Analysis of protein binding to the Sma/Cla DNA repeat in pathogenic *Neisseriae*

Leslie A. Wainwright[†], Joseph V. Frangipane[§] and H. Steven Seifert^{*}

Northwestern University Medical School, Department of Microbiology–Immunology, 303 E. Chicago Avenue, Chicago, IL 60611, USA

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ABSTRACT

Antigenic variation of the pilus is an essential component of *Neisseria gonorrhoeae* pathogenesis. Unidirectional recombination of silent pilin DNA into an expressed pilin gene allows for substantial sequence variation of this highly immunogenic surface structure. While the RecA protein is required for pilin gene recombination, the factors which maintain the silent reservoir of pilin sequences and/or allow unidirectional recombination from silent to expression loci remain undefined. We have previously shown that a conserved sequence at the 3' end of all pilin loci (the Sma/Cla repeat) is required to be present at the expression locus for efficient recombination from the silent loci. In this study, the binding of gonococcal proteins to this DNA sequence was investigated. Gel mobility shift assays and competition experiments using deletion derivatives of the repeat, show that multiple activities bind to different regions of the Sma/Cla repeat and define the boundaries of the binding sequences. Moreover, only the pathogenic *Neisseria* harbor proteins which specifically bind to this repeat, suggesting a correlation between the expression of these DNA binding proteins and the potential to cause disease.

INTRODUCTION

Gonococcal (Gc) pili are essential for bacterial colonization of the human epithelium (1,2). The pilus, along with other gonococcal surface constituents (opacity proteins and lipooligosaccharide), undergo structural variation (3–6), which produces numerous antigenic forms of the bacterium. Antigenic variation is important in gonococcal pathogenesis since it promotes evasion of host immune responses (7,8), and may also change functional characteristics of the bacterial cell (9–11).

Pilus antigenic variation occurs via changes in the pilin amino acid sequence (12–16). The chromosome of the gonococcal strain MS11 harbors either 1 or 2 complete, expressed pilin genes (*pilE*) and 17 transcriptionally silent copies of partial pilin information (*pilS*) (12–18). Antigenic variation of the pilin protein occurs after segments of silent-copy pilin DNA recombine into the

expressed pilin gene (13–15). The transfer of an entire silent copy is unusual. Instead, recombination of 30–150 silent copy nucleotides is more common, exponentially increasing the number of potential expressed pilin gene sequences (13–15,19). Although recombination between *pilS* and *pilE* is facilitated by the RecA protein (20), gonococcal pilin gene recombination reactions predominantly generate non-reciprocal recombination products (14,15). The mechanism(s) responsible for promoting non-reciprocal recombination between *pilS* and *pilE* are unknown, although two models have been proposed. The extracellular model proposes that the non-reciprocal nature of the recombination reactions can result from homologous recombination of *pilS* sequences donated from lysed neighbor cells into the *pilE* locus of a live cell, through the natural exchange mechanism of DNA transformation (21–24). The intracellular recombination model postulates that the *pilS* and *pilE* sequences which recombine to produce a variant pilin gene are present within the same cell. The intracellular model necessitates more complex recombination mechanisms, requiring additional factors to ensure the unidirectional transfer of *pilS* DNA (25,26). Regardless of which model is utilized to generate recombinant *pilE*, we postulate that proteins in addition to RecA may function to: (i) limit the extent of recombination tracts within *pilE*; (ii) limit *pilS* to *pilS* recombination to maintain a stable reservoir of variant pilin DNA; and/or (iii) ensure the non-reciprocal nature of *pilS* to *pilE* recombination.

All pilin loci possess a conserved DNA sequence at their 3' end (the Sma/Cla repeat). We have previously shown that the Sma/Cla repeat is important for pilin gene recombination since removal of this DNA sequence from *pilE* reduces *pilS* to *pilE* recombination (27). This finding suggests that either the deletion blocks the action of one or more proteins that act by binding this repeat, or the deletion reduces the amount of flanking homology available for *pilS* to *pilE* recombination. The first hypothesis is favored for two reasons. First, specific binding of multiple gonococcal proteins to the Sma/Cla repeat has been observed (27). Second, several silent loci contain multiple silent copies (e.g. *pilS1* of strain MS11 has six copies), yet studies that examine the source of variant *pilS* DNA show no preference for silent DNA adjacent to the Sma/Cla repeat (14,28–30). To determine how this DNA sequence functions in antigenic variation, we need to know more about the specificity of protein binding to the repeat, and which portions of the repeat are important in allowing efficient recombination.

