Removal of the periplasmic DNase before electroporation enhances efficiency of transformation in the marine bacterium *Vibrio alginolyticus*

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We have established a reliable procedure for electroporation in the marine bacterium *Vibrio alginolyticus*. Plasmids carrying the P15A replicon were found to be stably maintained in the *Vibrio* cells, and chloramphenicol, kanamycin or tetracycline were used for selection. Since we found that the *Vibrio* cells excrete DNase into the culture medium, cells were subjected to osmotic shock before extensive washing in order to remove the DNase from the periplasmic space. This manipulation resulted in about a 10-fold increase in the efficiency of transformation. In addition, cells were washed in the presence of 5–10 mM Mg²⁺ in order to stabilize the outer membrane. The efficiency of transformation was found to be optimal when cells were harvested at early stationary phase, and when electroporation was carried out at an electric field strength between 5.0 and 7.5 kV cm⁻¹. Under optimal conditions, about 10⁵ transformants per µg of input DNA were reproducibly obtained, which is tolerable for cloning.

Keywords: *Vibrio alginolyticus*, transformation, electroporation, osmotic shock

INTRODUCTION

Vibrio alginolyticus and the closely related species *V. parahaemolyticus* are slightly halophilic Gram-negative bacteria mainly found in estuarine or coastal areas (Kaneko & Colwell, 1973). In seawater, the cell is rod-shaped and propelled by a single polar sheathed flagellum. When the cell attaches to an animate or inanimate surface, it differentiates into a multinucleate and highly elongated cell, which migrates over viscous surfaces by numerous unsheathed lateral flagella (Allen & Baumann, 1971; Shinoda & Okamoto, 1977; McCarter & Silverman, 1990).

Recently, Atsumi *et al.* (1992) found that the polar flagellar motor is driven by sodium-motive force across the cytoplasmic membrane, whereas the lateral flagellar motor is driven by proton-motive force. A single cell possessing two types of motors with two different coupling ions provides an excellent experimental system for studying

the bioenergetics of bacterial flagellar motors. These organisms are also intriguing in the following respects: (i) increase in viscosity is the signal for induction of the lateral flagellar genes (Belas *et al.*, 1986) and the polar flagellum is proposed to function as the viscosity sensor (McCarter *et al.*, 1988; McCarter & Silverman, 1990); (ii) they have a respiration-coupled primary sodium pump, NADH:quinone oxidoreductase (Tokuda & Unemoto, 1982; Tsuchiya & Shinoda, 1985); (iii) when swarming, they migrate as tufts of elongated cells (Henrichsen, 1972; McCarter & Silverman, 1990; Allison & Hughes, 1991); (iv) in the marine ecosystem, they are major decomposers of chitin, a ubiquitous form of marine biomass (Yu *et al.*, 1991).

In order to investigate any of the aspects described above, it is vital to analyse the genes involved. Transposon insertion mutagenesis has been carried out in *V. parahaemolyticus* and other marine *Vibrio* spp. (Belas *et al.*, 1984, 1986; McCarter & Silverman, 1987; McCarter *et al.*, 1988) and the genes of interest have been cloned into *Escherichia coli* using the inserted transposons as markers. In addition, triparental mating has been used to disrupt a gene on the *V. parahaemolyticus* chromosome (McCarter *et al.*, 1988). Furthermore, two generalized transducing

†Professor Yasuo Imae, who had devoted himself to studying various aspects of bacterial motility and behaviour, died suddenly of a cerebral haemorrhage at the age of 53 during this work. This article is dedicated to him by the rest of authors with deep sorrow, professional respect, and personal affection.

phages in *V. parahaemolyticus* and *V. alginolyticus* have been isolated and characterized (Muramatsu & Matsumoto, 1991).

