ELECTROPORATION OF *Neisseria meningitidis* WITH PLASMID DNA

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ABSTRACT

In this paper we describe an electroporation protocol for *Neisseria meningitidis*. Plasmid vectors up to 12.5 kb, containing a selection marker, flanked by genomic regions, were constructed and used to select electroporation conditions. When nonpiliated B385 cells, suspended in electroporation buffer (glycerol 15%; $MgCl_2$ 1 mM; Sucrose 272 mM; HEPES 1 mM; pH 7.2), were mixed with 200 ng of plasmid DNA and a single pulse of 11.5 kV/cm, 400 Ω and 25 mF was applied (in a BioRad Gene Pulser II), transformation efficiencies up to 6.5 x 10^4 transformants/μg and survival rates of up to 80% were reached. The generation with this method, of an *lpdA* neisserial knock-out mutant, is also shown. To date this is the first electroporation report in meningococci.

Keywords: electroporation, *N. meningitidis*, transformation efficiency

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RESUMEN

En este artículo describimos un protocolo de electroporación para *Neisseria meningitidis*. Varios plasmidios de hasta 12,5 kb, conteniendo regiones genómicas interrumpidas por un marcador de selección, fueron construidos y usados para establecer las condiciones de electroporación. Cuando se mezclaron células de B385 no piliadas, suspendidas en el tampón de electroporación (15% glicerol; 1 mM $MgCl_2$; 272 mM sacarosa; 1 mM HEPES; pH 7,2), con 200 ng de ADN plasmídico, se obtuvieron eficiencias de transformación que alcanzaron 6,5 x 10^4 transformantes/μg con porcientos de supervivencia alrededor de 80. También se muestra la generación, a través de este método, de un mutante de *N. menigitidis* para el gen *lpdA*. Este constituye el primer reporte describiendo la técnica de electroporación aplicada a meningococo.

Palabras claves: electroporación, *N. meningitidis*, eficiencia de transformación

Introduction

Transformation is currently the most feasible option for exploring the genetics of *Neisseria meningitidis*, given that transducing phages, or efficient conjugative systems, are not available for the *Neisseriaceae* [1]. Like other bacterial species, this organism is naturally competent for genetic transformation. However, standard molecular techniques for transformation, used in *Escherichia coli*, are hardly applicable for *N. meningitidis*. The *in vitro* transformation of meningococci is dependent on a number of bacterial functions, most notably: the host piliation status, and the presence of species-specific sequence motifs in the transforming DNA. Both factors affect the first stages of DNA transfer, during which nucleic acids traverse the bacterial envelope [2, 3]. Unfortunately, even as few as two rounds of laboratory culture passages can render neisserial strains refractory to transformation, due to the quick appearance and take over of nonpiliated, non competent variants [4]. This situation is sometimes compounded by the absence of uptake motifs in the genetic constructions under study.

Previously reported transformation in *Neisseria gonorrhoeae*, using electroporation, support this technique as well suited for overcoming the above limitations [5]. Many species have been transformed by using this methodology [6, 7]. Campylobacter jejuni [8] and *E. coli* [9] have served as models for field strength, time constant (pulse duration), temperature, host cells, electroporation media, and DNA concentration optimization. However, despite the relatively simple concepts that apply to electroporation, the relatively complicated, or unknown parameters, inherent to each biological system under study demand a close monitoring of electroporation conditions. In this article we describe the development of a novel and simple electroporation protocol to transform nonpiliated variants of the *N. meningitidis* strain B385.

Materials and Methods

Bacterial strains and culture conditions

Bacterial strains used in this study included XL-1 Blue, an *E. coli* K12 strain, and B385p-, a nonpiliated variant from *N. meningitidis* strain B385 (B4:P1.15). Meningococcal cells were grown in a Brain Heart Infusion (BHI) broth, or on BHI agar plates, at 37 °C in a candle jar. XL-1 Blue was grown in a Luria-Bertani (LB) broth. When needed, Ampicillin, or Kanamycin, was added at a final concentration of 50 μg/mL.

Recombinant DNA techniques

The methods used for *in vitro* manipulation of DNA (plasmid purification, restriction analysis, Southern blots) were performed according to Sambrook, *et al.* [10]. Chromosomal DNA extraction from meningococci was performed following the procedure described by Cornelissen *et al*, 1992 [11].

Immunological methods

Expanded colonies from the transformation plates, were inoculated into 5 mL of BHI cultures overnight at 37 °C. The following day, the cultures were

1. Facius D, Fussenegger M, Meyer TF. Sequential action of factors involved in natural competence for transformation of *Neisseria gonorrhoeae*. FEMS Microbiol Lett 1996;137:159-64.

