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Research article

Variations in gene organization and DNA uptake signal sequence in the *folP* region between commensal and pathogenic *Neisseria* species

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Abstract

Background: Horizontal gene transfer is an important source of genetic variation among *Neisseria* species and has contributed to the spread of resistance to penicillin and sulfonamide drugs in the pathogen *Neisseria meningitidis*. Sulfonamide resistance in *Neisseria meningitidis* is mediated by altered chromosomal *folP* genes. At least some *folP* alleles conferring resistance have been horizontally acquired from other species, presumably from commensal *Neisseriae*. In this work, the DNA sequence surrounding *folP* in commensal *Neisseria* species was determined and compared to corresponding regions in pathogenic *Neisseriae*, in order to elucidate the potential for inter-species DNA transfer within this region.

Results: The upstream region of *folP* displayed differences in gene order between species, including an insertion of a complete Correia element in *Neisseria lactamica* and an inversion of a larger genomic segment in *Neisseria sicca*, *Neisseria subflava* and *Neisseria mucosa*. The latter species also had DNA uptake signal sequences (DUS) in this region that were one base different from the DUS in pathogenic *Neisseriae*. Another interesting finding was evidence of a horizontal transfer event from *Neisseria lactamica* or *Neisseria cinerea* that introduced a novel *folP* allele to the meningococcal population.

Conclusion: Genetic recombination events immediately upstream of *folP* and horizontal transfer have resulted in sequence differences in the *folP* region between the *Neisseria* species. This variability could be a consequence of the selective pressure on this region exerted by the use of sulfonamide drugs.

Background

Horizontal gene transfer via natural transformation is believed to be the main mechanism to generate genetic diversity among *Neisseriae* [1-3]. Horizontal transfer has for example contributed to the spread of resistance to pen-

icillin in pathogenic *Neisseriae*, where the origin of the transforming DNA was traced to commensal *Neisseria* species [4]. Sulfonamide resistance in some strains of *Neisseria meningitidis* (the meningococcus) has also been acquired by horizontal transfer [5]. As evidence, a mosaic
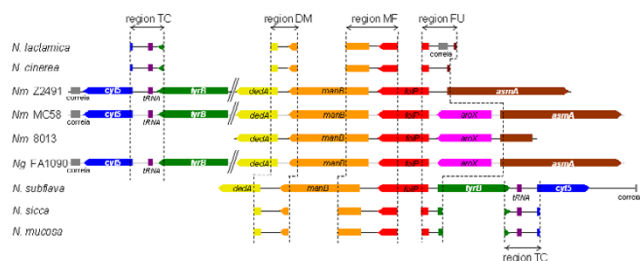
Figure 1
Comparison of gene arrangements in human *Neisseriae*. The *N. subflava* sequence (accession number AJ581792 in the EMBL database) is displayed in alignment with corresponding parts from other *Neisseria* species. Open reading frames are visualized as colored block arrows (the tip of each arrow symbolizing the stop codon) and labeled with their designations according to the text. Non-coding regions are visualized as solid lines. The *dedA* gene and the Correia element in *N. subflava* were only partially sequenced. The symbol // means that 13 kb sequence separating *tyrB* from *dedA* has been omitted in the figure. The intergenic regions are defined by dotted vertical lines and designated TC (*tyrB-cyt5*), DM (*dedA-manB*), MF (*manB-folP*) and FU (*folP*-upstream), respectively.

Figure 2
PCR amplification of the region between *folP* and *asmA* in *Neisseriae*. A 1293 base pair product denotes the presence of *aroX* between *folP* and *asmA*. M: molecular marker; a: *Neisseria meningitidis* (Nm) strain 952 (Su[S]); b: Nm 1014 (Su[S]); c: Nm BT054 (Su[S]); d: Nm 418 (Su[R]); e: Nm 3976 (Su[R]); f: Nm BT227 (Su[R]); g: Nm MO035 (Su[R]); h: Nm MO124 (Su[R]); i: *Neisseria lactamica* (Su[S]); j: *Neisseria cinerea* (Su[S]). Su[S] = sulfonamide-susceptible, Su[R] = sulfonamide-resistant.

pattern of fragments with high sequence divergence was detected in the chromosomal *folP* gene, the sulfonamide resistance determinant. The origin of the transforming DNA is still unknown, but presumable donors are commensal *Neisseria* species.

We have previously demonstrated that commensal *Neisseria* isolates from healthy carriers exhibit high level sulfonamide resistance, most likely due to sulfonamide-resistant variants of *folP* [6]. These commensal *folP* genes differ substantially from previously described *folP* genes, but the findings suggest that sulfonamide resistance is a property carried among species of the commensal flora. Thus, the commensal flora can be a reservoir of sulfonamide resistance genes that might be transferred to pathogenic species.

Efficient transformation is dependent upon a reasonable sequence similarity between donor and recipient to facilitate homologous recombination. In addition, DNA uptake in pathogenic *Neisseriae* is markedly enhanced by the presence of a DNA uptake signal sequence (DUS) in the donor DNA [7,8]. Thus, the sequence within and immediately surrounding *folP*, especially the presence of DUS in that region, should influence how efficient commensal *Neisseria* species may transfer sulfonamide resistance to *Neisseria meningitidis*. This work presents the gene organization and the distribution of DUS in the *folP* region in some commensal *Neisseria* species and compares these to the corresponding parts of the genomes
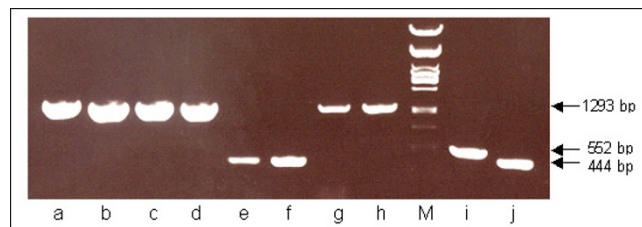
from three *Neisseria meningitidis* strains and *Neisseria gonorrhoeae* FA1090.