^{*}To whom correspondence should be addressed. Tel: +1 312 503 9788; Fax: +1 312 503 1339; Email: h-seifert@nwu.edu

Present addresses: [†]University of Maryland Medical School, Center for Vaccine Development, 685 W. Baltimore Street, Baltimore, MD 21201, USA and

[§]Amersham Life Science, 2636 S. Clearbrook Drive, Arlington Heights, IL 60005, USA

Figure 1. DNA sequence of the *Sma*/*Cla* repeat. (A) (center line) shows the 66 bp of the gonococcal (Gc) *Sma*/*Cla* repeat that are contained between the *Sma*I and *Cla*I sites. DNA sequence 1 shows the *Salmonella* Hin protein binding site *hixR*. DNA sequences 2 and 3 show the transposon *Tn3* transposase and resolvase binding sites, respectively (31). The “.” shows the between the *Sma*/*Cla* repeat and the other DNA sequences indicate nucleotides which are conserved. (B) DNA sequences of four *Sma*/*Cla* deletion derivative that were generated to localize protein binding. The nomenclature for each subclone indicates the end of the *Sma*/*Cla* repeat which is present (5' or 3') and the number of base pairs which are present. (C) The 66 bp of the meningococcal (Mc) *Sma*/*Cla* repeat that are contained between the *Sma*I and *Cla*I sites. Identical bases to the Gc sequence are in uppercase letters and divergent bases are represented by lower case letters.

The DNA sequence encoded by the Sma/Cla repeat shows a high degree of sequence similarity to recombination protein binding sites from other systems. These include: (i) the recognition site (*hixR*) for the *Salmonella* recombination protein Hin; (ii) the recognition sequence (Tn3 IR) for the Tn3 transposon transposase and (iii) the recognition sequence (Tn3 res) for the Tn3 transposon resolvase (31; Fig. 1). In these systems, DNA binding proteins function to cut or nick double stranded DNA to promote subsequent recombination. The sequence similarities between the Sma/Cla repeat and these defined recombination protein binding sites may indicate a conserved function, lending further support to the hypothesis that proteins involved in pilin gene recombination bind to the Sma/Cla repeat.

This report describes the continued characterization of the Sma/Cla repeat and its interactions with DNA binding proteins. The primary goal was to identify the portions of this repeat that bind to gonococcal proteins. To this end, competition experiments with DNA fragments containing portions of the Sma/Cla repeat have identified three regions of the Sma/Cla repeat which show differential effects on protein binding to the Sma/Cla repeat. Also, mobility shift experiments using proteins isolated from both pathogenic and commensal *Neisseriae* demonstrated that only the pathogenic species express the full range of proteins that specifically bind to the repeat, lending further support to for a role of the Sma/Cla repeat and its binding proteins in neisserial pathogenesis.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Neisseria gonorrhoeae: VD300-*recA6* (P⁺, Δ *pilE2*, *recA6*) (Seifert H.S. 1997, in press). Other *Neisseriae*: *N.meningitidis* (strain FAM18) (32); *N.cinerea* (strain NNRL9); *N.lactamica* (strain NNRL30011); and *N.sicca* (strain NRL9989). All commensal strains were a generous gift from J. Cannon. *Escherichia coli*: DH5 α MCR (Gibco).

Neisseriae were grown either: (i) on Gc medium agar base (GCB) (Difco) plus Kellogg supplements (33) at 37°C in 5% CO₂; or (ii) in Gc liquid growth medium (GCLB) (1.5% Protease Peptone #3; 0.4% K₂HPO₄; 0.1% KH₂PO₄; 0.1% NaCl) plus Kellogg supplements and 0.042% NaHCO₃ at 37°C in a shaking water bath.

Molecular cloning

Deletions of the 66 bp *SmaI*–*ClaI* fragment were produced in plasmid pUCSC1, which has a *SmaI*–*HindIII* fragment from pNG1312 (14) containing the *Sma*/*Cla* repeat inserted between the same sites of pUC18. All enzymes were from New England Biolabs except as noted. Enzyme were used according to manufacturers' directions except as noted.

