Introduction of pBIN19 Plasmid Gene into the Marine Origin Pseudomonas SC9 Using Electroporation

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Introduction of new functions to a marine bacterium was aimed at by using a plasmid vector. The plasmid pBIN19, derived from the broad host range plasmid RK2, was introduced into marine isolated Pseudomonas strain SC9 by electroporation. Despite the low transformation efficiency of the bacterial conjugation method reported previously, a transformation efficiency of 1.81 × 10⁴ transformants/μg of pBIN19 was obtained for the strain SC9 by electroporation. The introduced pBIN19 was not detected in plasmid form in transformed cell. Southern blot hybrids appeared in genomic DNA under 50 kb using crossed field gel electrophoresis, which indicated introduction and recombination of pBIN19 into genomic DNA of Pseudomonas SC9.

Key words: bacteria, electroporation, gene, Pseudomonas SC9, plasmid

In crustacean culture ponds, larvae are infected by pathogenic organisms mostly because of the poor environmental conditions. In this biotope, for example, the pathogenic bacteria Vibrio spp., which has been suggested to cause fish disease, prevails. Antibiotics might repress the growth of these pathogens but eventually multidrug-resistant strains would appear. To reduce the number of pathogens without using drugs and improve the growth of shrimp and crab in culture, several useful bacterial strains have been isolated.

In this study, our aim was to transfer useful functions to the above marine bacteria using a plasmid vector. As a model, a broad host range plasmid vector was suitable to demonstrate introduction of a new function to bacteria. Thus, plasmid pBIN19, derived from the Inc P1 plasmid RK2 was chosen as the vector, as it replicates in diverse Gram-negative bacteria.

Many reports on the transformation of Escherichia coli and other Gram-negative bacteria have shown that electroporation exhibits considerably higher efficiency than conjugation or chemical methods and also the electroporation efficiency was reported to be 50 to 80 times higher than natural transformation of competent cells in Methanococcus voltae. In marine bacteria, a few strains such as Caulobacter crescentus and some pseudomonads were successfully transformed by electroporation although the procedure has not been established in detail. The requirement of the complete removal of ions from the cell suspension seems to be one of the main problems to be faced during the electroporation procedure, because many marine bacteria might be inactive or lyse in salt-free solution. However, the adaptation of bacteria to ion starvation has been reported. Consequently, the Pseudomonas strain SC9 might be adapted to salt-free conditions required for the high voltage necessary for electroporation to transform with plasmid pBIN19.

The host strain Pseudomonas SC9 was chosen from isolated bacteria from the sea for it showed sensitivity to the vector marker antibiotics. It also showed a tolerance to low salt concentration, which made it possible for the experiment to carry on.

In this report we described the detailed procedures for electroporation applied to the marine isolated Pseudomonas strain SC9 and also the successful results in the introduction of plasmid genes into the strain SC9.

Materials and Methods

Bacterial Strains and Plasmid

Marine derived bacterial strains for selection as the host strain of gene introduction were provided from Microbial Biology Section in National Research Institute of Aquaculture. Marine derived bacterial strain SC9 which was identified as genus Pseudomonas by the providers according to the method of Shimidu was isolated from a sea cucumber Stichopus japonicus intestine. The other origins and strains provided are, rotifer (Brachionus plicatilis, Strains BP-4 and BP-6), swimming crab (Portunus trituberculatus, Strain SC-1), sea cucumber (Stichopus japonicus, Strains SC-7 and SC-9) and top shell (Turbo cornutus, Strains TS-3, TS-5 and TS-9).

Escherichia coli MC1022 containing the plasmid pBIN19 was obtained from CLONTECH Laboratories (USA), and E. coli XL1-Blue and JM109 were purchased from Takara Co. Ltd. (Japan). Plasmid pBIN19 was introduced into XL1-Blue by electroporation. Transformation of competent JM109 with pBIN19 was done by the mixing procedure.

Culture of Bacteria

Marine derived bacteria were grown in ZoBell 2216 medium provided as Marine Broth (Difco Laboratories, USA) at 25°C unless otherwise stated. E. coli strains were grown in L broth (1% Bactotryptone, 0.5% Bacto yeast extract,
1% NaCl, pH 7.2) at 37°C. Several salt concentrations (0, 0.6, 3.5%) of ZoBell medium was made in distilled water with an addition of NaCl instead of using seawater. Other chemicals were of commercial products used without further purification.

