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Analysis of the Filamentous Bacteriophage Genomes Integrated Into *Neisseria gonorrhoeae* FA1090 Chromosome

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A b s t r a c t

Bioinformatic analysis of the genome sequence of *Neisseria gonorrhoeae* revealed presence of four specific prophage islands. Based on the similarity with other DNA phage sequences they seem to belong to the filamentous ssDNA phages group. Phages belonging to this group are also present in the genome of *Neisseria meningitidis*. The nucleotide and amino acids sequence of NgoΦ6 and NgoΦ7 show similar genetic organization and high homology on DNA and amino acid level. The NgoΦ9 contains only part of the genomes of the NgoΦ6-8 prophages. Several functionally same genes of different origin are duplicated, with no homology to their counterparts in phages NgoΦ6, NgoΦ7 and NgoΦ9. The prophage sequences of nucleotides of NgoΦ6 and NgoΦ7 contain specific blocks of genes responsible for phage DNA replication and structural proteins. Comparative analysis at nucleotide and amino acid level suggests that these sequences can encode functionally active phages. The genetic organization of the NgoΦ6 suggests that it can serve as a prototype of filamentous phage of *N. gonorrhoeae*. Presence of the genomic ssDNA of these phages in the cultures of *N. gonorrhoeae* confirms this conclusion.

K e y w o r d s: prophage sequences, ssDNA bacteriophages

Introduction

The sequencing of the bacterial genomes revealed that presence of integrated viral genomes (prophages) is a common phenomenon. As much as 51 of the 82 genomic sequences published since year 2003 (Casjens, 2003) carry prophages, and these contain 230 recognizable putative prophages (for review see, Casjens, 2003; Canchaya *et al*., 2004; Brüssow *et al*., 2004). Prophages can constitute up to 10–20% of bacterium's genome and are major contributions between individual species (Casjens, 2003). The acquisition of prophages would be an irrelevant process for the evolution of bacteria if phages did not transfer genes useful to the lysogens (Brüssow *et al*., 2004). A lot of these genes play an important role in the pathogenicity or in general fitness of the bacterial host (Brüssow *et al*., 2004; Canchaya *et al*., 2004; Casjens, 2003). This influence of the presence of prophages on phenotypic properties is termed lysogenic conversion. The important role is played by so called morons, defined as extra genes in the prophage sequences; they do not have a phage function but may act as fitness factors for the lysogen. The list of such putative and known morons of phages and phage-like elements found in pathogenic bacteria is very long and representing a broad group of genes (Brüssow *et al*., 2004). Among them there are type III effector proteins or CT, the principal virulence factor of *Vibrio cholerae* (Brüssow *et al*., 2004).

The analysis of the prophages suggested that after being integrated into bacterial genomes, they undergo a complex decay process which involve inactivating point mutations, genome rearrangements, modular exchanges, invasion by further mobile DNA elements, and massive DNA deletions (Canchaya *et al.*, 2004; Casjens, 2003). However, the final answer whether the particular gene is in fact inactivated has to be proven experimentally, since it was shown that the demaged by deletion genes encoding the RM system in *Neisseria gonorrhoeae* can be biologically active (Piekarowicz *et al.*, 2001).

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The presence of the Mu-like prophage sequences was detected in the genomes of serogroup A strains of the epidemic subgroups I, III, IV-1 and VI of *Neisseria meningitidis* (Masignani *et al*., 2001; Morgan *et al*., 2002; Klee *et al*., 2000). A ~35-kb region designated Pnm1, is inserted within a putative gene encoding an ABC-type transporter. This region contains 46 open reading frames, 29 of which are collinear and homologous to the genes of *Escherichia coli* Mu phage. The two additional Mu-like sequences were found in *N. menigitidis* serotype A (Morgan *et al.*, 2002) and the sequence homologous to Pnm1 was found in the genome of serogroup B *N. meningitidis*, *Haemophilus influenzae* which is being named FluMu and *Deinococcus radiodurans,* but not in genome of *N. gonorrhoeae* FA1090 (Masignani *et al.*, 2001). Recently, the presence of filamentous prophages genomes was detected in *N. menigitidis* and it was shown that these prophages could be excised resulting in the production of the biologically active phages (Bille *et al*., 2005).

In this report, we present the data indicating the presence of filamentous bacteriophages genomes integrated into *N. gonorrhoeae* strain FA1090 chromosome. These are able to produce active phages whose genomes can be detected in the cultures of *N. gonorrhoeae* strains.

Experimental

Materials and Methods

Bacterial strains, plasmids and growth conditions. *Escherichia coli* K-12 strain XL-1 Blue MRF' [Δ(*mcrA*)*183*Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F' *proAB lacI*^{q}*Z* ΔM15Tn*10*(Tet^{r})]] was used during manipulation with pH17 plasmid. This strain was grown at 37°C in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989). Antibiotics included in media were used at the following final concentrations (μg/ml): ampicillin 100, tetracycline 10. Neisseria strains were grown in standard gonococcal medium (designated GCP if broth and GCK if agar) (Difco laboratories) plus growth supplements (White and Kellogg, 1965) and 0.042% sodium bicarbonate if in broth or in a 37°C CO_2 incubator. *N. gonorrhoeae* FA1090 strain was obtained from Dr D.C. Stein's collection. Chromosomal DNAs of *N. gonorrhoeae*, *Neisseria lactamica* and *N. meningitidis* strains were obtained from Dr D.C. Stein (University of Maryland, USA). Plasmid pUC19 was purchased from Fermentas.

