Plasmid Diversity in Neisseriae†

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Horizontal gene transfer constitutes an important force in prokaryotic genome evolution, and it is well-known that plasmids are vehicles for DNA transfer. Chromosomal DNA is frequently exchanged between pathogenic and commensal neisseriae, but relatively little is known about plasmid diversity and prevalence among these nasopharyngeal inhabitants. We investigated the plasmid contents of 18 *Neisseria lactamica* isolates and 20 nasopharyngeal *Neisseria meningitidis* isolates. Of 18 *N. lactamica* strains, 9 harbored one or more plasmids, whereas only one *N. meningitidis* isolate contained a plasmid. Twelve plasmids were completely sequenced, while five plasmid sequences from the public databases were also included in the analyses. On the basis of nucleic acid sequences, mobilization, and replicase protein alignments, we distinguish six different plasmid groups (I to VI). Three plasmids from *N. lactamica* appeared to be highly similar on the nucleotide level to the meningococcal plasmids pJS-A (>99%) and pJS-B (>75%). The genetic organizations of two plasmids show a striking resemblance with that of the recently identified meningococcal disease-associated (MDA) plasmid, while four putative proteins encoded by these plasmids show 25% to 39% protein identity to those encoded by the MDA plasmid. The putative promoter of the gene encoding the replicase on these plasmids contains a polycytidine tract, suggesting that replication is subjected to phase variation. In conclusion, extensive plasmid diversity is encountered among commensal neisseriae. Members of three plasmid groups are found in both pathogenic and commensal neisseriae, indicating plasmid exchange between these species. Resemblance between plasmids and MDA plasmid may be indicative of dissemination of phase-related sequences among pathogenic and commensal neisseriae.

The genus *Neisseria* includes species that may be pathogens to their human host, such as *N. meningitidis* and *N. gonorrhoeae*, the causative agents of meningococcal disease and gonorrhea, respectively. Other members of this genus, such as *N. lactamica*, *N. sicca*, *N. flavescens*, and various others, do not cause disease but can colonize the human nasopharynx and have been involved in antibiotic resistance transfer to *N. meningitidis* (21, 28). Linz and coworkers have established that commensal neisseriae and *N. meningitidis* frequently exchange chromosomal DNA (17), consistent with the notion of a shared neisserial global gene pool (18). In a more recent study, we have shown that *N. lactamica* also contains putative virulence-associated sequences (32a).

Although few plasmids of neisseriae have been sequenced, different plasmids have been identified in both pathogenic and commensal neisseriae, albeit with a bias to antibiotic resistance plasmids (7, 22, 24). This limited amount of sequence data restricts insight into neisserial plasmid diversity and evolution. Notable exceptions are the gonococcal plasmids pJD1 (15) and pJD4 (6), the meningococcal plasmids pJS-A (12) and pJS-B (5), and the commensal neisseriae plasmids pNL1 from *N. lactamica* (32a) and pMDG2830 from *N. flavescens* (19).

In order to obtain more insight into the role of plasmids in horizontal gene transfer among neisseriae, we evaluated plasmid content and diversity and nucleotide composition.

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† Supplemental material for this article may be found at http://iai.asm.org/.

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**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Eighteen different *N. lactamica* isolates and 20 *N. meningitidis* isolates from the collection of the National Reference Laboratory for Bacterial Meningitis (RLBM), Academic Medical Center/RIJVM, Amsterdam, The Netherlands, were used in this study and were obtained from healthy carriers in The Netherlands during the summer of 2004 (see Table 1). *N. meningitidis* and *N. lactamica* were differentiated by lactose fermentation, β-galactosidase activity, and aminopeptidase tests. Three *N. lactamica* strains isolated from healthy carriers known to harbor plasmids were additionally selected. Neisseriae were grown on heat-sterilized chocolate agar plates or in liquid tryptic soy broth (Difco) medium at 37°C in a humidified atmosphere of 5% CO2.

**Plasmid DNA preparation, digestion, and sequence analysis.** Isolation of plasmid DNA from neisseriae was carried out using the Wizard kit (Promega), which allows efficient DNA isolation for plasmids up to 20 kbp in size. Restriction endonuclease digestion (both for restriction fragment length polymorphism pattern analyses and subcloning procedures) and subsequent heat inactivation were carried out according to the manufacturer’s instructions (Roche). We subcloned (amplified) restriction fragments into the pCR2.1 vector (Invitrogen), or a dephosphorylated SmaI-digested pUC18 vector (Promega) according to each respective manufacturer’s instructions. Escherichia coli DH5α was transformed by the standard heat shock procedure. Plasmid inserts were sequenced using standard M13 primers or primer walking on cloned fragments or neisserial plasmid DNA according to the manufacturer’s instruction (AB). Sequences were analyzed with CodonCode Aligner program (CodonCode Corporation) and analyzed with BLASTN or TBLASTX using the BLOSUM-62 substitution matrix (http://www.ncbi.nlm.nih.gov/BLAST/), the annotation tool Artemis software (www.sanger.ac.uk/), and the web tool NEBcutter version 2 (New England Biolabs, Inc.) (33).