**Antibiotic Susceptibility Test**

The cells preincubated overnight in ZoBell medium were inoculated to the same solid media or liquid media containing various concentrations of kanamycin, and minimum inhibitory concentration (MIC) was determined at 20°C. To select for the transformed cells resistant to kanamycin, MIC and 2 to 16 times higher concentrations were added to the plates.

**Bacterial Preparation for Electroporation**

The strain SC9 was incubated in ZoBell 2216 medium with 0.6% of salt concentration to mid-late log growth phase as \( \Delta \text{OD}_{600} = 0.5-0.8 \), followed by chilling on ice for 30 min. All subsequent steps were carried out on ice. Cells were harvested and washed twice with cold MgCl\(_2\)-CaCl\(_2\) solution (10 mM MgCl\(_2\) and 5 mM CaCl\(_2\)), and washed once with cold 10% glycerol solution, followed by resuspension with 10% glycerol solution to a final concentration of 1 x 10\(^9\) cells/ml. The suspension was divided in 250 \( \mu \)l portions and stored at –80°C.

**Plasmid Extraction**

Plasmids in bacterial strains were extracted by alkaline lysis\(^{21}\) using a QIAGEN plasmid kit (QIAGEN, Germany). Extracts were subjected to 0.7% agarose gel electrophoresis. DNA concentration was determined by measurement of absorbance at 260 nm using a UV photometer Shimadzu UV-240.

**Electroporation Conditions**

Cell suspensions, 40–250 \( \mu \)l, were thawed on ice and mixed with 2–50 \( \mu \)l of pBIN19 (100 ng/\( \mu \)l) in 0.1-cm cuvettes before electroporation. Bio-Rad Gene Pulser apparatus was set at 2.5 kV and 25 \( \mu \)Farad (\( \mu \)F), and the pulse controller settings varied from 400 to 600 \( \Omega \). Immediately after the impulse, the contents were mixed with 1 ml SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl\(_2\), 10 mM MgSO\(_4\) and 20 mM glucose) and incubated for more than 1 h at 25°C with shaking at 180 rpm according to the method of Diver et al. (1990).\(^{16}\) The cultures were diluted and inoculated onto ZoBell plates containing kanamycin at concentrations ranging from 50 to 800 \( \mu \)g/ml. To determine the cell survival rate during electroporation, dilutions of the cultures were plated on kanamycin-free ZoBell plates.

**Plasmid Identification**

Detection of plasmids introduced into bacterial cells was carried out by the method described in Maniatis et al.\(^{21}\) Further analysis by Southern blot hybridization\(^{21,22}\) was performed to confirm the presence of the original plasmid. The gel after electrophoresis was depurinated and set on a ATTO genopirator chamber with an AE-6680 pump. Hybond-N\(^+\) membrane (Amersham, UK) was used for blotting, and the buffer (10 x SSC: 0.15 M sodium citrate, 1.5 M NaCl) was aspirated for 60 min. DNA blotted on the membrane was cross-linked by UV at 1200 \( \mu \)W/cm\(^2\) using a spectrolinker XL-1500 (Spectronics Corporation, USA). Hybridization was then performed according to the method described by Maniatis et al.\(^{21}\). The radioactive marker [\( \alpha ^{32}\)]P dCTP was used to mark the probe plasmid pBIN19.