2. Gibbs CP, Reimann B-Y, Schultz E, Kaufmann A, Haas R, Meyer TF. Reassortment of pilin genes in *Neisseria gonorrhoeae* occurs by two distinct mechanisms. Nature (London) 1989;338: 651-2.

3. Goodman SD, Scocca JJ. Factors influencing the specific interaction of *Neisseria gonorrhoeae* with transforming DNA. J Bacteriol 1988;173:5921-3.

4. Biswas GD, Lacks SA, Sparling PF. Transformation-deficient mutants of piliated *Neisseria gonorrhoeae*. J Bacteriol 1989;171:657-64.

5. Genco CA, Chen C-Y, Arko RJ, Kapczynski DR, Morse SA. Isolation and characterization of a mutant of *Neisseria gonorrhoeae* that is defective in the uptake of iron from transferrin and haemoglobin and is avirulent in mouse subcutaneous chambers. J Gen Microbiol 1991; 137:1313-21.

6. Manivasakam P, Schiestl RH. High efficiency transformation of *Saccharomyces cerevisiae* by electroporation. Nuc Acids Res 1993;21:4414-5.

7. Faber KN, Haima P, Harder W, Veenhuis M, Geert AB. Highly-efficient electrotransformation of the yeast *Hansenula polymorpha*. Curr Genet 1994;25:305-10.

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harvested and 20 µg of total cellular proteins were used for SDS-PAGE [12] and Western blotting [13] with MAb114, a monoclonal antibody elicited against the C-terminal region of the LpdA protein. The immune complexes were visualized by using 3-amino-9 ethyl-carbazole.

Plasmids

pM-150: pUC18-derived recombinant plasmids obtained from subcloning a 9 kb *KpnI-SalI* library fragment from *N. meningitidis*, interrupted by a 1.2 kb Kanamycin resistance marker from pUC4K [14].

pM-110: pUC18-derived recombinant plasmid containing one copy of the 1.2 kb Kanamycin resistance marker, from pUC4K, flanked by 207 and 253 bp from 5' and 3' regions of the *lpdA* gene.

pM-84: Recombinant plasmid containing one intact copy of the *lpdA* gene, in pUC18.

Electroporation protocol

300 mL of the BHI broth were inoculated from a fresh plate of B385p- to a final OD_{640nm} = 0.1 (in Photoelectric colorimeter, Model AE330, EOW, LTD) and grown at 37 °C to exponential phase (OD_{640nm} = 0.8-0.9). Cells were harvested by centrifugation (6 000 rpm, 4 °C, 5 min), suspended in 150 mL of ice-cold electroporation buffer (EB: glycerol 15%; $MgCl_2$ 1 mM; Sucrose 272 mM; HEPES 1 mM; pH 7.2), and washed twice with 40 and 20 mL in the same EB. The cells were suspended in a final volume of 400 µL EB. High density aliquots (over 10^9 colony forming units (cfu)/mL) of cell suspension (40 µL), were frozen at -70 °C or mixed with 1 µL of undigested plasmid DNA in a TE 0.1X solution, and transferred immediately to a 0.2 cm electrode gap pre-chilled Gene Pulser cuvette (0.1 cm electrode gap cuvettes were used to reach field strengths above 12.5 kV/cm), just prior to the electric pulse with the BioRad Gene Pulser II. The parameters were set in the following ranges: Voltage, 8.0-14 kV/cm; Capacitance, 25-50 µF; Resistance, 100-600 Ω. After pulsing, 1 mL of BHI was immediately added to the cells, followed by 3 h of incubation at 37 °C before plating and selection for Kanamycin resistance.

The cells were plated both onto a selective medium for the detection of plasmid recipients and onto a non selective medium for the determination of survival rate. Transformants were visible 16-20 h after plating, and individual colonies were further isolated by serially streaking twice onto a selective medium before additional analysis.

Results

Determination of succeeding electroporation parameters

The effect of different field strengths on the electroporation efficiency of B385p- was determined using 1 µg of the plasmid pM-150, a single pulse with a capacitance of 25 µF and a resistance of 400 Ω resulting in pulse lengths of 6-9 ms.

