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Transformation of an Alkaline Protease Overproducer, *Vibrio metschnikovii* Strain RH530, and Improvement of Plasmid Stability by the *par* Locus

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Abstract *Vibrio metschnikovii* strain RH530 is a non-pathogenic, industrially-important alkaline protease producer which has been isolated from wastewater. In this paper, we report on the transformation of this strain by using the method of electroporation. A field strength of 7.5 $kVcm^{-1}$ and 25 μF, and using a 0.2-cm cuvette, appeared to be the optimal conditions for electroporation of the cells with the recombinant pSBCm plasmid carrying the *vapK* alkaline protease gene and the ColE1 replicon. Cells were subjected to osmotic shock in order to remove extracellular DNase, and adding 200 mM of sucrose to electroporation buffer cells showed an increased transformation efficiency. Maximum efficiency of transformation was obtained at an early exponential growth phase. Using all of the conditions mentioned above, we routinely obtained a transformation efficiency of more than 10^4 (μg plasmid DNA)$^{-1}$. The stability of the plasmid pSBCm in *V. metschnikovii* RH530 was 25% after 18 h of growth (27 generations) in the medium without antibiotic selection. The insertion of the *par* locus to the pSBCm increased the stability of the plasmid up to 42% without selective pressure. The increase in plasmid stability was accompanied by the increase in the productivity of alkaline protease in the recombinant *V. metschnikovii* strain RH530. Determining optimal conditions for the transformation of the industrially-important, nonpathogenic *Vibrio* strain, and the improvement of plasmid stability by introducing the *par* locus into the high copy number plasmid vector, will allow the development of procedures involved in the genetic manipulation of this strain, particularly for its use in the production of industrial enzymes such as alkaline protease.

Key words: *Vibrio metschnikovii*, transformation, electroporation, plasmid stability, *par* locus

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Most studies conducted on the transformation of the *Vibrio* species have focused on clinically-important pathogenic strains such as *V. cholerae* [17], *V. vulunificus* [13], and *V. alginoliticus* [6]. However, to our knowledge, there was no report on the genetic transformation of the industrially-important, nonpathogenic *Vibrio* species. Recently, *V. metschnikovii* RH530 (VmR), known to produce six alkaline serine proteases including VapK and a metalloprotease, has been isolated from wastewater [11]. The alkaline protease overproducing mutants were isolated and characterized together with the detergent properties of the protease [2]. Due to a wide use of alkaline protease in industry, we attempted to overproduce this enzyme by transforming VmR with the recombinant plasmid pSBCm carrying the ColE1 replicon and *vapK* gene, which codes for one of the major proteases, VapK [11]. Additionally, the VmR strain has been found to secrete DNase, which is a major barrier of transformation. Thus, to overcome such a barrier during VmR transformation, optimal conditions were used as a preliminary step in the development of recombinant VmR strains. Here, we report the results of efficient transformation of recombinant plasmids to VmR cells by electroporation.

The maintenance of the transformed plasmid in recombinant VmR without supplement of antibiotics in the culture medium is an important parameter for a large production of the alkaline protease for industrial use. It was necessary to develop a method to maintain recombinant plasmids in VmR cells. Hence, we report the improvement of plasmid stability by introducing the *par* locus (*par*=partition) [14, 15, 16, 18] in the recombinant plasmid. The *par* locus is responsible for the stable inheritance of low copy number plasmids such as pSC101 [14, 18]. The *par* function has been mapped to a short DNA region of 370 bp in the immediate vicinity of the origin of DNA replication of pSC101, but the mechanism by which this sequence affects the correct partition of plasmids is still unknown

[18]. By introducing this *par* locus to the recombinant plasmids, we obtained about a two-fold improvement of the plasmid stability in recombinant VmR cells.