**Data analysis.** Clustering analysis of the different open reading frames (ORFs) was carried out using MEGA (Molecular Evolutionary Genetics Analysis) (16) (available from www.megasoftware.net), and genomic dissimilarity analyses of the different plasmids were carried out using sp-web as described previously (32). Tandem repeats and inverted repeats were detected using the Tandem (and Inverted) Repeats Finder at http://tandem.bu.edu/trf/trf.html (3). Nucleotide sequence accession numbers. The nucleotide sequence data of the various plasmids are available in the EMBL/GenBank/DDBJ nucleotide sequence databases under accession numbers DQ223897 through DQ223899.
TABLE 1. Neisserial plasmid classification scheme

| Plasmid group | Plasmid   | Size (bp) | Plasmid accession no. | Species origin | Similarity based on:
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*a* The protein family (pfam) or clusters of orthologous groups (COG) numbers on which the grouping was based are shown. Although some pfam numbers occur in different groups, proteins from members of a group are substantially more similar than proteins from members of different groups. Mob is a mobilization protein, and Rep is a replication protein.

*b* NS, not sequenced. Restriction patterns were identical to a plasmid from the same plasmid group.

RESULTS

Six different neisserial plasmid groups. Of 18 *N. lactamica* strains, 9 carried one or more plasmids <20 kbp in size. In contrast, we identified only one plasmid-carrying isolate among 20 *N. meningitidis* throat isolates (Table 1; see Tables S1 and S2 in the supplemental material).

Three additional *N. lactamica* isolates, isolated prior to 2004 and known to carry plasmids, were included (Table 1). From this collection of *N. lactamica* isolates, a total of 12 plasmids were completely sequenced. Together with sequenced neisserial plasmids available in public databases (pJS-A, pJS-B, pJD1, pNL01, and pMIDG2830 [accession numbers are given in Table 1]), six distinct groups of neisserial plasmids (I to VI) could be distinguished on the basis of nucleotide identity and replication protein similarity. The grouping of the plasmids was supported by intragroup tandem repeat similarity (Table 2). In contrast to groups I and II, groups III and IV comprise plasmids heterogeneous in size, while groups V and VI are represented by only one plasmid each. The single *N. meningitidis* plasmid identified, pNM12, was approximately 4.2 kb in size on the basis of restriction fragment patterns, which were highly similar (>90% identical fragments) to that of pNL01 (group IV [data not shown]). An AluI restriction fragment of 259 bp was sequenced and was nearly identical (257/259 bp) to the *N. lactamica* pNL01 plasmid, which is 4,150 bp in size; therefore, pNM12 was not sequenced further. Three *N. lactamica* plasmids, one plasmid each from groups I, II, and IV, were not sequenced, as their AluI and RsaI restriction enzyme digestion patterns were identical to that of a putative protein on the 2-kbp cryptic plasmid from *N. lactamica*.

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Coexistence of a group II plasmid and a group IV plasmid was found in two *N. lactamica* isolates, and the combination of a group III plasmid and a group IV plasmid was found in one isolate (see Table S1 in the supplemental material).

Different groups of neisserial plasmids. (i) Group I. Group I consists of four plasmids almost identical in size (1,986 bp) and sequence, three of which have been isolated from *N. lactamica* strains isolated in 1975, 1993, and 2004 (pNL750149, pNL932024, and pNL12, respectively). Previously, pJS-A (AJ238491) was identified in Germany in *N. meningitidis* sub-group IV isolates exclusively (between 1986 and 1988) (12). The largest ORF, encoding a putative replication initiation factor (likely a topoisomerase) of 336 amino acids (pfam02486, E = 2 × 10⁻⁹), shows 31% identity (93/294 amino acid residues) to a putative protein on the 2-kbp cryptic plasmid from the ammonia-oxidizing bacterium *Nitrosomonas* (34), but no nucleotide identity has been found.

