NarE: a novel ADP-ribosyltransferase from *Neisseria meningitidis*

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Summary

Mono ADP-ribosyltransferases (ADPRTs) are a class of functionally conserved enzymes present in prokaryotic and eukaryotic organisms. In bacteria, these enzymes often act as potent toxins and play an important role in pathogenesis. Here we report a profile-based computational approach that, assisted by secondary structure predictions, has allowed the identification of a previously undiscovered ADP-ribosyltransferase in *Neisseria meningitidis* (NarE). NarE shows structural homologies with *E. coli* heat-labile enterotoxin (LT) and cholera toxin (CT) and possesses ADP-ribosylating and NAD-glycohydrolase activities. As in the case of LT and CT, NarE catalyses the transfer of the ADP-ribose moiety to arginine residues. Despite the absence of a signal peptide, the protein is efficiently exported into the periplasm of *Neisseria*. The *narE* gene is present in 25 out of 43 strains analysed, is always present in ET-5 and Lineage 3 but absent in ET-37 and Cluster A4 hypervirulent lineages. When present, the gene is 100% conserved in sequence and is inserted upstream of and co-transcribed with the lipoamide dehydrogenase E3 gene. Possible roles in the pathogenesis of *N. meningitidis* are discussed.

Introduction

Mono ADP-ribosylation is a post-translational modification of proteins, shared by eukaryotes and prokaryotes, which

modulates protein function (Ueda and Hayaishi, 1985). Mono-ADP-ribosyltransferases (ADPRTs) catalyse the transfer of the ADP-ribose group of β-nicotinamide adenine dinucleotide (NAD⁺) to a specific amino acid in target protein acceptors with the simultaneous release of nicotinamide (Nam) (Althaus and Richter, 1987).

In pathogenic bacteria, proteins known to belong to this class of enzymes are generally classified as toxins, as they are able to modify or disrupt essential functions of eukaryotic cells (Wels *et al.*, 1992; Ward *et al.*, 1994). Well-known examples are represented by cholera toxin (CT) (Mekalanos *et al.*, 1983), *E. coli* heat-labile enterotoxin (LT) (Yamamoto *et al.*, 1984) and pertussis toxin (PT) (Locht and Keith, 1986), which interfere with signal transduction by ADP-ribosylating regulatory G proteins, and diphtheria toxin (DT) (Pappenheimer, 1977), and *Pseudomonas* exotoxin A (PAETA) (Gray *et al.*, 1984), which inactivate protein synthesis by ADP-ribosylation of elongation factor 2 (EF-2).

A second family of related proteins, which contains *Clostridium botulinum* C2 toxin (Aktories *et al.*, 1986), *C. perfringens* iota toxin (Schering *et al.*, 1988), and *C. difficile* toxin (Popoff *et al.*, 1988) use actin as the acceptor molecule, and interfere with actin polymerization.

All these toxins belong to the class of AB proteins, where the A domain is the toxic moiety that carries the active site, whereas the B domain is required for receptor-binding and for translocation of fragment A across the host's cell membrane. The enzymatic and receptor-binding domains can be located on the same polypeptide, as in the case of DT and PAETA, or on different subunits, as in the case of LT, CT and PT. When located on different subunits, the A and B polypeptides are expressed with signal peptides and are exported across the cytoplasmic membrane. After cleavage of the signal peptides, the two proteins are released into the periplasmic space, where they assemble into the mature holotoxin (Hirst and Holmgren, 1987). Whereas LT remains in the periplasm, CT and PT are released in the culture supernatant.

Another group of ADP-ribosylating proteins, named the 'A-only' toxins, lacks the B domain and follows a different pathway to be delivered to host cells. This is the case of exoenzyme S (ExoS) of *P. aeruginosa*, which has been shown to be directly injected into eukaryotic cells by a specialized type III secretion system (Frithz-Lindsten *et al.*, 1997), exoenzyme C3 of *Clostridium botulinum*

(Nemoto *et al.*, 1991), EDIN of *Staphylococcus aureus* (Sugai *et al.*, 1990) and the toxins of *Bacillus cereus* (Just *et al.*, 1995) and of *Clostridium limosum* (Just *et al.*, 1992) for which the mechanism of cell entry is still unknown.

Despite the lack of significant primary sequence homology among members of this family of prokaryotic and eukaryotic enzymes, the crystal structures of several of them have revealed a common topology of the enzymatic domain, accompanied by the strict requirement of a few key catalytic residues (Douglas and Collier, 1987; Antoine *et al.*, 1993; Domenighini *et al.*, 1994; Han *et al.*, 1999). On the basis of sequence analysis, the catalytic subunits have been divided into two distantly related groups: the DT-group, characterized by conserved His and Glu catalytic residues, which includes diphtheria toxin, Pseudomonas exotoxin A and the human PARPs, and the CT-group, characterized by conserved Arg and Glu catalytic residues, which includes LT, PT and all the other known ADPRTs, most of them of bacterial origin (Domenighini and Rappuoli, 1996; Pizza *et al.*, 1999).

Because these toxins play a key role in pathogenesis and are often the main virulence factors produced by bacteria, they have been extensively characterized for their activity and have been used as component of vaccines able to prevent each related disease. Moreover, because of their ability to profoundly affect cellular processes, they have found wide application in cellular and molecular biology including the killing of specific cells and the identification of specific target proteins involved in signal transduction and cytoskeleton functions (Pizza *et al.*, 1989; Rappuoli, 1997; Douce *et al.*, 1998; Koch-Nolte *et al.*, 2001).

For all these reasons and encouraged by the availability of a growing number of sequenced bacterial genomes, a series of studies have recently come out, which aimed at the computer-based identification of novel members of this family of enzymes (Otto *et al.*, 2000; Pallen *et al.*, 2001).

The strategy used for these analyses was based on the screening of non-redundant protein databases and of Glimmer-predicted ORFs from unfinished genomes by means of the position-specific iterative BLAST (PSI-BLAST) algorithm (Altschul *et al.*, 1997) using as input the whole-length sequences of known ADP-ribosyltransferases.

As a result, more than 20 new putative ADP-ribosyltransferases have been identified both in Gram-positive and Gram-negative finished and unfinished genomes, including five from *Pseudomonas syringae*, five from *Burkholderia cepacea*, two from *Enterococcus faecalis* and one each from *Salmonella typhi, Streptococcus pyogenes*, *Mycoplasma pneumoniae, Streptomyces coelicolor*, *Bacillus halodurans* and *Vibrio parahaemolyticus*. With the exception of the protein detected in Salmonella, which is adjacent to an ORF similar to the S2 subunit of

pertussis toxin, all the other genome-derived putative ADPRTs lack a predicted receptor-binding domain.

