Kumar *et al.*, 1993). It may be concluded that the -10 extension motif is rarely used in *E. coli* as the sole adjunct to the -10 hexamer. Its role may be limited to strengthening promoters showing homology to consensus promoters.

Studies in other systems have shown that extended -10 regions occur frequently in gram-positive organisms (Graves and Rabinowitz, 1986). The comparison of promoters from different organisms such as *Bacillus subtilis*, *Staphylococcus aureus* and *Clostridium pasteuranium* revealed the conservation of the TG residues at positions -15 and -16, thus demonstrating the existence of an extended -10 region in these bacteria (Graves and Rabinowitz, 1986). Furthermore, mutations in the sequence TGTG, extending from positions -18 to

-15 (-16 region) in the σ^A promoter of the *amyP* gene in *B. subtilis* caused a decrease in promoter activity (Voskuil *et al.*, 1995). A G to C transversion at position -16 was the most detrimental mutation. Taking into account the similarity between *E. coli* σ^{70} and *B. subtilis* σ^A promoters, these results suggest that the -16 might be an additional region of the promoter in *Bacillus subtilis* (Voskuil *et al.*, 1995).

Another organism containing an extended -10 region is *Streptococcus pneumoniae*. Its *DpnII* restriction modification system gives rise to a polycistronic transcript which encodes both methylase genes *dpnM* and *dpnA*, as well as an endonuclease gene *dpnB* (Sabelnikov *et al.*, 1995). Base-substitution and deletion analysis have shown that the promoter for this transcript consisted of an extended -10 site, TaTGgTATAAT, with no required -35 site (Sabelnikov *et al.*, 1995).

2.1.2. Mycobacterial and streptomycete promoters

A study of the mycobacterial transcriptional apparatus has also revealed the lack of a conserved -35 site for transcriptional initiation (Bashyam *et al.*, 1996). Analysis of randomly isolated promoters from *Mycobacterium smegmatis*, *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG showed that their -10 regions are highly similar to those of *E. coli*, contrary to their -35 regions, which tolerate more sequence variety. The lack of a conserved -35 region therefore appears to be a feature of mycobacterial promoters. Such features in transcriptional signals have also been reported for *Streptomyces* species. A study of 139 streptomycete promoters (Strohl, 1992) has shown that although some share sequences at the two hexamers and display similarities to the *E. coli* consensus sequence, a majority (80%) do not function in *E. coli*, do not have a consensus -35 region and do not show any homology in the -35 hexamers to *E. coli* promoters. The heterogeneity in the -35 regions of *Streptomyces* is explained by the presence of multiple sigma factors (Buttner, 1989; Westpheling

et al., 1985) and the potential for a high degree of transcriptional flexibility. A comparison of the binding domains of the principal sigma factors of *M. smegmatis, Streptomyces aureofaciens* and *E. coli* showed that all three had nearly identical -10 binding domains. The -35 domain, however, is nearly identical in the *M. smegmatis* and *S. aureofaciens* sigma factors, but vastly different from the one in *E. coli* (Bashyam *et al.*, 1996). The broad ranges of metabolism encompassed by the promoters described by Strohl (1992) suggest that sequences other than the -10 and -35 regions are important for transcriptional regulation.

1.2.3. Other promoters

Analysis of promoters from *Clostridium xyli* subsp. *cyodontis*, a gram-positive endophytic coryneform plant bacterium, also revealed differences to the *E. coli* consensus promoter (Haapalainen *et al.*, 1996). Examination of the DNA sequences at the -10 and -35 regions showed a variety of possible sequences that define these regions. There were no similarities to the *E. coli* promoters observed. Although the *Clostridium xyli* subsp. *cyodontis* promoters are different from *Streptomyces* promoters, a resemblance to the -35 regions of individual promoters sharing a consensus sequence in *Streptomyces griseus* and *Streptomyces clavuligerus* was noted (Haapalainen *et al.*, 1996).

In *Chlamydia trachomatis*, the plasmid coutertranscript (PCT) promoter of the cryptic 7.5 kb plasmid functions with *E. coli* RNAP *in vivo* and *in vitro* (Fahr *et al.*, 1992, Ricci *et al.*, 1993). The PCT promoter contains an *E. coli*-like consensus sequence with a -10 (TATAAT) box and a -35 (TTGACA) box separated by 18 bp. Although these sequences show a perfect match to the *E. coli* consensus, many single mutations in the -10 and the -35 region did not alter promoter function in *C. trachomatis*, while multiple mutations in both hexamers rendered the promoter inefficient or ineffective. The sequence requirement for chlamydial promoters therefore differs from that of *E. coli* and chlamydial RNAP was shown

to be able to tolerate more variety at the two hexamers than did *E. coli* (Mathews *et al.*, 1994).

2.2. Analysis of sequences upstream and downstream of the promoter

The analysis of DNA sequences upstream of the transcriptional start site of *sapA*, a spore coat gene in *Streptomyces coelicolor*, did not show any similarities to previously described promoters (Im, 1995). Sequences 5' of position -8 were found to be not essential for *sapA* promoter function, with transcription initiation still maintained faithfully, although greatly reduced, over the course of the life cycle of the bacterium in the presence of a -8 to +10 fragment. The finding that the *sapA* promoter may not utilize the typical prokaryotic -35 and -10 hexamers can once again be explained by the large number of σ factors found in *S. coelicolor*. Maximal activity of the *sapA* promoter is also maintained by two putative activation sites located 50 to 200 bp upstream and downstream of the transcription start site (Im, 1995).

The UAR of *rrnB* P1 in *E. coli* was defined as extending from approximately -154 to -50 with respect to the transcription start site (Gourse *et al.*, 1986) and having an AT-rich sequence (Koo *et al.*, 1986). The UAR can function in a factor-independent way by increasing the association of RNAP and *rrnB* P1 (Leirmo and Gourse, 1991) or in a Fis-dependent manner (Ross *et al.*, 1990). Josaitis *et al.*, (1996) found that a C to T change at position -37 in *rrnB* affected promoter activity while leaving the UAR fully functional. A substitution of bases -38 to -40 maintained normal UAR activity, while the deletion of those bases severely reduced UAR-mediated activity (Josaitis *et al.*, 1996). It appears that UAR function is dependent on its position relative to the RNAP binding site, suggesting that a particular spatial geometry may be necessary for UAR-dependent activation to occur.

Pseudomonas putida contains a TOL plasmid, that has a σ^{70}-dependent promoter, Pm, and is stimulated by a XylS regulator. XylS is activated either by a benzoate effector (Franklin *et al.*, 1981; Inouye *et al.*, 1981; Ramos *et al.*, 1986) or by its overproduction (Inouye *et al.*, 1987, Mermod *et al.*, 1987). Like most positively regulated promoters, Pm does not exhibit -35 sequences similar to those of *E. coli* and has a regulator binding sequence adjacent to the -35 region. Deletion and transcription analyses have shown that this binding sequence appears to be organized as two T(C or A)CAN$_4$TGCA motifs located between positions -46 and -57 and between positions -67 and -78 (Gallegos *et al.*, 1996).

PART II: THE NEISSERIAE

Neisseria gonorrhoeae and *Neisseria meningitidis* are members of a small group of closely related gram-negative bacteria of the genus *Neisseria*.

These two diplococci are the only human pathogens in this group and they have no other known reservoir. In addition, other *Neisseria* species are commensal organisms that colonize humans. *Neisseria lactamica* and *Neisseria sicca* are commonly found in the mouth and upper respiratory tract of man. Although generally non-pathogenic, pigmented forms of *Neisseria flava* and *Neisseria perflava* have frequently been isolated in the nasopharynx and have been occasionally associated with disease (Gotschlich, 1980).

1. *Neisseria meningitidis* and meningitis

Neisseria meningitidis represents a major public health problem because of its epidemic nature and high mortality rate (Achtman, 1990). This human pathogen, also termed meningococcus, was first identified as a causative agent of bacterial meningitis by Anton Weichselbaum in Vienna in 1887 (Branham, 1956). It is also responsible for bacteremia, sometimes with septic shock, which results in death in about 20% of cases (Jones, 1995). Today, capsulated organisms such as *Neisseria*, *Streptococcus*, *Haemophilus*, *Staphylococcus* and *Pseudomonas* are recognized as important causes of mortality in developing countries.

1.1. Epidemiology and classification system

It has been estimated that more than 310,000 persons per year in developing countries suffer from infections caused by *N. meningitidis*, resulting in approximately 35,000 deaths (Robbins and Freeman, 1988). In the US and Canada, the incidence rates are at about 0.2-4 cases per 100,000 population (Jones, 1995). Steady low-incidence rates were reported in Canada between the

1940s and mid 1980s (Wang *et al.* 1993; Whalen *et al.* 1995). The mortality rates of meningococcal meningitis are approximately 7%, with age-specific death rates highest in infants and the elderly (Jones, 1995). In the early 1980s, the World Health Organization started a program of vaccine development for the prevention of endemic and epidemic disease in infants (Robbins and Freeman, 1988).

The meningococcus has been extensively studied and a large body of knowledge about its epidemiology, pathogenesis, surface components and host defense mechanisms is presently available.

Antigenic variability of *N. meningitidis* is attributable to four different surface structures on the bacterium (Verheul *et al.*, 1993): capsular polysaccharides (CPS), outer membrane proteins (OMP), lipopolysaccarides (LPS)/lipooligosaccharides (LOS) and pili (surface appendages). Meningococcal LPS was found to contain no O-antigen; therefore more recent studies refer to this component as LOS. The first three components provide the basis for classification of bacterial strains into serogroups, based on differences of the CPS, serotypes and serosubtypes, based on differences of the OMP, and finally immunotypes, based on differences in the oligosaccharide structure of LOS.

N. meningitidis strains belonging to serogroups A, B and C are responsible for the largest number of cases of meningococcal meningitis (Verheul *et al.*, 1993). Group A is the predominant group during both epidemic and endemic situations in Africa, whereas groups B and C are prevalent in the western world during endemic periods and localized outbreaks of the disease (Verheul *et al.*, 1993). Epidemiological analyses based on serotyping and serosubtyping have been used to identify different meningococcal strains of specific clonal types associated with disease in several geographical locations (Cruz *et al.* 1990; Ringuette *et al.* 1992; Sacchi *et al.* 1992). Outbreaks in each location have been associated with the emergence of specific strains of a certain clonal type (Arhin *et al.*, 1998).

1.2. Vaccine development

During the course of meningococcal carriage or infection, bactericidal antibodies directed against both the capsular polysaccharides and the outer membrane proteins are produced (Frasch, 1983). Immunization with either one of these antigens would therefore be expected to provide immunity to subsequent infections. A commercial tetravalent vaccine based on purified capsular polysaccharides (CPS) from meningococcal serogroups A, C, Y and W-135 is currently available and it provides protection against four of the five major disease-causing serogroups (Hankins *et al.*, 1982; Peltola *et al.*, 1985). Another vaccine, a bivalent polysaccharide vaccine, provides protection against serogroups A and C (Frasch, 1995). Protection by both vaccines however is effective only in adults and children over two years of age; infants, who are more prone to disease, are not protected (Reingold *et al.*, 1985; King *et al.*, 1996). The tetravalent (CPS) vaccine is routinely administered to military personnel in the United States and it is recommended for travelers going to areas with recent epidemic disease, such as the meningitis belt in Africa (Verheul *et al.*, 1993).

There is currently no vaccine available against serogroup B, one of the predominant cause of meningococcal disease in temperate climates. The polysaccharide of this serogroup is known to be poorly immunogenic, even in adults (Wyle *et al.*, 1990). This may be due to its cross-reaction with polysialic antigens present in human tissues during embryonal development (Finne *et al.*, 1983).

The limitations of the polysaccharide vaccines have drawn attention to the use of other approaches to develop effective vaccines, which include the use of OMPs as potential vaccine components (van der Ley and Poolman, 1992).

2. Outer membrane proteins of *Neisseria meningitidis*

2.1. Description and typing value of OMPs

The meningococcal outer membrane contains a limited number of major proteins, which have been divided into five distinct classes on the basis of their molecular mass M_r. *N. meningitidis* strains usually express four of the five major outer membrane proteins or OMPs (Tsai *et al.*, 1981): the class 1 protein (PorA; M_r 42,000); class 2 or the class 3 protein (PorB; M_r 30,000 - 35,0000); the class 4 protein (M_r 33,000) and the class 5 protein (M_r 28,000). PorB and PorA form the basis of strain identification by serotyping and serosubtyping, respectively.

2.1.1. Class 5 OMPs

Class 5 OMPs elicit bactericidal antibodies during infection (Poolman *et al.*, 1983) but exhibit intrastrain and interstrain antigenic variation. The class 5 proteins have been divided into opacity (Opa) proteins, which may be expressed in both *N. meningitidis* and *N. gonorrhoeae* (formerly called PII protein) (Sarkari *et al.*, 1994) and Opc proteins (formerly called 5C), the expression of which is restricted to *N. meningitidis* (Sarkari *et al.*, 1994). Class 5 proteins play a role in attachment to human cells, with Opa proteins mediating attachment to epithelial cells in the early stages of disease and Opc proteins mediating attachment to both endothelial and epithelial cells (Virji *et al.*, 1993). Because of their multiplicity and intrastrain antigenic variation, class 5 OMPs do not have any typing value.

2.1.2. Class 4 OMP

The class 4 OMP is antigenically stable and is thought to play a role in cellular attachment. It is encoded by the *rmpM* gene and is related to protein III of *N. gonorrhoeae* (Lytton *et al.*, 1986). A comparison of the DNA sequences of *rmpM* and *rmpG*, which encodes the gonoccoccal Protein III, showed a 95.9% homology. Furthermore, OMP4 shared a limited homology with *E. coli* OmpA

(Klugman *et al.*, 1989). It has been shown to induce antibodies capable of blocking the bactericidal activity of human immune serum against serum-resistant gonococci (Rice *et al.*, 1990). Because of its conservation among *N. meningitidis* strains, class 4 OMP does not have any typing value.

2.1.3. Class 2/3 OMPs

All meningococci express a class 2 or class 3 OMP. The expression of these two classes is antigenically stable and mutually exclusive within a strain. These proteins are therefore regarded as "alleles" of a single gene locus, *porB* (McGuinness *et al.*, 1993). Class 2 and class 3 proteins function as porins and are considered to be the equivalents of the gonococcal porin PIA, which forms anion-selective pores in the outer membrane through which small hydrophilic solutes can pass in a diffusion-like process (Murakami *et al.*, 1989). Despite their common function and their antigenic stability within a strain, class 2 and class 3 OMPs exhibit antigenic diversity between different strains, which is the basis of the serotype classification of meningococci (Frasch *et al.*, 1985).

2.1.4. Class 1 OMP

Virtually all isolates express a class 1 OMP, but variation in the levels of expression are observed (van der Ende *et al.*, 1995). Although the antigenic diversity of this protein forms the basis of serosubtyping of meningococcal strains, OMP1 shows antigenic stability within a single strain (Barlow *et al*, 1989). OMP1 is a pore-forming protein with cationic selectivity (Tommassen *et al.*, 1990) and is the product of the *porA* gene locus (Barlow *et al.*, 1989). Sequence comparison of *porA* and the gene encoding the gonococcal porin PIB has identified several regions which exhibit a high degree of homology (Barlow *et al.*, 1989).

The detailed study of the primary structure of OMP1, as well as that of other proteins, has advanced the understanding of the molecular determinants of their

antigenic variability and the identification of regions common to different strains. The transmembrane parts of OMP1, 2 and 3 are the parts which are highly conserved. The strain-variable domains, which play a role in host immune response, are located on three surface-exposed loops (Poolman *et al.*, 1995). This is of considerable interest in vaccine development, and also in devising new methods for serotyping and subtyping based on the amino acid sequence of the variable domains of OMP1 (Arhin *et al.*, 1998). Another method to characterize *N. meningitidis* strains is by DNA fingerprinting of *porA* and the assignment of a Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) pattern to isolates (Peixuan *et al.*, 1995; Speers and Jelfs, 1997).

2.2. Use of OMPs as vaccine components

In addition to their importance as serological markers, the outer membrane proteins provide potential targets for a protective immune response. Complement-mediated bactericidal killing of meningococci can be activated by monoclonal antibodies directed against OMPs. These antibodies show type- or subtype specificity (Verheul *et al.*, 1993). This has encouraged the development of new vaccines based on lipopolysaccharide-depleted outer membrane proteins (Milagres *et al.*, 1994) in order to avoid the toxic side effects of the lipid A component of LPS. The proteins are first isolated in the form of soluble outer membrane vesicles (OMVs) and are subsequently treated with detergent to remove LPS. To improve specificity, the proteins are complexed with one or more capsular polysaccharides (CPS) (Verheul *et al.*, 1993). Efficacy trials with such vaccines have recently been conducted in Chile and Norway, where the efficacy levels were approximately 50%. Trials in Cuba showed efficacy levels around 80% (Verheul *et al.*, 1993). These OMV-derived vaccines proved to be immunogenic in both young children and adults and the antibodies induced activated complement-mediated bactericidal killing of meningococci (Verheul *et al.*, 1993).