(ii) Group II. Of 18 *N. lactamica* strains isolated in 2004, 5 contained a plasmid approximately 2.25 kbp in size. These almost identical plasmids, making up plasmid group II, display two noteworthy features. First, all contain a long homopoly-
meric guanine tract of variable length (10, 12, 12, and 15 guanine residues for plasmids pNL18.1, pNL7.1, pNL15, and pNL11, respectively). This homopolymeric G tract is located upstream of an ORF, p153, encoding a putative protein of 153 amino acids and therefore could be involved in transcriptional phase variation (26, 29). However, it is doubtful whether the p153 sequence encodes a protein, as there is no significant similarity to NCBI database entries, and no putative promoter domains could be identified.

Second, these four plasmids all contain two inverted neisserial DNA uptake sequences (DUS) (shown underlined) with two adenosine residues in between the DUS (TTCAGACGGCAAGCCGTCTGAA). However, this sequence has the reverse orientation in plasmid pNL15.

(iii) Group III. Group III consists of three different plasmids, of which two have been isolated from N. meningitidis isolates (pJS-B and pNL9 [this study]) and one from N. meningitidis (pJS-B [5]). The remaining plasmid was previously identified among isolates from a cluster of hypervirulent N. meningitidis ET-37 (plasmid pJS-B, which can also occur as a chromosomal integration [5]). These plasmids vary considerably in size (ranging from 4 kbp [pNL3.2] to 8.3 kbp [pNL9]), and the genetic organization of the plasmids resembles that of the recently described meningococcal disease-associated (MDA) phage (4) (Fig. 1), as well as that of the recently described Eikenella corrodens virulence-associated plasmid pMU1 (1). Their putative replication-associated protein shows similarity to phage replication proteins (COG2946, E. coli 6). In addition, pJS-B and pNL9 both contain an ORF (pJS-B_8 and pNL9-8, respectively) with a conserved domain of the zonular occludens toxin Zot (pfam05707, E. coli 48) family of proteins, which is 39% identical to its MDA homolog NMA1799 (Table 3).

Upstream of the genes encoding the putative phage replication proteins (pNL9-3, pJS-B_8, and pNL3.2_2) on the different plasmids of group III, a polymeric cytidine tract of variable length in the sequence C_6–10N_10G_7 is found. With its flanking sequences, this sequence shows a striking resemblance with the phase-variable porA promoter sequence (30) (Fig. 2).

(iv) Group IV. Plasmids in group IV are heterogeneous in size and were isolated from different bacterial species, N. gonorrhoeae, N. meningitidis, and N. lactamica (Table 4). pJD1 has been isolated from N. gonorrhoeae (15), which displays 72.3%
nucleotide identity to pNL01 from \textit{N. lactamica} (32a). However, on the basis of plasmid size, mobilization protein similarity, and replication protein similarity, four different subclasses (A to D) can be identified (Table 4). Some of these plasmids harbor combinations of these genes, suggestive of hybrid plasmids. Indeed, we were able to identify hybrid plasmids (pNL14 and pNL871104) based on nucleotide sequence similarity. Alternatively, the other plasmids evolved after several deletion events.

(v) Groups V and VI. Group V and group VI plasmids (pMIDG2830 (accession number AY174058) and pJD4 (accession number NC_002098), respectively) consist of plasmids sequenced by other groups and are different from all previously mentioned neisserial plasmid groups on the basis of replication or mobility proteins.

\textbf{Genome signature comparisons between plasmid DNA and chromosomal DNA display large differences.} The genome signature, which is the set of dinucleotide relative abundance values (13, 14), is one of the parameters available to identify putative horizontally transferred DNA. We compare the genome signature dissimilarity scores ($\delta^*$) between the different neisserial plasmids identified in this study and a representative genome sequence (for \textit{N. lactamica} we use the preliminary \textit{N. lactamica} sequence, available as contigs at the Sanger Institute) as described previously (32). Briefly, the $\delta^*$ of the plasmids are compared to the $\delta^*$ of identical-sized fragments of the

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Comparison of the genetic organizations of \textit{N. meningitidis} plasmids pNL3.2, pNL9, and pJS-B and the meningococcal disease-associated island with the ORFs NMA1792 to NMA1800. Similar colors indicate similar protein-coding ORFs (except for white). The \textit{Eikenella corrodens} pMU1 plasmid is depicted at the top of the figure.}
\end{figure}
host genomes. High compositional dissimilarity between plasmid and host genome is reflected by a large percentage of genomic fragments with a lower 5\(^{-}\) than the 5\(^{+}\) for the plasmid. We find high compositional dissimilarity between plasmid DNA and chromosomal DNA for most plasmid groups (Table 5), as noted previously for a large collection of prokaryotic plasmids from the Plasmid Database (31). However, group II plasmids do not display a high genomic dissimilarity with the \textit{N. lactamica} genome sequence.