MATERIALS AND METHODS

Strains and Plasmids

VmR was isolated from wastewater that produced six alkaline proteases and one metalloprotease extracellularly [9, 11]. Among the proteases, two major proteases, VapT and VapK, were purified and characterized [9]. The gene encoding *vapT* has been cloned and characterized [10], and *vapK* (2.9 kb) was recently cloned in our laboratory (unpublished result). VmR was used as a host in the transformation of the recombinant plasmid. Based on a number of reports made concerning the transformation of *E. coli* plasmids into the *Vibrio* species [3, 5, 6, 7, 12, 13, 17], we used plasmids carrying the ColE1 replicon for transformation of VmR cells by electroporation. The *E. coli* plasmid pT7T3 19U (2.6 kb, Ap^r, Promega Co.) and pKF3 (2.2 kb, Takara Co.) containing the chloramphenicol resistance gene (Cm^r) were used as cloning vectors. The plasmid pEYR (3.96 kb, Ap^r, our lab.) has a *par* locus originating from pSC101 that was constructed by joining the 0.37 kb *Ava*I-*Hinc*II fragment of the pSC101 plasmid to the corresponding site of pBR322. Recombinant plasmid pSB1 (5.9 kb) contains the *vapK* gene encoding an alkaline protease VapK in the *Hin*dIII site by joining the 2.9 kb DNA fragment from VmR chromosomal DNA to the corresponding site of pT7T3 19U. Plasmid pSBCm (5.1 kb) was derived from pSB1 by subcloning *vapK* to the *Hin*dIII site of pKF3. To construct plasmid pSP1 (6.0 kb), the *Pvu*II-*Hinc*II fragment of pEYR containing the *par* locus (1 kb) was inserted into the *Pvu*II site of pSBCm (Fig. 1). Plasmids in VmR and *E. coli* were isolated using the alkaline lysis method [1].

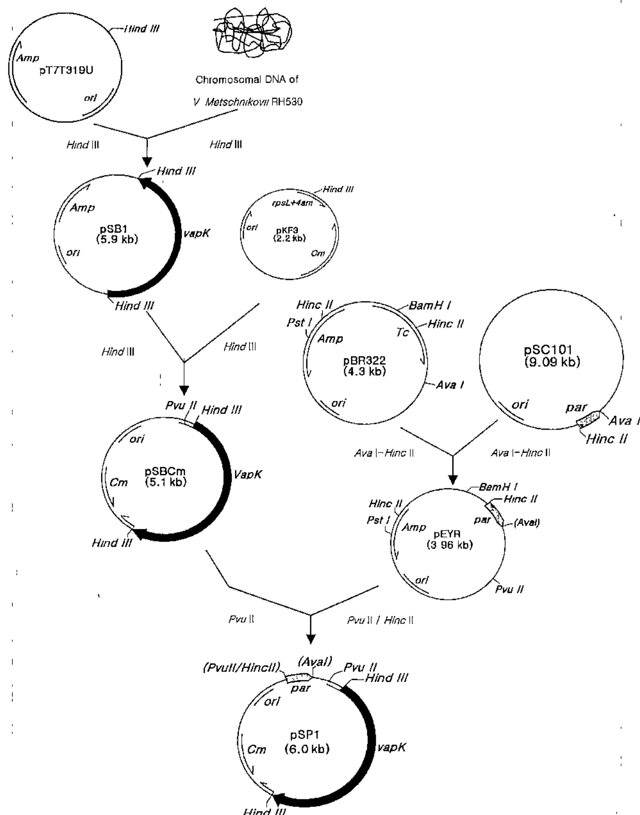

Fig. 1. Construction of recombinant plasmids.
To construct a recombinant pSP1 plasmid, a DNA fragment (1 kb) containing the *par* locus from *Pvu*II-*Hinc*II digested plasmid pEYR was inserted to the *Pvu*II site of pSBCm. Other details for the construction of recombinant plasmids are described in Materials and Methods.

Media and Culture Conditions

VmR cells were cultured at 30°C in the LSC medium containing 0.5% yeast extract, 1% bacto-tryptone, and 1% sodium chloride in 100 mM of sodium carbonate buffer (pH 10.5) or the LB medium. To detect alkaline protease activity by halo formation, 3% skim milk was added to the LSC agar medium. Chloramphenicol (Cm, 12.5 μg/ml), ampicillin (Ap, 50 μg/ml), tetracycline (Tc, 12.5 μg/ml), and kanamycin (Km, 25 μg/ml) were added separately to the respective LSC media. To detect extracellular DNase production by VmR, cells were grown on DNase test agar (Difco) followed by spreading with 0.1 M HCl to precipitate the unhydrolyzed DNA.

