Conjugative Plasmids of Neisseria gonorrhoeae

Emilia Pachulec1,2, Chris van der Does1,2,3

1 Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Haren, The Netherlands, 2 Zernike Institute for Advanced Materials, University of Groningen, Haren, The Netherlands, 3 Department of Ecophysiology, Max Planck-Institute for Terrestrial Microbiology, Marburg, Germany

Abstract

Many clinical isolates of the human pathogen Neisseria gonorrhoeae contain conjugative plasmids. The host range of these plasmids is limited to Neisseria species, but presence of a tetracycline (tetM) determinant inserted in several of these plasmids is an important cause of the rapid spread of tetracycline resistance. Previously plasmids with different backbones (Dutch and American type backbones) and with and without different tetM determinants (Dutch and American type tetM determinants) have been identified. Within the isolates tested, all plasmids with American or Dutch type tetM determinants contained a Dutch type plasmid backbone. This demonstrated that tetM determinants should not be used to differentiate between conjugal plasmid backbones. The nucleotide sequences of conjugative plasmids with Dutch type plasmid backbones either not containing the tetM determinant (pEP5233) or containing Dutch (pEP5289) or American (pEP5050) type tetM determinants were determined. Analysis of the backbone sequences showed that they belong to a novel IncP1 subfamily divergent from the IncP1a, β, γ, δ and ε subfamilies. The tetM determinants were inserted in a genetic load region found in all these plasmids. Insertion was accompanied by the insertion of a gene with an unknown function, and rearrangement of a toxin/antitoxin gene cluster. The genetic load region contains two toxin/antitoxins of the Zeta/Epsilon toxin/antitoxin family previously only found in Gram positive organisms and the virulence associated protein D of the VapD/VapX toxin/antitoxin family. Remarkably, presence of VapX of pJD1, a small cryptic neisserial plasmid, in the acceptor strain strongly increased the conjugation efficiency, suggesting that it functions as an antitoxin for the conjugative plasmid. The presence of the toxin and antitoxin on different plasmids might explain why the host range of this IncP1 plasmid is limited to Neisseria species. The isolated plasmids conjugated efficiently between N. gonorrhoeae strains, but did not enhance transfer of a genetic marker.

Introduction

The obligate human pathogen Neisseria gonorrhoeae colonizes mucosal tissues in the urogenital tracts to cause the sexually transmitted disease gonorrhea [1]. Although gonorrhea can still be treated with antibiotics it has progressively accumulated resistance against many antibiotics like e.g. penicillin, ciprofloxacin, tetracycline, azithromycin and cefixime, and currently not many new antibiotics are available [2]. The rapid spread of antibiotic resistance is caused by the high rate of horizontal gene transfer in N. gonorrhoeae, resulting in a panmictic population structure [3]. Horizontal gene transfer in N. gonorrhoeae is driven by its high rates of natural transformation and recombination. DNA for horizontal gene transfer is most likely derived from lysis, but transfer frequencies are approximately 500 fold increased in strains which secrete DNA via the Type IV secretion system found within the recently characterized Gonococcal Genetic Island (GGI) [4]. Horizontal gene transfer also occurs via conjugative plasmids, which can not only transfer their own DNA, but often can also mobilize chromosomal or plasmid DNA. Currently three types of gonococcal conjugative plasmids have been described in N.gonorrhoeae; a 24.5 MDa plasmid with no detectable marker, and two 25.2 MDa plasmids which contain the tetM determinant [3]. The 24.5 MDa plasmid (also called pLE2451) was first found in 1974 in the United States in clinical isolates of non-penicillinase and penicillinase producing N. gonorrhoeae [6,7,8]. The host range of the 24.5 MDa plasmid is limited to N. gonorrhoeae and to some strains of Neisseria cinerea [9]. The 24.5 MDa plasmid was shown not to be involved in the mobilization of genomic DNA. In 1982, 25.2 MDa conjugative plasmids containing tetM determinants were identified in clinical isolates from the United States [10]. TetM determinants are transposon-borne determinants found in many organisms like e.g., Streptococcus [11], Mycoplasma hominis [12], Ureaplasma urealyticum [13], Enterococcus spp [14] and Gardnerella vaginalis [5,15] and are responsible for high levels of tetracycline resistance. The tetM determinants within the 25.2 MDa plasmids are derived from a so-called class II Tn916-like transposon insertion which means that large parts of the Tn916-like transposon are deleted but that the tetM determinant is maintained [16]. Nowadays, gonococcal isolates resistant to high doses of tetracycline (MIC>8 µg/ml) carrying 25.2 MDa plasmids have been isolated worldwide [5,17,18,19]. Restriction endonuclease mapping and Southern blotting of conjugative plasmids from different isolates revealed two different 25.2 MDa conjugative plasmids [18], which were named the “American” and “Dutch” type plasmids [20]. The restriction map of the Dutch type plasmid strongly resembled the restriction map of the 24.5 MDa conjugative plasmid and it was proposed that the Dutch type 25.2 MDa plasmid is a derivative of the 24.5 MDa plasmid by an insertion of the tetM determinant [3,21]. Early studies proposed
that the American type plasmid might be similar to both the conjugal and the Dutch type plasmid in areas of conserved functions like replication, incompatibility and transfer function [10], but restriction endonuclease mapping demonstrated large differences between American and Dutch type plasmids [22]. Sequencing of the tetM regions of American and Dutch type plasmids also revealed differences within the two tetM determinants, and it was proposed that the tetM determinant found in the American type conjugal plasmids has a different origin from the tetM determinant present in the Dutch type conjugal plasmids [23]. In a different study 13 American tetM determinants were linked to the restriction maps of American type conjugal plasmids [20]. Based on the different tetM sequences PCR primers were developed which could differentiate between the 2 different tetM determinants. The tetM determinant has been generally linked to the conjugal plasmid type, although some tetM determinants have also been identified in plasmids with restriction maps that differ from the initially identified American and Dutch types, indicating that either the American and Dutch type plasmids are still evolving or that the different tetM determinants have been inserted into different families of conjugal plasmids [20]. Unfortunately, it is unclear in many of the studies whether Dutch or American type plasmids were used, and whether differences would be observed between these two types of plasmids.

25.2 MDa plasmids showed a broader host range then the 24.5 MDa plasmid and could be transferred to other Neisseria species like Neisseria cinerea, Neisseria meningitidis, Neisseria mucosa, Neisseria flavescens, Neisseria subflava, and Neisseria perflava [17]. Plasmids carrying the tetM determinant were also identified in tetracycline-resistant Neisseria meningitidis, Kingella denitrifans and Eikenella corrodens while Neisseria subflava biovar perflava, Neisseria sicca and Neisseria mucosa isolates carried the tetM determinant in the chromosome [24]. The 25.2 MDA conjugal plasmid can also be transferred to Escherichia coli, but can not be maintained in this species [25].

The conjugal plasmids were also shown to be involved in the mobilization of small non-selftransmissible β-lactamase gonococcal plasmids [26]. Mobilization by the conjugal plasmids was observed after short mating to other Neisseria species, to Haemophilus influenzae and to restriction-deficient Escherichia coli [27]. Mobilization occurs either via the oriT-binding Moba mobilization protein [28] or with lower efficiency via co-integration with the conjugal plasmid [25]. Interestingly some plasmids can only be mobilized by a 25.2 MDA conjugal plasmid and not by the 24.5 MDA plasmid [29].

These gonococcal conjugal plasmids have been studied for many years, and in this paper we demonstrate that within our isolates both Dutch and American type tetM determinants are found in plasmids with a Dutch type backbone. The sequence of the Dutch type backbone, and the sequences of the genetic load regions in which the Dutch and American type tetM determinants were inserted were determined. Based on these obtained sequences the plasmids were further characterized, and their involvement in DNA transport was compared to the type IV DNA secretion system encoded within the Gonococcal Genetic Island.