Detection of the prophage and phage nucleotides sequences. The plasmid pH17 carrying the 5430 bp HindIII fragment of *N. gonorrhoeae* FA1090 was obtained by chance during cloning of the genes encoding MTases (Piekarowicz *et al*., 1991). Sequencing of the insert fragment showed that it contains the internal fragment of the NgoΦ6 spanning from the 1 082 100 to 1 087 500 bp. The dot blot hybridization was carried out using this fragment of DNA as a probe. The total DNA (0.1 μg) extracted from the bacterial cells was used in the hybridization experiments for the detection of the filamentous phage DNAs. The DNA was separated in 0.8% agarose gel and then transferred onto previously charged nylon membranes as described in Sambrook *et al*. (1989). The hybridization was carried out using DIG labeled HindIII fragment obtained from the pH17 DNA as a probe and under standard Southern blotting conditions, final washing was carried out for 30 min in 2×SSC buffer (all manipulations were performed at room temperature). The DIG labeling, hybridization and detection was carried out according to the manufacturer's instructions (La Roche). Presence of the prophage sequences was determined by PCR reactions, that were carried out using Pfu polymerase (MBI Fermentas) and according to the manufacturer's instructions. The reaction mixture (25 μl) contained template chromosomal DNA or "crude phage preparations" DNA; reaction buffer; dATP, dGTP, dCTP and dTTP (0.2 mM each); forward and reverse primers (0.2 μM each) and 0.5 units of polymerase. Sequences of the primers used to amplify the fragment of prophage DNA were: 5'-AACTTTCGACGTGTCCGGGAACTTATGGAGACGGA-3' and 5'-AAGGCGCGGTGCTGATTGTTGGCGAAGCGCACTA-3'. PCR conditions were as follows: 5 min at 95, followed by 2 cycles of 30 sec at 94°C, 45 sec at 48°C, and 2 min at 72°C, followed by 29 cycles of 30 sec at 95°C, 45 sec at 62°C, and 3 min at 72°C (with final elongation – 10 min at 72°C). The PCR product (1050 bp) corresponds to the chromosomal sites localized within the NGO1138 sequence which encodes the homologue of CTXΦ Zot protein. The specificity of this product was confirmed by sequencing.

The primers used for PCR amplification were obtained from IBB (Warsaw, Poland). All routine cloning procedures was carried out in accordance to protocols described by Sambrook *et al*. (1989).

Enzymes and chemicals. Restriction enzymes were purchased from MBI Fermentas and New England Biolabs. T4 DNA ligase, Pfu DNA polymerase and DNA size markers were purchased from MBI Fermentas. Kits for the DNA clean-up and plasmid DNA isolation were purchased from A&A Biotechnology, Gdansk, Poland. All the chemicals used were reagent grade or better and they were obtained from Sigma and ICN, unless otherwise noted.

Computer analysis. DNA and protein sequences were compared with the GenBank and SWISS-PROT databases on the BLAST server, hosted by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast). The *N. gonorrhoeae* strain FA1090 genomic sequence was obtained from the University of Oklahoma's Advanced Center for Genome Technology (http://www.genome.ou.edu/gono.html). Both the *N. meningitidis* strain Z2491 (serogroup A) (http://www.sanger.ac.uk/Projects/N_meningitidis) and *N. meningitidis* strain FAM 18 (http://www.sanger.ac.uk/Projects/N_meningitidis) sequences were obtained from Sanger Institute. Other comparisons were performed using the BLAST tools at the NCBI web site (http://www.ncbi.nlm.nih.gov/BLAST) or http://www.cbs.dtu.dk/services/TMHMM.

Phage techniques. The secreted form of phage and its DNA was prepared by standard phage preparation techniques (Sambrook *et al.*, 1989). Bacteria were sedimented by centrifugation from 200 ml of culture exponentially growing in GC medium, and a total DNA extracted from the cells was dissolved in 200 μl of TE (10 mM Tris-HCl, 1 mM EDTA-Na pH, 8.0) buffer. After filtration through 0.45 μm filters, the supernatant was treated with DNase I and RNaseA (25 μg/ml each) for 3 h at 20°C. Particles were precipitated by addition of NaCl to final concentration 1 M and PEG 8000 to a final concentration of 10%. The mixture was incubated at 4°C overnight and the phage particles were precipitated by centrifugation at 12 000×g for 30 min. DNA from the presumptive phage particles was extracted with phenol, and the precipitated material was redisolved in 200 μl of TE buffer.

Results

Identification of the prophage sequences in the genome of *N. gonorrhoeae* FA1090. Annotations of the complete genome sequence of *N. gonorrhoeae* FA1090 (Accession number: AE004969) revealed four probable prophage islands, designated as NgoΦ6 to NgoΦ9, containing several ORFs with striking amino acid similarities to known functional phage proteins (Table I). The nucleotide sequences as well as amino acid sequences of NgoΦ6–9 show high similarity to the filamentous ssDNA phages, especially to those able to integrate into the chromosome of the host (Davis and Waldor, 2003; Bille *et al*., 2005).