**DISCUSSION**

In this study we describe the isolation and identification of 12 plasmids from \textit{N. lactamica} isolates. Only 1 of 20 \textit{N. meningitidis} throat isolates carried a plasmid, which is in accordance with the previous results of Backmann and colleagues (2). They found plasmids in 2 of 119 invasive meningococcal strains and in 1 of 50 meningococcal strains from the respiratory tract (2). In contrast, we found half of the \textit{N. lactamica} isolates harboring a plasmid. Among gonococci, an even larger proportion of the isolates (96\%) harbor plasmids (23), and multiple plasmids may be present in individual strains (6). Three \textit{N. lactamica} strains were also found to carry more than one plasmid. In each case, the different plasmids belong to different plasmid groups (see Tables S1 and S2 in the supplementary material).

The reason for this erratic distribution of plasmids among different neisserial species remains unknown, but the erratic distribution suggests either limited establishment of plasmids in \textit{N. meningitidis} or more efficient exchange between \textit{N. lactamica} and \textit{N. gonorrhoeae} or successful acquisition by \textit{N. lactamica} and \textit{N. gonorrhoeae}. Limited establishment might result if plasmids more readily integrate into the chromosome in \textit{N. meningitidis}. Integration of plJS-B into the meningococcal chromosome was shown by Claus and colleagues (5). Differences in the ability to take up extracellular DNA could explain differences in plasmid acquisition. Although most neisseriae were found to be naturally competent, DUS-mediated transformation has been established only for the pathogenic species (9), and to our knowledge, natural transformation of \textit{N. lactamica} has not yet been reported. However, plasmid transfer may follow a DUS-independent route for most plasmid groups, as only the compositionally nonamalgamous group II plasmids contain copies of the DUS. Finally, different restriction barriers between the different species could influence successful establishment. Only a few restriction and modification systems that are present in \textit{N. meningitidis} and have no isoschizomer in \textit{N. lactamica}, are known notably NmeBI, NmeRI, and NmeSI. Only two plasmids (pNL3.1 and pJD4) contain sites for these restriction and modification systems, so it is unlikely that restriction barriers are the main reason for the observed differ-

![FIG. 2. Analysis of the upstream region of the phage-associated replication proteins on plasmids from group III (pJS-B, pNL9-3, and pNL3.2) [see also Table 3]. In the comparison we included the upstream region of the putative phage-associated protein from the meningococcal disease-associated gene cluster (NMA1792), which is thought to be inactive, and the phase-variable promoter from the \textit{porA} gene (from \textit{N. meningitidis} MC58 [NMB1429]). The boxes represent regions of similarity between the \textit{porA} promoter and plasmid sequences. The –35 and –10 domains of the \textit{porA} promoter are underscored. The putative –35 region of the (inactive) NMA1792 promoter seems to have degenerated.](image-url)
The nucleotide sequences of the different neisserial plasmids display substantial diversity both in length and composition within the relatively small number of tested isolates. Together with the neisserial plasmids with chromosomal sequences, these in occurrence of all plasmids in *N. meningitidis* and *N. lactamica*.

The broad spectrum of plasmids described in this study should harbor suitable vectors for *N. lactamica*, which may be of interest, as *N. lactamica* is currently under consideration for its vaccine potential against meningococcal disease (10, 11).

In group III, the different plasmids vary in length considerably. The genetic organizations of the ORFs encoding putative phage-associated proteins (including a putative toxin and a phage replication-associated protein) on the larger plasmids resemble that of the recently identified, integrated, MDA phage from *N. meningitidis* Z2491 (4). It is tempting to hypothesize that these *N. lactamica* plasmids may in fact be phage replicative forms isolated during the plasmid DNA isolation procedure. Further experiments are to be performed to confirm or refute this hypothesis. The identification of a putative phage toxin-encoding gene on *N. lactamica* plasmids highly similar (39% identical) to that of the MDA phage raises questions concerning the association between the MDA phages and pathogenic potential, as found recently in a study by Bille and coworkers (4). Obviously, *N. lactamica* lacks other virulence factors important in the pathogenicity of invasive disease (the capsule is one clear example).

The presence of disease-associated sequences in commensal

tables in public databases, we identified six distinct plasmid groups (I to VI) on the basis of nucleotide identity, restriction fragment length polymorphism, and protein similarity. Additionally, we considered intragroup genome signature resemblance and tandem repeat similarity. Group IV plasmids could be subdivided on the basis of the presence or absence of genes encoding Rep and Mob proteins. Plasmids pNL14 and pNL871104, harboring combinations of these genes, appear to be hybrid plasmids on the basis of sequence comparisons. As a consequence, these plasmids may have extended their host range, as they are found in three species, *N. meningitidis*, *N. lactamica*, and *N. gonorrhoeae*.

