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Deletion of the Correia element in the *mtr* gene complex of *Neisseria meningitidis*

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The *mtr* gene complex in *Neisseria meningitidis* encodes an efflux pump that is responsible for export of antibacterial hydrophobic agents. The promoter region of the *mtrCDE* operon harbours an insertion sequence known as a Correia element, and a binding site for the integration host factor (IHF) is present at the centre of the Correia element. It has been suggested that the expression of the *mtrCDE* operon in meningococci is subject to transcriptional regulation by the IHF and post-transcriptional regulation by cleavage in the inverted repeat of the Correia element. The promoter region of the *mtrCDE* operon as well as the association of changes at that point with decreased susceptibility to antimicrobial drugs in 606 *Neisseria meningitidis* strains were analysed in this study. Two different deletions were present in the analysed region. The first one, found in seven strains, corresponded to absence of the Correia element. The second one, affecting the −10 region and first 100 bp of the *mtrR* gene and present in 57 isolates, was only found in ST-1624 isolates. None of the deletions were associated with decreased susceptibility to antimicrobial drugs. Although most of the meningococcal strains carry the Correia element at that position, its deletion is not an exception.

INTRODUCTION

The *mtr* gene complex encodes an energy-dependent efflux pump that is responsible for export of hydrophobic agents, including those with a non-ionic detergent-like activity, antibiotics and antibacterial peptides. This efflux pump is composed of the MtrC, MtrD and MtrE proteins, which are encoded by the tandemly linked *mtrCDE* genes. In *Neisseria gonorrhoeae*, the *mtrCDE* operon is negatively regulated by the product of the adjacent but divergent *mtrR* gene positioned 250 bp upstream. Mutations in the *mtrR* gene or in its promoter enhance *mtrCDE* gene expression, leading to gonococcal clinical isolates that express elevated levels of resistance to hydrophobic agents (Dewi *et al.*, 2004; Rouquette-Loughlin *et al.*, 2004).

The emergence of meningococcal strains with reduced susceptibility to ciprofloxacin has only been reported occasionally (Enríquez *et al.*, 2008). This reduced susceptibility has been associated with point mutations in the quinolone-resistance determining regions (QRDRs) of the target sites for the fluoroquinolones, and particularly with changes in the GyrA subunit of DNA gyrase (Shultz *et al.*, 2005). However, point mutations at that locus or in the other QRDRs (*parC*, *gyrB* and *parE*) were not detected in two meningococcal strains isolated in Argentina and Spain

Abbreviations: CC, clonal complex; IHF, integration host factor; MLST, multilocus sequence typing; QRDR, quinolone-resistance determining region; ST, sequence type.

showing an MIC for ciprofloxacin of 0.12 and 0.25 mg l^{-1}, respectively. Both strains [characterized as serogroup Y and assigned to sequence type (ST)-1624 from the ST-167 clonal complex (CC)] showed a 154 bp deletion affecting the *mtrR* gene from the *mtrRCDE* gene complex. This deletion was suggested as responsible for reduced susceptibility to ciprofloxacin in these strains (Enríquez *et al.*, 2008; Corso *et al.*, 2005).

Two independent genome sequencing projects have verified the presence of the Mtr efflux system in *Neisseria meningitidis* (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000) and recently Rouquette-Loughlin *et al.* (2004) reported that meningococci have a functional MtrCDE efflux system but its expression is independent of MtrR. The authors analysed the promoter region of the *mtrCDE* operon in several meningococcal strains and found it contained a 155 bp and 159 bp insertion sequence that corresponded to the previously described Correia element placed immediately downstream of the *mtrCDE* promoter (Rouquette-Loughlin *et al.*, 2004). Correia elements are small insertion sequences (100–155 bp in length) of unknown function that have long-terminal inverted repeats and a target site duplication, and have been identified elsewhere in the gonococcal and meningococcal genomes (Delahay *et al.*, 1997; Buisine *et al.*, 2002). Buisine *et al.* (2002) reported the presence of an integration host factor (IHF) binding site at the centre of the Correia element (Buisine *et al.*, 2002). The IHF was found to bind specifically to this site and deletion of the IHF binding site within the Correia element enhanced *mtrCDE* transcription. Post-transcriptional regulation of the *mtrCDE* transcript by cleavage in the inverted repeat of the Correia element has also been reported.

