A New Family of Secreted Toxins in Pathogenic Neisseria Species

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Abstract

The genus Neisseria includes both commensal and pathogenic species which are genetically closely related. However, only meningococcus and gonococcus are important human pathogens. Very few toxins are known to be secreted by pathogenic Neisseria species. Recently, toxins secreted via type V secretion system and belonging to the widespread family of contact-dependent inhibition (CDI) toxins have been described in numerous species including meningococcus. In this study, we analyzed loci containing the maf genes in N. meningitidis and N. gonorrhoeae and proposed a novel uniform nomenclature for maf genomic islands (MGI). We demonstrated that mafB genes encode secreted polymorphic toxins and that genes immediately downstream of mafB encode a specific immunity protein (MafI). We focused on a MafB toxin found in meningococcal strain NEM8013 and characterized its EndoU ribonuclease activity. maf genes represent 2% of the genome of pathogenic Neisseria, and are virtually absent from non-pathogenic species, thus arguing for an important biological role. Indeed, we showed that overexpression of one of the four MafB toxins of strain NEM8013 provides an advantage in competition assays, suggesting a role of maf loci in niche adaptation.

Introduction

The growing number of sequenced bacterial genomes has led to the computer-based prediction of numerous novel bacterial factors possibly involved in virulence. As a result, many novel putative bacterial toxins have been identified by sequence-homology criteria. However, very few of these bacterial proteins have been tested for their toxic activity. Using in silico analysis, Aravind and colleagues have recently described widespread genes encoding putative secreted multi-domain toxins grouped under the name of bacterial polymorphic toxin systems (or polymorphic toxin-immunity systems) [1–3]. In silico analysis identified over 150 distinct toxin domains in these systems including many putative peptidase, nuclease or deaminase domains. Immunity genes found immediately downstream of the toxin genes encode highly variable proteins that protect bacteria from their own toxins or from toxins secreted by neighboring cells [4–6]. Immunity genes are a characteristic of polymorphic toxin systems that distinguishes them from host-directed toxins (i.e. cholera toxin or pertussis toxin) [7]. The polymorphic toxin systems are typically encoded on hypervariable chromosomal islands with characteristics of horizontal gene transfer [1]. These systems are found in both Gram-negative and positive bacteria [8]. The dominant hypothesis is that polymorphic toxin systems are primarily involved in conflict between related bacterial strains. The N-terminal domain of the toxin is typically related to trafficking whereas the C-terminal domain carries the toxic activity [1]. In a defined family of polymorphic toxins, the N-terminal domains are similar, while the C-terminal domains are highly variable. Toxins are potentially secreted by Type II, V, VI, or VII (ESX) secretion systems [1]. Toxins are encoded in loci that also contain standalone cassettes and immunity genes. Cassettes encoding alternative C-termini could promote diversity of toxic activities through genetic recombination [1,9,10].

The recently described contact-dependent growth inhibition (CDI) systems are a subgroup of polymorphic toxin systems [1,11]. Toxins encoded by CDI systems are large filamentous proteins that exhibit Rhs (rearrangement hotspot) or filamentous haemagglutinin repeats in their central region [4,6,8]. Rhs proteins are likely to be exported through the Type VI secretion machinery [6], whereas toxins with filamentous haemagglutinin repeats are exported through the Type 5 Secretion System (T5SS) [8,12]. The first CDI toxin secreted by a T5SS was reported in Escherichia coli EC93 (CdiAEC93) [11]. E. coli EC93 was found...
Author Summary

Many bacteria are able to secrete toxins targeted against neighboring cells. In order to protect themselves against their own toxin, they also express an “immunity” protein. In silico analysis of bacterial genomes predicts that numerous genes could encode potential new toxin-immunity systems. The recently described CDI system is involved in contact-dependent inhibition of growth and confers to its host strain a significant advantage in competitive ecosystems such as the gastro-intestinal tract. Indeed, an Escherichia coli CDI strain is able to out compete CDI strains and to become predominant. Here, we show that a large family of genes found in pathogenic Neisseria species, encodes a toxin-immunity system. We demonstrate that a toxin named MafB is able to abolish the toxicity. MafB toxins exhibit highly variable toxic domains. This variability of secreted toxins could be important to compete against bacteria of different species sharing the same reservoir. Since a strain may contain numerous toxin-immunity systems that can all play a role in interbacterial competition, deciphering interactions between these systems will allow a better understanding of complex bacterial communities.

to inhibit the growth of other E. coli strains (i.e. E. coli K-12) in co-culture experiments. The growth inhibition mediated by E. coli EC93 required a direct contact between toxic and target cells. In CDI systems, the toxin CdiA is secreted by an outer membrane transporter named CdiB. CdiA and CdiB are part of a two-partner secretion protein family (type Vb). Subsequently, several studies have demonstrated that CDI systems are present in many species including Neisseria meningitidis [4,5,13]. Moreover, it has been recently demonstrated that the two-partner system TpsAB is involved in contact-dependent inhibition of growth and RNA and show that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibits the growth of E. coli by degrading RNA and that the immunity protein MafB inhibit
Fig. 2A). MGI-2 is located at the 3' end of a tRNA-Pro gene and at the 3' end of a conserved gene encoding a putative Trk system potassium uptake protein (Fig. 1B, Fig. 2B). MGI-3 is located at the 3' end of pyrH (encoding an uridylate kinase) and at the 5' end of a truncated IS1016 element (Fig. 1B, Fig. 2C). MGI-1, MGI-2 and MGI-3 are present in N. meningitidis and in N. gonorrhoeae. N. gonorrhoeae genomes contain two additional MGIs: i) MGI-4 is flanked by two truncated IS1016 elements that are located between a small ORF encoding a putative protein and a malP pseudogene (Fig. 1B), ii) MGI-5 is located at the 3' end of an IS1016 and at the 5' end of relA (encoding a ppGpp synthase) (Fig. 1B).

To designate a gene located in a MGI, we propose to abandon the nomenclature from the initial genome annotation which was solely based on the order of appearance of maf genes in the genome (i.e. in NEM8013: mafB1 = NMV_0410,

Fig. 1. Organization and location of maf genomic islands in pathogenic Neisseria species. A) Schematic depiction of maf genomic island (MGI). By definition, a GI containing a mafBI module is a MGI. Each mafBI module is composed of two genes: mafb (green) and mafI (red). Additional genes (on a grey background) are mafa (pink), ORFs encoding alternative C-terminal domains of MafB (mafB-CT, green) associated with their cognate mafI gene (red). Black box at the 5' end of mafB-CT indicates a region potentially involved in antigenic variation of MafB. In class 1 MafBs, this region encodes a bacterial intein-like (BIL) domain whereas in class 3 MafBs, this region encodes a DWVKN motif. B) Simplified genomic organizations and flanking genes of the 5 MGIs found in pathogenic Neisseria. MGIs 1, 2 and 3 are found in N. meningitidis and N. gonorrhoeae while MGIs 4 and 5 are only found in N. gonorrhoeae. MGI-1 flanking genes encode an Anhydro-N-acetylmuramic acid kinase (AnmK) on the 5' end of the island and a hypothetical periplasmic protein on the 3' end. MGI-2 flanking genes encode a Proline tRNA on the 5' end of the island and Trk system potassium uptake protein on the 3' end of the island. A cluster of genes, which is only present in some strains, is represented on a grey background. MGI-3 flanking genes encode an uridylate kinase (PyrH) and a truncated IS1016 element. MGI-4 is flanked by two truncated IS1016 elements. MGI-5 flanking genes encode an IS1016 and a ppGpp synthase (RelA). Color code: conserved flanking genes (white), mafa (pink), mafb and mafB-CT cassettes (green), mafI (red), location of sequence encoding BIL domain (blue box), location of sequence encoding DWVKN motif (salmon rose box). The number (1, 2 or 3) inside the 5' end of mafB genes indicates the corresponding class of MafB (Class 1, 2 or 3 respectively). The dotted outline indicates a mafB-CT gene without initiation codon. C) A schematic representation of the 3 classes of MafB proteins. All MafBs contain a signal peptide (SP, light grey), a N-terminal conserved domain named DUF1020 (white) and a C-terminal variable region (dark grey). Class 1 MafBs contain a VSGDF motif at the end of the N-terminal conserved domain, and between the conserved and variable regions a bacterial intein-like (BIL) domain can be inserted (blue box). Class 2 MafBs contain a VKYDT motif at the end of the N-terminal conserved domain. Class 3 MafBs contain a DWVKN motif (salmon rose box) at the end of the N-terminal conserved domain.

