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Characterization of a spermine/spermidine transport system reveals a novel DNA sequence duplication in *Neisseria gonorrhoeae*

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One sentence summary: A functional transporter of specific polyamines was identified in *Neisseria gonorrhoeae*, and linked to a previously uncharacterized sequence duplication element.

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ABSTRACT

During infection, *Neisseria gonorrhoeae*, the causative agent of the sexually transmitted disease gonorrhea, comes into contact with numerous host compounds including polyamines (e.g. spermine and spermidine). Here, we show that spermine and spermidine concentrations in the growth medium decrease to undetectable levels in the presence of gonococci over time, but not when proteins of the putative polyamine transport system are lost due to mutation. We propose that gonococci have a functional and sole polyamine transport system (PotFGHI) that specifically imports spermine and spermidine. Bioinformatics and molecular analyses showed that the transporter's *potGHI* genes are organized as an operon while the gene encoding the necessary cognate periplasmic polyamine-binding protein (PotF) is located elsewhere on the chromosome. Interestingly, within the *potGHI* locus, we identified a novel duplicated sequence, which we term the Pot-Gene-Associated-Duplication-Element, present in variable copy numbers in different gonococcal strains that was likely formed from the 5' and 3' ends of the coding sequences of the tandemly linked *potH* and *potG* genes, respectively.

Keywords: gonococci; polyamine transport; gene duplication

INTRODUCTION

Neisseria gonorrhoeae is the causative agent of gonorrhea, a sexually transmitted disease of increasing public health concern, affecting over 100 million women and men worldwide (Velicko and Unemo 2009). Gonorrhea is increasingly resistant to first-line antibiotics and could become untreatable (Ohnishi et al.

2011; Bolan, Sparling and Wasserheit 2012; Camara et al. 2012). Untreated gonorrhea can lead to pelvic inflammatory disease and ectopic pregnancies in women, infertility in both sexes and blindness if transmitted to newborns at birth (Woods 2005).

During infection of the human urogenital tract, gonococci encounter a multitude of physiological compounds that can

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Figure 1. Structural and genomic organization of *pot* genes. (A) Schematic representation of the PA transporter PotFGHI. PotF is the periplasmic PA-binding protein, PotH and PotI are the permeases nested in the IM and PotG is the ABC that promotes import of PAs. The black box inside PotF represents a PA. (B) Schematic genomic organization of the genes putatively involved in PA transport in *N. gonorrhoeae* FA19. The bent arrow represents the putative promoter site.

influence the progression of the disease. Among these compounds, polyamines (PA) are present at high concentrations in the human urogenital tract and, notably spermine and spermidine are found at millimolar levels (Bachrach 1970). For instance, we showed that spermine and spermidine at physiological concentrations decrease susceptibility to human- and bacteria-derived cationic antimicrobial peptides (CAMPs) [LL-37 and polymyxin B (PMB), respectively], to killing by normal human serum (Goytia and Shafer 2010), and block biofilm formation (Goytia, Dhulipala and Shafer 2013), under laboratory conditions. Several bacteria were shown to actively transport PAs from the extracellular environment or synthesize them *de novo* (Igarashi, Ito and Kashiwagi 2001), and use intracellular PAs to modulate several important physiological processes (e.g. nucleic acid synthesis and translation) (Tabor and Tabor 1984). In Gram-negative bacteria, PAs diffuse through outer membrane porins and enter the cytoplasm through active transport. In *Escherichia coli*, several transport systems are responsible for the movement of PAs across the inner membrane (IM) (Igarashi, Ito and Kashiwagi 2001), such as ATP-binding cassette (ABC) transporters (PotDABC, PotFGHI) and antiporters (PotE, CadB, PuuP, AdiC). For instance, PotDABC specifically transports spermine and spermidine, and to a lesser extent putrescine (Sugiyama et al. 1996), while PotFGHI specifically transports putrescine (Pistocchi et al. 1993; Sugiyama et al. 1996; Vassilyev et al. 1998a,b).