Electroporation of VmR Cells

Electroporation of VmR was carried out by the procedure described by Dower *et al.* [4] with some modifications. Briefly, cells were grown in 1 l of LSC medium at 30°C with vigorous shaking until an OD_{600} of 0.7 was reached. Cells were then chilled and harvested by centrifugation (4,000 ×g) for 10 min at 4°C. The cells were washed twice with 1/20 vol of ice-cold EP buffer (137 mM sucrose, 1 mM HEPES, 10% glycerol, pH 8.0), and then resuspended in 1/100 vol of the same buffer. A 100 μl aliquot of the cell suspension was used as competent cells for electroporation. The competent cells were mixed with plasmid DNA (typically 1 μg) and they were subjected to electroporation by using the Gene Pulser II (Bio-Rad Lab) with 0.2 cm electrode gap cuvettes. One-ml of fresh LSC medium was subsequently added to the cells followed by incubation at 30°C for 1 h with gentle shaking. The cells were plated on selective medium containing appropriate antibiotics and skim milk. The transformed cells showing both resistance to antibiotics and halo-formation were selected.

Curing of Plasmid

Curing of the plasmid from VmR cells was carried out as described by Kawakami *et al.* [17] with some modifications. VmR cells harboring plasmids were grown overnight in

20 ml of LSC medium, and 20 μl of the culture was incubated overnight in 1 ml of fresh LSC medium containing 200 μg/ml of acridine orange (Sigma), followed by plating on the LSC medium. The colonies were analyzed for plasmid curing by detecting the loss of plasmid-encoded function, such as antibiotic resistance, as well as by plasmid isolation.

DNase Test of VmR and VmRc Cells

To test and characterize DNase secreted by the VmR and VmRc, both cells were grown in 50 ml of LSC media at 30°C, overnight. One of each culture was re-inoculated to 100 ml of fresh LSC. From the cultures, 50 ml of the broth was harvested after 2 h of growth corresponding to an OD_{600} of 0.6, and the remaining cells were continuously grown for 18 h, followed by harvest. Both parts of the cells were prepared by using the same procedure for electroporation that was finally suspended in 500 μl of the EP buffer. One-hundred μl of the cells were mixed with 1 μg of plasmid pSBCm which was isolated from *E. coli* cells. The reaction mixture was incubated at 37°C for 30, 50, and 70 min. After removing the cells by centrifugation, the supernatant containing plasmid DNA was analyzed using agarose gel electrophoresis.

Determination of Plasmid Stability

The plasmid stability at 18 h of growth (27 generations) of VmR was important, because the production of alkaline proteases achieved a maximum level at that time. The plasmid stability at that time was determined by the viability of the cells in LSC medium. Briefly, 0.5 ml of the seed culture that was grown overnight at 30°C in the LSC medium with Cm was re-inoculated in 100 ml of fresh LSC medium without Cm. By growing the cells at 30°C, the same number of cells were harvested at an indicated time point, and they were spread both on the agar media that contained Cm (W-medium) or without Cm (WO-medium). The ratio of the number of viable cells appearing on W-medium to WO-medium after an overnight incubation was defined as plasmid stability.

Protease Assay

Alkaline protease activity was measured as described elsewhere [9, 10]. One unit of protease activity (PU) was defined as the amount of enzyme which catalyzes an increase in the absorbance of 0.1 per 10 min by releasing tyrosine.