Results

Isolation and sequencing of a conjugal plasmid with a Dutch type tetM derminant

We set-out to study the conjugal plasmids of N. gonorrhoeae. A collection of 60 strains from low-passage clinical isolates was obtained from the Municipal Health Service, GG&GD in Amsterdam, The Netherlands. The strains were obtained from the proctum, the cervix, the urethra, and the tonsil. This collection was a selection of strains resistant to the fluoroquinolones: ciprofloxacin and oxacillin, tetracycline, penicillin, and strains resistant to all these antibiotics (see table 1). These strains were tested for the presence of the Dutch and American type tetM determinants by two different PCR amplification methods described previously [30,31]. The results for both PCR methods agreed for all samples. Within 42 isolates tested 6 contained the American type tetM determinant and 6 contained the Dutch type tetM determinant. As expected, the tetM determinants were only detected in the strains with resistance above 1 µg/ml tetracycline. Neisseria strain 5289 carrying the Dutch type tetM determinant was chosen for plasmid isolation. Analysis of the isolated plasmid by gel electrophoresis identified products running at ~40 kb, ~10 kb and ~4 kb. Both the 40 kb and 10 kb products hybridized with a Dutch type tetM probe after Southern blotting, demonstrating that they are two differently coiled forms of the plasmid. The product at ~4 kb was pJD1, a 4.207-bp cryptic plasmid, found in most N. gonorrhoeae strains [32]. To confirm that the isolated plasmid was the Dutch type plasmid, digestion with BglII was performed and the obtained digestion pattern was similar to the previously observed pattern for a Dutch type plasmid [20]. To test the efficiency of transfer of the plasmid of strain 5289, the plasmid was first conjugated to our laboratory strain MS11, and overnight matings were performed in liquid medium and on filters. The mating efficiency was high (10^7 transconjugants/donor) and liquid mating was as efficient as surface mating. The plasmid was sequenced via the shotgun method with at least 6 times coverage followed by gap closure based on PCR amplification. Complete sequencing of the plasmid (pEP3289) resulted in a sequence of 42,004 bp. The plasmid has a G+C content of 48%, compared to 52.7% for the N. gonorrhoeae chromosome and 44% for the horizontally acquired Gonococcal Genetic Island. The sequence was identical to contig NZ_ABZP01000196 of N. gonorrhoeae strain SK-92-67 obtained during the recent sequencing of 15 different N. gonorrhoeae strains. The plasmid contained 3 DNA Uptake Sequences (DUS) which is a 14-fold lower frequency then observed for the N. gonorrhoeae chromosome. Manual annotation revealed 48 open reading frames (see table 2). Analysis of the annotated open reading frames, revealed a modular structure typical for the IncP1 plasmids. The plasmid is organized in modules for replication initiation (Rep), conjugal DNA-transfer (Tra), mating-pair-formation (Tyr), stable plasmid inheritance and control (Cig) (see figure 1). Furthermore, the plasmid contains a ‘genetic load’ module containing several genes inserted between the Tyr and Tra regions.

Analysis of the sequence of conjugal plasmids with the American tetM determinant

Since the restriction patterns of the Dutch and American type conjugal plasmids showed some overlap [18], we tried to detect the presence of the different regions of the Dutch type plasmid in the isolates containing the American type tetM determinant. Region specific PCRs targeting the conjugal DNA-transfer (traE, traD and traI), mating-pair-formation (tbb, tbbC, tbbD, tbbE and tbbF), replication (tgfA and sgb), stable plasmid inheritance and control (korC, traN, traO) regions and the regions used for gap closing of the initial sequences gave PCR fragments for all 6 isolates containing the American type tetracycline plasmid. Sequencing of the above regions revealed that these regions were 100% similar with the sequences determined for the plasmid with the Dutch tetM determinant. Isolation and restriction with BglI of the plasmids with the American type tetM determinant confirmed that these plasmids indeed contained the plasmid backbone of
Dutch type conjugative with the American type tetM determinant integrated (data not shown). The region from the zeta_1 gene to the ngorSK11390 gene of a plasmid (pEP5050) with an American type tetM determinant and a Dutch type plasmid backbone isolated from clinical isolate 5050 was amplified and sequenced. Sequencing of this region revealed that the only difference between the two plasmids is the presence of a region with homology to the transposon Tn916 in the plasmid with the American type tetM determinant (see figure 2). This difference was reported previously for plasmids with the American type tetM determinant in an American type conjugative plasmid [16].

Table 1. Characteristics of clinical isolates used in this study (S-sensitive).

| Isolate nr | Material source | Ciprofloxacin MIC μg/ml | Tetracyclin MIC μg/ml | Penicillin MIC μg/ml | GGI presence | type of plasmid |
|------------|----------------|------------------------|----------------------|----------------------|--------------|----------------|
| 4314       | urethra        | 2                      | S                    | S                    | --           | Conjugative    |
| 4465       | proctum        | 4                      | S                    | S                    | +            | Conjugative    |
| 4485       | urethra        | 3                      | S                    | S                    | --           | Conjugative    |
| 4511       | proctum        | 3                      | S                    | S                    | --           | Conjugative    |
| 4518       | urethra        | 0.5                    | S                    | 0.75                 | +            | Conjugative    |
| 4603       | proctum        | 6                      | S                    | S                    | --           | Conjugative    |
| 4890       | urethra        | 1.5                    | S                    | S                    | --           | Conjugative    |
| 4892       | urethra        | 1                      | S                    | S                    | --           | Conjugative    |
| 4894       | proctum        | 0.75                   | S                    | S                    | --           | Conjugative    |
| 5308       | urethra        | 8                      | S                    | S                    | --           | Conjugative    |
| 4393       | proctum        | 5                      | 1                    | 2                    | --           | American       |
| 4703       | cervix         | S                      | 6                    | >32                  | +            | Dutch          |
| 4731       | urethra        | S                      | 0.38                 | S                    | +            | Not detected   |
| 5050       | urethra        | S                      | 64                   | S                    | --           | American       |
| 5135       | proctum        | S                      | 16                   | S                    | +            | American       |
| 5289       | urethra        | S                      | 24                   | S                    | +            | Dutch          |
| 5291       | cervix         | S                      | >256                 | S                    | --           | American       |
| 5351       | urethra        | S                      | 24                   | S                    | +            | Dutch          |
| 5371       | urethra        | S                      | 16                   | >32                  | --           | Dutch          |
| 4290       | urethra        | S                      | 8                    | 0.125                | +            | American       |
| 4415       | tonsil         | S                      | S                    | 3                    | --           | Conjugative    |
| 4458       | cervix         | S                      | S                    | 12                   | +            | Conjugative    |
| 4547       | urethra        | S                      | 24                   | S                    | +            | Dutch          |
| 4635       | urethra        | S                      | 2                    | >32                  | --           | Conjugative    |
| 4722       | urethra        | S                      | S                    | 2                    | +            | Not detected   |
| 4993       | urethra        | S                      | 2                    | 0.19                 | +            | Conjugative    |
| 5224       | urethra        | S                      | 16                   | 24                   | +            | American       |
| 5233       | urethra        | S                      | S                    | 6                    | +            | Conjugative    |
| 5259       | proctum        | S                      | S                    | 0.75                 | +            | Conjugative    |
| 5343       | urethra        | S                      | 8                    | 24                   | +            | Dutch          |
| 4305       | proctum        | S                      | S                    | S                    | +            | Not detected   |
| 5312       | proctum        | S                      | S                    | S                    | +            | Not detected   |
| 4392       | tonsil         | S                      | S                    | S                    | +            | Not detected   |
| 4308       | urethra        | S                      | S                    | S                    | +            | Not detected   |
| 4745       | urethra        | S                      | S                    | S                    | +            | Not detected   |
| 4818       | tonsil         | S                      | S                    | S                    | +            | Conjugative    |
| 5020       | urethra        | S                      | S                    | S                    | --           | Not detected   |
| 5065       | proctum        | S                      | S                    | S                    | +            | Not detected   |
| 5066       | urethra        | S                      | S                    | S                    | +            | Conjugative    |
| 5067       | cervix         | S                      | S                    | S                    | +            | Not detected   |
| 5284       | urethra        | S                      | S                    | S                    | +            | Not detected   |
| 5375       | urethra        | S                      | S                    | S                    | +            | Not detected   |