**Genomic DNA Extraction and Detection**

Extraction of genomic DNA for Southern blot analysis was carried out by the method described by Kido.\(^{23}\) DNA samples for crossed field gel electrophoresis\(^{24}\) were prepared in LcT agarose gels (FM Bio Products, USA) according to the method of Smith and Cantor\(^{25}\) modified as described below. Overnight cultures of SC9 and SC9-29 (transformed SC-9) were incubated with addition of 100 \( \mu \)g/ml chloramphenicol for 1 h, and 1.5 ml aliquots were centrifuged. Pellets were washed in 300 \( \mu \)l of 1 M NaCl-10 mm Tris·HCl (pH 7.6) and resuspended in the same buffer. After addition of an equal amount of 2% (w/v) in Cert agarose, portions were poured into the gel formation mold and cooled at 4°C. The cells were disrupted by setting solid gels in EC buffer (6 mm Tris·HCl (pH 7.6), 1 mm NaCl, 100 mm EDTA (pH 7.6), 1% sarkosyl and 1 mg/ml lysozyme) at 37°C for 22 h. Proteins were digested by 1 mg/ml proteinase K (EC 3.4.21.14, Wako Pure Chemical, Japan) at 37°C for 3 days. The activity of proteinase K was inhibited by incubating gels twice into freshly prepared 1 mm phenylmethylsulfonyl fluoride in Tris·EDTA buffer (TE; 1990).\(^{16}\) The cultures were diluted and inoculated onto ZoBell plates containing kanamycin at concentrations (1990). Kanamycin resistance test was carried out for E. coli MC1022 carrying pBIN19 and the other 10 bacterial strains isolated from various sea regions as described in materials and methods. The results are shown in Table 1. Kanamycin at a concentration of 50 \( \mu \)g/ml was applied in this test. Growth of BP6 and SC9 was totally inhibited in kanamycin supplemented medium during incubation for 3 days. BP3 also showed sensitivity to kanamycin, but its lag
Table 1. Growth of the various strains isolated from the marine environment using the kanamycin supplemented medium

<table>
<thead>
<tr>
<th>Incubation days</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>7</th>
<th>8</th>
<th>9</th>
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<tr>
<td>medium</td>
<td>Z</td>
<td>ZK</td>
<td>Z</td>
<td>ZK</td>
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</tr>
<tr>
<td>Strains</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MC1022</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BP3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>BP4</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>SC1</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SC7</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SC9</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TS1</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>TS2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TS3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Z stands for ZoBell medium, ZK stands for ZoBell medium supplemented with 50 \( \mu \)g/ml kanamycin. --, growth turbidity between 0.3-1.0; +, growth turbidity over 1.0.

Phase was as long as 3 days. Other strains grew on kanamycin supplemented medium. All the strains were found to have no plasmids (Fu and Maeda, unpublished data), and SC9 was finally chosen as the recipient strain for transformation because its growth was faster than that of BP6 in kanamycin-free ZoBell medium.

Plasmid Extraction

Plasmid pBIN19 was extracted from 5 ml of the *E. coli* MC1022 culture. The size of the plasmid was 10 kb, had a single *EcoRI* restriction site, but had no restriction site for *Not* I (data not shown).

Conditions for Bacterial Preparation

Bacterial solution (10 μl) of the strain SC9 stored in ZoBell medium with 15% glycerol at -80°C was directly inoculated into 5 ml of ZoBell medium. Salt included in inoculants changed salt concentration of culture of 0, 0.6, and 3.5% salt ZoBell medium to 0.005, 0.604, and 3.498% salt, respectively. As is shown in Fig. 1, the growth amount after 50 h of incubation under essentially salt-free conditions (0.005%), was about 67% of that at 3.498% salt concentration. For electroporation, cells were harvested after 20 h in medium with a salt concentration of 0.6%, which prevented the cells from lysis in the completely salt-free solution in the electroporation cuvette. When the cells were put into a salt free solution in the cuvette after culture in 3.5% salt medium, the cells lysed.

Conditions for Selection of Transformants

The strain SC9 showed growth inhibition by kanamycin at the concentration of 200 μg/ml and the transformants were selected at this concentration. On the other hand, *E. coli* MC1022 containing the plasmid pBIN19 showed almost complete resistance to 200 μg/ml kanamycin.

Conditions for Electroporation

Electrotransformation of *Pseudomonas* SC9 was performed in 0.2 cm cuvettes at 2.5 kV, 600 Ω. Most cells burst during washing in sterilized distilled water. When the washing medium was changed to MgCl₂-CaCl₂ solution after incubation as written in Materials and Methods, no cell lysis occurred. Also instead of using 0.2 cm wide cuvettes, 0.1 cm cuvettes were adopted and a reduced voltage of 1.25 kV was applied, because the cell suspension lysed under high voltage conditions. Resistance was also reduced to 400 Ω and pulse durations were 2.3 to 2.4 ms. Finally, in these conditions transformants were obtained as shown in Table 2. Selection was found successful when the colonies of electroporated SC9 were produced on the ZoBell plates with 200 μg/ml kanamycin because cells did not grow on the plate containing kanamycin without applying electroporation.