RESULTS

Electroporation of VmR

It was necessary to determine which antibiotics could be used in the selection of VmR transformants. We tested various concentrations of antibiotics such as Ap, Tc, Km, and Cm. The cells showed resistance to 50 μg/ml of Ap, 12.5 μg/ml of Tc, and 25 μg/ml of Km. Only Cm (12.5 μg/ml) inhibited the growth of the cells. Therefore, plasmid pKF3 containing a Cm^r gene was used as the cloning vector. The recombinant plasmid pSBCm (Fig. 1) was used for the transformation of VmR cells. The electroporation of VmR cells was initially carried out by using the conditions for *E. coli* (2.5 $kVcm^{-1}$, 25 μF, 50 Ω) [6] with 1 μg of pT7T3 19U plasmid, followed by plating on LSC media which contained 100 μg/ml of Ap and 3% skim milk. Only one transformant was obtained as shown by the formation of a clear halo and resistance to a high concentration of Ap. It was suspected that this result was caused by the extracellular secretion of DNase in VmR cells, so that almost all the added DNA were degraded by the enzyme (Fig. 2A). To obtain a suitable host for the transformation, plasmid pT7T3 19U was cured with acridine orange [8, 16] from the transformed VmR cells. The curing was confirmed by detecting the loss of antibiotic resistance along with the deficiency of the plasmid from the transformed VmR cells. The resulting cured VmR cells, designated as VmRc, were then efficiently transformed with pT7T3 19U, pKF3, pSB1, pSP1 (data not shown), and pSBCm. Furthermore, all the plasmids which were isolated from the transformed VmRc cells caused a high efficiency of transformation even in the VmR (more than 10^3 transformants per μg DNA). These results suggested that DNase secretion in the VmRc was reduced and some modifications may have occurred in the plasmid DNA in the VmRc. The results of the DNase test of VmRc are shown in Fig. 2A. Plasmid pSBCm, incubated with 2 h-grown VmR cells for 30 min, was easily digested by the extracellular DNase (Fig. 2A, lane 1). After 70 min, almost all of the plasmids were completely digested by the VmR cells (Fig. 2A, lane 9). However, plasmids incubated with VmRc was not degraded for 2 h-grown cells, even after 70 min of incubation (Fig. 2A, lane 10). All the plasmids incubated with 18 h-grown VmR and VmRc cells were degraded after 30 min and almost completely degraded after 70 min. These results confirmed that the mutant VmRc cells secreted less amounts of extracellular DNase compared to the wild-type VmR, resulting in an increase in the transformation efficiency. The restriction patterns of the plasmid DNA isolated from the transformed VmR and VmRc were the same as those from *E. coli* (Fig. 2B). Therefore, we used plasmid pSBCm that was isolated from VmRc cells for subsequent transformations of VmR. All the values shown in the figures and tables represent mean values of results from three independent experiments.

Effects of Electric Field Strength on the Efficiency of Transformation

In order to study the effects of electric field strength on electroporation, field strengths of 2.5, 5, 6, 7.5, 9, and 10 $kVcm^{-1}$ with 25 μF were applied to VmR cells mixed