It is also important to establish a host-vector system and an efficient transformation procedure in marine *Vibrio* spp., since a gene of interest can then be cloned by complementation and analysed in the *Vibrio* cells. Hamashima *et al.* (1990) reported that using electroporation, several strains of *V. alginolyticus* and *V. parahaemolyticus* could be transformed with the *E. coli* plasmids pACYC184, pBR322 and pHSG398 (a pUC-type plasmid with the pMB1 replicon and the chloramphenicol resistance gene). The efficiency of transformation, however, was too low (about 10^2 transformants per μg DNA) for routine use.

In this paper, we report that the removal of DNase from the periplasmic space by osmotic shock and washing the cells in the presence of Mg^{2+} results in reasonably efficient transformation.

METHODS

Bacterial strains. *V. alginolyticus* strain VIK2, which was used as the standard recipient for electroporation, is a rifampicin-resistant derivative of the wild-type strain 138-2 (Tokuda *et al.*, 1981). *E. coli* strain DH5 α (Grant *et al.*, 1990) was used as the host for isolation and construction of plasmids and as a control in the DNase test.

Plasmids. The P15A plasmids used in this study were: pACYC184 (Chang & Cohen, 1978) with chloramphenicol resistance (Cm^r) and tetracycline resistance (Tc^r) genes; pSU18 and pSU21 (Bartolomé *et al.*, 1991), both of which have the Cm^r gene and the 5' part of the *lacZ* gene; and pSU38 and pSU41 (Bartolomé *et al.*, 1991), both of which have the kanamycin resistance (Km^r) gene and the 5' part of the *lacZ* gene. The pMB1 plasmids used in this study were: pBR322 (Bolivar *et al.*, 1977) with the ampicillin resistance (Ap^r) and Tc^r genes; pUK21 (Vieira & Messing, 1991) with the Km^r gene and the 5' part of the *lacZ* gene; pUC4K (Vieira & Messing, 1982) with the Ap^r and Km^r genes; and pHSG398 (Takeshita *et al.*, 1987) with the Cm^r gene and the 5' part of the *lacZ* gene.

Media and conditions of culture. *V. alginolyticus* cells were cultured at 30 °C in complex medium (0.5% polypeptone, 0.5% yeast extract, 0.4% K_2HPO_4 , 3% NaCl; Tokuda, 1986) supplemented with 0.2% glucose (named VC medium). When necessary, chloramphenicol, kanamycin, rifampicin and tetracycline were added at final concentrations of 1.25, 100, 50 and 1.25 $\mu\text{g ml}^{-1}$, respectively. *E. coli* cells were cultured in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl; Silhavy *et al.*, 1984). For *E. coli*, antibiotics were used at the following concentrations ($\mu\text{g ml}^{-1}$): ampicillin, 50; chloramphenicol, 25; kanamycin, 50; and tetracycline, 10.

To examine whether DNase was secreted, cells were grown on DNase Test Agar (Difco), supplemented with 0.4 M NaCl for *V. alginolyticus*, and 0.1 M HCl was poured to precipitate unhydrolysed DNA.

Standard method of preparation of cells for electroporation in *E. coli*. Electroporation in *E. coli* was carried out according to Dower *et al.* (1988), as follows. Cells were grown in 1 litre of LB medium at 37 °C with vigorous shaking to an OD_{600} of 0.5–0.8. Then the cells were chilled and harvested by centrifugation (4000 g) for 15 min at 0 °C. The pellets were rinsed first with 1

litre ice-cold water, then with 20 ml ice-cold 10% (w/v) glycerol, and the cells were resuspended to a final volume of 2–3 ml in ice-cold 10% (w/v) glycerol. A 40 μl sample of the cell suspension was used in one electroporation.

