Neisseria meningitidis RTX Proteins Are Not Required for Virulence in Infant Rats

Stanislav Forman,[1] Irena Linhartova,[1] Radim Osicka,[1] Xavier Nassif,[2]
Peter Sebo,[1] and Vladimir Pelicic[2]*

*Laboratory of Molecular Biology of Bacterial Pathogens, Institute of Microbiology of the Czech Academy of Sciences,
CZ-142 20 Prague 4, Czech Republic,[1] and INSERM U570, Faculté de Médecine
Necker-Enfants Malades, 75015 Paris, France[2]*

RTX cytotoxins play an important role in virulence of numerous gram-negative pathogens. Unexpectedly, however, we show here that the RTX proteins of *Neisseria meningitidis* are dispensable for virulence in the infant rat model of infection.

The ability of *Neisseria meningitidis* to cause fulminant septicemic infections and large epidemics justifies the fear inspired by meningococcal disease. Moreover, with more than 500,000 cases each year, accounting for 50,000 deaths and significant morbidity, meningococcal infections remain one of the most serious medical emergencies worldwide (13). Paradoxically, *N. meningitidis* colonizes the nasopharynx of a substantial proportion of the world population, approximately 10%, without causing any symptoms (14). Occasionally, however, meningococci can cross the nasopharyngeal mucosa and gain access to the bloodstream, where they thrive by circumventing the host immune defenses and by acquiring iron from scavenger proteins such as hemoglobin, transferrin, and lactoferrin (6). Meningococcemia may further lead to crossing of the blood-brain barrier by the bacterium and development of life-threatening meningitis.
