Yonsei Medical Journal
Vol. 39, No. 2, pp. 141~147, 1998

# Factors Affecting Transformation Efficiency of BCG with a *Mycobacterium-Escherichia coli* Shuttle Vector pYUB18 by Electroporation

**Sang-Nae Cho[1], Jin-Hee Hwang[1], Sun Park[1], Yunsup Chong[2], Sung-Kyu Kim[3], Chul-Yong Song[4], and Joo-Deuk Kim[1]**

*BCG has been one of the vehicles for multi-recombinant vaccine. However, low transformation efficiency of BCG with plasmid DNA hampered studies involving expression of foreign antigens in BCG. In an effort to determine the optimal conditions, this study was initiated to investigate factors involved in the transformation of BCG with a* Mycobacterium-Escherichia coli *shuttle vector, pYUB18, by electroporation.* Mycobacterium bovis *BCG (strain 1173P2) was grown in Middlebrook (M) 7H9 broth containing albumin-dextrose-catalase and 0.05% tween 80, and transformed BCG was grown in M7H10 agar containing kanamycin for counting viable cells. Pretreatment of BCG with 10 mM $CaCl_2$ improved the transformation efficiency, but overnight incubation of BCG with 1% glycine did not. The transformation efficiency in BCG also varied depending on voltage, resistance, and DNA concentration. The maximum transformation efficiency was obtained when the infinity resistance, 12.5 Kv/cm, and 100 ng of DNA were used, and reached 1.4 x $10^5$ CFU/μg of plasmid DNA, which is about 3~100 times greater than those from previous reports. The transformation conditions described in this study, therefore, will give us a better position for employing BCG as a vehicle for developing multi-recombinant vaccines.*

**Key Words:** BCG, transformation, electroporation, pYUB18, shuttle vector

Tuberculosis is still a major public health problem in most parts of the world, killing more than three million people each year. One of the approaches to control the disease is to develop an effective vaccine. Although the BCG vaccine has been available for a long time, its protection efficiency has varied markedly between field trials (Ten Dam, 1984) and it is still controversial. One of the strategies to improve BCG vaccine is to over-express protective antigens of *Mycobacterium tuberculosis* in BCG or human cytokines, thus augmenting the host immunity against tuberculosis (Jacobs Jr *et al.* 1987; Stover *et al.* 1992). For example, the MPT70 antigen from *M. tuberculosis* H37Rv was expressed in BCG (Matsumoto *et al.* 1995), and human IL-2 in BCG (Kong and Kunimoto, 1995).

In addition, BCG has been one of the ideal vehicles for multi-recombinant vaccines against other infectious diseases. For example, foreign genes including tetanus toxin fragment C (Stover *et al.* 1991), *OspA* of *Borrellia burgdorferi* (Stover *et al.*

Received December 31, 1997
Accepted February 3, 1998
[1]Departments of Microbiology, [2]Clinical Pathology, and [3]Internal Medicine, Yonsei University College of Medicine, Seoul and [4]Department of Biology, Chung-Ang University, Seoul, Korea
This study was supported by a development project grant and faculty funds (92-3) of Yonsei University College of Medicine for 1992.
Address reprint request to Dr. S.N. Cho, Department of Microbiology, Yonsei University College of Medicine, C.P.O. Box 8044, Seoul 120-752, Korea

1993), nef of human immunodeficiency virus (Winter *et al.* 1991), and gp63 of *Leishmania major* (Abdelhak *et al.* 1995) were expressed in BCG.

One of the major obstacles in cloning and the expression of foreign genes in BCG, however, was the difficulty in delivering vectors containing recombinant molecules to the organisms mainly due to a rather complex cell wall structure of mycobacteria (Draper, 1982). The transformation efficiency in BCG with *Mycobacterium-Escherichia coli* shuttle vectors by electroporation ranged from $10^2$ to $10^4$ CFU/μg DNA, which were markedly lower than $10^8$ CFU/μg in *E. coli* (Lugosi *et al.* 1989; Garbe *et al.* 1994).