Electroporation in *V. alginolyticus*. The 'Gene Pulser' electroporation apparatus (Japan Bio-Rad Laboratories, Tokyo) was used throughout this study. The electric field strength was varied between 2.5 and 12.5 kV cm^{-1} . Capacitance and resistance were fixed at 25 μF and 200 Ω , respectively, and cuvettes with a 0.2 cm electrode gap were used. The bacteria were grown with shaking in 20 ml VC medium at 30 °C to late-exponential phase, and harvested by centrifugation (5000 g) for 5 min at room temperature. The pellet was chilled, and washed twice with 10 ml ice-cold 10 mM MgSO_4 and once with 5 ml ice-cold 10% glycerol containing 5 mM MgSO_4 . The pellet was suspended in 120 μl ice-cold 10% glycerol, and used immediately for electroporation or kept frozen at -80 °C. Forty microlitres of the cell suspension, kept at 0 °C, was mixed with an appropriate amount (typically 0.1 μg) of plasmid DNA and was subjected to electroporation in a chilled cuvette. Immediately after electroporation, 1 ml VC medium was added and the cell suspension was transferred to a test tube and then incubated at 30 °C for 50 min with shaking. The cells were precipitated, resuspended in an appropriate volume of VC medium, and plated on a selection plate. The plate was then incubated at 30 °C overnight.

Transformation by the osmotic shock method. The procedure of osmotic shock for *V. alginolyticus* cells was modified from the method of Neu & Heppel (1965) for *E. coli* cells. The cells were cultured in 20 ml VC medium, harvested and washed with an equal volume of motility medium [50 mM HEPES/Tris (pH 7.0), 0.4 M NaCl, 10 mM MgSO_4 , 5 mM glucose]. The pellet was resuspended in 10 ml SNT medium [30 mM Tris/HCl (pH 8.0), 20% sucrose, 0.4 M NaCl], to which EDTA was added (final 1 mM). The cell suspension was then incubated for 10 min at 30 °C with gentle shaking. The cells were precipitated and resuspended in 10 ml ice-cold 10 mM MgSO_4 . After incubation on ice for 10 min, the cells were precipitated and washed as described in the previous section.

RESULTS

Electroporation in *V. alginolyticus*

To determine which antibiotics could be used for selection, we examined the effects of various concentrations of ampicillin, chloramphenicol, kanamycin and tetracycline on the growth of *V. alginolyticus* strain 138-2 and its rifampicin-resistant derivative VIK2. Two microlitres of overnight culture was spotted on VC plates containing various concentrations of one of the antibiotics and the plates were incubated at 30 °C. Growth of both the strains was inhibited by 1.0 μg chloramphenicol ml^{-1} , 100 μg kanamycin ml^{-1} , and 0.5 μg tetracycline ml^{-1} . In the case of kanamycin, however, spontaneous resistant colonies appeared even at 200 $\mu\text{g ml}^{-1}$. Ampicillin, even at 1 mg ml^{-1} , did not inhibit colony formation. We therefore used genes responsible for resistance to chloramphenicol (Cm^r), kanamycin (Km^r), or tetracycline (Tc^r) as selective markers.

We first applied the standard electroporation procedure for *E. coli* (see Methods) to *V. alginolyticus*. Transformation of 138-2 and VIK2 cells with plasmids pACYC184, pSU18, pSU21, pSU38, pSU41, pBR322, pUK21, pUC4K and pHSG398 was attempted using chloramphenicol,

tetracycline or kanamycin at concentrations of 1.25, 1.25 or 100 $\mu\text{g ml}^{-1}$, respectively. No transformants were found with any of these combinations of host strains and plasmids, and so we explored various other conditions for the electroporation protocol.

When cells were harvested at late-exponential phase rather than early-exponential phase and the wash media included 10 mM MgSO_4 , we obtained tens to hundreds of transformants of 138-2 or VIK2 with pSU21 (containing the P15A replicon and Cm^r gene) in one electroporation. That the plasmid was maintained in the host cells in the original form was verified by extracting the plasmid DNA and analysing the restriction enzyme digestion pattern by agarose gel electrophoresis (data not shown). Furthermore, even in the absence of chloramphenicol, VIK2 retained the plasmid as stably as *E. coli* strain DH5 α did for at least seven generations (data not shown).

