Unveiling electrotransformation of *Moraxella catarrhalis* as a process of natural transformation

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Abstract

The human respiratory tract pathogen *Moraxella catarrhalis* is a naturally competent microorganism. However, electrotransformation has long been used to introduce foreign DNA into this organism. This study demonstrated that electrotransformants obtained with linear or circular nonreplicating plasmid DNA originated exclusively from natural transformation processes taking place during the recovery phase after the application of current. Only replicating plasmid DNA could be introduced into *M. catarrhalis* by electrotransformation, in a type IV pilus-independent manner. Electrotransformation with homologous genomic DNA indicated that restriction of double-stranded DNA was independent of type III restriction-methylation systems. Nontransformability of *M. catarrhalis* by electrotransformation was observed using double- as well as single-stranded DNA. In addition, the study showed that natural competence is a very constant feature of *M. catarrhalis*.

Introduction

Moraxella catarrhalis has now been recognized as a major human respiratory tract pathogen, most frequently associated with otitis media in children (Gehanno *et al.*, 2001; Kilpi *et al.*, 2001) and infectious exacerbations of chronic obstructive pulmonary disease in adults (Murphy, 1998). Research activities over the past two decades have mainly focused on the characterization of vaccine candidates (McMichael, 2000) including outer membrane proteins and lipo-oligosaccharides. This research requires manipulations of the *M. catarrhalis* genome.

Although *M. catarrhalis* is naturally competent (Marrs & Weir, 1990), transformation of this organism is often performed by electroporation. Electroporation (electrotransformation) allows DNA penetration through brief permeabilization of the bacterial cell surface (Kimoto & Taketo, 1997) and has been successfully used to introduce DNA into bacterial (Dower *et al.*, 1988), plant (Fromm *et al.*, 1985) and mammalian cells (Chu *et al.*, 1987). Recently, Luke *et al.* (2004) reported the presence of a type IV pilus apparatus in *M. catarrhalis* and showed that the PilA and PilQ proteins were essential for natural transformation of this organism.

A number of unusual observations during the construction of knock-out mutants prompted us to investigate the transformation process of *M. catarrhalis*. Electrotransformation was compared with natural transformation and analyzed with regard to the nature of the transforming DNA. The influence of restriction/modification systems was investigated.

Material and methods

Bacterial strains and growth conditions

Moraxella catarrhalis strains O35E and O35E.1 have been described (Aebi *et al.*, 1997). *Moraxella catarrhalis* was grown routinely on brain–heart infusion (BHI) agar plates at 37 °C in an atmosphere containing 5% CO_2 or in BHI broth. *Escherichia coli* strains harboring plasmids pEMCJH04 and pEMCJH04ΩlacZ (Hays *et al.*, 2005) were either grown on Luria–Bertani (LB) agar plates at 37 °C or in LB broth at 37 °C, 200 r.p.m., ambient air. Media used for strain O35E.1 and *E. coli* were supplemented with 20 and 100 mg L^{-1} kanamycin, respectively.

Transforming DNA

The double-stranded linear DNA fragment Δ*uspA1::kan*^{R} was amplified by PCR from strain O35E.1 using the primers U1F1 (5-CGGGATCCCCGTGAAGAAAAATGCCGCAGGT-3) and U1M (5-CCGCTCGAGCGGCGTCGCAAGCCGATTG-3) and Taq Polymerase (Roche). Single-stranded DNA was obtained using the above-described primers and an asymmetric two-step amplification protocol as described (Kaltenboeck *et al.*, 1992). Plasmids pEMCJH04 and its derivative pEMCJH04ΩlacZ were constructed as described (Hays *et al.*, 2005). Plasmid pF1MKan (unable to replicate in *M. catarrhalis*) is a pBluescript KS II (Stratagene) derivative containing Δ*uspA1::kan*^{R}, amplified as described above, ligated into the *Bam*HI and *Xho*I restriction sites of the multiple cloning site. Homologous linear genomic DNA was obtained as follows: DNA was extracted from strain O35E.1, digested with *Xba*I and purified using the MinElute PCR purification kit (Qiagen).