In contrast to the well-studied systems in *E. coli*, functional PA transporters have not yet been characterized in gonococci. And, since intracellular PAs can influence important intracellular metabolic processes of bacteria, we sought to define the PA transport system(s) in gonococci. We now propose that, in contrast to *E. coli*, gonococci produce a single PA transport system, which appears similar to the PotFGHI transporter in *E. coli* (Fig. 1A), for active influx of spermine and spermidine, which can be present at high levels in

genital fluids (Bachrach 1970) encountered by gonococci. We also describe a novel DNA sequence duplication that we term the Polyamine-Gene-Associated-Duplication-Element (PGADE), which was likely formed by an in-frame fusion of the 5' and 3' ends of the tandemly linked *potH* and *potG* genes, respectively.

MATERIALS AND METHODS

Bacterial strains, culture conditions and chemical compounds

Neisseria gonorrhoeae strain FA19 was used as the wild-type (WT) strain for PA uptake studies and to construct mutants. All gonococcal strains (Table 1) were routinely grown at 37°C in supplemented GCB liquid broth (Difco Laboratories, Detroit, MI) with 0.043% (v/v) sodium bicarbonate or passed on supplemented GCB agar grown at 37°C under 5% CO₂, as described previously (Shafer et al. 1984a,b). *Escherichia coli* DH5-α was grown on Luria-Bertani (LB) agar or in LB broth (Difco Laboratories, Detroit, MI) at 37°C.

All hydrochloride salt PAs and antibiotic agents were obtained from Sigma-Aldrich (St. Louis, MO), except for ciprofloxacin (Fluka BioChemika, Steinheim, Germany). PAs were dissolved in double distilled H₂O (ddH₂O), and then filtered on 0.45 μm PES filters.

Construction of mutant strains

The oligonucleotide primers and the plasmids used are listed in Table S1 (Supporting Information). PA permease mutant strain FA19 *potHI::kan* mutant was described previously (Goytia and Shafer 2010). Complemented strain FA19 *potHI::kan* C'HI was constructed using primers allowing amplification of the entire open reading frames (ORFs) of *potH* and *potI*. The amplified sequence was digested (see Table S1, Supporting Information), purified and inserted in the pGCC4 vector (Mehr and Seifert 1997) digested with the same restriction enzymes, downstream of the *lacZ* promoter controlled by isopropyl β-D-1-thiogalactopyranoside (IPTG). When needed, FA19 *potHI::kan* C'HI mutant was grown as described above but in the presence of 1 mM of IPTG.

Periplasmic PA-binding protein (*potF*) mutants were constructed using the strategy previously described (Goytia and Shafer 2010), using specific primers and selectable marker cassettes for insertion/deletion mutations (see Table S1, Supporting Information).

Determination of *potGHI* operon, *potF* and PGADE transcripts

RNA was extracted from gonococcal strain FA19 using the Trizol reagent (Invitrogen, Carlsbad, CA) following manufacturer's instructions, followed by RQ1 DNase treatment (Promega, Madison, WI). First-strand cDNA was synthesized using SuperScript reverse transcriptase II (Invitrogen) and a specific primer annealing to the 3' region of *potI*, *potI*-RTR (Table S1, Supporting Information), or random hexamers, following manufacturer's instructions. To determine whether *potGHI* genes were in an operon, cDNA synthesized with *potI*-RTR was amplified using specific primers annealing to the 5' region of *potG* (i.e. PE-*potGR3* and *rpS15*-RTF). To confirm transcription of *potF* genes, cDNA synthesized from random hexamers was amplified

Table 1. Bacterial strains used in the study.

