Electroporation of *Haemophilus influenzae* is effective for transformation of plasmid but not chromosomal DNA

Marilyn A. Mitchell, Krzysztof Skowronek¹, Leszek Kauc¹ and Sol H. Goodgal*

Department of Microbiology, The School of Medicine, University of Pennsylvania, Philadelphia, PA 19104–6076, USA and ¹Institute of Microbiology, University of Warsaw, Warsaw, Poland

Received March 18, 1991; Revised and Accepted June 5, 1991

ABSTRACT

Electroporation of plasmid and chromosomal DNAs were tested in *Haemophilus influenzae* because of an interest in introducing DNA into mutants that are deficient in competence for transformation. The initial experiments were designed to investigate and optimize conditions for electroporation of *H. influenzae*. Plasmid DNA was introduced into the competence proficient strain Rd and its competence-deficient uptake mutants *com-52*, *com-59*, and *com-88*, and the recombination deficient mutant *rec1*. Plasmid DNA could also be electroporated into the non-transforming strains Ra, Rc, Re and Rf. Plasmid DNA without sequences that are involved in tight binding (uptake) of DNA by competent cells of *H. influenzae* Rd was electroporated into both competent and non-competent cells. Competent cells were several orders of magnitude less efficient than non-competent cells for electroporation of plasmid DNAs. Electroporation of *H. influenzae* chromosomal DNA was not successful. Low levels of integration of chromosomal markers were observed following electroporation and these could be ascribed to transformation. The treatment of cells with DNaseI following electroporation separated the effects due to electroporation from those due to transformation. The DNaseI treatment did not affect the efficiency of plasmid incorporation, but severely restricted effects due to natural DNA transformation.

INTRODUCTION

Voltage shock transformation, electroporation, has been used to introduce a variety of genes into eukaryotic (1, 2, 3) and prokaryotic (4, 5, 6, 7, 8, 9) cells. This technique often increases the efficiency of transformation and permits the introduction of DNA into cells without known methods of competence development. *Haemophilus influenzae* serotype d is a bacterium whose naturally occurring competence and mechanism of DNA uptake are relatively well known (10, 11). A large number of *H. influenzae* mutants have been described that do not transform (12). Some of these competence mutants (*com*[–]) have been characterized as being unable to integrate or recombine donor

DNA while others have been characterized as being unable to bind donor DNA. *H. influenzae* Ra, Rc, Re and Rf are known to be non-transformable, or naturally competence-deficient, since, to date, all efforts to introduce transforming DNA into these strains have failed. The original goal of this project was to transform competence-deficient cells of *H. influenzae*, however, it soon became apparent that chromosomal transformation by electroporation would be extremely inefficient. The introduction of plasmids or plasmids with chromosomal inserts was highly successful.

MATERIALS AND METHODS

Bacterial strains and plasmids

H. influenzae strains used in this study were Ra, Rb, Rc, Re, Rf, RdV23, three uptake-deficient mutants of Rd, *com-52*, *com-59* and *com-88* (12), and a recombinant-defective mutant of Rd, *rec1* (13). The plasmid *pJ1-8* was obtained from N. Notani (14). The derivatives *pJ1-8(2s)*, *pJ1-8(4s)*, and *pER194* contain inserts of *H. influenzae* Rd DNA and were prepared in this laboratory. Plasmid DNAs were isolated by an alkaline lysis procedure (15).

Electroporation

For the electroporation experiments cells were made competent by the MIV procedure (16, see below) and non-competent cells were grown in supplemented brain heart infusion broth (sBHI) to OD₆₅₀=0.16. The cells were ice-chilled for 30 min, transferred to ice-chilled centrifuge tubes, harvested by centrifugation at 4,200×g in a Sorvall RC2B centrifuge for 5 min, and thoroughly washed 5 times with PSG buffer (15% glycerol, 272 mM sucrose, 2.43 mM K₂HPO₄, 0.57 mM KH₂PO₄, pH 7.4) or SG buffer (PSG minus phosphate) at 4°C. The washes were centrifuged at 4,200×g for 20 minutes. The first two washes were performed with buffer volumes equal to the volume of the culture, and for the next 3 washes the volumes were reduced by half for each wash. The last cell pellet was drained well and suspended, without vortexing, in PSG or SG buffer to provide a 100-fold increase in concentration or an OD₆₅₀ of 12 for a 1 cm path. The cells were aliquoted into 40 µl samples. In some early experiments the cells were not as