In this work we report the results of a novel type of computer-based analysis, where criteria of amino acid pattern recognition have been used cooperatively with secondary structure prediction methods to highlight putative ADP-ribosylating enzymes in bacterial genomes.

By this novel two-step strategy, not only have we found most of the hits previously detected by Pallen and colleagues, but also other gene products that very likely belong to this family of enzymes and that were missed by previous searches.

Here we describe a novel ADP-ribosyltransferase identified in strain MC58 of *N. meningitidis,* serogroup B. By biochemical characterization of the '*in silico*' predicted ADP-ribosyltransferase we show that NarE (*Neisseria* ADP-*r*ibosylating *e*nzyme) is able to hydrolyse NAD and to transfer the ADP-ribose group to small guanidino compounds like agmatine and arginine analogues. Interestingly we found that, despite the absence of a predicted leader peptide, NarE efficiently accumulates in the periplasm of Meningococcus. Finally, we found that the *narE* gene is present only in a subset of hypervirulent lineages of Meningococcus. The possible role of this novel toxin-like molecule of Meningococcus is discussed.

Results

Identification in Neisseria meningitidis *B of NarE, a protein sharing motifs and topology of ADP-ribosylating toxins*

Bacterial ADP-ribosylating enzymes do not share extended amino acid conservation but they have a conserved scaffold of the active site. This is composed by a beta-strand followed by a slanted alpha-helix, which form the floor and the ceiling of the catalytic cavity respectively (Fig. 1A, region 2). This central domain, which binds NAD, is flanked by two antiparallel beta-strands (Fig. 1A, regions 1 and 3), which carry the two catalytic residues, an arginine (a histidine in the DT group), and a glutamic acid, which is conserved in all toxins (Domenighini and Rappuoli, 1996). In the case of the ADP-ribosylating toxins of the CT group, region 2 has an additional conserved region characterized by the consensus Ser-Thr-Ser/Thr preceded by an aromatic and an hydrophobic residue. ADP-ribosylating enzymes of the DT-group have a different consensus, characterized by two conserved Tyr spaced by 10 amino acids.

Pallen and colleagues have adopted a whole-length homology-based strategy to search for distantly related members of this family of bacterial toxins in finished and unfinished genomes.

We have applied a novel strategy designed to take into account only the constant features of known bacterial
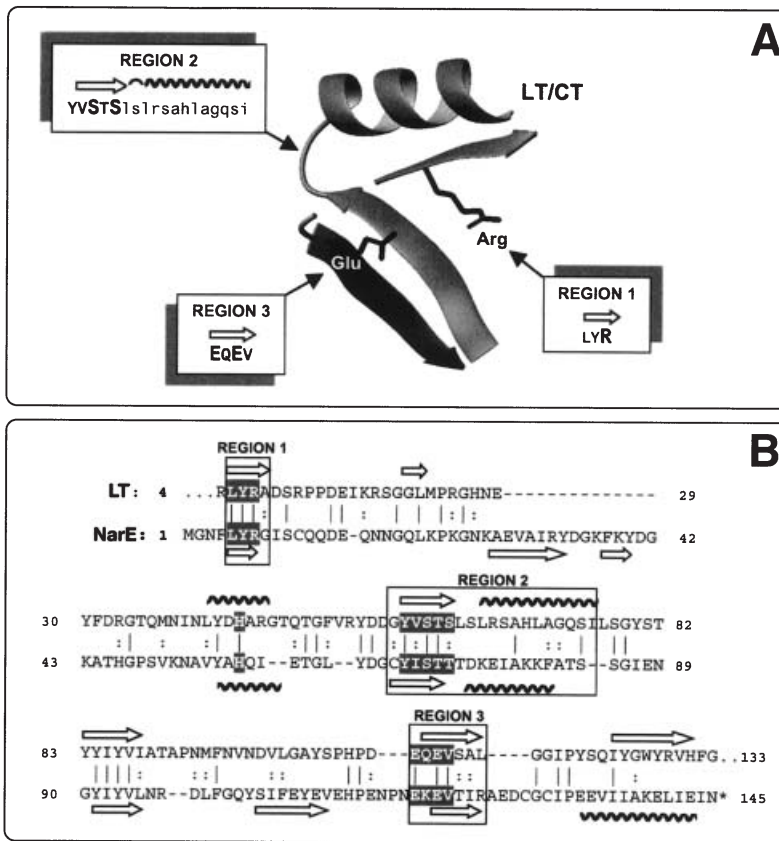
Fig. 1. Catalytic site of LT and sequence alignment with NarE.
A. Three-dimensional structure of the three regions that make up the conserved catalytic site of bacterial ADP-ribosyltransferases (as a reference, only the LT structure is reported here). The catalytic Arg and Glu residues are shown, whereas the whole primary sequences of the three domains are reported in boxes. The strictly conserved residues that we have used to build the amino acid consensus are indicated in bold.
B. Primary and secondary structure alignment of NarE with the sequence and observed secondary structure of the A subunit of toxin LT.

ADP-ribosyltransferases, regardless of overall sequence similarity. These features include the primary sequence conservation of the active site residues, their relative distance along the protein sequence, and the maintenance of the secondary structure elements where the catalytic residues are localized.

Based on the consensus motifs generated from the alignments of the CT- and DT-group of toxins (Pizza *et al.*, 1999) we designed two sequence patterns (see *Experimental procedures*) that have been used to screen several bacterial genomes. By this strategy we have identified most of the genes already described by Pallen and colleagues. Furthermore, when the patterns were used to search the genome of *N. meningitidis* strain MC58 (Tettelin *et al.*, 2000), the CT-group derived consensus found three hits. These were the twitching motility protein NMB0051, the type IV pilus assembly protein NMB0329, and NMB1343, annotated as hypothetical protein.

Analysis of the secondary structure of these proteins showed that only in NMB1343 the predicted catalytic residues are localized on secondary structure elements compatible with the formation of a properly folded active site cleft. The other two hits are therefore to be regarded as false positives. The full sequence of the NMB1343 protein, which we have named NarE (*Neisseria* ADP-*r*ibosylating enzyme), is shown in Fig. 1B, aligned with the catalytic subunit of the LT toxin. As shown in this figure, the structural features of the three regions forming the catalytic site are conserved in NarE, including the Arg residue of region 1 located in position 7 within a predicted short beta strand (followed by a segment of weak sequence homology to LT), and the Glu118-Glu120 located in region 3 on a predicted beta strand. Furthermore, another amino acid which is conserved in NarE is the histidine in position 57, which corresponds to His 35 in PT and to His 44 in LT and CT (Fig. 1B). This residue is located near the beginning of the beta-strand which forms the floor of the cavity and has been considered important for catalysis and associated with recognition of the acceptor substrate (Pizza *et al.*, 1989; Xu *et al.*, 1994; Jobling and Holmes, 2001). Surprisingly, differently from LT and from most ADP-ribosylating toxins, NarE lacks a predicted signal peptide.