Preparation of electrocompetent cells and electroporation

The protocol was adapted from Helminen *et al.* (1993). Bacteria were grown in BHI broth to an optical density $OD_{600\,nm}$ of 1.0, harvested and washed first in 1 volume, second in 1/2 volume and finally resuspended in 1/10 volume of ice-cold glycerol 10%. Electrocompetent cells were aliquoted into 1 mL portions and stored at −70 °C. Before electroporation, frozen cells were thawed on ice, harvested and resuspended in 100 μL of supernatant. Twenty microliter portions of competent cells were mixed with 5 μL of water containing 1 μg of DNA. The mix was immediately transferred to 0.1 cm electroporation cuvettes (Bio-Rad) and placed in the Gene pulser II system (Bio-Rad). The electroporation conditions were as follows: field strength, 12.5 and 25 kV cm^{-1}; resistance, 200 Ω; and capacity, 25 mF. After application of current, bacteria were immediately transferred to 1 mL of BHI broth and incubated for 1 h at 37 °C with shaking at 200 r.p.m. Selection was performed overnight on BHI agar plates containing 20 mg L^{-1} of kanamycin. Bacteria were also cultivated on antibiotic-free BHI agar plates in order to assess the total number of viable bacteria. All experiments were performed in independent triplicates. For certain experiments, bacteria were treated as described above, but not actually exposed to current.

Preparation of competent cells and natural transformation

The protocol was adapted from Furano & Campagnari (2003). Briefly, *M. catarrhalis* cells were grown to an optical density $OD_{600\,nm}$ of 0.2, glycerol was added to a final concentration of 20% and 200 μL aliquots of the naturally competent cells were stored at −70 °C until required. Competent cells were thawed on ice. Aliquots (100 μL) were spread on BHI agar plates and air-dried. Two circles (2 cm diameter) were marked on each plate. Either 30 μL of distilled water or 30 μL of water containing 100 ng of DNA was dropped onto these circles, followed by incubation at 37 °C, 5% CO_2. After 5 h, bacteria within each circle were harvested with a cotton swab and resuspended in 1 mL of BHI broth. Serial dilutions were prepared and spread on BHI agar plates for total viable cell counts, or on BHI agar plates containing kanamycin at 20 mg L^{-1}. After 24 h of incubation at 37 °C in 5% CO_2, the number of CFUs per experiment was determined and transformation frequencies were calculated. All experiments were performed in independent triplicates.

DNase treatment

Cells were made electrocompetent as described above. Immediately after application of current, bacteria were resuspended either in 1 mL of BHI or in 1 mL of BHI containing 20 mg L^{-1} of DNase and were incubated for 1 h at 37 °C and 200 r.p.m. Selection was then performed as described above.

Results and discussion

Electrotransformation versus natural transformation

In both natural and electrotransformation experiments, 100 or 1000 ng of DNA, respectively, were used to transform *M. catarrhalis* O35E. These amounts of DNA were previously determined to be optimal for both methods of transformation (data not shown). *Moraxella catarrhalis* strain O35E was transformed with various forms of DNA, and comparative transformation frequencies are reported in Table 1. Transformants could be established by both methods and

Table 1. Electrotransformation versus natural transformation of *Moraxella catarrhalis*

		Transformation frequency*	
Transforming DNA	Form of DNA	Electrotransformation	Natural transformation
Δ*uspA1::kan*^{R}*ds*	Linear double-stranded	3×10^{-4}	2.4×10^{-1}
Δ*uspA1::kan*^{R} *ss*	Linear single-stranded	8.4×10^{-5}	4.7×10^{-3}
pEMCJH04ΩlacZ	Circular replicating	4×10^{-4}	1.6×10^{-3}
pF1Mkan	Circular nonreplicating	3×10^{-5}	1.5×10^{-1}

*Transformation frequency reports the ratio of kanamycin-resistant transformants to the total of surviving bacteria.

all forms of DNA, although natural transformation yielded more transformants than electrotransformation (Table 1).

Effect of DNase treatment and current on electrotransformation

In a second set of experiments, electrotransformed bacteria were treated with DNase immediately after electroporation. After addition of DNase, no transformants of *M. catarrhalis* could be observed, when Δ*uspA1::kan*^{R} or the plasmid pF1MKan (unable to replicate in *M. catarrhalis*) were used (Table 2). However, electrotransformation with the plasmid pEMCJH04ΩLacZ (replicating in *M. catarrhalis*) yielded transformants in DNase-treated as well as in DNase-untreated reactions.

In a third set of experiments, electroporation was performed with or without applying electric current to electrocompetent bacteria. Only the double-stranded linear PCR product was used for these experiments. Transformants could be isolated from both experiments. Transformation frequencies were 4.2×10^{-4} with and 4.1×10^{-4} without application of electric current, respectively, and similar numbers of bacteria were recovered (1.0×10^9 and 1.8×10^9 CFU mL^{-1}, respectively).