We also obtained Cm^r colonies when pHSG398 (which has the replicon of pMB1) was used. The subsequent restriction analysis, however, revealed that some kind of rearrangement had occurred. Selections using kanamycin were unsuccessful at this stage since none of the Km^r colonies contained plasmid DNA. The other plasmids with the P15A replicon and Cm^r gene also gave transformants, suggesting that the P15A replicon functions in the *V. alginolyticus* cell. Strains 138-2 and VIK2 exhibited similar efficiencies of transformation.

We therefore used VIK2 as the standard host and pSU21 as the standard vector in the subsequent experiments. To avoid digestion by *Vibrio* restriction systems, plasmid DNA was extracted from VIK2 cells carrying pSU21 obtained in the previous electroporation experiment and used in the subsequent experiments.

Effects of electric field strength, amount of plasmid DNA, and cell growth phase on the efficiency of transformation

First, the electric field strength of the pulse upon electroporation was varied from 2.5 to 12.5 kV cm^{-1} . As shown in Fig. 1, the efficiency of transformation was optimal when the electric field strength was set between 5.0 and 10.0 kV cm^{-1} .

Second, with the electric field strength fixed at 5.0 kV cm^{-1} , the amount of plasmid DNA was varied. As shown in Fig. 2, the number of transformants increased with increasing amount of pSU21 DNA, whereas the number of transformants per μg was not much changed (maximal when 0.1 μg of DNA was applied).

Third, the growth phase at which cells were harvested was varied. Cells were harvested when the Klett value at 660 nm reached 50, 150 and 300 and when cultivation lasted for 5, 7 and 9 h. Volumes of culture harvested were varied, so that the total cell numbers were similar in all sampling: e.g. 60 ml of the culture at Klett value 50 was harvested, whereas at Klett value 150, only 20 ml was collected. These cells were precipitated and washed as described in Methods, and electroporation was carried out with 0.1 μg pSU21 DNA and an electric field strength

Fig. 1. Effect of electric field strength on the efficiency of transformation when electroporating *V. alginolyticus* strain VIK2. A 0.1 μg sample of plasmid pSU21 DNA was added to 40 μl of a suspension of VIK2 cells harvested when the Klett value (at 660 nm) reached 150.

Fig. 2. Effect of the amount of DNA on the efficiency of transformation when electroporating *V. alginolyticus* strain VIK2. The electric field strength was set at 5.0 kV cm^{-1} and the other conditions were the same as Fig. 1.

of 7.5 kV cm^{-1} . We repeated this experiment several times and a typical result is presented in Fig. 3. The highest efficiency of transformation was obtained when the cells were harvested at early stationary phase.

V. alginolyticus secretes DNase to the culture medium

It has been reported that *V. cholerae* cells secrete DNases (Newland *et al.*, 1985; Focareta & Manning, 1987; Marcus *et al.*, 1990). So we examined whether this is also the case in *V. alginolyticus*. An overnight culture of VIK2 cells was streaked on a DNase Test Agar plate supplemented with 0.4 M NaCl and the plate was incubated at 30 °C. After overnight incubation, 10 ml 1 M HCl was poured onto the plate in order to precipitate unhydrolysed DNA. As shown in Fig. 4(a), a large zone of clearing around the

Fig. 3. Effect of the growth phase at which the cells were harvested on the efficiency of transformation when electroporating *V. alginolyticus* strain VIK2. Electroporation was carried out with 0.1 μg pSU21 DNA and an electric field strength of 7.5 kV cm^{-1} . $\square$, Klett value (660 nm) of the culture; $\circ$, number of Cm^r transformants per μg DNA.