The hypervariability of the class 5 OMP as well as the antibody blocking effects of the class 4 OMP make these two proteins unsuitable candidates for vaccine components. Although OMP2 and OMP3 were shown to be immunogenic, they are mutually exclusive within a strain and display considerable diversity among strains, especially in serogroup B (Frasch, 1995). The *in vitro* bactericidal activity and infant rat protection capacity of a pool of monoclonal antibodies revealed a hierarchy of protective capacity, with OMP1> OMP2 or 3 (van der Ley and Poolman, 1992), thus showing that, among the major outer membrane proteins, OMP1 may be the most suitable candidate for a vaccine. Furthermore, OMP1 displays less antigenic diversity than OMP2 and OMP3. However, the use of OMP1 as a vaccine component is not without shortcomings. The antibodies induced by this protein are type-specific and are not protective against all *N. meningitidis* strains. To overcome this problem, Claassen *et al.* (1996) used a hexavalent OMV vaccine containing 6 different class 1 epitopes, thereby inducing broadly protective antibodies.

Although these results are encouraging, the discovery of the lack of OMP1 expression in some clinical isolates (van der Ende *et al.*, 1995; Arhin *et al.*, 1997a) has prompted the search for alternative approaches to vaccine development, such as the use of different cell surface antigens as vaccine candidates. These antigens include detoxified LPS/LOS (Frasch, 1995) as well as minor outer membrane proteins such as NspA (Martin *et al.*, 1997), which has been shown to elicit the production of bactericidal antibodies in mice.

3. Genetic analysis of the *Neisseriae*

The pathogenic properties of *N. meningitidis* and *N. gonorrhoeae* have been extensively studied. Since *Neisseria* species are exclusively human pathogens, no appropriate animal models exist for bacteria-host cell interactions. In the last few years, however, some insights into mucosal cell invasion were obtained by

cell culture systems (Stephens, 1989). Several bacterial components that promote the adherence and entry of *N. meningitidis* into epithelial cells have been identified. Pili are required for initial adherence (Stephens and Farley, 1991) and Opa and Opc proteins promote the entry of the bacteria into the host cell at a later stage (Virji *et al.*, 1993). The capsular polysaccharides enhance resistance to complement-mediated bacteriolysis and phagocytosis (Cross, 1990).

The use of genetic methods has fueled investigations of the biology of the Neisseriae because the tools to conduct molecular genetic studies have begun to be developed. The molecular characterization of virulence factors will provide more information on their regulation and their possible use as potential vaccine components.

3.1. Molecular techniques

Neisseria strains are naturally competent for DNA transformation. Transformation frequencies of 1% are easily attainable in recipient cells. Their transformation system resembles that of *Haemophilus influenzae* in that both bacteria only take up DNA containing species-specific transformation uptake sequences (Goodman and Scocca, 1988). Restriction endonucleases act on plasmid DNA during transformation if the DNA is isolated from a heterologous strain (Stein *et al.*,1988). Furthermore, chromosomal transformation occurs at a higher frequency than plasmid transformation and it occurs at much greater frequencies if there are resident, homologous DNA sequences in the recipient (Seifert and So, 1991). For these reasons, most transformations that introduce new DNA sequences into the bacterium rely on the insertion of loci into the chromosome by homologous recombination (Seifert and So, 1991). For many studies, chromosomal transformation is preferable to plasmid transformation since stable, single-copy insertions are produced. This is of particular importance

when studying gene expression where the particular number of elements encoded by these genes may influence the results (Seifert and So, 1991).

DNA transformation can be separated into two steps: DNA uptake and recombination. The first event requires that a 10 bp recognition sequence (5'-GCCGTCTGAA -3') be present on the DNA. Gonococcus and meningococcus can transform each other, suggesting that their DNA share the same uptake sequence. One uptake sequence is sufficient to introduce DNA into an organism. (Seifert and So, 1991). Competence for DNA transformation relies on the piliated status of the recipient bacterial cell. Transformation of non-piliated strains can however be achieved if higher amounts of DNA are used, but at much lower transformation frequencies than those detected for piliated strains (Seifert *et al.*, 1990).

Recombination into the chromosome involves *recA*. The identity of other enzymatic activities is unknown, but several mutations have been shown to interfere with different steps in transformation. For example, mutations in an 11 kDa outer membrane protein have been shown to interfere with uptake of DNA (Dorward and Garon, 1989). Furthermore, plasmid DNA is opened at random sites during uptake into the cell, which implicates a nonspecific nuclease activity during uptake into the cell (Seifert and So, 1991).

3.2. Promoter analysis and gene regulation

3.2.1. Gene regulation of lipooligosaccharides (LOS)

Lipooligosaccharides (LOS) are outer membrane glycolipids involved in meningococcal attachment and colonization of the human nasopharynx (Kahler *et al.*, 1996a). These components also influence resistance to complement-mediated killing in the bloodstream (Moran *et al.*, 1994). Many of the genes involved in LOS biosynthesis in *Neisseria* species are dispersed around the chromosome. The *lgtABCDE* glycosyltransferase gene cluster is an exception and it encodes the five genes necessary for the construction of the lactoside and

digalactoside oligosaccharide α chains of several LOS immunotypes (Kahler *et al.*, 1996a). Two glycosyltransferase genes in *N. meningitidis*, *rfaK*, an α-1,2-N-Acetylglucosamine (GlcNac) transferase gene (Kahler *et al.*, 1996b) and *lgtF*, a UDP-glucose: LOS - β -1,4 glucosyltransferase, constitute the lipooligosaccharide *ice* (inner core extension) biosynthesis operon and are co-transcribed as a polycistronic message. Analysis of the region upstream of the *lgtF* did not reveal any σ^{70} promoter sequences but showed the presence of a tRNA sequence. A consensus σ^{70} promoter (5 out of 6 matches in both the -10 and the -35 hexamers and an optimal spacing of 17 bp) was found upstream of the tRNA, suggesting that *lgtF* and *rfaK* are also transcribed from this promoter and regulated by the conditions that control tRNA expression (Kahler *et al.*, 1996a).

3.2.2. Promoter analysis of capsular polysaccharides

The disease-associated meningococcal serogroups B, C, Y and W-135 all have sialic acid in their capsular polysaccharides which confers resistance to host complement-mediated attack mechanisms (Poolman *et al.*, 1995). Serogroups B and C, which predominate in the Northern hemisphere, are homopolymers of sialic acids with α-2,8 and α-2,9 linkages, respectively (Hammerschmidt *et al.*, 1996a). Sialic acid is also found as a modification of the meningococcal LOS, with the substitution of the terminal galactose residue of LOS with sialic acid (Mandrell *et al.*, 1990). LOS sialylation is considered an important virulence factor in *N. meningitidis*. The lack of a vaccine against group B meningococcus and the importance of LOS sialylation and the polysialic acid capsule in the pathogenesis of this organism have encouraged the active study of the genetic basis for expression of these molecules.

Frosch *et al.* (1989) identified a 24 kb *cps* gene complex, composed of 5 distinct regions (E, C, D, A and B) as capable of expressing group B meningococcal capsule in *E. coli*. Region B contains two genes, *lipA* and *lipB*, which encode proteins proposed to add a phospholipid-anchoring group onto the

reducing ends of the capsule polysaccharide chain (Frosch and Müller, 1993). Region D contains *galE*, which is involved in LOS biosynthesis (Lee *et al.*, 1995), as well as other genes which were shown to be involved in the synthesis of rhamnose in *N. gonorrhoeae*. Region C was found to contain genes *ctrA, B, C* and *D*, encoding proteins which transport phospholipid substituted polysialic acid across the inner and outer membranes (Frosch *et al*, 1991). The *ctr* gene products show amino acid homology with the ABC superfamily of ATP-dependent active transporters (Frosch and Müller, 1993). Region A is involved in capsule biosynthesis and includes four linked genes, *synX*, *synC*, *synB* and *synD* (Stephens *et al.*, 1991; Swartley and Stephens, 1994), also called *siaA*, *siaB*, *siaC* and *siaD* (Edwards and Frosch, 1992; Edwards *et al.*, 1994).

The intergenic region separating the serogroup B capsule biosynthesis genes and the capsule transport genes contains the *ctrA* to *-D* and the *synX to -D* promoters which drive the transcription of these gene clusters in opposite directions (Swartley *et al.*, 1996). The promoter of the *synX to -D* operon has a perfect -10 region and 3 out of 6 nucleotides in its -35 hexamer match the consensus -35 region. However, the *ctrA- D* operon did not show the presence of a consensus -35 region in its promoter. Examination of its -10 region revealed a sequence resembling an extended -10 promoter.

The same intergenic organization was observed for the other sialic-acid containing serogroups C, Y and W-135 capsules (Swartley *et al.*, 1996; Swartley *et al.*, 1997). The genetic cassette responsible for the expression of the non-sialic acid containing serogroup A capsule has also been defined and was shown to be located between the *ctrA* gene, a homolog of *ctrA* in serogroup B, C, Y and W-135, and the *galE* gene, which encodes a UDP-glucose-4-epimerase (Swartley *et al.*, 1998). Although this intergenic region does not show any homology with the region found in sialic acid capsular serogroups, it contains two overlapping and divergent σ^{70} promoters that initiate transcription of the biosynthetic operon and the capsule transport operon (Swartley *et al.*, 1998). No extended -10 region was found in the serogroup A *ctrA- D* operon.

These findings suggest that this intergenic region is an important control point for the regulation of capsule expression in all serogroups.

3.2.3. Analysis of iron acquisition

Iron is an essential nutrient for all bacteria. Pathogens have to deal with extremely low concentrations of free Fe^{3+} in the extracellular fluids of mammals because most Fe^{3+} is bound to transferrin in serum (Bullen *et al.*, 1974) or to lactoferrin on mucosal surfaces (Masson *et al.*, 1966). Iron acquisition therefore constitutes another important virulence factor in bacteria.

Most Gram-negative bacteria such as *E. coli* have developed a system of small, organic chelators called siderophores used to acquire iron. *N. meningitidis,* however, is able to sequester iron directly from human transferrin (Mickelsen *et al.*, 1981) and lactoferrin (Mickelsen *et al.* , 1982) via specific receptors on the outer membrane called transferrin-binding proteins and lactoferrin-binding proteins.

3.2.3.1. Transferrin-binding proteins in *N. meningitidis*

Competitive binding studies and affinity isolation experiments (Legrain *et al.*, 1993) demonstrated that separate receptors for lactoferrin and transferrin exist in *N. meningitidis*. Two receptor proteins for transferrin, transferrin-binding protein A (TbpA) and transferrin-binding protein B (TbpB), have been identified (Cornelissen *et al.*, 1992; Legrain *et al.*, 1993).

The two genes encoding these proteins, *tbp1* and *tbp2*, were isolated from two *N. meningitidis* group B strains and were shown to be organized in tandem association (*tbp2* followed by *tbp1*), with an 87 bp intergenic region (Legrain *et al.*, 1993). Upstream from the putative *tbp2* start codon, sequences with homologies to *E. coli* -35/-10 consensus motifs were found. A putative Fur-binding site was also identified overlapping the -10 hexamer. This is an interesting finding because the Fur (ferric uptake regulation) protein, in conjunction with Fe^{2+}, represses the transcription of several iron-regulated genes

in *E. coli* (Hennecke, 1990) by binding to this sequence. This observation can be correlated with the observed stimulation of TbpB production by *N. meningitidis* under iron-starvation conditions.

TbpA and TbpB possibly form a complex in the outer membrane (Cornelissen and Sparling, 1996). TbpB has been shown to be a lipoprotein, most probably exposed to the surface (Anderson *et al.*, 1994) and TbpA protein showed homology to siderophore receptors of *E. coli*, which are in direct contact with receptor proteins in the outer membrane (Postle, 1990). Both proteins were shown to be immunogenic in humans and antibodies against TbpB were shown to be cross-reactive between different strains (Ala'Aldeen *et al.*, 1994). However, these antibodies are still not known to be bactericidal. Furthermore, the antigenic variability of TbpA and TbpB may limit their use as potential vaccine candidates.

3.2.3.2. Lactoferrin receptors in *N. meningitidis*

The first lactoferrin-binding protein originally identified in *N. meningitidis* (Schryvers and Morris, 1988) was shown to have a high degree of similarity to TbpA. The structural gene for this protein, designated *lbpA*, has been characterized (Biswas and Sparling, 1995) and a sequence weakly homologous (12/19 bp) to a Fur binding site upstream of *lbpA* (Pettersson *et al.*, 1993) was observed. However, iron regulation of *lbpA* expression could not be demonstrated when this gene was cloned in *E. coli* (Pettersson *et al.*, 1994). Although several putative -35 and -10 regions could be allocated in the 5' flanking region of *lbpA*, these promoter elements show in no case the appropriate spacing of 17+/-1 nt. Furthermore, part of an open reading frame (ORF) was identified upstream of the gene.

The product of this ORF, called LbpB, was shown to have an amino acid sequence with a considerable degree of similarity to the TbpB protein. The stop and start codons of *lbpB* and *lbpA* overlap, suggesting that these genes are most probably organized as a single operon (Pettersson *et al.*, 1994). In the promoter area of *lbpB*, putative -10 and -35 hexamers could be discerned. Furthermore, a

putative Fur-binding site was also found overlapping the -10 region. This site is believed to regulate the expression of both genes (Pettersson *et al.*, 1998).

Previous studies have shown that all tested meningococcal strains could use lactoferrin as a sole iron source (Mickelsen *et al.*, 1982) and lactoferrin-binding proteins are assumed to be present in all wild-type meningococcal strains (Pettersson *et al.*, 1993). Lactoferrin is therefore probably an essential iron source for meningococci, thus making LbpA and LbpB interesting vaccine candidates.

3.2.4. Phase variation

Bacteria regulate the production of many cellular components and have the ability to vary the immunogenicity of important structures in order to compete effectively with the normal flora and survive host defense systems. Such changes often lead to the phenotypic variation of these components. Phenotypic variation includes phase variation, which is the differential regulation of the amounts of a molecule or defined structure, and antigenic variation, which is concerned with the absence or presence of a certain surface epitope (Robertson and Meyer, 1992). The Neisseriae undergo such changes and the molecular study of such modifications is of value in understanding the mechanisms by which these organisms escape the immune system.

3.2.4.1. Phase variation of pili

One component that has been extensively studied in *N. gonorrhoeae* is the pilus. This component is considered a major virulence factor because only piliated variants produce infection in healthy volunteers (Swanson *et al.*, 1987). *N. meningitidis* strains express one of two structurally distinct types of pili termed Class I and Class II pili. Gonococcal and Class I meningococcal pili are highly homologous, with pilin being the major subunit of the pilus and its expression controlled at the chromosomal *pilE* locus.

Phase and antigenic variation of pilin expression in *N. gonorrhoeae* result from recombination events in which variant sequences from one of the silent loci (*pilS*) that lack promoters are transferred to the expression locus (*pilE*) (Gibbs *et al.*, 1989). Pilin in meningococcus is also subject to phase and antigenic variation, which have been shown to modulate adhesiveness to human epithelial cells (Jonsson *at al.*, 1994).

Three putative promoters (P1, P2 and P3) were identified in the *pilE* locus of gonococci: P1 and P2 are σ^{70}-like promoters, with P1 having a perfect -10 sequence and lacking a consensus -35 region and P2 having a 2 out of 6 nucleotide match in both hexamers. P3 is a σ^{54} dependent promoter and it overlaps the P1 sequence. *N. meningitidis* was shown to have equivalents of P1, P2 and P3 (Carrick *et al.*, 1997). The P1 promoter contains an upstream sequence similar to a UAS and was shown to mediate transcription of the *pilE* gene in both *N. gonorrhoeae* and *N. meningitidis* (Carrick *et al.*, 1997). Although P3 is not used in both these organisms *in vitro*, it was shown to be functional in *P. aeruginosa* in the presence of an activator protein PilR (Carrick *et al.*, 1997). These results suggest that transcription from P3 in *Neisseria* may occur under conditions which provide the presence of such an activator (Carrick *et al.*, 1997).

The absence of a perfect -35 region has been shown to be characteristic of positively controlled bacterial promoters (Raibaud and Schwartz, 1984). This observation led to the identification of an additional mechanism for phase variation in gonococcus which consists of the regulation of *pilE* transcription by a two-component regulatory system encoded by the *pilA* and *pilB* genes (Taha *et al.*, 1988). These genes encode proteins which act in *trans* on the pilin promoter, with PilA protein having a putative DNA binding domain near its N-terminus (Taha and Giorgini, 1995). The regulation of *pilE* by *pilA* seems to also depend on a -24/-12 type promoter, which could be recognized by a σ^{54} subunit (Taha and Giorgini, 1995).

Two pilus-associated proteins, PilC1 and PilC2, encoded by two *pilC* genes and first described in gonococcus (Jonsson *et al.*, 1991) were shown to be key components in meningococcal adhesiveness as they have been identified as the pilus tip-located adhesins (Rudel *et al.*, 1992). Their expression in gonococcus was found to be controlled at the translational level by frameshift mutations in a run of guanosine residues positioned in the region encoding the signal peptide. A "switch ON and OFF" of PilC expression causes phase variation of pilus expression (Jonsson *et al.*, 1991).