Strain name	Relevant genotype	Reference
FA19	WT strain	Maness and Sparling (1973)
FA19v	FA19 with empty pGCC4 vector	This study
FA19 <i>potHI::kan</i>	FA19 with deletion of <i>potHI</i> , insertion of <i>aphA-3</i> cassette (kanamycin resistance)	Goytia and Shafer (2010)
FA19 <i>potHI::kan C'HI</i>	FA19 <i>potHI::kan</i> complemented with <i>potHI</i> inserted at a secondary site; <i>potHI</i> expression under IPTG control	This study
FA19 <i>potF1::spc</i>	FA19 with deletion of <i>potF1</i> , insertion of Ω cassette (spectinomycin resistance)	This study
FA19 <i>potF2::kan</i>	FA19 with deletion of <i>potF2</i> , insertion of <i>aphA-3</i> cassette (kanamycin resistance)	This study
FA19 <i>potF3::spc</i>	FA19 with deletion of <i>potF3</i> , insertion of Ω cassette (spectinomycin resistance)	This study
MS11	WT strain used for PGADE studies	Swanson (1973)
1291	WT strain used for PGADE studies	M.A. Apicella
F62	WT strain used for PGADE studies	Shafer et al. (1984a,b)
FA1090	WT strain used for PGADE studies	West and Clark (1989)
FA6140	WT strain used for PGADE studies	P.F. Sparling

using gene-specific primers annealing to the coding region of each of the *potF* genes. To confirm transcription of PGADE, cDNA synthesized from random hexamers was amplified using specific primers annealing to the junction of *potH*–*potG* sequence in PGADE and to a unique sequence of *potH*.

Minimal inhibitory concentration (MIC) and biofilm assays

MIC values for selected antibiotics and biofilm formation assays with or without added spermine to the culture medium were determined as previously described (Goytia and Shafer 2010; Goytia, Dhulipala and Shafer 2013).

Bioinformatics analysis of whole genome sequences (WGSs)

WGS reads from 63 clinical isolates of *N. gonorrhoeae* in FASTQ format were obtained using Illumina sequencing (Ezewudo et al. 2015). The NCBI Short Read Archive accession number for the data is SRA099559. Briefly, the incidence of each unique 30 nucleotide word ('30-mer') was counted in each FASTQ file using the Jellyfish software (Marçais and Kingsford 2011) and stored as a table. For the purposes of this study, we wrote a UNIX shell script to parse out the counts for specific 30-mers associated with unique sequences in *potG*, *potH* and PGADE.

PA content determination

After overnight growth on GCB agar plates, gonococci were re-suspended in 13 mL of supplemented GCB liquid broth containing sodium bicarbonate to approximately 10^5 CFU/mL and were grown with shaking at 37°C until stationary phase was reached. The cultures were centrifuged at $3500 \times g$ and the resulting supernatant (spent medium) was filtered through a 0.45- μ m filter unit (Millipore, Darmstadt, Germany) and stored at –80°C until further analysis. Extracellular PAs in fresh and spent growth media were measured by the method described by Hawel and Byus (2002) and levels were expressed as μ mol of PA/L of medium.

RESULTS

Identification of a putative PA transport system in *N. gonorrhoeae*

In order to determine if gonococci can import PAs, we quantified PA content in fresh broth and in spent growth media, harvested from a culture of strain FA19 at different time points of growth. We observed that extracellular concentrations of spermine and spermidine, in the culture medium, gradually decreased from 13 μ M in the fresh broth to undetectable levels (<10 pM) in spent media from FA19 cultures in stationary phase (Fig. 2). We also tested the fresh broth and the spent media for the contents of other PAs, putrescine and cadaverine. Interestingly, neither of the concentrations of these PAs were significantly changed (data not shown) over time, suggesting that gonococci can discriminate PAs in GCB broth.

Genomic identification and biological characterization of a putative gonococcal spermine/spermidine transport system

Bioinformatics analysis of the WGS of gonococcal strain FA19 (www.broadinstitute.org) revealed the presence of homologous genes to *E. coli*'s PA transporter *potFGHI* (Fig. 1A). In the gonococcal genome, we found three *potF* genes encoding putative periplasmic PA-binding proteins (vs 2 for *E. coli*), a pair of genes encoding putative IM permeases, *potHI* (vs 2 pairs for *E. coli*), and two ABC genes, *potA* and *potG* (as in *E. coli*). The genomic organization of the gonococcal *pot* genes is shown in Fig. 1B. Bioinformatics analysis of the sequence of the *potGHI* locus also suggested that the three genes are organized as an operon (Fig. 1B), which was confirmed by RT-PCR (Fig. S1A, Supporting Information). The deduced amino-acid sequences of the putative proteins encoded by the *potFGHI* genes revealed varying degrees of identity (40–54%) to their counterparts in *E. coli* (Table 2, and Fig. S2, Supporting Information). Interestingly, we could not find homologs in the gonococcal genome of other PA transport systems that have been described in *E. coli* (e.g. PotDABC, PotE, CadB, PuuP, AdiC, MdtJ) (Igarashi, Ito and Kashiwagi 2001)