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Fig. 2. Pairwise comparison of the genetic organization of MGI-1, MGI-2 and MGI-3 loci in 6 representative Neisseria genomes. Nucleotide comparison of MGI-1 (A), MGI-2 (B) and MGI-3 (C) from meningococcal strains Z2491, MC58 and NEM8013, gonococcal strains FA1090 and NCCP11945 and N. cinerea strain ATCC 14685. Genome comparisons were generated using BLASTn implemented in Easyfig 2.1 with a cutoff value of 80%. Grey vertical blocks indicate regions of shared similarity shaded according to BLASTn identity. The level of nucleotide identity is shown in the gradient scale for each MGI. Genes are indicated with arrows colored according to their predicted functions with the color code illustrated in the legend. Double-headed arrows connect mafB-CT genes that are identical to the 3′ region of a full-length mafB gene. The orientation of genes is as in the published genomic sequences, except for MGI-1_NEM8013, MGI-1_MCG38, MGI-2_NEM8013, MGI-3_Z2491 and MGI-3_FA1090 which have been reversed for clarity purpose. Genomic regions found in N. cinerea at the location of MGI-2 and MGI-3 are not considered as MGIs because they do not encode a full-length MafB toxin.

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mafB2 = NMV_1757 and mafB3 = NMV_2312, and to name a gene by its nature mafA, mafB or mafC followed in a lower index by the name of the genomic island where it is found and the name of the strain. For example, NMV_0410 is referred as MafB_NMVMG1-1NEM8013 and NMV_2312 is referred as MafB_NMVMG1-3NEM8013. Correspondence between locus tags, old and new nomenclature is summarized in S1 Table. If there are several mafB genes in a MGI, a number that refers to the position in the locus is added. This is especially the case for MGI-2 that has two mafB genes, the first mafB gene on the 5′end of the island is numbered 1. For example in MGI-2_NMVMG1-3NEM8013, NMV_1766 is now referred as MafB1_NMVMG1-2NEM8013 and NMV_1757 is referred as MafB2_NMVMG1-2NEM8013. The small ORF found immediately downstream of a mafB gene is termed mafI, i.e. MafI1_NMVMG1-2NEM8013 designates the gene found immediately downstream of mafB1_NMVMG1-2NEM8013.

Strikingly, there are few or no MGIs in commensal species, in contrast to strains of pathogenic species where multiple MGIs are present. MGI-1 is found in N. lactamica (strain 020-06), N. cinerea (ATCC 14685) and N. polysaccharea (ATCC 43768). N. cinaerea (ATCC 14685) and N. polysaccharea (ATCC 43768) do not contain additional MGI. N. lactamica is the non-pathogenic species that is the most closely related to the two pathogenic Neisseria species. In N. lactamica 020-06 a MGI-1 and a MGI-3 are present. A MGI-2 and a MGI-5 are also present but the corresponding mafB exhibit a frameshift in both of these MGIs. In the available unassembled genomes of commensal species N. subflava (N9703), N. mucosa (ATCC 25966 and C102 strains) or N. elongata subsp. glycolytica (ATCC 29315) we did not find a mafA gene or a full-length mafB gene. In N. flavescens (NRL 30031/H210), there is an incomplete MGI-5 with a mafA gene (NEFLA001129) and genes encoding C-terminal regions of mafB but without a full-length mafB gene.

Classification of MafB proteins based on their N-terminal domain. Sequence alignment of MafB proteins revealed that MafB has a N-terminal conserved domain of unknown function named DUF1020 (or PF06255 in PFAM Database) and a C-terminal (CT) variable region (Fig. 1C). DUF1020 is restricted to Neisseria genus and is preceded by a signal peptide domain (approximately 25 residues long). Amino acids alignment of 150 sequences of MafB proteins (see details in Materials and Methods section) revealed 3 classes of MafB with 3 conserved N-terminal regions (Fig. 1C). The percentage of sequence identity within N-terminal conserved regions of MafB proteins belonging to the same class is over 85% (90%, 97% and 89% for class 1, 2, 3, respectively) whereas the percentage of sequence identity between the conserved regions of different classes is approximately 35%.

Class 1 MafB proteins exhibit a conserved motif (VSGDF) located approximately 300 amino acids from the N-terminus (Fig. 1C and S1 Fig.). This motif is located at the transition between the conserved N-termini and the variable CT sequences. On the other hand, in class 2 and 3 MafB proteins, the conserved motifs found at the transition between the conserved and the variable regions are VKYDT and DWKVN, respectively (Fig. 1C, S2-S3 Fig.).

Some class 1 MafB proteins have a striking feature at the end of their N-terminal constant domain. Indeed, an A-type Bacterial Intein-Like (BIL) domain of approximately 140 amino acids is present at the junction between the conserved N-terminal region and the variable C-terminal region (i.e. in MGI-1_FA1090 (Fig. 1C and S1 Fig.). This BIL domain is located immediately after the conserved motif (VSGDF) (S1 Fig.).

All the MafB sequences, that we analyzed using the SignalP 4.1 program [36] showed a signal peptide (SP) recognized by type 1 signal peptidase. For example in NEM8013, MafBMG1-1NEM8013 and MafBMG1-2NEM8013 harbor a predicted cleavage site between amino acid residues 27 and 28, MafBMG1-2NEM8013 between amino acid residues 24 and 25 and MafBMG1-3NEM8013 between amino acid residues 26 and 27. Despite the presence of two consecutive arginine residues in the signal-peptide sequence of class 1 Mafs, PRED-TAT [37], TatP 1.0 [38] or TATFIND 1.4 [39] programs did not predict a putative tat (twin arginine translocation) signal. Thus, the translocation of the 3 classes of MafB proteins through the inner membrane is likely to occur via the Sec pathway.

In most cases, the variable domain of MafB shares no homology with protein of known function. Only in few cases a homology with CT extremities of polymorphic Cdi and Rhs toxins family can be noticed. However, in contrast with Cdi and Rhs toxins no repeat domains are found in the N-terminal region of MafB.

Gene content of the different maf genomic islands. In addition to the mafB-mafI module, a mafA gene is frequently found immediately upstream of genes encoding class 1 and class 3 MafB but not upstream of genes encoding class 2 MafB (Fig. 1A, 1B). A BLASTp search using MafA sequence as a query evidenced that mafA is also specific of the Neisseria genus. The Lipop 1.0 server [40] predicts that MafA exhibits a lipoprotein signal peptide without an aspartic acid in position +2 after the cleavage site and thus could be a lipoprotein attached to the outer membrane. It has been shown by immunoelectron microscopy [35] that MafA of gonococcal strain MS11 is surface exposed and is able to bind to glycolipids [35]. The location of MafA in the outer membrane and in outer membrane vesicles (OMV) has been confirmed by several proteomic studies [41–43].