In meningococcus, genic variation of both *pilC* and *pilE* also occurs (Taha *et al*; 1996). Furthermore, alleles similar to the PilA/PilB pleiotropic regulatory pathway in gonococcus have been found in *N. meningitidis* (Taha *et al.*, 1996), leading to the demonstration of another regulatory pathway of pilin expression in this bacterium. Results from Taha *et al.*, (1996), have shown that the PilA protein binds to the promoter region of *pilC1* but not of *pilC2*. The different organization of the regulatory regions of *pilC1* and *pilC2* is in good agreement with the different functions of these proteins. While *pilC2* can be expressed from a consensus -35/-10 promoter and is not modulated by PilA, *pilC1* can be transcribed from three different promoters. The promoter giving the strongest initiation of transcription has a sequence close to the -12/-24 consensus sequence, which is similar to the one used by PilA to control the transcription of gonococcal *pilE*. It is therefore likely that this promoter is the one controlled by PilA. This demonstrates that PilA modulates meningococcal adhesiveness via the transcription of *pilC1* and that in addition to phase variation, a more co-ordinate system mediates adhesiveness of meningococci in response to different environments (Taha *et al.*, 1996).

Class II pili form a structurally distinct group of pili expressed by some strains of *N. meningitidis*. The characterization of their *pilE* locus has revealed several potential transcription initiation signals located upstream of the start codon (Aho *et al.*, 1997). These include a possible -10 sequence, a potential extended -10 sequence as well as two regions with homology to the consensus -24/-12

promoter sequence. The presence of these regions provides further evidence of diversity among neisserial *pilE* promoters. Analyses of *pilE* loci from mutiple meningococcal strains are needed to further understand the degree of heterogeneity of the pilin gene family of *Neisseria*.

3.2.4.2. Phase variation of the capsule

In the sialic acid-containing CPS of meningococcal serogroups B and C, the polysaccharide capsules inhibit Opa and Opc-mediated invasion of cells, suggesting that capsule formation may need to be down-regulated during the first steps of infection (Hammerschmidt *et al.*, 1996a) by the switching between an encapsulated and a non-encapsulated bacterial phenotype. This enables the bacteria to pass the mucosal barrier and to resist the host's immune defense (Hammerschmidt *et al.*, 1996a). The mechanism which is responsible for the reversible inactivation of the meningococcal *siaA* gene involves the insertion/excision of an insertion sequence element termed IS*1301*, which is present in multiple copies on the chromosome of sialic acid-containing meningococcus (Hammerschmidt *et al.*, 1996a). This transposable element occurs in a large variety of meningococcal clinical isolates and is of importance for modulation of meningococcal virulence (Hammerschmidt *et al.*, 1996a). This element has not yet been identified in serogroup A, which does not contain sialic acid in its capsule (Hilse *et al.*, 1996). Another mechanism which is responsible for the switching between encapsulated and non-encapsulated meningococcus was identified in serogroup B organisms. It involves the insertion or deletion of a cytidine residue within a 7-nt poly(C) tract in the coding region of *siaD*. This alteration may result in a frameshift and a termination of translation (Hammerschmidt *et al.*, 1996b).

Recently, the identification of genetically similar meningococcal isolates which are either from serogroup B or serogroup C suggested that *N. meningitidis* strains can switch their capsule type (Swartley *et al.*, 1997). This switching is believed to occur as a result of transformation and horizontal DNA exchange *in*

vivo. The switching of the capsule from one type to another may be yet another way by which these pathogens escape the immune system (Swartley *et al.*, 1997).

3.2.4.3. Phase variation of outer membrane proteins

Phase variation is also exhibited by the outer membrane proteins of *N. meningitidis*. A single strain may express up to 3 antigenically different class 5 proteins during the course of infection (Poolman *et al.*, 1980; Tinsley and Heckels, 1986). The Opa and Opc proteins, which form the class 5 OMPs in meningococcus, are involved in the invasion of epithelial cells and the interaction with human leukocytes (Robertson and Meyer, 1992). *opa* genes have been identified in both *N. gonorrhoeae* and *N. meningitidis* and were shown to be highly conserved in their nucleotide sequence (Aho *et al.*, 1991). The DNA encoding the *opa* signal peptide contains a series of CTCTT pentameric repeats and phase variation occurs at the translational level via the addition or deletion of these base sequences (Belland *et al.*, 1989; Murphy *et al.*, 1989).

In the case of the Opc proteins, the other type of class 5 OMPs, phase variation occurs as a result of transcriptional regulation. The promoter of the *opc* gene has a perfect -10 consensus sequence (Sarkari *et al.*, 1994). It also has a sequence containing a variable number of non-alternating cytidine residues overlapping the -35 region (Sarkari *et al.*, 1994). The regulation of Opc expression was shown to occur through the variation of the number of these cytidine residues (Sarkari *et al.*, 1994) with Opc levels changing from moderate to high by a single nucleotide change.

This same mechanism of phase variation occurs in the regulation of OMP1 expression. The promoter of the *porA* gene was shown to contain a perfect -10 consensus region and a putative -35 region with only 2 out of 6 nucleotides matching the *E. coli* 5' TTGACA 3' (van der Ende *et al.*, 1995). Sequence analysis has revealed a variable number of contiguous G-residues between the

two hexamers (van der Ende *et al.*, 1995; Arhin *et al.*, 1998). OMP1 expression levels can vary from no protein levels, to moderate and high levels by single nucleotide changes in this poly(G) stretch (van der Ende *et al.*, 1995).

INTRODUCTION

Outer membrane proteins of meningococcus have attracted much interest because they form the basis of serotyping and serosubtyping (Frasch *et al.*, 1985) and because their potential as vaccine components is being explored (van der Ley and Poolman, 1992).

The system that is currently used for routine serosubtyping of *Neisseria meningitidis* relies on the use of whole cells in dot blot or enzyme-linked immunosorbent assays (ELISAs) where mAbs are directed against the class 1 OMP (OMP1) [Abdillahi and Poolman, 1987]. However, this procedure is not comprehensive due to the masking of epitopes by other macromolecules in whole cells (Wedege *et al.*, 1993), the presence of epitopes to which mAbs are currently unavailable (Suker *et al.*, 1994) as well as the absence of OMP1 in some isolates (Arhin *et al.*, 1997a). A study of 96 isolates obtained from various locations in the United States showed that only 44.8% of the isolates were completely typeable by mAb techniques and that some mAbs gave ambiguous results (Swaminathan *et al.*, 1996).

To address these limitations, other techniques for subtyping have been assessed. One possibility is the assignment of subtypes based on deduced amino acid sequences of the variable regions of OMP1 (Suker *et al.*, 1994; Feavers *et al.*, 1996; Arhin *et al.*, 1997b) from DNA sequencing of the variable regions of *porA*.

Between 1993 and 1994, 174 clinical isolates of *N. meningitidis* were collected in the province of Québec, Canada (Lorange, 1996). The majority belonged to serogroup C (47%) or to serogroup B (46%), the major serogroups associated with invasive disease in the Western hemisphere (Whalen *et al.*, 1995). Forty-eight of those 174 isolates were not serosubtypeable using routine

methods (Lorange, 1996). They were therefore characterized by OMP profiles as well as ELISAs using outer membrane vesicles (OMVs) in order to rid the OMPs of structures masking their epitopes (Arhin *et al.*, 1997a). Eight of these strains were found by SDS-PAGE not to express OMP1 (Arhin *et al.*, 1997a). Amplification of the *porA* gene from these strains as well as Southern hybridization analysis revealed the presence of *porA* in all strains (Arhin *et al.*, 1997a). Northern analysis failed to show the presence of *porA* mRNA in these strains (Arhin *et al.*, 1998), thus supporting an earlier report (van der Ende *et al.*, 1995) that regulation of *porA* expression in some isolates occurs at the level of transcription. Sequencing of the promoter region of all 48 non-subtypeable isolates revealed the presence of a poly(G) stretch between the -10 and the -35 regions. It has been suggested that the length of this poly(G) stretch determines promoter activity (van der Ende *et al.*, 1995). The eight isolates that did not express OMP1 had different numbers of G residues in the poly(G) stretch and seven of them had one of the Gs replaced by an adenosine residue (Arhin *et al.*, 1998). The other isolate which did not express OMP1 did not contain an adenosine but a mutation in the structural gene gave rise to a truncated protein (Arhin *et al.*, 1998).

These observations suggest that the number of residues in the poly(G) stretch between the -10 and the -35 regions may not be the only factor that contributes to different expression levels of *porA* and that the adenosine residue in the poly(G) stretch may play a role in the lack of *porA* expression.

We tested this hypothesis by inserting site-specific mutations in the spacer region of the *porA* promoter and expressing these constructs in *E. coli*.

MATERIALS AND METHODS

1. Bacterial strains, plasmids and growth media

Bacterial strains and plasmids used in this study are described in Table 1. The Québec meningococcal strains used in this study (19966, 21784 and 22857) were clinical isolates from hospitalized patients with invasive disease collected from 1993 to 1994 by the Laboratoire de Santé Publique du Québec. Strains 19966 and 22857 did not express the class 1 outer membrane protein whereas strain 21784 did express the class 1 outer membrane protein (Arhin *et al.* 1997a).

Neisseria meningitidis strains were routinely grown on brain-heart infusion (BHI; Oxoid) agar plates supplemented with 0.5% v/v horse serum or in BHI broth. They were maintained at -70°C in Greave's freezing solution: 5% w/v monosodium glutamate, 5% w/v bovine serum albumin and 10% v/v glycerol. Bacteria were propagated at 37°C on BHI agar plates supplemented with 0.5% v/v horse serum in an atmosphere of 5% CO_2. For liquid cultures, a single colony was inoculated into BHI and incubated overnight on a rotary shaker at 240 rpm and at 37°C. Media for *Escherichia coli* have been described (Sambrook *et al.* 1989). Antibiotic concentrations used for selection of plasmid markers after transformation of *E. coli* were 100 $\mu g.ml^{-1}$ of ampicillin, 10 $\mu g.ml^{-1}$ of tetracycline and 250 $\mu g.ml^{-1}$ of erythromycin. The antibiotic concentration used for selection of chromosomal markers after transformation of *N. meningitidis* was 2 $\mu g.ml^{-1}$ of erythromycin.

2. Molecular Biological Techniques

DNA-modifying and restriction enzymes were from Pharmacia Biotech (Baie d'Urfé, Québec). The β-galactosidase enzyme was from Boehringer Mannheim (Laval, Québec). Restriction endonuclease digestions, ligations and DNA

manipulations were performed as described by Sambrook *et al.* (1989). DH5α or MV1190 strains were used as hosts for isolation of plasmid DNA using the plasmid mini prep kit from QIAGEN (Mississauga, Ontario). DNA was purified from agarose gels using the Sephaglas kit (Pharmacia Biotech). Meningococcal genomic DNA was isolated using the DNAzol DNA isolation reagent (Gibco BRL, Gaithersburgh, Maryland) and following the manufacturer's protocol. The concentration and purity of the DNA samples were estimated spectrophotometrically (Sambrook *et al.* 1989). Dephosphorylation of the digestion products of pAErmC'G was done using shrimp alkaline phosphatase (SAP) (Boehringer Mannheim, Laval, Québec). To transform *E. coli*, cells were made competent with calcium chloride (Sambrook *et al.* 1989). Oligonucleotides primers for mutagenesis were synthesized by the Core Facility for Protein/DNA Chemistry, Queen's University, Kingston, Ontario. Oligonucleotide primers for PCR and for sequencing were synthesized by the Sheldon Biotechnology Centre, McGill University, Montreal, Québec.

3. Southern Hybridization

Plasmid DNA was isolated from *E. coli* strains using plasmid prep kits from QIAGEN. Genomic DNA from *N. meningitidis* was isolated using the DNAzol DNA isolation reagent and was digested overnight with *EcoR*I. DNA restriction fragments were separated electrophoretically in 0.8% agarose gels containing TAE (0.01 M Tris Acetate plus 0.01 M EDTA) buffer and transferred to Nytran hybridization membranes (Schleicher and Schuell, Keene, New Hampshire). Enzymes and the digoxigenen DNA labeling kit (The Genius system) for Southern hybridization were obtained from Boehringer Mannheim. Southern hybridizations were carried out with digoxigenen-labeled probes at 25 $ng.ml^{-1}$ of hybridization solution. Hybridizations were performed at 68°C after which the membranes were washed twice with 2x SSC containing 0.1% SDS for 15 min at room temperature. A final wash with 0.1x SSC containing 0.1% SDS at 68°C for 15 min was included to reduce background.

4. β-galactosidase Assay

E. coli cells were grown overnight on a rotary shaker at 240 rpm at 37°C in 5 ml of Luria-Bertani (LB) Broth [1% (w/v) tryptone, 1% (w/v) NaCl, 0.5% (w/v) Bacto yeast extract, pH 7.4]. Tetracycline was added at a concentration of 10 $\mu g.ml^{-1}$ to maintain propagation of the tetracycline-resistant plasmids. After growing overnight to saturation, 200 µl of culture was inoculated in fresh media and grown to log phase (A_{600}= 0.1-0.2). The cell concentration was adjusted on the spectrophotometer calibration of $3x10^8$/ml at A_{600}= 0.38 by spinning down 1 ml of culture and resuspending in the appropriate volume of 0.1 M sodium phosphate. 8 µl of chloroform and 4 µl of 0.1% sodium dodecyl sulphate were added to 200 µl of the above cell suspension. This mixture constituted cell extract. The following reagents were added to 60 µl of each cell extract: 1.5 µl of 10 M $MgCl_2$, 4.5 M 2-β-mercaptoethanol, 33 µl of 2-nitrophenyl-β-D-galactopyranosid (ONPG) and 55.5 µl of 0.1 M sodium phosphate. 0.5 µl of β-galactosidase (stock solution of 1,500 units/ml, diluted into 30 µl of 0.1 M sodium phosphate for a working solution of 25 units/ml) was added to the control sample. The samples were incubated for 30 minutes at 37°C and the reaction was stopped by adding 250 µl of 1 M Na_2CO_3. 100 µl of each sample was added to a different well in a 96 well plate and the absorbance was measured at a wavelength of 415 nm by using a wavelength of 595 nm as a reference. β-galactosidase enzyme units were calculated as follows (Miller, 1992):

$$\beta\text{-galactosidase in Miller units} = (1000 \times Abs_{600}) / (\text{time (min) x. vol (ml)} \times Abs_{600})$$
$$= (1000 \times Abs) / (30 \text{ x.} 0.06 \times 0.38)$$

5. Mutagenesis of Cloned Promoters

Site-directed mutagenesis of plasmids pUR19, pUR21 and pUR22 was achieved with the Unique Site Elimination (U.S.E.) Mutagenesis kit (Pharmacia Biotech) using mutagenic oligonucleotide primers. The kit is based on the unique site elimination procedure developed by Deng and Nickoloff (1992), and utilizes

a two-primer system to generate site-specific mutations in the above-mentioned plasmids. The U.S.E. selection primer contains a mutation in a unique non-essential restriction site. This primer eliminates the *Sca*I site and introduces an *Mlu*I site. The mutated plasmid DNA is rendered resistant to restriction by *Sca*I and sensitive to digestion by *Mlu*I, thereby providing the basis for selection. The desired target mutation is introduced into a defined sequence of the plasmid by the target mutagenic primer. Target primers used for the mutagenesis reactions are described in Table 2. After *in vitro* mutagenesis, the reactions were subjected to digestion by *Sca*I for selection of mutated plasmids and then transformed into *E. coli* NM522 *mutS*. Plasmids were isolated from transformants using the mini prep kit from QIAGEN and subjected to two more rounds of restriction digestion by *Sca*I. The reactions were finally transformed into *E. coli* MV1190. Plasmids were re-isolated from transformants and were digested with *Sca*I and *Mlu*I for selection of mutants. All candidates for each mutation were confirmed by sequencing of plasmid DNA.

6. DNA Sequence Determination

pUR mutants (pURp121 to pURp522, Table 1) were isolated using the mini prep kit from QIAGEN. Universal primer was used to sequence the desired mutated promoter regions. Sequencing was performed by the Sheldon Biotechnology Centre, McGill University.

7. Polymerase Chain Reaction

Polymerase Chain Reactions (PCR) were carried out using a programmable thermal controller from MJ Research Inc. (Watertown, Mass.). The primers used for the amplification were: *Sph*I-P1 (5' - GCATGCGCATGC CCGGTTTCAGCGGCAGCGTCCAATTCG- 3') and *Stu*I-P2 (5' - AGGCCT AGGCCTGCGGCATTAATTTGAGTGTAGTTGCC- 3'). These primers are specific for *porA* and would amplify a fragment of the 3'-end of *porA* of approximately 700 bp. *Sph*I-P1 contains two tandem recognition sites for the

restriction enzyme *Sph*I (underlined) at the 5' end and *Stu*I-P2 contains two tandem recognition sites for the restriction enzyme *Stu*I (underlined) at the 5'-end. The amplification reaction contained 2 μM of both primers, template genomic DNA from strain 19966 at 50 ng/μl, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% w/v gelatin, 200 μM each of dATP, dGTP, dTTP and dCTP, and 1 Unit of *Taq* polymerase in a total volume of 100 μl. The reaction was carried out for 30 cycles as follows: a denaturation step at 95°C for 2 min; an annealing step at 70°C for 2 min; and an extension step at 70°C for 2 min. Following the cycles, the reaction was further incubated for 3 min at 70°C. A sample containing genomic DNA from *N. meningitidis* strain 19966 and primers *Xba*-PorA7 and *Xba*-P2 (Arhin *et al.* 1998) was used as a control.