Results
Gene organization in commensal **Neisseriae**
The sequence surrounding *folP* was determined for *Neisseria subflava* by sequencing 6.8 kb of the genome. (*N. subflava* was chosen because sulfonamide-resistant commensal *Neisseriae* found in healthy carriers were phenotypically characterized as being most related to this species [6]). The corresponding sequences from *N. meningitidis* strains MC58 [9] and Z2491 [10], as well as *N. gonorrhoeae* FA1090 [11] were aligned with the *N. subflava* sequence. Included in this comparison was also a sequence from the on-going genome sequencing project of *N. meningitidis* strain 8013 (The Pasteur Institute, France). PCR primers designed from this alignment were then used to determine the gene order surrounding *folP* in one strain each of *N. sicca*, *N. cinerea*, *N. lactamica* and *N. mucosa*. The results are outlined in Figure 1 (see Additional file for sequence data). *N. subflava*, *N. sicca* and *N. mucosa* had identical gene order: directly downstream of *folP* was a putative phosphomanno-/phosphoglucomutase family gene (designated *manB*), followed by a putative membrane-associated gene belonging to the dedA family (*dedA*). Upstream of *folP* was an aromatic amino-acid amino-transferase gene (*tyrB*), followed by a *tRNA-Arg* gene, an open reading frame similar to a cytochrome c5 gene (*cyt5*) and a copy of the Correia repeat unit [12].

N. gonorrhoeae, *N. lactamica*, *N. cinerea* and the *N. meningitidis* strains had the same gene succession as *N. mucosa*/ *N. sicca*/*N. subflava* downstream of *folP*, but the genes found upstream of *folP* were completely different. Instead of being located upstream *folP*, the gene sequence *tyrB-tRNA-cyt5*-Correia was in these species located 14 kb
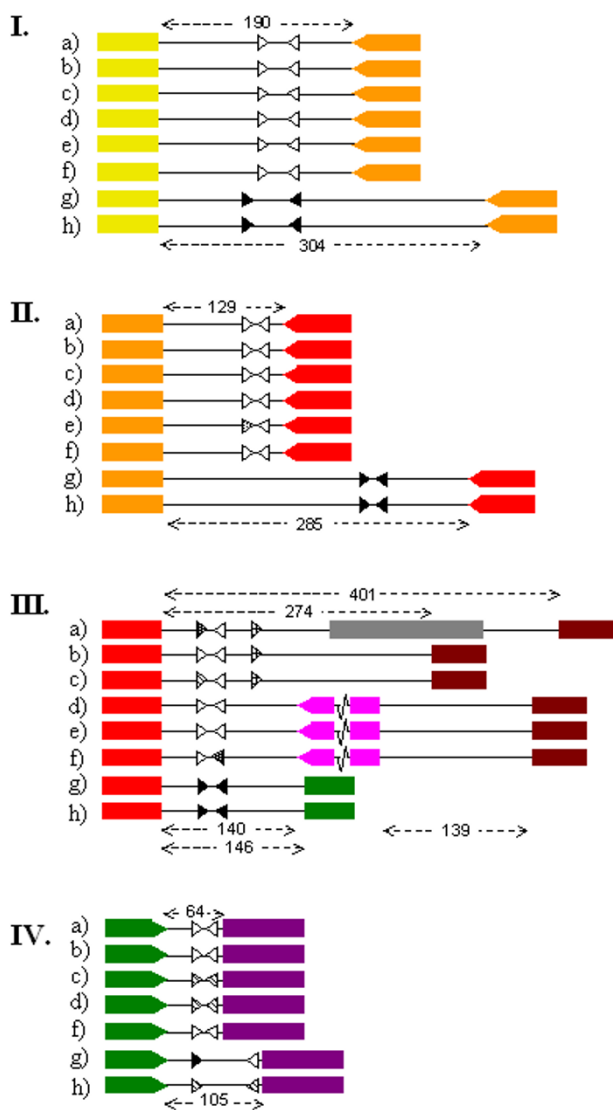
Figure 3
Enlargement of the intergenic regions from Figure 1.
I: region DM. II: region MF. III: region FU. IV: part of region
TC. The order of the strains is as in Figure 1: a) *Neisseria
lactamica*; b) *Neisseria cinerea*; c) *N. meningitidis* Z2491; d) *N.
meningitidis* MC58; e) *N. meningitidis* 8013; f) *N. gonorrhoeae*;
g) *Neisseria subflava*; h) *Neisseria sicca*. Numbers flanked by
dotted arrows specify distances in base pairs between adja-
cent start- and stop codons. Triangles illustrate various DNA
uptake signal sequences: ▶: GCCGTCTGAA; ▶:
G**T**CGTCTGAA; ▷: GCCGT**T**TGAA; ▷: GCCGTC**C**GAA; ▷:
GCCGT**A**TGAA; ▷: **A**CCGTCTGAA; ▷: **AT**CGTCTGAA.
Triangles pointing towards each other illustrate inverted
repeats. For the complete nucleotide sequences, see Addi-
tional file.

downstream of *folP*. The direction of *tyrB* and the follow-
ing genes in relation to *folP* was also the opposite com-
pared to the case in *N. subflava*/*N. sicca*/*N. mucosa*. This
indicates that the difference in gene organization was due
to an inversion of a genomic segment consisting of the
folP gene and the region between *folP* and *tyrB*. Another
interesting observation was that the distance between *folP*
and the upstream gene in *N. lactamica* was longer than in
other species, due to the insertion of a Correia element.
This Correia element was identical to some of the ele-
ments found in pathogenic *Neisseriae*. It had the typical
features for these repeats: it was flanked by TA repeats, had
a putative integration host factor binding site and its
length and inverted terminal repeats indicated that it
belonged to the α family of Correia elements [13].

