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Significance of *tagI* and *mfd* genes in the virulence of non-typeable *Haemophilus influenzae*

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Summary. Non-typeable *Haemophilus influenzae* (NTHi) is an opportunist pathogen well adapted to the human upper respiratory tract and responsible for many respiratory diseases. In the human airway, NTHi is exposed to pollutants, such as alkylating agents, that damage its DNA. In this study, we examined the significance of genes involved in the repair of DNA alkylation damage in NTHi virulence. Two knockout mutants, *tagI* and *mfd*, encoding *N*³-methyladenine-DNA glycosylase I and the key protein involved in transcription-coupled repair, respectively, were constructed and their virulence in a BALB/c mice model was examined. This work shows that *N*³-methyladenine-DNA glycosylase I is constitutively expressed in NTHi and that it is relevant for its virulence. [Int Microbiol 2014; 17(3):159-164]

Keywords: *Haemophilus influenzae* · alkylating agents · virulence · genes *tagI* and *mfd*

Introduction

Non-typeable *Haemophilus influenzae* (NTHi) is a commensal gram-negative bacterium well adapted to the human upper respiratory tract [7]. It has been implicated in the etiology of otitis media, conjunctivitis, sinusitis, pneumonia, and chronic bronchitis, and in the progression of chronic obstructive pulmonary disease (COPD) [19]. However, within its human host, this opportunistic pathogen is exposed to high levels of genotoxic stress in the form of airway pollutants. In a study

based on proteomic expression profiling of *H. influenzae* grown in pooled sputum from adults with COPD, both the expression of antioxidant activity and stress responses were shown to be important for NTHi survival in the airways [13].

DNA-damaging agents are ubiquitous. They are generated endogenously during cell metabolism and are present in the environment—in air, water and foods—although generally in low concentrations. For example, tobacco smoke contains a mixture of alkylating agents, some of which act directly (alkyl halides, acrolein, crotonaldehyde, ethylene oxide, propylene oxide, acrylonitrile, and acrylamide), while others act indirectly (requiring metabolic transformation to form reactive species) [15]. Moreover, human airway pollutants such as tobacco smoke damage not only eukaryotic cells but also the DNA of the respiratory tract microbiota.

The repair of DNA alkylation damage in bacterial cells has been mainly studied in *Escherichia coli*. As in other

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bacteria, *E. coli* has two specific mechanisms to remove alkyl radicals from its DNA: (i) via the constitutive expression of genes encoding the necessary repair enzymes and (ii) via the alkyl-induced expression of these proteins [16]. This adaptive response to the repair of DNA alkylation damage is regulated by the Ada protein, a positive transcriptional regulator that stimulates the expression of the *ada*, *alkA*, *alkB*, and *aidB* genes [5,16]. Bacteria also have two additional enzymes involved in the specific repair of DNA alkylation damage: Ogt (*O*⁶-meG-DNA methyltransferase) [10] and TagI (*N*³meA-DNA glycosylase I) [2]. In addition, two other systems are involved in the repair of DNA alkylation damage: the nucleotide excision repair (NER) [20] and the transcription-coupled repair (TCR) [17] systems. The latter system mediates the bulk repair of DNA damage via the Mfd protein, followed by the engagement of NER.

The aim of the present work was to determine the significance of *tagI* and *mfd* genes involved in the repair of DNA alkylation damage in NTHi virulence. Accordingly, knockout mutants in *tagI*, specific for DNA alkylation damage,

and *mfd*, involved in bulk DNA repair, were constructed and their virulence in a BALB/c mouse model was studied.

Materials and methods

Bacteria, media, and growth conditions. *Haemophilus influenzae* NTHi375, an otitis media isolate [4], was grown on chocolate agar + PolyViteX plates (PVX; BioMerieux), on brain heart infusion (BHI) medium with or without agar supplemented with 10 µg hemin ml⁻¹, and 10 µg NAD ml⁻¹ (sBHI). The cultures were grown at 37 °C for 18 h in an atmosphere of 5 % CO₂. *Escherichia coli* DY380 strain was grown in LB (Luria-Bertani) broth or on agar plates at 37 °C for 18 h. When necessary, 50 µg ampicillin ml⁻¹ and 50 µg spectinomycin ml⁻¹ were added.