8. Transformation of *Neisseria meningitidis* strains

The transformation assay was performed as described by Nassif *et al.* 1991. BHI media supplemented with horse serum was used instead of gonococcal base (GCB) media. The transformation assay was performed in a 24-well tissue-culture plate and 8 μg of linearized plasmid DNA were used for 400 μl of cells.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant Characteristics	Source
BACTERIAL STRAINS		
E. coli strains		
MV1190	*Δ(lac-proAB) thi supE Δ (srl-recA)306::Tn10(tetr) F'[traD36 proAB lac 1q lacZDM15]*	Life Technologies, Inc
DH5α	*supE44 ΔlacU169(ø80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bio-Rad
NM522	*thi, supE, Δ(hsdMS-mcrB)5, Δ(lac-proAB)[mutS::Tn10]F'[proAB+laciq lacZ ΔM15]*	Pharmacia Biotech
N. meningitidis strains		
19966	Clinical isolate - Serogroup C	LSPQ
21784	Clinical isolate - Serogroup B	LSPQ
22857	Clinical isolate - Serogroup C	LSPQ
Nm19	19966 wild-type transformed by pSP19	This study
PLASMIDS		
pUC119	Cloning vector, *bla*	Vieira and Messing, 1982
pUR19	pUC119 containing promoter from *N.m.* strain 19966	Arhin *et al.*, 1998
pUR21	pUC119 containing promoter from *N.m.* strain 21784	Arhin *et al.*, 1998
pUR22	pUC119 containing promoter from *N.m.* strain 22857	Arhin *et al.*, 1998
pSL1180	Cloning vector, *bla*	Pharmacia Biotech
pSR19	pSL1180 containing promoter from *N.m.* strain 19966	This study
pSR21	pSL1180 containing promoter from *N.m.* strain 21784	This study
pSR22	pSL1180 containing promoter from *N.m.* strain 22857	This study
pDN*lac*19Ω	*lacZ, tetA*	Totten and Lory, 1990
plac19	pDN19*la* cΩ containing promoter from *N.m.* strain 19966	This study
plac21	pDN19*la* cΩ containing promoter from *N.m.* strain 21784	This study
plac22	pDN19*lac*Ω containing promoter from *N.m.* strain 22857	This study
pURp121	pUC119 containing mutated promoter p1+21784	This study
pURp221	pUC119 containing mutated promoter p2+21784	This study
pURp321	pUC119 containing mutated promoter p3+21784	This study

Table 1. Bacterial strains and plasmids used in this study (continued)

Strain or plasmid	Relevant Characteristics	Source
	PLASMIDS	
pURp219	pUC119 containing mutated promoter p2+19966	This study
pURp522	pUC119 containing mutated promoter p5+22857	This study
pSRp121	pSL1180 containing mutated promoter p1+21784	This study
pSRp221	pSL1180 containing mutated promoter p2+21784	This study
pSRp321	pSL1180 containing mutated promoter p3+21784	This study
pSRp219	pSL1180 containing mutated promoter p2+19966	This study
pSRp522	pSL1180 containing mutated promoter p5+22857	This study
placp121	pDN19*lac*Ω containing mutated promoter p1+21784	This study
placp221	pDN19*lac*Ω containing mutated promoter p2+21784	This study
placp321	pDN19*lac*Ω containing mutated promoter p3+21784	This study
placp219	pDN19*lac*Ω containing mutated promoter p2+19966	This study
placp522	pDN19*lac*Ω containing mutated promoter p5+22857	This study
pAErmC'G	*lacZ, ermC'*, GC uptake sequence	Zhou and Apicella, 1996
pSA	pSL containing the *lacZ-ermC'* insert	This study
pSA19	pSR19 containing the *lacZ-ermC'* insert	This study
pSA21	pSR21 containing the *lacZ-ermC'* insert	This study
pSA22	pSR22 containing the *lacZ-ermC'* insert	This study
pSAp121	pSRp121 containing the *lacZ-ermC'* insert	This study
pSAp221	pSRp221 containing the *lacZ-ermC'* insert	This study
pSAp321	pSRp321 containing the *lacZ-ermC'* insert	This study
pSAp219	pSRp219 containing the *lacZ-ermC'* insert	This study
pSAp522	pSRp522 containing the *lacZ-ermC'* insert	This study
pSP19	pSA19 containing the 3' -end of *porA*	This study

Table 2. Primers used for mutations

Characteristics and Sequence	Source
Primer 1 - Eliminates 1 G in the poly(G) stretch of 21784 5' GTT TTT TCG GGG GGG GGG GGT ATA ATT G 3'	This study
Primer 2 - Converts A to G in the poly(G) stretch of 19966 5' GTT TTT TCG GGG GGG GGG GTA TAA TT G 3'	This study
Primer 3 - Converts A to G in the poly(G) stretch of 21784 5' GTT TTT TCG GGA GGG GGG GGT ATA ATT G 3'	This study
Primer 5 - Converts A to G in the poly(G) stretch of 22857 5' GTT TTT TCG GGG GGG GGG TAT AAT T G 3'	This study

RESULTS

1. The *porA* promoter of *Neisseria*: analysis in *Escherichia coli*

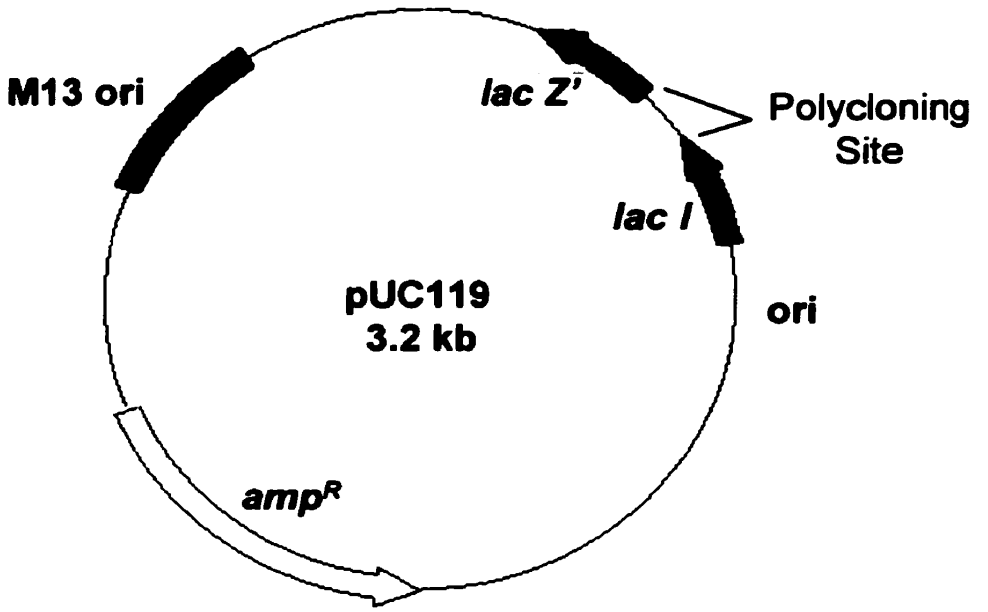
The OMP profiles of the 48 non-serosubtypeable clinical isolates of *Neisseria meningitidis* isolated in Québec (1993-94) confirmed the absence of OMP1 in eight isolates (Arhin *et al.*, 1997a). PCR analysis revealed the presence of the *porA* gene in all strains that were studied (Arhin *et al.*, 1997a), while Northern analysis did not show any *porA* mRNA in the isolates lacking OMP1 [Arhin *et al.*, 1998]. Selected promoter regions were amplified by PCR and the promoter products were subcloned as 0.4 kb *Xba*I fragments into the vector pUC119 (Fig. 1) [Arhin *et al.*, 1997b]. Sequencing of the *porA* promoter in these isolates, as well as in the others, showed the presence of a poly(G) stretch lying between the -10 and the -35 regions of the promoter (Fig. 2). In seven of the eight isolates that lack OMP1, an A-residue was incorporated in this poly(G) stretch (Fig. 2). One isolate did not express OMP1 because of the presence of a frame-shift mutation in the coding region of *porA*. DNA sequencing of the promoter in these constructs revealed a 12 nt poly(G) stretch in isolate 21784, which expresses OMP1, a 10 nt poly(G) stretch with an adenosine in isolate 19966 and a 9 nt poly(G) stretch with an adenosine in isolate 22857 (Fig. 2) [Arhin *et al.*, 1998]. These latter two isolates do not express OMP1.

The different lengths of the poly(G) stretches as well as the different expression levels of OMP1 led us to examine the strengths of the promoters of *porA* in these isolates in correlation to OMP1 expression.

Construction of the expression vectors

In order to determine the strengths of these different promoters, DNA was subcloned into a promoter-probe vector, pDN19*lac*Ω, which contains a promoterless *lacZ* gene downstream of an *Eco*RI-*Bam*HI cloning site (Fig. 3) (Table 1). To obtain compatible ends for cloning into pDN19*lac*Ω (plac), the

Figure 1. pUC119 vector.



Polycloning site

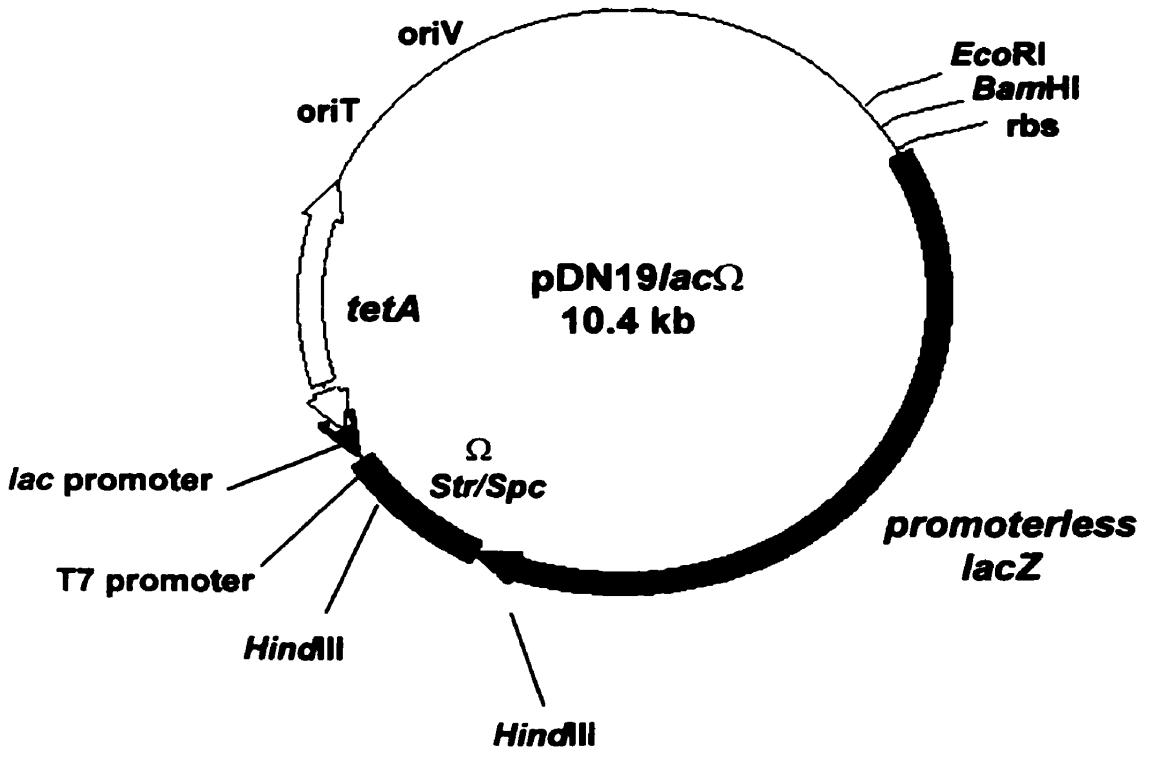
------ *Hind*III - *Sph*I - *Pst*I - *Sal*I/*Acc*I/*Hinc*II - *Xba*I - *Bam*HI - *Sma*I/*Xma*I - *Kpn*I - *Sac*I - *Eco*RI ------

Vieira and Messing, 1982

Figure 2. Nucleotide sequence alignment of promoter regions of *N. meningitidis* strains. The -35 and -10 regions are underlined. Class 1 OMP expression is indicated as + (expressed) or - (not expressed). The poly(G) track between the -35 and -10 regions is shown bold-face (Taken from Arhin *et al.*, 1998).

Strain of *N. meningitidis*	-35 ?	- 10	OMP1 Expression
19540	AAATGGTTTTTTGCGGAGGGGGGG_ _	TATAATTG	-
19594	AAATGGTTTTTTGCGGAGGGGGGG_ _	TATAATTG	-
19966	AAATGGTTTTTTGCGGAGGGGGGG_ _	TATAATTG	-
21292	AAATGGTTTTTTGCGGAGGGGGGGG_	TATAATTG	-
21509	AAATGGTTTTTTGCGGAGGGGGGGG_	TATAATTG	-
24966	AAATGGTTTTTTGCGGAGGGGGGGG_	TATAATTG	-
22857	AAATGGTTTTTTGCGGAGGGGGG_ _ _	TATAATTG	-
20253	AAATGGTTTTTTGCGGGGGGGGGGGG	TATAATTG	-
21784	AAATGGTTTTTTGCGGGGGGGGGGGG	TATAATTG	+
19749	AAATGGTTTTTTGCGGGGGGGGGG_ _	TATAATTG	+

Figure 3. pDN19*lac*Ω vector.



Totten and Lory, 1990

promoter regions were subcloned from pUC119 into pSL1180, a superlinker® plasmid containing multiple restriction sites. The 19966, 21784 and 22857 promoters were excised from pUR19, pUR21 and pUR22, respectively, by using the *Eco*RI and *Sal*I restriction sites in pUC119. The resulting *Eco*RI-*Sal*I fragments of 0.4 kb were subcloned into pSL1180 (Fig. 4), thus generating plasmids pSR19, pSR21 and pSR22 (Table 1). These promoter regions were then cleaved as *Eco*RI-*Bam*HI fragments and finally subcloned into the *Eco*RI-*Bam*HI restriction sites in pDN19*lac*Ω (Fig. 5), resulting in plasmids plac19, plac21 and plac22 (Table 1). To confirm cloning of the correct fragments into pDN19*lac*Ω, plac19, plac21 and plac22 were subjected to a *Hind*III- *Eco*RI digestion, which results in three digestion products: a *Hind*III-*Hind*III fragment and two *Eco*RI-*Hind*III fragments of different lengths. The *Hind*III-*Hind*III fragment is approximately 2.2 kb and the first *Eco*RI-*Hind*III fragment is about 5 kb. The second *Eco*RI-*Hind*III fragment shifts from 3.2 kb to 3.6 kb if the promoter region of 0.4 kb is properly inserted. These digestion products were run on an agarose gel along with plac which had also been subjected to a *Hind*III-*Eco*RI digestion. A *porA* promoter fragment from isolate 21784 served as a positive control. Southern analysis was performed on these samples using a *porA* probe (Fig. 6). Hybridizing signals were observed at 3.6 kb for all three promoter constructs and the *porA* promoter, while no signal was observed for plac. These results confirm that the promoters had been properly inserted in the *Eco*RI-*Bam*HI site upstream of the *lacZ* gene.

Measurement of the activity level of the wild type promoters

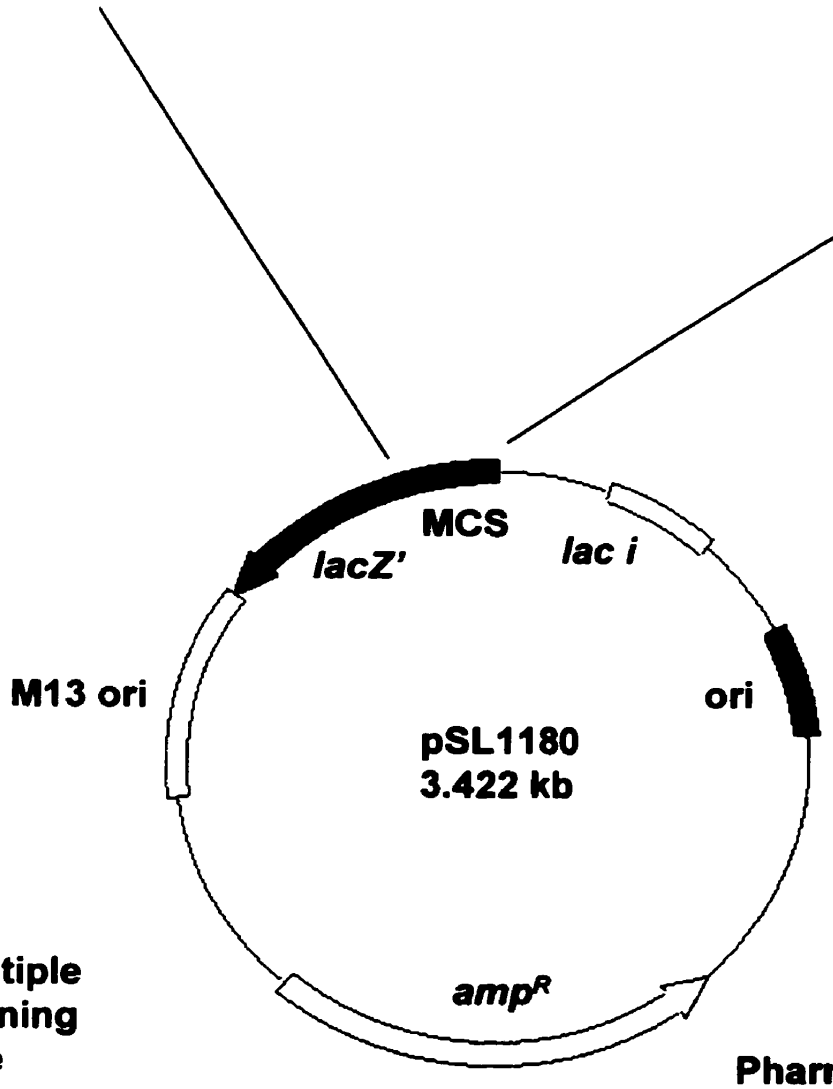
The *porA* promoter activity in each construct was measured by β-galactosidase activity. The assay was performed independently three times and the final value was taken as the mean of those measurements (Table 3). The activity levels of the two negative controls, DH5α cells without a plasmid and DH5α cells containing plac without a promoter, were also measured. plac21,

Figure 4. Cloning of promoter regions of *porA* of isolates 19966, 21784 and 22857 into the *Eco*RI-*Sal*I sites of pSL1180.