For better efficiency, two experiments were set with different concentrations of plasmid pBIN19 (2.5 μg and 0.1 μg). These concentrations of plasmid solutions were mixed with a suspension of the strain SC9. As higher as 3.2 times the amount of transformants appeared when 2.5 μg of plasmid was applied instead of 0.1 μg; the transformation frequency was 1.12 x 10⁻⁹ in the former case and 3.5 x 10⁻¹⁰ in the latter. However, transformation efficiency of 2.5 μg pBIN19 condition was lower, 3.5 x 10⁻³ transformants/μg DNA, than for 0.1 μg pBIN19, 1.8 x 10⁴ transformants/μg DNA (Table 3). Competent cells of the *E. coli* strain JM109 were also transformed by pBIN19 in which transformation efficiency was 1.25 x 10⁵ transformants/μg DNA.

Resistance Test for Transformants

Resistance to kanamycin of transformants was determined by the addition of kanamycin at concentrations ranging between 0 and 800 μg/ml to the medium and the number of colonies was counted. No growth inhibition was observed for the transformed strain SC9-29 by kanamycin at concentrations in the range from 50 to 400 μg/ml. Also kanamycin resistance of MC1022 carrying pBIN19 was examined. Strain MC1022 grew even at the kanamycin maximum concentration of 800 μg/ml (Table 4). In case of kanamycin sensitive strain of SC9, a few colonies appeared on 200 μg/ml kanamycin supplemented plate after incubation for 4 days. Based on these results, selection of transformants was carried out by 3 days of incubation to distinguish from the non transformed cells.

![Fig. 1. Growth of the strain SC9 versus various salt concentrations.](image-url)

Strain SC9 was grown on ZoBell medium of distilled water with addition of varied amount of NaCl instead of sea water. ○: 0.005%, ●: 0.604%, △: 3.498% (w/v) salt ZoBell medium (5 ml) with SC9 inoculum (10 μl).
Plasmid Introduction to Marine Derived *Pseudomonas*

Fig. 2. Hybridization analysis of plasmid DNA of electrotransformed strains of SC9.

Lane 1. *Hin d* III marker. $^{32}$P-labeled pBIN19 was hybridized to extracts (supposed to contain the plasmid pBIN19) of 5 ml culture by alkaline lysis of strains SC9-29 (lane 2) and SC9-30 (lane 3). Lane 4. pBIN19. Panel A, DNA profiles; panel B, Southern blot of panel A.

Detection of Introduced DNA

After selection by kanamycin resistance, plasmid extraction was carried out for the 48 electroporated strains, but no plasmid was detected. The amount of cells for plasmid extraction multiplied 40 times but still no plasmid was detected. Southern blot analysis was applied to extracts of plasmid extraction of 2 electroporated strains using pBIN19 probe in which no hybrids were detected on the gel (Fig. 2). However, the acquired kanamycin resistance was retained after 3 months storage at 4°C. To detect pBIN19 DNA in the transformants, genomic DNA from both transformed and non-transformed SC9 strain were extracted from 1.5 ml of overnight cultures and loaded onto a 0.7% agarose gel. Hybridization of SC9 genomic DNA with pBIN19 was affirmed by Southern blot analysis (Fig. 3). As shown in Fig. 3, intact and EcoRI digested genomic DNA from transformant SC9 hybridized to the pBIN19 probe in a wide range around 20 kb. These hybrids of genomic DNA were specific, as no hybrids were detected of genomic DNA of non transformed SC9 (Fig. 3B, lane 5 and 9). Because agarose gel electrophoresis resolves DNA sizes below 20 kb in general, larger DNA fragments could not be separated in this method. For detailed analysis of the size of hybrids observed in Fig. 3, crossed field gel electrophoresis was applied. Genomic DNA extraction in agarose plugs allowed minimum disruption of large DNA molecules over 1 Mbp. Genomic DNA plugs were digested with Not I or EcoRI. From the hybridization analysis using $^{32}$P labeled pBIN19 as a probe, a clear difference between transformants and non transformed cell of the

Fig. 3. Hybridization analysis of the DNA from electrotransformed strains and recipient strain SC9 for homology with pBIN19.