In this study, the presence of deletions in *mtrR* gene, their frequency in the meningococcal population and their potential association with the decreased susceptibility to antimicrobial drugs were analysed in 606 meningococcal strains from different CCs.

METHODS

***N. meningitidis* strains.** The analysis included 606 meningococcal strains associated with patients and also carriers, isolated in six countries (Spain, Argentina, Brazil, Chile, Colombia and Costa Rica) from 1992 to 2007. The strains belonged to different serogroups and represented different CCs (Table 1).

PCR amplification and DNA sequencing. Previously recommended primers mtr405 and mtr254 (Stefanelli *et al.*, 2001) were used to amplify a 1033 bp fragment corresponding to the first 27 bp of the *mtrC* gene, the *mtrR*/*mtrCDE* intergenic region containing the Correia element and the putative promoters of the *mtrR* and *mtrCDE* genes, and the first 800 bp of the *mtrR* gene (Fig. 1a). The PCR products were analysed by agarose gel electrophoresis for checking the presence of deletions by comparison of the sizes of the amplified fragments (Fig. 1b). Strains harbouring deletions were sequenced using the same pair of primers. Fifty-five strains without deletions were also sequenced for comparison.

Alignments were made using the MEGALIGN program (DNASTAR).

Antimicrobial susceptibility testing. MICs of erythromycin, ciprofloxacin, rifampicin, penicillin, ceftriaxone and cefotaxime for 418 strains which are part of the Spanish collection were determined by the agar dilution method as has been previously described (Enríquez *et al.*, 2008). *Streptococcus pneumoniae* ATCC 49619 and *Escherichia coli* ATCC 25922 were used as quality control organisms.

MICs of erythromycin, ciprofloxacin and rifampicin for a selection of 20 meningococcal strains isolated in Latin America (including strains with and without deletions in the amplified region) were determined by the Etest method (AB Biodisk) according to the manufacturer's recommendations.

The breakpoints used were those recommended by the CLSI for *Neisseria meningitidis* (CLSI, 2007).

Table 1. Meningococcal strains included in the screening for the *mtr* deletion

Serogroup	Country of isolation	No. of strains	*mtrR* deletion*	ST/CC†
A	Spain	7	0	
B	Spain	108	1 (152 bp)	4031/41/44 Lineage 3
C	Spain	96	1 (152 bp)	35/ST-35
29E	Spain	23	0	
Y	Spain	78	3‡ (152 bp)	23/ST-23
Y	Argentina	63	52 (154 bp)	1624/ST-167
Y	Brazil	44	2 (152 bp)	6533/ST-23
Y	Chile	23	5 (154 bp)	1624/ST-167
Y	Colombia	41	0	
Y	Costa Rica	16	0	
W135	Spain	96	0	
X	Spain	11	0	

*No. of strains with *mtrR* deletion and type of deletion.
†ST corresponds to the assigned sequence type; CC corresponds to the clonal complex in which the ST has been included.
‡Two strains were isolated from asymptomatic carriers.