NarE displays both ADP-ribosyltransferase and NAD-glycohydrolase activities

NarE was expressed in *E. coli* as a C-terminal Histidine-tag fusion protein. Under reducing conditions the protein migrates as single band of about 20 kDa (data not shown).

Table 1. ADP-ribosyltransferase and NAD-glycohydrolase activities of recombinant NarE.

	ADP-ribosylagmatine formed (nmoles h^{-1})	Nicotinamide released (nmoles h^{-1})
Without agmatine	N.D.	8.5 ± 0.35
Plus 20 mM agmatine	6.7 ± 0.87	15.2 ± 0.53
Plus 75 mM agmatine	18.1 ± 0.58	25.9 ± 0.44

Transferase activity of NarE was assayed in 300 µl of 50 mM potassium phosphate (pH 7.5) containing 0.1 mM radioactive NAD using 55.6 µg of purified recombinant protein with or without 20 or 75 mM agmatine for 1 h at 30°C. Data are means ± S.E. ($n = 3$). ND, not detectable.

The purified recombinant protein was used in the ADP-ribosylation assay, with agmatine as ADP-ribose acceptor. The results, reported in Table 1 show that the ADP-ribosylation activity was detectable, using either 20 mM or 75 mM agmatine. In addition, the rate of formation of ADP-ribosylagmatine closely correlates with the rate of release of nicotinamide. In absence of agmatine, the NarE was able to hydrolyse NAD and release nicotinamide. These data suggest that NarE has both ADP-ribosyltransferase and NAD-glycohydrolase activities and that, in presence of an ADP-ribose acceptor, it acts as a transferase whereas in absence of the acceptor it acts as a NAD glycohydrolase. Furthermore, as shown in Table 2, using increasing amounts of NarE, the nanomoles of nicotinamide released are almost identical to those of ADP-ribosylagmatine formed (molar ratio of 1) suggesting that NarE is able to perform ADP-ribosyltransferase and NAD-glycohydrolase activities with comparable efficiency.

To verify whether the ADP-ribosylation of agmatine was induced by an enzymatic, and not by a non-enzymatic addition of ADP-ribose, the reaction was performed in presence of 2 or 5 mM free ADP-ribose. ADP-ribosylation of agmatine was not affected by the addition of free ADP-ribose, consistently with the conclusion that the transfer of ADP-ribose to agmatine is due to an enzymatic reaction (data not shown).

Table 2. Formation of ADP-ribosylagmatine and release of nicotinamide at different concentrations of NarE.

NarE (µg)	NADase (nmoles of nicotinamide released)	ADPRT (nmoles of ADP-ribosylagmatine formed)	NADase/ADPRT (ratio)
7	0.8	0.8	1.0
14	1.9	2.0	0.9
28	4.2	4.5	0.9

The ADP-ribosyltransferase activity was assayed in a reaction mix containing 50 mM potassium phosphate at pH 7.5, with or without 20 mM agmatine in a total volume of 0.3 ml as described in the *Experimental procedures*. After 5 h at 30°C the radioactive compounds were purified as described in the *Experimental procedures*. All assays were run in duplicate.

Amino acid specificity and effect of DTT and Mg^{2+} on ADP-ribosyltransferase and NAD-glycohydrolase activities

Amino acid specificity of NarE was evaluated by monitoring the rate of release of nicotinamide induced in presence or absence of different amino acids as potential ADP-ribose acceptors. As shown in Fig. 2, the rate of nicotinamide release was increased in the presence of arginine and agmatine, further confirming that NarE catalyses the formation of ADP-ribose-linkage with arginine or arginine-analogues like agmatine. On the other hand, no difference was detected in the presence of glycine, serine, proline or, more interestingly, in the presence of histidine or lysine, which are known to be potential acceptors for ADP-ribose.
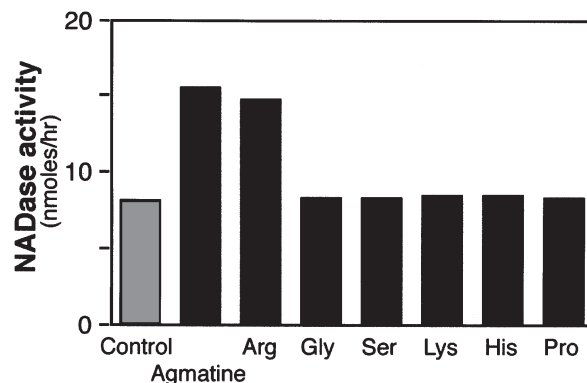
Fig. 2. ADP-ribose acceptors of NarE. NAD-glycohydrolase activity using different amino acids as acceptors. The activity was measured as amount of [carbonyl^{14}C]nicotinamide released from [carbonyl-^{14}C]NAD.

Many bacterial ADP-ribosylating toxins undergo enzymatic activation following reduction of a disulfide bridge (Mekalanos *et al.*, 1979). The presence of four cysteine residues in the primary structure of NarE prompted us to investigate whether it needs to be activated by sulphydryl agents and Mg^{2+}. The results of this experiment show that the formation of ADP-ribosylagmatine was not influenced by the presence of DTT or Mg^{2+} (data not shown), and therefore suggest that the ADP-ribosyltransferase is DTT- and Mg^{2+}-independent, in contrast to cholera (Moss *et al.*, 1980) and pertussis toxins (Moss *et al.*, 1983).

To evaluate the relative activity of NarE, purified cholera toxin was used in the assay. In presence of Mg^{2+} and DTT, the ADP-ribosylation activity of cholera toxin was 75-fold higher than NarE activity (data not shown). The lower activity of NarE could be due to a non-properly correct folding of the recombinant protein or to the requirement of ADP-ribosylation factors.

narE *is present in a subgroup of hypervirulent* Neisseria *clusters*

By computer search we were unable to identify *narE*

homologues either in the published genome sequence of serogroup A (Parkhill *et al.*, 2000), and in the complete sequence of the serogroup C FAM18 strain of Meningococcus (http://www.sanger.ac.uk/Projects/N_meningitidis/blast_server.shtml) suggesting that the gene is not ubiquitous in *Neisseria* species.

This finding prompted us to investigate the presence of *narE* in a more consistent number of meningococcal strains. For this purpose, we selected 43 *Neisseria* isolates, representative of different serogroups and hypervirulent clusters, isolated both from cases of invasive disease and from healthy carriers (Comanducci *et al.*, 2002). In particular, this panel of strains includes 12 strains belonging to the ET-5 complex, three to ET-37, four to Cluster A4, four to Lineage 3, one to subgroup III, 17 strains classified as 'others' and two strains of *Neisseria gonorrhoeae*.