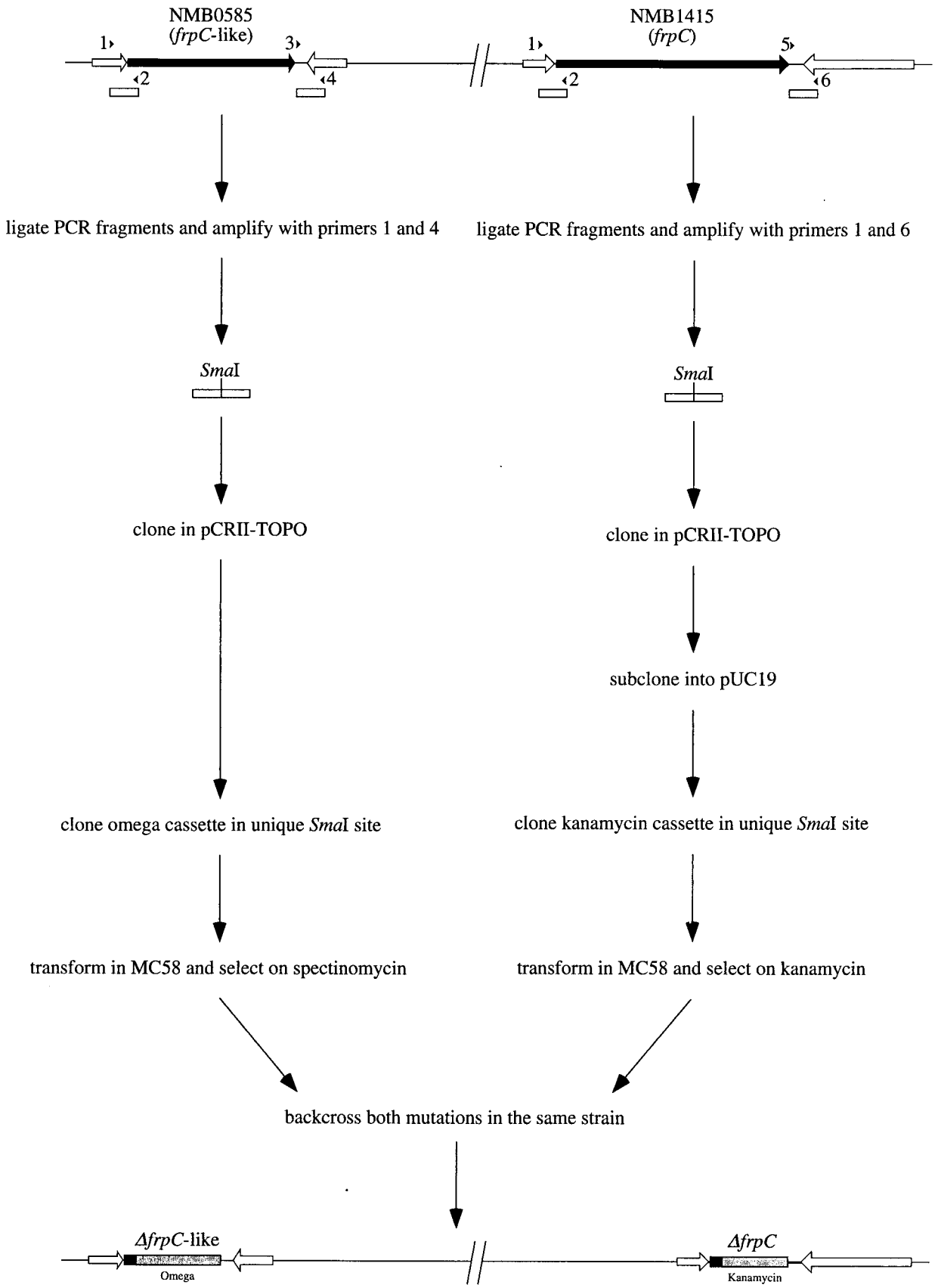
When bacterial pathogens are grown in iron-depleted conditions, similar to those encountered in the blood and mucosal secretions, where the levels of free iron are extremely low, the expression of a number of virulence factors is increased (6). Similarly, *N. meningitidis* expresses increased amounts of several proteins under iron-depleted conditions (2), most of which are involved in iron acquisition (6). Interestingly, in *N. meningitidis* FAM20, two of these Fe-regulated proteins (Frp), initially called FrpA and FrpC, were shown to be related to the RTX family of bacterial cytotoxins (10, 12). Like other members of this protein family, of which *Escherichia coli* alphahemolysin is the most studied prototype, these proteins contain, near their carboxy termini, copies repeated in tandem of a nonapeptide rich in glycine and aspartate (10, 12), which are usually implicated in binding of calcium and target cells. A further demonstration of their relationship to RTX toxins came from the observation that these proteins, which are found in the meningococcal outer membrane and the supernatant, can be secreted by the heterologous *E. coli* alphahemolysin secretory apparatus (11), which recognizes carboxy-