Electroporation momentarily disrupts the cell membrane by electric field pulses thus enabling foreign DNA to enter the cells, while factors affecting the transformation efficiency include voltage, resistance, and DNA concentration (Forster and Neumann, 1989; Chang *et al.* 1992). In addition, strains of BCG, culture media and duration, and pretreatment of culture affected the transformation of BCG with vectors (Lugosi *et al.* 1989; Garbe *et al.* 1994). However, no systemic studies on these conditions have been reported to date, and this study was therefore initiated to examine such variables to obtain maximum transformation efficiency of BCG with one of the *Mycobacterium-E. coli* shuttle vectors, pYUB18.

## MATERIALS AND METHODS

### Bacterial strains and cultures

BCG 1173P2 (WHO International BCG Reference Center, Statens Seruminstitut, Copenhagen, Denmark) was maintained in an Ogawa egg media (Chadwick, 1982). For use in transformation, BCG was cultured in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA) containing 0.05% (v/v) Tween 80 supplemented with albumin-dextrose-catalase (M7H9-ADC-Tw) (Sigma Chemical Co., St. Louis, MO, USA) at 37°C in a shaking incubator. For counting the colony forming unit (CFU), BCG was plated on a M7H10 agar plate containing cyclohexamide (100 μg/ml) (Sigma Chemical Co.) to prevent fungal growth during a long incubation period. In order to select transformed BCG, kanamycin (10 μg/ml) (Sigma Chemical Co.) was added to M7H10 agar containing ADC.

*Mycobacterium smegmatis* and *E. coli* were also included in this study as controls for comparing the transformation efficiency. *M. smegmatis* $mc^2155$ (Snapper *et al.* 1990) was provided by W.R. Jacobs, Jr. (Albert Einstein College of Medicine, Bronx, NY, USA) and cultured in M7H9-ADC-Tw broth at 37°C for preparation of competent cells. Kanamycin (25 μg/ml) was added to M7H10 agar to select the transformed cells. *E. coli* DH5α (gift from W.R. Jacobs, Jr.) was grown in LB broth (Luria-Bertani, Difco Laboratories), and kanamycin was used at 50 μg/ml in LB agar for selection of transformed cells.

### Preparation of cultures for electroporation

In order to prepare BCG cells for transformation, BCG was grown in M7H9-ADC-Tw broth at 37°C for 2 weeks in a shaking incubator. Then, 5 ml of culture was transferred to 500 ml of M7H9-ADC-Tw broth and cultured further for 6 to 7 days to reach readings of 0.4-1.0 at O.D. 600. BCG cultures were placed on ice for 1 h and harvested by centrifugation at 8,000 x *g* at 4°C for 15 min. The packed cells were re-suspended in one-tenth volume (50 ml) of 10% glycerol and centrifuged at 8,000 x *g* at 4°C for 15 min. This procedure was repeated 4 times. Finally, the packed cells were re-suspended in one-hundredth volume (5 ml) of 10% glycerol. *M. smegmatis* cultured in M7H9-ADC-Tw and *E. coli* in LB broth were prepared by the same procedure described above for electroporation.

### Cosmid DNA

A 12 kb *Mycobacterium-E. coli* shuttle cosmid vector pYUB18 (Jacobs Jr. *et al.* 1991), provided by W.R. Jacobs, Jr., was prepared from *E. coli* $ec^2$88 containing the cosmid vector by the alkaline-sodium dodecyl sulfate methods (Sambrook *et al.* 1989). The cosmid DNA was purified by the polyethylene glycol precipitation method, and its concentration was determined by the standard method (Sambrook *et al.* 1989).