Genes encoding class 1 MafB are present in MGI-1, MGI-5 and some MGI-2, whereas genes encoding class 3 MafB are present in MGI-3 and MGI-4 (Fig. 1B). Genes encoding class 2 MafB are only present in MGI-2 (Fig. 1B). Class 1 MafB encoded in the MGI-1_NMVMG1-3NEM8013 and MGI-1_MCG38 genes: i) mafB1_NMVMG1-2NEM8013, (mafB2_NMVMG1-2NEM8013) and MGI-2_MCG38 (mafB2_NMVMG1-2MCG38) differs from the class 1 MafB encoded in the MGI-1_NMVMG1-3NEM8013 and MGI-1_MCG38 by a deletion of 50 amino acids in their N-terminal region.

Examination of maf clusters downstream of the full-length mafB gene shows many genes encoding alternative MafB-C-terminal cassettes. All these mafB-CT genes are followed by at least one mafI gene (Fig. 1A, 1B). There are two types of mafB-CT genes: i) mafB-CTs starting with an initiation codon, which are potentially translated, and ii) mafB-CTs devoid of initiation codon (ATG, GTG, TTG, ATG or CTG), which are potentially silent cassettes.
Intriguingly, potentially expressed cassettes are only found in MGIs encoding class 1 MaßB whereas silent cassettes are only found in MGIs encoding class 3 MaßB (Fig. 1B). In MGIs encoding class 1 MaßB, the mafB-CTs that have an initiation codon encode an A-type BIL (Fig. 1B) and have no homologous region with the conserved region of the full-length class 1 MaßB (Fig. 1B) that could allow a recombination. The initiation codon for the translation of the BILs is a TTG and is the initiation codon of the mafB-CT. It should be pointed out that BILs of the MaßB-CTs lack the first 7 amino acids compared to complete BIL sequence. On the other hand, the full-length mafB genes located upstream of these mafB-CTs may contain a complete BIL sequence (i.e. in MGI-1FAM16).

In the MGI containing class 3 MaßB, the mafB-CTs are gene fragments that usually encode a conserved WDIVXX motif present in the full-length class 3 MaßB, but as mentioned above these mafB-CTs do not have an initiation codon (Fig. 1B).

The maf cluster harbors numerous small ORFs, designated mafI, immediately downstream of mafB. Similarly to the high variability of MaßB-CT, Maßf sequences are also highly variable, suggesting that the encoded proteins could specifically interact with cognate MaßB-CTs. In support of this hypothesis, when two mafB-CTs are almost identical their associated mafI gene is also identical. For instance, the C-terminal region of MaßBMgi-5Fa109 (NM0A524) and a cassette (NMB2107) found in MGI-3 of MC58 exhibit 100% amino acid identity (Fig. 2C). The corresponding immunity proteins MaßIMgi-2Zzq91 (NM0A323) and NMB2108 are also 100% identical (Fig. 2C). Similarly, MaßBMgi-5Fca1090 (NG01970) and a cassette (NMV_2314) found in MGI-3 of NEM8013 exhibit 90% amino acid identity (Fig. 2C). The corresponding immunity proteins MaßIMgi-5Fca1090 (NG01970) and NMV_2315 are 100% identical (Fig. 2C).

A search in the Conserved Domain Database (CDD) [44] of the NCBI revealed that many mafI genes encode proteins containing domains typically found in putative immunity proteins of bacterial polymorphic toxin systems [1,2]. These recently described domains include Imn17, Imn21, Imn22, Imn47, SUFU and SM11 domains. However, almost half of the small ORFs in MGIs do not contain any known domains. Small ORFs are considered immunity genes if they are located immediately downstream of a mafB-CT gene or if the corresponding amino acids sequence contain a predicted immunity domain in CD search database.

mafB-maff modules encode a new family of toxin-immunity

Analysis of the amino acid sequences of several MaßB-CT regions using the CDD server revealed homologies with putative or known toxic domains. For instance, proteins encoded by mafBMgi-1Nem8013 (NMV_0410) contains a domain belonging to the RNase EndoU-fold, mafBMgi-1Fa109 (NMG_01276) contains a domain belonging to a nucleotide deaminase superfamily and mafBMgi-5Fa1090 (NG01392) contains a domain belonging to the DNase HNH/EndoVII-fold. In this study, we decided to focus on the four putative MaßB toxins encoded in meningococcal strain NEM8013 which are MaßBMgi-1Nem8013, MaßBMgi-1Fa1090, MaßBMgi-1Fa1090 and MaßBMgi-1Fa1090 (formerly MaßB1, MaßB2, MaßB3 and MaßB-related respectively).

MaßBMgi-1Nem8013 and MaßBMgi-1Fa1090 proteins of strain NEM8013 inhibit cell growth when expressed in E. coli and expression of cognate Maßf counteracts MaßB toxicity. To assess the putative function of mafB-mafI modules, we expressed the four predicted MaßB toxins of strain NEM8013 in E. coli. We individually cloned the four mafB genes of strain NEM8013 into tightly controllable expression vector pBAD33 [45]. Induction of the expression of mafBMgi-1Nem8013 and mafBMgi-1Fa1090 was highly toxic for E. coli both on agar Luria-Bertani (LB) plates and in liquid LB culture (Fig. 3). Induction of the expression of mafBMgi-1Nem8013 and mafBMgi-1Fa1090 resulted in growth inhibition. On the other hand, induction of mafBMgi-2Nem8013 and mafBMgi-2Fa1090 genes did not alter growth of E. coli (Fig. 3A). Because some toxins targets are only present in the periplasmic space, we cloned mafBMgi-2Nem8013 and mafBMgi-2Fa1090 devoid of their own signal sequence in pET-22 and added peB-signal sequence at the N-termini of both proteins to direct them to E. coli periplasm. MaßBMgi-1Nem8013 and MaßBMgi-2Nem8013 remained non-toxic even when exported to E. coli periplasm (S4A Fig.). These results demonstrate that a toxic activity could be detected only for MaßBMgi-1Nem8013 and MaßBMgi-2Nem8013.

In order to test whether the toxicity of MaßB resides in its C-terminal domain, we cloned only the DNA region encoding the C-terminal domain or the N-terminal domain of MaßBMgi-1Nem8013 in E. coli using pBAD33. As expected, expression of the C-terminal domain of MaßBMgi-1Nem8013 was toxic for E. coli but expression of the N-terminal domain had no effect on E. coli growth (S4B–S4C Fig.).

To verify if the small ORF immediately downstream of the mafB gene was indeed an immunity gene, we cloned mafBMgi-1Nem8013 and mafBMgi-2Nem8013 under the control of the IPTG inducible promoter of pET-28 and pET-15 respectively. While the expression of mafBMgi-1Nem8013 or mafBMgi-2Nem8013 inhibited growth of E. coli, the co-expression of mafBMgi-1Nem8013 and mafBMgi-2Nem8013 or the co-expression of mafBMgi-1Nem8013 and mafBMgi-2Nem8013 did not impede growth of E. coli (Fig. 3B). There was no cross-protection conferred by MaßBMgi-1Nem8013 against MaßBMgi-1Nem8013 or by MaßBMgi-1Nem8013 against MaßBMgi-2Nem8013 (Fig. 3C). Thus, the small ORF adjacent to the toxin mafB gene encodes a protein providing immunity to the cognate toxin and preventing self-intoxication.

To explore the mechanism of toxin inactivation we investigated a potential direct toxin-antitoxin binding. For this, we cloned in the same vector (pColaDuet) under two different IPTG promoters, mafBMgi-1Nem8013 and mafBMgi-2Nem8013 in order to co-express in E. coli both proteins. Only MaßBMgi-1Nem8013 harbored a His6-tag. We tested whether MaßBMgi-1Nem8013 co-purified with His6-tagged MaßBMgi-1Nem8013 using Ni2+-affinity chromatography. Despite the very low expression of the MaßBMgi-1Nem8013 toxin in E. coli, we could co-purify MaßBMgi-1Nem8013 with His6-tagged MaßBMgi-1Nem8013 (Fig. 3D–E). Thus MaßBMgi-1Nem8013 is likely to inhibit MaßBMgi-1Nem8013 toxicity by a direct interaction.