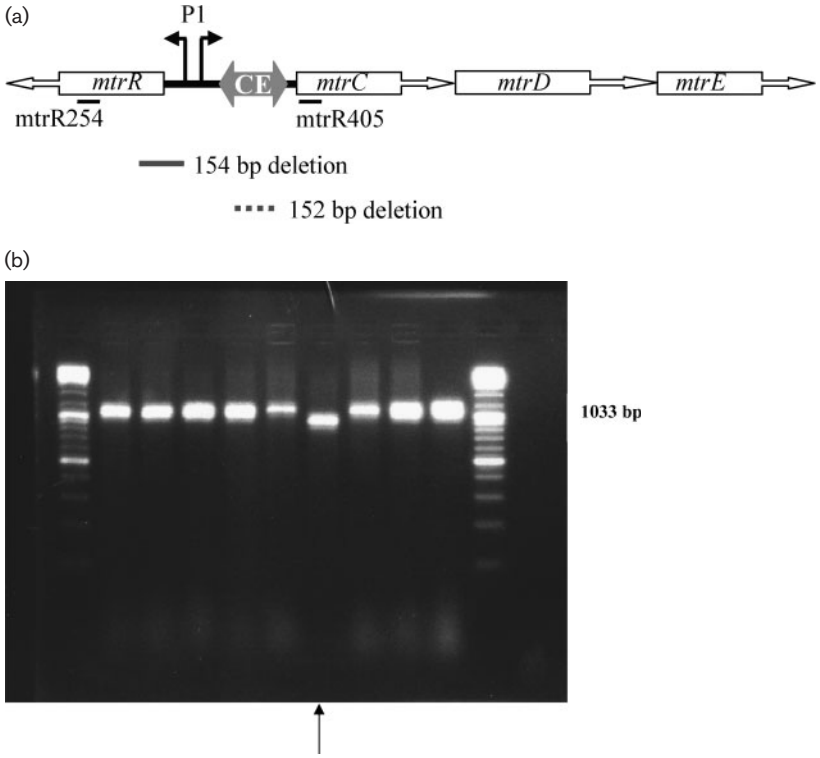

Fig. 1. (a) Schematic organization of the *mtr* locus in *N. meningitidis*. The solid arrows represent the putative promoters of the *mtrR* and *mtrCDE* genes (P1). CE, Correia element. The location of the primers used is shown with a solid line. The location of the two different deletions is also indicated. (b) Agarose gel showing the wild-type fragment and one strain with a deletion marked with an arrow.

Multilocus sequence typing (MLST). MLST was performed in 372 strains (all strains showing deletions and 308 randomly chosen strains) as described by Maiden *et al.* (1998). The ST and the CC designation was done according to the *Neisseria* MLST website (http://pubmlst.org/neisseria/). All STs already assigned to the ST-167 CC (to which ST-1624 belongs) on the Allelic Profile/ST Database hosted in http://pubmlst.org/neisseria were used to build an eBURST representation for that CC (Fig. 2).

RESULTS AND DISCUSSION

Mutations in the *mtrCDE* gene complex

The panel of meningococci included in the study corresponds to a quite diverse meningococcal population with strains showing seven serogroups (Table 1) and belonging to more than 164 STs (data not shown).

After analysis of the amplified region (Fig. 1b), deletions were found in 64 of the 606 *N. meningitidis* strains studied. The amplified region was sequenced in all strains (64) harbouring deletions and also in 55 strains without deletion for comparison, and two different deletions were identified (Fig. 1a). The first one, a 152 bp deletion found in seven strains, corresponded to absence of the Correia element on the intergenic region (GenBank accession no. GQ424833). The second one, a 154 bp deletion found in 57 strains, affected the −10 region and the first 100 bp of the *mtrR* gene (GenBank accession no. EF117896).

The 152 bp deletion, corresponding to absence of the Correia element, was found in seven strains showing different serogroups and belonging to different STs (Table 1): five strains were serogroup Y, three belonging to ST-23 (CC ST-23/Cluster A3) and two belonging to ST-6533 (CC ST-23/Cluster A3); one strain was serogroup B, ST-4031 (CC ST-41/44 Lineage 3); and the other strain was serogroup C, ST-35 (CC ST-35). Two of these seven strains were isolated from carriers so it is likely that this event can not be associated with strain virulence.

Several authors have identified the Correia element in the *mtrR*/*mtrCDE* intergenic region in meningococcal strains (Rouquette-Loughlin *et al.*, 2004; Parkhill *et al.*, 2000; Tettelin *et al.*, 2000; Abadi *et al.*, 1996), suggesting that this insertion sequence might be a common trait in meningococcal isolates, and Rouquette-Loughlin *et al.* (2004) have proposed that it could play a role in modulation of *mtrCDE* expression. The results presented here indicate that the Correia element is not present at this location in all meningococcal strains. Although most of the meningococcal strains may carry the Correia element at that position, the absence of this element is not an exception. The 154 bp deletion, affecting the −10 region and first 100 bp of the *mtrR* gene, was only found in serogroup Y strains belonging to ST-1624 (CC ST-167) (Table 1), all of them being isolated from patients. All the strains characterized as Y and ST-1624 showed this deletion. To find out whether the 154 bp deletion could be used as a ST-1624 or CC ST-167 marker, the deletion in 15 strains (belonging to the collection of 606 strains included in this study) belonging to 15 different STs from CC ST-167 (Fig. 2) was checked. None of the analysed strains