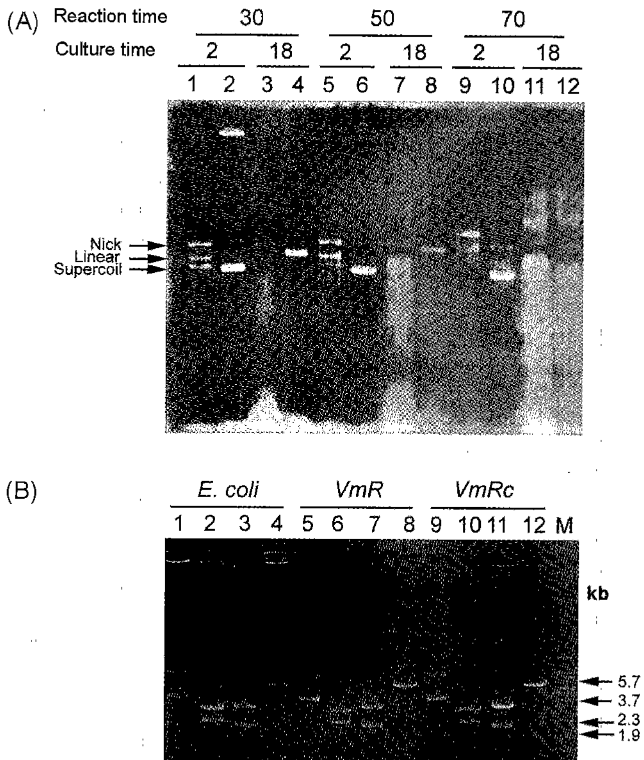

Fig. 2. Characterization of the VmR and VmRc strains for electroporation.
A. DNase test of VmR and VmRc cells. Plasmid pSBCm was incubated with 2 h-grown VmR cells (lanes 1, 5, and 9), 2 h-grown VmRc cells (lanes 2, 6, and 10), 18 h-grown VmR cells (lanes 3, 7, and 11), and 18 h-grown VmRc cells (lanes 4, 8, and 12) for the indicated time. After reaction as described in Materials and Methods, cells were removed by centrifuagtion and the supernatant was analyzed by agarose gel electrophoresis. B. Comparison of restriction patterns of plasmid pSBCm isolated from *E. coli*, *V. metschnikovii* strain RH530 (VmR), and cured VmR (VmRc). Lanes 1-4: isolated from *E. coli*; lanes 5-8: isolated from VmR; lanes 9-12: isolated from VmRc. Lanes 1, 5, and 9: supercoil; lanes 2, 6, and 10: digested with *Eco*RV; lanes 3, 7, and 11: digested with *Hin*dIII; lanes 4, 8, and 12: digested with *Pvu*II. M: size marker.

with 1 μg of plasmid pSBCm. The resistance was held constant at 50 Ω for all experiments. Cuvettes with a 0.2 cm electrode gap were used. As shown in Fig. 3, 7.5 $kVcm^{-1}$ of the field strength was optimal for the transformation of VmR. The conditions were different from those of *E. coli* whose optimal conditions for transformation were 2.5 $kVcm^{-1}$ of field strength with 25 μF.

Effects of Sucrose on the Efficiency of Transformation

The effect of sucrose concentration on electroporation was investigated by using VmR cells mixed with pSBCm. As shown in Fig. 4, sucrose in the EP buffer extensively affected the transformation efficiency of VmR. Without the addition of sucrose, all the VmR cells were destroyed by electric shock under the following conditions (7.5 $kVcm^{-1}$, 25 μF, and 50 Ω). The increase in the concentration of sucrose correspondingly appeared to increase the viability

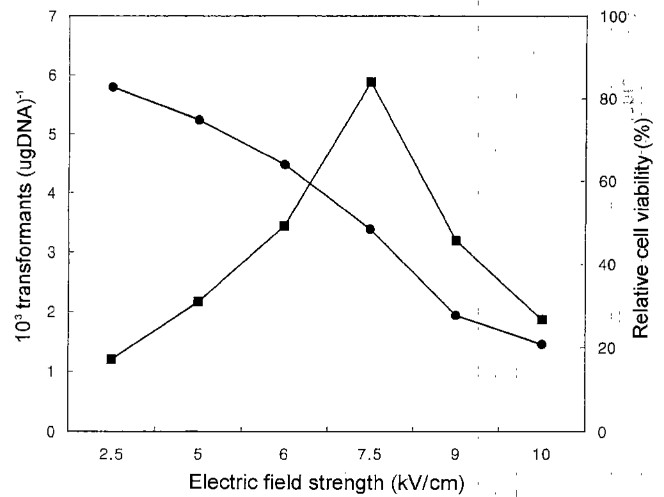

Fig. 3. Effect of electric field strength on the transformation efficiency.
Plasmid pSBCm (1 μg) was added to each suspension of VmR cells. Maximum efficiency of transformation was shown at 7.5 $kVcm^{-1}$. Symbols: ■, Number of transformants per μg of plasmid pSBCm; ●, Relative cell viability.

of cells, and the concentration higher than 137 mM did not affect the viability of VmR cells, but the efficiency of transformation was greatly enhanced by the increase of sucrose. Two-hundred mM of sucrose was optimal for the VmR cells, but the concentration above 200 mM decreased the efficiency of transformation. These results might be caused by the high osmotic pressure of the transformation buffer.