Figure 2. Growth medium's spermine and spermidine concentrations decrease over time as *N. gonorrhoeae* grows. (A) Growth curve of *N. gonorrhoeae* strain FA19 (filled diamonds) in supplemented GCB medium containing sodium bicarbonate. Samples were collected over time (indicated by an arrow) to measure concentrations of extracellular polyamines. (B) Concentrations of extracellular polyamines, spermine (filled squares) and spermidine (open squares), in the growth medium, decrease over time. The limit of detection of PA concentration using this methodology is 10 pmol/L of medium. This experiment is representative of three independent experiments. Error bars represent standard deviations. Statistically different (*) concentrations of spermine or spermidine in fresh media vs spent media at different time points were determined using Student's t-test with p -values < 0.05. Dagger denotes undetectable levels (< 10 pM) of spermine or spermidine. [PA]_e, extracellular PA concentration (μM); N/A: not assayed.

suggesting that gonococci express a single PA transport system, which was further tested by mutational and chemical studies described below.

To test whether the identified genes contribute to spermine and spermidine transport by gonococci, we constructed mutants of the three putative periplasmic PA-binding protein paralogs (*potF1::spc*, *potF2::kan*, *potF3::spc*) and of the putative permease genes *potHI* (*potHI::kan*). We were able to establish by RT-PCR that each one of these genes was transcribed (Fig. S1A and B, Supporting Information). These mutations did not impact the growth rate of gonococci (data not presented). Among the periplasmic PA-binding protein mutants, only spent media collected from strain FA19 *potF2::kan* cultures showed a significant difference of PA concentrations to spent media from WT. In the FA19 *potF2::kan* mutant, spermine and spermidine concentrations were similar in the fresh and spent media (Fig. 3A). In addition, bioinformatics analysis revealed that PotF2 in particular has conserved key amino acids for spermine and spermidine binding, similar to the well-characterized homologous PotD protein from *E. coli*. PotF1 and potF3 present conserved residues for PA binding to a lesser extent (Fig. S2C, Supporting Information). Together, the experimental data and bioinformatics results support a role for PotF2 as a critical binding protein for spermine and spermidine in gonococcal PA transport (Fig. 3A; Fig. 2C and D, Supporting Information).

Additionally, we assessed PA concentrations in spent media collected from cultures of the mutant of the putative PA permeases, FA19 *potHI::kan*, and its complemented variant (FA19

Table 2. Comparison of translated sequences of putative PA transport genes of *N. gonorrhoeae* strain FA19 to known PA transport proteins in *E. coli* strain K12.

Periplasmic PA-binding protein					
<i>E. coli</i>	PotD ^a	PotF ^a			
<i>N. gonorrhoeae</i>			PotF1	PotF2	
PotF1 (NGEG.00323)	32.2 ^b	37.8			60.4
PotF2 (NGEG.01921)	31.6	40.8	60.4		
PotF3 (NGEG.01368)	27.6	36.2	55.4		53.5
PA transporter permeases					
<i>E. coli</i>	PotB ^c	PotC ^c	PotH ^d	PotI ^d	
<i>N. gonorrhoeae</i>					PotH
PotH (NGEG.00311)	30.9 ^b	19.7	47.2	15.7	
PotI (NGEG.00312)	21.1	38.6	18.0	53.4	18.3
ATP-binding cassette					
<i>E. coli</i>	PotA ^e	PotG ^e			
<i>N. gonorrhoeae</i>			PotG		
PotA (NGEG.01780)	39.3 ^b	34.5	34.5		
PotG (NGEG.00310)	39.2	54.1			

^aPotD (P0AFK9) and PotF (P31133) from *E. coli* share 33% identities at the residue level.

