Improved Electroporation of *Rhodococcus equi*

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ABSTRACT. The condition of an electroporation method was re-evaluated for the introduction of foreign plasmid DNA into *Rhodococcus equi*. The method is based on an electroporation of the bacteria made competent by culturing in a broth containing glycine and by heat shock at 50°C. Transformation of *R. equi* could be achieved with a chloramphenicol-resistant shuttle vector originating from *Rhodococcus fascians* at an efficiency of about 10^4 transformants/μg DNA. The bacteria were also shown to become competent when they were incubated with a chemical transformation buffer prior to washing with an electroporation buffer. — KEY WORDS: electroporation, genetic transformation, *Rhodococcus equi*.

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Rhodococcus equi is a Gram-positive pathogen causing severe pneumonia and/or enteritis in foals [10]. We have previously reported that the virulent strains of *R. equi* produced plasmid-encoded 15- to 17-kDa surface antigens [15–17]. The gene responsible for production of these antigens locating on an 85-kb virulence-associated plasmid was cloned and sequenced [8, 11]. However, the analysis of the function of these antigens in the virulence of *R. equi* has been hampered by the lack of a system for introducing the cloned gene into the plasmid-cured derivatives. Several methods for transformation of *Rhodococcus* species have so far been described [2, 5, 12, 18]. However, preparation of competent cells with consistently high transformation efficiency is difficult in general, and we actually did not succeed in transformation of *R. equi* strains by the previously described methods [2, 5, 12, 18]. To achieve the efficient transformation of *R. equi*, we optimized conditions and parameters for the preparation of *R. equi* competent cells.

Plasmidless or plasmid-cured derivatives (P^-) of *R. equi* ATCC33701P^- [17], ATCC6939 [17], L1P^- [17], A2P^- [14], and A5P^- [14] were used for the transformation experiments. The strains were stored frozen with 20% glycerol in small aliquots at −80°C. The shuttle vectors used were pRF30, a chloramphenicol and ampicillin-resistant plasmid originated from a plasmid of *Rhodococcus fascians* [2], pBS305, a thiostrepton and ampicillin-resistant plasmid from *Rhodococcus* sp. [12], and pK4, a kanamycin-resistant plasmid from *Rhodococcus rhodochrous* [5]. The plasmid DNAs of the shuttle vectors were isolated from *Escherichia coli* by alkaline lysis, purified by CsCl-ethidium bromide density gradients [9], and dialyzed against distilled water. Electroporation buffer (EB) used was 2 mM potassium phosphate (pH 8.37) containing 10% sucrose. Chemical transformation buffer (CTB) used was 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, and 10 mM PIPES (pH 6.7) [7].

R. equi strains were grown in 100 m*l* of brain heart infusion (BHI) broth (Difco) supplemented with 0.4% glucose and, when necessary, some chemicals in a 500 m*l* flask in a rotary shaker (250 rpm) at 37°C for 12 to 14 hr. The cultures usually exhibited turbidities (OD_{600}) between 0.6 and 0.8 (middle log phase). The culture was heated at 50°C for 9 min and was immediately cooled in an ice water bath with vigorous shaking. After that, the cells were always kept on ice except for the centrifugation steps which were performed at 4°C until the electroporation was achieved. The cells were harvested by centrifugation (10,000 rpm, 10 min) and suspended in 30 m*l* of ice-cold EB by gentle vortexing. The cells were washed three times with EB. Any remaining supernatant was removed before resuspending the cells with 2 m*l* ice-cold EB containing 15% glycerol. The cell suspension contained 10^{10} colony forming unit/m*l* of the bacteria. Hundred-microliter aliquots were distributed into pre-cooled 1.5 m*l* Eppendorf tubes and stored at −80°C. Preparation of the competent cells with CTB treatment was also tested. In this case, the cells were washed once with 30 m*l* of CTB after harvesting from the culture, resuspended in 30 m*l* of CTB, placed on ice for 30 min, and pelletted again. The pellet was then washed 3 times with EB as described above.

The frozen cells were thawed on ice and mixed with 1 μg of plasmid DNA (0.5 to 1 μg/μ*l*). The mixture was then placed in a pre-chilled sterile electroporation cuvette (2 mm electrode gap, Bio-Rad), and pulsed immediately with a Bio-Rad Gene Pulser (set at 2.5 kV, 400 Ω, and 25 μF). The mixture was then diluted with BHI containing 20 mM $MgCl_2$, and incubated at 30°C for 1 hr. The cells were spread on BHI agar supplemented with 30% horse meat extract and appropriate antibiotics and incubated at 30°C. The antibiotics used were chloramphenicol (12.5 μg/m*l*), kanamycin (200 μg/m*l*), and thiostrepton (0.7 μg/m*l*).