Effects of Plasmid DNA Concentration and Growth Phase on the Efficiency of Transformation

The effect of plasmid DNA concentration on the transformation of VmR is shown in Fig. 5. The total number of transformants

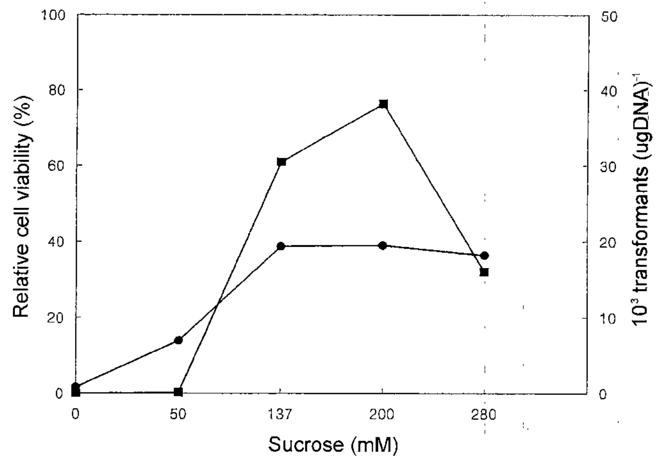

Fig. 4. Effect of sucrose on the efficiency of transformation.
VmR cells were mixed with 1 μg of plasmid pSBCm. Electroporation was carried out at 7.5 $kVcm^{-1}$, 25 μF, and 50 Ω with a 0.2-cm electrode gap cuvette. Symbols: ●, Relative cell viability; ■, Number of transformants per μg of plasmid pSBCm.

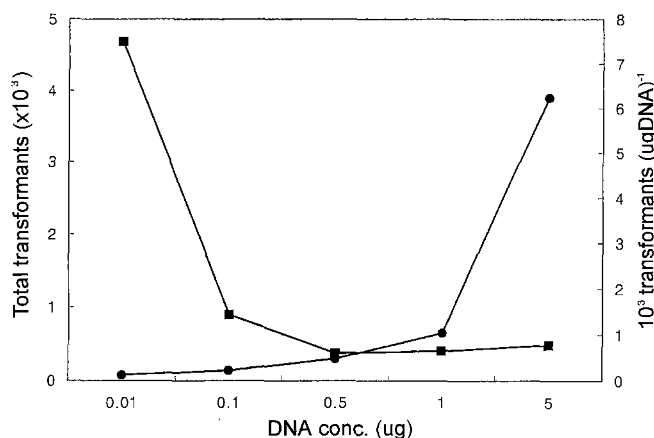

Fig. 5. Effect of concentration of plasmid DNA on the transformation efficiency.

The efficiency of transformation is expressed in terms of total number of transformant as well as the number of transformants per μg plasmid DNA. Plasmid pSBCm, in increasing amounts from 0.01 μg, was added to a suspension of VmR cells. Field strength of pulse, 7.5 $kVcm^{-1}$. Symbol: ■, Number of transformants per of plasmid pSBCm; ●, Total transformants.

was found to be dependent on the concentration of plasmid DNA, although the number of transformants per μg plasmid DNA remained constant above a concentration of 0.5 μg.

The effect of the growth phase at which the VmR cells were harvested on the efficiency of transformation was also studied. Cells were harvested at each time point as indicated in Fig. 6. The total number of cells for transformation was adjusted to an OD_{600} of 0.6 by varying the harvesting volume. Electroporation was carried out with 1 μg of pSBCm under the following conditions (7.5 $kVcm^{-1}$, 25 μF, and 50 Ω). The highest efficiency was obtained when the cells were harvested after 3 h of growth which corresponded to the early exponential phase (Fig. 6). After the early exponential phase of growth, the efficiency of transformation dramatically decreased. These results suggested that after the early exponential growth phase, a rapid increase in the DNase activity of VmR cells had occurred which degraded the added plasmid DNA.

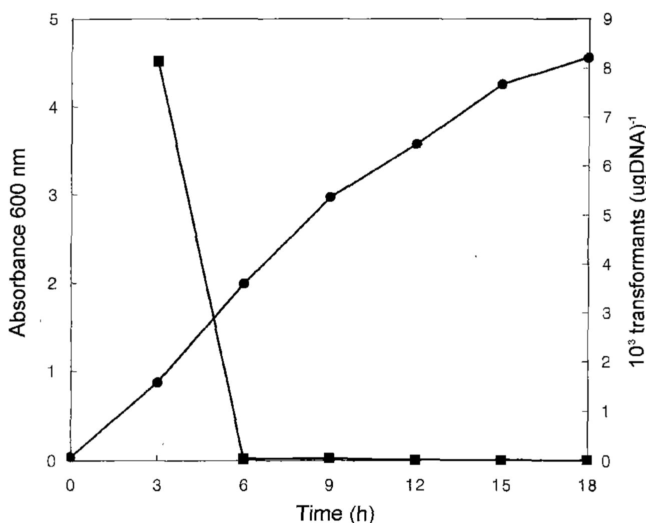

Fig. 6. Effect of growth phase on the efficiency of transformation of VmR cells by electroporation.