* To whom correspondence should be addressed

extensively washed and were stored in liquid nitrogen before use. These preparations were not as efficient for electroporation as extensively washed freshly prepared cells, probably due to the retention of electrolytes, since the time constants of the stored cells were consistently low (see below). DNA, 0.1 to 1 μ g, (1 to 4 μ l in water) was added to the ice-chilled cells, stored on ice for 1 min, and quantitatively transferred to cuvettes that had been kept at -20°C and warmed to approximately 0°C before electroporation. Some early experiments were also done at 23°C . Electroporation of the 40 μ l samples was performed in cuvettes with an 0.2 cm electrode gap using the Pulse-Gen apparatus (Bio-Rad). A variety of conditions were explored for all of the strains tested and except for *Ra* the maximum efficiency of electroporation was obtained at 2.5 kV (12.5 kV/cm), 400 ohms, and 25 μ F. The time constants in the early experiments were 7.5–8.0, and for later experiments in which the cells were more extensively washed, 9.0–9.3. After electroporation, the cells were immediately mixed with 1 ml of sBHI broth prewarmed to 37°C , incubated at 37°C for 1 hour, appropriately diluted and pour plated with 10 ml sBHI-agar. The plates were incubated at 37°C for 30 min to allow for expression of the ampicillin marker and 3 hrs for chromosomal markers. The plates were overlain with 10 ml sBHI-agar containing the selective antibiotic and incubated overnight at 37°C . The concentration of antibiotic in the overlay was 5 μ g/ml for ampicillin, 200 μ g/ml for streptomycin, 15 μ g/ml for kanamycin, and 20 μ g/ml for erythromycin or novobiocin. The yield of plasmid containing cells after electroporation was not greater than 1% of surviving cells, and was directly proportional to DNA concentration even at the highest concentrations used. For those experiments in which DNase treatment was performed, DNaseI (Sigma Chem. Co.) was added at 20 μ g/ml to 0.5 ml of the electroporated cells diluted with prewarmed sBHI broth.

Competence development

H. influenzae was made competent for DNA uptake with the MIV procedure (16) by growing cells diluted 1:100 from a frozen stock at ca. 2×10^9 cells per ml. Cells were grown to an optical density of 0.16 (optical density at 650 nm on a Coleman Junior spectrophotometer) equal to 2×10^9 cells per ml. The culture was centrifuged and washed twice with MIV, resuspended in an equal volume of MIV, and further shaken with aeration at 37°C for 100 min.

RESULTS

Electroporation of non-competent cells by plasmid DNA

The results of transformation by electroporation of non-competent strains of *H. influenzae* with *pJ1-8* DNA carrying an ampicillin marker are presented in Table 1. The strains tested included the rough derivatives of serotypes *a, b, c, e* and *f*, a non-competent culture of the competent proficient strain *Rd* and its competent deficient mutants *rec1*, *com-52* and *com-59*. All of the strains exhibited some level of electroporation except for *Ra*. In other experiments in which the conditions were modified, by lowering the resistance to 200 ohms, *Ra* yielded low levels of electroporation, about 10^3 /ml. Since the plasmid *pJ1-8* used for electroporation was amplified in *Rd* it was anticipated that the other serotypes would restrict the plasmid, and the levels of *pJ1-8* incorporation following electroporation suggested this was the case. As shown in Table 1, *pJ1-8* extracted from *Ra(pJ1-8)* markedly improved electroporation into *Ra*. However, these

Table 1. Electroporation of *Haemophilus* serotypes and competence deficient mutants of *Haemophilus influenzae Rd* with *pJ1-8* DNA^a