terminal secretion signals. Finaly, in a recent epidemiological study it was demonstrated that *frpC* alleles of various lengths were present in all invasive and most carrier strains of *N. meningitidis* (5). Moreover, the detection of FrpC-specific antibodies in the sera of patients recovering from invasive meningococcal disease demonstrated that FrpC-like protein is produced in vivo during infection (5).

Although their biological activity remains unknown, all the observations above pointed towards the involvement of FrpC-like proteins in meningococcal virulence. We therefore examined the capacity of an *frp* mutant to cause systemic infection in infant rats, the model of infection most widely used to study *N. meningitidis* genes required for disseminated infection (7, 8).

Construction of an MC58 *frpC* double mutant. The availability of the genomic sequence of *N. meningitidis* MC58 (9) made this serogroup B strain the obvious choice for our study. In this strain, six open reading frames (ORF)s, scattered around the genome, present homologies to *frpC* and constitute the paralogous FrpC family: NMB0365, NMB0585, NMB1403, NMB1405, NMB1409, and NMB1415. However, only two of the corresponding proteins contained the classical nonapeptide repeats near their carboxy termini and could therefore be considered bona fide RTX proteins. They were encoded by NMB0585 (3,906 nucleotides) and NMB1415 (5,487 nucleotides). In contrast with what was observed in FAM20 (10, 12), the two ORFs were almost identical throughout their entire sequence, except for three internal fragments missing in NMB0585, and therefore clearly represent two different *frpC* alleles. NMB1415 encodes the prototype FrpC protein, whereas NMB0585 encodes an FrpC-like protein. Therefore, in order to test the involvement of RTX proteins in meningococcal virulence, it was crucial to construct a double mutant where both *frpC* alleles would be disrupted. Due to the lengths of the target genes, it was preferable to construct mutants where the two genes would be precisely deleted in order to avoid any residual activity. To perform this, we used a PCR-ligation-PCR mutagenesis method (1) (Fig. 1). First, a 681-bp region upstream of each gene was amplified by using primer 1 (5′-GGTTTGGATAGCG TGGACGATA-3′) and primer 2 (5′-TACCCGGGTGCAACC

NMB0585
(*frpC*-like)

NMB1415
(*frpC*)

ligate PCR fragments and amplify with primers 1 and 4

ligate PCR fragments and amplify with primers 1 and 6

*Sma*I

*Sma*I

clone in pCRII-TOPO

clone in pCRII-TOPO

subclone into pUC19

clone omega cassette in unique *Sma*I site

clone kanamycin cassette in unique *Sma*I site

transform in MC58 and select on spectinomycin

transform in MC58 and select on kanamycin

backcross both mutations in the same strain

Δ*frpC*-like

Omega

Δ*frpC*

Kanamycin

CGATCAAATTTCTC-3′), which contained an underlined *Sma*I site overhang. It should be noted that the amplified regions were identical and comprised part of an ORF of unknown function, potentially cotranscribed with the *frp* genes. Next, we amplified the differing regions downstream from the two *frp* genes (Fig. 1). The 689-bp region downstream of the *frpC*-like allele was amplified by using primers 3 (5′-GGAGCCTAATTACATTCATGG C-3′) and 4 (5′-CGCTTGGGCAACTACCAAATG-3′), whereas the 684-bp region downstream of the *frpC* allele was amplified by using primers 5 (5′-GGAGCCTAATCACATTCATGGC-3′) and 6 (5′-GCCGCCGCAGCGACACCCTGCAAC-3′). The amplified fragments, upstream and downstream, for *frpC* and *frpC*-like, respectively, were ligated and subsequently cloned into the pCRII-TOPO vector (Invitrogen) (Fig. 1). A kanamycin resistance cassette was inserted between the two fragments amplified for the *frpC* allele, whereas the Ω fragment encoding resistance to streptomycin was inserted between the two fragments amplified for the *frpC*-like allele (Fig. 1). This allowed the construction of individual knockout mutants of either of the *frp* alleles by allelic exchange in MC58. Finally the double mutant was constructed by backcrossing the *frpC*-like mutation in the *frpC* mutant and selecting on plates containing both kanamycin and spectinomycin (Fig. 1). PCR and Southern blot analyses confirmed precise deletions of both NMB0585 and NMB1415 genes (data not shown). In addition, the strains were analyzed by Western blotting with an FrpC-specific rabbit serum. This demonstrated that the double mutant does not produce any RTX protein (Fig. 2).

In vitro characterization of the double mutant. As expected, the double mutant was not affected for growth in liquid GC base medium, confirming that the *frp* genes were dispensable for growth (data not shown). In addition, simple in vitro assays showed that the Frp proteins were neither involved in meningococcal serum resistance nor in adhesion to human endothelial cells, two phenotypes that are essential for virulence (14). The resistance of the double mutant to the bactericidal activity of the complement was tested as follows, by using a human serum obtained from a single individual: bacteria, grown overnight on GCB agar plates, were inoculated in 5 ml of RPMI medium and grown for 2 h at 37°C. Serum, RPMI medium, and bacteria were mixed in four-well NUNC plates (POLYLABO) so that the final 400-μl suspension contained 2×10^5 CFU in 25% serum (vol/vol). The reactions were incubated at 37°C in an atmosphere containing 5% CO_2. Samples were taken from the reaction mixtures at different time points, and serial dilutions were plated on GCB. Colony counts were performed after overnight incubation of the plates. We first confirmed that an unencapsulated mutant, known to be serum sensitive, was killed within minutes (data not shown). In contrast, the double *frpC* mutant resisted in human serum as well as the wild-type MC58 strain (data not shown). Adhesion of meningococci to human umbilical vein endothelial cells was measured as described previously (3). This showed that, after a 5-h
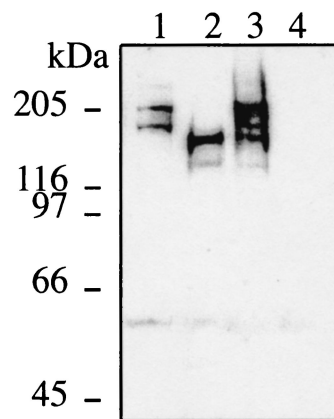