Presence of the aro**X** gene
Two of the meningococcal strains (MC58 and 8013) and
N. gonorrhoeae FA1090 had a putative chorismate mutase-
related gene (*aroX*) immediately upstream of *folP* fol-
lowed by a putative *asmA* gene (see Figure 1). The third
meningococcal strain (Z2491), *N lactamica* and *N. cinerea*
lacked *aroX* in this position and instead had *asmA* next to
folP. PCR analysis revealed that *aroX* was present upstream
of *folP* in six out of eight clinical strains examined (see Fig-
ure 2). Interestingly, the two clinical strains lacking *aroX*
both had *folP* genes identical to Z2491. This *folP* allele is
associated with sulfonamide resistance [5] and displays a
very high degree of sequence divergence surrounding the
start codon of the gene, compared to other meningococci:
26 altered positions in a 61 base pairs sequence. In fact,
this divergent part was almost identical to corresponding
sequences in *N. cinerea* and *N. lactamica*, suggesting that
horizontal transfer from one of these commensal species
removed the *aroX* gene and simultaneously altered the
"wild type" meningococcal sequence immediately
upstream of, and the first part of, *folP*.

Difference in DNA uptake signal sequences
The complete 6.8 kb sequence obtained from *N. subflava*
and the intergenic regions amplified by PCR from *N. sicca*,
N. lactamica and *N. cinerea* were examined for presence of
the neisserial DNA uptake signal sequence (DUS) 5'-
GCCGTCTGAA-3'. The meningococcal and gonococcal
genomes contain almost 2000 copies of this sequence,
which makes an average of one copy every 1,100 base
pairs. The intergenic sequences from *N. lactamica* and *N.
cinerea* were highly similar to the corresponding regions
from the pathogenic species, including the numbers and
positions of DUS (see Figure 3). In contrast, only one copy
of the neisserial DUS was found in the *N. subflava*
sequence and none was found among the *N. sicca* inter-
genic sequences. However, an altered variant where the
second base is a thymine (5'-G**T**CGTCTGAA-3') was more
prevalent in the latter species: *N. subflava* displayed as

Table 1: Oligonucleotides used as PCR primers.

name	sequence (reference)
cytup	5'-TAACGATACCGCTCACAAGGG-3'
dedAup	5'-GTCAAATCTTCGGGAATCGG-3'
NM10	[42]
NM11	CCGCCGCCACGCTGCCGTGT
NM7	TTGGCAGGCAGGACGGTTTG
NmAsmup	5'-CATATTTCAGACGGTATTTGTGG-3'
NmdusCrev	CCTT<u>TTCAGACGGC</u>ATGAAGTATGTGAACC
Nmdusfwd	GTTTTTTATTTTGGGAGGAGTTC
NmdusTrev	CCTT<u>TTCAGACGA</u>CATGAAGTATGTGAACC
NmdusXrev	CCTT<u>TTGTGAGTGC</u>ATGAAGTATGTGAACC
Nsfcytokrom1	5'-TATCGCGCAAGGCTTCGATAC-3'
Nsfcytokrom2	5'-TAACGCCATGCCTGCAAAAG-3'
Nsfcytokrom3	5'-AGAAACCCTGCACAAACACG-3
Nsfcytokrom5	5'-AACGCCATGCCTGCAAAAG-3'
Nsfdownsense3	5'-CAGGTTTCCCGCTGTTGATCG-3'
Nsfmutasdown2	5'-TGCGCATCCAAAAAGGTCAG-3'
Nsfmutasdown3	5'-AAAGACGTGTTGGCGGAAGTG-3'
Nsfmutasdown4	5'-GGGAACGGTGGTACAGTGGAT-3'
Nsfmutasdown5	5'-CCATGCCGGTTTAAGGTTAAT-3'
Nsfmutasfwd6	TATTCCGACAACGGCATCAAATTC
Nsfmutasfwd7	5'-GTGAAGCTGAGCGATGAAATCGAG-3'
Nsfmutasrev5	5'-GTCGATTTTGGCTTCGATTTCC-3'
NsftyrBdown1	5'-GATTGAGCGTGGAACAAGTCCG-3'
NsftyrBdown2	5'-ATTCCGGCAGGATGTGCGTC-3'
NsftyrBfwd1	5'-ACCGGCGTGGATATGTCGG-3'
NsftyrBup	5'-GGTCGTTTTTGAAGGTTTCGAC-3'
Nsfupanti	5'-GTTTGGGCGTTTTGCGAATA-3'

many as 10 copies of this altered DUS (see Figure 3; three of the altered DUS are located outside of the displayed sequences) and 6 copies was found in *N. sicca*. The occurrence of the neisserial and the altered DUS in the *folP* region of various *Neisseria* species is listed in the left part of Table 2.

To extend the analysis beyond the folP region, all sequences from human Neisseria species that were larger than 400 base pairs and deposited in the GenBank as of October 2005, were screened for both types of DUS described above. As a comparison, we also searched for another variant of DUS, where the third nucleotide was a thymine (5'-GCTGTCTGAA-3'). The results are summarized in the right part of Table 2. The altered DUS 5'-GTCGTCTGAA-3' was detected in the pathogenic Neisseria genomes and in sequences from N. cinerea, N. subflava, N. mucosa and N. elongata, but not as frequent as found around folP in N. subflava and N. sicca. Furthermore, all species also had several copies of the neisserial DUS, present at a much higher density than the altered variant. This indicates that the neisserial DUS is the prevalent one in all Neisseria species, and contradicts our finding from the folP region in N. subflava and N. sicca. When looking at this region in other species, several different variants of DUS can be identified (see Figure 3). For exam-

ple, the region upstream of folP displays four variants of DUS: the neisserial DUS, the altered DUS and two other variants. The last column in Table 2 lists the prevalence of a DUS with an altered third base: this variant was also found in most Neisseria species. Obviously, the genomes of Neisseria species contain multiple variants of DUS, which seem to be scattered in variable densities throughout the genomes.