When the bacterial cells were treated with CTB, *R. equi* ATCC33701P^- could be transformed using a shuttle vector pRF30 with a low efficiency. Presence of the plasmids in the transformants was confirmed by an agarose gel electrophoresis (data not shown). This result demonstrated that the chemical induction of cell competence could be achieved and the competence could be preserved after washing with EB. Then we optimized the conditions of the

transformation. Transformation efficiencies of the competent cells cultured in various medium containing various growth supplements were then compared (Table 1). The addition of 1% glycerol and 0.2% Tween 80 to BHI broth increased the transformation efficiency by approximately 10^2-fold. Furthermore, the incorporation of 2% glycine in addition to 1% glycerol and 0.2% Tween 80 had an additive effect. However, the efficiency of transformation of the cells without CTB treatment was higher than that of the cells with the treatment (Table 1). Glycine is known to be incorporated into the Gram-positive cell wall in place of D-alanine which leads to a less tightly cross-linked structure [4], resulting in increasing the efficiency of transformation [6, 13]. The results obtained in this study also suggest that the addition of glycine to BHI medium might modify the cell surface structures resulting in increasing the transformation frequency. On the other hand, although the addition of glycine to the culture media could be replaced by the chemical induction, the CTB treatment may be applicable to the other bacteria whose growth is inhibited by the supplements used in this study.

A heat shock treatment has sometimes been introduced to render bacterial cells competent [1]. This has usually been done after mixing the cells and plasmid DNAs. On the other hand, it was reported that the competence of the cells enhanced by heat shock could be preserved if the cells were kept cooling on ice [1]. Therefore, we heated the cultures at the first step of the preparation of the competent cells. The effect of the temperature on the efficiency of transformation was evaluated by heating the culture at various temperatures ranging from 37°C to 55°C. The results obtained in this study showed that heating at 50°C was the best condition and increased the efficiency of transformation by about 10-fold compared with the results obtained by heating at 37°C.

The effect of variation of the applied voltage and the resistance on the transformation efficiency of *R. equi* was tested. Optimal results were obtained when the voltage applied to the cuvette was 2.5 kV and the resistance was set at 400 Ω. When the resistance was set at 200 Ω, the time constant obtained during electroporation was lower, resulting in a lower number of transformants. At 600 Ω, the viability of the bacteria was reduced, resulting in a decrease of the number of transformants (data not shown). The optimized condition of electroporation was almost the same as that of the conventional method [3]. This suggests that the most important factors affecting transformation may be the culture condition and heating of the cells.

Electroporation of *R. equi* ATCC33701P$^-$ using the other shuttle vectors was performed by the optimized condition. However, no transformant could be obtained with pBS305 and pK4. When the several strains of *R. equi* listed above were used for the experiment, all the strains could be transformed by the optimized condition using pRF30. The efficiencies of transformation among the strains ranged from 1.0×10^2 to 1.2×10^4 transformants/μg DNA, and the most efficient transformation occurred when the strain A2P$^-$ was used.

Achieving highly efficient transformation of bacteria is particularly important when the source of the DNA is from heterologous species, as restriction and modification barriers, which cause drastic decreases in the number of transformants and would limit the application of shuttle vectors, may exist between *E. coli* and *R. equi*. Although the efficiency of transformation using pRF30 was high enough to analyze the gene function by complementation *in trans*, the efficiency seemed to be still low when the gene with some modifications was to be introduced in the corresponding genetic region by a gene replacement via homologous recombination. The results obtained in this study revealed that pRF30 was the only shuttle vector applicable for transformation of *R. equi* among the vectors tested. For a conduction of the gene replacement, development of a novel vector, perhaps originated from *R. equi*, is necessary to improve the efficiency of transformation.

In this study, all the strains used have successfully been transformed. It works for at least 5 *R. equi* strains, and possibly for any other *R. equi* strains. The transformation method described in this study will aid genetic analysis of virulence and function of the virulence-associated antigens in *R. equi*.

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Table 1. Effect of growth supplements added to a culture medium on transformation efficiency of *R. equi*

Growth supplements	CTB treatment	Efficiency (transformants/μg DNA)
none	+	7.5×10
1% glycerol	+	1.5×10
0.2% Tween 80	+	2.5×10^3
1% glycerol + 0.2 % Tween 80	+	7.3×10^3
1% glycerol + 0.2% Tween 80 + 2% glycine	+	9.1×10^3
1% glycerol + 0.2% Tween 80 + 2% glycine	–	1.7×10^4

R. equi ATCC33701P$^-$ strain was cultured in various media with various growth supplements, and the transformation was carried out by the electroporation using a shuttle vector pRF30. CTB treatment: The cells were incubated with CTB prior to washing with EB as described in the text.

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