FIG. 2. Immunodetection of FrpC and FrpC-like proteins in MC58 wild-type and *frp* mutant strains. Strains were ΔNMB0585 (lane 1), ΔNMB1415 (lane 2), wild-type MC58 (lane 3), and *frp* double mutant (lane 4). The intermediate band (150 kDa) probably results from proteolytic processing, since meningococcal RTX proteins are known to be highly susceptible to proteolysis (12). A loopful of bacteria, grown overnight on GCB plates, was resuspended in 500 μl of RPMI medium, and 100 μl of this suspension was inoculated in 2 ml of RPMI medium. After 150 min of static culture at 37°C in an atmosphere containing 5% CO_2, 200-μl aliquots were inoculated in 2 ml of RPMI medium, to which the iron chelator desferrioxamine (desferal) was added at a 200 μM final concentration in order to induce the expression of the *frp* genes. After 2 h, the bacteria were harvested by centrifugation, resuspended in loading buffer containing 8 M urea, boiled for 5 min, and loaded on a sodium dodecyl sulfate–7.5% polyacrylamide gel. After electrotransfer to nitrocellulose membrane, the Frp-derived proteins were detected by using an Frp-specific rabbit serum raised against purified recombinant FrpC (5) and were revealed by enhanced chemiluminescence detection with an anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Amersham Biosciences).

contact with a monolayer of human umbilical vein endothelial cells, approximately 1.5×10^8 CFU ml^{-1} could be recovered for both the double *frpC* mutant and the MC58 parental strain, confirming that the *frpC* alleles are not important for adhesion to human endothelial cells.

In vivo analysis of the double mutant virulence. To determine whether the FrpC and FrpC-like RTX proteins were required for meningococcal virulence, the capacity of the double mutant to cause disseminated disease was assessed in the infant rat model of infection and compared to that of the parental MC58 strain. Lewis female rats with their litters of six or seven 4-day-old infants were obtained from Elevage JANVIER (France). Bacteria grown overnight on GCB agar plates were resuspended in 0.9% NaCl at different concentrations and were injected intraperitoneally, in 100-μl samples, to a total of six or seven infant animals coming from two different litters. The mortality rate, which was monitored over a 24- to 30-h course, was repeatedly found to be comparable in rats