Fig. 4. Secretion of DNase by *V. alginolyticus* strain VIK2 (a) but not by *E. coli* strain DH5 α (b). Cells were grown on DNase Test Agar, supplemented with 0.4 M NaCl in the case of *V. alginolyticus*. After overnight incubation at 30 $^{\circ}\text{C}$, the DNase activity was detected by precipitating the unhydrolysed DNA with 1 M HCl.

lawn of VIK2 cells was observed, indicating that DNase is indeed secreted. A similar result was obtained for the wild-type strain 138-2 (data not shown). On the other hand, the lawn of *E. coli* DH5 α cells was not surrounded by such a clearing zone (Fig. 4b).

We then examined whether covalently closed circular plasmid DNA is degraded by the secreted *Vibrio* DNase. Plasmid pSU21 DNA was mixed with the supernatants of

Fig. 5. Effect of osmotic shock treatment before electroporation and the presence of carrier DNA or RNA during electroporation on the efficiency of transformation. Electroporation using osmotically shocked (a) or unshocked (b) VIK2 cells was carried out in the absence (TE) or presence of 1 μg of chromosomal DNA extracted from *V. alginolyticus* (DNA) or 25 μg of tRNA^{Phe} from *S. cerevisiae* (RNA). The electric field strength was set at 7.5 kV cm^{-1} and 0.5 μg pSU21 DNA was used.

cultures (Klett values about 150) of 138-2, VIK2 and DH5 α at either 37 $^{\circ}\text{C}$ or 0 $^{\circ}\text{C}$. The resultant DNA was precipitated with 70% ethanol and analysed by agarose gel electrophoresis (data not shown). Significant degradation of the plasmid DNA was detected even at 0 $^{\circ}\text{C}$ when incubated with the supernatants of the 138-2 and VIK2 cultures. The supernatant of the DH5 α culture, however, showed no degrading activity. We therefore suspect that by the procedure described in the previous section DNase may not be washed out from the periplasmic space and/or the outer membrane and that this may cause the low efficiency of transformation in *V. alginolyticus*.

Osmotic shock before electroporation significantly improves efficiency of transformation

Degradation of plasmid DNA might be avoided or reduced by removal of DNase from the periplasmic space and/or by adding carrier DNA or RNA. Cells were thus subjected to osmotic shock before electroporation as described in Methods. Electroporation using shocked or unshocked cells was carried out in the presence or absence of 1 μg chromosomal DNA extracted from *V. alginolyticus* 138-2 or 25 μg of tRNA^{Phe} from *Saccharomyces cerevisiae* (Boehringer Mannheim Yamanouchi, Tokyo). As shown in Fig. 5, the osmotic shock treatment resulted in a 10-fold increase in the efficiency of transformation. This suggests that DNase is effectively removed from the periplasmic space of the cell. Addition of carrier DNA or RNA, however, showed no effect on the transformation efficiency of either shocked or unshocked cells (Fig. 5).

Using osmotic shock before electroporation, the effects of electric field strength, amount of plasmid DNA, and growth phase at which cells were harvested were examined again. As shown in Fig. 6, the tendencies were

Fig. 6. Effects of electric field strength (a), amount of plasmid DNA (b), and growth phase (c) on the efficiency of transformation. Osmotically shocked VIK2 cells were used in all experiments, and electroporation was carried out under the following conditions: (a) Klett value of the culture when harvested was 150 and the amount of pSU21 DNA was 0.5 μg ; (b) Klett value was 150 and the electric field strength was set at 5.0 kV cm^{-1} ; (c) 0.1 μg pSU21 was used and the electric field strength was set at 7.5 kV cm^{-1} . $\square$, Klett value (660 nm) of the culture; $\circ$, number of Cm^r transformants per μg DNA.

very similar to those obtained without osmotic shock (Figs 1–3).