Transformation with DNA containing altered DUS
The transformation efficiency for DNA containing the neisserial DUS (5'-GCCGTCTGAA-3') and the altered DUS (5'-GTCGTCTGAA-3') was determined for *N. meningitidis* strain 952. Transformation frequencies were highest for DNA containing the neisserial DUS and around 50% lower for DNA with the altered DUS. However, the absolute transformation frequencies varied between experiments, so there was no statistically significant difference between the two DUS variants (see Table 3). DNA with a distorted DUS variant, where four bases had been replaced, scored significantly lower values, indicating that the DNA uptake was indeed sequence dependent, as expected in *N. meningitidis*. The conclusion from these experiments was therefore that a one-base-substitution in the DUS did not produce a significant reduction in transformation frequency in this laboratory setting.

Discussion
The *Neisseria* species are believed to be non-clonal bacteria with a high degree of genetic transfer within and between different species [2,3,14-18]. Previous reports of transformation in *N. meningitidis* have suggested that different regions of the genome may have different recombination rates [19,20]. This work identified the region upstream of *folP* as particularly active in recombination events. The most obvious example is the insertion of a complete Correia element, upstream of *folP* in *N. lactamica*. Correia elements are small putative transposable elements, around 152 base pairs long with inverted repeats at the ends [12]. Hundreds of Correia copies are scattered around the genomes of pathogenic *Neisseriae* [13,21,22] and they have also been reported in *N. lactamica* [23]. We detected another copy of a Correia repeat in the *N. subflava* sequence, suggesting that these elements are ubiquitous in the *Neisseria* genus and possibly was introduced before the divergence into different species.

Another example of recombination events is the apparent inversion of a genomic segment in *N. subflava*, *N. sicca* and *N. mucosa*, with one breakpoint upstream of *folP*. A third recombination event in the same location is the deletion of *aroX* from some strains. This gene is obviously not essential since it is absent from the genome of *N. meningitidis* Z2491, but was nevertheless present in the majority of strains examined in this work. The sequences between

folP and *aroX* from all these latter strains are highly similar and include two DUS downstream of *aroX*, arranged as an inverted pair followed by a stretch of thymidines. This arrangement is characteristic of transcription terminators and is a general configuration for DUS in meningococcal and gonococcal genomes [8,24]. The strains lacking *aroX* had retained this putative termination signal, although located between two divergently operating start codons and hence without function. Furthermore, all commensal strains examined in this work also had inverted repeated DUS between the start codons of *folP* and the upstream gene (*tyrB* or *asmA*). These observations support the proposed genetic rearrangements in these species: a deletion of *aroX* in *N. lactamica*, *N. cinerea* and some meningococcal strains, and an inversion of a genomic segment in *N. subflava*, *N. mucosa* and *N. sicca*.

The sequence differences and similarities detected in this work could divide the examined species into two groups, where the pathogenic species, *N. lactamica* and *N. cinerea* fall in one group and *N. subflava*, *N. sicca* and *N. mucosa* in a second group. This is in agreement with the classic taxonomy of *Neisseria* species based on ribosomal RNA hybridization [25,26] and phenotypic tests [27]. The inversion of the *folP-tyrB* genomic fragment and the accumulation of the altered DUS likely occurred in a common ancestor to *N. subflava*,*N. sicca* and *N. mucosa*. Similarly, the deletion of *aroX* probably occurred in a common ancestor to *N. lactamica* and *N. cinerea*, but after the separation of commensal species from pathogenic species. The removal of *aroX* created a novel intergenic sequence that was later transferred to a meningococcus, removing *aroX*

also in this strain. The fact that all meningococcal strains we found lacking *aroX* were sulfonamide-resistant and had identical *folP* genes can be explained in three ways: either, the recipient was already sulfonamide-resistant at the time of transfer; the recipient acquired resistance after the transfer; or the donor was a sulfonamide-resistant commensal strain that transferred the resistant *folP* allele together with the upstream region to the meningococcus.

The DNA uptake signal sequence (DUS) is crucial for efficient transformation in pathogenic *Neisseriae* [7,8]. The tendency to preferentially take up DNA from closely related species was noted in early experiments with gonococci [28-31]. Further experiments established that this was due to the presence of an uptake signal sequence in the donor DNA, and this sequence was subsequently determined to be 5'GCCGTCTGAA-3' [7,8,32]. The meningococcal and gonococcal genomes contain close to 2,000 copies of this neisserial DUS, but they also contain several variants of this sequence with one or a few base substitutions. The altered variants of DUS are frequently located in pairs as perfect inverted repeats after open reading frames, just as the neisserial DUS. This could indicate that this arrangement is important for its function and therefore support the hypothesis of DUS as being transcription terminators [24]. The DUS variant with a thymine in second position was highly prevalent in the *folP* region of *N. subflava* and *N. sicca*, where it seemingly had replaced the neisserial DUS, and was also fairly abundant in pathogenic strains: 165 and 118 copies in *N. meningitidis* MC58 and *N. gonorrhoeae* FA1090, respectively. The results from our transformation experiments, albeit per-

Table 2: Presence of DNA uptake signal sequences in human *Neisseria* species.