### Electroporation

BCG cells were transformed with the cosmid pYUB18 DNA by electroporation using a Gene Pulser (Bio-Rad Laboratories, Inc., Richmond, CA, USA) as described by Lugosi *et al.* (1989) with minor modifications. Briefly, 0.4 ml of concentrated BCG cells were mixed with 5 μl of pYUB18 DNA. The mixture was added to 0.2 cm electrode gap pulser cuvette, and the cuvette was then placed on ice for 10 min. After electroporation in various conditions, the contents were washed out with 1.0 ml of M7H9-ADC-Tw broth and incubated at 37°C for 1 h. The cells were diluted by 10-fold, and 50 ml from each dilution was spread on the M7H10-ADC plate containing 25 μg/ml of kanamycin in triplicate and incubated at 37°C in a $CO_2$ incubator. After 3 to 4 weeks, the number of colonies were counted. Transformation efficiency was expressed as the number of BCG colonies (i.e., transformed cells) per 1 μg of DNA per $10^7$ viable BCG cells, in which viable cells after pulse were determined in non-selection media without kanamycin.

### Statistical analysis

Each experiment was conducted 3 times, and the paired Student's *t*-test was employed for comparison between treatment groups. Statistical significance was set at $p < 0.05$. Data were expressed as mean + standard errors of mean.

## RESULTS

### Pretreatment of BCG cells

In order to increase permeability of BCG cell walls, BCG cultures were treated with 1% glycine for 24 h before harvesting. For modified $CaCl_2$ treatment, the harvested cells were mixed with one-tenth of the original volume of transformation buffer consisting of 30 mM potassium acetate, 50 mM $MnCl_2$, 10 mM $CaCl_2$, 15% glycerol and placed on ice for 15 min, followed by washing 4 times with 10% glycerol. Control cells had no pretreatment after washing with 10% glycerol as described above. pYUB18 DNA (500 ng) was added to each cuvette, and the electroporation conditions for voltage, resistance and capacitance were 12.5 kV/cm, 1,000 ohms, and 25 μFD, respectively. The pretreatment of BCG cultures with 1% glycine for 24 h killed more than 70% of the organisms (data not shown) and resulted in lower transformation efficiency than control (Fig. 1). On the other hand, $CaCl_2$-treated BCG cells gave a higher transformation efficiency compared to control, although there was no statistical significance ($p > 0.05$). Therefore, $CaCl_2$-

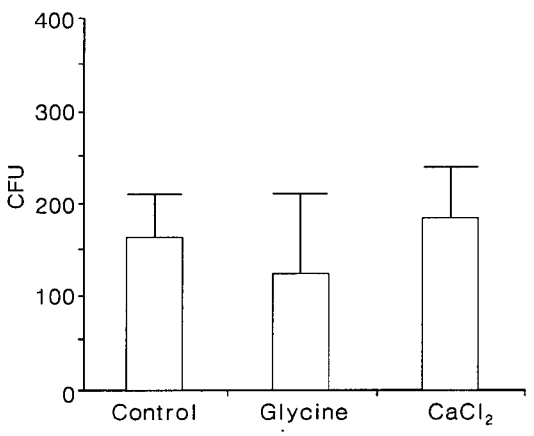


*Fig. 1. Effect of pretreatment of BCG cells on transformation efficiency. Results are expressed as the mean (plus standard deviations) of colony forming units transformed with the pYUB18 vector in three experiments.*

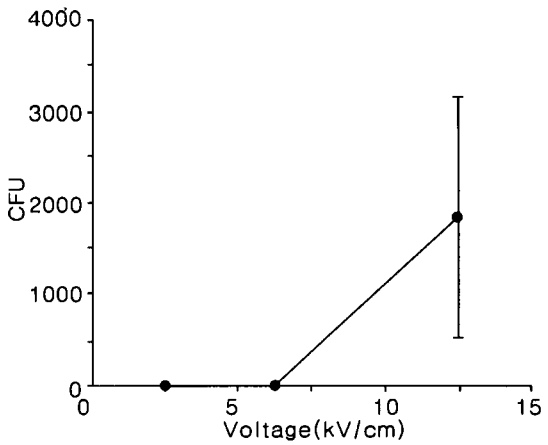


*Fig. 2. Effect of electroporation voltage on transformation efficiency. Results are expressed as the mean ± standard deviations of colony forming units transformed with the pYUB18 vector in three experiments.*

treated cells were used in the rest of the experiments.