^bpercent identity at the residue level. Putative protein sequences from *N. gonorrhoeae* FA19 are translated from the genomic sequences. The highest percent identities for each of the translated sequences from *N. gonorrhoeae* to the *E. coli* sequences are in bold.

^cPotB (P0AFK4) and PotC (P0AFK6) from *E. coli* share 18% identities at the residue level.

^dPotH (P31135) and PotI (P0AFL1) from *E. coli* share 16% identities at the residue level.

^ePotA (P69874) and PotG (P31134) from *E. coli* share 33% identities at the residue level.

potHI::kan C'potHI) expressing *potHI* from a second chromosomal site in the presence of IPTG. Unlike the WT and complemented strain supernatants, FA19 *potHI::kan* supernatant showed spermine and spermidine concentrations similar to fresh media concentrations (Fig. 3B). Accordingly, we suggest that the gonococci imports spermine and spermidine specifically through the PotF2GHI transporter, and that this is the main mechanism by which gonococci deplete these PAs from the extracellular environment.

Our previous work revealed that the presence of spermine and spermidine in the growth media can render WT gonococci less susceptible to cationic antimicrobial peptides and less able to form a biofilm. Accordingly, we tested the *pot* gene mutants (described above) for differences in these properties compared to WT FA19. We observed that the mutant and WT strains had the same level of susceptibility to PMB and the same ability to form a biofilm in the absence of spermine. Similarly, the WT and mutant strains showed decreased antimicrobial susceptibility levels and were impaired to form biofilms in the presence of spermine (data not shown).

Identification of the variable PGAE sequence in gonococci

While studying the genomic organization of the *potGHI* operon in gonococcal strains commonly used in the lab (e.g. FA19, MS11, FA1090), we noticed a variation in the nucleotide sequence between *potG* and *potH* in the WGS of multiple gonococcal strains.

Figure 3. *Neisseria gonorrhoeae* uses PotF2GHI to import spermine and spermidine. (A) The periplasmic PA-binding protein PotF2 is essential to deplete spermine and spermidine from the growth medium. PA concentrations of spermine and spermidine in the fresh broth and in the growth medium were tested in the supernatant of several strains, grown to stationary phase, with one of the putative periplasmic PA-binding proteins deleted. (B) PA contents from fresh and spent media from FA19 WT, FA19 *potHI::kan* mutant and the complemented strain FA19 *potHI::kan* C'HI were grown in the absence (left panel) or presence of IPTG (1 mM) (right panel). When IPTG is present in the medium, complemented strains express *potHI*, and regain the WT phenotype. FA19, WT strain; FA19v, FA19 with the empty vector pGCC4; FA19 *potHI::kan*, FA19 with deleted region in *potHI*; and FA19 *potHI::kan* C'HI, complemented strain FA19 *potHI::kan* with *potHI* genes integrated in the genome at a secondary site under IPTG control. Results represent averages of an experiment run in triplicate. This experiment is representative of three independent experiments. Error bars represent standard deviations. Statistically different (asterisk) concentrations of spermine or spermidine in fresh media vs spent media were determined using Student's t-test with *p*-values < 0.05. Dagger denotes undetectable levels (<10 pM) of spermine and spermidine. [PA]_e, extracellular PA concentration (μM); N/A, not assayed.

Using the genome sequence accessible online of strain FA19 as an example, we observed that this intergenic region contained a duplication of the sequence between the 3' end of *potG* and the 5' end of *potH* (Fig. 4A). This variation was characterized by a 144-bp sequence that begins at the first 74 nucleotides of *potH* and is fused in-frame to the last 70 nucleotides of *potG*; we termed this sequence as the PGADE (Fig. 4B). Furthermore, the duplication also contains the 5' untranslated intergenic region preceding the *potH* ORF, with a putative Shine–Dalgarno sequence, located 8 nucleotides upstream of the translational start codon, promoting the initiation of translation of *potH*. WGSs from gonococcal clinical isolates were analyzed for the relative presence of the PGADE sequence to flanking chromosomal sequences, not associated with PGADE (Fig. 4B). Bioinformatics analysis of WGSs revealed that 10 of 63 isolates (16%) contained approximately one PGADE sequence per flanking chromosomal sequence (1:1 ratio). Three additional isolates displayed ratios of PGADE sequences to flanking chromosomal sequences that varied (1:50, ~1:100 and 1:120). It is possible that these samples include a mixed population of gonococcal cells, some of which contained the PGADE sequence, while others did not. Indeed, we would not expect a typical Illumina sequence error to create these sequences as they are a fusion of two separate DNA regions. The remainder

50 strains analyzed by whole-genome sequencing lacked the PGADE sequence.