Table I
Localization of the filamentous bacteriophages integrated into *Neisseria gonorrhoeae* FA1090 genome

Phage	Nucleotide sequence coordinates[a]	Length of the DNA sequence (bp)	ORF annotations (Acc. No AE004969)	Number of ORFs
NgoΦ6	1080185 – 1088060	8 235	NGO1137 – NGO1146	13
NgoΦ7	1215967 – 1222383	7 416	NGO1262 – NGO1270	12
NgoΦ8	1103017 – 1109444	6 427	NGO1164 – NGO1170	8
NgoΦ9	1599378 – 1606537	6 754	NGO1641 – NGO1648	9

[a] Coordinates are relative to the DNA sequence contained at GenBank Acc. No AE004969

Nucleotide sequence and genomic organization. The DNA and amino acid sequences of both NgoΦ6 and NgoΦ7 show similar genetic organization and high homology (50–90% of identity) to each other. NgoΦ8 lacks an equivalent of ORF1 (NGO 1146), ORF2 and ORF3 (present in NgoΦ6 and NgoΦ7) show lower level of homology (Fig. 1A). However, the ORF2 and ORF3 of NgoΦ6 lack the homology (both on DNA and amino acid levels) to their equivalents present in NgoΦ7 and NgoΦ8 (Fig. 1B). The NgoΦ9 contains only part of the genomes of the NgoΦ6–8 prophages. Several functionally same genes of different origin are duplicated, with no homology to their counterparts in phages NgoΦ6, NgoΦ7 and NgoΦ9. (Fig. 1B). The genetic organization of the NgoΦ6 suggests that it can serve as a prototype of filamentous phage of *N. gonorrhoeae*. The nucleotide sequence of NgoΦ6 consists of 8240 nucleotides and has mean G+C content of 46.0%, which is lower than G+C content of the host *N. gonorrhoeae* (54%). In the chromosome of *N. gonorrhoeae* the 5' end of the NgoΦ6 prophage is flanked by the gene encoding glucosyl transferase (ORF1136) while the 3' is flanked by the ORF1149 encoding O-serin-succinylohomosulfohydrolase. The NgoΦ7, NgoΦ8 and NgoΦ9 prophage sequences are flanked on 5' end by the 136 bp long sequence that is also present in NgoΦ6 prophage DNA (Fig. 1A) but in different localization. The ORF11 (NGO1137) of NgoΦ6 encoding integrase, has the opposite orientation than the corresponding genes in other prophages and the 136 bp sequence is present on the other flank of this gene. On the 3' end of the NgoΦ9 sequence the ORF11 (NGO1648) encoding transposase is followed by the ORF1649 encoding the putative dsDNA phage protein. The 3' ends of NgoΦ7 and NgoΦ8 sequences are flanked by NGO1273 encoding tetraadenosinophosphate and NGO1173 encoding VSR protein, respectively. We were unable to determine any homology between the 3' ends of all four prophage sequences and between 3' and 5' ends of each sequence.

The CTXΦ and VGJΦ phages, prototypes of the lysogenic filamentous phages, contain three modules essential for phage production (Davis *et al.*, 2000; Campos *et al*., 2003). The five classical core genes of CTXΦ encode proteins needed for virion structure and assembly, the replication module encodes RstA, RstR and RstB proteins; RstA and RstR mediate replication of phage DNA and regulation of *rstA* expression respectively (Kimsey and Waldor*,* 2004*;* Mekalanos, 1983). The role (if there is any) of RstB in CTXΦ virion production has not yet been determined; it was only shown that RstB contributes to phage integration (Waldor and Mekalanos, 1996). The third module, the assembly one, encodes several genes needed for phage maturation. Their corresponding genes in VGJΦ are represented by ORF81, ORF44, ORF29, ORF493, ORF80, ORF384 (virion structure and assembly); ORF359, ORF112, ORF58, ORF67, ORF136, ORF 154 (replication and expression). The CTXΦ genome lacks an integrase; instead, its integration depends on the chromosome encoded tyrosine recombinases XerC and XerD (McLeod and Waldor, 2004). The NgoΦ6 – NgoΦ9 prophage nucleotide sequences posses the ORFs that encode putative transposase. The gene shares homology with Pfam 02371 transposase 9 and Pfam 01548 transposase 20 families (Table II). *N. gonorrhoeae* contains several copies of this gene that may encode a site-specific recombinase and play