### Voltage and resistance

The effect of voltage on the transformation efficiency was determined by transforming CaCl2-treated BCG cells at different voltages in fixed conditions of 1,000 ohms, 25 μFD, and 500 ng of DNA/cuvette. As shown in Fig. 2, transformation efficiency increased as voltage did, and 12.5 kV/cm gave the highest transformation ($p<0.05$). Likewise, when the effect of resistance was examined at the fixed conditions of 500 ng DNA/cuvette, 12.5 kV/cm, and 25 μFD, transformation efficiency increased as the resistance did, and the infinity resistance gave the highest transformation ($p<0.05$) (Fig. 3).

### DNA concentration

pYUB18 DNA concentration may be one of the factors affecting the transformation efficiency of BCG. In order to examine the effect of DNA concentration on transformation efficiency, $CaCl_2$-treated BCG cells were transformed with 50 to 1,000 ng/cuvette at the fixed electroporation conditions of 12.5 kV/cm, 1,000 ohms, and 25 μFD. In BCG cells harvested from 6-day cultures, 100 ng DNA/cuvette gave the highest transformation efficiency of $1.1\times10^5$ CFU/μg DNA (Fig. 4).

### Comparison of transformation efficiency in BCG with *E. coli* and *M. smegmatis*

For comparison of the transformation efficiency of BCG with that of other organisms, the optimal conditions for transformation were examined in *M. smegmatis* and *E. coli* as well as in BCG. As shown in Table 1, the transformation efficiency in *E. coli* was 1.6 x $10^8$ CFU/μg DNA at the conditions of 100 pg DNA/cuvette, 12.5 kV/cm, 200 ohms, and 25 μFD. *E. coli* DNA over 1 ng/cuvette and the resistance over 200 ohms resulted in a marked reduction

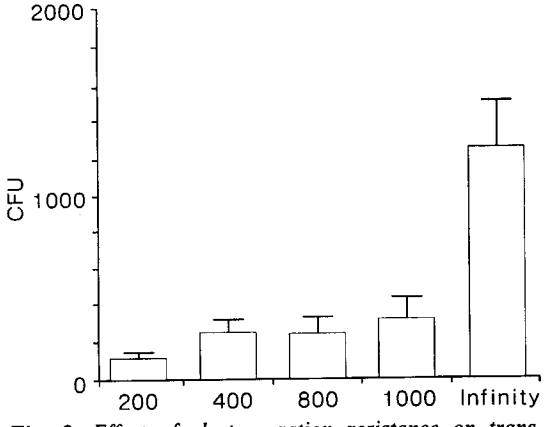


*Fig. 3. Effect of electroporation resistance on transformation efficiency. Results are expressed as the mean (plus standard deviations) of colony forming units transformed with the pYUB18 vector in three experiments.*

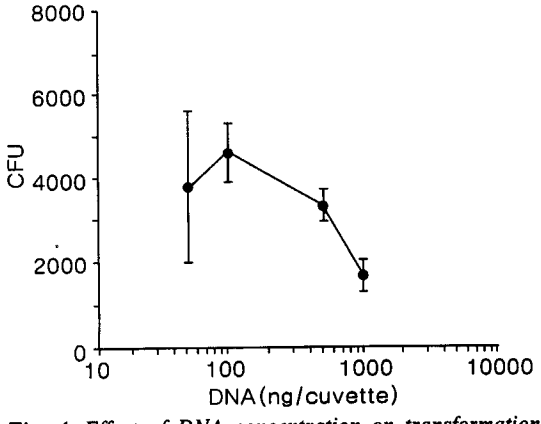


*Fig. 4. Effect of DNA concentration on transformation efficiency. Results are expressed as the mean ± standard deviations of colony forming units transformed with the pYUB18 vector in three experiments.*