doi:10.1371/journal.pone.0009962.t001
| gene  | length (bp) | homologue of putative protein | identity/range (aa) | function of homologue |
|-------|-------------|------------------------------|----------------------|------------------------|
| trbA  | 366         | TrbA of *Pseudomonas aeruginosa* | 50%/116              | transcriptional repressor |
| trbB  | 999         | TrbB of *Legionella pneumophila* str. Corby | 55%/292              | VirB11 like conjugal transfer ATPase (VirB11) |
| trbC  | 384         | TrbC of plasmid pB3           | 72%/112              | Prepilin (putatively circularized by TraF) |
| trbD  | 318         | TrbD of *Xylella fastidiosa*  | 59%/103              | conjugal transfer |
| trbE  | 2565        | TrbE of Birmingham IncP-alpha plasmid | 60%/853              | VirB4-like conjugal transfer ATPase |
| trbF  | 708         | TrbF of *Azoarcus sp. EbN1*   | 55%/230              | DNA transfer |
| trbG  | 867         | TrbG of *Azoarcus sp. EbN1*   | 53%/275              | VirB9-like Core complex component |
| trbH  | 411         | TrbH of IncP-1 plasmid pJK5   | 33%/151              | VirB7-like Core complex component |
| trbI  | 1440        | TrbI of *Acidovorax sp. JS42* | 46%/477              | VirB10-like Core complex component |
| trbJ  | 777         | TrbJ of Bordetella pertussis  | 48%/226              | VirB5 |
| trbK  | 141         |                              |                      |                        |
| trbL  | 1629        | TrbL of *Azoarcus sp. EbN1*   | 46%/215              | VirB6-like inner membrane protein |
| trbM  | 579         | TrbM of *Bordetella pertussis*| 48%/154              | conjugal transfer |
| trbN  | 600         | TrbN of *Sphingomonas* sp. A1 | 49%/207              | Lytic transglycosylase |
| vapD  | 333         | VapD of *Haemophilus somnus*  | 73%/84               | Virulence-associated protein D |
| ResA  |             | resolvase of *Azotobacter vinelandii* DJ | 56%/183              | Resolvase |
| MarR  | 432         | ESA_01699 of *Enterobacter sakazakii* ATCC BAA-894 | 28%/27              | transcriptional regulator, MarR family |
| Zeta-1| 1206        | CAMGR0001_1552 of *Campylobacter gracilis* RM3268 | 50%/280          | Zeta toxin |
| Epsilon-1| 186       |                              |                      | Epsilon antitoxin |
| Zeta-2| 1209        |                              |                      | Zeta toxin |
| Epsilon-2| 255       | Bcen2424_6818 of Burkholderia cenocepacia | 30%/69              | Epsilon antitoxin |
| tetM  | 1935        | TetM of *Streptococcus pneumoniae* | 98%/644               | Tetracycline resistance |
| 11375 | 522         | GCWU000324_02316 of *Kinecella oralis* ATCC 51147 | 40%/77              | Hypothetical |
| 11390 | 474         | Bcen2424_6818 of Burkholderia cenocepacia | 29%/72              | Epsilon antitoxin? |
| 11395 | 897         | YplP275_pipT202_0130 of *Yersinia pestis* biovar Orientalis | 63%/297 | DNA modification methylase |
| 11400 | 342         | NmuCA2_00935 of *Neisseria mucosa* ATCC 25996 | 36%/103              | Hypothetical |
| 11405 | 369         | HMPREF0530_1319 of *Lactobacillus paracasei* | 36%/100              | Hypothetical |
| 11410 | 360         | NgonSK11410 of *Neisseria gonorrhoeae* SK-92-679 | 100%/119             | Hypothetical |
| yegA  | 558         | YegA of *Neisseria gonorrhoeae* NCCP11945 | 40%/163             | Conserved hypothetical with TAT signal sequence and DUF88 domain |
| 11420 | 471         | DR_0894 of *Deinococcus radiodurans* R1 | 28%/88              | Transcription elongation factor |
| traC  | 3000        | TraC of IncP-1 plasmid pKJK5 | 46%/1032             | DNA primase |
| traD  | 165         | BACUNI_04470 of *Bacteroides uniformis* ATCC | 56%/25              | Conjugal transfer |
| traE  | 2196        | TraE of Birmingham IncP-alpha plasmid | 58%/734             | DNA topoisomerase III family |
| traF  | 513         | TraF of Bordetella pertussis  | 40%/166              | Conjugal preplin peptidase |
| traG  | 1890        | TraG of Bordetella pertussis  | 69%/621              | Conjugal coupling protein |
| traI  | 2394        | TraI of plasmid pB3           | 34%/809              | DNA relaxase |
| traJ  | 378         | TraJ of plasmid pKH54        | 45%/109              | oriT-binding protein |
| traK  | 420         | XfasM23_2251 of *Xylella fastidiosa* | 30%/113             | DNA transfer |
| traL  | 726         | TraL of *Xylella fastidiosa* Dixon | 48%/241              | Conjugal transfer |
| traM  | 483         | TraM of *Legionella drancourtii* LLAP12 | 22%/110             | DNA transfer |
| kfcC  | 537         | KFC of *Pseudomonas aeruginosa* | 48%/148             | Plasmid partitioning |
| krbC  | 357         | KraC of Bordetella pertussis  | 41%/86               | Plasmid partitioning |
| korB  | 918         | ParB of *Xylella fastidiosa* Dixon | 48%/327             | Plasmid partitioning DNA binding protein |
| incC  | 756         | IncC2 of Birmingham IncP-alpha plasmid | 54%/257            | Plasmid partitioning ATP binding protein |
| kleE  | 246         | KleE of Bordetella pertussis  | 28%/76               | Plasmid partitioning |
| korC  | 348         | KorC of Bordetella pertussis  | 36%/75               | Plasmid partitioning, regulator |
| trfA  | 879         | TrfA of *Nitrosomonas eutropha* C91 | 47%/265             | Plasmid replication, oriV activator |
| ssb   | 384         | Ssb of *Xylella fastidiosa* M23 | 37%/115             | Single-strand DNA binding protein |
present in the Dutch type plasmid, while the other genes were 100% identical (see Figure S1). Dutch type conjugative plasmids with an American type tetM determinant have been observed before at low frequency [33], but our results show that in our Dutch isolates, they occur with a very high frequency. Important-
ly, this shows that the tetM determinant is not a good marker to
differentiate between conjugative plasmids with American and
Dutch type backbones.

Plasmids with tetM determinants are derived from the
24.5 MDa conjugative plasmid
Since it was proposed that the 25.2 MDa plasmid with a Dutch
type backbone is a derivative of the 24.5 MDa conjugative
plasmid by an insertion of the Dutch type tetM determinant [5,21],
it was tried to detect the presence of the different regions of the
Dutch type plasmid described above in the isolates which were not
positive for the presence of the Dutch and American
tetM determinants. Remarkably, in 19/30 isolates the presence of the
different regions could be detected, showing that 31/42 of our
isolates contain a conjugative plasmid with a Dutch type backbone
(see table 1). Again, sequencing of the above regions revealed that
these regions were 100% similar with the sequences determined
for the plasmid with the Dutch
tetM determinant. This
demonstrated that the conjugative plasmids with Dutch and
American type tetM determinants are derived from the 24.5 MDa
conjugative plasmid by insertion of the tetM determinant.

When the antibiotic resistance spectrum of the different clinical
isolates was compared with the presence or absence of the
conjugative plasmids, it was remarkable that all strains with
resistance against the fluoroquinolones ciprofloxacin and oxacillin
contained a conjugative plasmid. Resistance to fluoroquinolones is
generally a result of point mutations in the DNA gyrase gyrA or the
topoisoenzyme IV parC genes [34]. However, there is no clear
indication in the plasmid sequence that would explain a

correlation between presence of the conjugal plasmid and the
resistance to fluoroquinolones.

To determine the exact position of the insertion of the tetM
determinants in the conjugative plasmid, the region between the
zeta_1 and the ngoSK11390 gene of plasmid pEP5233, a
conjugative plasmid with a Dutch type backbone isolated from
clinical isolate 5233 was amplified and sequenced. Alignment of
the sequences (see Figure S1) showed that sequences outside the
epsilon_1 and the ngoSK11390 genes are conserved between this
plasmid (pEP5233) and the plasmids with the
tetM determinant (pEP5289 and pEP5050) but that differences are found in the
region between these genes (see figure 2). Conservation of 300 and
109 bp long regions in all three plasmids suggests that three
insertions/rearrangements have taken place: I) insertion of the
tetM determinant (with or without the Tn916 transposon), and II)
insertion of the ngoSK11375 gene and III) a rearrangement/
mutagenesis of the zeta_2/epsilon_2 genes to the zeta_3/epsilon_3 genes. A more detailed analysis of the genetic load regions of these
plasmids will be given below.

The conjugative plasmids with the Dutch type backbone
are phylogenetically divergent from the other IncP1 sub-
families
The modular arrangement of the conjugative plasmid and
derivates thereof, the absence of several genes often found in the
IncP1-α subfamily, and an insertion of an additional module suggested that these plasmids are members of the IncP1-β subfamily. To verify placement of the plasmids in the IncP1-β subfamily, a phylogenetic analysis was performed on the TraI and TraG proteins encoded in the Tra region, the TrbE and TrbC proteins encoded in the Trb region, and the TrfA and KorB proteins encoded within the Rep and Ctl regions. Neighboring tree containing representative members of the different IncP1 plasmid subfamilies (α, RP4; β, pADP-1; γ, QKH54; δ, pEST4011 and ε, pKJK5) and the neisserial conjugative plasmids with the Dutch type backbone were created (See figure 3). Remarkably this showed that these neisserial conjugative plasmids fall within the IncP1 family, but are phylogenetically divergent from the other IncP1 sub-families, and are members of a new subfamily.