Specific primers were used to amplify the gene from chromosomal DNA of the 43 strains. The presence or absence of the gene was discriminated on the basis of the size of DNA fragments obtained by PCR, of 660 and 190 bp respectively (data not shown). Southern blot experiments were performed on the same panel of strains to rule out the possibility that the *narE* gene could be located somewhere else in the genome.

The results show that in Meningococcus the gene is present in seven out of the 17 strains classified as 'other' or not ET-typed, it is always present in strains belonging to the ET-5 complex and Lineage three hypervirulent clusters, but always absent in strains belonging to cluster A4 and ET-37 complex. This is consistent with the absence of the gene in the genome sequence of serogroup C strain FAM18, which belongs to the ET-37 complex.

When present, the gene was sequenced in all strains and shown to be 100% conserved. In the case of Gonococcus, although the gene is present and conserved in all strains at a level of 99% identity, the duplication of a tetra nucleotide (TTAT) occurs 12 bases downstream the predicted ATG site, thus causing the premature interruption of the gene after eight codons. This result suggests that *narE* could represent a pseudogene in Gonococcus.

To verify the level of expression of NarE in different strains, total cell extracts of five PCR positive strains, one PCR negative strain and one gonococcus strain were analysed by Western blot analysis. The results of this analysis showed that NarE is always expressed when its coding gene is present, without significant variation in the expression levels whereas as expected, the protein is absent in the PCR negative strain and in Gonococcus, consistently with the sequence data (data not shown).

narE *gene locus*

In MC58, the *narE* gene is located between the genes

nmb1342 and *nmb1344*, which code for the dihydrolipoyl acetyltransferase (E2) and the dihydrolipoyl dehydrogenase (E3) respectively.

A comparative analysis was performed on the region encompassing the *narE* gene between the positive MC58 and the negative Z2491 strains (Fig. 3). The results of this analysis revealed that the MC58 strain has an insertion of 548 bp, which includes the 438 bp *narE* coding region flanked upstream by a 93 bp segment containing the putative promoter, and downstream by 17 bp containing the putative ribosome-binding site (RBS, 5′-AAGG-3′) of the following *nmb1344* gene. No predicted RBS could be identified upstream of *narE*.

The whole insertion is characterized by a GC content of 35%, surprisingly low with respect to the average GC composition of 51.5% calculated for *N. meningitidis*. The sequence of this region in the other strains carrying the gene, also validated by Southern Blot data, confirms that when present, *narE* is always located at the same genome locus.

In strain Z2491, where the gene is absent, the whole segment is replaced by a stretch of 77 bp, which contains two inverted repeats and a putative ribosome-binding site (5′-AAGA-3′) for the *nmb1556* gene, slightly different from the one detected in MC58 (Fig. 3). Surprisingly, a comparison of the 77 bp of strain Z2491 with the corresponding region of FAM18 (the serogroup C strain), which also lacks the gene, has revealed that this element is poorly conserved (73% identity).

Analysis of the sequence immediately adjacent to the *narE* insertion, indicates the presence of a pair of 5 bp direct repeats (5′-TATAA-3′), which superimpose with the stop codon of *nmb1342* and with the start codon of gene *nmb1344* respectively. In strain Z2491, where the insertion is not present, only one disrupted pentanucleotide can be identified.

Nmb1342 and *nmb1344*, together with *nmb1341,* the gene coding for pyruvate dehydrogenase/decarboxylase (E1), constitute the three components of the pyruvate dehydrogenase multienzymatic complex, conserved in several prokaryotic and eukaryotic organisms. The genes coding for these components, also known as E1, E2 and E3, are generally adjacent on bacterial chromosomes, however, they are not always co-transcribed, as it has been shown in the case of *E. coli*, where an independent promoter has been mapped upstream of the E3 coding gene (Stephens *et al.*, 1983). On the other hand, *Rhodobacter capsulatus* produces a unique transcript for the pyruvate dehydrogenase multienzyme complex (Dastoor *et al.*, 1997).

Here, the position of *narE* and the identification of putative −10 and −35 promoter elements upstream of the 5′ end of the gene are consistent with the possibility that
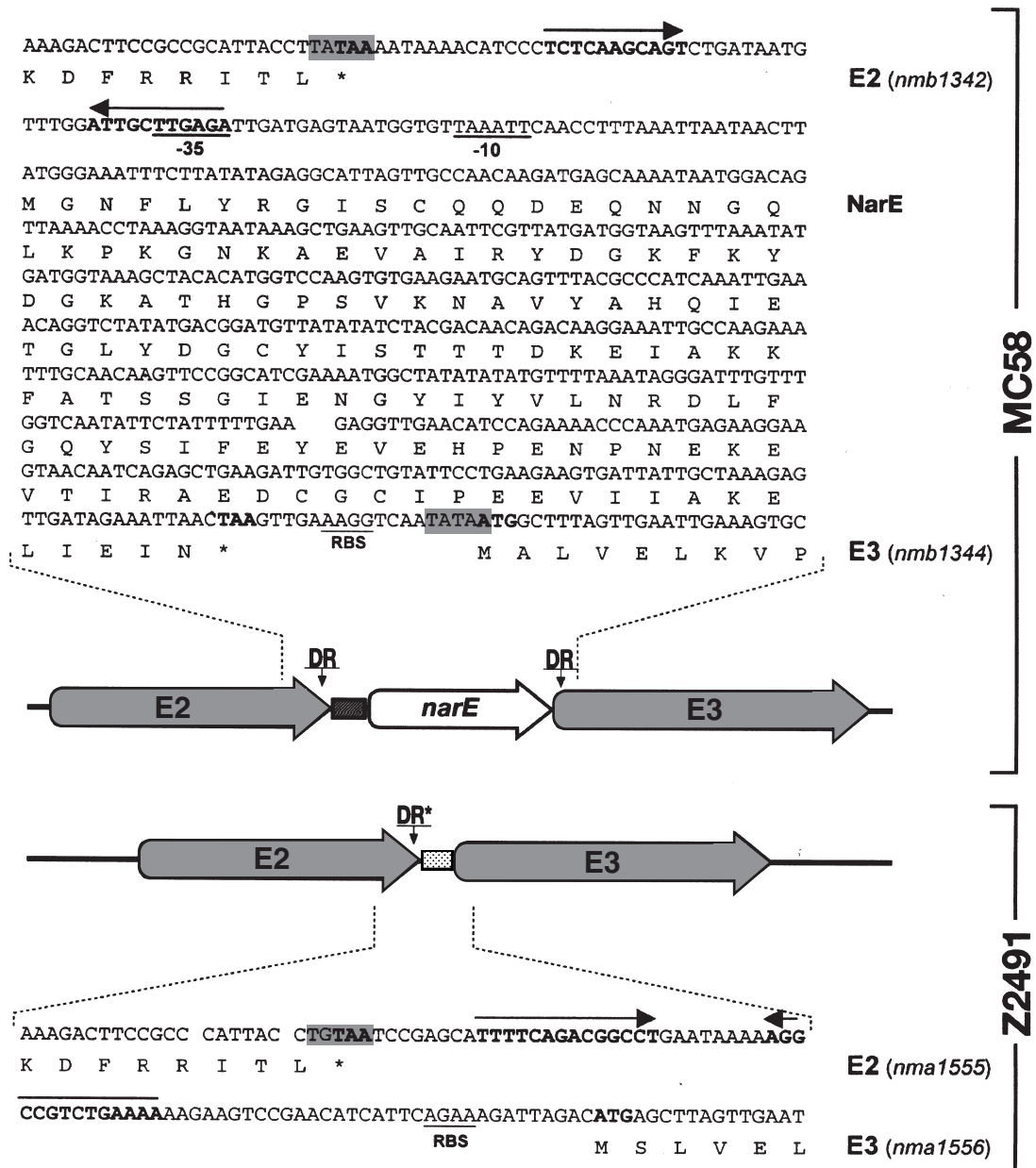
Fig. 3. Genetic organization and schematic representation of the intergenic region between E2 and E3 in MenB strain MC58 and MenA strain Z2491 genomic sequences. Putative −10 and −35 promoter elements are indicated. The inverted repeats downstream of *nmb1342* (E2) are in bold. The pentanucleotide direct repeats (DR) possibly involved in the mechanism of recombination are shaded and indicated by arrow in the scheme (in Z2491 only a single disrupted copy is left, DR*). The predicted ribosome binding site (RBS) of *nmb1344* (E3) is shown.