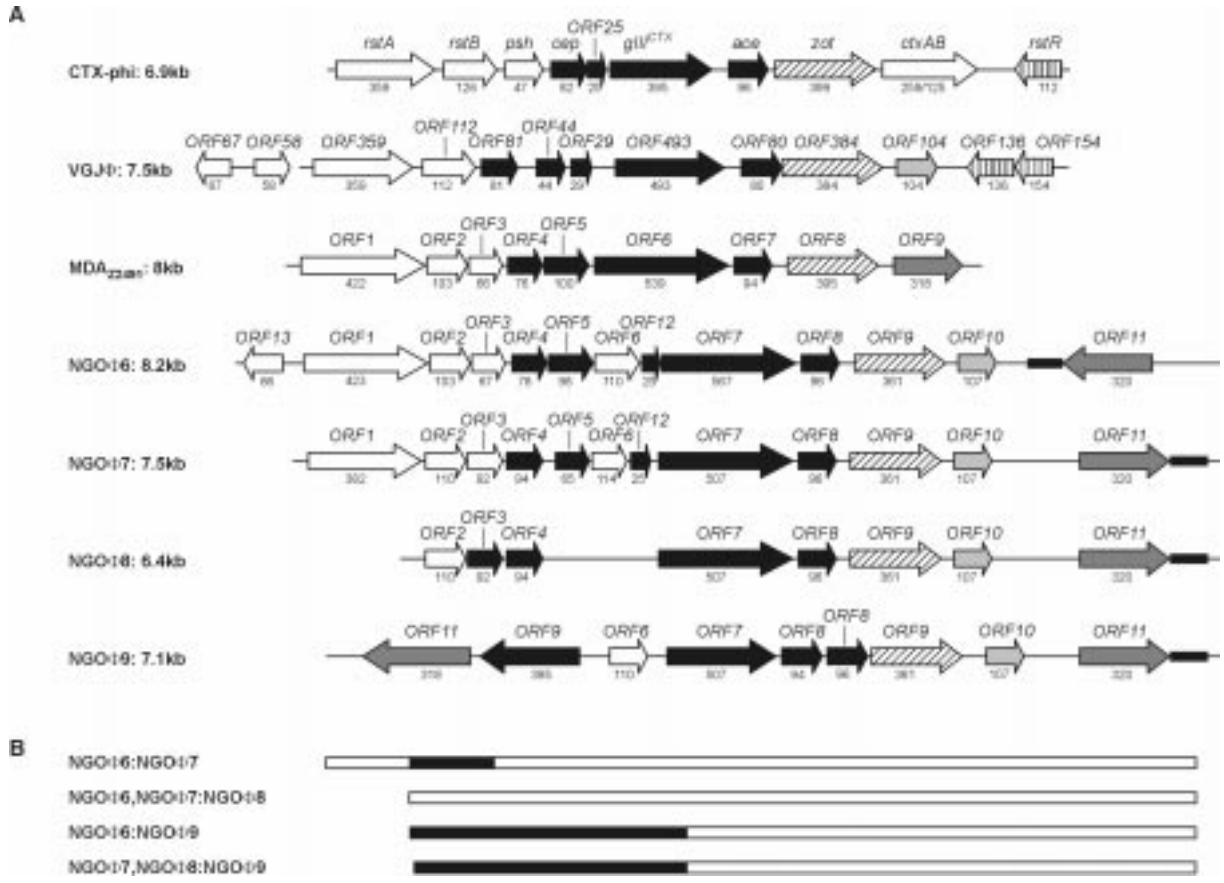

Fig. 1. Comparison of the organization of the NgoΦ6-NgoΦ9 prophages with that of *Vibrio cholerae* bacteriophages CTXΦ and VGJΦ *N. meningiditis* MDA_{Z2491}.

(A) Linear maps of CTXΦ, VGJΦ, MDA_{Z2491} and NgoΦ6-9 phages, aligned by using the first base of the replication initiator gene of CTXΦ as an orientation point. Arrows oriented in the direction of transcription represent ORFs or genes. Open arrows represent the replication module, solid arrows represent the structural module, arrows with left cross-hatching represent an assembly module, an arrow with black dots represents the integrase and the arrows with vertical lines represent the ORFs encoding the repressors. The arrow with right cross-hatching represents the ORFs with unknown function. The ORFs of VGJΦ, CTXΦ genes and MDA_{Z2491} are designated according to the published papers (Campos *et al*., 2003; Bille *et al*., 2005). The lengths of the products encoded (in amino acids) are indicated below each arrow. The solid boxes represent 136 bp sequence present on one flank of ORFs encoding integrase gene common for all four NgoΦ phages.

(B) Similarity between the DNA nucleotide sequences of filamentous phages of *N. gonorrhoeae*. The open boxes represent the region of high homology (99% of identity), the solid boxes the regions of lack of homology (below 5% of identity).

a role in the integration and the excision of the ssDNA phage genome within the chromosome of *N. gonorrhoeae*. Its homologues, present in the genome of filamentous prophage in *N. meningitidis*, are responsible for their integration and excision from the chromosome (Bille *et al.*, 2005).

A computer-aided homology search was performed for the ORFs presumed to encode functional viral genes (designation presented in Table II). In NgoΦ6 it showed the same type of genetic organization as in other filamentous phages, like *Vibrio* phages CTXΦ, VGJΦ or *N. meningitidis* MDA (ssDNA prophage) sequence (Campos *et al*., 2003; Bille *et al.*, 2005). The common property of these phages is the ability to integrate into the host chromosome. The function of these genes was also deduced from the homology to genes of other filamentous phages and by comparison of the presence of intermembrane motives (TM) (Table III).

Within the putative replication module, (Fig. 1A; note that the maps of the NgoΦ6-9 on this figure are presented in the opposite orientation than in the chromosome of *N. gonorrhoeae* in order to aid in comparison with other phages) we identified ORF1 (NGO1146). The peptide encoded by ORF1 is homologous to the RstA protein of CTXΦ (26% identity and 44% positives over 234 residues), which is necessary for phage replication (Waldor *et al*., 1997). ORF1 is also similar in terms of size and position to genes of other filamentous phages, which were mapped at the same relative position as the *gII* gene in Ff phages (data not shown). ORF1 could be homologous to PII protein of filamentous phages necessary for rolling-circle replication of phage genomes (Model and Russel, 1988). There is no homologue of ORF NGO1 in NgoΦ8 or NgoΦ9. We were unable to identify the peptide that would be homologous to *rstB* or gene encoding ssDNA

Table II
Properties of the NgoΦ6 ORF's amino acid sequences

Gene	Protein Length (aa)	Protein homologies (BlastP)	Conserved domains (BlastP)	Presence and number of transmembrane domains (TMHMM)
ORF1, NGO 1146	423	pMU1_p3 (*Eikenella corrodens*) plasmid (YP_245390), 1×10^{-103}; RstA1 protein [*Vibrio cholerae* Prophage] (NP_231106.1), 1×10^{-11}	replication ini- tiation factor (pfam02486), 8×10^{-38}; phage replication protein RstA (COG2946), 4×10^{-143}	N[a]
ORF2, NGO 1145	103	NS[b]	NS	N
ORF3, NGO 1144	67	NS	NS	N
ORF4, NGO 1143	78	NS	NS	Y[c] (2)
ORF5, NGO 1142	98	NS	NS	
ORF6, NGO 1141	110	NS	NS	N
ORF7, NGO 1140	506	NS	*neisserial* TspB virulencefactor	Y (1)
ORF8, NGO 1139	107	NS	NS	Y (2)
ORF9, NGO 1138	361	pUM1_p7 (YP_245394.1), 2×10^{-32}	zonular occludens toxin (Zot) [*Eikenella corrodens*] (pfam05707), 7×10^{-39}	Y (1)
ORF10	107	NS	NS	
ORF11, NGO 1137	320	transposase [*Escherichia coli* IS621], (BAC768887.1), 3×10^{-37}	transposase (COG3547), 1×10^{-17}	
ORF12	23	NS	NS	Y (1)
ORF13	86	NS	NS	