MaßBMgi-1Nem8013, MaßBMgi-1Fa1090 and MaßBMgi-2Nem8013 proteins are toxic in their original NEM8013 Neisseria strain. In a first attempt to assess the toxicity of MaßB proteins in meningococci, we tried to generate mutants of the immunity gene located downstream of each of the four mafB genes in NEM8013. It was impossible to delete the immunity gene of mafBMgi-1Nem8013, mafBMgi-2Nem8013 and mafBMgi-3Nem8013 (respectively mafI1Mgi-1Nem8013, mafI1Mgi-2Nem8013 and mafI1Mgi-3Nem8013). Every attempt of mutagenesis led to duplication of the gene. On the other hand, the replacement of mafI2Mgi-2Nem8013 immunity gene by a chloramphenicol resistance cassette was possible. We next tried to insert an ectopic copy of the four mafB genes in the NEM8013 genome using pGCC constructs (see Materials and Methods section) [46]. Similar results as above were obtained and no strain carrying an additional copy of mafBMgi-1Nem8013, mafBMgi-2Nem8013 and mafBMgi-3Nem8013 could be obtained. On the other hand, it was possible
to insert an additional copy of \( mafB_2 \) in the wild-type (WT) strain and in a \( mafI_2 \) deleted mutant. On the other hand, it was possible to insert an additional ectopic copy of the four whole modules (\( mafB \) with \( mafI \)) using \( pGCC4 \) constructs. These results evidenced that all MafB proteins of strain 8013 except MafB2 have a toxic effect in NEM8013, unlike what was observed in \( E. coli \) where MafB1 and MafB2 were non-toxic.

Overexpression of the toxin MafB1 gives a competitive advantage. Since Cdi and Rhs toxins have been involved in inter-bacterial competition, we searched for a role of MafB in competition assays. We used strain NEM8013 and tested the impact of the overexpression of the four MafB proteins in competition assays. Constructions overexpressing each of the four MafB toxins with their cognate MafI proteins, in strain NEM8013, were used as inhibitor cells. As it has been shown previously that expression of a capsule could block CdiA mediated toxicity [47], we used an unencapsulated derivative of meningococcal strain NEM8013 as target cell. Competition assays were performed overnight on a solid media at an inhibitor to target ratio of 10:1 (see Materials and Methods). Under these experimental conditions, we evidenced a significant competitive advantage only for the strain overexpressing MafB1 (Fig. 4). The advantage was lost when target cells overexpressed the cognate immunity Mafi1. These data suggest that MafB1 could be employed by strain NEM8013 to outcompete strains that do not possess or express at a sufficient level the cognate immunity. Altogether these data demonstrate that MafB1 has a toxic effect on a non-capsulated derivative of strain NEM8013.

MafB toxins are secreted in a signal-peptide dependent manner. We focused on MafBMGI-1 (Class 1 MafB) and MafBMGI-3 (Class 3 MafB) of strain NEM8013 and we
engineered NEM8013 strains chromosomally expressing a FLAG-tagged version of these proteins (Fig. 5A). Using Western blotting, we were able to detect MafBMGI-1NEM8013 (Fig. 5B) in the culture supernatant. MafBMGI-1NEM8013 was no longer detected in the supernatant of a strain expressing MafBMGI-1NEM8013 devoid of its signal sequence (Fig. 5B). Similar results were obtained after overexpression of the wild type sequence of MafBMGI-1NEM8013 instead of the FLAG-tagged version using anti MafBMGI-1NEM8013 antibodies (S5 Fig.). Thus, the first step of MafB secretion across the inner membrane involves signal peptide recognized by the general secretory pathway. To gain access to the culture medium MafB has to cross the outer membrane, potentially through an outer membrane secretin. As PilQ is the only characterized outer membrane secretin in Neisseria sp, we overexpressed MafBMGI-1NEM8013 in a pilQ mutant strain NEM8013. The lack of PilQ secretin had no impact on MafBMGI-1NEM8013 level in the supernatant when the protein was produced from a pET vector (S6C Fig.). Taken together, these data suggest that secretion of MafB requires a Neisseria-specific factor that is neither PilQ nor MafA.

MafBMGI-1NEM8013 is a bacterial EndoU nuclease that can degrade multiple sources of RNA. Previous bioinformatics studies conducted by Zhang et coll. (in Supplementary material of [2]) suggested that MafB2MGI-2Nm053442 (encoded by gene NMCC_0602 GI: 161869586) could contain a nucleasic toxin domain of the EndoU fold. Amino acid sequences comparison between MafBMGI-1NEM8013 and MafB2MGI-2Nm053442 showed 95% identity over the entire length and 100% identity over the CT region. A search in the GenBank database with the BLASTp program using the CT domain of MafBMGI-1NEM8013 protein as a query confirmed the presence of a putative EndoU catalytic domain with two conserved histidine residues (Fig. 6A).

First, we examined total RNA from E. coli cells expressing MafBMGI-1NEM8013 for evidence of RNA degradation. Gel analysis of total RNA isolated from cells before and after induction of MafBMGI-1NEM8013 production revealed that expression of "pGCC4-mafBMGI-1NEM8013FLAG (S6A-S6B Fig.). Besides, we could not evidence the presence of MafBMGI-1NEM8013 in E. coli supernatant when the protein was produced from a pET vector (S6C Fig.). Together, these data suggest that secretion of MafB requires a Neisseria-specific factor that is neither PilQ nor MafA."
MafBMGI-1NEM8013 led to the non-specific cleavage of most cellular RNAs (Fig. 6B). To further characterize MafB MGI-1NEM8013 ribonuclease activity, we purified a recombinant His 6-tagged-MafBMGI-1NEM8013 protein and incubated it with various nucleic acids substrates. Recombinant MafB MGI-1NEM8013 was able to degrade purified total RNA from *E. coli*, *N. meningitidis* and human epithelial cells (Fig. 6C). Thus, MafBMGI-1NEM8013 is a ribonuclease. Incubation of MafBMGI-1NEM8013 with a synthetic RNA confirmed the RNAse activity (Fig. 6D). Addition of MafIMGI-1NEM8013 blocked the toxic activity of MafBMGI-1NEM8013 towards RNA (Fig. 6D). In order to determine whether MafBMGI-1NEM8013 ribonuclease had an uridylate specific activity, characteristic of the EndoU fold, we obtained a similar synthetic RNA where all the Us were replaced by As. This synthetic RNA was no longer cleaved even when incubation was extended up to 1 hour (Fig. 6E). Our results evidenced that MafBMGI-1NEM8013 preferred cleavage sites contain uridylates. Thus, with MafBMGI-1NEM8013, we characterized the first bacterial EndoU ribonuclease.