**Table 1. Electroporation conditions for the highest transformation efficiency**

| Organism | DNA (ng/cuvette) | Resistance (ohm) | Voltage (kV/cm) | Time constant (s) | Efficiency (CFU/μgDNA) |
|---|---|---|---|---|---|
| *E. coli* | 0.1 | 200 | 12.5 | 4.6 | $1.6\times10^8$ |
| *M. smegmatis* | 100 | 1000 | 12.5 | 17.0 | $3.8\times10^6$ |
| BCG | 100 | infinity | 12.5 | 45.0 | $1.4\times10^5$ |

in transformation efficiency (data not shown). In *M. smegmatis*, the transformation efficiency of 3 8 x $10^6$ CFU/$\mu$g DNA was obtained when the electroporation was carried at 100 ng DNA/cuvette, 12.5 kV/cm, 1,000 ohms, and 25 $\mu$FD. Unlike in BCG cells, the resistance of infinity gave a lower transformation efficiency in *M. smegmatis* (data not shown). The maximum efficiency of transformation of BCG with pYUB18 DNA reached $1.4 \times 10^5$ CFU/$\mu$g DNA at the conditions of pretreatment with $CaCl_2$, 100 ng DNA/cuvette, 6-day cultivation, 12.5 kV/cm, infinity resistance, and 25 $\mu$FD. The time constant also differed markedly from 4.6 msec in *E. coli* to 17.0 msec in *M. smegmatis* and to 45.0 msec in BCG reflecting the difference in cell wall structure.

## DISCUSSION

This study shows that there are numerous factors affecting the transformation efficiency of BCG with the pYUB18 cosmid vector by electroporation. Electroporation is a simple and rapid procedure by which target cells have momentary membrane breakdowns or pores through which DNA enters the cells (Weaver and Powell, 1989; Chang *et al.* 1992). Due to its simplicity, this procedure has been widely applied to both eucaryotic and procaryotic cells. Since the high voltage of electric pulse gave a physical effect on the cell wall or membrane, its constituents and integrity are major factors affecting the efficiency of DNA delivery by electroporation (Neumann *et al.* 1992; Weaver and Barnett, 1992). Thus electroporation conditions vary markedly depending on the cell wall structure, even among procaryotes. The mycobacterial cell wall, including BCG, consists of a large content of lipid moiety (over 60% of dry weight) thus giving it very high hydorphobic nature (Draper, 1982), which interferes with membrane transport of hydrophilic molecules such as DNA.

In order to loosen the BCG cell walls, Lugosi *et al.* added glycine at 1% one-to-two days before harvesting cells (Lugosi *et al.* 1989). In this study, however, pretreatment with 1% glycine resulted in reduction of viable cells by more than 70% and gave a lower transformation efficiency. On the other hand, the $CaCl_2$ treatment, which has been widely used for preparing competent cells for transformation with foreign DNA (Sambrook *et al.* 1989), gave a greater transformation efficiency compared to control cells. But control BCG cells, i.e., BCG cells just washed with 10% glycerol, also showed comparable transformation efficiency. Thus, depending on the purpose of the experiment both $CaCl_2$ treated cells and cells washed with 10% glycerol can be used for transformation with foreign DNA.

The next factors examined in this study were voltage and resistance. Snapper *et al.* used 6.25 kV/cm for transformation of BCG, but in this study, the maximum efficiency was obtained at 12.5 kV/cm (Snapper *et al.* 1988). Likewise, 12.5 kV/cm gave the highest transformation efficiency in animal cells and *E. coli* (Chernomordik, 1992). Resistance was also one of the major factors in the electroporation model (Weaver and Barnett, 1992). In our study, anything over 200 ohms was detrimental to *E. coli* (data not shown), while 1000 ohms and infinity resistance were required to obtain the maximum efficiency in *M. smegmatis* and BCG, respectively.