Cells were harvested at indicated times (h). Electroporation was carried out with 1 μg of pSBCm DNA and an electric field strength of 7.5 $kVcm^{-1}$. Symbols: ■, Number of transformants per μg of plasmid DNA; ●, Absorbance of cells at OD_{600}.

Stability of Plasmid in VmR and Its Improvement by Introducing the *par* Locus

The stability of the plasmid pSBCm in VmR was investigated as described in Materials and Methods. As shown in Table 1, the stability of plasmid pSBCm in the VmR cells after 18 h of growth (which corresponds to 27 generations) was approximately 25%. These results suggested that most of the pSBCm in VmR cells were lost during the cultivation process in the WO-medium. By contrast, almost all the cells grown in W-medium showed some resistance to Cm, indicating that plasmid pSBCm was maintained without loss under the selection pressure (data not shown).

To improve the stability of pSBCm in VmR without the selection pressure, the *par* locus was introduced in the pSBCm to generate pSP1 (Fig. 1). After transformation and cultivation, the stability of pSP1 was compared to that of pSBCm (Table 1). About a two-fold increase in the stability was observed in pSP1 compared to pSBCm by showing 42% viability. The pSP1 plasmid was maintained fairly constant even after 27 h of growth (41 generations)

Table 1. Plasmid stability of pSBCm and pSP1 in *V. metschnikovii* RH530 (VmR) cells.

Culture time (h)[a]/ Generation (N)	Growth (O.D. at 600 nm)[b]			Plasmid stability (%)[c]	
	VmR	VmR (pSBCm)	VmR (pSP1)	VmR (pSBCm)	VmR (pSP1)
6/4.5	2.41	2.16	2.25	51.72	64.83
12/18	4.27	3.66	3.32	37.72	62.38
18/27	5.31	4.00	3.42	24.56	41.99
27/40.5	5.98	4.41	4.04	22.00	37.07

[a]VmR cells were routinely harvested after 18 h of growth (which corresponds to 27 generations) for the production of alkaline protease using the strain. At that time, the production of alkaline protease was at a maximum level (unpublished data). Plasmid stability was measured at that time point.

[b]Cells were inoculated in 100 ml of LSC medium and cultured at 30°C. Whenever necessary, antibiotic Cm (12.5 μg/ml) was added. Cells were collected at the indicated time point and their absorbance was measured at 600 nm.

[c]The same number of cells was plated on the LSC agar medium with or without Cm. Plasmid stability was defined as the ratio of the number of cells which appeared on the agar medium with antibiotics to the number of cells present on the medium without antibiotics.

Table 2. Comparison of the alkaline protease productivity in *V. metschnikovii* RH530 (VmR) cells harboring recombinant plasmid pSBCm and pSP1.

Strain	Plasmid	Growth[a] (O.D. at 600 nm)	Protease productivity[b] (PU/ml/O.D.)	Relative productivity (%)
	-	4.9	120	100
VmR	pSBCm	3.5	486	405
	pSP1	3.3	596	497

[a]Cells were grown in LSC media containing Cm for 18 h.
[b]Alkaline protease productivity was determined by dividing the enzyme activity (PU/ml) by the optical density of cells measured at 600 nm.

by showing 38% viability. The increase in the plasmid stability was accompanied by the increase in the productivity of alkaline protease from the recombinant VmR harboring the plasmid pSP1 compared to VmR cells harboring pSBCm (Table 2). These results concluded that the *par* locus inserted in pSBCm might play a role in the partition and segregation of plasmids in VmR cells.