Recipient Strains	Amp ^R Cells		Survival
	Elec.	Non-Elec.	
<i>Ra</i>	$< 10^2$	$< 10^2$	0.90
<i>Ra</i> ^b	3.0×10^3	$< 10^2$	n.d.
<i>Rb</i>	1.0×10^2	$< 10^2$	0.76
<i>Rc</i>	4.5×10^5	$< 10^2$	0.79
<i>Re</i>	1.0×10^6	$< 10^2$	0.40
<i>Rf</i>	1.4×10^5	$< 10^2$	1.0 ^c
<i>Rd com-52</i>	1.4×10^7	$< 10^2$	0.71
<i>Rd com-59</i>	1.2×10^6	$< 10^2$	0.67
<i>Rd rec1</i>	3.1×10^7	$< 10^2$	0.32
<i>Rd</i>	1.4×10^7	1×10^2	0.65

^aThe plasmid was extracted from *Rd(pJ1-8)* and the concentration of DNA was 0.2 μ g/40 μ l sample. The cell concentration before electroporation was 8×10^9 /40 μ l sample.

^bThe *pJ1-8* DNA was extracted from *Ra(pJ1-8)*.

^cThe high level of survival of *Rf* can be accounted for by its growth in chains.

levels were still far lower than those for electroporation of *Rd* by *pJ1-8(Rd)* indicating that some parameter other than restriction-modification must also be important for the efficiency of electroporation. The survival of electroporated cells in this experiment was greater than 50% for most strains which was 2 to 5 fold higher than earlier experiments in which cells were only washed twice. Higher levels of survival were associated with more extensive washing of cells prior to electroporation. Electroporants could not be detected in the control experiments in which the DNA was omitted, and few, if any, in experiments in which *pJ1-8* DNA was included but the electric shock was omitted. Because of the background with ampicillin selection the lowest number of transformants that could be detected in these experiments was 10^2 per 10^{10} cells.

The plasmid *pJ1-8* (14) has been shown to exist in two forms, monomeric and multimeric, due to a mutation in the plasmid (Roszczyk and Goodgal, unpublished observation). The multimeric form transforms with single-hit kinetics compared to the two-hit requirement for the monomeric form. It is likely that nuclease activity in competent cells prevents the establishment of the single monomeric plasmid, and that the multimeric form can recombine to yield a functional replicon. Since it is also far less efficient in transformation of *H. influenzae Rd*, the monomeric form has been used to construct a DNA library based on the increased efficiency of uptake of plasmids containing inserts of *H. influenzae* DNA with strong uptake sites. The library could be deficient in DNA fragments without uptake sites. To circumvent this problem electroporation was used to introduce monomeric *pJ1-8* plasmids with DNA inserts that do or do not contain the 9 bp uptake sequence AAGTGCGGT. A comparison of the electroporation of plasmids with and without uptake sequences (see below) is shown in Tables 2 and 4 and demonstrates that the presence or absence of strong uptake sites was not a barrier for electroporation.

During the procedure for electroporation there was induction of low to moderate levels of competence in competent proficient strains of *Haemophilus*. The *Rd* cells that had no capacity for transformation with plasmids or chromosomal DNA did become somewhat competent after preparation for electroporation and incubation in broth prior to selection. As a result of treatment of non-electroporated cells with plasmid DNA, Table 2, a low level of transformation of *Rd* was observed with *pJ1-8*(4s) which

Table 2. Electroporation of *Rc*, *Rd* and *Rd* competence deficient mutants with chromosomal and plasmid DNAs^a