Construction of *tagI* and *mfd* knockout mutants. The *tagI* knockout mutant was constructed from strain NTHi375 using a previously described method [18]. Briefly, the entire gene targeted for deletion was PCR-amplified from the genomic DNA of NTHi375 strain (Table 1, Fig. 1A), cloned into pGEM-T (Promega), and electroporated into *E. coli* DY380. Strain DY380 harboring the plasmid with the *tagI* gene was selected by plating onto LB agar plates supplemented with 50 µg ampicillin ml⁻¹. Then, with plasmid pRSM2832 [18] as template, PCR was used to generate an amplicon

Table 1. Oligonucleotide primers used in this study

Primer	Sequence (5'-3')
Primers used to obtain the mutants	
TagI_F	cgggtgctgcagcaatca
TagI_R	tctgtgaaagccttatgtgaactc
Mfd_F	tacactatgctcctcaatttacaca
Mfd_R	acaatgatcgggctctttttatg
P1-TagI	<u>ggttgccgaacaatctatttataattgattatcatgacaaaggaatggggaagcctgaattcgacagccaaaagctatttgattccggggatccgctcgacc</u>
P2-TagI	<u>aatcatttaaatgatcgtccaccagccccatagattgcataaacgcatagcacgtggtttcgccaataaagacgaaacatgtaggctggagctgcttcg</u>
P1-Mfd	<u>cattttaaaggaaatgactgttttcggtgagacggaaggtcgcgagagactttgcttgatttgccttaccctttaaattccgggatccgctcgacc</u>
P2-Mfd	<u>tattaagcgttcgaggaattgctgtgcccgttagcgttaagaatcgcataattgcacgaagctgtttgattttctctttttaggctggagctgcttcg</u>
Primers used for RT-qPCR assays	
trpA F	cttcgtgccgttcgttacc
trpA R	tgaccgcactttttccaatagt
Tag F	cgccaataagctttcgcgat
Tag R	cggggctttcgtggatta
recA F	cagtgcggcaacggagtc
recA R	cgcaaaaagcaggaaaaacc

Forward primer, up; reverse primer, dw. Underlined text corresponds to the 80 nucleotides of the 5' and 3' ends (H1 and H2) of the NTHi gene to be deleted.