[a] N, lack of TM domains; [b] Y, presence of TM domains; [c] NS, no significant homologies were found

Table III
Comparison of the predicted transmembrane helix motives in some of the proteins encoded by filamentous phages

Phage/protein (aa)	Number of predicted TMHs	Outside	TMhelix 1	Inside	TMhelix2	Outside
CTXΦ, Zot (399)	0	1 – 399	–	–	–	–
VgJΦ, ORF384 (Zot) (384)	1	1– 213	214 – 233	234 – 384	–	–
NgoΦ9, ORF9* (395)	1	225–395	205–224	1–204	–	–
NgoΦ6, ORF 9361	0	1 – 361	–	–	–	–
CTXΦ, *gIII*^{CTX} (395)	1	1 – 374	375 – 394	395 – 395		
VgJΦ, ORF493 (493)	1	30 – 493	7 – 29	1 – 6	–	–
NgoΦ6, ORF7 (507)	1	1 – 483	484 – 506	507 – 507	–	–
CTXΦ, *gIII*^{CTX} (395)	1	1 – 374	375 – 394	395 – 395		
VgJΦ, ORF493 (493)	1	30 – 493	7 – 29	1 – 6	–	–
NgoΦ6, ORF7 (507)	1	1 – 483	484 – 506	507 – 507	–	–
CTXΦ, *Ace* VgJΦ, ORF8	2	1 – 17	15 – 37	38 – 64	65 – 87	88 – 96
NgoΦ6, ORF8 (80)	1	1 – 43	44 – 60	67 – 80	–	–
NgoΦ9, ORF8 (98)	2	1 – 17	18 – 40	41 – 60	61 – 83	84 – 98

* NgoΦ9 encodes two copies of ORF9, which differ slightly in number of amino acid residues.

binding protein within the putative replication module. The ORF2 and ORF3 are located in the position of the *rstB* and *psh* genes of CTXΦ phage that participate in DNA replication (Waldor *et al*., 1997). These two genes do not show the presence of transmembrane domains (data not shown), this property is common for the structural and assembly proteins of the filamentous phages. Thus, it is possible that they are in fact engaged in the replication process. In addition, the ORF6 that does not have TM domain, is flanked by the ORF5 and ORF12 with such domains. Instead, this ORF shows the presence of Zinc-finger motive what could suggest its participation in replication or transcription control processes (Klug and Schwabe, 1995).

Within the putative structural module of NgoΦ6, we identified the ORFs 4, 5, 12, 7 and 8; sizes and positions of these sequences are similar to those of genes encoding capsid structural proteins of Ff phages, CTXΦ, VGJΦ and other previously described filamentous phages (Campos *et al*., 2003). The ORF4 and 8 could play the same role as the *cep* and *ace* genes of CTXΦ phage (Canchaya *et al.*, 2004). These ORFs are predicted to encode proteins with transmembrane domains. The results support the idea that they form the module of structural genes (Fig. 1A) which encode the capsid proteins of NgoΦ6. ORF7 (NGO1140) is homologous to *N. meningitidis* and *N. gonorrhoeae* TspB protein belonging to pfm05616 family. This family consists of several TspB virulence factor proteins. This ORF is located at the same relative position as *gIII* of the Ff phages and $gIII^{CTX}$ of CTXΦ phage and its homologue (ORF384) in VGJΦ phage. Its size is comparable to the sizes of these genes that encode pIII, a minor capsid protein that recognizes and interacts with the receptors and co receptors of these phages (Armstrong *et al*., 1981; Heilpern and Waldor, 2003; Lubkowski *et al.*, 1999). Therefore, ORF7 (NGO1140) could be a homologue of *gIII* in CTXΦ. The ORF7 (NGO1140) shows very high homology (92% identity) to homologous genes in NgoΦ7–9 phages. ORF8 (NGO 1139) shows some degree of identity (about 25% identity, 50% positives over 70 residues) to other ssDNA phages of *Acetinobacter*, *Nitrosomonas europea*, *Xylella fastidiosa* and *Pseudomonas* phage Pf3).

Within the third putative module of NgoΦ6, the assembly module, we found ORF9 (NGO1138) belonging to pfam05707 of proteins, whose product is homologous to the pI protein of *Pseudomonas* phage Pf3 (27% identity over 344 residues) and to the Zot protein of CTXΦ (19% identity and 39% positives over 209 residues) as well to ORF6 of MDA_{Z2491}. Based on its size and position, ORF9 also corresponds to the *gI* gene, which encodes pI in Ff phages, needed for assembly and secretion of viral particles (Marvin, 1998; Russel, 1995; Russel *et al.*, 1997; Campos *et al*., 2003; Bille *et al.*, 2005).