**Discussion**

In this study, we have demonstrated that *mafB* encodes a functional polymorphic toxin and *mafI*, the downstream gene, a specific immunity protein. Focusing on MafBMGI-1NEM8013, we were able to characterize its EndoU ribonuclease activity. Toxins carrying EndoU activities are predicted to be widespread among diverse polymorphic toxin systems, however no EndoU activity carrying toxins had been previously experimentally confirmed in bacteria. Polymorphic toxins encompass numerous families distributed in all bacterial lineages. Nevertheless, very few of these systems have been identified.
been experimentally characterized except the Cdi and the Rhs families in Gram-negative bacteria [6,9] and the PF04740 family from Gram-positive bacteria [40]. CdiA and Rhs toxins are the best characterized and can be found in many species including E. coli, Versinia pesci, Dickeya dadantii or Burkholderia pseudomallei [9]. CdiA and Rhs toxins are large filamentous proteins (over 1000 amino acids) with multiple repetitive elements. In contrast, MafB toxins are predicted to have a globular structure. Organization of the loci encoding CdiA and Rhs toxins shares similarities with MGIs organization. In particular, the presence of genes encoding CT cassettes/immunity modules downstream of the toxin gene is a common feature [8]. Interestingly, mafB genes are restricted to the genus Neisseria, which is very unusual in polymorphic toxin systems [1]. A mafA gene, encoding a surface exposed outer membrane lipoprotein, is frequently found immediately upstream of mafB. mafA is also specific of the Neisseria genus. It has been shown that gonococcal MafA binds to glycopilids but its biological function remains elusive [33]. Unlike CDI two-partner secretion system in which CdiB mediates the secretion of CdiA [49], the mafA gene located 5’ of mafB shares no homology with known secretion systems. Furthermore, the sequence of MafA offers no other clues to its function.

As observed for mafA genes, there are several loci bearing haemagglutinin related genes in meningococcal genomes. Haemagglutinin related genes are termed tpsA and tpsB genes. tpsA encodes a secreted filamentous haemagglutinin and tpsB encodes its dedicated transporter. Note of haemagglutinin related genes are only present as pseudogenes in gonococcal genomes [50]. Analysis of the sequences of the TpsA proteins encoded in N. meningitidis genomes revealed the presence of three distinct groups [51]. Group 1 is found in all meningococcal genomes whereas tpsA genes of group 2 and 3 are overrepresented in disease isolates compared to carriage isolates [51]. In meningococcus, the number of tpsA genes range from 1 to 5 [52]. Genomes of strains FAM18, B16B6 and Z2491 contain only one tpsA gene of group 1 system while strain MC38 contains five tpsA genes (NMB0493, NMB0497, NMB1214, NMB1768 and NMB1779) belonging to the three different groups [51].

The tps locus of meningococcal strain FAM18 is referred as a cdi locus in the comparative genomic study conducted by Poole et al. in 2011 [4]. Indeed, the FAM18 tpsA gene (NMC0444 also termed cdaA by Poole et al.) encodes a protein that exhibits a filamentous haemagglutinin family N-terminal domain and a Pretoxin domain with a VENN motif, which is located before the putative C-terminal toxin domain. Since this study, several recent works have been published on the meningococcal tps loci. It has been shown that meningococcal strain B16B6 had an advantage in competition assay against a B16B6 deletion mutant lacking the cognate immunity gene of tpsA [14]. The tpsA gene of strain B16B6 (HQ420265) encodes a protein with >99% sequence identity with that of FAM18 [14] confirming the prediction of Poole et al. [4] that meningococcal TpsA proteins from group 1 constitute functional CDI systems. It remains to be investigated whether TpsA proteins from group 2 and 3 are also able to mediate growth inhibition.

The six secretion pathways identified in Gram-negative bacteria can be classified in two categories, either the pathways transporting proteins in a single-step across both inner and outer membranes (i.e. type I, III, IV and VI) or the two-step secretion pathways (i.e. type II and V), where proteins are first targeted to a machinery that recognizes their N-terminal signal peptide [53–55]. Type V secretion systems encompass auto-transporter proteins (type Va) and two-partner secretion systems (type Vb) [54]. Cdi toxins belong to the type Vb subclass. Only type I, Va and Vb secretion pathways are found in N. meningitidis, whereas only type IV and Vb secretion pathways are found in N. gonorrhoeae [56]. Thus, the only common secretion pathway shared by pathogenic Neisseria species is autotransporters pathway. Autotransporters are single polypeptides that consist of a surface-exposed variable N-terminal domain (“passenger domain”) that can be released from the cell surface by a proteolytic cleavage and a C-terminal domain (“translocator domain”) folded into a β-barrel structure in the outer membrane. The β-barrel of translocator domains is in most cases composed of 14 β-strands [57]. The IgA protease of N. gonorrhoeae was the first described autotransporter [58]. Since MafB toxins possess an N-terminal signal peptide and are found in N. meningitidis and N. gonorrhoeae, they are likely to be secreted by a two-step secretion pathway found in both species and not yet identified. Indeed, according to their domains organization and the lack of β-barrel structure prediction (using TBBpred server [59] and HMM-TM server [60]), MafB toxins do not belong to the autotransporters family. Besides, secreted proteins known to be targeted to a TpsB transporter of two-partners systems exhibit a TPS secretion domain adjacent to the signal peptide sequence [61]. There is no such TPS domain in the N-terminal region of MafB that could target the toxin to a TpsB transporter. Thus, in contrast to Cdi toxins, MafB toxins are unlikely to be secreted via type V secretion systems. Moreover, in contrast to Rhs toxins that can be secreted through type VI secretion system, there is no type VI secretion system in pathogenic Neisseria species.

In addition to these six types of secretion systems, Gram-negative bacteria, including Neisseria pathogenic species, constitutively produce outer membrane vesicles (OMV) during their normal growth [62,63]. OMVs are mainly composed of outer membrane and periplasmic components. OMVs enable the secretion of virulence factors to the surrounding environment or directly to neighboring bacteria [64,65] or to eukaryotic cells [66,67]. For instance, OMVs derived from P. aeruginosa is able to kill other bacterial species [64] by the release of murine hydrolases capable of degrading the peptidoglycan of other species. P. aeruginosa is also able to deliver multiple virulence factors directly into the host cell cytoplasm by fusion of OMV with host cell membrane lipid raft [67]. Thus, OMVs can interact both with competing bacteria and with host cell to promote bacterial colonization of the host or pathogenesis. Pathogenic Neisseria are well known for their release of OMVs [56,62]. Natural and engineered OMVs have recently gained interest for use as vaccine or adjuvants. The OMV vaccine strategy has been successfully used during several clonal outbreaks of serogroup B meningococcal strains in Cuba, Norway, and New Zealand [68]. As a consequence, several recent proteomic studies analyzed the Neisseria OMVs content. A proteomic study of naturally released OMVs isolated from four gonococcal strains (FA1090, F62, MS11, and 1291) revealed the presence of MalA and MalB,MGI-2 (corresponding to ORFs NGO00225, NGNG_00563, NGPG_00362 and NGAG_00430) in OMVs of 4 strains [43]. In addition, supplemental data published by Zielke et al. in the same proteomic study suggest that four other MalB toxins produced by these gonococcal strains could also be present in OMVs. Since MalA has been previously described as an adhesin able to bind cellular glycolipids [35], the presence of MalA in OMVs could mediate attachment of OMVs to eukaryotic cells. This suggests that OMV-mediated release could be a mean for delivery of MalB toxins to neighboring bacteria or to eukaryotic cells. The presence of MalB toxins in OMVs and its potential implications for Neisseria pathogenesis have to be further explored.

A hallmark of MGIs is the presence of numerous mafB-CT genes. Genetic recombination, resulting from the replacement of
the 5’ end of a full-length mafB gene by an alternative CT cassette is supported by genome comparison both for MGI encoding class 1 or class 3 MaBs. For example in MGI-1s, the mafB-CT cassette found in MGI-1NEM8013 (NVM_0408) encodes the same CT region that mafB_{MGI-1FA1090} (Fig. 2A). In MGI-3, the mafB-CT cassette found in MGI-3NEM8013 (NVM_2314) encodes the same CT region that mafB_{MGI-3FA1090} (Fig. 2C). Genetic recombination of polymorphic toxins has been recently demonstrated for CdiA in N. meningitidis [14] and for Rhs in Salmonella enterica serovar Typhimurium [10].