DNA concentration for optimal transformation efficiency was also variable depending on mycobacterial species. Lugosi *et al.* examined DNA concentration ranging from 50 ng to 2 $\mu$g/cuvette and reported the maximum efficiency of 1.0 - $2.7 \times 10^4$ CFU/$\mu$g DNA when DNA was used at the concentration of 100 and 500 ng/cuvette for transformation of BCG (Lugosi *et al.* 1989). Our study also showed the highest efficiency at 100 ng/cuvette and a much lower efficiency at the DNA concentration of 1000 ng/cuvette in BCG transformation. This differed markedly from the previous report by Garbe *et al.* in which the maximum efficiency was obtained at 1.25 $\mu$g DNA for transformation of BCG and *M. vaccae* and 5 $\mu$g DNA for *Mycobacterium* w (Garbe *et al.* 1994).

Transformation efficiency also varied depending on DNA size and types, i.e., DNA libraries, plasmids, and the nature of plasmids. This implies that each plasmid or DNA library should be examined to determine its optimal concentration for transformation of BCG or other mycobacteria. For example, the transformation efficiencies of $4 \times 10^3$ CFU/$\mu$g DNA of p16K1 plasmid (Garbe *et al.* 1994)

and $1 \times 10^3$ CFU/$\mu$g DNA of pBAK14 (Zhang *et al.* 1991) in *M. smegmatis* have been reported. These were markedly lower than our results, which had an efficiency of $3.8 \times 10^6$ CFU/$\mu$g DNA of pYUB18 in *M. smegmatis*. When BCG was previously used as the host cell, the maximum efficiency had been 8 $\times 10^3$/$\mu$g DNA for p16R1 (Garbe *et al.* 1994) and $4.2 \times 10^4$/$\mu$g DNA for pYUB13 (Lugosi *et al.* 1989). Thus, the efficiency of $1.4 \times 10^5$/$\mu$g DNA of pYUB18 in our study was three times as high as that reported by Lugosi *et al.* (1989). To reach this efficiency level in BCG, $CaCl_2$-treated competent BCG cells were used and the electroporation conditions were 12.5 kV/cm, infinity resistance, 100 ng of pYUB18 DNA/cuvette, and 25 $\mu$FD in capacitance, respectively.

This study therefore clearly suggests that the optimization of electroporation conditions is necessary to have the maximum transformation efficiency for each host organism and DNA type.

## ACKNOWLEDGMENTS

We thank W.R. Jacobs, Jr. for providing *Mycobacterium-Escherichai coli* shuttle vector pYUB18 and *M. smegmatis* $mc^2155$ used in this study.

## REFERENCES

Abdelhak S, Louzir H, Timm J, Blel L, Benlasfar Z, Lagranderie M, Gheoghiu M, Dellagi K, Gicquel B: Recombinant BCG expressing the leishmania surface antigen Gp63 induces protective immunity against *Leishmania major* infection in BALB/c mice. *Microbiology 141: 1585-1592, 1995*

Chadwick MV: Mycobacteria. London, Wright, PSG, *1982, 33*

Chang DC, Saunders JA, Chassy BM, Sowers AE: *Overview of electroporation and electrofusion.* In Chang DC, ed. *Guide to Electroporation and Electofusion.* New York, Academic Press, Inc., *1992, 1-6*

Chernomordik LV: *Electropores in lipid bilayers and cell membranes.* In Chang DC, ed. *Guide to Electroporation and Electofusion.* New York, Academic Press, Inc., *1992, 63-76*

Draper P: *The anatomy of mycobacteria.* In Ratledge C, Stanford J, eds. *The Biology of the Mycobacteria,* vol. 1. London, Academic Press, *1982, 36-43*

Forster W, Neumann E: *Gene transfer by electroporation: A practical guide.* In Neumann E, ed. *Electroporation and Electrofusion in Cell Biology,* New York, Plenum Press, *1989, 299-318*

Garbe TR, Barathi J, Barnini S, Zhang Y, Abou-Zeid C, Tang D, Mukherjee R, Young DB: Transformation of mycobacterial species using hygromycin resistance as selectable marker. *Microbiology 140: 133-138, 1994*

Jacobs WR Jr, Kalpana GV, Cirillo JD, Pascopella L, Snapper SB, Udani RA, Jones W, Barletta RG, Bloom BR: Genetic systems for mycobacteria. *Methods Enzymol 204: 537-555, 1991*