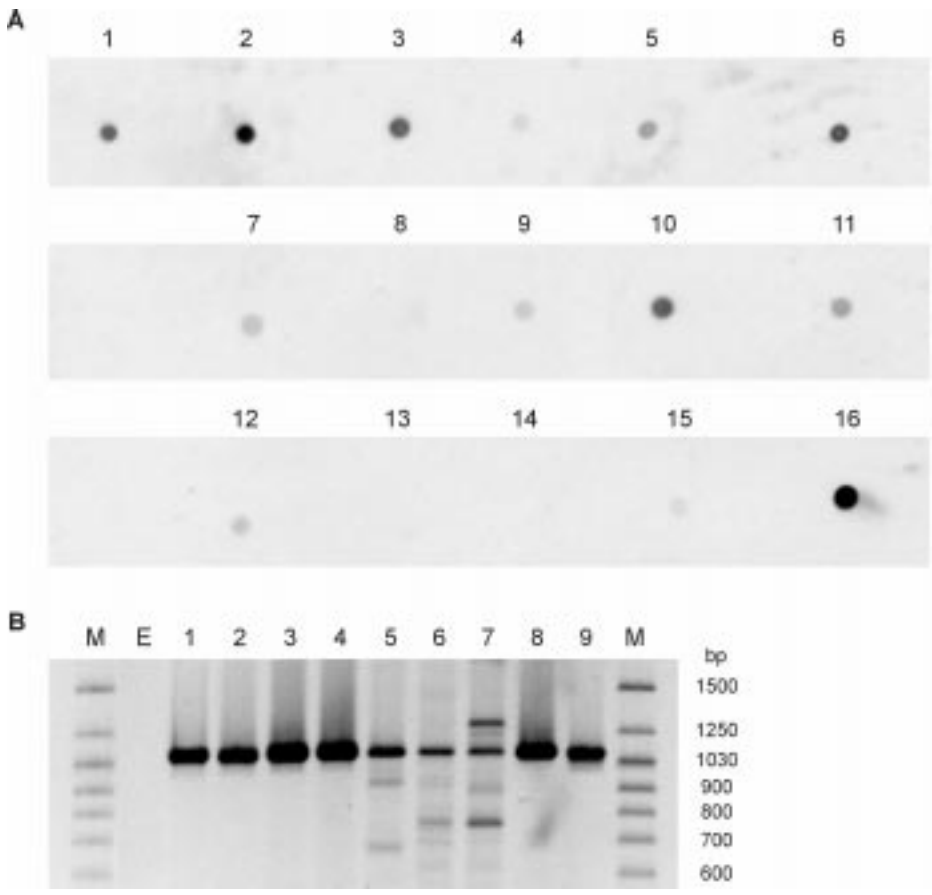

Fig. 2. Detection of the presence of filamentous DNA sequences in different strains of *Neisseria*.

(A) Dot blot hybridization of the NgoΦ6 probe with a total DNA isolated from different *Neisseria* strains. 1; *N. gonorrhoeae* 220, 2; *N. gonorrhoeae* FA1090, 3; *N. gonorrhoeae* WR302-1, 4; *N. gonorrhoeae* WR302, 5; *N. gonorrhoeae* MS11, 6; *N. gonorrhoae* MS11 (different isolate), 7; *N. gonorrhoeae* Pgh3-2, 8.; *N. lactamica* 5841, 9; *N. meningitidis* 13113, 10; *N. meningitidis* 53414, 11; *N. gonorrhoeae* FA5100, 12; *N. gonorrhoeae* 220 Rif^RNal^R, 13; *N. gonorrhoeae* WR302 Rif^RNal^R, 14; *N. gonorrhoeae* 220 Rif^RNal^R (different isolate), 15; *N. lactamica* 5841, 16; *N. gonorrhoeae* 1291. (B) Detection of the presence of phage sequences by PCR methods. 1; *N. gonorrhoeae* 1291, 2; *N. gonorrhoeae* 220, 3; *N. gonorrhoeae* FA1090, 4; *N. gonorrhoeae* WR302-1, 5; *N. gonorrhoeae* WR302, 6; *N. lactamica* 5841, 7; *N. meningitidis* 13113, 8; positive control, pH17, 9; *N. gonorrhoeae* MS11.

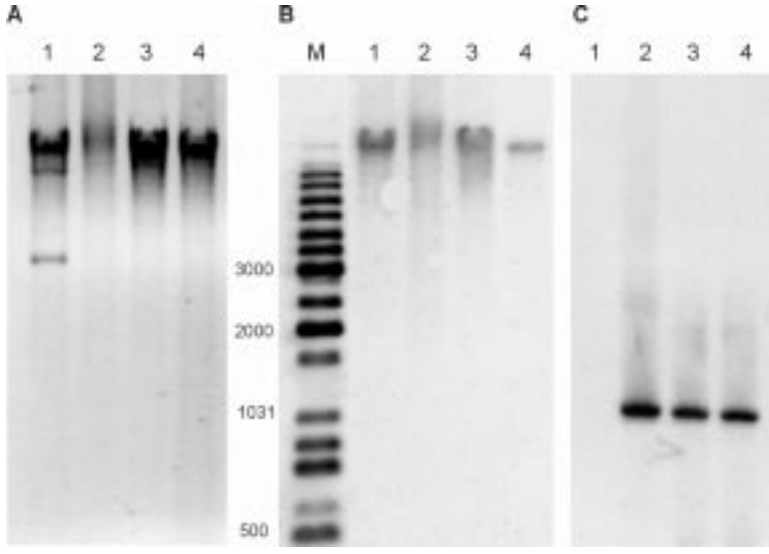

Fig. 3. Detection of the extrachromosomal replicative and extracellular forms of phage DNA.