In this study, we demonstrated a functional role for one of the four MaB toxins of NEM8013. Indeed, we showed that an unencapsulated derivative of NEM8013 was outcompeted by a strain overexpressing MaB1_{MGI-2NEM8013}. Several studies have evidenced that meningococci frequently become acapsulated in the nasopharynx as a result of phase variation [69,70] or by down-regulation of the genes involved in capsule biosynthesis [71,72]. Thus, both capsulated and unencapsulated strains are likely to compete with each other for colonization of the nasopharyngeal niche. The function of the three other mafB genes of this strain remains unknown. Given the diversity of the CT extremities of MaBs, it is also plausible that they exhibit various biological targets such as other bacterial species or eukaryotic cells. Thus, biological function of maf genes remains a challenging question. Since these genes represent 2% of the genome of pathogenic Neisseria, but are virtually absent from non-pathogenic species, it is likely that they play important biological roles, including in pathogenesis. Regulation of the expression of maf genes could give clues on their physiological role. It has been recently demonstrated by Fagnocchi et colleagues that NadR is a regulator of maf operons [73]. The NadR regulon in meningococcal MC58 strain comprises nadA (encoding an adhesin), and the operons NMB0375-374 (encoding MaA and MaB in MGI-1\_{MCG5}) and NMB0652-654 (encoding MaA, MaB and MaF in MGI-2\_{MCG5}). In the presence of human saliva (or the small metabolite 4HPA that is secreted in human saliva) Fagnocchi et colleagues showed that maf genes are repressed, while nadA is induced in a NadR-dependent manner. This coordinate regulation indicates an adaptation of the bacteria in response to the signal molecules present in saliva, and suggests a role in colonization of the port-of-entry.

It has been suggested that, in addition to growth-inhibiting function, Rhs and Cdi might have a broader role in interbacterial communication. CdiA- or Rhs-CTs could serve as signal molecules when translocated in neighboring cells protected by the cognate immunity protein, in a manner similar to quorum sensing. Furthermore, recent studies showed that Cdi plays a role in biofilm formation in Burkholderia thailandensis [13,74,75]. Indeed, CDI systems might be a mechanism allowing bacteria to discern kin versus non-kin within a complex population (e.g. in a polymicrobial biofilm). Only the cells expressing the same set of toxins and of immunity proteins will be able to live in close proximity. However, it is difficult to predict the evolution of a complex community since a strain may contain numerous toxin systems (such as Maf, Rhs, Cdi, bacteriocins or recently described type VI secretion systems effectors) that can all play a role in interbacterial competition. Deciphering interactions between these systems is a challenging question for future studies.

Materials and Methods

Bioinformatic analysis

We used 6 fully sequenced and annotated genomes of N. meningitidis (Z2491, MC58, FAM18 and NEM8013) and N. gonorrhoeae (FA1090 and NCCP11945) present in the MicroScope database with curated annotations from the NeMeSys project [19,76–80]. We also used N. gonorrhoeae MS11 genome sequence available from the Broad Institute, N. cinerea ATCC 14605 genome sequenced by Washington University and the following meningococcal strains: H44/76 [81], M04-240196 [82], M01-240355 [82], G2136 [82], M6190 [82], NZ-05/33 [82], WUE2594 [83] and 053442 [20]. We used BLASTp with default parameters to search for orthologs of the Maf proteins in other Neisserial strains present in the NCBI non-redundant protein database.

To identify the different classes of MaB proteins, we downloaded 150 sequences stored on the PFAM database server [84] that have been used to build the DUF1020 (PF06255) family. The alignment was performed with ClustalW [http://www.genome.jp/tools/clustalw/] [85,86] using default parameters and a rooted phylogenetic tree (UPGMA) was generated.

Multiple alignments were also performed using MUSCLE [87] or Clustal Omega [88] with default parameters and shaded using the BoxShade server (http://www.ch.embnet.org/software/BOX_form.html). The LipoP 1.0 server [40] and SignalP server version 4.1 [36] was used to predict the presence of a signal sequence with default options for Gram-negative bacteria. PRED-TAT [37], TatP 1.0 [38] or TATFIND 1.4 [39] servers were used to identify putative tat (twin arginine translocation) signal. β-barrel structure prediction were performed with TTBpred server [59] and HHM- TM server [60] and conserved domains were identified with the Conserved Domain Database (CDD) [44] of the NCBI server. Pairwise genome comparisons were visualized using Easyfig [89].

Accession numbers of proteins mentioned in the text with corresponding locus tags are listed in the supplementary information (S1 Table).

Bacterial strains and growth conditions

All strains used in this study can be found in S2 Table. Meningococci NEM8013 and N. cinerea were grown at 37°C in a moist atmosphere containing 5% CO2 on GCB (« Gonococcal Broth »; Difco) agar plates containing Kellogg’s supplements and appropriate antibiotics (100 μg/ml kanamycin, 6 μg/ml chloramphenicol and/or 4.5 μg/ml erythromycin for NEM8013 or 9 μg/ml erythromycin for N. cinerea). E. coli TOP10 (Life technologies) or BL21(DE3) (Life technologies) were grown at 37°C in liquid or solid Luria-Bertani (LB) medium (Difco), which contained appropriate antibiotics (50 μg/ml ticarcillin, 10 μg/ml chloramphenicol and/or 50 μg/ml kanamycin).

Vectors construction for toxicity assays in E. coli BL21(DE3)

All vectors and primers used in this study can be found in S3 Table and S4 Table.

Full-length mafB genes including the putative signal peptide sequence or only the 5’ or 3’ regions of mafB genes from NEM8013 were cloned in pBAD33. Briefly, PCR products of mafB_{1MGI-2NEM8013}, mafB_{MGI-1NEM8013}, the 5’ region of mafB_{MGI-1NEM8013} (the first 1020 nucleotides), the 3’ region of mafB_{MGI-1NEM8013} (the last 477 nucleotides) and the 3’ region of mafB_{MGI-3NEM8013} (the first 339 nucleotides) were digested by SacI and XhoI, whereas PCR products of mafB_{2MGI-2NEM8013}, mafB_{MGI-1NEM8013} and the 5’ region of mafB_{MGI-3NEM8013} (the first 1107 nucleotides) were digested by SmaI and XhoI. The digested PCR products were ligated to pBAD33 under the control of arabinose-inducible PBAD promoter. mafB_{2MGI-2NEM8013} and mafB_{1MGI-2NEM8013} amplified without their putative signal peptide sequence (without the first 93 nucleotides for mafB_{2MGI-2NEM8013} and the first 120 nucleotides for mafB_{1MGI-2NEM8013}.

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were also cloned in pET22 using BamHI and XhoI to obtain proteins containing PelB peptide signal. mafB1MGI-2NEM8013 was cloned in pET28 using NcoI and XhoI and mafB1MGI-3NEM8013 was cloned in pET15 using XhoI and BamHI. pBAD33-mafB or pET22-mafB constructs were transformed in *E. coli* BL21(DE3) with or without pET-mafi constructs to perform toxicity assays.

All vectors constructed were verified by PCR and sequencing.

**Vectors construction for protein production in *E. coli* BL21(DE3)**

In order to produce and purify MafB1MGI-1NEM8013, the operon mafB1MGI-1NEM8013 (NMV_0410-NMV_0409) without the sequence of the signal peptide of MafB1MGI-1NEM8013 was cloned downstream of an IPTG inducible promoter in pET15 using NcoI and XhoI. The resulting MafB protein harbors hexahistidine N-terminal tag.

In order to produce and purify MafB1MGI-1NEM8013, pGCC4-NMV_0409 was cloned downstream of an IPTG inducible promoter in pET28 using NcoI and XhoI. The resulting MafB protein harbors hexahistidine C-terminal tag.