narE and the gene for E3 (*nmb1344*) might be co-transcribed in MC58 (Fig. 3).

To verify this prediction, Northern blot analysis has been performed using the full-length *narE* gene as probe on the total RNA prepared from three positive and two negative *N. meningitidis* strains. The three positive strains were of the ET-5 complex (72/00, 220173i and MC58), whereas of the two negative strains, one was of Cluster A4 (2996), and the other was an unclassified strain (8047).

As shown in Fig. 4, in the positive strains (lanes 1–3) the length of the *narE* transcript is around 2.37 kb, consistent with the hypothesis that *narE (*438 bp) and *nmb1344* (1785 bp) are co-transcribed in these strains. As expected, no signal is present in the two strains that lack the *narE* gene (lanes 4 and 5).

The fact that no gene coding for putative receptor binding domain could be identified in the vicinities of *narE*, suggests that this putative ADP-ribosyltransferase might
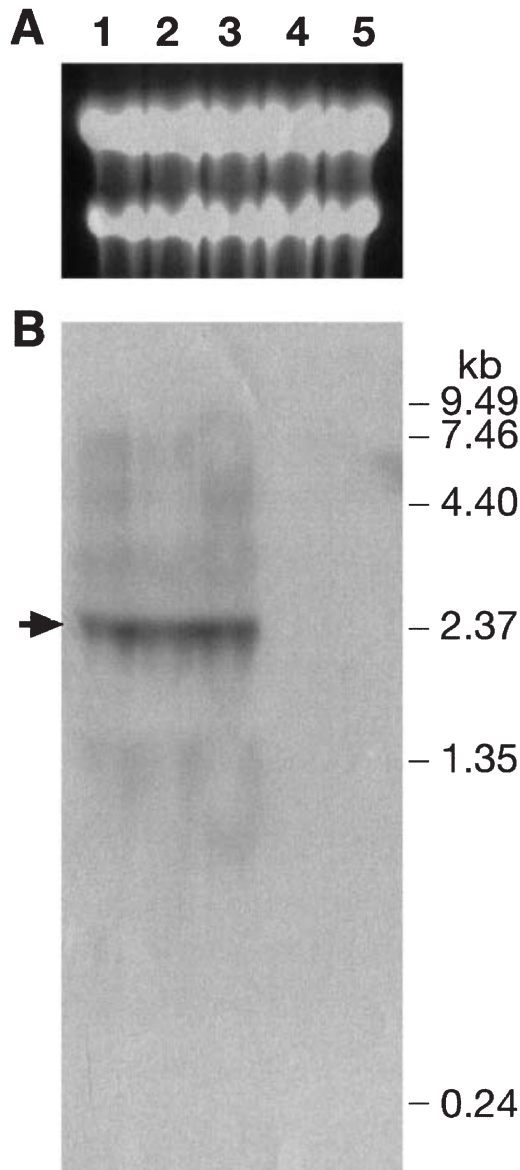
Fig. 4. Analysis of *narE* mRNA in *N. meninigitidis*. Northern blot analysis of *narE* specific transcript in five *N. meningitidis* strains: lane1, strain 72/00; lane 2, strain 220173i; lane 3, strain MC58; lane 4, strain 2996; lane 5 strain 8047. The arrow indicates the band corresponding to the mRNA transcript for *narE* plus *nmb1344*. Total RNAs were isolated from these strains and 40 µg were separated in denaturing agarose gel (A), blotted and hybridized to a *narE* specific probe (B). The positions of a 0.24–9.5 kb RNA marker (Invitrogen) are indicated on the right.

belong to the 'A-only' type of toxins, as is the case of the *Pseudomonas aeruginosa* exoenzyme S (ExoS) and of other toxins belonging to this family.

NarE is secreted in the periplasmic space

To assess the cellular localization of NarE in Meningococcus, Western blot analyses were performed on cellular extracts of selected strains where the gene is or is not present. In particular, MC58 was taken as representative of a *narE* positive strain, whereas 2996 was chosen as negative control. For both isolates, whole-cell lysates, cytoplasmic, periplasmic and culture supernatant fractions obtained from bacteria in the exponential phase of growth ($OD_{620} = 0.45$) were probed using an antiserum raised against the recombinant NarE protein. As expected, reactive bands were detected in the total extract and cytoplasmic preparations of MC58 (Fig. 5A, lanes 3 and 4) at the same molecular weight of the purified NarE (Fig. 5A, lane 1), whereas no corresponding band was present in the whole-cell lysate of the negative strain 2996 (Fig. 5A, lane 2). Interestingly, a band of the same size was also detected in the periplasmic fraction of MC58, while the supernatant was clean (Fig. 5A, lanes 5 and 6). The two bands of higher molecular weight, present also in the negative strain 2996, represent unidentified cross-reacting proteins.