Analysis of the open reading frames of the IncP backbone

The conjugative DNA-transfer (Tra) region contains the transfer proteins, encoded in two oppositely transcribed traCDEF-GHIJ and trkLM operons, and is similar to the other IncP-1 family plasmids. The origin of transfer (oriT), including the nic site is in plasmids of the IncP family located between these two operons [35]. This region also contains an inverted repeat sequence, which is the binding sequence for TraJ. The sequence in this region of the Neisserial conjugative plasmids differs significantly from the sequences observed in other IncP1 subfamilies. A putative nic site and an inverted repeat sequence are present, but the sequence of the inverted repeat to which TraJ putatively binds seems not to be conserved (See figure 4). The proteins encoded within the mating-pair-formation region, TrbA-TrbN, are similar to the proteins of the other IncP1-α, β, γ and ε plasmids. In pEST4011, a member of the IncP1-δ subfamily most of the Trb region (the C-terminal half of TrbE and the TrbF to TrbN proteins) is missing, but the TrbA-TrbD proteins are similar to the pTetM proteins. A difference with the other IncP1 subfamilies is found in TrbK. The traK gene is the only non essential gene in the tra operon, and normally encodes a small lipoprotein involved in entry exclusion [36]. The protein located between TrbJ and TrbL in the Neisserial conjugative plasmids is a small protein with a signal sequence followed by a cysteine residue for lipid anchoring, but has very little homology in its mature part to the known TrbK proteins. However, the genetic location of the protein, the small size and the presence of the lipoprotein signal sequence suggests that this protein also is an entry exclusion protein, and we have therefore also named it TrbK. Similar to representatives of the IncP1-β and IncP1-γ subfamilies, the TrbO and TrbP proteins present in the IncP1-α and IncP1-γ families are not found in neisserial conjugative plasmids. The origin of replication (oriV) is in IncP1 plasmids generally located next to the replication initiation region. However in many plasmids insertions occur in this region, separating the oriV and the replication initiation region. Similar to the other IncP1 plasmids, the Rep region of the neisserial conjugative plasmids contains the tfdA and tsh genes, which encode the oriV activator, and a single stranded DNA binding protein, respectively. No insertions have occurred between the Rep region and the oriV. The oriV region of IncP1 plasmids is approximately 400 bp long and consists of several direct repeats called iterons (17-mers) to which TrfA binds. Indeed the tfdA gene is followed by 8 5’-GCTATGGT-GTAATCCG-3’ repeats in the next 400 bp. Comparison with the iterons of the other IncP1 subfamilies shows that the TrfA binding motif differs significantly from the other TrfA binding motifs (see figure 5). The inheritance and control region is relatively small compared to the other IncP1 plasmids, and contains only 6 genes. The IncC and KorB proteins are the ParA ATPase, and the ParB DNA binding protein homologues of the active partitioning system. KorB of the RK2 plasmid recognizes a 13 bp inverted repeat sequences (5’-TTTAGCCCGCTAAA-3’), which was found 12 times on the RK2 plasmid [37]. This sequence is found 6 times on the neisserial conjugative plasmid, and 4 out of the 6 repeats were extended to 19 bp inverted repeats (5’-AATTTAGCCGGCTAAAT-3’). Remarkably the putative ParB site located in the genetic load region, shows homology over 52 bp (with 86% identity) with the putative ParB site between KorC and KlcE. Remarkably even though the genetic load region around this ParB site seems a spot for insertions of gene sequences, the ParB binding site in the genetic load region was conserved in all three conjugative plasmids, suggesting an important function for this region. The control region further contains the accessory stability components KfrB, KfrC, and KlcE, and the KorC regulator. This region does not contain a post-segregational killing system, but a remarkable combination of such systems was found in the genetic load region.

Analysis of the genetic load region

The genetic load region of the conjugative plasmid contains 14 open reading frames, putatively located in three larger operons, and two single genes (ngoSK11390 and vapD). The plasmids with a tetM determinant contain next to the tetM determinant a second inserted region which contains the ngoSK11375 gene. The ngoSK11375 gene encodes a protein with an unknown function. The first putative operon consists of the ngoSK11395, ngoSK11400 and ngoSK11405 genes and contains the ngoSK11395 gene which

![Figure 2. Comparison of the genetic loads regions of the conjugative plasmid, and the plasmids with the American and Dutch tetM determinants.](Image 58x21 to 76x41)

**Neisserial Conjugative Plasmid**
has homology to DNA modification methylases and two hypothetical proteins. This operon is separated from the operon including the \( \text{yegA} \) and \( \text{ngonSK11420} \) genes by a large 76 bp inverted repeat which functions putatively as a terminator. The \( \text{yegA} \) gene encodes a conserved protein with a Twin Arginine Translocation (TAT) signal sequence and a DUF88 domain, while the \( \text{ngonSK11410} \) and \( \text{ngonSK11420} \) genes encode hypothetical proteins. A homolog of the \( \text{yegA} \) gene is found within the Gonococcal Genetic Island, but also the function of this gene is unknown. The \( \text{zeta}_1, \text{epsilon}_1, \text{zeta}_2 \) and \( \text{epsilon}_2, \text{marR} \) and \( \text{resA} \) genes also form a putative operon. In the conjugative plasmid the \( \text{zeta} \) and \( \text{epsilon} \) genes located at the positions of the \( \text{zeta}_2 \) and \( \text{epsilon}_3 \) genes in the tetM containing plasmids have obtained extended mutations without affecting the reading frame, and were named \( \text{zeta}_3 \) and \( \text{epsilon}_3 \). The \( \text{Zeta} \) and \( \text{Epsilon} \) proteins encode components of a toxin-antitoxin system which was found in plasmid maintenance systems of Gram positive organisms which use a post segregation killing mechanism. Normally, plasmids encoding \( \text{Zeta} \) and \( \text{Epsilon} \) toxin-antitoxin proteins are part of a three-component system which also contains a regulator (\( \text{omega} \)). The \( \text{marR} \) gene also encodes a regulator of the MarR family, but this regulator comes from a different family than the \( \text{omega} \) regulators. The specific mechanism of the \( \text{Zeta} \) toxin is still unknown, but overexpression of the \( \text{Zeta} \) toxin of plasmid \( \text{pSM19035} \) of \( \text{Streptococcus pyogenes} \) in \( \text{Bacillus subtilis} \) and \( \text{Escherichia coli} \) inhibited replication, transcription, and translation, and eventually lead to cell death [38]. This is the first \( \text{Zeta}/\text{Epsilon} \) toxin-antitoxin system identified in a Gram negative organism and the first \( \text{Zeta}/\text{Epsilon} \) toxin-antitoxin system in which two different copies of the toxin and of the antitoxin are located on one plasmid. Interestingly the genetic load region also contains the \( \text{vapD} \) gene that encodes the virulence associated protein \( \text{D} \). VapD was shown to function as a toxin and is generally counteracted by an antitoxin protein called VapX. VapD/VapX systems have been reported in pathogens like \( \text{Rhodococcus equi} \) [39], \( \text{Dichelobacter nodosus} \) [40], \( \text{Xylella fastidiosa} \) [41], and \( \text{Haemophilus influenzae} \) [42]. It has been shown that expression of VapD enhances the invasion and survival within both human epithelial and endothelial cells [42]. Remarkably, no open reading frame encoding a VapX antitoxin could be identified within the sequence of the conjugative plasmid. A VapD/VapX toxin-antitoxin system was previously identified on \( \text{pJD1} \), the cryptic plasmid which is present in 96% of the \( \text{N. gonorrhoea} \) strains [32]. It was tested whether the VapX antitoxin encoded on the cryptic plasmid \( \text{pJD1} \) of \( \text{N. gonorrhoea} \) might fulfill the role of antitoxin. Indeed when overnight conjugation experiments between \( \text{N. gonorrhoea} \) containing the conjugative plasmid with the Dutch type \( \text{tetM} \) determinant and \( \text{Escherichia coli} \) were performed, tetracycline resistance was transferred much more efficiently to \( \text{E.coli} \) strains which expressed the VapX antitoxin of \( \text{pJD1} (2 \times 10^2 \text{tetracycline resistant clones per recipient}) \) then to \( \text{E.coli} \) strains that did not contain the antitoxin (1 \times 10^1 \text{tetracycline resistant clones per recipient}). However, the conjugative plasmids could not be further maintained in \( \text{E.coli} \) for more then a few generations. The initial colonies did not grow in liquid medium, and could only be transferred once to new plates. This demonstrates that the conjugative plasmid can be conjugated to

---

**Figure 3. Phylogenetic analysis of genes of different IncP1 plasmids.** The TraI (A), TrbE (B), TraG (C) and TrfA (D) proteins of the neisserial conjugative plasmids and of representative members of different IncP1 plasmid subfamilies (a, RP4; b, pADP-1; c, QKH54; d, pEST4011 and e, pJK55) representative for different regions of the conjugative plasmids were used to create phylogenetic trees of the IncP1 plasmid family. (For details see materials and methods).

doi:10.1371/journal.pone.0009962.g003
E. coli, but that the plasmid can not be further maintained. This suggests that the VapX antitoxin of the cryptic pJD1 plasmid might function as antitoxin of the VapD protein encoded on the conjugative plasmid, and might explain why the host range of the IncP1 conjugative plasmids of *N. gonorrhoeae*, is limited to *N. gonorrhoeae* and other neisserial species. This further suggests that the limitation of the host range could have resulted in a loss of the ability of the plasmid to be maintained in other species.

Comparison with the type IV DNA secretion system encoded within the gonococcal genetic island

To examine whether there was a relation between the occurrence of the GGI and the conjugative plasmids, the presence of the GGI was determined for all strains. Within 27 of the 42 strains the presence of the GGI was detected. Conjugative plasmids were present in 14 of the 15 strains which do not contain the GGI and in 17 of 27 strains that contain the GGI. Thus the conjugative plasmids are present in strains which contain the GGI and in strains that do not contain the GGI. In a final step the involvement of the type IV DNA secretion systems encoded in the GGI and the conjugative plasmid in the spread of chromosomal markers was studied. Strain with and without the GGI, and with and without the conjugative plasmid were grown in the presence of acceptor strains with and without the GGI. In all donors strains the erythromycin marker was inserted within the *recA* gene to ensure one directional transfer, while the recipient strains contained the chloramphenicol marker. As was observed previously [4], the transfer of a chromosomal marker was increased 500-fold in donor strains that contained the type IV DNA secretion systems encoded in the GGI, whereas no influence was observed of the presence of the conjugative plasmids (see figure 6). This demonstrates that the type IV DNA secretion system encoded in the GGI is responsible for an increase in the spread of chromosomal markers, whereas no effects were observed for the conjugative plasmids.