Recipient Cells	DNA	Transf to Str ^R		Amp ^R Cells		Cell Surv
		Elec	Non-Elec	Elec	Non-Elec	
<i>Rd</i>	H.i.SCE	1.3×10^{5b}	2.4×10^{6b}	—	—	0.65
<i>Rd</i>	<i>pJI-8(4s)</i>	2.2×10^3	1.0×10^5	4.9×10^7	1.4×10^3	0.65
<i>Rd com-52</i>	<i>pJI-8(4s)</i>	$< 10^1$	$< 10^1$	6.0×10^7	$< 10^2$	0.67
<i>Rd com-59</i>	<i>pJI-8(4s)</i>	$< 10^1$	$< 10^1$	1.4×10^7	$< 10^2$	0.70
<i>Rd</i>	<i>pJI-8(194)</i>	—	—	1.4×10^6	3.6×10^5	0.65
<i>Rd com-59</i>	<i>pJI-8(194)</i>	—	—	3.4×10^5	$< 10^2$	0.70
<i>Rc</i>	<i>pJI-8(194)</i>	—	—	3.2×10^5	$< 10^2$	n.d.

^aThe concentration of *H. influenzae* DNA was 1 µg/40 µl sample and for plasmid DNA 0.5 µg/40 µl sample. The cell concentration before electroporation was 8×10^9 /40 µl sample.

^bSimilar numbers of transformants were also obtained for the erythromycin (E) and novobiocin (C) resistance markers.

Table 3. Electroporation of competent and non-competent cells with *pJI-8* DNA^a

Recipient Cells	Amp ^R Electroporants			
	Competent Cells		Non-Competent Cells	
	Elec	Non-Elec	Elec	Non-Elec
<i>Rd</i>	5.6×10^3	1.7×10^3	6.0×10^6	$< 10^2$
<i>Rd com-52</i>	2.0×10^2	$< 10^2$	1.8×10^6	$< 10^2$
<i>Rd com-59</i>	1.2×10^4	$< 10^2$	6.0×10^6	$< 10^2$
<i>Rd com-88</i>	1.0×10^2	$< 10^2$	1.2×10^6	$< 10^2$

^aThe concentration of *pJI-8* DNA was 0.25 µg/40 µl sample, and the viable count of the cells before electroporation was 2.0×10^{11} /ml. After electroporation the cells were diluted 26 fold and measured for electroporants and viable cells. The survival after electroporation was 0.10 to 0.30.

Table 4. Effect of DNase on plasmid and chromosomal markers following electroporation^a

Recipient Cells	H.i. DNA Markers ^b		Plasmid DNA Amp ^R		Plasmid
	w/o DNase	w DNase	w/o DNase	w DNase	
<i>Rd</i>	1.2×10^5	1×10^2	4.8×10^7	3.0×10^7	<i>JI-8</i>
<i>com-52</i>	$< 10^2$	$< 10^2$	1.5×10^7	1.0×10^7	<i>JI-8</i>
<i>com-59</i>	$< 10^2$	$< 10^2$	7.6×10^6	8.8×10^6	<i>JI-8</i>
<i>Rd</i>	—	—	1.2×10^8	1.0×10^8	<i>JI-8(4s)</i>
<i>Rd</i>	—	—	9.0×10^7	1.0×10^8	<i>JI-8(2s)</i>

^aThe *H. influenzae* DNA concentration was 1 µg/40 µl sample and the plasmid concentrations were 0.5 µg/40 µl sample for *pJI-8* and 1.0 µg/40 µl sample for *pJI-8(4s)* and *pJI-8(2s)*. The cell concentrations were 10^{10} /40 µl before electroporation and the survivals after electroporation were 0.55–0.71.

^b*H. influenzae* chromosomal DNA contained the markers for streptomycin, novobiocin, erythromycin, and dalacin (streptovaricin) resistance. Marker efficiencies were all consistent and corrected to the marker efficiency of the *str^R* marker.

has only weak binding sites and monomeric *pJI-8(2s)* that has a single strong binding site. A significantly greater amount was observed with *pER194* a multimeric plasmid that contains at least 3 strong uptake sites. Non-electroporated *com-52*, *com-59* and *Rc* that were deficient in DNA uptake receptors failed to show transformation by any plasmid DNA. When *pER194* DNA was used to electroporate strain *RdV23*, the strong binding sites of *pER194* and the high level of spontaneous competence development among the *H. influenzae* *RdV23* cells (10^5 to 10^6 transformants/µg DNA), at first, prevented us from distinguishing the electroporants from naturally transformed cells. However, uptake deficient strains and DNaseI treatment (see below) separated the two processes.