To assess the genuine nature of the periplasmic preparations we used both positive and negative controls. As shown in Fig. 5B, antibodies raised against the periplasmic protein DsbA (NMB0407) recognized a band of the expected molecular weight in the total extracts and in the periplasm of MC58, but not in the supernatant. On the other hand, to exclude the possibility of non-specific lysis of bacteria and of contamination from membrane components, the same samples were tested using antibodies against the cytoplasmic protein Fur (NMB0205) and the outer membrane protein PorA (NMB1429) (Fig. 5B). In both cases, no reactivity could be observed both in the periplasmic preparations and in the culture supernatants. This finding is consistent with the observation that when expressed as a full-length gene in *E. coli*, NarE is clearly compartmentalized in the periplasm of *E. coli* (data not shown). Taken together, these data indicate that, despite the lack of a clear signal for secretion, NarE is able to cross the inner membrane and to localize in the periplasm both in *E. coli* and *Neisseria*. This observation would suggest that the exportation system, although non-canonical, is conserved between the two species. However, the molecular mechanism involved in the process of secretion is not clear and will be the subject of further investigation.

Discussion

ADP-ribosyltransferases represent a class of enzymes which are usually very important for the pathogenesis of the microorganisms that produce them. The availability of the sequence of bacterial genomes has allowed the identification of several new putative members of the family using conventional methods of homology search (Pallen *et al.*, 2001). However, given the importance of this class

Fig. 5. Cellular localization of NarE in *N. meningitidis*.
A. Lane 1, purified NarE; lanes 2 and 3, whole cell extracts of 2996 and MC58; lanes 4, 5 and 6, cytoplasmic, periplasmic and culture supernatant preparations from MC58. All samples were probed using the anti-NarE antiserum.
B. Insert 1. Total extract (lane 1), periplasmic (lane 2) and supernatant (lane 3) fractions of MC58 probed with antibodies raised against DsbA. Insert 2. Total extract (lane 1), cytoplasmic (lane 2), periplasmic (lane 3) and supernatant (lane 4) fractions of MC58 probed with antibodies raised against Fur. Insert 3. Total extract (lane 1), periplasmic (lane 2) and supernatant (lane 3) fractions of MC58 probed with antibodies raised against PorA.

of enzymes, we have decided to use more sophisticated algorithms to identify novel members of the family.

For this purpose, we have developed a novel pattern-based strategy, which, flanked by secondary structure prediction analysis, has allowed to identify putative bacterial ADP-ribosylating enzymes that were missed by previously used homology-based approaches. In *N. meningitidis*, although three hits were found with the amino acid pattern, only one could match the secondary structure requirements. This result confirms that the two steps of the computer approach are both required and equally important for the prediction of novel ADP-ribosyl-transferases.

In this work, we have described the sequence analysis and enzymatic properties of NarE, a novel ADP-ribosylating enzyme detected in the MC58 genome of *N. meningitidis.* The protein has all the important features of the enzymes, including the conserved catalytic residues (arginine in position 7 and a glutamic acid in position 120) and a conserved region 2. In addition, the histidine in position 57, corresponds to His35 of PT and to His44 of CT and LT. Interestingly, this amino acid, present only in a subset of ADP-ribosylating toxins, has been proposed to be required for catalysis in the group of proteins where it is present and has been associated with recognition of the acceptor substrate. This could imply that NarE acts on the same class of substrates used by PT, CT and LT.

In vitro, the protein has ADP-ribosylation and NAD-glycohydrolase activities and is able to transfer the ADP-ribose moiety specifically to an arginine residue. However, it is not obvious which is the target of the enzyme *in vivo*.

Interestingly, the gene coding for NarE is carried on a DNA segment characterized by an extremely low GC content. This observation and the finding that *narE* is present in a subset of Meningococcal strains indicates that this gene could have been acquired by unrelated bacterial sources via a mechanism of lateral gene transfer. This is consistent with the fact that several bacterial ADP-ribosylating toxins are coded within mobile genetic elements, as in the case of the DT and CT encoding genes, which are carried on bacteriophages, and the LT-I encoding gene, which is located on a plasmid. Furthermore, the strict conservation of the nucleotide sequence of *narE* would suggest either a recent acquisition of the gene and/or a remarkably high selective pressure in keeping the same protein sequence (Caugant, 1998). The *narE* gene is present only in a subset of hypervirulent clusters: it is always present in ET-5 and Lineage 3 complexes, whereas it is absent from strains of ET-37, cluster A4 and from other unrelated isolates. These bacteria might be descendants of an ancestral strain that had not yet imported this region or otherwise could represent the result of a further deletion event. Interestingly, the same finding has been

described for the genes coding for NadA (Comanducci *et al.*, 2002) and for the OpcA invasin (Zhu *et al.*, 1999).

On the other hand, Gonococcal strains appear to encode a *narE* pseudogene, as it happens for other possibly virulence-related *Neisserial* proteins, such as the recently identified serine protease AspA (Turner *et al.*, 2002) and PorA (Feavers and Maiden, 1998). Furthermore, the few polymorphisms detected between the meningococcal and gonococcal *narE* genes would suggest that the silencing event was relatively recent.

As already mentioned, the *narE* gene is inserted between the genes coding for the E2 and E3 components of the lipoamide dehydrogenase, a multienzymatic complex that is widespread in bacteria. Interestingly, short hypothetical genes can be mapped between the genes coding for E2 and E3 also in other genomes, such as *Ralstonia solanacearum* (Salanoubat *et al.*, 2002), *Alcaligenes eutrophus* and *Zymomonas mobilis* (Hein and Steinbuchel, 1994; de Kok *et al.*, 1998). Although the function of these genes, which are totally unrelated to NarE, is unknown, this finding might suggest that this intergenic region represents a possible hot spot for recombination.

The fact that NarE lacks a leader peptide as well as a gene coding for the translocation/receptor-binding subunit B, raises the question as to whether and by which mechanism this putative toxin is secreted and can achieve contact with host cells. The unexpected finding that the protein is secreted in the periplasmic space of both *E. coli* and Meningococcus would suggest the hypothesis that a conserved mechanism of exportation is present in both species. Because the protein is relatively small, a possible explanation could be that the translocation across the cytoplasmic membrane goes through natural membrane pores. However, in order to exert a toxic activity, NarE has to achieve contact with host cells. For this reason, once in the periplasm, a second step would be required to allow the extrusion of the protein–alone or complexed with some other factor- and therefore to make it available for further interaction with cells.