The results of assays testing both the effects of DNase treatment and application of electric current led to the conclusion that transformants obtained by electroporation with linear double-stranded DNA in fact result from natural transformation processes occurring during the recovery phase after the application of the electric pulse. Results obtained by electroporation of a *pilA* mutant of *M. catarrhalis* strain O35E (O35E.pilA) corroborated these results. Strain O35E.pilA could only be electrotransformed with the replicating plasmid pEMCJH04ΩLacZ (transformation frequency 1.7×10^{-4}), but not with linear DNA (transformation frequency $< 10^{-8}$). Using natural transformation, O35E.pilA could not be transformed with any form of DNA (transformation frequencies $< 10^{-8}$). PilA mutants of *M. catarrhalis* have a defect in the type IV pilus apparatus and are therefore not transformable by natural transformation (Luke *et al.*, 2004) (P. Stutzmann Meier, K. Bögli, N. Heiniger, R. Troller, R. Coimbra, J.P. Hays and C. Aebi, own unpublished results).

The differences in the transformation frequencies between natural and electrotransformation may actually reflect the variation in methodology used between the two protocols. Specifically, natural transformation was performed on agar plates, bringing together 10^{10} DNA molecules and 10^6 bacteria for 5 h, while in the electrotransformation methodology 10^9 bacteria were exposed to 10^{11} DNA molecules in a 1 mL culture for 1 h.

Inability to transform bacteria with linear DNA using electroporation has been demonstrated in other bacteria such as *Haemophilus influenzae* (Mitchell *et al.*, 1991) and *Streptococcus pneumoniae* (Lefrancois & Sicard, 1997; Lefrancois *et al.*, 1998). In *S. pneumoniae*, double-stranded linear DNA penetrated the cells upon electrotransformation, but was fully restricted after penetration (Lefrancois & Sicard, 1997). In contrast, *Haemophilus ducreyi* was easily electrotransformed with linear DNA (Hansen *et al.*, 1992).

The addition of DNase after the application of current hardly had an influence on transformation with a replicating plasmid. This finding indicates that replicating plasmids can be introduced into *M. catarrhalis* by electrotransformation in a type IV pilus apparatus-independent way.

Transformation of *M. catarrhalis* with single-stranded DNA

It has been well demonstrated in both gram-positive and gram-negative bacteria that during natural transformation, one strand of the DNA is degraded during the uptake process and that the remaining single strand is transferred to the cytoplasm (Chaussee & Hill, 1998). We tested the ability of single-stranded DNA to transform *M. catarrhalis* either by natural or by electrotransformation. The results (Tables 1 and 2) demonstrated that *M. catarrhalis* could be transformed with single-stranded DNA but only by natural transformation as seen with double-stranded DNA. Transformation efficiencies were lower for single-stranded DNA compared with double-stranded DNA ($10\times$ for electroporation and $100\times$ for natural transformation). This is in accordance with experiments performed in *Pseudomonas stutzeri* and *H. influenzae*, which also demonstrated that single-stranded DNA is transformable, although less efficient than double-stranded DNA (Postel & Goodgal, 1966; Meier *et al.*, 2002).

DNA restriction in *M. catarrhalis*

Restriction-modification systems have been described in *M. catarrhalis* (Seib *et al.*, 2002). In particular, two distinct

Table 2. Effect of DNase on electrotransformation of *Moraxella catarrhalis*

Transforming DNA	Form of DNA	Transformation frequency*	
		− DNase	+DNase
Δ*uspA1::kan*^{R}*ds*	Linear double-stranded	2.8×10^{-4}	$< 10^{-8}$
Δ*uspA1::kan*^{R}*ss*	Linear single-stranded	1.9×10^{-5}	$< 10^{-8}$
pEMCJH04ΩlacZ	Circular replicating	1.4×10^{-4}	7.4×10^{-5}
pF1Mkan	Circular nonreplicating	9.5×10^{-5}	$< 10^{-8}$
O35E.1 × *Xba*I	Linear genomic	7.5×10^{-5}	$< 10^{-8}$

*Transformation frequency reports the ratio of kanamycin-resistant transformants to the total of surviving bacteria.

genetic loci of type III restriction-methylation systems have been found and demonstrated to be phase variable.

We tested DNA restriction using plasmid pEMCJH04. Plasmid pEMCJH04 isolated from *E. coli DH5*α cells transformed *M. catarrhalis* O35E at a low transformation frequency (1×10^{-6}) using natural transformation, whereas the identical plasmid isolated from the isogenic *M. catarrhalis* parent O35E isolate yielded *c.* 100-fold higher transformation frequencies (1.6×10^{-4}). These results indicate that restriction-modification systems are indeed active in *M. catarrhalis* and play a part in *M. catarrhalis* transformability.