Effect of DNaseI on markers following electroporation

Since many *Haemophilus* strains do not transform it was of some interest to test for the electroporation of chromosomal DNA into

non-transforming strains. The tests performed on the strains listed in materials and methods were uniformly negative as illustrated in Table 3 for the uptake deficient mutants *com-52* and *com-59*. The uptake proficient strain *Rd*, however, did yield transformants but as shown in Table 4 they were sensitive to DNase added to the broth medium in which the cells were diluted following electroporation. On the other hand, plasmid transformation was not affected by the addition of DNase. Although the efficiency of electroporation in this experiment was lower for *com-52* compared to *Rd*, other data (see Tables 1, 2 and 3) suggest that their efficiencies were comparable but *com-59* usually exhibited a lower efficiency of electroporation. The plasmid *pJI-8(4s)* contains an insert with the streptomycin resistance marker that is not expressed from the plasmid but only after integration into the chromosome. Electroporation with *pJI-8(4s)* of *Rd* cells that were not specifically induced to competence produced about 5×10^4 Str^R cells, but they were eliminated by treatment with

DNaseI following electroporation, and suggest that these transformations were due to competence development and uptake of *pJ1-8(4s)* DNA after electroporation.

Electroporation of competent and non-competent cells

The process of electroporation with plasmid DNA by-passes the normal process of uptake associated with transformation, and appears to be a simple function of the displacement of a volume of the surrounding solution into the cell. However, the efficiency of the process was dependent upon the condition of the recipient. For non-competent cultures levels of $2 \times 10^8/\mu\text{g}$ DNA were readily attainable, but for competent cells electroporation efficiencies relative to non-competent cells were greatly reduced (Table 3). Non-electroporated *Rd* competent cells were transformed at low levels by *pJ1-8*, however, there were no transformants in the non-electroporated uptake mutants *com-52*, *com-59* and *com-88*. The *Amp^R* cells observed after electroporation of competent cultures of *Rd* were in part due to transformation, and most of these were eliminated by the addition of DNase after electroporation. Since the competence deficient mutants do not transform naturally, all their *Amp^R* cells were due to electroporation. These results demonstrated the markedly greater efficiency of electroporation of non-competent compared to competent *H.influenzae*.

DISCUSSION

Electroporation is an efficient method for the incorporation of plasmid DNAs without strong binding (uptake) sites and for introducing plasmid DNAs into non-transformable strains or non-competent *H.influenzae*. With this procedure it was possible to transform plasmid DNA into the non-transforming serotypes *Ra*, *Rc*, *Re*, *Rf* and competent deficient strains of *H.influenzae Rd*. The efficiencies of electroporation were 4 or more orders of magnitude greater than for transformation with plasmids. However, the efficiencies for different strains varied considerably, and could be due, in part, to restriction-modification (17, 18). Plasmids reextracted from electroporants of *Ra* (also *Rc* and *Re* for which the data are not shown) were incorporated more efficiently into recipients of the same serotype, but not as efficiently as *Rd* plasmids into *Rd* suggesting that restriction-modification was not the only mechanism affecting the efficiency of electroporation.

It should be emphasized that there are no reports of successful electroporation with chromosomal DNA in procaryotes. Preliminary experiments suggested that low levels of transformation could be obtained with non-competent *Rd* following electroporation, however, the procedure for the preparation of non-competent *Rd* cells resulted in the stimulation of low levels of competence during the incubation period following electroporation. Treatment of cells with DNase during this period eliminated the normal transformants, and provided a procedure to separate the effects of electroporation from normal transformation. Furthermore, there were no transformants by electroporation with chromosomal DNA in the uptake deficient mutants *Rd com-52* or *com-59*, the recombination defective mutant *Rd rec1*, or serotypes *Ra*, *Rc*, *Re* and *Rf*. Since the length of the average chromosomal DNA fragment is at least an order of magnitude greater than that of the cell it would be difficult to enclose it in a volume of solution that enters the cell by electroporation.