Because the protein is not freely released in the culture medium, as shown by the Western blot experiments, a possible hypothesis would be that NarE follows a pathway similar to that recently described for LT (Horstman and Kuehn, 2002). According to the model proposed for LT, after secretion via the GSP apparatus, the toxin binds to LPS on the cell surface. As a consequence, vesicles released from the cell display LT on their surface. Finally, internalization of vesicles and their associated LT cause intoxication of the host cells. Alternatively, the export through the outer membrane could be activated by cell contact or could just happen upon lysis of the bacterium.

Many bacterial pathogens alter host cell signalling pathways to exert their toxic activity or to promote their uptake by host cells (Dramsi and Cossart, 1998). Major consequences of these signalling cascades include modifications of G proteins and morphological change of the host cell surface resulting from the reorganization of the actin cytoskeleton. Interestingly, Pujol and colleagues before (Pujol *et al.*, 1997), and Hoffmann and Eugene more recently (Hoffmann *et al.*, 2001; Eugene *et al.*, 2002), have demonstrated that, following type IV pilus-mediated adhesion, *N. meningitidis* bacteria perform an intimate attachment, which generates profound actin cytoskeleton rearrangements. This pathway is required for the efficient internalisation of Meningococcus into endothelial cells and entails the activation of the small GTP-binding proteins of the Rho family RhoA and Cdc42. The fact that several ADP-ribosylating toxins, such as *P. aeruginosa* ExoS, *C. difficile* exoenzyme C3 and Toxin B, exert their cytotoxic effects on the cytoskeleton through an inactivation of the Rho GTPases of the Ras superfamily would suggest that a protein with ADP-ribosyltransferase activity could be likely involved in the described mechanism of bacterial internalisation in Meningococcus. On the other hand, the ability of Meningococcus to invade meningi and cause inflammation could be mediated by alteration of regulatory proteins of the signal transduction pathway. The mechanism and the bacterial factors responsible for signalling events in *N. meningitidis* have not yet been elucidated. The possible role of this newly identified ADP-ribosylating enzyme in the virulence and pathogenesis of meningococcus needs further investigation.

Experimental procedures

Data base search and sequence analysis

The GCG Wisconsin Package suite of programs has been used for computer analysis. Two sequence patterns have been designed based on the consensus motifs generated from the alignments of the CT- and DT-group of toxins (Domenighini and Rappuoli, 1996; Pizza *et al.*, 1999): '(A,I,V,F,L,M)(F,Y,L)R(G,S,R,A,W,Y)x{20,80}ST(S,T)x{10,60}(Q,E)xE' for the CT-group and '(Y,F,W)HGx{20,50}GxYxxxxxxxxxxYx{60,100}E', for the DT-group. The two patterns have been used to screen the genomic sequence of MenB MC58 using the program FindPatterns.

Secondary structure predictions were carried out using three different programs: PHD (Rost and Sander, 1993), HNN (http://npsa-pbil.ibcp.fr/) and PSIPRED (McGuffin *et al.*, 2000) and the results obtained by the three methods have been superimposed to give a consensus result. Finally, the three-dimensional structures have been displayed and handled using the program PDBVIEWER (Guex and Peitsch, 1997).

Strains and plasmid construction

Escherichia coli DH5α and BL21(DE3) were obtained from Invitrogen and used as cloning strain and expression host,

respectively. *Escherichia coli* strains were cultured at 30°C or 37°C in Luria–Bertani (LB) medium and supplemented with 100 μg ml^{-1} ampicillin.

The *Neisseria* strains used in this study were described by Comanducci *et al.* (2002).

To produce a recombinant NarE as Histidine-fusion protein in *E. coli*, NarE DNA coding sequence, devoided of the STOP codon, was amplified by PCR from *N. meningitidis* strain MC58. Oligonucleotides used for amplification of the NarE gene incorporated an *NdeI* or *XhoI* site in the forward or reverse primers, respectively, which were used to clone the gene into the pET21b$^+$ vector. We used 20 pmol of the following primers, NarE-for-CGCGGATCCCATATGG GAAATTTCTTATATAGAGGCATTAGTTGC and NarE-rev-CCCGCTCGAGGTTAATTTCTATCAACTCTTTAGCAAT and 100 μg of genomic DNA from MC58 strain as template. Polymerase chain reaction mixture was performed according to the application protocol of PWO-DNA polymerase (Boerhinger-Mannheim). The mixture was subjected to five cycles at the following conditions: 94°C for 40 s, an annealing cycle of 40 s at 52°C, and extension at 72°C for 40 s; and after 30 cycles at the following conditions: 94°C for 40 s, an annealing cycle of 40 s at 64°C, and extension at 72°C for 40 s. The PCR product was digested with the appropriate enzymes and ligated into pET21b$^+$ vector to obtain the plasmid pET-ADPRT. In order to express the gene, this plasmid was introduced into *E. coli* BL21(DE3).

Polymerase chain reaction and nucleotide sequencing

narE gene was amplified by means of primers *narE*-A (AGCT GTTGAAAGACTTCCG) and *narE*-B (GCAAATGAAGTTG GCGCTA), mapping 120 bp upstream the start codon and 35 downstream the stop codon respectively. Ten ng of each strain chromosomal DNA underwent 30 PCR cycles as follows: 94°C for 30 s (denaturation), 52°C for 30 s (annealing), 68°C for 1 min (extension). Polymerase chain reaction products were analysed on 1% agarose gel and compared with the 1Kb-Plus DNA Ladder molecular weight marker (Gibco). The amplified fragments were then purified by Qiaquick columns (Qiagen), then automatically cyclo-sequenced (Perkin-Elmer 377 model) by means of the same amplification primers. The sequence were assembled and analysed using the Gene-Jokey sequence processor (Biosoft).

Expression and purification of the NarE His$_6$ fusion protein

Escherichia coli BL21 (DE3) harbouring the NarE were grown at 30°C to an A$_{550}$ of 0.6–0.8 in Luria broth medium containing ampicillin 100 mg ml^{-1}. Expression of recombinant protein was induced with 1 mM isopropyl-b-D-thiogalactopyranoside (IPTG-Sigma) and the culture shaken for 3 h at the same temperature. Following induction, cells were collected by centrifugation at 8000 *g* for 15 min at 4°C and then the pellet was resuspended in 50 mM phosphate buffer pH 8 containing 300 mM NaCl, and 10 mM imidazole and a complete, EDTA-free protease inhibitor (Roche, Mannheim, Germany). All subsequent procedures were performed at 4°C. Cells were disrupted by high-pressure homogenisation using a continuous-fill French pressure cell (SLM, Aminco) operating at 10 000 p.s.i. Debris and membranes were pelleted by

centrifugation at 16 000 *g* for 30 min and then discarded, the supernatant was loaded on a metal-chelate affinity chromatography column (MCAC) The column was extensively washed with 10 mM imidazole; 20 mM imidazole; 50 mM imidazole in the same buffer used for the pellet resuspension and the NarE protein was purified in a single step elution with 250 mM imidazole in the same buffer.