	Prevalence (density[1]) of DNA uptake signal sequences					
	in intergenic parts of the *folP* region (Fig. 3)			in database sequences		
Species	GCCGTCTGAA	G**T**CGTCTGAA	**Sequence length**	GCCGTCTGAA	G**T**CGTCTGAA	GC**T**GTCTGAA
N. meningitidis[2]	6	0	2,272,300	1935 (1,174)	165 (13,772)	47 (48,347)
N. gonorrhoeae	7	0	2,153,894	1965 (1,096)	118 (18,253)	31 (69,480)
N. lactamica	7	0	51,533	40 (1,288)	0	0
N. cinerea	8	0	12,158	15 (811)	3 (4,053)	4 (3,040)
N. subflava	1	7	13,523	16 (845)	1 (13,523)	0
N. sicca	0	6	3,114	3	0	1
N. mucosa	n.a	n.a	4,492	3	1	1
N. polysaccharea	n.a	n.a.	22,903	25 (916)	0	2 (11,452)
N. flavescens	n.a	n.a	4,844	8	0	0
N. perflava	n.a	n.a	1,392	2	0	0
N. elongata	n.a	n.a	7,064	8	1	0

[1] Density as the average distance between two DUS. Density was only calculated when the (accumulated) sequences included more than 10,000 base pairs.
[2] Strain MC58

Table 3: Transformation frequencies

Type of DUS	Average transformation frequency per 10,000 cells (mean ± SD)
neisserial DUS (GCCGTCTGAA)	56 ± 21
altered DUS (G**T**CGTCTAA)	27 ± 15
distorted DUS (CG**AC**TC**AC**AA)	4 ± 2

formed with synthetic PCR-products under conditions different from the natural habitat of these bacteria, indicated that this one-base difference in DUS only marginally reduced the transformation frequency. Perhaps this can explain why this particular variant of DUS seems so prevalent in neisserial genomes: this alteration may be well tolerated by the DNA uptake mechanism.

It is not clear why some naturally competent species have a sequence-specific DNA uptake mechanism. One explanation describes their presence as a mere consequence of a preferential uptake of specific sequences during natural transformation [33]: DNA uptake processes that favor a specific sequence automatically lead to an accumulation of this preferred sequence in the genome, because DNA containing this sequence is more likely to be integrated than other DNA. Another theory is that DUS may be beneficial for the bacterial population since it directs the DNA uptake to sequences from closely related bacteria that can be used for creation of genetic variability or for DNA repair. A recent study report a higher frequency of DUS in genome maintenance genes compared to genes of other function, thus supporting the DNA repair explanation [34]. If DUS evolved as a mechanism to obtain homologous DNA for repair purposes it would make sense that only closely related species shared the same DUS. This hypothesis could therefore justify why the neisserial DUS was found in *N. lactamica* and *N. cinerea*, whereas an altered version of DUS was detected in the more distantly related *N. subflava* and *N. sicca*. On the other hand, this distinction was only true for the *folP* region: when examining sequences from other regions the neisserial DUS dominated in all species. However, the sequences available for analysis from several of the commensal species, including *N. subflava* and *N. sicca*, are in short supply and not representative for the whole genomes. Obviously, analysis of more sequences from these commensals is required to address the distribution of various DUS within the genomes of commensal *Neisseria* species.

Conclusion
This work compared the sequence surrounding the *folP* gene in various *Neisseria* species. The results lead to a general differentiation of the examined species into two groups, where *N. subflava*, *N. mucosa* and *N. sicca* belonged to one group and *N. cinerea*, *N. lactamica*, *N. meningitidis* and *N. gonorrhoeae* to another group. The

major differences between these two groups were an inversion of DNA with one breakpoint in the upstream region of *folP*, and a one base pair difference in the DNA uptake signal sequences present in this genomic region. The variability within the *folP* region may reflect the ongoing selection of different *folP* alleles by the selective pressure of sulfonamide drug use.

Methods
Neisseria *strains*
The *Neisseria meningitidis* clinical isolates used in this work were 418, 1014 [35], MO035 [36], 952, BT054, BT227, MO124 [37] and 3976 [38]. The commensal strains were *Neisseria lactamica* ATCC23970, *Neisseria mucosa* ATCC19696, *Neisseria sicca* ATCC9913, *Neisseria subflava* ATCC19243 and *Neisseria cinerea* ATCC14685. Bacteria were cultivated on Brain Heart Infusion agar plates (Difco Laboratories, USA) in 10% CO_2.

Reagents
Oligonucleotides (listed in Table 1) were purchased from Thermo Hybaid, Germany. Restriction enzymes and other molecular biology reagents were purchased from Roche Diagnostics, Germany, if not otherwise stated. Ligation mixtures were transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen™, The Netherlands). PCR products were purified from agarose gels using MinElute Gel Extraction Kit (QIAGEN, Germany) and cloned into pCR®2.1-TOPO® vector (Invitrogen™, The Netherlands) according to the manufacturer's instructions. Plasmids were purified using QIAprep Spin Miniprep (QIAGEN, Germany). DNA was sequenced on ABI PRISM 310 Genetic Analyzer (Applied Biosystems, USA) using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Sweden).

Determination of Neisseria subflava *sequence*
PCR primers NM7 and NM11 were used to amplify 734 base pairs of *folP* from *N. subflava* ATCC19243. Flanking sequences were determined using a chromosome walking method [39]; Restriction enzyme digested chromosomal DNA (approximately 2 µg) was inserted into vector pUC19 with T4 DNA Ligase (New England Biolabs, Beverly, MA) at 16°C. The unknown sequence was then amplified with the ligation mixture as template and one vector-specific primer, directed towards the insert, and a sequence-specific primer, directed towards the unknown

sequence. The polymerase chain reactions contained 0.2 units Taq Polymerase (Fermentas, Lithuania), 0.5 µM of each primer, 0.2 mM of each deoxynucleotide and 100 ng of DNA in a 20-µl reaction. PCR products were cloned and sequenced as described above. The chromosome walking was repeated stepwise in both directions until a 6808 base pair region of the chromosome had been sequenced. Nested PCR was performed in some of the steps to achieve specific products.