DNase treatment following electroporation had essentially no effect on plasmid establishment and demonstrated that the cell surface rapidly recovered from electric shock. What was surprising was the relatively low efficiency of electroporation with competent cells compared to non-competent cells. This effect was not due to differential survival since these levels were quite similar, and it was not due to differences in the DNA uptake process since uptake deficient mutants induced by the MIV competence procedure behaved similarly. One possible explanation is that nucleases are induced in competent cells that inactivate the donor plasmid. Another interpretation is that the DNA is compartmentalized differently in competent and non-competent cells. It may be worth noting that the efficiency of plasmid and phage DNAs for transformation of competent *H.influenzae* is 3 or 4 orders of magnitude lower than chromosomal DNA transformation and that the efficiency of plasmid or phage DNA but not chromosomal DNA can be enhanced by the addition of high concentrations of glycerol following DNA uptake (19).

Although it was not possible to directly introduce linear chromosomal DNA by electroporation into *H.influenzae Ra* and *Re*, compatible plasmids containing chromosomal DNA inserts were readily established in these strains. For some markers subsequent recombination led to donor marker integration into the chromosome (data not presented) by a mechanism similar to that for plasmids in *H.influenzae Rd* (20).

ACKNOWLEDGEMENTS

This work was supported by the Department of Public Health and Human Services Grant GM24915 from the National Institutes of Health. We wish to thank Dr Khotso Mokhele for reading the manuscript.

REFERENCES

1. Chu, G., H. Hayakawa and P. Berg (1987) *Nucl. Acids Res.* **15**, 1311–1326.
2. Fromm, M., L. P. Taylor, and V. Walbot (1985) *Proc. Natl. Acad. Sci. USA* **82**, 5824–5828.
3. Karube, I., E. Tamiya and H. Matsuoka (1985) *FEBS Lett.* **182**, 90–94.
4. Chassy, B. M. and J. L. Flickinger (1987) *FEMS Microbiol. Lett.* **44**, 173–177.
5. Dower, W. J., J. F. Miller and W. Ragsdale (1988) *Nucl. Acids Res.* **16**, 6127–6145.
6. Fiedler, S. and R. Wirth (1988) *Anal. Biochem.* **170**, 38–44.
7. MacNeil, D. J. (1987) *FEMS Microbiol. Lett.* **42**, 239–244.
8. Miller, J. F., W. J. Dower and L. S. Tompkins (1988) *Proc. Natl. Acad. Sci. USA* **85**, 856–860.
9. Taketo, A. (1988) *Biochem. Biophys. Acta* **949**, 318–324.
10. Goodgal, S. H. (1982) *Annu. Rev. Genet.* **16**, 169–192.
11. Smith, H. O., D. B. Danner and R. A. Deich (1981) *Ann. Rev. Biochem.* **50**, 41–68.
12. Caster, J. H., E. H. Postel and S. H. Goodgal (1970) *Nature* **227**, 515–517.
13. Setlow, J. K., M. E. Boling, K. L. Beattie and R. F. Kimball (1972) *J. Mol. Biol.* **68**, 361.
14. Joshi, V. P. and N. K. Notani (1984) *Indian J. Exp. Biol.* **22**, 625–628.
15. Maniatis, T., E. F. Fritsch and J. Sambrook (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
16. Herriott, R. M., E. M. Meyer and M. Vogt (1970) *J. Bacteriol.* **101**, 517–524.
17. Piekarczyk, A. and J. Kalinowska (1974) *J. Gen. Microbiol.* **81**, 405–411.
18. Piekarczyk, A., L. Kauc and S. W. Glover (1974) *J. Gen. Microbiol.* **81**, 391–403.
19. Stuy, J. H. and Walter, R. B. (1986) *Mol. Gen. Genet.* **203**, 296–299.
20. Setlow, J. K., N. K. Notani, D. McCarthy, and N. L. Clayton (1981) *J. Bacteriol.* **148**, 804–811.