Jacobs WR Jr, Tuckman M, Bloom BR: Introduction of foreign DNA into mycobacteria using a shuttle phasmid. *Nature 327: 532-535, 1987*

Kong D, Kunimoto DY: Secretion of human interleukin 2 by recombinant *Mycobacterium bovis* BCG. *Infect Immun 63: 799-803, 1995*

Lugosi L, Jacobs WR Jr, Bloom BR: Genetic transformation of BCG. *Tubercle 70: 159-170, 1989*

Matsumoto S, Matsuo T, Ohara N, Hotokezaka H, Naito M, Minami J, Yamada T: Cloning and sequencing of a unique antigen MPT70 from *Mycobacterium tuberculosis* H37Rv and expression in BCG using *E. coli*-mycobacteria shuttle vector. *Scand J Immunol 41:281-287, 1995*

Neumann E, Sprafke A, Boldt E, Wolf H: *Biophysical considerations of membrane electroporation.* In Chang DC, ed. *Guide to Electroporation and Electofusion.* New York, Academic Press, Inc., *1992, 77-90*

Sambrook J, Fritsh EF, Maniatis T: *Molecular Cloning: a Laboratory Manual.* 2nd ed. Cold Spring Harbor, Cold Spring Habor Laboratory Press, *1989, 1.25-1.41*

Snapper SB, Lugosi L, Jekkel A, Melton RE, Kieser T, Bloom BR, Jacobs WR Jr: Lysogeny and transformation in mycobactria: stable expression of foreign genes. *Proc Natl Acad Sci USA 85: 6987-6991, 1988*

Snapper SB, Melton RE, Mustafa S, Kieser T, Jacobs WR Jr: Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis. Mol Microbiol 4: 1911-1919, 1990*

Stover CK, Bansal GP, Hanson MS, Burlein JE, Palaszynski SR, Young JF, Koenig S, Young DB, Sadziene A, Barbour AG: Protective immunity elicited by recombinant bacille Calmette-Guerin (BCG) expressing outer surface protein A (OspA) lipoprotein: a candidate Lyme disease vaccine. *J Exp Med 178: 197-209, 1993*

Stover CK, De la Cruz VF, Bansal GP, Hanson MS, Fuerst TR, Jacobs, WR Jr, Bloom BR: *Use of recombinant BCG as a vaccine delivery vehicle.* In Ciardi JE, ed. *Genetically Engineered Vaccines.* New York, Plenum Press, *1992, 175-182*

Stover CK, De la Cruz VF, Fuerst TR, Burlein JE, Benson LA, Bennett LT, Bansal GP, Young JF, Lee MH, Hatfull GF, Snapper SB, Barletta RG, Jacobs WR Jr, Bloom BR: New use of BCG for recombinant vaccines. *Nature 351: 456-460, 1991*

Ten Dam HG: Research on BCG vaccination. *Adv Tuberc Res 21: 79-106, 1984*

Weaver JC, Barnett A: *Progress toward a theoretical model for electroporation mechanism: Membrane electrical behavior and molecular transport.* In Chang DC, ed. *Guide to Electroporation and Electofusion.* New York, Academic Press, Inc., *1992, 91-117*

Weaver JC, Powell KT: *Theoray of electroporation.* In Neumann E, ed. *Electroporation and Electrofusion in Cell Biology.* New York, Plenum Press, *1989, 111-126*

Winter N, Lagranderie M, Rauzier J, Timm J, Leclerc C, Guy B, Kieny MP, Gheorghiu M, Gicquel B: Expression of heterologous genes in *Mycobacterium bovis* BCG: induction of a cellular reponse against HIV-1 Nef protein. *Gene 109: 47-54, 1991*

Zhang Y, Lathigra R, Garbe T, Catty D, Young D: Genetic analysis of superoxide dismutase, the 23 kilodalton antigen of *Mycobacterium tuberculosis. Mol Microbiol 5: 381-391, 1991*