The best transformation efficiency was obtained using a single pulse of 10-12.5 kV/cm. When the above conditions were used, strain B385p- consistently gave 10^3 transformants/µg with a 60% survival rate. Higher field strengths (13-15 kV/cm) resulted in survival rates lower than 30% and thus a decrease in overall transformation efficiency. Lower values (8-9.5 kV/cm), while yielding higher survival rates (90%), were unable to achieve the same electroporation frequency, even when longer pulse lengths were used (data not shown).

Figure 1 shows the influence of pulse length in transformation efficiency, using the above conditions and a constant field strength of 12.5 kV/cm. The best pulse time peaks were around 8.31 ms, with a drop in transformation efficiency above 8.78 ms.

To ascertain the dependence of the electroporation efficacy on the total amount of DNA, several assays were set up using a single pulse of 11.5 kV/cm with a capacitance of 25 µF and a resistance of 400 Ω. The results for varying amounts of DNA are shown

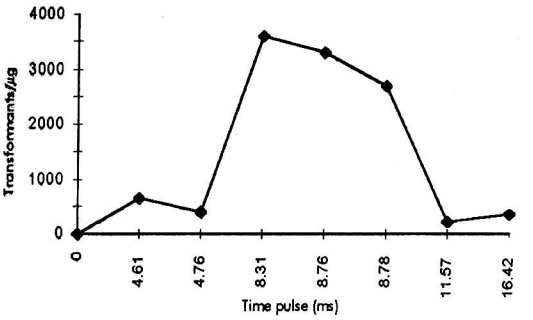

Figure 1. Effect of time pulse in transformation efficiency. Data was obtained using the following conditions: cells in early exponential phase (grown in a liquid BHI medium) were mixed with 1 µg of plasmid DNA and electroporated with a field strength of 12.5 kV/cm.

in Figure 2. As can be seen, there is a steep increase in efficiency which levels off at 50 ng and starts to decrease above 200 ng. Pulse lengths of 6.3 to 7.7 ms were reached within the useful 50-200 ng range. This further improvement allowed us to obtain more than 10^4 transformants/µg of plasmid DNA.

Generation of neisserial knock-out *lpdA* mutants

We applied these parameters to obtain neisserial *lpdA* knock-out mutants. A plasmid containing a Kanamy-

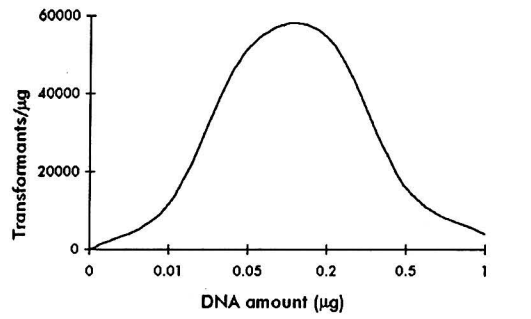

Figure .2 Effect of the amount of DNA on transformation efficiency.

8. Miller JF, Dower WJ, Tompkins LS. High-voltage electroporation of bacteria, Genetic transformation of *Campylobacter jejuni* with plasmid DNA. Proc Natl Acad Sci USA 1988;85:856-860.

9. Dower WJ, Miller JF, Ragsdale C. High efficiency transformation of *E. coli* by high voltage electroporation. Nuc Acids Res 1988;16:6127-6145.

10. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning. A laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.

11. Cornelissen CN, Biwas GD, Tsai J, Paruchuri DK, Thompson SA, Sparling PF. Gonococcal transferrin-binding protein I is required for transferrin utilization and is homologous to TonB-dependent outer membrane receptors. J Bacteriol 1992; 174:5788-5797.

12. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227: 680-685.

13. Towbin. H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrilamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979;76:4350.

14. Vieira J, Messing J. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 1982;19: 259-268.

cin resistance marker flanked by 207 and 253 bp from 5' and 3' regions of the *lpdA* gene (pM-110, see Materials and Methods) was used to electroporate B385p-. Since pM-110 does not replicate in meningococci, Kanamycin resistant transformants can be rescued only in the event of a single (insertion and gene duplication) or double (allelic replacement) recombination between the sequences flanking the selection marker and their genomic counterparts. All eight transformants tested by Western blot with MAb114 (Figure 3) failed to express the protein, suggesting that allelic replacement had occurred in all cases. This conclusion was confirmed by Southern blot analysis of 4 transformants (Figure 4) with an internal 300 bp *ClaI* fragment from the *lpdA* obtained from pM-84 as a probe.