Promoter 19966 *Eco*RI —— 5' ATGGTTTTTTGCGGAGGGGGGGTATAAT 3'—— *Sal*I

Promoter 21784 *Eco*RI —— 5' ATGGTTTTTTGCGGGGGGGGGGGGGTATAAT 3'—— *Sal*I

Promoter 22857 *Eco*RI —— 5' ATGGTTTTTTGCGGAGGGGGGTATAAT 3'—— *Sal*I



MCS= Multiple Cloning Site

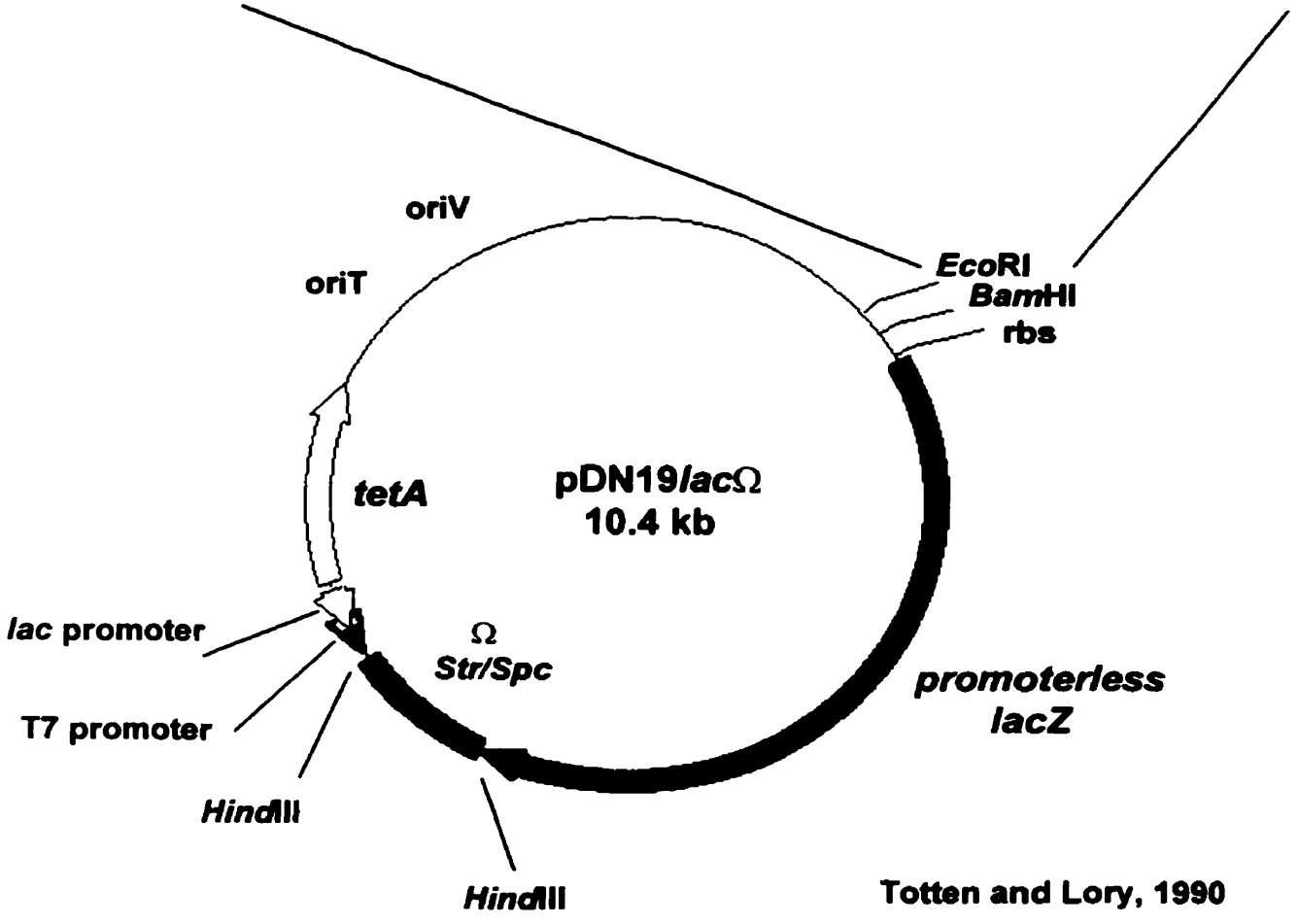
Pharmacia Biotech

Figure 5. Cloning of promoter regions of *porA* of isolates 19966, 21784 and 22857 into the *Eco*RI-*Bam*HI sites of pDN19*lac*Ω.

Promoter 19966 *Eco*RI ---- 5' ATGGTTTTTTGCGGAGGGGGGGTATAAT 3'---- *Bam*HI

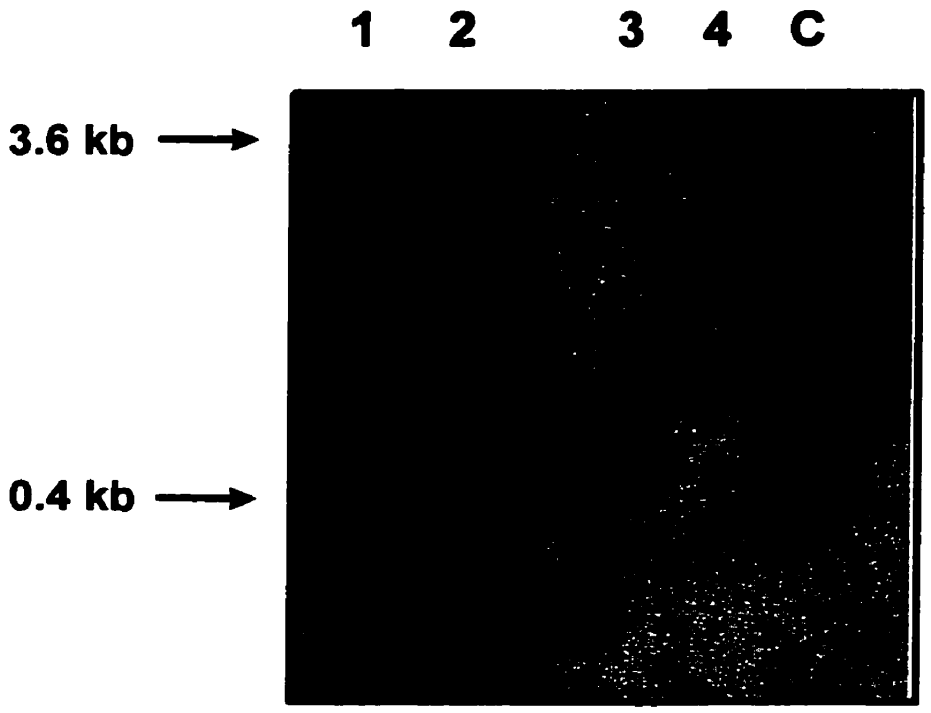
Promoter 21784 *Eco*RI ---- 5' ATGGTTTTTTGCGGGGGGGGGGGGGTATAAT 3'---- *Bam*HI

Promoter 22857 *Eco*RI ---- 5' ATGGTTTTTTGCGGAGGGGGGTATAAT 3'---- *Bam*HI



Totten and Lory, 1990

Figure 6. Southern hybridization of plac19, plac21 and plac22. A *porA* promoter probe was used to identify the promoter inserts in the plasmid constructs. The positive control is a labeled PCR product of the 21784 *porA* promoter.



1 plac

2 plac19

3 plac21

4 plac22

C positive control

which contains the promoter from isolate 21784, showed the highest activity level. This promoter does not contain an adenosine residue and has a 12 nt poly(G) stretch. It allows for expression of OMP1 in *Neisseria meningitidis.* The other two constructs, plac19 and plac22, which contain promoters from strains 19966 and 22857, respectively, exhibited much lower activity levels (Table 3). Both 19966 and 22857 contain an adenosine residue in a promoter poly(G) stretch of 10 nt and 9 nt, respectively, and do not express OMP1. The activity levels of these promoters were then compared to the positive control, which is the activity level of the β-galactosidase enzyme. This was done by expressing the different values obtained for the promoter constructs as a percentage of the value obtained for the β-galactosidase enzyme (Fig. 7). The lower activities of the 19966 and 21784 promoters can be explained either by the shorter poly(G) stretches (van der Ende *et al.*, 1995) or by the presence of the adenosine residue or both. We tested both possibilities by the insertion of site-specific mutations in this poly(G) stretch.

Mutational analysis of the promoter regions in *Escherichia coli*

Five different site-specific mutations were inserted in the poly(G) stretch of these promoters (Table 4). The first two mutations, p1+21 and p2+21, tested the importance of the length of the poly(G) stretch in isolate 21784 by the modification of the number of nucleotides. The last three mutations, p3+21, p2+19 and p5+22, tested the importance of the adenosine residue in isolates 21784, 19966 and 22857, respectively, by the substitution of an adenosine for a guanosine or vice-versa. They were performed in the pUC119 constructs, pUR19, pUR21 and pUR22 instead of the pDN19*lac*Ω derivatives because the kit does not allow for the use of plasmids containing the *tet* gene. This is because the NM522 *mutS* cells that are used for the transformation of these

Table 3. β-galactosidase assay of wild-type constructs

Plasmid	Sequence	OMP 1 Expression	β-gal Levels
DH5α	No promoter insert	-	22
plac	No promoter insert	-	21
plac21 (plac+pro21)	5′ GTT TTT TGC GGG GGG GGG GGG TAT AAT TG 3′	+	1868
plac19 (plac+pro19)	5′ GTT TTT TGC GGA GGG GGG GTA TAA TTG 3′	-	199
plac22 (plac+pro22)	5′ GTT TTT TGC GGA GGG GGG TAT AAT TG 3′	-	66
β-gal	No promoter insert	-	926

Levels are expressed in standard Miller units. Each assay was performed independently three times. The levels shown are the means of these determinations.

\+ indicates the presence of OMP1 in the isolate containing the promoter

\- indicates the absence of OMP1 in the isolate containing the promoter

Figure 7. Histogram showing the β-galactosidase results of the wild-type constructs as a percentage of the positive control.

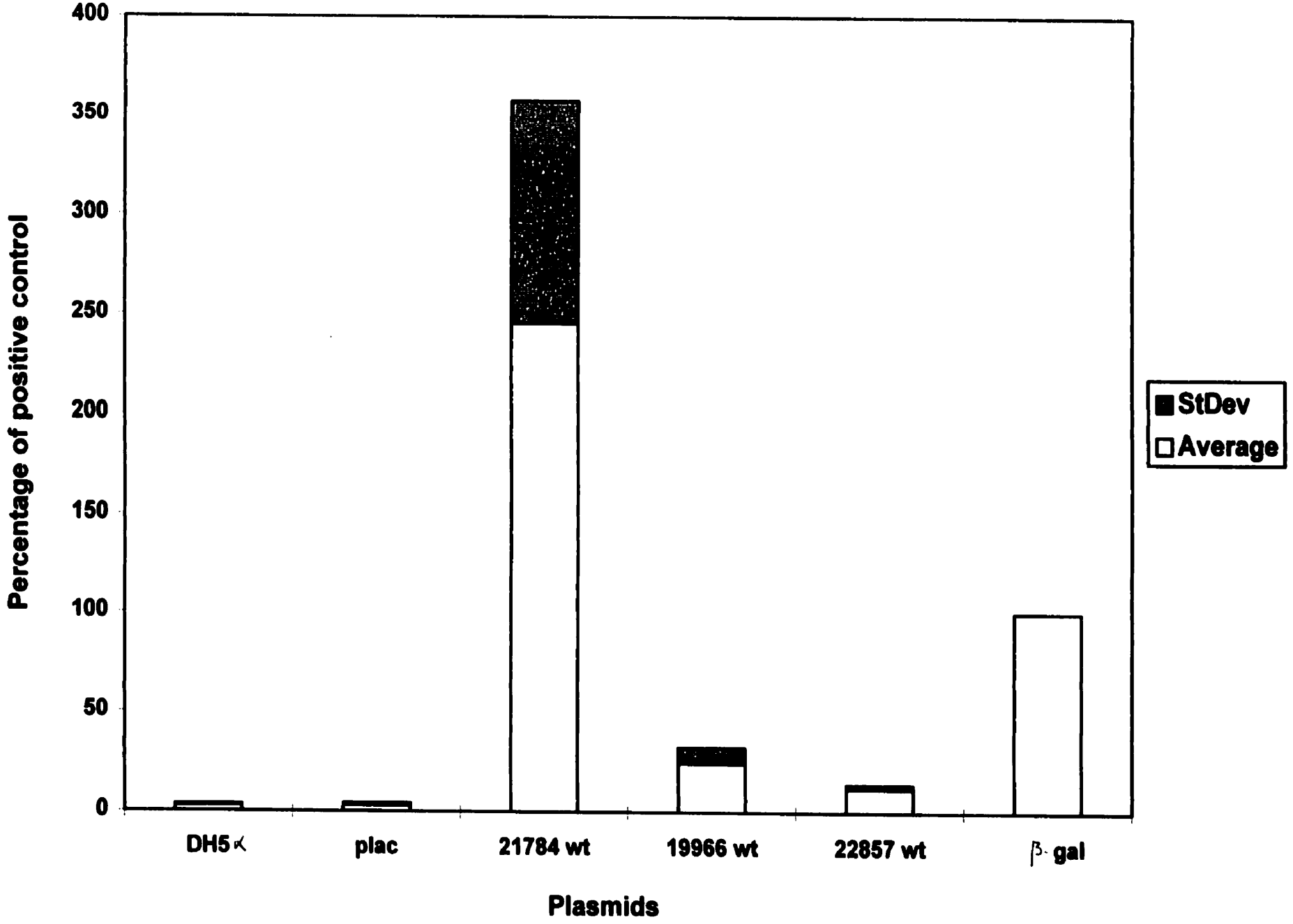

400
350
300
250
200
150
100
50
0
Percentage of positive control
DH5α
plac
21784 wt
19966 wt
22857 wt
β-gal
Plasmids
StDev
Average

Table 4. Summary of mutations inserted in promoters of *porA* in *N. meningitidis*

P1 + 21 (Eliminates 1 G in the G-stretch of 21784) Wild-type 5′ GTT TTT TGC **GGG GGG GGG GGG** TAT AAT TG 3′ Mutant 5′ GTT TTT TGC **GGG GGG GGG GGT** ATA ATT G 3′
P2 + 21 (Eliminates 2 Gs in the G-stretch of 21784) Wild-type 5′ GTT TTT TGC **GGG GGG GGG GGG** TAT AAT TG 3′ Mutant 5′ GTT TTT TGC **GGG GGG GGG GTA** TAA TTG 3′
P3 + 21 (Converts G to A in the G-stretch of 21784) Wild-type 5′ GTT TTT TGC **GGG GGG GGG GGG** TAT AAT TG 3′ Mutant 5′ GTT TTT TGC **GGA GGA GGG GGG** TA TAA TTG 3′
P2 + 19 (Converts A to G in the G-stretch of 19966) Wild-type 5′ GTT TTT TGC **GGA GGG GGG GTA** TAA TTG 3′ Mutant 5′ GTT TTT TGC **GGG GGG GGG GTA** TAA TTG 3′
P5 + 22 (Converts A to G in G-stretch of 22857) Wild-type 5′ GTT TTT TGC **GGA GGG GGG** TAT AAT TG 3′ Mutant 5′ GTT TTT TGC **GGG GGG GGG** TAT AAT TG 3′

mutants are tetracycline-resistant. After the mutagenesis reactions (Figure 8), the plasmids isolated from the transformants were subjected to screening for the presence of the desired mutations by digestion with both *Mlu*I and *Sca*I. The wild-type plasmids pUR19, pUR21 and pUR22 contain a *Sca*I site but not an *Mlu*I site. Successful mutagenesis is predicted to abolish the *Sca*I site and to introduce an *Mlu*I site in these constructs. Therefore, digestion of the mutated plasmids with *Mlu*I will linearize them (Figure 9) while digestion with *Sca*I will not (Fig. 10). The linearized pUR derivatives have an approximate length of 3.6 kb (3.2 kb of pUC119 + 0.4 kb promoter fragment). Therefore successful linearization of these plasmids by digestion results in a migration at 3.6 kb on an electrophoresis gel. The presence of these mutations was further confirmed by sequencing the promoter region.