In order to assess the presence of MafB in the supernatant of *E. coli*, the operon mafB1MGI-1NEM8013 (NMV_0410-NMV_0409) was cloned downstream of an IPTG inducible promoter in pET28 using NcoI and XhoI.

To assess the potential co-purification of MafB1MGI-1NEM8013 and MafB1MGI-3NEM8013, mafB1MGI-1NEM8013 and mafB1MGI-3NEM8013 were cloned in two different multiple cloning site (MCS) under two IPTG inducible promoters in pcolaDUET. mafB1MGI-1NEM8013 was cloned in MCS2 using BgIII and KpnI and mafB1MGI-3NEM8013 was cloned in MCS1 using BgIII and HindIII.

All vectors constructed were verified by PCR and sequencing.

**Vector construction for mafABI deletion in *N. cinerea***

PCR reactions were used to amplify 400 bp upstream of mafA (NEICl_01_50108) and 770 bp downstream of mafA (NEICl_01_50110). The resulting products were digested by EcoRI/BamHI and BamHI/HindIII respectively and cloned into pUC19 digested by EcoRI/HindIII. A kanamycin resistance cassette *apha*-3 was then inserted in the BamHI site. The final construct containing kanamycin resistance cassette *apha*-3 flanked by homologous regions for recombination was amplified by PCR using primers with DNA uptake sequence. Amplicon was introduced in *N. cinerea* by transformation and transformants were verified by PCR and sequencing.

**Vectors construction for maf genes expression in NEM8013 or *N. cinerea***

mafB genes, mafs or mafB-mafI operons of NEM8013 were cloned in pGCC4 under the control of an IPTG inducible promoter using Pael and ScoI. pGCC4-mafB or pGCC4-mafB-mafI were transformed in NEM8013, pGCC4-mafI-MGI-2NEM8013 was transformed in NEM8013[ctvA][80] to insert mafB, mafs or mafB-mafI in the meningococcal intergenic region between lacP and asPC. NEM8013 strains harbouring IPTG inducible mafB-mafI operons have been used in competition assays. pGCC4-mafB1MGI-1NEM8013 was also transformed in NEM8013[pilQ][90] to assess the role of PilQ on MafB secretion.

In order to express a FLAG-tagged MafB1MGI-1NEM8013 protein in NEM8013, the 5’ region of mafB1MGI-1NEM8013, with or without the sequence of its signal peptide, was amplified with a reverse primer encoding the FLAG epitope [(DYKDDDDK)]. The resulting PCR product was cloned in pGCC4 using Pael and ScoI. pGCC4-mafB1MGI-1NEM8013FLAG or pGCC4-mafB1MGI-1NEM8013FLAG-SP was transformed in NEM8013. pGCC4- mafB1MGI-1NEM8013FLAG was also transformed in *N. cinerea* and in *N. cinerea* ΔmafABI to assess the role of MafA on MafB secretion.

All strains constructed were verified by PCR and sequencing.

**Toxicity assays in *E. coli***

Toxin activity was assessed by growing *E. coli* BL21(DE3) carrying pBAD33-mafB1MGI-1NEM8013, pBAD33-mafB2MGI-1NEM8013, pBAD33-mafB2MGI-2NEM8013 or pBAD33-mafB1MGI-3NEM8013 at 37°C on LB agar plates with or without 0.2% L-arabinose to induce gene expression. Growth curves were performed in LB broth at 37°C (200 rpm) and toxin expression was induced either by addition of L-arabinose to a final concentration of 0.2% (pBAD33-mafB) or by 1 mM IPTG (pET22-mafB).

To assess the protective role of mafI, *E. coli* BL21(DE3) were co-transformed with pBAD33-mafB and either cognate or non-cognate pET-mafi. Growth curves were performed with 0.01 mM IPTG to induce the antitoxin production throughout the experiment whereas L-arabinose (0.2%) was added 2 h post-inoculation to induce the toxin production. Growth was monitored every hour with OD 600 nm. Viability was assessed in parallel of the growth curves by spotting 5 microliters of bacterial cultures onto LB agar plates containing D-glucose (0.2%) before and after the induction of toxin expression.

**Purification of proteins***

Toxin MafB1MGI-1NEM8013 and cognate immunity protein MafI1MGI-1NEM8013 were expressed in *E. coli* BL21 [DE3] using pBAD33-mafB resulting in the production of N-terminal hexahistidine-tagged toxin and untagged immunity protein. Protein expression was induced for 2 h at 37°C with 1 mM IPTG. Proteins were purified using NiNTA metal affinity resin (Qiagen) in denaturing conditions. Bacterial pellets were lysed by sonication in lysis buffer (100 mM NaH2PO4, 10 mM TrisHCl, 8 M urea, pH 8) and centrifuged for 20 min at 10 000 g. Supernatants were incubated with Ni-NTA resin and loaded onto columns. The resin was washed with denaturing wash buffer (100 mM NaH2PO4, 10 mM TrisHCl, 8 M urea, pH 6.5) to first remove untagged immunity protein and then, MafB1MGI-1NEM8013 was eluted using the same buffers by lowering pH (pH 5.9 and pH 4.5). Renaturation of MafB1MGI-1NEM8013 was achieved by serial dialysis against buffers containing decreasing concentrations of urea.

Immunity MafI1MGI-1NEM8013 was expressed in *E. coli* BL21(DE3) using pBAD33-mafB resulting in the production of a C-terminal hexahistidine-tagged immunity protein. Protein expression was induced for 1 h at 37°C with 1 mM IPTG. Proteins were purified using NiNTA resin in native conditions. Bacterial pellets were lysed by sonication in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8) and centrifuged for 20 mn at 10 000 g. Supernatants were loaded on NiNTA resin columns. The resin was washed with denaturing wash buffer (50 mM NaH2PO4, 300 mM NaCl, 20 mM and 50 mM imidazole, pH 8) and MafI1MGI-1NEM8013 was eluted using a buffer containing 250 mM imidazole (pH 8).

MafB1MGI-1NEM8013 and MafI1MGI-1NEM8013 were co-expressed in *E. coli* BL21(DE3) using pcolaDUET. Protein expression was induced for 2 h at 37°C with 1 mM IPTG. Proteins were purified using NiNTA resin in native conditions as described above for MafB1MGI-1NEM8013 alone. Bound complexes were eluted with native elution buffer containing 250 mM imidazole (pH 8) for SDS-PAGE analysis and immunoblotting.
In vivo RNase activity

*E. coli* Top10 carrying pBAD33 or pBAD33-mafBMGI-1NEM8013 were grown in LB containing chloramphenicol at 37°C, 200 rpm, until OD600 reached 0.2, then L-Arabinose was added to a final concentration of 0.2%. Total RNA from *E. coli* was isolated before induction (T0) and 30 min after addition of L-arabinose using TRIzol reagent (Life Technologies) according to manufacturer’s instructions. Total RNAs were analyzed by denaturing gel electrophoresis (5% polyacrylamide/8 M urea) and visualized by staining with ethidium bromide.

In vitro RNase activity

Purified MafBMGI-1NEM8013-His6 alone (3 μM) and/or MafBMGI-1NEM8013-His6 (100 μM) were incubated with 4 μg of total RNA isolated from different sources with TRIZol reagent method (N. meningitidis NEM8013, E. coli Top10 and human epithelial cells FaDu). Each reaction was performed for 30 min at 37°C in Tris-EDTA buffer and run on native 1% agarose gels containing ethidium bromide. To assess the role of divalent cations, buffers containing Mg2+ (10 mM Tris-Cl, 2.5 mM MgCl2) or Mn2+ (25 mM HEPES pH 7.4, 50 mM NaCl, 5 mM MnCl2, 1 mM DTT) have been used instead of Tris-EDTA buffer.