N. gonorrhoeae total DNA separated in 0.8% agarose gel (A) and hybridized (B) with the NgoΦ6 probe. 1; *N. gonorrhoeae* 220, 2; *N. gonorrhoeae* FA1090, 3; *N. gonorrhoeae* WR302-1, 4; *N. gonorrhoeae* WR302. M; DNA marker, (C). The prophage DNA is secreted in the nuclease-resistant form. Treatment with DNaseI had no significant effect on the material amplifiable by the NgoM6 primers. PCR amplification was performed on DNA from phage preparation. 1; phage DNA (*N. gonorrhoeae* FA1090) treated with S1 nuclease, 2; phage DNA (*N. gonorrhoeae* FA1090) treated with DNaseI, 3; phage DNA (*N. gonorrhoeae* FA1090) treated with AciI, 4; phage DNA (*N. gonorrhoeae* FA1090).

The Zot protein encoded by ORF9 shows only partial homology to the Zot proteins of NgoΦ7 and 8 (37% identity, 55% positive over 350 residues) and also with both Zot proteins of NgoΦ9 (395 residues). What is more important, all of these Zot proteins show the homology to phage CTXΦ encoded Zot protein only at N-terminal end and not at the C-terminal ends. However, only NgoΦ9-encoded Zot protein do possess TM motive (Table III).

Most of the structural and assembly proteins of NgoΦ6 show the overall structures of TM domains similar to the homologous protein of other filamentous phage (data not presented). However, in some cases they show important differences. The proteins ORF7 (NgoΦ6), gIII (f1), $gIII^{CTX}$ and ORF493 (VGJΦ) all have one TM domain but in the ORF493 it is located in N-terminal end of protein while in all others at the opposite end. The ORF 8 (NgoΦ6), ace (CTXΦ) and ORF80 (VGJΦ) proteins have two TM domains while their homolog in f1 phage (*gVI*) three domains (Table III). The proposed assembly and secretion module of NgoΦ6 do not have a homologue of the g*IV* of the Ff phages, similarly to VGJΦ (Campos *et al.*, 2003) and MDA_{Z2491} (Morgan *et al.*, 2002). Function of this gene in the development of CTXΦ , which is critical for phage morphogenesis (Opalka *et al.*, 2003) is substituted by cell function of the gene *EpsD* (Campos *et al.*, 2003).

We were unable to identify the fourth, regulatory module. The ORF13 which is located in the position corresponding for the repressor gene encoded by CTXΦ phage but does no show homology to any of the known protein. Like the *rstR* of CTXΦ the ORF13 is transcribed in opposite direction of transcription of the rest of the phage genes. RstR protein is a repressor that regulates transcription of the initiator replication protein, RstA, and in turn regulates the expression of all phage genes (Davis *et al.*, 2002; Kimssey and Waldor, 2004). We do not know the function of the ORF10, which is present in the genome of all Ngo ssDNA phages and in VGJΦ, but was not identified in MDA_{Z2491} (Fig. 1A).

Presence of the prophage sequences in different *N. gonorrhoeae* strains. In *N. meningitidis* the phages homologous to NgoΦ6 are present predominantly in the hypervirulent isolates (Bille *et. al.*, 2005). On the other hand, the Mu-like prophage sequences are present in all *N. meningitidis* strains (Masignani *et al.*, 2001). The *N. gonorrhoeae* is the causative agent of the sexually transmitted disease called gonorrhoea. In the male, this is typically associated with a purulent discharge from the urethra. However, in women, infection of the cervix is often asymptomatic while the spread of the gonococci up the urinary tract or invasion across the epithelial layers can cause additional complications such as pelvic inflammatory diseases (PID) and disseminated gonococcal infection (DGI) (Jordan *et al*., 2005). There are experimental strains of *N. gonorrhoeae* commonly used in the laboratory, which were isolated either from the patient with DGI (FA1090) or with UG (F62 and MS11) (Jordan *et al.*, 2005).

The dot blot hybridization experiment (Fig. 2), where the 5.5 kb fragment of NgoΦ6 sequence was used as a probe, indicated that probably all strains of *N. gonorrhoeae*, except some mutants, contain the phage sequence, either the whole or its fragment. This sequence was not present in non-pathogenic strain of *Neisseria lactamica*. The presence of the NgoΦ6 was also confirmed by the formation of the PCR products specific for the *zot* gene of NgoΦ6 (Fig. 2B).

Presence of the filamentous phages in *N. gonorrhoeae* cultures. The replication of the CTXΦ family of phages proceeds without the excision of the integrated genome in the form of the circular intracellular form of DNA (Waldor *et al.*, 1997). The presence of such phage DNA form inside the lysogenic cells may serve as an indicator of the presence of the biologically active phage. The hybridization studies carried out with the total DNA isolated from the *N. gonorrhoeae* FA1090 cells failed however to show the presence of the prophage sequences in the form of free DNA molecules inside the cells (Fig. 3B). The failure to detect the presence of free phage DNA inside the cells could be due to limited sensitivity of our hybridization method or due to very low level of the phage DNA replication.

In *N. meningitidis,* the genomic DNA of the filamentous phages was also detected as a single positive strand present in "crude phage preparations" (Bille *et al.*, 2005), indicating the secretion of these phages from the cells and the functionality of the phage genome. In similar experiments, we have also shown the presence of free ssDNA specific for NgoΦ6 (Fig. 3C). This result indicates that also in *N. gonorrhoeae* FA1090, the prophage sequences of the filamentous phages can replicate and produce the progeny. Our attempts to obtain propagation of these phages on different strains of *Neisseria* have failed so far.