The mutated promoters were transferred to the pSL1180 vector as *Eco*RI-*Sal*I fragments as previously described and were then subcloned into plac as *Eco*RI-*Bam*HI fragments. The resulting constructs, placp121, placp221, placp321, placp219 and placp522 included the promoter regions containing mutations p1+21784, p2+21784, p3+21784, p2+19966 and p5+22857, respectively.

Measurement of the activity level of the mutants

The activity levels of these new promoters were measured by the β-galactosidase assay. The assay was performed independently three times and the final value was taken as the mean (Table 5). The background levels of DH5α cells and plac were measured.

Comparison of the β-galactosidase activities in plasmids placp121 and placp221 with the wild-type plasmid plac21, which contains the wild-type promoter from strain 21784, showed a decrease in activity in both placp121 and placp221. The first mutation, p1+21784, removed one guanosine in the poly(G) stretch, thus giving 11 nt instead of 12 nt, and resulted in 1.5 fold decrease in

Figure 8. Schematic of mutagenesis protocol.

(Taken from U.S.E. Mutagenesis kit instructions manual, Pharmacia Biotech).

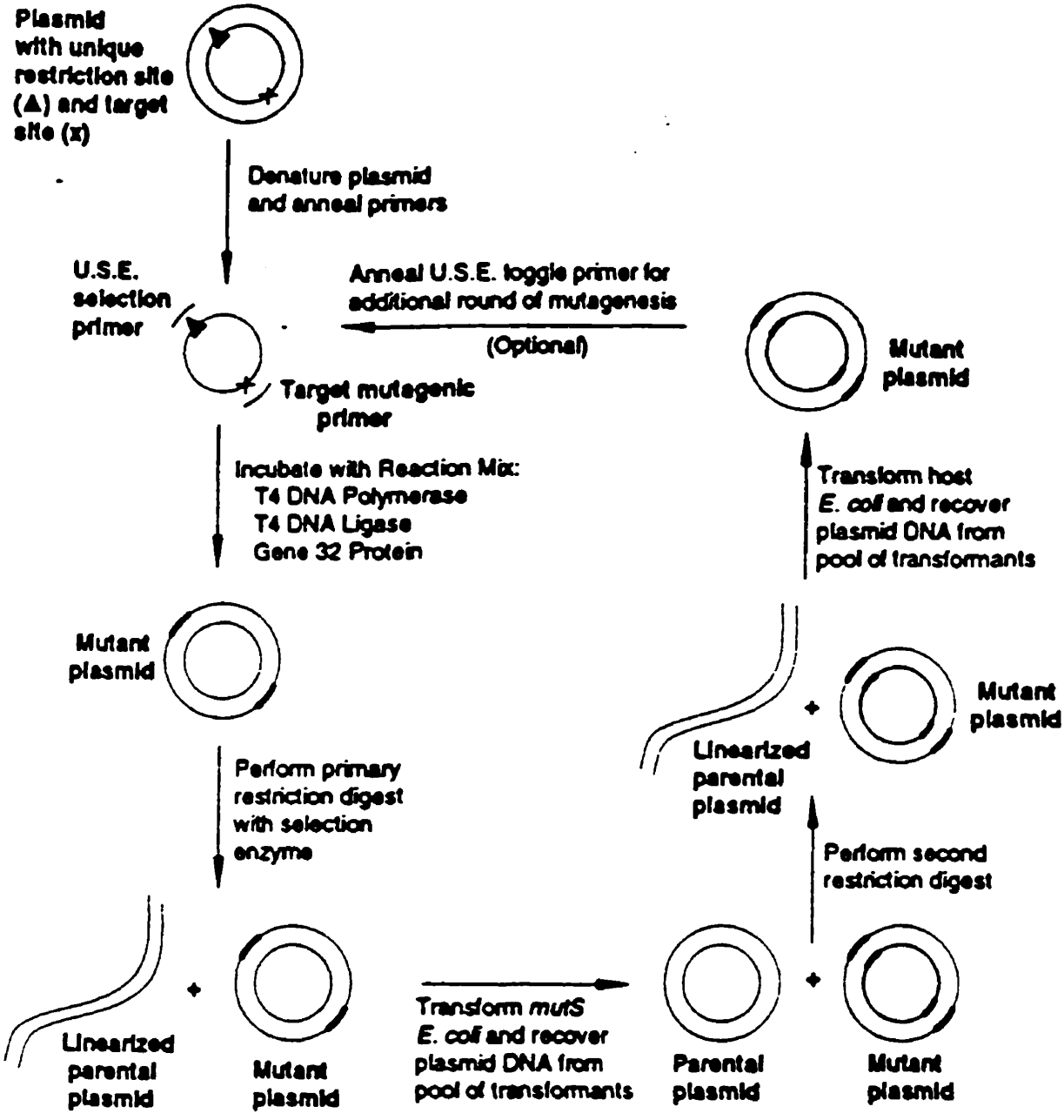
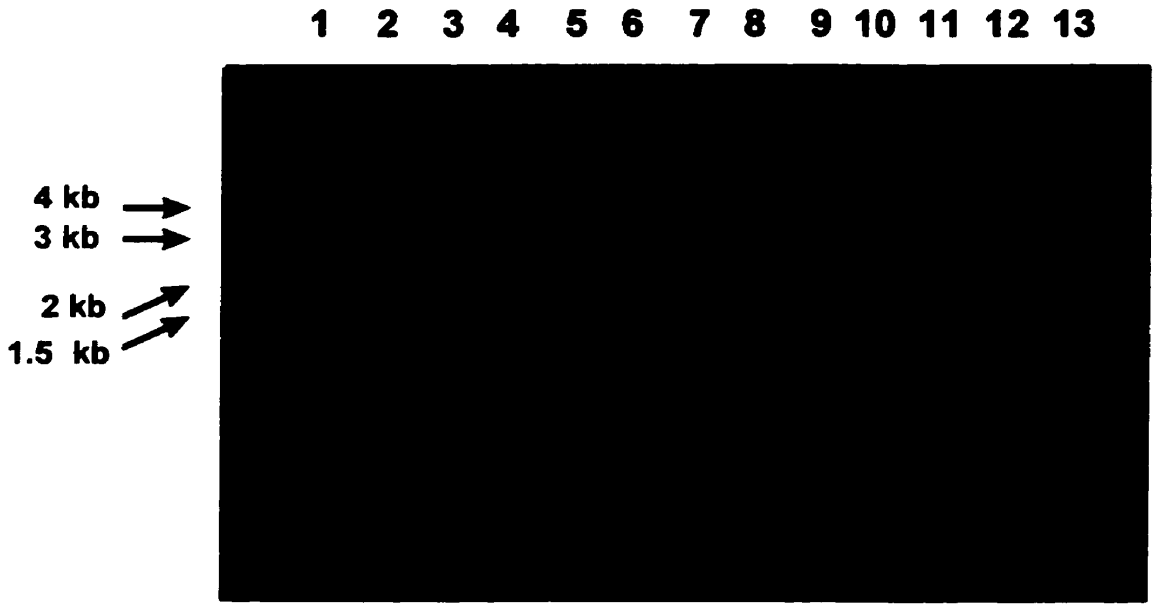
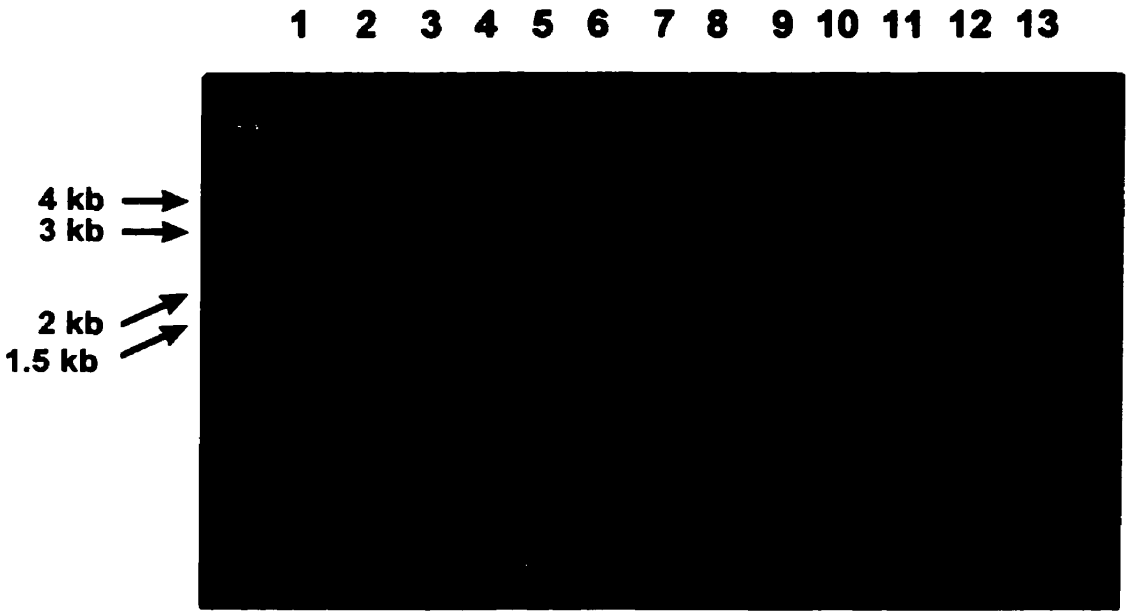

Plasmid with unique restriction site (▲) and target site (x)
Denature plasmid and anneal primers
U.S.E. selection primer
Anneal U.S.E. toggle primer for additional round of mutagenesis
(Optional)
Target mutagenic primer
Mutant plasmid
Incubate with Reaction Mix:
T4 DNA Polymerase
T4 DNA Ligase
Gene 32 Protein
Transform host E. coli and recover plasmid DNA from pool of transformants
Mutant plasmid
Mutant plasmid
Perform primary restriction digest with selection enzyme
Linearized parental plasmid
Perform second restriction digest
Linearized parental plasmid
Mutant plasmid
Transform mutS E. coli and recover plasmid DNA from pool of transformants
Parental plasmid
Mutant plasmid

Figure 9. *Mlu*I digests of pUR clones.



1 undigested pUC119
2 undigested pUR19
3 undigested pUR21
4 undigested pUR22
5 digested pUC119
6 digested pUR19
7 digested pUR21
8 digested pUR22
9 digested pURp121
10 digested pURp221
11 digested pURp321
12 digested pURp219
13 digested pURp522

Figure 10. *Sca*I digests of pUR clones.



1 undigested pUC119
2 undigested pUR19
3 undigested pUR21
4 undigested pUR22
5 digested pUC119
6 digested pUR19
7 digested pUR21
8 digested pUR22
9 digested pURp121
10 digested pURp221
11 digested pURp321
12 digested pURp219
13 digested pURp522

promoter activity as compared to the wild-type. The second mutation, p2+21784, removed two guanosines, thus resulting in 10 nt instead of 12, and showed a greater than three-fold decrease as compared to the wild-type. The plotting of these results as a percentage of the positive control and a measure of the standard deviation of the three independent assays (Fig. 11) shows the significance of these decreases. These results are consistent with the observation that the length of the poly(G) stretch has an influence on *porA* promoter activity (van der Ende *et al.*, 1995).

The next two mutations, p2+19966 and p5+22857, replace the adenosine in the poly(G) stretch by a guanosine in 19966 and 22857, respectively, while the last mutation, p3+21784, replaces the third guanosine in the poly(G) stretch by an adenosine in 21784. In the placp321 construct, which contains the mutation p3+21784, a 10-fold decrease in promoter activity (Table 5; Fig. 11) was observed. Although the wild type promoter of 12 nt displayed high activity, this nucleotide change in the promoter region was sufficient to cause a very high decrease in expression level. In the case of placp219, where the adenosine in the wild type 19966 promoter region was replaced by a guanosine, the activity level was increased by two-fold (Table 5; Fig. 11). However, in placp522, the modification of the adenosine did not cause a significant change in activity (Table 5; Fig. 11). Van der Ende *et al.* (1995) observed that a poly(G) stretch of 9 nt in the *porA* promoter does not exhibit any promoter activity. Our experiments showed that constructs placp221 and placp219 have the same sequence in their poly(G) stretch and thus exhibit about the same promoter activity.

In summary, we conclude that all four constructs in which the poly(G) stretch does not contain an adenosine display promoter activity levels that increase with an increase in the length of the poly(G) stretch: 12 guanosine residues show higher levels than 11 guanosine residues and 10 guanosine residues. In the case of the three constructs that have an adenosine in the promoter region, the promoter activity is extremely low in all three cases.

Table 5. β-galactosidase assay of mutant constructs

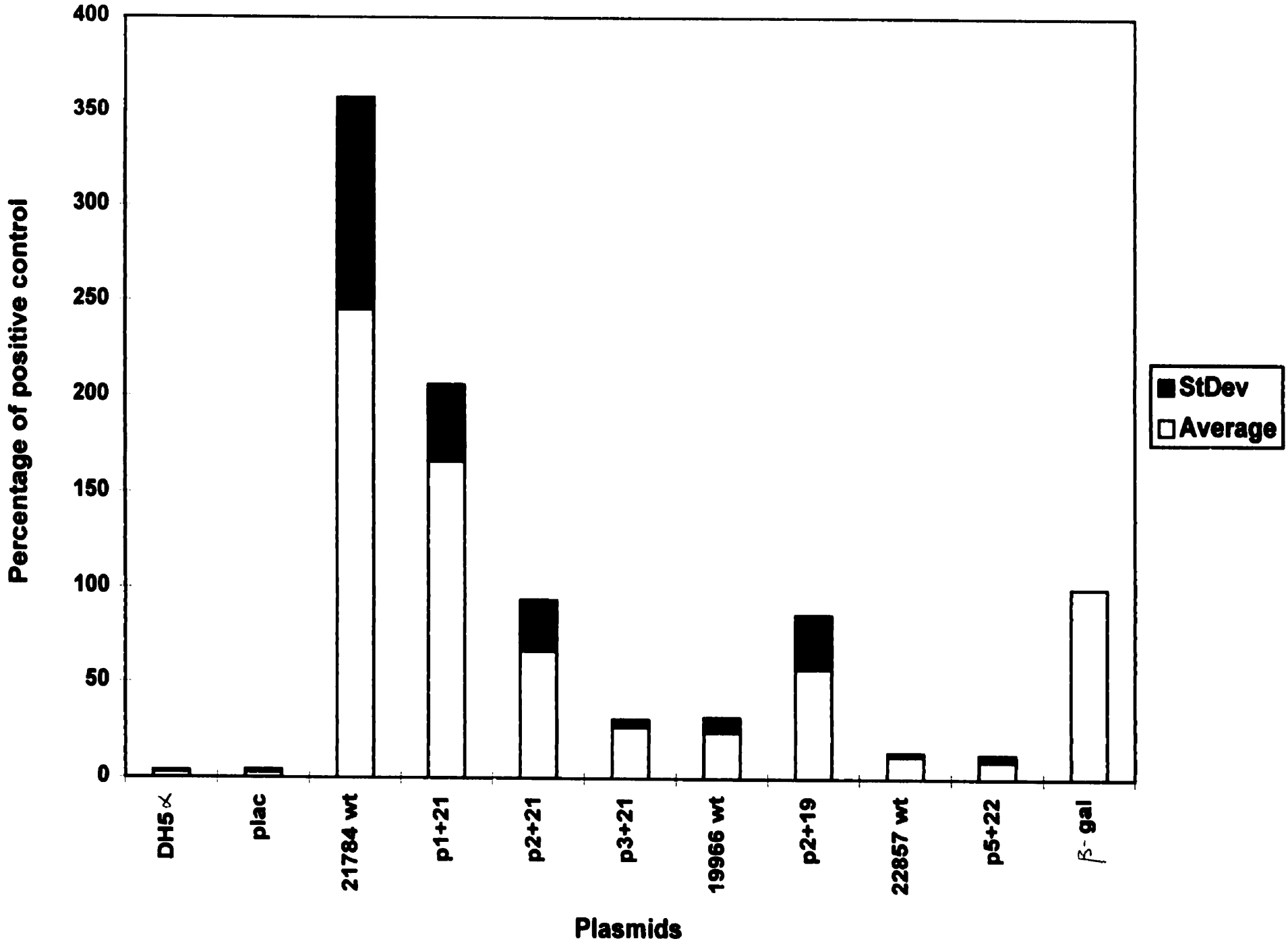
Plasmid	Sequence	OMP 1 Expression	β-gal Levels
DH5α	No promoter insert	-	22
plac	No promoter insert	-	21
plac21	5′ GTT TTT TGC GGG GGG GGG GGG TAT AAT TG 3′	+	1868
p1+21	5′ GTT TTT TGC GGG GGG GGG GG TAT AAT TG 3′		1452
p2+21	5′ GTT TTT TGC GGG GGG GGG GTA TAA TTG 3′		672
p3+21	5′ GTT TTT TGC GGA GGG GGG GGG TAT AAT TG 3′		221
plac19	5′ GTT TTT TGC GGA GGG GGG GTA TAA TTG 3′	-	199
p2+19	5′ GTT TTT TGC GGG GGG GGG GTA TAA TTG 3′		576
plac22	5′ GTT TTT TGC GGA GGG GGG TAT AAT TG 3′	-	66
p5+22	5′ GTT TTT TGC GGG GGG GGG TAT AAT TG 3′		81
β-gal	No promoter insert	-	926

Levels are expressed in standard Miller units. Each assay was performed independently twice. The levels shown here are the means of these determinations.