Deletions from the 5' end of *Sma*/*Cla* were generated by treating 8 μ g aliquots of *Sma*I digested pUCSC1 with 2 U of Exonuclease III at 30°C for various times. The reactions were stopped by addition of cold S1 nuclease reaction mixture (final concentration: 1 U S1 nuclease [USB], 30 mM NaOAc pH 5.0, 50 mM NaCl, 1 mM ZnSO₄). Cleavage of single stranded overhangs with S1 nuclease was performed at room temperature for 30 min. S1 nuclease was inactivated by addition of 0.1 vol of 300 mM Tris (pH 8.0), 50 mM EDTA, and incubation at 70°C for 30 min. The DNA was purified from buffers and proteins by spin dialysis through Sepharose CL-6B (Sigma). After *Not*I linker ligation, the plasmid DNA was digested with *Not*I, spin-dialyzed through CL-6B, self-ligated with T4 DNA ligase, and transformed into DH5 α MCR. Deletions were sized by digesting resultant plasmid with *Not*I and *Cla*I and analyzing the smaller DNA fragment on 4% Metaphor (FMC)-TBE gels, and further defined by fMol (Promega) cycle sequencing using standard M13 primers.

Deletions from the 3'-end of Sma/Cla were constructed as follows. Aliquots of 7 μ g *Cla*I digested pUCSC1 were treated with 1 U of BAL31 nuclease at 22°C for various times and the reactions stopped with 20 mM EGTA. This was followed by spin-dialysis through Sepharose CL-6B, repair of ends with DNA polymerase I large fragment, ligation of *Not*I linkers, digestion with *Not*I, self-ligation with T4 DNA ligase, and transformation into DH5 α MCR.

For use as competitors in mobility shift experiments, the *Sma*/*Cla* deletion subclones were digested with *Sma*I and *Not*I, each fragment was separated from the vector by electrophoresis through 4.0% Metaphor agarose-TBE gels, and purified using β -agarase I followed by phenol/chloroform extraction. Concentration of each fragment was determined by running 2-fold dilutions on a 4% Metaphor gel and comparing band intensities to known standards.

Figure 2. Analysis of gonococcal proteins that bind to the Sma/Cla repeat. (A) Coomassie stained proteins separated by SDS-PAGE from a selected fraction of 200 mM (200), 400 mM (400) and 800 mM fractions. (B) Gel mobility shift analysis of each fraction from a DEAE-column. Each protein fraction from the DEAE-column are marked above the autoradiogram from 1 to 20. Fractions 1–7 are from the 200 mM elution, fractions 8–14 from the 400 mM elution and fractions 15–20 from the 800 mM fractions. The arrow indicates the position of the unshifted, radiolabeled Sma/Cla fragment (*Sma/Cla). The mobility shifted Sma/Cla fragments have been numbered based on their relative mobility (I, II and III). In some cases, multiple shifted fragments have similar mobility through PAGE gels. These shifts were distinguished from one another with an early 'E' or late 'L' designation.

Preparation of Gc protein lysates and mobility shift experiments

Lysate preparations and gel mobility shift experiments were performed essentially as described by Wainwright *et al.* (27). Briefly, total Gc proteins were isolated from 1 l of 18 h-old, piliated gonococci by sonication in the presence of 250 µg/ml PefablocSC and 1.0 µg/ml Leupeptin protease inhibitors (Boehringer Mannheim). Proteins were fractionated over DEAE-Sephadex anion exchange resin as described (27). Fractions from the 200 or 800 mM steps that showed similar gel mobility shift patterns were combined, and four to five rounds of concentration and desalting accomplished using Centricon 15 filter units (Amicon) to reduce salt to >10% of starting. The concentrated fractions were resuspended in 1 ml of TE and stored at 4°C. BCA assays (Pierce) were used to equalize total protein from each fraction. For comparison between experiments, the relative mobility (r.m.) was determined by dividing the distance from the wells of the protein–DNA complex by the distance from the wells of the unshifted DNA fragment and multiplying by 10. Shift I had an r.m. = 1.8–2.3, shift II showed an r.m. = 4.1–4.7, and shift III had an r.m. = 5.7–6.1.