With this reasonably high efficiency of transformation (about 10^5 transformants per μg DNA input) obtained, we attempted to transform VIK2 cells with plasmids pIO1 ($\text{Cm}^r \text{ Km}^r$), which consists of pSU21 and the Km^r gene cassette from pUC4K, and pACYC184 ($\text{Cm}^r \text{ Tc}^r$) (data not shown). All 50 colonies tested on the plates containing 100 μg kanamycin ml^{-1} after electroporation with pIO1 were also found to be resistant to 2.5 μg chloramphenicol ml^{-1} . Similarly, when pACYC184 was introduced, 50 out

of 50 Tc^r transformants were found to be Cm^r . Thus, these P15A plasmids as well as pSU38 and pSU41 can be maintained in the *V. alginolyticus* cells and kanamycin (100 $\mu\text{g ml}^{-1}$) and tetracycline (1.25 $\mu\text{g ml}^{-1}$) can be used for selection of transformants.

DISCUSSION

In this study we established a reliable procedure for electroporation in *V. alginolyticus*. When a plasmid with the P15A replicon and the Cm^r gene is used as a vector, about 10^5 transformants per μg of input DNA are reproducibly obtained under optimal conditions. Major differences from the standard procedure for *E. coli* (Dower *et al.*, 1988) are as follows. (i) Cells are harvested at early stationary phase rather than early-exponential phase. (ii) Cells are subjected to relatively mild osmotic shock before extensive washing. (iii) Cells are washed in the presence of 5–10 mM MgSO_4 . (iv) Electroporation is performed using a pulse with an electric field strength of between 5.0 and 7.5 kV cm^{-1} .

We found that the culture medium of *V. alginolyticus* cells contains considerable DNA-degrading activity, whereas that of *E. coli* does not. The *V. alginolyticus* DNase can degrade covalently closed circular forms of plasmid DNA and is active even at 0 $^\circ\text{C}$. Permeabilization of the outer membrane by osmotic shock was carried out to remove the DNase which otherwise would remain in the periplasmic space even after thorough washing of the cells. This manipulation resulted in about a 10-fold increase in the efficiency of transformation.

V. alginolyticus cells are easily lysed when exposed to low ionic strength; the presence of magnesium ions prevents this cell lysis, probably by stabilizing the outer membrane (Unemoto *et al.*, 1973; Unemoto & MacLeod, 1975). Therefore 5–10 mM MgSO_4 was used throughout the washing process. The presence of higher concentrations (up to 50 mM) did not significantly increase the efficiency of transformation (data not shown).

It was found that plasmids which have the P15A replicon can be stably maintained in *V. alginolyticus* cells even in the absence of selective pressure. Chloramphenicol (1.25 $\mu\text{g ml}^{-1}$), kanamycin (200 $\mu\text{g ml}^{-1}$) and tetracycline (1.25 $\mu\text{g ml}^{-1}$) can be used for selection of transformants. However, in the case of pHSG398, a Cm^r -carrying plasmid containing the replicon of pMB1, the original form of DNA from the Cm^r transformants was not recovered, although Hamashima *et al.* (1990) reported that the same plasmid can be maintained in the *Vibrio* cells. This discrepancy might be due to the difference in the recipient strains. We wished, however, to use strain 138-2 and its derivatives since 138-2 has been well characterized in terms of the bioenergetics of the respiration-coupled sodium pump (Tokuda, 1989; Unemoto & Hayashi, 1989) and the flagellar motors (Tokuda *et al.*, 1988; Atsumi *et al.*, 1992).

The efficiency of transformation of *V. alginolyticus* under the optimal conditions in this study is still lower than that of *E. coli*, but is not too low for routine use in experiments

such as shot-gun cloning. We have already isolated mutants defective in the polar and lateral flagella (Pof⁻ Laf⁻) from the wild-type strain 138-2 (Y. Maekawa, I. Kawagishi, Y. Imae & M. Homma, unpublished). Since these mutants cannot make swarm colonies on 0.3% agar plate, we expect to clone genes responsible for the formation of polar or lateral flagella by introducing libraries (with pSU21 as vector) of *V. alginolyticus* chromosome DNA into the Pof⁻ Laf⁻ strains and by selecting transformants which recover the ability to form swarm colonies.

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