\+ indicates the presence of OMP1 in the isolate containing the promoter

\- indicates the absence of OMP1 in the isolate containing the promoter

Figure 11. **Histogram showing the β-galactosidase results of the mutant and wild-type constructs as a percentage of the positive control.**


400
350
300
250
200
150
100
50
0
Percentage of positive control
StDev
Average
DH5∝
plac
21784 wt
p1+21
p2+21
p3+21
19966 wt
p2+19
22857 wt
p5+22
β-gal
Plasmids

The presence of this critical adenosine leads to a decrease in expression levels independent of the length of the poly(G) stretch.

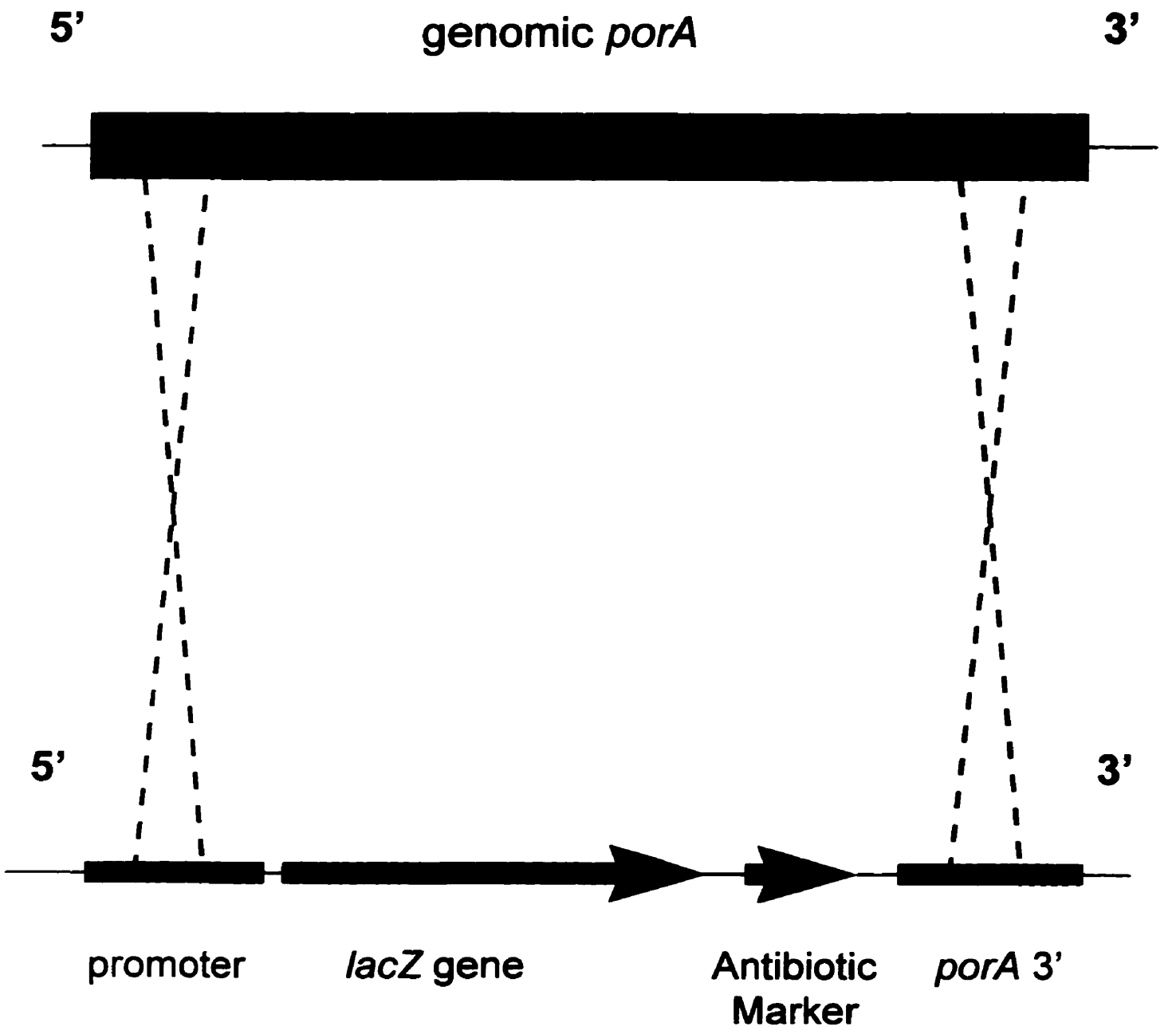
2. The *porA* promoter of *Neisseria*: analysis in *Neisseria meningitidis*

The next step in the analysis of the *porA* promoter was the study of the promoter activity of the modified promoter sequences in *N. meningitidis.*

Initial attempts to transfer the plac derivatives from *E. coli* to *N. meningitidis* by conjugation proved to be unsuccessful. Because of the highly recombinogenic nature of *N. meningitidis*, we chose to study the promoter regions by transformation of the organism by homologous recombination.

To obtain an efficient transformation, the plasmid used should contain the following features: a GC uptake sequence, both a portion of the 5'-end and a portion of the 3'-end of the target gene to be replaced as well as an antibiotic marker (Seifert and So, 1991). In our case, an additional element would be the *lacZ* gene downstream of the *porA* promoter regions. In summary, our plasmid would include the promoter region which constitutes the 5'-end of the *porA* gene followed by the *lacZ* gene, the antibiotic marker and a portion of the 3'-end of *porA*. The GC uptake sequence would also be present on this construct. The region containing *lacZ* and the antibiotic marker and flanked by the promoter and the 3'-end of *porA* would replace *porA* in *N. meningitidis* by gene knockout (Fig. 12). A plasmid containing part of the required elements (GC uptake sequence, *ermC'* gene and the *lacZ* gene) is the pAErmC'G plasmid (Zhou and Apicella, 1996) [Fig. 13]. The *ermC'* gene, which encodes the erythromycin resistance gene, is an appropriate gene to insert in *N. meningitidis* because erythromycin is not used for treatment of meningococcus. The region containing the *lacZ* gene, the *ermC'* gene and the GC uptake sequence (called the *lacZ-ermC'* insert and measuring approximately 4.7 kb) was cleaved as a *Bam*HI fragment, subject to dephosphorylation and cloned downstream of the *porA* promoter regions in the *Bgl*II site of the pSR derivatives. The resulting constructs (Table 1; Fig. 14) are

Figure 12. Recombination event between the fragment containing the promoter, the *lacZ* gene, the antibiotic marker, the 3'-end of *porA* and the GC uptake sequence and the *porA* gene in *N. meningitidis*.

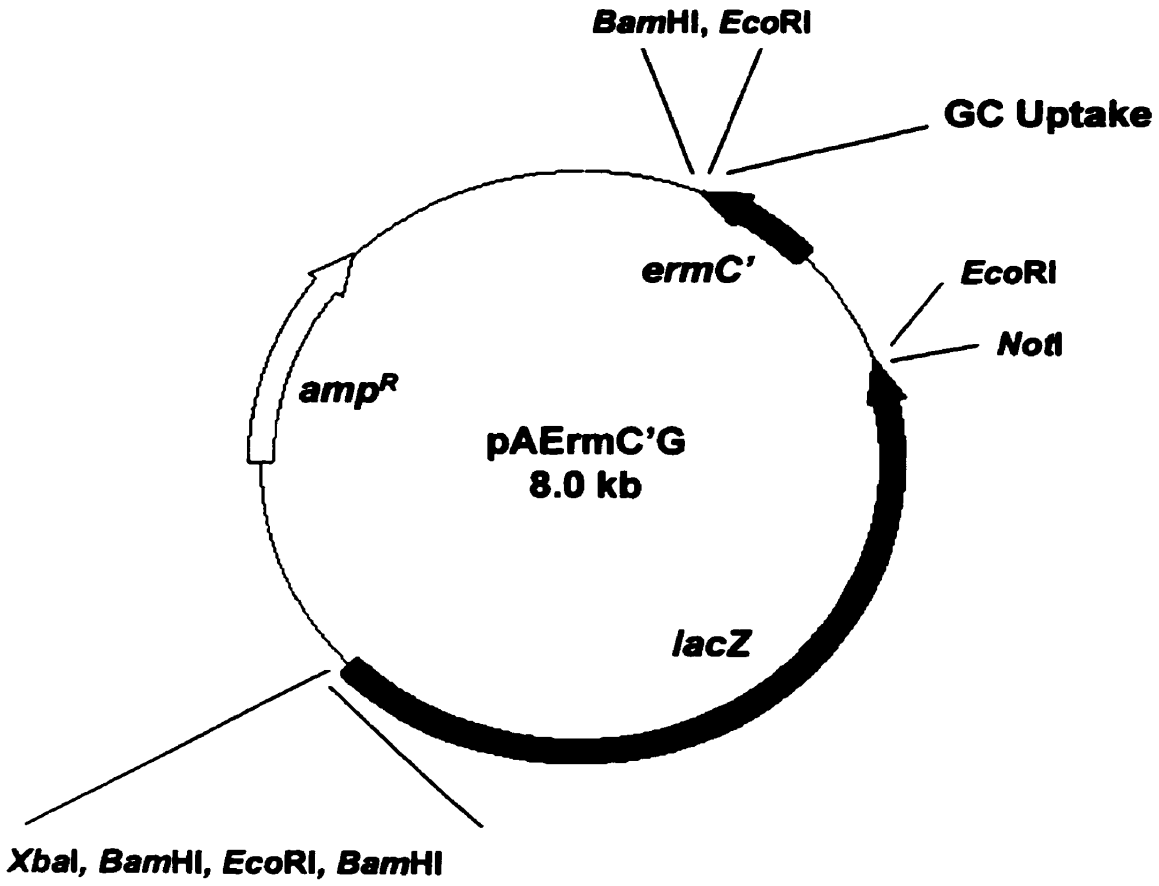

5'
genomic porA
3'
5'
3'
promoter
lacZ gene
Antibiotic Marker
porA 3'

the pSA derivatives and were then transformed in *E. coli* DH5α cells. Selection for transformants was on erythromycin plates. The dephosphorylation of the *lacZ-ermC'* insert ensured that it would not religate and that it would not be selected. Selection with erythromycin ensured that pUR derivatives that do not contain the *lacZ-ermC'* insert are also not selected. These transformants were further screened by *Not*I digestion in order to ensure that the *lacZ-ermC'* insert was cloned in the desired direction: *lacZ* downstream of the promoter region. This digestion produces two *Not*I fragments; in the right orientation, the fragments are of sizes 7.3 kb and 1.2 kb (Fig. 15). pSA derivatives were made for all wild-type and mutant promoters.

The next step was to clone the 3'-end of the *porA* gene downstream of the *ermC'* gene in the pSA derivatives. This was done only for the construct containing the 19966 wild-type promoter, pSA19. The restriction sites *Stu*I and *Sph*I are present downstream of the cloned *ermC'* gene and are unique sites in the pUR derivatives. They were thus selected as appropriate sites for cloning the 3'-end of the *porA* gene. A portion of the 3'-end of *porA* was PCR-amplified using the *Sph*I-P1 and *Stu*I-P2 primers, resulting in a *porA* product flanked by a *Stu*I and an *Sph*I restriction sites. pSA19 was digested with *Sph*I and *Stu*I and the restricted PCR product was cloned downstream of the *lacZ-ermC'* insert (Fig. 14). The plasmid was then linearized with *Nru*I, which has a unique restriction site in this construct, and then introduced by transformation into the *N. meningitidis* isolate 19966 to replace *porA*. Southern analysis was performed on the resulting transformant Nm19 in order to confirm the recombination of the desired fragment. Restricted genomic DNA from the wild-type 19966 and the transformant were run on a gel and probed with both *lacZ* and *ermC'*. The *lacZ* probe was obtained from plac and the *ermC'* probe was obtained from pAErmC'. Hybridizing signals were observed for the transformant when probed with both genes while no signals were observed for the wild-type when probed with both genes (Fig. 16; data shown only for *lacZ* probing). These results confirmed the

successful transformation of *N. meningitidis* using pSP19 but are not sufficient to show successful knockout of the *porA* gene. Additional PCR and sequence analysis are required in order to demonstrate the latter.

Figure 13. pAErmC'G vector. The *Eco*RI *ermC'* fragment is approximately 1.1 kb in length and the *lacZ-ermC'* fragment is approximately 4.7 kb in length. The GC uptake sequence is 5'- TTCAGACGGC -3'.



Zhou and Apicella, 1996

Figure 14. Cloning events to generate pSA19 and pSP19. The *porA* fragment cloned is a 0.7 kb fragment at the 3'-end of the *porA* gene.

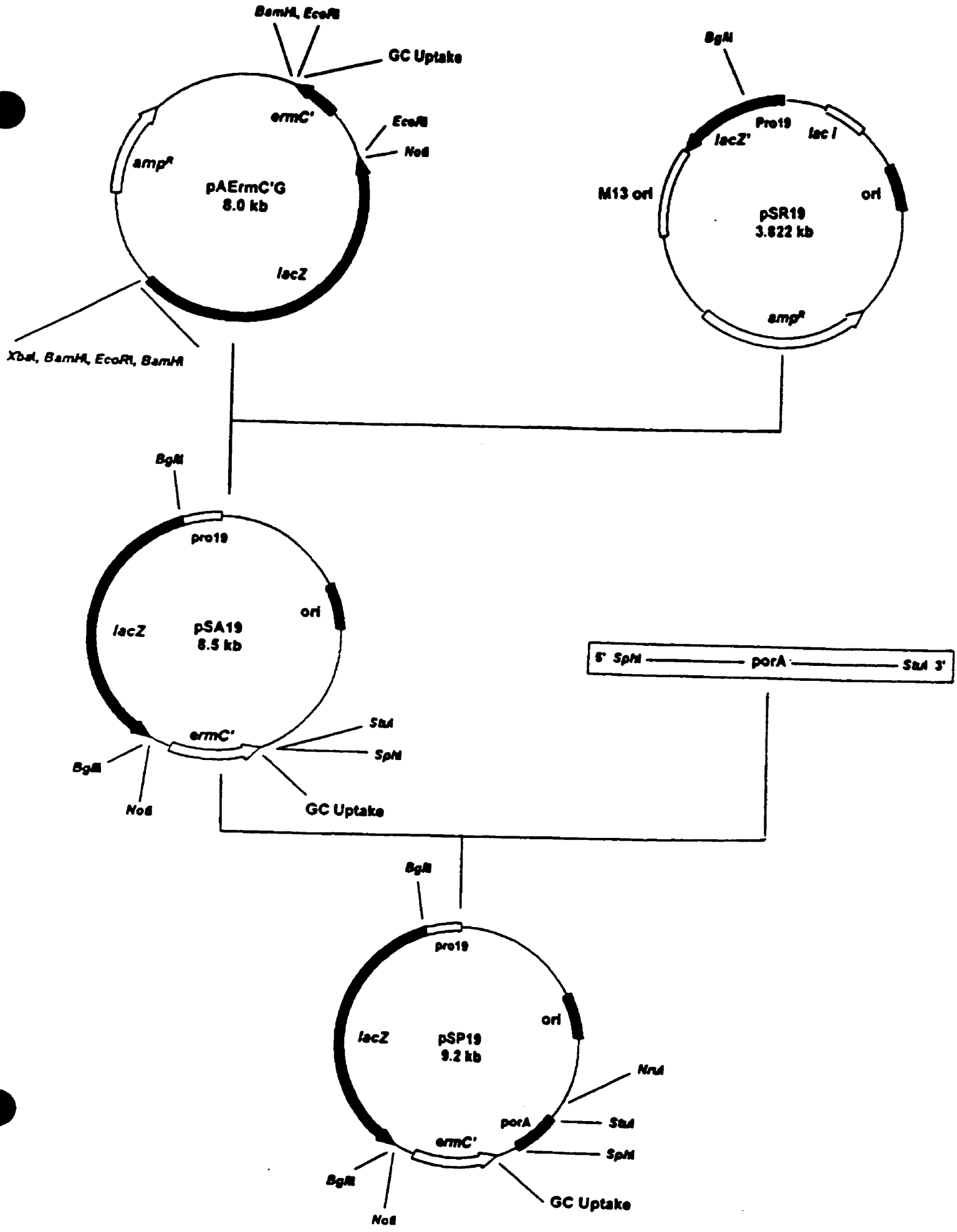
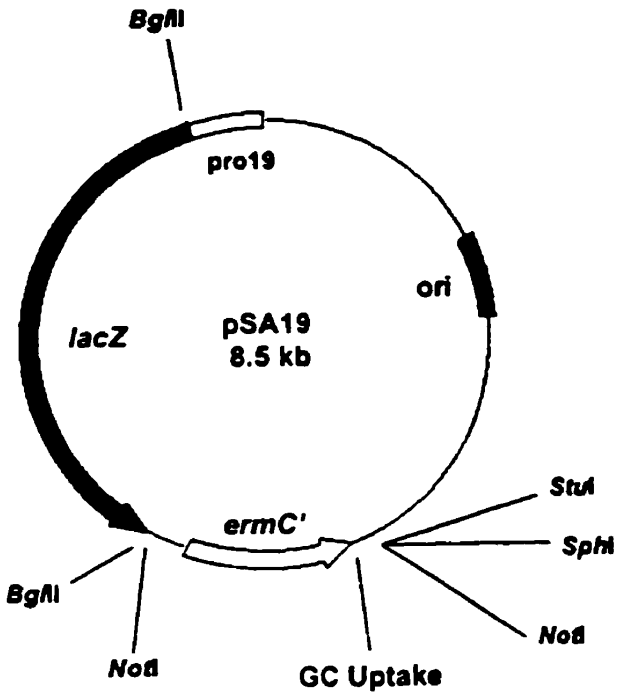

BamHI, EcoRI
GC Uptake
ermC'
EcoRI
NotI
ampR
pAErmC'G
8.0 kb
lacZ
XbaI, BamHI, EcoRI, BamHI
BglII
lacZ'
Pro19
lac I
M13 ori
ori
pSR19
3.822 kb
ampR
BglII
pro19
ori
lacZ
pSA19
8.5 kb
ermC'
StuI
SphI
BglII
NotI
GC Uptake
5' SphI
porA
StuI 3'
BglII
pro19
ori
lacZ
pSP19
9.2 kb
NruI
porA
StuI
ermC'
SphI
BglII
NotI
GC Uptake

Figure 15. The two possible plasmids resulting from the cloning of the *lacZ-ermC'* insert into pSR19. The digestion of these two plasmids results in two *Not*I fragments. A. pSA19, which is the correct clone, results in a 1.2 kb and a 7.3 kb fragments. B. pSAxx, which is the undesired clone, results in a 3.5 kb and a 5.0 kb fragment.