Moreover, we were able to show by PCR in strains frequently used in the laboratory (e.g. strains FA19, FA1090 and F62) that PGADE can be present in variable copy numbers ranging from 1 to 3 (summarized in Fig. 4C and D). Inspection of the PGADE nucleotide sequence suggests that it could encode a 47-amino-acid peptide with a putative Shine–Dalgarno sequence (5'-AAGG-3') positioned 8 nucleotides upstream of the translational start codon and complementary to the 16S rRNA. In order to determine if the PGADE sequence can be expressed, we used RT-PCR to detect a transcript. As is shown in Fig. S3 (Supporting Information), we were able to generate a cDNA using an oligonucleotide primer that anneals to *potI* (downstream of PGADE), *potI*-RTR, and then amplify the cDNA across the junction of the *potH* and *potG* sequences within PGADE, with specific primers.

DISCUSSION

Herein, we report that *N. gonorrhoeae* can deplete specific PAs from growth media and suggest that this is due to PA import by the Pot transport system described herein. Based on this hypothesis, we further suggest that gonococci could use this Pot transport system to acquire PAs present in the human urogenital tract. Intracellular PAs can significantly impact important physiological processes (Karatan, Duncan and Watnick 2005; McGinnis et al. 2009; Potter and Paton 2014) and pathogenic mechanisms of a number of bacteria (Nastri and Algranati 1996; Mathur and Waldor 2004; Kwon and Lu 2006a, b; Patel et al. 2006). Accordingly, we asked whether these cationic compounds can be actively transported as observed for other bacteria. We describe major transport proteins such as PotF2 and PotHI in gonococci that impact PA content in the medium. Due to the similarity of these potential proteins to the well-characterized PA transport system in *E. coli*, we suggest that PotF2GHI is the main PA transport system possessed by gonococci that selectively imports spermine and spermidine, but not putrescine and cadaverine, from growth media. Bioinformatics analyses of gonococcal WGSs support that PotF2GHI is the main, if not sole, PA transport system possessed by gonococci, since the other characterized bacterial PA transporters seem absent from the gonococcal genome. It is of interest that this transporter displays specificity for spermine and spermidine in gonococci, while the PotFGHI transporter in *E. coli* is specific for putrescine. This difference in selectivity may reflect key amino acid differences between the PA-binding component of the transporters (Sugiyama et al. 1996; Vassilyev et al. 1998a,b) responsible for recognition of PAs in the periplasmic space (Fig. 1; Fig. S2C and D, Supporting Information). We suggest that these key residues present in PotF2 are important for determining PA substrate specificity and future studies will be conducted to test this hypothesis. The function of the other putative PotF-like proteins is presently unknown. As such, we cannot draw conclusions regarding the affinity or specificity of PotF1 or PotF3 for PAs, or whether they work in conjunction with PotHI. Further biochemical and bioinformatics analysis of the function of PotF1 and PotF3 will provide greater detail on the key residues of PA binding and specificity.

We previously showed that in the presence of spermine WT and mutant strains display the same decreased susceptibility level to PMB and reduced biofilm formation compared to growth medium without additional spermine. This result suggests that an active PA transporter is not necessary to decrease susceptibility to PMB and biofilm formation. Hence, further analyses need