Determination of gene order
Primers binding within genes but directed towards non-coding regions were utilized to amplify intergenic sequences from chromosomal DNA from all *Neisseria* strains listed above using the Expand™ High Fidelity PCR System (Roche, Germany). Primers dedAup and Nsfmutasdown3 were used to amplify the region between *dedA* and *manB*; Nsfmutasrev5 and NM10 to amplify the region between *manB* and *folP*; Nsfupanti and NmAsmAup between *folP* and *asmA*; Nsfupanti and NsftyrBup between *folP* and *tyrB*; and NsftyrBdown2 and cytup between *tyrB* and *cyt5*. PCR products from *N. lactamica*, *N. cinerea* and *N. sicca* were cloned and sequenced as described above.

Transformation frequencies
The complete *folP* gene from the sulfonamide-resistant *N. meningitidis* strain MO035 was amplified from chromosomal DNA using the forward primer Nmdusfwd and one of three reverse primers. NmdusCrev was used to incorporate the meningococcal DUS in the PCR-product, Nmdus-Trev to incorporate the subflava variant of DUS and NmdusXrev to make a control DNA with equal length but with a disrupted DUS. PCR was performed with the Expand™ High Fidelity PCR System (Roche, Germany) according to the manufacturer's instructions and the products were purified with the MinElute PCR Purification kit (QIAGEN, Germany). The sulfonamide-susceptible *N. meningitidis* strain 952 was suspended to $OD_{600} = 0.1$ in a rich broth containing Brain-Heart-Infusion Blood-Agar Base (Difco Laboratories, USA), 10 mM $MgCl_2$, 2× Kellogg's supplement and 0.042% $NaHCO_3$ [40]. Exactly 1 µg of purified PCR-products was added to 200 µl bacterial suspension and the mixture was incubated at 37°C in 5% CO_2 until $OD_{600} = 0.3$. The diluted transformation mixtures were incubated on ISA plates (Oxoid, England) with or without 0.25 mM sulfathiazole (Sigma-Aldrich, Sweden).

Sequence data analysis
The complete genome sequences from pathogenic *Neisseria* species were extracted from GenBank: AL157959 (strain Z2491), NC003112 (MC58) and AF00969 (FA1090). The 5 kb sequence from *N. meningitidis* strain 8013 was provided by Christophe Rusniok and Philippe Glaser at the Pasteur Institute, France. The following entries from commensal *Neisseria* species were extracted from GenBank: *N. cinerea*: AF158604, Z17308-Z17310, X64869, AJ223935, AJ223913-AJ223915, AJ223890-AJ223892, AY831725, AJ704731, AY360352, AF470674, AF319536, AF306836, AF139614, AF216744, AJ247236, AJ239300, AJ239299, AJ239287, AF121876, AF029360, U82861, U82845, AF85340, AJ223869-AJ223871, U57906, U57710, X59540; *N. elongata*: AF121870, DQ007933, AJ223877-AJ223882, AJ223921-AJ223926, AJ223898-AJ223903, AY821559, AY167422, AF542173, AJ247252-AJ247254, AJ239302, AJ239303, AJ239297, AJ239278, AF121870, L06171; *N. flavescens*: M26645, AY174058, Z22955, X64872, AJ223938, AJ704736, AJ79434, AJ704735, AJ704733, AJ239280, U82863, U82847, AF987677, Y09310, Y09311, U57907, U57711, L06168; *N. lactamica*: DQ015867, Y11818, Y11819, Y10876, Y10877, X54485, X64871, AJ288888-AJ288893, AJ223909, AJ223945-AJ223947, AJ223910, AJ223907, AJ223908, AJ223884-AJ223887, AJ223863-AJ223866, NC006968, AY857302-AY857306, AY532623-AY532628, AY532630, AY54849, AY654848, AJ704746-AJ704748, AJ704737, AY438701, Y134876, AF542177, AJ270921-AJ270932, AJ270918, AJ270906-AJ270908, AJ270902, AJ270900-AJ270904, AF470682, AF470683, AF470673, AY038932, AF319537, AJ270919, AJ270920, AF312972, AF216855, AF158603, AJ247241, AJ247242, AJ239313, AJ239305, AJ239296, AJ239283-AJ239286, U82843, U82858, AF085689, AJ001737-AJ001740, Y09308, U57709, Y57905, U59398, X74901, X65533, U06074; *N. mucosa*: AJ223875, X59635, X64873, AJ223937, AJ223919, AJ223896, AJ223875, AJ704738, AY462277, AJ247255-AJ247260, AJ239279, AJ239282, AF121872, U82860, U82848, L47156, U57908, U57901; *N. perflava*: AJ223920, AJ223906, AJ223897, AJ223883, AJ223876, AJ223862, AY837569, AF312973, AJ247246, AJ247247, AJ239295; *N. polysaccharea*: DQ007935, Y11814-Y11817, Y10878-Y10881, X59626, X64870, AJ223944, AJ223940-AJ223943, AJ704761, AJ704762, AJ704743, AJ704739-AJ704744, AY134878, AY072807-AY072809, AY099335, AF542178, AF216858, AJ239314, AJ239315, AJ239298, AJ239289, AJ011781, U82844, U82859, Y09309, U57708, U57904, L06167; *N. sicca*: DQ007936, X76285, AJ223916, AJ223872, AJ223893, AY857301, AJ704730, AF312974, AJ247248, AJ239292-AJ239294, Y09307; *N. subflava*: DQ007937, AJ288894, AJ223918, AJ223895, AJ223874, AY522860, AJ704745, AY134877, AF216745, AF240672, AF241526, AJ247249, AF470684, AF479577, AF479578, AJ239291. The search for DUS and Correia elements was performed using the DNA Pattern Find algorithm in the Sequence Manipulation Suite [41].

Authors' contributions

YQ participated in the planning of this study, performed all experimental work and drafted the manuscript. GS participated in the planning, coordinated the study, and assisted in writing the manuscript. Both authors have approved the final manuscript.