A


BglII
pro19
ori
lacZ
pSA19
8.5 kb
StuI
ermC'
SphI
BglII
NotI
NotI
GC Uptake

B

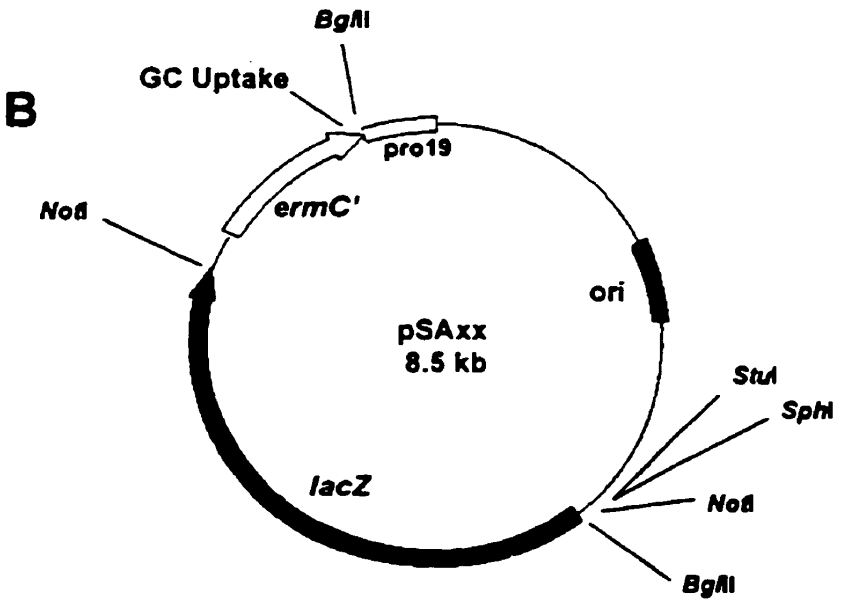
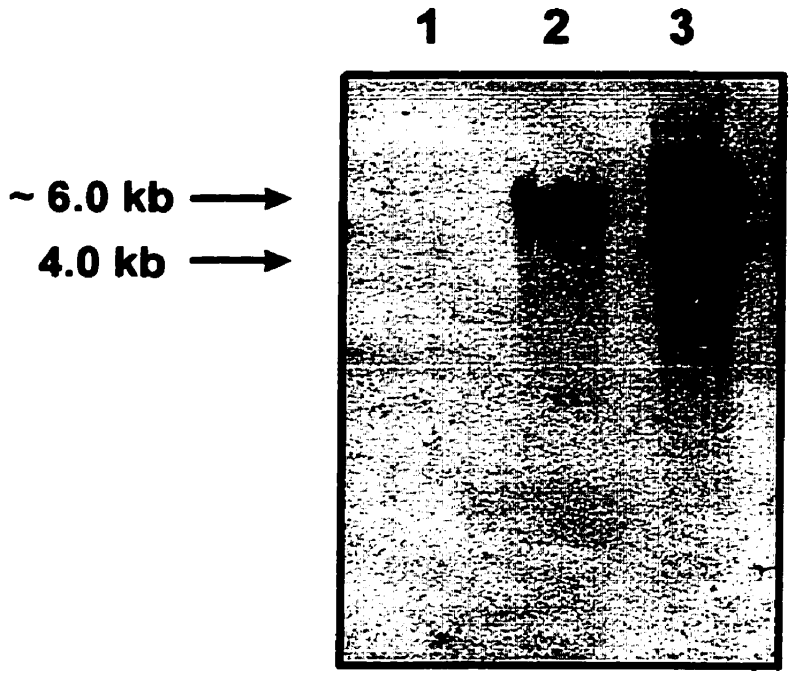

BglII
GC Uptake
pro19
NotI
ermC'
ori
pSAxx
8.5 kb
StuI
SphI
lacZ
NotI
BglII

Figure 16. Southern hybridization of the wild-type 19966 isolate and the transformant Nm19. A *lacZ* probe was used to identify the *lacZ-ermC'* insert in the transformant.



1 19966 wild type

2 Nm19

3 pAErmC'G

DISCUSSION

Phase variation of OMP1 in *Neisseria meningitidis* is attributed to variations in the length of a poly(G) stretch between the -10 and the -35 regions of the *porA* gene (van der Ende *et al.*, 1995). Forty-eight clinical isolates of *N. meningitidis* were obtained from the Laboratoire de Santé Publique du Québec (1993- 94) and were subjected to serosubtyping by ELISA using OMP and by OMP1 profiles (Arhin *et al.*, 1997a). The *porA* promoters of these isolates were sequenced and were shown to contain the poly(G) stretch described by van der Ende *et al.* (1995), which varied between 9 and 15 nucleotides in length (Arhin *et al.*, 1998). Seven isolates which did not express OMP1 (Arhin *et al.*, 1997a) had different poly(G) stretches and an intact *porA* gene. They were also shown to possess an adenosine residue in this poly(G) stretch (Arhin *et al.*, 1998). These results suggest that the length of the poly(G) stretch may not be the sole factor that contributes to different levels of expression of *porA*. The presence of this adenosine residue may contribute to the absence of promoter activity.

We tested the importance of the length of the poly(G) stretch and the presence of the adenosine residue by the insertion of site-specific mutations in the promoter region. The promoter regions of isolates 19966, 21784 and 22857 were cloned into the promoter-probe vector pDN19*lac*Ω and then expressed in *E. coli*. The observation of β-galactosidase activity in the plac21 construct confirmed that the promoter region of the *porA* gene determined by sequence analysis (van der Ende *et al.*, 1995) reflects transcriptional activity.

OMP1 levels have been correlated to the length of the poly(G) stretch in several isolates (van der Ende *et al.*, 1995; Arhin *et al.*, 1998). Isolates with a stretch of 9 nt or less do not express OMP1 and isolates with 10 Gs were shown to have intermediate levels of OMP1. Van der Ende *et al.* (1995) observed high OMP1 expression for isolates with an 11 nt poly(G) stretch. The promoter of the 21784 isolate has 12 nt in its poly(G) stretch and this isolate expresses OMP1.

The β-galactosidase levels of the plac21 and placp121 constructs which have 12 Gs and 11 Gs, respectively, and no adenosines, reflect the activities observed in isolates that express high levels of OMP1. A slight decrease in *lacZ* activity was observed when the length of the poly(G) stretch was reduced from 12 to 11 Gs, but high activity was maintained. This is because these lengths still allow for transcription of *porA* and expression of OMP1. When the length of the poly(G) stretch was decreased to 10 Gs, the activity level of the promoter was decreased by more than 3-fold in comparison to the promoter with 12 Gs. This was observed in both placp221 and placp219 which have a 10 nt poly(G) stretch in the promoter region. In this case, a reduction of the number of guanosine residues in the poly(G) stretch down to 10 nt is sufficient to limit *porA* promoter activity to intermediate levels, as was described previously (van der Ende *et al.*, 1995), thus resulting in a larger decrease in the β-galactosidase levels. In summary, the mutations that reduce the poly(G) stretch from 12 to 11 to 10 Gs result in promoter activity levels that decrease with decreasing length of the poly(G) stretch.

We noticed a 10-fold decrease in promoter activity in placp321 as compared to plac21. However, in placp522, the modification of the adenosine did not cause a significant change in activity. A poly(G) stretch of 9 nt was in fact shown to exhibit no class 1 OMP (van der Ende *et al.*, 1995). In placp219, where the adenosine in the wild-type 19966 promoter was replaced by guanosine, the activity level was modestly increased by two-fold. The three constructs that contain adenosine in their poly(G) stretch (plac19, placp321 and placp522) all displayed low activity as compared to the positive control. This is observed regardless of the length of the poly(G) stretch. One conclusion is that the presence of an adenosine residue in this poly(G) stretch leads to a decrease in *porA* expression levels below a threshold and is independent of the length of the poly(G) stretch.

Phase variation is exhibited by several components in *N. meningitidis* and *N. gonorrhoeae* in order to escape the host's immune system. This phenomenon

has been correlated to the different expression levels of the genes encoding these components. A correlation was established between the length of a poly(G) stretch in the 5'region of the *pilE* gene and the expression levels of the pilus in *N. gonorrhoeae*, which exhibits translational phase variation (Jonsson *et al.*, 1991). In the case of OMP5, both Opc and Opa proteins were shown to exhibit phase variation. This variation in Opa is the result of regulation at the translational level by the alteration of the number of CTCCT pentameric repeats in the *opa* signal peptide, causing a shift in the translation frame (Belland *et al.*, 1989; Murphy *et al.*, 1989). Opc proteins, like OMP1, exhibit transcriptional phase variation via the addition or deletion of cytidine residues in a poly(C) stretch just upstream of the -10 region and overlapping the -35 region (Sarkari *et al.*, 1994). A single nucleotide change from 13 Cs to 14 Cs was sufficient to cause a shift from high Opc levels in the outer membrane to barely detectable levels (Sarkari *et al.*, 1994).

DNA regions containing such mononucleotide stretches are considered hot spots for slipped-strand mispairing during replication and are independent of the RecA protein (Belland *et al.*, 1989; Murphy *et al.*, 1989). Such oligonucleotide regions or short tandem repeats have been correlated with translational and transcriptional regulation in other bacterial systems (Robertson and Meyer, 1992). Several of these systems were described by Sarkari *et al.* (1994). For example, in *Haemophilus influenzae*, variation in the expression of lipopolysaccharide epitopes is caused by alterations in the number of CAAT repeats which occur in the 5'end of the coding region of the *lic* loci (Weiser *et al.*, 1989, Maskell *et al.*, 1992). Phase variation of the *bvgS* gene in *Bordetella pertussis* is caused by alterations in a stretch of six C residues within the open reading frame (Stibitz *et al.*, 1989). Furthermore, a single base deletion in the poly(A) stretch of the *yopA* gene in *Yersinia pestis* leads to a shift in the reading frame and truncation of the *yopA* gene product (Rosquist *et al.*, 1988). Base alterations in promoter regions affecting gene regulation have also been described. The fimbriae genes (*hifA* and *hifB*) of *H. influenzae* have a poly(TA)

stretch present in their combined promoter (van Ham *et al.*, 1993), while promoters of fimbriae genes (*fim*) in *Bordetella pertussis* (Willems *et al.*, 1991) contain a poly(C) stretch. The promoter of the variant lipoprotein genes (*vlp*) of *Mycoplasma hyorhinis* contain a poly(A) stretch (Yogev *et al.*, 1991). Changes in the lengths of these stretches were associated with phase variation of the gene products.

In *E. coli* no evidence for specific contacts between the spacer DNA and the RNA polymerase have yet been established. The main role of the spacer region is thought to be limited to maintaining the -10 and -35 hexamers in the proper orientation to allow efficient binding of the RNA polymerase (Warne and deHaseth, 1993). Although the length of the spacer region (17 +/- 1) was originally thought to be the sole determinant of this region, there is now evidence that the sequence of the promoter region affects the overall structure of the DNA, which in turn affects gene regulation (Warne and deHaseth, 1993). DNA containing poly-purine/pyrimidine tracts can adopt unusual non-B-DNA conformations *in vitro* (Wells *et al.*, 1988; Kohwi and Kohwi-Shigermatsu, 1991). Transitions from one conformation to another puts strain on the superhelicity of the DNA and plays a role in gene regulation. In *E. coli*, the substitution of regions of the spacer DNA devoid of RNA polymerase contact points with non-alternating poly(G) and poly (C) 9 nt sequences resulted in the DNA adopting an A-helical structure (Warne and deHaseth, 1993) This structure causes a reduction in the twist and length of the DNA and impairs promoter activity (Warne and deHaseth, 1993). A spacer region of 16 nt containing this substitution had a much larger decrease in promoter activity than an 18 nt region (Warne and deHaseth, 1993). Poly(dG)-poly(dC) sequences can also adopt a different structure, known as H-DNA, under superhelical strain. This structure consists of an intramolecular dGdGdC tripled region and can augment gene expression when placed 5' to a promoter (Kohwi and Kohwi-Shigermatsu, 1991).

The poly(G) stretch in the *porA* gene in *N. meningitidis* might be adopting an H-DNA conformation which increases the *porA* expression. We notice that in

plac21, which exhibits optimal activity, the spacer region is of 18 nt, while in placp221, which exhibits much lower activity, the spacer region is of 16 nt. These results are in parallel with those of Warne and deHaseth, (1993), who showed that, as compared to a B-DNA conformation, an A-DNA conformation with a spacer of 16 nt shows a lower promoter activity than a spacer of 18 nt. Therefore, it is probable that the poly(G) stretch confers an H-DNA conformation upon the helix and the length of this stretch plays a role in compensating for lack of the B-DNA conformation. Additional analysis is required to see if alternate nucleotides in the poly(G) stretch will also modify promoter activity. The adenosine nucleotides may be disturbing the harmony of this poly(G) stretch, thus causing a sharp decrease in promoter activity. DNA bends have been observed at adenine-thymine tracts of 5 or 6 nucleotides (Koo *et al.*, 1986). It is unlikely that this is the case in this single nucleotide. However, 3 oligo-T-tracts are present upstream of the putative -35 region in *porA* (van der Ende *et al.*, 1995) as well as in the region upstream of the poly(G) stretch and overlapping the -35 hexamer (Arhin *et al.*, 1998). It would be timely to study the relationship, if any, of these oligo(T) tracts and the lone adenosine residue. The promoter of the *porA* gene does not display a typical *E. coli* consensus sequence. Although its -10 region displays a perfect (TATAAT) sequence, there is only a 2 out of 6 match in its -35 region (van der Ende *et al.*, 1995). The absence of a good -35 region suggests that some other regulatory region or protein might compensate for the lack of this hexamer. The *hifA* and *hifB* genes of *H. influenzae* have a perfect consensus -10 region, but their -35 region has only a 3 out of 6 nucleotide match with the consensus *E. coli* (van Ham *et al.*, 1993). The same feature is observed for the *fim* genes of *B. pertussis* (Willems *et al.*, 1990). Although the *vlp* genes of *M. hyorhinis* contain a -10 and a -35 region, the latter is contiguous and partially overlaps the poly(A) stretch (Yogev *et al.*, 1991). The *opc* gene of *N. meningitidis* has a consensus -10 sequence but lacks a -35 region altogether (Sarkari *et al.*, 1994). The lack of a perfect -35 region has been shown to be characteristic of positively regulated promoters (Raibaud and

Schwartz, 1984). Although nucleotide residues in the spacer region are thought not to play a role in RNAP binding, the fact that base substitutions and deletions in these poly-nucleotide stretches have an effect on promoter activity and gene expression suggests that this region may be critical. In the *E. coli fadD* gene, which encodes acyl coenzyme A synthetase, an operator site was identified between the -10 and -35 regions of the promoter (Black *et al.*, 1992). This poly(G) stretch in *porA* may therefore constitute an important cis-factor for a yet unidentified accessory protein.

In conclusion, results of this thesis provide conclusive experimental support for activity of the promoter of *porA* of *N. meningitidis* as defined by van der Ende *et al.* (1995). We have also shown that both the length of the poly(G) stretch as well as the identity of the nucleotides in this region contribute to promoter activity of the *porA* gene and result in phase variation of OMP1. The successful transformation of *N. meningitidis* will allow us to analyze these mutant promoters in *N. meningitidis* and enhance our understanding of this differential gene regulation.

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