To assess the ability of MafBMGI-1NEM8013 to cleave synthetic RNA in *vivo*, purified MafBMGI-1NEM8013-His6 alone (3 μM) and/or MafBMGI-1NEM8013-His6 (100 μM) were incubated with 3 μM of a synthetic oligoribonucleotide in Tris-EDTA buffer for 15 min at 37°C. Two synthetic oligoribonucleotides (synthesized by Integrated DNA Technologies) were used: 5'-CAGGUGUUUAAG-GAAUGCGCCAGAGGCGCCAGAUGAAAA-3' (mRNA U) and 5'-CCAGAAGAAAAGAGAGAGAGCGACGCGAGCG-AAGAAAA-3' (mRNA V). The reactions were stopped by the addition of an equal volume of Gel Loading Buffer II (95% formamide, 18 mM EDTA, 0.025% SDS; Ambion) and incubation for 5 min at 95°C. The reaction products were separated by electrophoresis in 14% polyacrylamide/8 M urea and were visualized by ethidium bromide staining.

Immunoblotting

Preparation of protein samples, SDS-PAGE separation, transfer to membranes and immunoblotting were performed using standard molecular biology techniques. Proteins were quantified using NanoDrop, following manufacturer’s instructions.

We raised polyclonal antibodies in rabbits against purified recombinant protein MafBMGI-1NEM8013 and against a synthetic peptide (KNSNIEHEKNYGRD) of the COOH-terminal region of MafBMGI-1NEM8013 protein (Proteognex). We used rabbit polyclonal anti-FLAG antibody directed against DYKDDDDK epitope (Cell Signaling Technology) and mouse monoclonal antibody directed against NADP-dependent glutamate dehydrogenase. Bound primary antibodies were detected by goat Anti-Rabbit or Anti-Mouse HRP-linked antibodies (Cell Signaling Technology) using ECL Plus detection reagents (Pierce).

Preparation of supernatants from meningococcal and *E. coli* cultures

Overnight cultures of *N. meningitidis* grown on GCB agar plates were used to inoculate RPMI 1640 medium (PAA) containing Kellogg’s supplements and 1 mM IPTG. Overnight cultures of BL21(DE3) grown on LB agar plates were used to inoculate LB medium containing appropriate antibiotic. When OD 600 reached 0.2, IPTG was added to a final concentration of 1 mM. When OD 600 reached 0.5, the cells were harvested by centrifugation (3 000 × g for 30 min), and supernatants were passed through a 0.22-μm pore size filter unit. Supernatant proteins were concentrated by ultrafiltration (Amicon, Ultra-15, 3 kDa cutoff) according to the manufacturer’s instructions. The pellets and the concentrated supernatants were used for immunoblotting analysis.

Efficiency of protein expression is calculated as the ratio of output counts to input counts and the competitive index (CI) was calculated as the output divided by the initial inhibitor/target ratio. The data from three independent experiments were examined for significance using a two-tailed Student’s t-test. A p-value < 0.05 was considered significant.

Supporting Information

**S1 Fig**  Amino acid alignment of class 1 MafBs. Protein sequences were aligned using Clustal Omega (1.2.1), with default parameters and shadded using the BoxShade server. Residues that are identical or similar in all sequences are shaded with black or grey background respectively. The GenBank locus tag was used to identify each protein. The end of the conserved N terminal region is aligned to show the VSGDF motif which demarcates the beginning of the variable C terminal region in NMB0633, NMV_1757, NMA2113, NMB0374 or the beginning of the bacterial iniein in NGO1392, NGO1585, NGK_1637, NGK_1886. (TIF)

**S2 Fig** Amino acid alignment of class 2 MafBs. Protein sequences were aligned using Clustal Omega (1.2.1), with default parameters, and shadded using the BoxShade server. Residues that are identical or similar in all sequences are shaded with black or grey background respectively. The GenBank locus tag was used to identify each protein. The end of the conserved N terminal region is aligned to show the VKYDT motif that demarcates the beginning of the variable C terminal region. (TIF)

**S3 Fig** Amino acid alignment of class 3 MafBs. Protein sequences were aligned using Clustal Omega (1.2.1), with default parameters and shadded using the BoxShade server. Residues that are identical or similar in all sequences are shaded with black or grey background respectively. The GenBank locus tag was used to identify each protein. The end of the conserved N terminal region is aligned to show the WDWVKN motif that demarcates the beginning of the variable C terminal region. (TIF)

**S4 Fig** MafB1MGL-1NEM8013 and MafB2MGL-1NEM8013 are not toxic in *E. coli* periplasm and the toxicity of
MaBMGI-1NEM013 resides in its C-terminal domain. A) Growth curves of BL21(DE3) cells transformed with vector pET22 carrying mafl1MGI-1NEM013 or mafl2MGI-1NEM013 genes. Toxin expression was induced by adding 1 mM IPTG in LB broth 2 h after inoculation (arrow). B) Effect of MaBMGI-1NEM013 N-terminal domain over-expression on E. coli growth in the presence of 0.2% L-arabinose (Ara+). BL21(DE3) cells were transformed with vector pBAD33 carrying the 5’ end of maflBMGI-1NEM013 gene. C) Effect of MaBMGI-1NEM013 C-terminal domain over-expression on E. coli growth in the presence of 0.2% L-arabinose (Ara+). BL21(DE3) cells were transformed with vector pBAD33 carrying the 3’ end of maflBMGI-1NEM013 gene. Inhibition of growth due to the toxin is counteracted by cognate immunity protein co-expression. LB agar plates contain 0.01 mM IPTG to induce expression of maflBMGI-1NEM013 cloned in pET28. Control strains contain empty vectors.

S5 Fig MaBMGI-1NEM013 is a secreted toxin. MaBMGI-1NEM013 (MaB) is detected in the whole-cell lysates (WC) and in the supernatant of NEM8013 strain expressing MaBMGI-1NEM013 under an IPTG inducible promoter. NEM8013 parental strain is used as a control (ctrl). When the background strain was a pBAD-derivative of NEM8013, MaBMGI-1NEM013 was also detected in the supernatant. Antibodies used for immunoblotting of WC and supernatants were Anti-NADP-GDH (NADP-dependent glutamate dehydrogenase, as a cytoplasmic marker protein) and an Anti-peptide that recognizes a C-terminal epitope of MaBMGI-1NEM013.

S6 Fig MaA is not required for MaB secretion and MaB is not secreted by E. coli. A) C-terminal FLAG-tagged MaBMGI-1NEM013 (MaB) is detected in the whole-cell lysate (WC) and in the supernatant of N. cinerea strain (Nc) expressing MaB under an IPTG inducible promoter. This IPTG inducible construction has been inserted in an intergenic region of N. cinerea chromosome using pGCG4 vector. N. cinerea parental strain is used as a control. B) MaBMGI-1NEM013 is detected in the whole-cell lysate and in the supernatant of a N. cinerea mutant where the whole maflABI locus (MGI-1Nc14685) has been replaced by a kanamycin resistance cassette. Antibodies used for immunoblotting of whole-cell lysates and supernatants were Anti-NADP-GDH (NADP-dependent glutamate dehydrogenase, as a cytoplasmic marker protein) and Anti-FLAG to detect C-terminal, FLAG-tagged MaB. A non-specific band detected with Anti-NADP-GDH antibody is indicated with an arrow. C) MaBMGI-1NEM013 is detected in the whole-cell lysate but not in the supernatant of E. coli BL21(DE3) transformed with pET28maflBMGI-1NEM013. Antibodies used for immunoblotting of whole-cell lysates and supernatants were Anti-MBP (Maltose Binding Protein, as a periplasmic marker protein) and an Anti-peptide that recognizes a N-terminal epitope of MaBMGI-1NEM013.

(SIF)

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