FIG. 1. Scheme of the construction of an MC58 *frpC* double mutant by PCR-ligation-PCR mutagenesis (1). The unique *Sma*I site was present within primer 2. The omega cassette was amplified from pT1Ω1E (4), whereas the kanamycin casette was obtained by amplifying the *aphA-3* gene. The subcloning step, for the construction of the *frpC* mutant, was necessary because the pCRII-TOPO vector contains a kanamycin resistance gene.
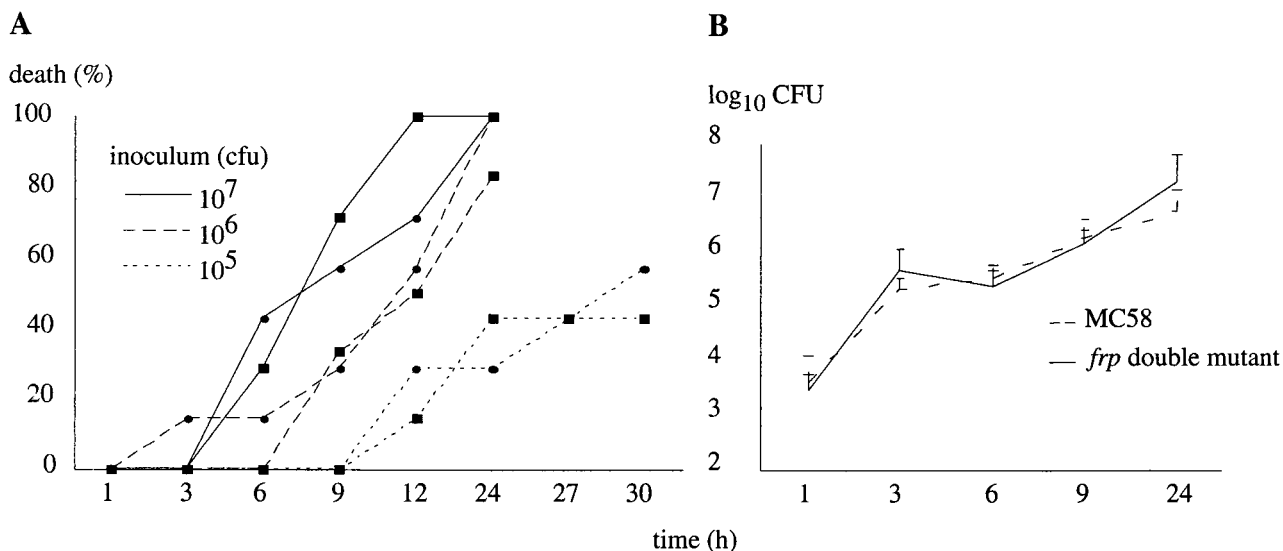
A

B

FIG. 3. Time course of death (A) and meningococcemia (B) in 4-day-old infant rats inoculated with different doses of the MC58 parental strain (■) and the isogenic *frp* double mutant (●). Mortality experiments, performed on a total of six or seven infant animals, were repeated twice, and representative results are shown. For the meningococcemia quantification experiment, six animals were injected with inocula of 7.7×10^5 and 6×10^5 CFU for the wild-type and the *frp* double mutant, respectively. Bars indicate standard errors of the means.

inoculated by the parental strain and the double *frpC* mutant (Fig. 3A). In parallel, the kinetics of meningococcemia caused by each strain was determined by counting the number of bacteria recovered from the blood of the infected animals after 3, 6, 9, and 24 h of infection. Five-microliter blood samples were taken from the tail of the surviving animals at the different time points and resuspended in 95 μl of phosphate-buffered saline, and 25 μl of serial dilutions was plated on GCB. Colony counts were performed after overnight incubation of the plate contents. As shown in Fig. 3B, no obvious difference in the course of bacteremia was seen between the wild-type strain and the double *frpC* mutant.

Despite numerous clues that point to a role in pathogenesis for the FrpC and FrpC-like proteins, we show here that an *N. meningitidis* MC58 isogenic double mutant, which does not produce these RTX-like proteins, is as virulent as the wild type in an infant rat model of infection. This suggests that these proteins do not appear to possess a toxin activity affecting the complement and/or phagocyte activities of the host, which would be necessary for systemic infection, because such activities would have been most likely detected in the used animal model. However, we cannot exclude that FrpC proteins might play a role in other steps essential for meningococcal virulence, such as colonization and dissemination in the nasopharynx or escape from the endocytic vacuoles, which could not be unraveled in the infant rat model of septicemia. Hence, it remains possible that in a more sensitive or adequate animal model, which is still sorely missing, a subtler contribution of the FrpC proteins to meningococcal pathogenesis could be detected. Therefore, if new animal models of infection for *N. meningitidis* become available in the future, the role of the RTX proteins in meningococcal virulence should be assessed again.

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