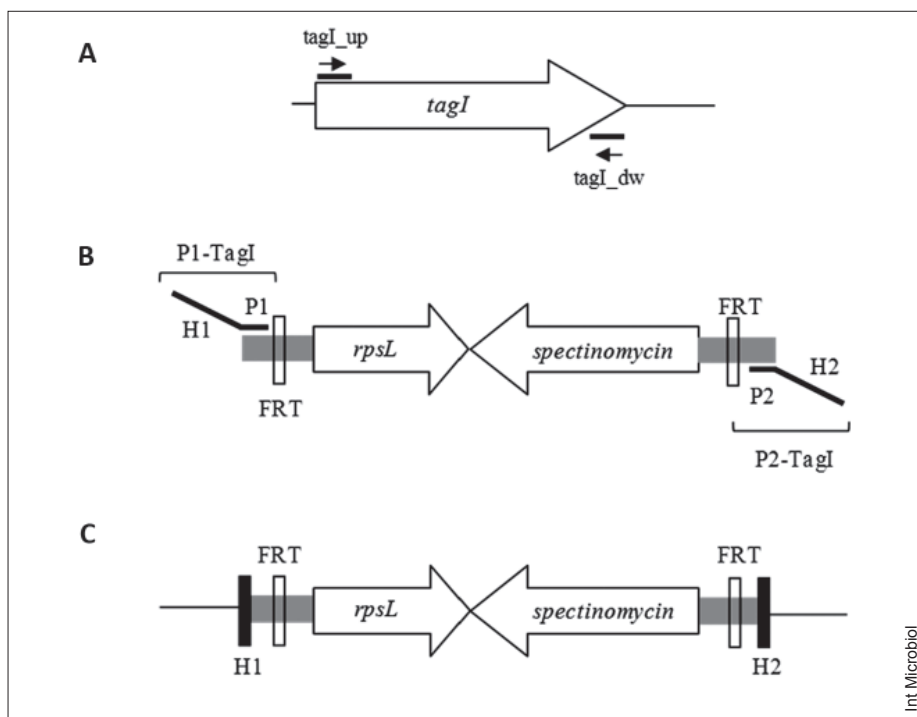


Fig. 1. Construction of *tagI* mutant. (A) The PCR-amplified *tagI* gene from NTHi375 was cloned into pGEMT and electroporated into *E. coli* DY380. (B) An amplicon containing a cassette with both the *rpsL* and the spectinomycin resistance genes, flanked by each of the FRT regions, was obtained by PCR of pRSM2832 with primers P1-TagI and P2-TagI (see Table 1). Homology arms H1 and H2 are 80-nucleotide (nt) sequences of the 5' and 3' ends of the *tagI* gene to be deleted, and P1 and P2 are 20-nt sequences of DNA homologous to the 5' and 3' ends of the cassette, respectively. (C) The inserted region of the amplicon of panel B in the chromosome of the NTHi375 knockout *tagI* mutant.

containing a cassette with both the *rpsL* and the spectinomycin resistance genes, flanked by FRT (FLP recombinase target) sites. In addition, the design of the primers produced an amplicon that contained 80 nucleotides (nt) of the 5' and 3' ends of the *tagI* gene to be deleted, flanking each of the FRT regions (Table 1, Fig. 1B). The amplicon was electroporated into *E. coli* strain DY380 harboring pGEM-T carrying the *tagI* gene. After induction of the recombinase genes of strain DY380 by heat shock at 42°C, spectinomycin-resistant clones were isolated by plating the transfectants onto LB agar plates supplemented with 50 µg spectinomycin ml⁻¹. One of these positive clones was chosen for further use after PCR and sequencing to confirm that it harbored a plasmid with the correct insertion. This plasmid was digested with *NcoI* and *NsiI* restriction enzymes, and the fragment with the appropriately sized insert was recovered and used to transform NTHi375 strain by the MIV method, as previously described [12], to obtain the desired construct (Fig. 1C). Spectinomycin-resistant clones were isolated by plating onto BHI agar plates supplemented with 200 µg spectinomycin ml⁻¹ followed by incubation at 37°C. One of these clones was isolated for further use after PCR and DNA sequencing to confirm that it contained the desired mutation and that it did not harbor any remnants of the plasmid. The same procedure was used to obtain the *mfd* mutant. Afterwards, pRSM2947, a temperature-sensitive replicon appropriate for NTHi and harboring both the FLP recombinase under the control of the *tet* regulatory system and a kanamycin resistance marker, was transformed by electroporation into the knockout mutants, to remove the cassette containing both *rpsL* and the spectinomycin resistance genes.

NTHi infection BALB/c model. To infect the mice, the bacteria were recovered with 1 ml of PBS from a chocolate-agar plate grown for 16 h, yielding a bacterial suspension of $\sim 5 \times 10^9$ colony-forming units (CFU)/ml. Twenty microliters of bacteria ($\sim 10^7$ CFU) were inoculated into the nares of 5- to 7-week-old female BALB/c mice (Harland Iberica). After 48 h of infection, the mice were killed by cervical dislocation and their lungs were rapidly dissected for the determination of bacterial load. The dissected lungs were homogenized on ice in 500 µl of PBS using an Ultra-Turrax TIO basic homogenizer (IKA). Bacteria from the homogenates and from serial dilutions thereof were recovered on chocolate-agar plates. The results are reported as log CFU per gram of tissue. In each case, clones recovered from the mice were confirmed by PCR.

The mice were treated in accordance with the Directive of the European Parliament and of the Council on the protection of animals used for scientific purposes (Directive 2010/63/EU) and in agreement with the Bioethical Committee of the University of the Balearic Islands. This study was approved by the Bioethical Committee of the University of the Balearic Islands under authorization number 1748.

Reverse transcription-quantitative real-time PCR. RNA from strain NTHi375 grown in sBHI and treated or not with 1.5 µg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) ml⁻¹ for 1 h was extracted using the RNeasy minikit (Qiagen) and DNase treatment (Ambion). Reverse transcription-quantitative real-time PCR (RT-qPCR) was performed in a 20-µl reaction mixture with Lightcycler RNA Master SYBR Green I (Roche) on a

Lightcycler 480 instrument (LC480; Roche), following the manufacturer's instructions and using suitable oligonucleotide primer pairs for each gene (Table 1). The relative mRNA concentration obtained from the *tag* gene was determined according to a standard curve generated by amplifying an internal fragment of the *trpA* gene, which is not affected by MNNG treatment. Similarly, the *recA* gene served as the positive control for the induction of gene expression by MNNG. The expression factor was calculated as the ratio between the mRNA concentrations obtained from genes expressed in MNNG-treated NTHi 375 cells with respect to those from untreated cells.