DISCUSSION

Thus far, many studies conducted on the transformation of the *Vibrio* species have been reported. However, all the strains used in the past studies were generally restricted to clinically-important pathogens, such as *V. cholerae* [17], *V. vulnificus* [13], *V. parahaemolyticus* [6], and *V. alginolyticus* [7]. In this study, we attempted to transform the industrially-important *V. metschnikovii* RH530 (VmR) strain which had been isolated from wastewater [9, 11]. The strain produced large amounts of several alkaline proteases including the major alkaline protease, VapK, secreting into the extracellular medium [9, 10, 11]. As one of the attempts to develop an alkaline protease overproducing strain with VmR, recombinant DNA techniques were used in this study. To generate a recombinant strain for the overproduction of alkaline protease, it was essential to optimize the transformation of VmR by using the recombinant plasmid pSBCm with the *vapK* gene. However, as reported elsewhere [12], the *Vibrio* species secretes DNase and has a restriction-modification system. These are major barriers to transform plasmid into the cells. Several methods were reported to overcome the DNase barrier such as incubation of plasmid with the cells at extremely low temperatures [17], adding sucrose into the electroporation buffer to remove DNase from the periplasmic space by osmotic shock [5], incubation of cells with EDTA to inactivate the DNase [17], and simply by using a high concentration of DNA [3]. As the VmR strain used in this experiment secreted large amounts of DNase, the osmotic shock and high concentration of plasmid DNA were applied to reduce the degradation of plasmid by DNase in competent VmR cells. However, we could not obtain a high transformation efficiency with 1 μg of plasmid pT7T3 19U by using VmR as a host. Only one transformant was obtained by using the *E. coli* plasmid pT7T3 19U. The transformed VmR cells were treated by using acridine orange to cure plasmid pT7T3 19U. The resulting VmR cells (VmRc) showed a high efficiency of transformation with plasmid pSBCm and its derivatives. By analyzing the cells, VmRc cells secreted less amounts of DNase than the wild-type VmR (Fig. 2A). Using the conditions developed in this study, more than 10^4 transformants per μg plasmid DNA were routinely obtained. In spite of the high concentration of DNA, treatment of cells by osmotic shock, and using less DNase-secreting VmRc cells, it was impossible to completely avoid the degradation of plasmid DNA by the DNase. The low viability of VmR cells by electric shock is also another factor that cannot be ignored.

In conclusion, the differences in the optimized conditions for electroporation of VmR cells from the procedures of several clinically-important *Vibrio* pathogens are: (i) harvesting the cells for electroporation at an early exponential stage rather than in the early stationary phase [7], (ii) washing cells without $MgSO_4$ in contrast to *V. alginolyticus* [7], (iii) special treatment of the competent cells with acridine orange for the removal of DNase, (iv) using high concentrations of DNA and treatment of cells with sucrose for osmotic shock, and finally, (v) electroporation by 7.5 $kVcm^{-1}$, 25 μF, and 50 Ω with a 0.2-cm electrode gap cuvette.

The stability of the plasmid is a very important parameter in the production of industrial proteins with a large culture volume. Therefore, the stability should be highly maintained during cultivation without any selective pressure. The stability of the plasmid pSBCm in VmR was about 25% at 18 h of growth (27 generations). This low stability of plasmid might be due to the high copy number, a large size of plasmid pSBCm (5.1 kb), and overproduction of alkaline proteases. To increase plasmid stability, the *par* locus, which has been known to play a role in the partition and segregation during the replication of low-copy plasmids, such as pSC101 [14, 15, 16, 18], was introduced to pSBCm to generate pSP1 (Fig. 1B). As a result, the stability of plasmid pSP1 increased about two-fold (42%). Furthermore, the plasmid was maintained even after 27 h of growth (41 generations) by showing 37% viability (Table 1). Although the increase in plasmid stability in this study was not satisfactory, the introduction of the *par* locus contributed significantly to improving the stability, even in a high copy vector without the selection pressure.

In spite of the low efficiency of transformation in this study, this report suggests the first successful electroporation protocol for the nonpathogenic and industrially-important *Vibrio* strain. The optimized conditions mentioned herewith constitute useful procedures in the genetic manipulation of the nonpathogenic *Vibrio* strain. In addition, the strategies

of introducing a *par* locus to a high copy number recombinant plasmid can be generally applied in the maintenance of plasmids during cultivation of recombinant cells for a large production of commercially useful materials.

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