Figure 4. PGADE in the intergenic region of *potG*–*potH*. (A) Representation of the hypothetical duplication event that formed PGADE. PGADE is formed by the fusion in-frame of the 5' coding sequence of *potH* (white bar) and the 3' coding sequence of *potG* (gray bar). In this example, we represent a common ancestor and the examples of strains FA19, MS11 (both carry 1 copy of PGADE), 1291 and FA6140 (do not carry PGADE). (B) The sequence spanning the 3' region of *potG*, PGADE, the 5' region of *potH* and intergenic regions are shown. The 144-nucleotide sequence of PGADE in FA19 is boxed (one-letter code amino acid sequence is shown below the nucleotide sequence). The first 74 nucleotides of *potH* and of PGADE are identical (underlined by a white bar). The last 70 nucleotides of *potG* and of PGADE are identical (underlined by a gray bar). The putative Shine–Dalgarno sequence (SD) is underlined. The start codons of PGADE and *potH* are shown with a methionine residue (M) underneath. The stop codons of PGADE and *potG* are shown with an asterisk underneath. The 30-mer nucleotides (flanking chromosomal sequence) used for bioinformatics analyses of WGS of clinical isolates are underlined with a thick line; they span unique sequences in *potG* and in *potH*, and the unique sequence that spans the junction in PGADE. (C) Schematic representation of the intergenic region of *potG*–*potH* in several representative strains containing variable copy numbers of PGADE. (D) PCR amplification of *potG*–*potH* intergenic region demonstrating the copy number variation in several *N. gonorrhoeae* strains (lane 1, F62; lane 2, FA1090; lane 3, FA19; lane 4, FA6140; lane 5, 1291; M, molecular weight marker 1 kb ladder, Invitrogen).

to be completed to determine the impact on gene regulation by PA transport in the gonococcus.

Perhaps the most intriguing feature of the gonococcal PA transporter system is our finding of the novel PGADE, which appears to be an in-frame fusion of sequences derived from the 5' and 3' ends of the *potH* and *potG* coding sequences, respectively (Fig. 4). This new feature in the gonococci genome could potentially be expressed as a 47-amino-acid peptide. Indeed, the intergenic region preceding the PGADE ORF is identical to the intergenic region preceding the *potH* ORF. This intergenic region is highly conserved in the *Neisseria* genus. PGADE

is variably present between *potG* and *potH* in different gonococcal strains and up to three copies can exist in the intergenic region, as shown by the PCR amplification of the *potG*–*potH* intergenic region (Fig. 4C and D). Moreover, within a given gonococcal strain (e.g. FA19), we found by PCR that PGADE is stably maintained (data not presented) in both sequence and repeat number. Indeed, we could detect only a single copy of PGADE in frozen stocks of strain FA19 that have been maintained in our laboratory since 1982 (data not presented). It is important to note, however, that we detected by whole-genome sequencing that 3 out of 13 samples have an underrepresentation of

Figure 4. – continue

the PGADE sequence as compared to the background distribution of flanking chromosomal sequences in the genome. Our interpretation of this result is that these three samples included a mixed population comprising cells with and without PGADE, but all of which contained the flanking sequences. The mechanisms by which PGADE may be lost or acquired remain unknown. Nevertheless, in that PGADE can be found in laboratory-maintained gonococcal strains as well as recent clinical isolates (20.6%) suggests to us that its presence is a reflection of the genomic diversity and plasticity of gonococcal strains circulating in the community. To our knowledge, a PGADE-like sequence has not been described in other bacteria including other *Neisseria* spp. We believe that PGADE is restricted to the gonococcus as preliminary bioinformatics analysis on the genomes of other *Neisseria* species (*N. meningitidis* [22 strains], *N. lactamica* [5 strains], *N. elongata* [3 strains], *N. mucosa* [3 strains], *N. polysaccharaea* [2 strains], *N. sicca* [4 strains]), accessible on the NCBI site (taxid:482), indicated that although they uniformly contain the *potGHI* locus, they lack the PGADE sequence. We hypothesize that, in the gonococcus, PGADE is generated at a recombination hotspot, although we have not yet been able to identify specific DNA patterns or structures that would support this idea, and that PGADE can become fixed in the population ei-

ther by random drift or through selective advantage. Continued studies are underway to determine how PGADE is formed, if the predicted 47-amino-acid peptide is produced (and what function that peptide might perform) and if possession of PGADE provides gonococci with a competitive advantage over strains lacking this unique sequence.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

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Conflict of interest. None declared.

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