Lane 1. *Hin d* III marker. $^{32}$P-labeled pBIN19 was hybridized to genomic DNA of the transformed strains SC9-29 (lane 2), SC9-30 (lane 3), SC9-31 (lane 4) and non transformed SC9 (lane 5). Eco RI digested genomic DNA of SC9-29 (lane 6), SC9-30 (lane 7), SC9-31 (lane 8), and non transformed SC9 (lane 9) were also hybridized. Panel A, genomic DNA profiles; panel B, Southern blot of panel A.
strain SC9 was demonstrated; that is, there were no hybrids in genomic DNA of non-transformed cell despite large amount of DNA existed on the gel (Fig. 4). On the other hand, hybrids occurred below 50 kb only for transformants genomic DNA (Fig. 4).

Discussion

Transformation of the marine derived Pseudomonas SC9 was successful by electroporation. Dower et al. reported that electroporation requires higher DNA concentrations for higher efficiency than the chemical transformation. Since our aim was to obtain transformants, we applied a large amount of plasmid DNA according to Dower et al. The amount of cell suspension was 6 times higher than in the previously reported method.

Gilchrist and Smit reported that the highest electrical resistance of the electroporation apparatus induced the highest transformation efficiency. However, the cell suspension lysed under high voltage of 2.5 kV and 600 Ω when using 0.2 cm-wide cuvettes. Thus instead of the 0.2 cm-wide cuvettes, 0.1 cm cuvettes were adopted and the reduced voltage of 1.25 kV was applied. Resistance was also reduced to 400 Ω. With these modifications lysis was prevented.

Marine isolated bacteria grow in 3.5% (w/v) salt water. On the other hand, no salt condition in the poration

Table 2. Transformants obtained by the electroporation process

<table>
<thead>
<tr>
<th>Strains</th>
<th>Number of cells per plate Km conc. (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>charged pBIN19 + SC9*1</td>
<td>3.33 x 10^2</td>
</tr>
<tr>
<td>charged SC9*1</td>
<td>8.32 x 10^2</td>
</tr>
<tr>
<td>uncharged SC9*2</td>
<td>8.90 x 10^3</td>
</tr>
</tbody>
</table>

*1 treated by electroporation.
*2 not treated by electroporation.

Table 3. Number of transformants of SC9 obtained under the various conditions of electroporation

<table>
<thead>
<tr>
<th>pBIN19 (µg)</th>
<th>Incubation time (h)</th>
<th>Transformation frequency</th>
<th>Transformation efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>1</td>
<td>1.12 x 10^-9</td>
<td>3.48 x 10^3</td>
</tr>
<tr>
<td>0.1</td>
<td>1</td>
<td>3.53 x 10^-10</td>
<td>1.81 x 10^4</td>
</tr>
<tr>
<td>2.5</td>
<td>24</td>
<td>1.49 x 10^-9</td>
<td>2.97 x 10^3</td>
</tr>
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</table>

Transformation frequency was calculated as number of transformants/number of total survival cells. Transformation efficiency was calculated as number of transformants/amount of plasmid pBIN19 (µg).

Table 4. Expression of kanamycin resistance of pBIN19 in different host strains

<table>
<thead>
<tr>
<th>Km conc. (µg/ml)</th>
<th>0</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>400</th>
<th>800</th>
<th>Incubation days</th>
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<tr>
<td>Strains</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MC1022</td>
<td>7.0 x 10^6</td>
<td>—</td>
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<td>3.3 x 10^6</td>
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<tr>
<td>SC9-29</td>
<td>2.9 x 10^6</td>
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<td>1.4 x 10^4</td>
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<td>1.7 x 10^6</td>
<td>4.4 x 10^6</td>
<td>4</td>
</tr>
<tr>
<td>JM109</td>
<td>1.3 x 10^6</td>
<td>3.3 x 10^4</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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</tr>
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</table>

Fig. 4. Crossed field gel electrophoresis patterns at pulse time 40 sec of the strain SC9 and its electrotreated strain SC9-29 and hybridization analysis.

Band patterns of Eco RI digests (lane 1), Not I digests (lane 2) and intact genomic DNA (lane 3) of SC9, those of Eco RI digests (lane 4), Not I digests (lane 5) and intact genomic DNA (lane 6) of the transformed strain SC9-29 are displayed. Lane 7, DNA ladders. 32P-labeled pBIN19 was hybridized to genomic DNA samples. Panel A, genomic DNA profiles; Panel B, Southern blot of panel A.

Table 4. Expression of kanamycin resistance of pBIN19 in different host strains

<table>
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<tr>
<th>Km conc. (µg/ml)</th>
<th>0</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>400</th>
<th>800</th>
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<tr>
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References