Competition experiments

A quantity of 5.4 or 10.8 µg total protein was mixed with binding cocktail (27), 50 µg/ml p[d(I-C)] carrier DNA, and increasing molar concentrations (0, 30-, 100-, 300- and 900-fold molar excess) of competitor DNA. The reactions were incubated at 4°C for 10 min on a platform shaker. An aliquot of 0.5 ng of isolated, undeleted SmaI–NotI fragment (labeled at the NotI site with [α -³²P]dGTP and [α -³²P]dCTP using DNA polymerase I large fragment) was added to the binding reaction and incubated for an

additional 10 min at 4°C. The binding reactions were resolved through 6% PAGE as described (27).

Computer analysis

Densitometry was performed on the dried radioactive PAGE gels using the Fuji BAS 2000 phosphorimager using whole band counting analysis and subtracting background from an area of the gel not containing any radioactive counts. Autoradiographs were scanned using a BioRad Imaging Densitometer and Molecular Analyst 1.1.1 for Windows Software (BioRad). Scanned images were processed using Photoshop 2.5 for Window (Adobe) and Corel Draw 6 (Corel).

RESULTS AND DISCUSSION

Characterization of Sma/Cla binding proteins

Our preliminary analysis of protein binding to the Sma/Cla repeat showed that different salt fractions from a DEAE-column contained Gc proteins that retarded the mobility of the Sma/Cla repeat. To more specifically examine these Sma/Cla binding activities, a late log-phase liquid Gc culture was partially fractionated over DEAE–anion exchange resin. Examination of eluted proteins on SDS–PAGE showed changes in the protein profile with increasing salt concentrations indicating successful fractionation (Fig. 2D), although each fraction contained numerous protein species. The protein fractions were assayed for Sma/Cla repeat binding activity in the presence of p[d(I-C)] carrier DNA, and were resolved through PAGE to separate the bound and unbound DNA (Fig. 2B). Results from this analysis identified several DNA binding proteins present in these fractions and five distinct mobility shifts were defined. The first DNA binding activities were designated IE (relative mobility I, early

Figure 3. Dose response of competition using Sma/Cla deletion subclones. Increasing molar amounts of partial Sma/Cla fragments were utilized to compete for radiolabeled Sma/Cla binding activity. Each experiment shows averaged data from three to five different gel mobility shift experiments from at least two separate fractionations. (A) Competition profile of the IE shift. These proteins eluted at lower salt concentrations in fractions 1–6. (B) Competition profile of the IIE shift. These proteins eluted at lower salt concentrations in fractions 1–6. (C) Competition profile of the IL shift. These proteins eluted at lower salt concentrations, fractions 9–20. (D) Competition profile of the IIL shift. These proteins eluted at higher salt concentrations, fractions 9–20.

fractions) and IL (relative mobility I, late fractions). Two additional activities that retarded the fragment's migration to different but similar intermediate levels were identified in the first salt fractions IIE (relative mobility II, early fractions) and in the later salt fractions IIL (relative mobility II, late fractions). The final activity eluted at relatively high salt concentrations and showed the least change in mobility relative to the unbound fragment (relative mobility III). Selected fractions that harbored the representative binding activity(s) from the 200 and 800 mM fractions were chosen and combined, and the proteins present in these fractions were desalted and concentrated such that equal amounts of total protein could be used in each binding reaction.

To define the region of the Sma/Cla repeat recognized by the different gonococcal proteins, exonuclease-derived, deletion subclones were constructed that contained different portions of the Sma/Cla repeat. Four subclones containing either the first 33 or 27 nucleotides of the 5' end of the Sma/Cla repeat (5–33 and 5–27, respectively) or 31 or 26 nucleotides of the 3' end of the repeat (3–31 and 3–26, respectively) were used for this study (Fig. 1). In mobility shift assays using radiolabeled deletion fragments, each of these fragments was bound by one or more proteins present in the Gc lysate fractions and produced gel mobility shifts. However, it was not possible to correlate these

changes in binding pattern between the mobility shifts of intact and deleted Sma/Cla fragments (data not shown).

Distinct Sma/Cla binding activities are revealed by competition experiments

To determine the DNA sequences of the repeat responsible for the protein binding, fractionated, concentrated and desalted protein samples were incubated with increasing molar-excess amounts of the four deletion derivatives in the presence of radiolabeled, full-length Sma/Cla fragment. Results from this analysis showed that the different protein species isolated from DEAE fractionation had bound to different regions of the Sma/Cla repeat. To quantitate the results from the competition experiments, several repeats were performed and the averaged data reported as the percent of binding of radiolabeled Sma/Cla DNA in the presence of competitor relative to the binding of radiolabeled Sma/Cla DNA in the absence of competitor (Fig. 3).