Statistical analysis. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Bonferroni contrasts. $P < 0.05$ was considered statistically significant. The analyses were performed using Prism4 for PC (GraphPad Software).

In silico analysis. To identify the proteins of *H. influenzae* involved in DNA alkylation repair, *E. coli* protein sequences implicated in this system were scanned for homologues by using BLASTP [http://blast.ncbi.nlm.nih.gov/Blast.cgi] against the published genomes of *H. influenzae* strains.

Results and Discussion

The present work was designed to determine the significance of DNA alkylation damage repair in NTHi virulence. *In silico* analysis revealed that the adaptive response to the repair of DNA alkylation damage was missing in *H. influenzae* because Ada, AlkA, AlkB, and AidB proteins were absent. However, this bacterium contains the genes encoding *ogt* and *tagI* as well as the genes involved in the NER and TCR systems. Based on these results, we studied the importance of *N*³-meA-DNA glycosylase I, encoded by the *tagI* gene, and the Mfd protein involved in transcription-coupled repair. To achieve this, we constructed both *tagI* and *mfd* knockout mutants, as detailed in Fig. 1 for the construction of the *tagI* mutant. However, *rpsL* and the spectinomycin resistance genes could not be removed from the knockout mutants. It seems that the plasmid pRSM2947 was not compatible with strain NTHi375 because transformed cells were not obtained even when the cells were incubated at the non-restrictive temperature. Thus, spectinomycin resistance was conserved in the mutants.

Investigation of the virulence of the two mutants in a BALB/c mouse model showed that after infection of the mice, both the *mfd* mutant and the wild-type strain were recovered at the same concentration; while the recovery of *tagI* cells was dramatically lower ($P = 0.023$) (Fig. 2). This effect was not due to differences in growth of the *tagI* mutant because its growth rate was similar to that of the wild-type strain (Fig. 3).

Based on these results, we considered whether *tagI* expression was inducible by DNA alkylation damage. Cultures of NTHi375 were treated with a sublethal concentration (1.5

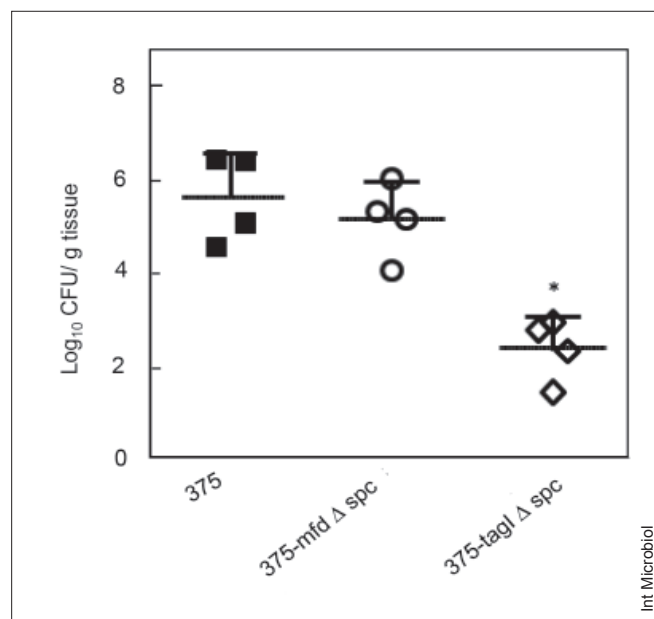


Fig. 2. Bacterial loads of NTHi in the lungs of BALB/c mice after 48 h of infection with the wild-type strain and the *mfd* and *tagI* mutants. (Standard deviations are shown.)

$\mu\text{g/ml}$) of MNNG for 1 h after which the expression of *tagI* was determined by RT-qPCR. Expression of the *recA* gene served as a positive control. The results showed that, as in other bacteria [13], the expression of NTHi375 *tagI* was constitutive because it was not further induced by MNNG treatment, whilst the expression of *recA* gene was induced by a factor of 3.6 (Fig. 4).