Discussion

Conjugative plasmids of *Neisseria gonorrhoeae* were found in a large percentage of clinical isolates obtained from the Public Health Laboratory in Amsterdam (The Netherlands). These plasmids all contained a Dutch type backbone. Remarkably, within the clinical isolates tested, no plasmids with an American type backbone could be detected. Within these plasmids with a Dutch type backbone insertions of both Dutch and American *tetM* determinants were found. Next to the insertion of the different *tetM* determinants, the only difference between the strains with the *tetM* determinant (25.2 MDa plasmids) and strains without the *tetM* determinant (24.5 MDa plasmids) was the insertion of the *ngoSK11375* gene. Previously several differences have been observed between the 25.2 and 24.5 MDa plasmids, e.g. 25.2 MDa plasmids showed a broader host range [17] and some plasmids could only be mobilized by a 25.2 MDa conjugative plasmid [29]. It is difficult to explain these differences based on the presence of the *tetM* determinant or the *ngoSK11375* gene, and it might be possible that these results have been obtained with 25.2 MDa plasmids with an American type plasmid backbone.

The conjugative plasmids with the Dutch type backbone are members of a novel IncP1 subfamily which similarly to other IncP1 plasmids contains backbone modules for replication initiation, conjugative DNA-transfer, mating-pair-formation, stable plasmid inheritance and control. The genes encoded in the
backbone modules of the Dutch type neisserial conjugative plasmids are phylogenetically divergent from the other IncP1 subfamilies, but since they belong to the broad host range IncP1 family, it is most likely that they have been acquired from other organisms. The limited host range compared to the normally broad host range of these plasmids might have allowed these plasmids to diverge from the other sub families.

The Dutch type neisserial conjugative plasmids contain a "genetic load", module not found in other IncP1 plasmids. Remarkably three different putative toxin/antitoxin systems are located within this region. Two of the toxin/antitoxin systems belong to the Zeta/Epsilon toxin/antitoxin family. After insertion of the tetM determinant, several mutations occurred in one of the Zeta toxin/Epsilon antitoxin systems, generating mutated Zeta toxin and Epsilon antitoxin proteins. Zeta/Epsilon toxin/antitoxin systems have only been identified in Gram positive organisms [43,44,45] and always as a single copy. Although the structure of the Zeta/Epsilon toxin/antitoxin complex contained a phosphotransferase fold [46], the mechanism of the Zeta/Epsilon toxin/antitoxin system is still unknown. The functionality and the interactions between the different putative Zeta/Epsilon toxin/antitoxin systems encoded within the neisserial conjugative systems still need to be investigated.

The third toxin/antitoxin system identified on the neisserial conjugative plasmid belongs to the VapD family. The vapD gene has been previously identified in plasmids of Rhodococcus equi [39], Actinobacillus actinomycetemcomitans [47], Rieumerella anatipestifer [48] and N. gonorrhoeae [32], and on the chromosomes of Haemophilus influenzae [49] and Xylella fastidiosa [50]. In Dichelobacter nodosus, vapD was found both on the chromosome and on a plasmid [51]. The vapD gene has been associated with virulence in several of these facultative intracellular microorganisms [40,42,49,52] but its precise role is still unknown. In H influenzae the vapX gene was identified in the same operon as vapD. The VapX protein was shown to function as an antitoxin for VapD [42]. A homologue of the VapX protein was also identified next to the vapD gene on the cryptic pJD1 plasmid of N. gonorrhoeae [32]. Remarkably, the Dutch type conjugative plasmid of N. gonorrhoeae contains only the vapD gene. Since expression of the VapX antitoxin protein of pJD1 in E.coli acceptor cells strongly increased the transfer of the conjugative plasmid from N. gonorrhoeae to E.coli, we hypothesise that VapX protein located on pJD1 might function as an antitoxin for VapD on the conjugative plasmid. This arrangement most likely prevent the conjugative plasmid to transfer to other cells that do not contain the cryptic pJD1 plasmid, limiting the host range of these conjugative plasmids to the host range of the pJD1 cryptic plasmid. Currently no information is available about a possible role of the VapD proteins encoded on the pJD1 and the conjugative plasmid on conjugation.

Multiple toxin/antitoxin systems have been found in many genomes, and some genomes contain more than 50 putative toxin/antitoxin systems [53]. Many different functions have been proposed for the multiple chromosomal toxin/antitoxin systems [54]. Most plasmids however contain only a single toxin/antitoxin system, and the advantages of having three different toxin/antitoxin systems on one conjugative plasmid also remains unclear.

Figure 5. Comparison of the oriV iterons of different members of the IncP1 subfamilies. Weblogos were created to compare the 13 bp iterons of different members of the IncP1 subfamilies. The height of each base represents its conservation. doi:10.1371/journal.pone.0009962.g005

Figure 6. Transfer of chromosomal markers via the Type IV DNA secretion system is not influenced by the presence of the conjugative plasmid. Strains with the GGI (GGI+) or without the GGI (GGI-) and/or with (pEP5289+) or without (pEP5289-) the conjugative plasmid with the Dutch type tetM determinant and the Dutch type backbone were mixed, and grown at 37°C under 5% CO2. Donor strains contained the erythromycin marker in the recA gene, while recipient Neisseria strains contained the chloramphenicol marker. After 5 h of growth, serial dilutions were spread on selective media. Transfer frequencies were calculated as CFU of transconjugants per CFU of donor. doi:10.1371/journal.pone.0009962.g006


Materials and Methods

Bacterial strains and growth conditions

The genomic DNA of clinical isolates and gonococcal strains used in the study were obtained from the GG&GD Municipal Health Service, Public Health Laboratory in Amsterdam. The strains were characterized for their Minimal Inhibitory Concentrations for ciprofloxacin, tetracycline and penicillin as described in [55]. N. gonorrhoeae was grown on GCB (Difco) plates containing Kellogg’s supplements [56] or in GCBL liquid medium (GCBL) containing 0.042% NaHCO3 [57] and Kellogg’s supplements. For N. gonorrhoeae, tetracycline was used at 5 μg/ml, chloramphenicol was used at 10 μg/ml and erthyromycin at 10 μg/ml. For E. coli, tetracycline was used at 5 μg/ml, erthyromycin was used at 500 μg/ml and ampicillin at 100 μg/ml. For further N. gonorrhoeae and E. coli strains used in this study see table 3.

Isolation of chromosomal DNA

Bacterial isolates were typically harvested for DNA isolation from pure culture plates, taking up to five morphologically identical and single colonies depending on the size of the colonies [55]. If no separate colonies grew on the pure culture plate, N. gonorrhoeae colonies were harvested from a tertiary plate. Colonies were lysed in 100 μl of 5 M guanidine thiocyanate buffer (BioMérieux, the Netherlands) containing 0.04 mg/ml glycogen (Roche Diagnostics, the Netherlands), and stored at 4°C until DNA isolation was performed. Chromosomal DNA was extracted from lysates by addition of an equal volume (700 μl) chilled (−20°C) isopropanol, followed by centrifugation for 20 minutes at 14,000 rpm. The pellet was subsequently washed twice with 500 μl 70% ethanol. Precipitated total DNA was dissolved in 50 μl 10 mM Tris/HCl pH 8.0 and diluted 500 times in this buffer for most PCR reactions.

Alternatively, chromosomal DNA for PCR analysis was obtained by rapid cell lysis. Shortly, a smear of cells from overnight growth on GCB plates was resuspended in 25 μl of lysis solution (50 mM Tris/HCl pH 8.0, 20 mM EDTA, 50 mM NaCl). For each 25 μl PCR reaction 3 μl of the lysate was used. All sequencing reactions of PCR products were performed by Service XS (Leiden, the Netherlands).

Determination of the tetM determinant and the presence of the Gonococcal Genetic Island

The presence of the tetM determinant was detected via PCR analysis as previously described. Shortly, either primers Tet_4F (universal forward) and Tet_AR (American reverse), or Tet_4F and Tet_DR (Dutch reverse) were used to amplify the tetM determinants, giving PCR fragments of 778 bp and 443 bp for the American and Dutch type determinants, respectively [31]. (For primers used in this study see table 4). Alternatively, primers RM4 and G1 were used to amplify the tetM determinant giving PCR fragments of 1600 and 700 bp for the American and Dutch type determinants, respectively [30]. The presence of the Gonococcal Genetic Island was detected via PCR analysis using the primers GGI-21F and GGI-22R (369 bp) and primers GGI-27F and GGI-28R (453 bp), amplifying regions of traK and topB, respectively. PCR products were analyzed by electrophoresis in 1% w/v agarose and visualized after ethidium bromide staining.