Discussion

We searched for an efficient electrotransformation protocol of *N. meningitidis* as a feasible way for the generation of knock-out insertional mutants of chromosomal genes. As a model we used a 9 kb *KpnI-SalI* library fragment from *N. meningitidis* B385 interrupted by a Kanamycin resistance marker. With pM-150, the best results were obtained by using cells grown on a liquid BHI medium, harvested during the early exponential phase, washed and suspended in the referred electroporation buffer, in aliquots of cell density above 10^9 cfu/mL, which were mixed with 50-200 ng of DNA in a low conductivity solution. A single electric pulse of 11.5 kV field strength reaching nearly 8 ms in length with a capacitance and resistance of 25 μF and 400 Ω, respectively, was applied.

Field strength and pulse length were selected according to a compromise between transformation efficiency and survival rate. Extreme values of these electrical parameters drastically reduced cell viability or failed to induce high DNA acquisition, respectively.

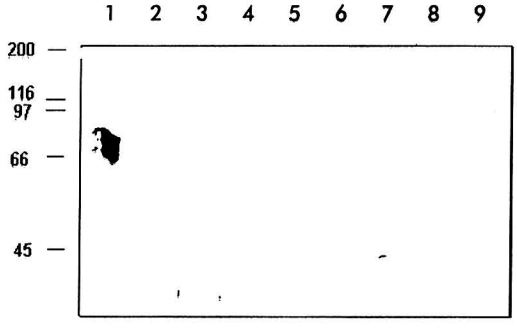

Figure 3. Western blot of whole neisserial cells for LpdA protein detection. Lane: 1, B385 wild type; 2-9, electroporated colonies from BHI Kanamycin agar plates. The double band present in the control is due to crossreactivity of the common lipoil binding domain present, at least, in two different dehydrogenases in *Neisseria*.

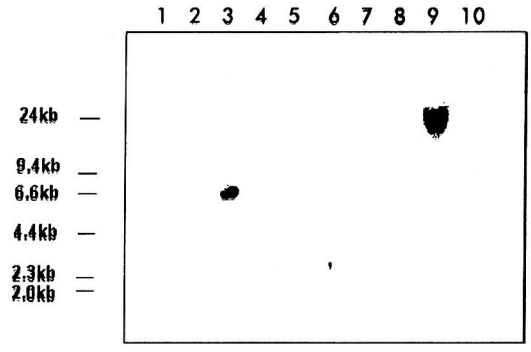

Figure 4. Southern-blot of *N. meningitidis lpdA* strains: 1, 2, 4, 5, *HindIII* digested chromosomal DNA of mutant electroporated strains for *lpdA* gene; 6, 7, 8, 10, *BamHI* digested chromosomal DNA of mutant electroporated strains for *lpdA* gene; 3,9, B385 wild type chromosomal DNA digested *BamHI* or *HindIII*. An 300 bp *lpdA* fragment from pM-84 *ClaI*-digested was used as probe.

No transformants were obtained when older cultures were used (data not shown). A similar decrease in transformation efficiency has been reported for *Streptococcus pyogenes* [15]. Negative results were also obtained when cells were taken directly from plates for electroporation, probably because lower viable cell densities were reached (data not shown).

The number of transformants dropped dramatically when amounts of DNA higher than 200 ng were added. A possible explanation to this phenomenon could be the occurrence of cotransformation events affecting cell physiology. However, we can not rule out the presence of contaminating elements in our DNA preparations as a probable cause, since the influence of alternative DNA purification methods was not evaluated.

Using the conditions established in this work (50-200 ng of DNA, 11.5 kV/cm, 400 Ω, 25 μF in a BioRad Gene Pulser II), transformation efficiencies of up to 6.5×10^4 transformants/μg and survival rates of up to 80% were reached. It should be noted that the efficiency could be even higher if replicative vectors are used [16]. Another important point to investigate is the influence of size, topology, and source of DNA (linear DNA, single stranded DNA, PCR-derived DNA, etc.), in this process.

The generation of *lpdA* mutants by electroporation was extremely easy, avoiding a large process of selection for piliated competent variants, which is the alternative method. Our results prove the feasibility of electroporation as a methodology for the genetic manipulation of laboratory-adapted strains of *N. meningitidis*, where standard molecular techniques for transformation are hardly applicable and neither transducing phages nor efficient conjugative systems are available. The development of genetic tools for this species can be very helpful in the construction of new strains for vaccine development.

15. Simon D, Ferretti JJ. Electrotransformation of *Streptococcus pyogenes* with plasmid and linear DNA. FEMS Microbiology Letters 1991;82:219-24.

16. Iborra F. High efficiency of *Kluyveromyces marxianus* by a replicative plasmid. Curr Genet 1993;24:181-3.

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