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The presence of disease-associated sequences in commensal

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* a Neisserial plasmids are compared to the collection of *N. lactamica* (Niac) contigs obtained from the Sanger Institute (June, 2004), the genome signature of *N. meningitidis* (Nmen) MC58 or of *N. gonorrhoeae* (Ngon) FA1090 (for the applicable plasmids). For the species from which each plasmid was isolated, see Table 1.

b % GFa and % GFb are the percentages of genomic fragments with identical length that have a G+C percentage lower than that of the plasmid (% GFa) or that have less genomic dissimilarity to the genome as a whole (measurement of dissimilarity was calculated). (32).

c These plasmids have not been sequenced and are subsequently not tested in this compositional analysis.

d Plasmid pMIDG2830 was isolated from a *Neisseria flavescens* isolate. As no representative genomic sequence is available, we display the genomic dissimilarity scores for both *N. lactamica* and *N. meningitidis* as the scores for *N. gonorrhoeae*.
bacteria indicates that commensal strains may form a reservoir of latent virulence factors, which may contribute to the variability of these factors in pathogenic neisseriae. As a clear example, Linz and coworkers demonstrated the frequent exchange of *tpbB* sequences between commensal neisseriae and *N. meningitidis*, which contributed significantly to the antigenic variation of the transferrin binding protein TbpB in meningococci (17). In a more recent study, we have shown that *N. lactamica* also contains genomic islands encoding putative virulence-associated genes with high similarity to genes on genomic islands in meningococci (32a).

The phase variation-associated sequence C$_{6-16}$N$_p$G$_7$ upstream of the ORF encoding phase replication-associated protein is another feature in group III plasmids. The variation among the different plasmids in its homopolymeric cytidine tract and the striking resemblance with the phase-variable *porA* promoter in *N. meningitidis* (30) are indicative of a phase-variable expression of the Rep protein on these plasmids. Of note, a similar phase variation-associated sequence was previously identified upstream of NMA1792 by Snyder and coworkers (27). In vitro verification of phase variation for the putative phase replication proteins is required, but these sequences indicate that plasmids (or replicative forms of phages), neisserial phages, and even *Eikenella corrodens* plasmids potentially contain phase-variable genes.

A homopolymeric nucleotide tract of variable length was also identified in group II plasmids. Association of the expression of the ORF downstream of this homopolymeric tract and the striking resemblance with the phase-variable *porA* promoter in *N. meningitidis* (30) are indicative of a phase-variable expression of the Rep protein on these plasmids. Of note, a similar phase variation-associated sequence was previously identified upstream of NMA1792 by Snyder and coworkers (27). In vitro verification of phase variation for the putative phase replication proteins is required, but these sequences indicate that plasmids (or replicative forms of phages), neisserial phages, and even *Eikenella corrodens* plasmids potentially contain phase-variable genes.

Most of the plasmids have very high genomic dissimilarity scores compared to their respective genomic contexts. How this relates to the plasmid host histories is unclear. A high genomic dissimilarity score may reflect either a recent acquisition of the mobile element, a bias supporting extracellular stability, or an atypical genetic organization required to maintain its selfish and/or mobile behavior (31). In contrast, plasmids of group II have a genome signature similar to that of the *N. lactamica* genome. Also, group II plasmids contain a dyad neisserial DUS, as well as tandem repeat sequences that are relatively overrepresented in the meningococcal, gonococcal, and preliminary *N. lactamica* genome sequences, in contrast to plasmids of other groups. Together, these results suggest that plasmids of group II may have a longer evolutionary relationship with their host than plasmids of the other plasmid groups.

Carriage of *N. lactamica* may assist in the development of natural immunity to *N. meningitidis* by induction of cross-reactive antibodies (8). Moreover, the incidence of meningococcal disease is lower in communities with high *N. lactamica* carrier rates (20). It has been previously established that commensal and pathogenic neisseriae share a common gene pool (17, 18). Our data indicate that commensal neisseriae may function as a reservoir for putative virulence-associated sequences located on plasmids (e.g., those of group III) and on chromosomal genomic islands (32a). Possibly, during carriage of *N. lactamica* early in life, these pathogenicity-associated factors may contribute to the natural immunity to meningococcal infection later in life (25).

**REFERENCES**