Plasmid DNA isolation from N. gonorrhoeae

Neisseria gonorrhoeae clinical isolates 5050, 5233 and 5289 were chosen for isolation of their conjugative plasmids. The isolation was performed by a cleared lysate method followed by double acetate precipitation adapted from the Genome Sequencing Center, Washington University for N. gonorrhoeae as follows. Nopililated colonies of Neisseria gonorrhoeae were selected and transferred into 3 ml of GCBL medium GCBL liquid medium (GCBL) containing 0.042% NaHCO3 and Kellogg’s supplements with a final concentration of tetracycline (5 μg/ml) for plasmids containing the tetM determinant. After incubating for 3 hours at 37°C under 5% CO2 in a shaking incubator at 200 rpm, the culture was diluted to an OD600 of 0.2 in 6 tubes of 3 ml of GCBL containing 0.042% NaHCO3 and Kellogg’s supplements and growth was

Table 3. Strains used in this study.

| strain       | Genotype                                                                 | source or references |
|--------------|--------------------------------------------------------------------------|----------------------|
| E.coli TOP10F- | F- mcrA Δ(mrr-hsdRMS-mcrBC) q80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ− | Invitrogen           |
| MS511A       | N. gonorrhoeae strain                                                    | [62]                 |
| ND500        | MS511AΔGGI                                                              | [4]                  |
| S289         | low passage clinical isolate of Neisseria gonorrhoeae                  | GGD                  |
| S5050        | low passage clinical isolate of Neisseria gonorrhoeae                  | GGD                  |
| 4393         | low passage clinical isolate of Neisseria gonorrhoeae                  | GGD                  |
| 4703         | low passage clinical isolate of Neisseria gonorrhoeae                  | GGD                  |
| S571         | low passage clinical isolate of Neisseria gonorrhoeae                  | GGD                  |
| S291         | low passage clinical isolate of Neisseria gonorrhoeae                  | GGD                  |
| S233         | low passage clinical isolate of Neisseria gonorrhoeae                  | GGD                  |
| EP006        | MS511A ΔrecA::Erm                                                       | This study           |
| EP030        | ND500 ΔrecA::Erm                                                        | This study           |
| EP015        | MS511A lctP::Cm::caspC                                                  | This study           |
| EP029        | ND500 lctP::Cm::caspC                                                   | This study           |
| EP006/5289   | ΔrecA::Erm pTetM Dutch type                                             | This study           |
| EP030/5289   | ΔrecA::Erm pTetM American type                                          | This study           |

doi:10.1371/journal.pone.0009962.t003
Table 4. Plasmids used in this study.

| Plasmid | properties | source or references |
|---------|------------|---------------------|
| pTrc99A | *E. coli* expression vector (AmpR) | [63] |
| pEP086  | derivative of pTrc99A with mutated trc promoter | This study |
| pEP087  | derivative of pEP086 containing vspX | This study |
| pIND1   | *E. coli* insertion duplication mutagenesis vector for *N. gonorrhoeae*; contains ermC | [61] |
| pKH35   | *E. coli* complementation vector for *N. gonorrhoeae* (CmrR) | [4] |
| pEP013  | derivative of pIND1 carrying fragment of recA | This study |
| pEPS050 | *N. gonorrhoeae* conjugative plasmid with American type tetM determinant and Dutch type backbone isolated from clinical isolate 5050 | This study |
| pEPS233 | *N. gonorrhoeae* conjugative plasmid with Dutch type backbone isolated from clinical isolate 5233 | This study |
| pEPS289 | *N. gonorrhoeae* conjugative plasmid with Dutch type tetM determinant and Dutch type backbone isolated from clinical isolate 5289 | This study |

doi:10.1371/journal.pone.0009962.t004

continued for 4 h. Then, all 18 ml of the pre-culture was added to 1 liter of GCBL containing 0.042% NaHCO3 and Kellog’s supplements and incubated overnight at 37°C under 5% CO2 in a shaking incubator at 200 rpm. After harvesting the cells by centrifugation at 8,000 rpm in a JLA-16.25 rotor for 15 minutes, the cells were resuspended in 40 ml of 10 mM of EDTA, pH 8.0. After mixing gently, the solution was incubated at room temperature for 3 minutes. 80 ml of alkaline lysis solution (0.2 N NaOH and 1% SDS) was added and after gentle swirling until the solution became homogenous, it was incubated for 5 minutes at room temperature. Then, 60 ml cold, 3 M KOAc pH 5.5 was added and again mixed gently by swirling the bottle several times. Consecutively, the bottle was frozen at −20°C overnight. After thawing, the lysate was clarified from precipitated SDS, proteins, membranes, and chromosomal DNA by centrifugation at 10,000 rpm in a JLA-16.25 rotor for 15 minutes. Prior to re-centrifugation, the cleared supernatant was filtered to remove any floating material. Then, remaining insoluble material was removed by an additional centrifugation at 10,000 rpm in a JLA-16.25 rotor for 15 minutes. To the supernatant an equal volume of isopropanol was added and mixed by swirling. After centrifugation at 5,000 rpm in a JLA-16.25 rotor for 15 minutes, the supernatant was decanted and the pellet drained. The second volume of isopropanol was added and mixed by swirling. After centrifugation at 5,000 rpm in a JLA-16.25 rotor the pellet was washed with 30 ml of 70% ethanol and dried. Each pellet was resuspended in 1 ml of 10 mM Tris pH 8.0 and 0.1 mM EDTA and incubated at 37°C for 2 hours to ensure all the high molecular weight DNA was dissolved completely. The DNA was further stored at 4°C. Approximately 1.2 µg of plasmid DNA was obtained from 1 liter of *N. gonorrhoeae*. 15 ug of plasmid DNA obtained from 13 liters of *N. gonorrhoeae* clinical isolate 5289 was send to the Macrogen Corporation (Seoul, South Korea) to obtain the plasmid sequence via shotgun cloning and sequencing.

Southern blot and restriction analysis

Whole plasmid DNA was transferred to nitrocellulose membranes by the method of Southern [58] followed by the hybridization of DIG-labelled tetM. The tetM-specific probe (448 bp) was PCR-amplified using primers Tet_4F and Tet_DR with purified plasmid as a template. The Dutch-type plasmid has been verified by the restriction analysis as follows. 10 µg of the purified Dutch type plasmid was digested for 14 h with *BglII* (0.1 U/µg) (Fermentas) followed by electrophoresis in 1% w/v agarose and visualized after ethidium bromide staining.

DNA sequence analysis and annotations

The nucleotide sequence of the pEP5289 plasmid of *Neisseria gonorrhoeae* clinical isolate 5289 was determined via the shotgun method by Macrogen Inc. (Seoul), resulting in 7 contigs with at least 6 times coverage. Gap closure of the sequences was achieved by PCR amplification with the following primer combinations: trfA_F and sbb_R, trbC_F and trbC_R, korC_R and kleE_1F, 11390_F and 11395_R, res_R and res_F, res_F and zetaR. The analysis of predicted open reading frames was performed manually. The predicted open reading frames and their products were analyzed using the BLAST algorithm [59] and manually annotated. To determine the region in which the tetM determinant had putatively inserted into the 24.5 Mda conjugative plasmid (pEP5233) PCR reactions were performed with the following primers combinations: 11375_F and 11390_dw_R, 11375_F and 11395_R, Tet_up_1F and 11390_dw_R, Tet_up_1F and 11390_R, Tet_up_4F and 11390_dw_R, Tet_up_4F and 11390_R, Tet_up_6F and 11390_R, Tet_up_6F and 11390_R, Tet_up_6F and 11390_R. The resulting PCR products were send for sequencing either directly or after cloning in pGEM-T-Easy (Promega). To compare the plasmid core of the Dutch (pEP5289) and American (pEP5050) *tetM* type plasmids, with the 24.5 Mda conjugative plasmid (pEP5233) PCR reactions were performed with the primers used for the gap closure, and the additional PCR primer pairs: traO_R and traN_F for the traN-traO region, traL_R and traF_R for the traL gene, trbB_F and trbB_R for the trbB gene, trbF_F and trbF_R for the trbF, kleE_2F and traA_R for the replication region, traC_C and traE_R for traC operon and traD_F and traE_R for traD operon. The templates for PCR reactions were the Dutch (pEP5289) and American (pEP5050) *tetM* type plasmids and the 24.5 Mda conjugative plasmid (pEP5233). All PCR products were purified by gel elution (Sigma) and sequenced. The sequence of the conjugative plasmid with the Dutch type *tetM* determinant
### Table 5. Primers used in this study.