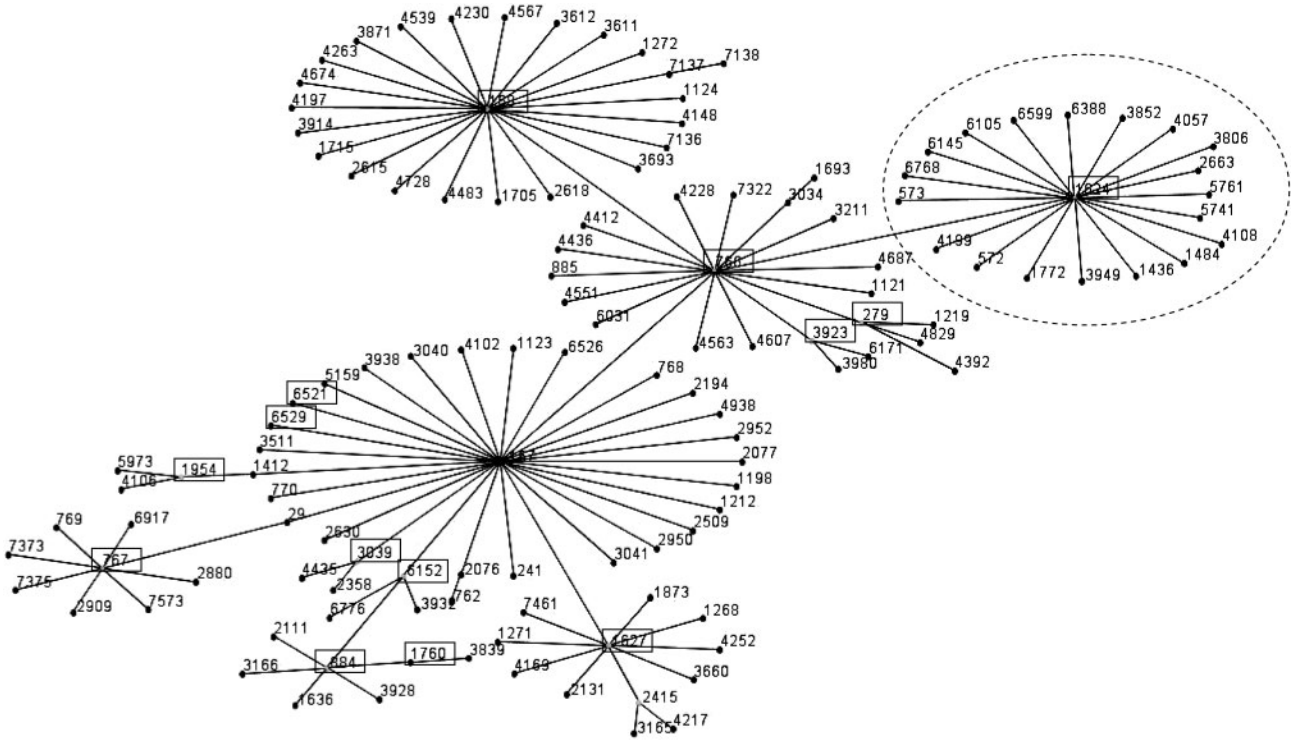

Fig. 2. eBURST representation of the ST-167 CC. Those STs in which at least one strain was analysed appear framed with a solid line. ST-167 and STs potentially evolving from ST-167 appear framed with a broken line.

belonging to the potential ancestors for ST-1624 presented the deletion. We do not have strains of the STs evolving from ST-1624, so we can not say whether the deletion is present in all STs potentially descending from ST-1624. Therefore, we can conclude that this particular deletion might have arisen in ST-1624 and it is a special feature of this ST.