The bound Sma/Cla repeat responsible for shift IE was competed equally well with either the 3–31 fragment or the entire Sma/Cla repeat (data not shown) with 100-fold molar excess of competitor fragment, producing a 50% reduction in binding of full-length, radiolabeled Sma/Cla DNA (Fig. 3A). In contrast,

Figure 4. Binding profile of proteins from other *Neisseriae* to Sma/Cla repeat or control DNA. Total protein was extracted from *N.meningitidis*, *N.cinerea*, *N.lactamica* and *N.sicca* as described in the Materials and Methods. Lysates from each of the different species were fractionated as described, and were tested to identify protein fractions which harbored DNA binding activity. Once fractions were identified, representative fractions were selected and desalted as described. The amount of protein present in each concentrated fraction was determined, and equal amounts of total protein were used in all binding reactions. Binding to both the radiolabeled Sma/Cla DNA (*Sma/Cla) or a radiolabeled similar-sized DNA fragment isolated from plasmid, pHSS6 (*control). The numbers above each lane indicate the salt fraction which the concentrated protein sample originated. '1' represents the unbound Sma/Cla fragment; '2' represents the 200 mM salt fraction; '3' represents the 400 mM salt fraction; '4' represents the 800 mM salt fraction; and '5' represents the 1200 mM salt fraction. Binding profiles of (A) *N.meningitidis*, (B) *N.cinerea*, (C) *N.lactamica* and (D) *N.sicca*.

shift IE required a 450–500-fold molar excess of 3–26, 5–33 or 5–27 to inhibit binding by 50%. These data suggest that the proteins responsible for shift IE recognize DNA sequences present in the 3–31 portion of the Sma/Cla repeat, and that the competition observed with the other three fragments was nonspecific. This shows that the additional 5 bp from the 5' end of 3–31 are required for the binding that produces the IE shift. The elution profile of the proteins responsible for IIE mirrored that of IE (Fig. 2), suggesting that IE and IIE may be different forms of the same protein species instead of two distinct protein complexes. The dependence of both these shifts for sequences contained entirely in the 3–31 fragment supported this conclusion (Fig. 3A and B). The 50% inhibition level was similar among the other three fragments tested, with >900-fold molar excess competitor required to compete for 50% of full-length Sma/Cla binding. The competition curves for IIE were quite different from the curve observed with IE (Fig. 3A versus Fig. 3B) with the other three fragments barely competing for radiolabeled Sma/Cla binding. Therefore, both the difference in mobility and the different competition curves with non-binding fragments suggest that

while the proteins responsible for shifts IE and IIE share common attributes, they are not identical.

Because of the elution profile of the Sma/Cla binding activities (Fig. 2), we assumed that two different protein species were responsible for shifts IE and IL. Competition experiments utilizing the Sma/Cla deletion subclones supported this hypothesis. In contrast to that observed for shift IE, both 3–31 and 3–26 fragments competed equally well for radiolabeled Sma/Cla binding to IL, with 50% inhibition occurring at 200-fold molar excess (Fig. 3C). These data localize the DNA sequence responsible for IL totally to the 3–26 fragment. Not surprisingly, neither of the 5-fragment deletion derivatives competed well for protein binding, since even in the presence of 900-fold molar excess of competitor, 50% inhibition was never obtained.

Analysis of the IL and IIL shifts demonstrated that different protein species are responsible for these shifts. Shift IIL showed modest competition profiles, with 50% inhibition occurring at 600-fold molar excess of competitor DNA generated from either the 5' or 3' end of the Sma/Cla repeat (Fig. 3D), and the smaller deletion derivatives showed similar levels of competition (data

not shown). This level of competition is similar to that observed with the 5' fragments and the 3–26 fragment with the IE shift. Thus, it appears that while the protein(s) responsible for this shift are specific for the Sma/Cla repeat, (no binding occurs when lysate fractions are tested with a radiolabeled control DNA fragment; ref. 27), neither half of the repeat contains the sequences required for protein binding. These data suggest that DNA sequences contained in the middle of the repeat are responsible for IIL protein binding. The Sma/Cla deletion derivatives currently available can not address this possibility. Additional clones which are deleted from both ends will have to be constructed to explore this finding.