| Oligo name | Oligo name |
|------------|------------|
| Tet_4F     | 5'-CTCGAACAAGAGGAAACG-3' |
| Tet_AR     | 5'-GCATCCAGCTTCACCAAAC-3' |
| Tet_DR     | 5'-TGACGACAGGGAGG-3' |
| Tet_1R     | 5'-CAGCTGATTATAATGAGGCTGGC-3' |
| Tet_7F     | 5'-TGCCAGCCCGCGTCAATAAGTGCGTATG-3' |
| Tet_up_3F  | 5'-ATAATCAATTTGATAGTGAGGCGG-3' |
| Tet_dw_1R  | 5'-CCATATATATATAATACATAATACATACTAGT-3' |
| Tet_8R     | 5'-ACCTTCGTTGGTTAATCC-3' |
| Tet_9F     | 5'-TAGCTCAATATATAGGAGAGCCG-3' |
| Tet_3F     | 5'-GGAAATTTGGCAATCCCAATCT-3' |
| Tet_6R     | 5'-TACAAATCTCCTGTCACAGCAAT-3' |
| Tet_2R     | 5'-TGTCTCGGGTGTCTACTGAT-3' |
| trib_R     | 5'-GTAACGAGCTCCGGGAGAT-3' |
| tribN_F    | 5'-CTTGCCGAGTACCGAAAGGGGA-3' |
| ssb_R      | 5'-AGGCCGCAAAGGCGCTAGG-3' |
| tribF      | 5'-AGAGGAACAGGGGCAATTT-3' |
| kcoR       | 5'-ATGGCTATGGTTTCTTATG-3' |
| trfA_F     | 5'-TAATCTCAAAGCTTGCTAACTA-3' |
| klee_1F    | 5'-CCCCATACAGTACGAGCAATGAG-3' |
| 11395_R    | 5'-GATCAATCGGAAACGCGGCTAGAAGC-3' |
| traA_R     | 5'-GACCGATGTTCTGACACGTG-3' |
| traN_F     | 5'-AGCGTTCAGCCTTGATTTTG-3' |
| tral_R     | 5'-TATCATCTGATTCTTCTCTTGCG-3' |
| tral_F     | 5'-AGCAGCTGATCTTCTCTCTCTCTCTCT-3' |
| trbB_F     | 5'-AGCGTTCAGGTTGGAAATTAG-3' |
| trbR_R     | 5'-GCGATTTTCCATGACGTG-3' |
| trbL_F     | 5'-TGACCTGGTGGCAAGCAACT-3' |
| trbL_R     | 5'-TGATGGTAAAAGCGCTGGCCAC-3' |
| Tet_10R    | 5'-CCACATCTGATCTTCTCTCT-3' |
| 11390_R    | 5'-GGCGCCGATACCTCGTGTATT-3' |
| Zeta_F     | 5'-TTTCTCCCGCTGCTGCTGGGAT-3' |
| Res_R      | 5'-AAGGACGAAATGTGCGCCGCA-3' |
| 11375_F    | 5'-CTTGAGGCTTTTGTTCG-3' |
| Tet_up_1F  | 5'-CCAGGTTCTTCTGTCATG-3' |
| Tet_up_4F  | 5'-CGGAAACAAATATGATGCTTCT-3' |
| Tet_up_6F  | 5'-CAGTGGAAATAGGCTTCTGATT-3' |
| 11390_R    | 5'-AGGCTGCTGCGAGGATGAGG-3' |
| 11390_dw_R | 5'-ACCAACCCCGTCCAAGCAGAAATTT-3' |
| Tet_up_5F  | 5'-TTTCTTGGACGCTCTTCTGTGAG-3' |
| Res_S      | 5'-GACTTCCTCAACCCAGCTTG-3' |
| Zeta_R     | 5'-GGGCAACACTGCAACGCAAAC-3' |
| Zeta_F     | 5'-CCAGGATTTTCCATCCCGAC-3' |
| Tet_up_2R  | 5'-AGTTGCCTGCTGCTGCTGGGAT-3' |
| 45M        | 5'-CTAATCTCCTTCTCTGCTG-3' |
| G1         | 5'-ATACCTACAGTTAATT-3' |
| 11355_F    | 5'-ATGACATCATCGAATTCGTAATTAT-3' |
| pTrc99a    | 5'-TTCCTTGAGGGAATGGTGGAGGCGGATAC-3' |
| mut_connected | 5'-CGAGCGGATGAAATTGTCACAAC-3' |

### Table 5. Cont.

| Oligo name | Oligo name |
|------------|------------|
| 477 klee_2F | 5'-CTCCGCTCCCGCATATATGTTG-3' |
| 478 trfA_R  | 5'-GGCGACCGCGTATTGAAATACG-3' |
| 79 trbC_F  | 5'-CCGACGAGCCATTCCTCTCTCTCTCTCTCT-3' |
| 480 trbE_R  | 5'-GGCGGCTCAATTTCCATACAC-3' |
| 481 tral_D  | 5'-TACCGGACTCCCGTCGATTGCTC-3' |
| 482 tral_R  | 5'-TGTCGGTGAGGCAATAGTAT-3' |
| 503 vapX_F  | 5'-TGCGCAGATGAAACCCCCATCTACCCCCCG-3' |
| 504 vapX_R  | 5'-CGGCTCAGGATGCGTTTGCTGACAA-3' |
| GGI-21F    | 5'-GGGGAAGGAACCTGCGAATCCGAGAACTG-3' |
| GGI-22R    | 5'-GAAAGGATCGTGATGCGCAGCGATCATAGA-3' |
| GGI-27F    | 5'-CGCGGATCGGGCTGTCGTTGCGAATAT-3' |
| GGI-28R    | 5'-GAAAGTACTCTGGCGTAAATACCTGAGGAT-3' |
| 400 recA_F | 5'-GAGCTCCTGGGCTGCCTTCTTGCTGATG-3' |
| 401 recA_R | 5'-GTACCTGGTTCTGCTGCTGCTGCTGCTG-3' |

**Phylogenetic analysis**

To generate a phylogenetic tree, homologues of the TraI, TrbE, TraG and TraA proteins of representative members of the different IncP1 plasmid subfamilies (α, RP4; β, pADP-1; γ, QKH54; δ, pEST4011 and ε, pJKJ5) and the Dutch type plasmids were obtained using BLAST [59] against the NCBI non redundant protein database using expect values lower than 1E-10 for TraI and TrfA, and expect values lower than 1E-100 for TraG and TrbE. Duplicates and incomplete proteins were removed, and multiple alignments were constructed with the CLUSTALX program [60] and an initial phylogenetic tree was created using the MEGA 4 package. Sequences of the IncP1 plasmid and of the Dutch type plasmids grouped in a small section of the phylogenetic tree. The sequences of this small section were selected, realigned with CLUSTALX, and Neighbor-joining trees based on the distance parameter were constructed using the MEGA 4 package. Bootstrap values from 1000 replicates were also acquired.

**Construction of strains and plasmids used in this study**

Gonococcal strains were constructed as follows. Strains EP06 and EP030 were generated via the insertion-duplication method as described previously [61]. Briefly, an internal fragment of the recA gene was cloned in pND1 plasmid [61] followed by transformation to strain MS11A and its derivative ND500 (MS11/DGG1), respectively. Conjuction of the tetracycline plasmid pTetM from non-piliated strain 5289 to piliated strains EP06 and EP030 resulted in piliated and tetracycline resistant strains EP06/5289 and EP030/5289, respectively. Strains EP015 and EP030 were constructed by transformation of strain MS11A and its derivative ND500 (MS11/DGG1), respectively. Construction of the trc promoter of the pJD1 plasmid contained in strain 5289 using primers 503 (vapX_F) and (pEP5289), and of the genetic load regions of the conjugative (pEP5233) and the plasmid with the American type tetM determinant (pEP5050) were deposited under genbank numbers GU479464, GU479465 and GU479466.

Neisserial Conjugative Plasmid
504 (capX_R). pEP086 is derived from vector pTrc99A. In pEP086, expression was reduced after mutagenesis of the promoter by PCR amplification of plasmid pTrc99A with primers 475 (pTrc99A mut-10_F) and 476 (pTrc99A mut-10_R). Plasmids used and created in this study are listed in table 5.

**Conjugation to Escherichia coli**

Non-piliated gonococcal donor strain 5289 was streaked from O/N grown plates and suspended in 3 ml of GCBL (Difco) medium supplemented with Kellogg’s supplements and 0.042% NaHCO₃. Cultures were diluted till an OD₆₀₀ of 0.2 and was grown with shaking for 4 h at 37°C in a 5% CO₂ atmosphere. The recipient strain *Escherichia coli* TOP10 was diluted 50-fold from an overnight culture and was grown for 3 h. Equal portions of donor and recipient were mixed, centrifuged and pellets were resuspended in 100 µl of GCBL. The suspensions were placed on GCB plates in the presence or absence of 0.5 mM IPTG. Plates were incubated at 37°C under 5% CO₂ overnight. After the incubation cells were suspended in 100 µl of LB medium, and mating was interrupted by vigorous vortexing for 5 s and serial dilutions were plated on selective media (5 µg/ml of tetracycline for transconjugants selection). The mating efficiency was calculated as a CFU of transconjugants per CFU of recipient.

**Neisseria gonorrhoeae conjugation assay**

Donors and recipients from plates inoculated overnight were suspended in 3 ml of GCBL (Difco) medium supplemented with Kellogg’s supplements and 0.042% NaHCO₃. Cultures were diluted till an OD₆₀₀ of 0.2 and were grown for 4 h at 37°C with 5% CO₂. Equal portions of donor and recipient were mixed, centrifuged and pellets were resuspended in 100 µl of GCBL. The suspensions were placed on GCB plates supplemented with Kellogg’s supplements. Plates were incubated at 37°C under 5% CO₂ for 24 hours. After the incubation cells were suspended in 1 ml of GCBL (Difco) medium supplemented with Kellogg’s supplements and 0.042% NaHCO₃ and serial dilutions were plated on selective media. Recipient *Neisseria gonorrhoeae* strains contained the chloramphenicol marker while donors were tetracycline resistance. All donors had the erythromycin insertion within the recA gene to ensure one directional transfer. Transfer frequencies were calculated as a CFU of transconjugants per CFU of donor.

**Transformation assay**

Donors and recipients from plates inoculated overnight were suspended in 3 ml of GCBL (Difco) medium supplemented with Kellogg’s supplements and 0.042% NaHCO₃. Cultures were grown for 2.5 h at 37°C with 5% CO₂ for both donor and recipient cultures were centrifuged and pellets were resuspended in 5 ml of GCBL. 0.5 ml of donor and 0.5 ml of recipient were inoculated in 3 ml of GCBL for 3 h at 37°C with 5% CO₂ (both shaking and non shaking conditions). Serial dilutions were spread on selective media. Recipient *Neisseria* strains contained the chloramphenicol marker while donors were tetracycline resistance. All donors had the erythromycin marker inserted within the recA gene to ensure one directional transfer. Transfer frequencies were calculated as CFU of transconjugants per CFU of donor.