Additional material

> ### Additional File 1
> *Intergenic sequences from commensal* Neisseriae. *This document lists the nucleotide sequences from the commensal species that are schematically illustrated in Figure 3.*
> Click here for file
> [http://www.biomedcentral.com/content/supplementary/1471-2180-6-11-S1.htm]

Acknowledgements

References

1. Holmes EC, Urwin R, Maiden MC: **The influence of recombination on the population structure and evolution of the human pathogen Neisseria meningitidis.** *Mol Biol Evol* 1999, **16(6):**741-749.
2. Feil EJ, Maiden MC, Achtman M, Spratt BG: **The relative contributions of recombination and mutation to the divergence of clones of Neisseria meningitidis.** *Mol Biol Evol* 1999, **16(11):**1496-1502.
3. Linz B, Schenker M, Zhu P, Achtman M: **Frequent interspecific genetic exchange between commensal Neisseriae and Neisseria meningitidis.** *Mol Microbiol* 2000, **36(5):**1049-1058.
4. Bowler LD, Zhang QY, Riou JY, Spratt BG: **Interspecies recombination between the penA genes of Neisseria meningitidis and commensal Neisseria species during the emergence of penicillin resistance in N. meningitidis: natural events and laboratory simulation.** *J Bacteriol* 1994, **176(2):**333-337.
5. Fermer C, Kristiansen BE, Skold O, Swedberg G: **Sulfonamide resistance in Neisseria meningitidis as defined by site-directed mutagenesis could have its origin in other species.** *J Bacteriol* 1995, **177(16):**4669-4675.
6. Qvarnstrom Y, Swedberg G: **Sulphonamide resistant commensal Neisseria with alterations in the dihydropteroate synthase can be isolated from carriers not exposed to sulphonamides.** *BMC Microbiol* 2002, **2(1):**34.
7. Elkins C, Thomas CE, Seifert HS, Sparling PF: **Species-specific uptake of DNA by gonococci is mediated by a 10-base-pair sequence.** *J Bacteriol* 1991, **173(12):**3911-3913.
8. Goodman SD, Scocca JJ: **Identification and arrangement of the DNA sequence recognized in specific transformation of Neisseria gonorrhoeae.** *Proc Natl Acad Sci U S A* 1988, **85(18):**6982-6986.
9. Tettelin H, Saunders NJ, Heidelberg J, Jeffries AC, Nelson KE, Eisen JA, Ketchum KA, Hood DW, Peden JF, Dodson RJ, Nelson WC, Gwinn ML, DeBoy R, Peterson JD, Hickey EK, Haft DH, Salzberg SL, White O, Fleischmann RD, Dougherty BA, Mason T, Ciecko A, Parksey DS, Blair E, Cittone H, Clark EB, Cotton MD, Utterback TR, Khouri H, Qin H, Vamathevan J, Gill J, Scarlato V, Masignani V, Pizza M, Grandi G, Sun L, Smith HO, Fraser CM, Moxon ER, Rappuoli R, Venter JC: **Complete genome sequence of Neisseria meningitidis serogroup B strain MC58.** *Science* 2000, **287(5459):**1809-1815.
10. Parkhill J, Achtman M, James KD, Bentley SD, Churcher C, Klee SR, Morelli G, Basham D, Brown D, Chillingworth T, Davies RM, Davis P, Devlin K, Feltwell T, Hamlin N, Holroyd S, Jagels K, Leather S, Moule S, Mungall K, Quail MA, Rajandream MA, Rutherford KM, Simmonds M, Skelton J, Whitehead S, Spratt BG, Barrell BG: **Complete DNA sequence of a serogroup A strain of Neisseria meningitidis Z2491.** *Nature* 2000, **404(6777):**502-506.
11. **Neisseria gonorrhoeae Genome Sequencing Strain FA 1090** [http://www.genome.ou.edu/gono.html]
12. Correia FF, Inouye S, Inouye M: **A family of small repeated elements with some transposon-like properties in the genome of Neisseria gonorrhoeae.** *J Biol Chem* 1988, **263(25):**12194-12198.
13. Buisine N, Tang CM, Chalmers R: **Transposon-like Correia elements: structure, distribution and genetic exchange between pathogenic Neisseria sp.** *FEBS Lett* 2002, **522(1-3):**52-58.
14. Feil EJ, Holmes EC, Bessen DE, Chan MS, Day NP, Enright MC, Goldstein R, Hood DW, Kalia A, Moore CE, Zhou J, Spratt BG: **Recombination within natural populations of pathogenic bacteria: short-term empirical estimates and long-term phylogenetic consequences.** *Proc Natl Acad Sci U S A* 2001, **98(1):**182-187.
15. Smith JM, Smith NH, O'Rourke M, Spratt BG: **How clonal are bacteria?** *Proc Natl Acad Sci U S A* 1993, **90(10):**4384-4388.
16. Zhu P, Klutch MJ, Bash MC, Tsang RS, Ng LK, Tsai CM: **Genetic diversity of three lgt loci for biosynthesis of lipooligosaccharide (LOS) in Neisseria species.** *Microbiology* 2002, **148(Pt 6):**1833-1844.
17. Maiden MC, Malorny B, Achtman M: **A global gene pool in the neisseriae.** *Mol Microbiol* 1996, **21(6):**1297-1298.
18. Morelli G, Malorny B, Muller K, Seiler A, Wang JF, del Valle J, Achtman M: **Clonal descent and microevolution of Neisseria meningitidis during 30 years of epidemic spread.** *Mol Microbiol* 1997, **25(6):**1047-1064.
19. Feil E, Zhou J, Maynard Smith J, Spratt BG: **A comparison of the nucleotide sequences of the adk and recA genes of pathogenic and commensal Neisseria species: evidence for extensive interspecies recombination within adk.** *J Mol Evol* 1996, **43(6):**631-640.
20. Claus H, Frosch M, Vogel U: **Identification of a hotspot for transformation of Neisseria meningitidis by shuttle mutagenesis using signature-tagged transposons.** *Mol Gen Genet* 1998, **259(4):**363-371.
21. Liu SV, Saunders NJ, Jeffries A, Rest RF: **Genome analysis and strain comparison of correia repeats and correia repeat-enclosed elements in pathogenic Neisseria.** *J Bacteriol* 2002, **184(22):**6163-6173.
22. Mazzone M, De Gregorio E, Lavitola A, Pagliarulo C, Alifano P, Di Nocera PP: **Whole-genome organization and functional properties of miniature DNA insertion sequences conserved in pathogenic Neisseriae.** *Gene* 2001, **278(1-2):**211-222.
23. De Gregorio E, Abrescia C, Carlomagno MS, Di Nocera PP: **Asymmetrical distribution of Neisseria miniature insertion sequence DNA repeats among pathogenic and nonpathogenic Neisseria strains.** *Infect Immun* 2003, **71(7):**4217-4221.
24. Smith HO, Gwinn ML, Salzberg SL: **DNA uptake signal sequences in naturally transformable bacteria.** *Res Microbiol* 1999, **150(9-10):**603-616.
25. Guibourdenche M, Popoff MY, Riou JY: **Deoxyribonucleic acid relatedness among Neisseria gonorrhoeae, N. meningitidis, N. lactamica, N. cinerea and "Neisseria polysaccharea".** *Ann Inst Pasteur Microbiol* 1986, **137B(2):**177-185.
26. Rossau R, van den Bussche G, Segers P, Grosch H, Göthe E, Mannheim W, de Ley J: **Ribosomal ribonucleic acid cistron similarities and deoxyribonucleic acid homologies of Neisseria, Kingella, Eikenella, Simonsiella, Alysiella, and Centers for Disease Control groups EF-4 and M-5 in the emended family Neisseriaceae.** *Int J Syst Bacteriol* 1989, **39:**185-198.
27. Barrett SJ, Sneath PH: **A numerical phenotypic taxonomic study of the genus Neisseria.** *Microbiology* 1994, **140 (Pt 10):**2867-2891.
28. Dougherty TJ, Asmus A, Tomasz A: **Specificity of DNA uptake in genetic transformation of gonococci.** *Biochem Biophys Res Commun* 1979, **86(1):**97-104.
29. Roberts M, Piot P, Falkow S: **The ecology of gonococcal plasmids.** *J Gen Microbiol* 1979, **114(2):**491-494.