Discussion

Recent discovery of the biologically active filamentous phages in *N. menigiditis* and the results described in this paper indicate the presence of a new group of such phages. Comparison of the deduced sequence of NgoΦ6 with the published genetic maps of variuos filamentous phages integrated into the chromosome of *N. meningitidis* reveals their great similarity to the CTXΦ and other phages of *Vibrio* (Davis *et al.*, 2000; Campos *et al.*, 2003). Both groups of phages have a modular genome organization in which three modules can be distinguished: the replication module, the structural module and the assembly module. We were not able to identify the presence of the gene encoding phage repressor. Such repressor gene is present in all other filamentous phages able to integrate into the chromosome of the host like CTXΦ, VGJΦ, Vf33 or Cf1c (Brüssow *et al.*, 2004) but absent in non-lysogenic phages. Its presence is required for maintenance of the lysogenic state and regulation of the gene expression (Kimsey and Waldor, 2004). The NgoΦ6 has a distinctive region of about 800 nucleotides that is not significantly homologous to any entry in databases and is absent in *N. meningitidis* filamentous phage genomes (Bille *et al.*, 2005). Such region is present in VGJΦ phage genome; it encodes the ORF of unknown function. This region can be homologous to intergenic (IG) region present in non lysogenic phages containing signals for replication, packaging of DNA and the strong transcription terminator (Webster, 1994).

Integration of CTXΦ DNA and probably several other lysogenic phages (VGJΦ, Cf1c, Cf16-v1) depends on the activity of the host encoded tyrosine recombinanses XerCD (Huber and Waldor, 2002; Campos *et al*., 2003) and the presence of the *attP* and *attB* sites within the phage genome and within the chromosome of bacteria, respectively. While one XerCD binding site in *att*P spans the short core region, the other site is approximately 80 bp away. Although integration occurs at the core XerCD binding site in *att*P, the second site is required for CTXΦ integration (McLeod and Waldor, 2004). In the chromosome of *N. meningitidis* the sequences corresponding to ssDNA prophages are inserted into 20 bp inverted repeats (dRS3) and are flanked by the sequence ATTCCCNC and GNGGGGAAT. These sequences can represent analogous integration core region as characterized for CTXΦ phage. No such sequences were found on the flanks of *N. gonorrhoeae* ssDNA prophages. However, the same 136 bp DNA sequence is present at the end of the ORFs encoding the phage integrase in all filamentous *N. gonorrhoeae* phages. This sequence can serve as a region responsible for binding of the integrase and the site of recombination.

Like in *N. meningitidis* there are several ssDNA phage islands within the genome of *N. gonorrhoeae* strain FA1090. The same explanation given by Bille *et al.* (2005) can be valid for this phenomenon. These are as follows: (i) genomic rearrangements that result in duplication, (ii) horizontal transfer by transformation and insertion through recombination into the same type of repeat sequence (iii) better induction of immunity to superinfection. The presence of several copies of different types of prophage sequences acquired during evolution and coding for several restriction-modification systems gave them a total protection against

phage infection. This can be an evolutional method for shifting the balance between phages and bacteria for favors of the host.

Is there any biological role of the filamentous prophage sequences in *N. gonorrhoae*? The simpliest explanation would be, as in case of *V. cholerae* strains, that phage proteins increases the pathogenic potential of the bacteria. In *N. meningitidis*, the homologous filamentous prophages are mainly integrated into chromosomes of strains that show increased invasiveness (Bille *et al*., 2005). In *N. gonorrhoae,* the strains that seem not to possess such phages are not recognized as less pathogenic than those that have these sequences.

There is one similarity in sequence between CTXΦ and the genomes of the *N. meningitidis* and *N. gonorrhoeae* filamentous phages; it lies within the genes encoding the Zot and Zot homologue proteins. The details analysis show that only the N-terminal end (from residue 9 to 196) of ORF9 (NGO1138) exhibits the homology with N-terminal end of Zot of CTXΦ (residues 9 to 205). The enterotoxin function of Zot has been assigned to its C-terminal region (Di Perro *et al.*, 2001) that has no homology to the C-terminal end of ORF9 (NGO1138). This fact would argue with enterotoxic properties of the ORF9. The Zot protein encoded by ORF9 (NGO1138) (361 residues) is smaller then Zot of CTXΦ (399 residues) what can be responsible for the lack of homology between these two proteins. However, the C-terminal region of the ORF9 (NGO1647) in NgoΦ9 phage that has the larger number of amino acids (399) does not share homology with the C-terminal end of CTXΦ Zot protein. It does not preclude the possibility that the Zot encoded by ORF9 (NGO1138) has other toxic properties different than Zot of classical CTXΦ. This suggestion is supported by the observation that the Zot protein (361 aa) encoded by the filamentous NgoΦ phages, similarly to the Zot of CTXΦ, and contrary to the Zot of VgJΦ does not show the presence of TM motive (Table III), what could suggest that their structures are similar. The low homology between the Zot proteins encoded by NgoΦ6–9 phages suggests that their acquisition by *N. gonorrhoeae* was in fact several independent procesess that happened several times in the evolution of the bacteria. The two essential phage genes of CTXΦ phage, *cep* and *ace* that have their homologs in NgoΦ6 (ORFs 4 and 8), encode a core pilin. The products of these two genes do not function as toxins during *V. cholerae* infection (Canchaya *et al*., 2004) what may also be true for *N. gonorrhoeae* and its filamentous phages.

To our knowledge it is the first description of the filamentous bacteriophage genomes integrated into *N. gonorrhoeae* but their role for *N. gonorrhoeae* pathogenicity is still not known.

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