**Additional information**

Weblogos were created at http://weblogo.berkeley.edu/.

**Supporting Information**

**Figure S1** Alignment of the region between the zeta_1 and ngoSK11390 of the genetic load region of the neisserial conjugative plasmids. Depicted are the conjugative plasmid with the American type tetM determinant (A), the Dutch tetM determinant (D) and the plasmid without a tetM determinant (C). The different genes are indicated with different colors. Arrows above the genes indicate the orientation of the genes.

**Acknowledgments**

We especially thank Arnold J.M. Driessen and the department of molecular microbiology, in which department these experiments have been performed and the Groningen Biomolecular Sciences and Biotechnology Institute for continuous support. We thank Sylvia M. Bruisten from the Public health laboratory of GGD Amsterdam for providing *Neisseria gonorrhoeae* strains and DNA isolates with antibiograms from clinical samples. We thank Santa Jain, Maria Zweig and Eva-Maria Heller for discussions and critical reading of the manuscript.

**Author Contributions**

Conceived and designed the experiments: EP CvdD. Performed the experiments: EP CvdD. Analyzed the data: EP CvdD. Contributed reagents/materials/analysis tools: CvdD. Wrote the paper: EP CvdD.

**References**

1. McGee ZA, Johnson AP, Taylor-Robinson D (1981) Pathogenic mechanisms of *Neisseria gonorrhoeae*: observations on damage to human fallopian tubes in organ culture by gonococci of colony type 1 or type 4. J Infect Dis 143: 413-422.
2. Livermore DM (2009) Has the era of untreatable infections arrived? J Antimicrob Chemother 64 Suppl 1: i29–36.
3. Smith JM, Smith NH, O’Rourke M, Spratt BG (1993) How clonal are bacteria? Proc Natl Acad Sci U S A 90: 4384–4388.
4. Hamilton HL, Dominguez NM, Schwartz KJ, Hackett KT, Dillard JP (2005) Transformation assay

Transformation of plasmids of *Neisseria gonorrhoeae* to other Neisseria species: potential reservoirs for the beta-lactamide resistance plasmid. J Infect Dis 150: 397–401.

5. Morse SA, Johnson SR, Biddle JW, Roberts MC (1986) High-level tetracycline resistance in *Neisseria gonorrhoeae*: observations on damage to human fallopian tubes in organ culture by gonococci of colony type 1 or type 4. J Infect Dis 143: 413-422.

6. Mayer LW, Holmes KK, Falkow S (1974) Characterization of plasmid deoxyribonucleic acid from *Neisseria gonorrhoeae*. Infect Immun 10: 712–717.

7. Stoffier PW, Lerner SA, Bohnhoff M, Morello JA (1973) Plasmid deoxyribonucleic acid in clinical isolates of *Neisseria gonorrhoeae*. J Bacteriol 122: 1293-1300.

8. Roberts M, Plot P, Falkow S (1979) The ecology of gonococcal plasmids. J Gen Microbiol 114: 491–494.

9. Genco CA, Knapp JS, Clark VL (1984) conjugative plasmids of *Neisseria gonorrhoeae* to other Neisseria species: potential reservoirs for the beta-lactamase plasmid. J Infect Dis 156: 397–401.

10. Knapp JS, Zenzulian JM, Biddle JW, Perkins GH, DeWitt WE, et al. (1987) Frequency and distribution in the United States of strains of *Neisseria gonorrhoeae* with plasmid-mediated, high-level resistance to tetracycline. J Infect Dis 155: 819-822.

11. Burdett V, Inamine J, Rajagopalan S (1982) Heterogeneity of tetracycline resistance determinants in Streptococcus. J Bacteriol 149: 995–1004.

12. Roberts MC, Koutsky LA, Holmes KK, LeBlanc DJ, Kenny GE (1985) Tetracycline-resistant *Mycoplasma hominis* strains contain streptococcal tetM sequences. Antimicrob Agents Chemother 28: 141–143.

13. Roberts MC, Kenny GE (1986) TetM tetracycline-resistant determinants in *Ureaplasma urealyticum*. Pediatr Infect Dis 5: S338–340.

14. Fletcher HM, Marr L, Dancovoo Moore L (1989) Transposon-T616-like elements in clinical isolates of *Enterococcus faecium*. J Gen Microbiol 135: 3067–3077.

15. Roberts MC, Hillier SL, Hale J, Holmes KK, Kenny GE (1986) Tetracycline resistance and tetM in pathogenic urogenital bacteria. Antimicrob Agents Chemother 30: 810–812.

16. Swarzyns KS, McAllister CF, Hajeh RA, Heinrich DW, Stephens DS (1993) Deletions of T616-like transposons are implicated in tetM-mediated resistance in pathogenic *Neisseria* strains. Mol Microbiol 10: 299–310.

17. Roberts MC, Knapp JS (1988) Host range of the conjugative 25.2-megadalton tetracycline resistance plasmid from *Neisseria gonorrhoeae* and related species. Antimicrob Agents Chemother 32: 408–491.
10. Gascoyne-DM, Heritage J, Hawkey PM, Turner A, van Klugtenber E (1991) Molecular evolution of tetracycline-resistance plasmids carrying TetM found in Neisseria gonorrhoeae from different countries. J Antimicrob Chemother 28: 173–183.

11. Ison CA, Tokki N, Gill MJ (1993) Detection of the tetM determinant in Neisseria gonorrhoeae. Sex Transm Dis 20: 329–333.

12. Chalhule L, Jane van Rensburg MN, Matthee PG, Ison CA, Botha PL (1997) Plasmid analysis of Neisseria gonorrhoeae isolates and dissemination of TetM genes in southern Africa 1993–1995. J Antimicrob Chemother 40: 917–922.

13. Johnson SR, Morse SA (1991) The relationship between the 39 kb tetracycline-resistance plasmid and the 36 kb conjugative plasmid of Neisseria gonorrhoeae. J Antimicrob Chemother 27: 864–867.

14. Gascoyne-Binzi DM, Heritage J, Hawkey PM (1993) Nucleotide sequences of the tetM genes from the American and Dutch type tetracycline resistance plasmids of Neisseria gonorrhoeae. J Antimicrob Chemother 32: 667–676.

15. Knapp JS, Johnson SR, Zenilman JM, Roberts MC, Morse SA (1988) High-level tetracycline resistance resulting from TetM in strains of Neisseria spp., Kingella denitrificans, and Eikenella corrodens. Antimicrob Agents Chemother 32: 765–767.

16. Scharbaar-Vazquez R, Candelas T, Torres-Bauza LJ (2007) Mobilization of the gonococcal 3.2 kb beta-lactamase plasmid pGB2 into Escherichia coli by cointegration with several gram-negative conjugative plasmids. Plasmid 57: 156–164.

17. Eisenstein BI, Sex T, Biswas G, Blackman E, Sparling PF (1977) Conjugal transfer of the gonococcal penicillinase plasmid. Science 195: 998–1000.

18. Blet F, Humphreys GO, Saunders JR (1981) Intraspecific and intergeneric mobilization of non-conjugative resistance plasmids by a 24.5 megadalton conjugative plasmid of Neisseria gonorrhoeae. J Gen Microbiol 125: 123–129.

19. Rodriguez-Bonano NM, Torres-Bauza LJ (2004) Molecular analysis of oriT and MobA protein in the 7.4 kb mobilizable beta-lactamase plasmid pJF7 from Neisseria gonorrhoeae. Plasmid 52: 89–101.

20. Marquez C, Xia M, Borthagaray G, Roberts MC (1999) Conjugal transfer of the 3.05 beta-lactamase plasmid by the 25.2 Mda plasmid in Neisseria gonorrhoeae. Sex Transm Dis 26: 157–159.

21. Turner A, Gough KR, Leeming JP (1999) Molecular epidemiology of tetM genes in Neisseria gonorrhoeae. Proc Soc Exp Biol Med 219: 301–315.

22. Scharbaar-Vazquez R, Bortagaray G, Roberts MC (1995) Detection of two groups of 25.2 M Da T M plasmids by polymerase chain reaction of the downstream region. Mol Cell Probes 9: 327–332.

23. Korch C, Haghghom P, Ohman H, Goransson M, Normark S (1985) Cryptic plasmid of Neisseria gonorrhoeae: complete nucleotide sequence and genetic organisation. J Bacteriol 163: 430–438.

24. Gascoyne-Binzi DM, Hawkey PM, Heritage J (1994) The distribution of variants of the Tet M determinant in tetracycline-resistant Neisseria gonorrhoeae. J Antimicrob Chemother 33: 1011–1016.

25. Gianhazadeh B, Akhras M, Unemo M, Wretlind B, Nyren P, et al. (2005) Detection of gyrA mutations associated with ciprofloxacin resistance in Neisseria gonorrhoeae by rapid and reliable pre-programmed short DNA sequencing. J Mol Microbiol Biotechnol 7: 523–531.

26. Meinhart A, Alonso JC, Strater N, Saenger W (2003) Crystal structure of the plasmid maintenance system epsilon/omega and inactivation by epsilon/omega complex formation. Proc Natl Acad Sci U S A 100: 1661–1666.

27. Katz ME, Rood JI (1992) Molecular characterization of a Neisserial Conjugative Plasmid.