30. Graves JF, Biswas GD, Sparling PF: **Sequence-specific DNA uptake in transformation of Neisseria gonorrhoeae.** *J Bacteriol* 1982, **152(3):**1071-1077.
31. Mathis LS, Scocca JJ: **Haemophilus influenzae and Neisseria gonorrhoeae recognize different specificity determinants in the DNA uptake step of genetic transformation.** *J Gen Microbiol* 1982, **128(5):**1159-1161.
32. Burnstein KL, Dyer DW, Sparling PF: **Preferential uptake of restriction fragments from a gonococcal cryptic plasmid by competent Neisseria gonorrhoeae.** *J Gen Microbiol* 1988, **134(3):**547-557.
33. Bakkali M, Chen TY, Lee HC, Redfield RJ: **Evolutionary stability of DNA uptake signal sequences in the Pasteurellaceae.** *Proc Natl Acad Sci U S A* 2004, **101(13):**4513-4518.
34. Davidsen T, Rodland EA, Lagesen K, Seeberg E, Rognes T, Tonjum T: **Biased distribution of DNA uptake sequences towards genome maintenance genes.** *Nucleic Acids Res* 2004, **32(3):**1050-1058.
35. Salih MA, Danielsson D, Backman A, Caugant DA, Achtman M, Olcen P: **Characterization of epidemic and nonepidemic Neisseria meningitidis serogroup A strains from Sudan and Sweden.** *J Clin Microbiol* 1990, **28(8):**1711-1719.
36. Kristiansen BE, Radstrom P, Jenkins A, Ask E, Facinelli B, Skold O: **Cloning and characterization of a DNA fragment that confers sulfonamide resistance in a serogroup B, serotype 15 strain of Neisseria meningitidis.** *Antimicrob Agents Chemother* 1990, **34(11):**2277-2279.
37. Radstrom P, Fermer C, Kristiansen BE, Jenkins A, Skold O, Swedberg G: **Transformational exchanges in the dihydropteroate synthase gene of Neisseria meningitidis: a novel mechanism for acquisition of sulfonamide resistance.** *J Bacteriol* 1992, **174(20):**6386-6393.
38. Kristiansen BE, Fermer C, Jenkins A, Ask E, Swedberg G, Skold O: **PCR amplicon restriction endonuclease analysis of the chromosomal dhps gene of Neisseria meningitidis: a method for studying spread of the disease-causing strain in contacts of patients with meningococcal disease.** *J Clin Microbiol* 1995, **33(5):**1174-1179.
39. Kneidinger B, Graninger M, Messner P: **Chromosome walking by cloning of distinct PCR fragments.** *Biotechniques* 2001, **30(2):**248-249.
40. Gunn JS, Stein DC: **Use of a non-selective transformation technique to construct a multiply restriction/modification-deficient mutant of Neisseria gonorrhoeae.** *Mol Gen Genet* 1996, **251(5):**509-517.
41. Stothard P: **The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences.** *Biotechniques* 2000, **28(6):**1102, 1104.
42. Qvarnstrom Y, Swedberg G: **Additive effects of a two-amino-acid insertion and a single-amino-acid substitution in dihydropteroate synthase for the development of sulphonamide-resistant Neisseria meningitidis.** *Microbiology* 2000, **146 (Pt 5):**1151-1156.