Susceptibility to antimicrobial agents

There were no differences when the MICs of erythromycin, ciprofloxacin or rifampicin among strains both with and without deletions were compared (Table 2). All the analysed strains were susceptible to these antibiotics. Therefore, neither of the two deletions found can be associated with decreased susceptibility to antimicrobial agents.

A full-length MtrR protein is needed for regulation of *mtrCDE* in gonococci (Rouquette-Loughlin *et al.*, 2004). While the MtrR protein acts as a negative transcriptional regulator in *N. gonorrhoeae* and mutations in the *mtrR* gene or in its promoter lead to resistance to hydrophobic agents (Dewi *et al.*, 2004; Rouquette-Loughlin *et al.*, 2004), the deletion of 154 bp affecting the −10 region and first 100 bp of the *mtrR* gene observed was not associated with reduced susceptibility to antimicrobial agents. Rouquette-Loughlin *et al.* (2004) analysed the promoter region of the *mtrCDE* operon in a panel of meningococcal strains and they reported the presence of a 155–159 bp insertion sequence element, known as the Correia element, placed immediately downstream of the *mtrC* promoter in all meningococcal strains tested. However, it seems to be a rare phenomenon in gonococci. The authors concluded that the presence of a Correia element within the *mtrR/mtrC* intergenic region is a common trait in meningococcal isolates. However, the 152 bp deletion found in seven of the strains analysed in this study revealed that the Correia element is not always present at this location in the meningococcal population.

Post-transcriptional regulation of the *mtrCDE* transcript by cleavage in the inverted repeat of the Correia element has been proposed (Rouquette-Loughlin *et al.*, 2004). The 152 bp deletion, corresponding to absence of the Correia element, also involved the IHF binding site at the centre of the Correia element that has been suggested as critical for modulating expression of MtrCDE. Those seven strains harbouring the deletion did not show decreased susceptibility to the antimicrobial agents tested. We have not checked the level of expression of MtrCDE in those meningococci showing absence of the Correia element at that position. The lack of an MIC increase does not necessarily prove that the *mtr* efflux system in meningococci is not subject to transcriptional regulation by IHF. The deletions described might have resulted in changes in the expression of the MtrCDE system without translation into a significant MIC change in meningococci. Additional

Table 2. MICs of erythromycin, ciprofloxacin and rifampicin in a selection of meningococcal strains with and without deletions in the *mtr* gene complex

ID number	Deletion	MICs (mg l^{-1})		
		Erythromycin	Ciprofloxacin	Rifampicin
AR930	154 bp	0.25	0.006	0.003
1055	–	1	0.006	0.012
1730	154 bp	0.5	0.006	0.006
1964	154 bp	0.25	0.006	0.003
2096	154 bp	0.25	0.006	0.006
2151	154 bp	0.25	0.006	0.003
2289	154 bp	0.5	0.006	0.003
2432	154 bp	0.25	0.006	0.006
2508	154 bp	0.5	0.006	0.006
N21/05	–	0.5	0.006	0.003
N71/00	–	0.5	0.006	0.006
N207/05	–	0.5	0.006	0.003
N683/05	152 bp	0.5	0.006	0.012
N689/05	152 bp	0.5	0.006	0.012
13977	152 bp	0.25	0.006	0.006
14203	152 bp	0.5	0.006	0.012
15103	–	0.25	0.006	0.025
15320	–	0.25	0.006	0.006
15596	–	0.5	0.006	0.012
15656	152 bp	1	0.006	0.012
15701	–	0.5	0.006	0.012
15970	152 bp	1	0.006	0.025
422N	–	0.5	0.006	0.012
513N	–	0.25	0.006	0.006
M12/02	154 bp	0.25	0.003	0.012
M25/01	154 bp	0.25	0.003	0.012
M102/04	154 bp	0.25	0.003	0.012

studies on the expression of the efflux system in those meningococci are necessary to elucidate the exact meaning of the deletions found. However, the hypothetical association of the 154 bp deletion with quinolone resistance in strains without changes in QRDRs (Corso *et al.*, 2005; Enríquez *et al.*, 2008) is rejected by this study. Alternative mechanisms for quinolone resistance in those meningococcal isolates should be considered and further analysed.

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