Multiple attempts were made to quantitatively assess the changes in binding of the protein(s) responsible for shift III in the presence of competitor DNA. Since this shift migrated very close to the radiolabeled Sma/Cla fragment (Fig. 2 and data not shown), it was impossible to obtain accurate densitometry values. The elution profile of the shift III activity was distinct from the other late shifts (Fig. 2) and its higher mobility relative to the other shifted fragments suggests that it contains protein(s) distinct from those responsible for the other shifts.

From the data presented in Figures 2 and 3, we have determined that four specific DNA-binding activities produce the five mobility shifts observed. From this data we cannot determine whether the four activities defined in this study share protein components or are discrete proteins or protein complexes. Identification of the proteins responsible for binding to the repeat will determine the protein composition of the four activities identified here, and their functions.

Specific Sma/Cla binding proteins are only found in pathogenic *Neisseria*

Horizontal genetic exchange is significant in the epidemiology and evolution of *Neisseria* species. Evidence exists to suggest that a continuous flow of genetic material affects the chromosomal composition of both pathogenic and commensal *Neisseria* (34,35). To assess the distribution of Sma/Cla binding proteins in the *Neisseria* genus, protein fractions were generated from the pathogenic *N.meningitidis*, and the non-pathogenic *N.cinerea*, *N.lactamica* and *N.sicca*, and tested in mobility shift experiments. Experiments that examined protein binding to either a radiolabeled Sma/Cla repeat or a radiolabeled control DNA fragment showed specific binding in only the pathogenic *Neisseriae*. As was observed with the gonococcus, *N.meningitidis* (Mc, Fig. 4) produced a complex pattern of DEAE-fractionated proteins binding to the Sma/Cla repeat as measured by gel mobility shifts. The proteins present in the Mc lysate produced shifts of similar elution profile and r.m. to the Gc shifts, IL and IIL and III. The proteins producing shifts IE and IIE were not detected in the Mc lysates. Consistent to what was observed with Gc (27), the Mc proteins did not produce detectable binding to the radiolabeled control DNA fragment.

Binding was also observed with both the Sma/Cla repeat and the control DNA in the commensal species; however, it was largely non-specific (Fig. 4). *Neisseria sicca* harbored proteins which bound to both the Sma/Cla repeat and the non-Sma/Cla control fragment (indicated by * and +, Fig. 4D), showing that the proteins were not specific Sma/Cla binding proteins. Similarly, *N.lactamica* also expressed proteins which bound to both DNAs (indicated by > and #, Fig. 4C). While low level binding to the

Sma/Cla repeat was detected in a single fraction from both *N.cinerea* and *N.lactamica* (Fig. 4B, *Sma/Cla-lane 4, and Fig. 4C, *Sma/Cla-lane 5), the low abundance suggests that these activities do not efficiently recognize the Sma/Cla repeat. Interestingly, comparative enzyme electrophoresis experiments have shown that both *N.cinerea* and *N.lactamica* are more closely related to the pathogenic *Neisseria* species than any other commensal species (36).

Southern blot analysis demonstrated that of the species tested, only pathogenic species harbored DNA that hybridized to both conserved pilin probes and probes specific for the Sma/Cla repeat (data not shown). Also, an *E.coli*-K-12, DEAE-fractionated cell lysate, exhibited no specific Sma/Cla binding activities (data not shown). Finally, Southern blot analysis showed that no DNA was present in the *E.coli* genome that hybridized with either Sma/Cla or conserved pilin oligonucleotide probes (data not shown).

The discovery that only pathogenic *Neisseria* harbor specific Sma/Cla binding is consistent with a function that is important for pathogenesis such as antigenic variation. Since Southern blot analysis showed that only Mc and Gc harbored pilin and Sma/Cla DNA, one could argue that the presence of both the Sma/Cla repeat and its binding proteins have been conserved through evolution. It is possible that the commensal species have DNA binding proteins specific for other repeat sequences different from the Sma/Cla, but since there is no evidence for a multi-locus antigenic variation system in the nonpathogenic species, it is unlikely they have need for such a system.

CONCLUSION

The gonococcus expresses four distinct activities that fractionate differentially and/or bind to different sequences of the Sma/Cla repeat. The meningococcus expresses detectable amounts of three of the four activities, while the commensal species tested do not express similar activities. The identification and definition of these Sma/Cla binding activities implies a complex series of protein–DNA interactions that influence the transfer of DNA sequences between pilin loci in Gc and Mc.

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