These observations indicated that the 3-methyladenine DNA glycosylase I activity encoded by the *tagI* gene is crucial for NTHi 375 survival during lung infection. Similar to AlkA, TagI is a monofunctional glycosylase of the base excision repair system; as such, it hydrolytically cleaves the glycosidic bond of alkylated purine bases. However, unlike AlkA, TagI has a very high specificity because it almost exclusively cleaves 3-methyladenine [2,6]. This specificity probably arises from the enzyme's unique aromatic-residue-rich 3-MeA binding pocket and the absence of the catalytic aspartate that is present in all other helix-hairpin-helix family members, including AlkA [6].

Our results contrast with those reported for *Salmonella enterica*, in which inactivation of the *ada*, *ogt*, *tag*, *uvrB*, and *mfd* genes is necessary to decrease bacterial virulence when the cells are orally inoculated in mice [1]. It has been suggested that the extensive alkylation repair system of *Salmonella* is involved in the survival of *Salmonella* cells outside the infected animal, enabling them to overcome the potentially massive

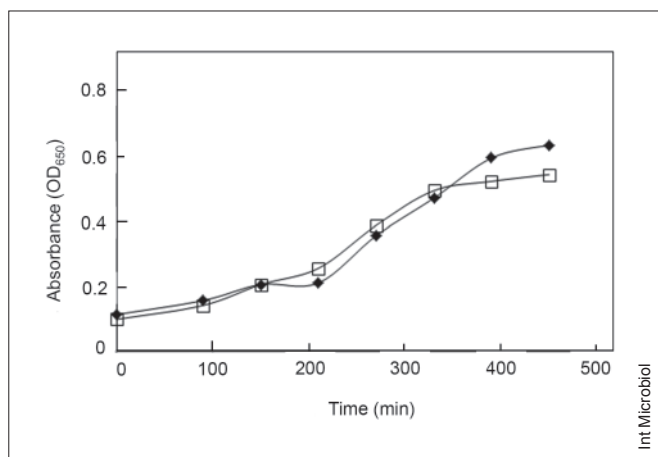


Fig. 3. In vitro growth of NTHi375 wild-type strain (◆) and *tagI* mutant (□) in SBHI.

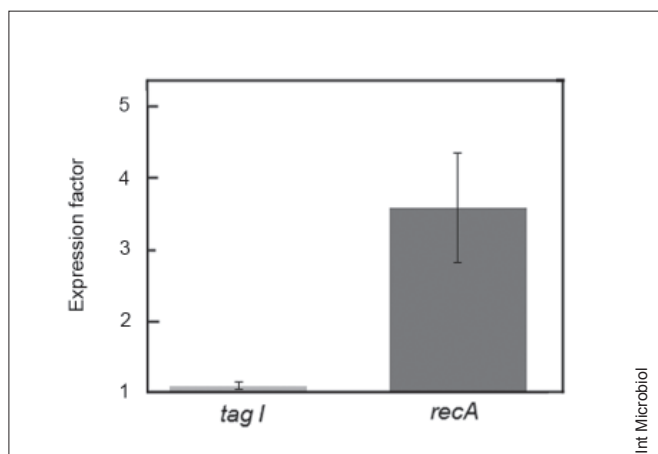


Fig. 4. Expression factor of the *tagI* and *recA* genes in the NTHi375 strain after treatment with MNNG at 1.5 µg/ml. The expression factor is the ratio of the mRNA concentration of each gene from the treated wild-type strain with respect to the non-treated. The amount of mRNA of each gene was determined by using a standard curve generated by the amplification of an internal fragment of the *H. influenzae trpA* gene (see Table 1 for primer sequences). Standard deviations of two independent experiments are shown.

DNA injuries induced by alkylating agents present in the environment [1]. By contrast, *H. influenzae* is a human obligate pathogen well adapted to the human upper respiratory tract, and with a low persistence outside the host [8], which would explain why it does not have the full complement of repair mechanisms needed to repair alkylation-type damage. Consequently, the deletion of a key protein in the repair of alkylation injuries must be more relevant for this species than it is for *Salmonella*. In this context, the role of *N*³-meA-DNA glycosylase I in NTHi survival in human airways must be emphasized, because this enzyme catalyzes the specific removal of *N*³-methyladenine, a mainly lethal insult that blocks DNA replication [3,9]. In the

setting of tobacco smoke and NTHi respiratory infections [10], the bacterial TagI protein would thus be critical in repairing DNA damage caused by the alkylating agents in cigarette smoke during the infective process of NTHi.

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Competing interests. None declared.

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