Assays of enzyme activity

The standard ADP-ribosyltransferase assay was carried out in 0.3 ml containing 50 mM potassium phosphate, pH 7.5, 20 or 75 mM agmatine (Sigma) and 0.1 mM [adenine-U-^{14}C]NAD (0.05 μCi) (Pharmacia). After incubation at 30°C, duplicate samples (100 μl) were applied to 1 ml columns of Dowex AG 1-X2 (Bio-Rad). [^{14}C]-ADP-ribosylagmatine was eluted for radioassay with 5 ml of H$_2$O and the radioactivity counted in a Packard mod counter. The NAD-glycohydrolase activity was evaluated with a radioactive assay using [carbonyl-^{14}C]NAD (Pharmacia). This assay was carried out in 50 mM potassium phosphate, pH 7.5, 0.1 mM [carbonyl-^{14}C]NAD (0.05 μCi) with and without 20 mM agmatine in a total volume of 0.3 ml. After incubation at 30°C duplicate samples (100 μl) were applied to 1 ml column of Dowex AG 1-X2, [carbonyl^{14}C]nicotinamide was eluted with 5 ml of H$_2$0 for liquid scintillation counting.

ADP-ribosylation of the different amino acids was evaluated using 20 mM of either agmatine, arginine, glycine, serine, lysine, histidine and proline, 100 μM [carbonyl-^{14}C]NAD (52465 c.p.m.) and 50 mM potassium phosphate (pH 7.5) in a total volume of 0.3 ml. Reactions were started with 7 μg of enzyme and incubated 4 h at 30°C. Two 0.1 ml samples were transferred to Dowex AG 1-X2 columns to isolate [carbonyl^{14}C]nicotinamide as described in *Experimental procedures* section. ADP-ribosyltransferase and NAD-glycohydrolase activities were also evaluated in presence of 20 mM DTT or 10 mM MgCl$_2$ using 7 μg of NarE. Cholera toxin (purified in our laboratories, Chiron Srl, Siena) was used as standard for the ADP-ribosylation experiments.

Cell fractionation and protein analysis

Neisseria meninigitidis strains MC58 and 2996 were grown at 37°C with 5% CO$_2$ in GC medium to mid-log phase. Samples were collected in order to prepare the whole cell lysates, the cytoplasmic fractions, the periplasmic fractions, and the culture supernatants.

Whole cell lysate. Bacterial cells were harvested and resuspended in 10 ml of 10 mM TrisHCl (pH 8.0) and heat killed at 56°C for 60 min. The samples were then sonicated on ice to disrupt membranes obtaining whole cell lysates.

Cytoplasmic fraction. An aliquot of the whole cell lysates were centrifuged for 30 min at 13 000 *g*. The supernatant part represents the cytoplasmic fraction.

Periplasmic fraction. Ten ml of the bacterial cultures were harvested by centrifugation and the pellet resuspended in 0.4 ml of 25% sucrose, 50 mM Tris-HCl (pH 8.0). Polymixin

B (SIGMA) was added to the cell suspension to a final concentration of 1 mg ml^{-1}. After a 1 h, RT incubation, the cells were removed by centrifugation (13 000 *g* for 30 min) at 4°C. The supernatant corresponds to the periplasmic fraction.

Culture supernatant. The culture supernatant was isolated by centrifugation of 10 ml bacterial culture, filtered using a 0.2-mm filter and 1 ml precipitated by the addition of 250 ml of 50% trichloroacetic acid (TCA). The sample was incubated on ice for 2 h, centrifuged at 13 000 *g* for 40 min at 4°C and the pellet washed with 70% ice cold ethanol, and resuspended in 10 ml of PBS.

Total protein concentrations of the different fractions were determined by the Bradford method using BSA as a standard.

For the Western Blot, separation of protein samples (3.5 mg of each fraction) was performed by SDS-PAGE through 12% NuPAGE gels (Invitrogen) using MES or MOPS as running buffers and standard protocols. After electrophoresis the gels were transferred to nitrocellulose membranes (Invitrogen). Mouse polyclonal anti-NarE antiserum (dilution 1:000), mouse polyclonal anti-DsbA (NMB0407) antiserum (dilution 1:800), mouse polyclonal anti-Fur (NMB0205) antiserum (dilution 1:2000) or mouse monoclonal anti-PorA (NMB1429) antibody (dilution 1:400) were used as primary antibodies. A secondary rabbit antimouse HRP-conjugated antibody (DAKO) was added at a dilution of 1:10 000. Bands were visualized with Super Signal Chemiluminescent Substrate (PIERCE).

RNA analysis

Neisseria meningitidis strains were grown at 37°C with 5% CO_2 in 25 ml of GC medium to mid-log phase. Cultures were harvested by centrifugation and bacterial cells were lysed in 3.7 ml of 100 mM Tris-HCl (pH 7.5), 2 mM Na2-EDTA, and 1% sodium dodecyl sulphate for 5 min at 95°C. After 10 min of incubation on ice in the presence of 80 mM KCl, cellular debris was removed by centrifugation at 8000 r.p.m. for 10 min in a JA20 rotor (Beckman). To 3.2 ml of supernatant, 4.0 g of CsCl was added, and the RNA was sedimented by centrifugation in an SW60 rotor (Beckman) for 20 h at 35 000 r.p.m. The RNA pellet was then resuspended in 500 μl of TE (10 mM Tris-HCl [pH 8], 1 mM Na$_2$-EDTA), extracted once with an equal volume of phenol-chloroform (1:1), ethanol precipitated, resuspended in 200 μl of TE, reprecipitated, and stored at −20°C.

RNA samples (40 μg) were separated on 1.5% agarose-formaldehyde gels and then dry blotted and UV cross-linked onto nylon membranes (Amersham). Hybridization was carried out at 65°C for 16 h in 7% SDS, 1 mM EDTA, 0.5 M Na$_2$HPO$_4$ (pH 7.2). The DNA probe used for detecting *narE* transcripts was obtained by amplifying the full-length *narE* coding sequence and labelling the PCR product with [α-^{32}P]dATP and [α-^{32}P]dCTP (800 Ci mM^{-1}) using a random priming method. After hybridization, membranes were washed at 65°C in 1% SDS, 1 mM EDTA, 40 mM Na$_2$HPO$_4$ (pH 7.2) and exposed to X-OMAT AR films (KODAK).

Acknowledgements

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