If restriction-modification systems are also responsible for the nontransformability of *M. catarrhalis* by linear DNA upon electrotransformation (due to their extra-Moraxella origin), then transformants should be obtained, when homologous linear genomic DNA is used. DNA was extracted from the *uspA1* mutant O35E.1, digested with *Xba*I, purified and used to electrotransform *M. catarrhalis* O35E. This mutant harbors the identical Δ*uspA1::kan*^{R} fragment that was used in the former experiments. In control experiments, electroporated cells were immediately resuspended in BHI medium containing DNase. The results are shown in Table 2. As seen with the Δ*uspA1::kan*^{R} PCR product, no transformants (transformation frequency $< 10^{-8}$) could be isolated in DNase-treated reactions, indicating that homologous double-stranded DNA was also restricted upon cell entry. The same observation has been made in *S. pneumoniae* and *H. influenzae*. Chromosomal markers of different sizes were used to electrotransform these organisms but none of them was transformed successfully (Mitchell *et al.*, 1991; Lefrancois *et al.*, 1998). These results indicate that the restriction process of incoming double-stranded DNA during electrotransformation is not triggered by a methylation pattern, but may be induced simply by the presence of double-stranded DNA, which the host cell is not able to recombine with its own DNA.

Natural competence of *M. catarrhalis* is a constant phenomenon

Our observations that bacteria in the early exponential growth phase, as used in natural transformation, as well as in the late exponential phase, as used in electroporation, could be transformed, prompted us to investigate the influence of the growth phase on the competence of *M. catarrhalis*. Bacteria were harvested at different optical densities, adjusted to the same number of cells (5×10^7 CFU) in all transformation experiments, and then exposed to the transforming DNA (Δ*uspA1::kan*^{R}). The results shown in Fig. 1 demonstrate that except in the very late stationary phase (overnight cultures), the cells remain competent in a constant manner. This contrasts with the behavior of *S. pneumoniae*. In this species, competence is induced only during a restricted period of time during the early exponential growth phase (Claverys *et al.*, 2000). In general, competence is either regulated by cell–cell signaling or in response to changes in nutritional conditions and is most consistently present during the late exponential growth phase (Solomon & Grossman, 1996).

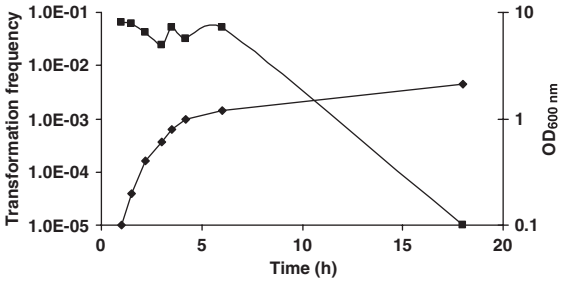

Fig. 1. Influence of growth phase on natural transformation of *Moraxella catarrhalis*. Bacteria were grown in BHI broth at 37 °C, 200 r.p.m., harvested at defined $OD_{600\,nm}$ values and adjusted to an $OD_{600\,nm}$ value of 0.2. Bacteria were spread on BHI agar plates, exposed to either DNA or distilled water on a defined area during 5 h. After incubation, bacteria were harvested from this area, resuspended, serially diluted and incubated on BHI agar plates with or without 20 mg L^{-1} kanamycin. Transformation frequency represents the ratio of kanamycin-resistant bacteria to the total of surviving bacteria. Both the transformation frequencies (closed squares) and the $OD_{600\,nm}$ values (closed diamonds) are shown with relation to time.

Conclusions

Moraxella catarrhalis is a naturally competent bacterium easily transformable by various forms of DNA. This gram-negative bacterium remains competent for a very long period of its growth cycle. In contrast, this study demonstrates that electrotransformation of *M. catarrhalis* is not an efficient method to introduce linear DNA, but can be used to establish replicating plasmids in a type IV pilus apparatus-independent way. Electrotransformants could not be obtained either by the use of double-stranded or single-stranded homologous DNA. Two explanations are possible. (i) In natural transformation, linear DNA is not only processed to single-stranded DNA during uptake but also altered or labeled, allowing subsequent recombination with the host DNA. (ii) Natural competence induces expression of yet unknown proteins necessary for the uptake and recombination of linear DNA and these proteins are not induced during electrotransformation.

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