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A rapid and efficient electroporation method for transformation of
Halomonas sp. O-1

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Abstract

Halomonas sp. O-1 is a halophilic bacterium with a high potential for industrial application due to its natural ability to produce polyhydroxyalkanoates (PHAs) using seawater-based media. However, a major barrier preventing industrial scale implementation of this organism is a lack of molecular methodologies capable of readily transforming members of the *Halomonas* genus. Currently, the only reliable method used for introducing DNA into *Halomonas* spp. is bacterial conjugation, a somewhat tedious and time-consuming technique compared to electroporation-based methodologies. Here we describe a rapid and reproducible method for the electroporation of *Halomonas* sp. O-1 with plasmid DNA. Electrocompetent cells were generated by growing *Halomonas* sp. O-1 in a yeast extract-tryptone medium with a final salinity of 3.5%, pH of 7.5, followed by several washes using 300 mM sucrose. Results show that plasmids containing chloramphenicol (Cm^R) and gentamicin (Gm^R) resistance cassettes are suitable antibiotic selection markers for transformation and yields of 10⁴ transformants per µg of DNA were obtained. This method is simple to perform and the materials used are readily available in most research labs. Additionally, this plasmid-based transformation procedure has the potential to be adapted for a number of applications including the creation of recombinant stains and the generation of deletion mutants of *Halomonas* spp.

Keywords: *Halomonas*; Competent Cells; Transformation; Electroporation

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49 **1. Introduction**

50 Halophiles are a class of extremophiles characterized by their ability to
51 thrive in high salt environments that are typically toxic to most other forms of life
52 (Oren, 2008). There has been increasing interest in the use of these microorganisms
53 for various biotechnology and industrial applications due to the overall enzymatic
54 stability required for survival in such harsh environments and a decreased risk of
55 contamination during processing as a result of these conditions (Aguilar, 1996). The
56 genus of Gram-negative halophilic bacteria known as *Halomonas* was first
57 established in 1980 with the discovery and morphological characterization of
58 *Halomonas elongata* (Vreeland et al., 1980). Since then, isolates of this genus have
59 been identified in a myriad of habitats including coastal sea waters (Ilham et al.,
60 2014), salt-mines (Cardinali-Rezende et al., 2016), saline lakes (Zhang et al., 2016),
61 salt-secreting leaves (Finkel et al., 2011), industrial brine (Carlson et al., 2016),
62 heavy metal polluted shores (Jain et al., 2016), rust found on the sunken RMS *Titanic*
63 (Sánchez-Porro et al., 2010), and even as part of the human skin microbiome
64 (Cosseau et al., 2016). New members of the *Halomonas* genus are being identified at
65 an extraordinary rate and the habitats in which they thrive are equally astounding.

66 Polyhydroxyalkanoates (PHAs) are a class of diverse polyesters that are
67 naturally produced by a variety of microorganisms to serve as intracellular carbon
68 storage molecules (Anderson and Dawes, 1990). PHAs have attracted great interest
69 as biobased and biodegradable replacements for petroleum-based plastics (Lu, et al.
70 2009) and as biomaterials for biomedical applications (Levine, et al. 2015). In

71 addition to the inherent environmentally friendly properties of PHA-based
72 polymers, studies have also shown that bacteria can be engineered to make these
73 materials from a variety of renewable resources and waste products such as woody
74 biomass and biodiesel glycerol, thus enhancing overall sustainability (Pan et al.,
75 2012; Salamanca-Cardona et al., 2014; Zhu et al., 2010). However, major barriers
76 that exist for wide-scale industrial application and integration of these processes
77 include the large amount of freshwater needed to sustain PHA production and the
78 associated energy costs to sterilize medium for bacterial growth (Chen, 2012).

79 A number of *Halomonas* isolates and recombinant strains have been shown
80 to be capable of producing a variety of PHAs, some at levels that exceed 80% cell dry
81 weight (Ilham et al., 2014; Quillaguamán et al., 2005; Tan et al., 2011; Yue et al.,
82 2014). *Halomonas* spp. have been gaining heightened attention as attractive
83 production strains for these materials because of their capacity to produce PHAs in
84 a continuous and open process that does not require fresh water or sterilized media
85 (Tan et al., 2011; Yue et al., 2014). A major limitation for further implementation at
86 an industrial-scale is an overall lack of molecular methodologies to genetically
87 manipulate these microorganisms to produce PHA derivatives at levels that can
88 compete with synthetic processes (Chen, 2012; Fu et al., 2014).

89 Currently, the most common and primary method for genetically modifying
90 *Halomonas* spp. is via bacterial conjugation, a process first established in this genus
91 in 1995 (Vargas et. al., 1995; Li et al., 2016). The only other transformational
92 method that has been described in detail for *Halomonas* spp. is transduction
93 (Seaman and Day, 2007), but is very much still in its infancy due a lack of available

phages capable of transforming these organisms (Yin et al., 2015). Additionally, there are two instances in which a *Halomonas* spp. is said to have been electroporated, but in both cases there is little description of the methodology used or the efficacy of transformation (Azachi et al., 1996; Burch et al., 2013).

The goal of this study was to develop an electroporation method for transformation of *Halomonas* spp. To develop this method, we used *Halomonas* sp. O-1, an isolate from the coast of Japan that shares 98% homology with *Halomonas* sp. MAN K22 (Ilham et al., 2014). *Halomonas* is typically grown in high-ionic strength media (5% NaCl), which can complicate electroporation due to the non-ionic wash buffers commonly employed for encouraging electrocompetency (Prasanna and Panda, 1997). A recent review of the increasing prevalent role of *Halomonas* spp. in industrial biotechnology has cited the preparation of electrocompetent cells as a major barrier preventing transformation of these organisms (Yin et al., 2015). Here we describe a simple and rapid electroporation method that reliably and reproducibly allows for the creation of viable electrocompetent cells of *Halomonas* sp. O-1 capable of uptaking broad-host range plasmid DNA containing antibiotic selectable markers.

2. Materials and Methods

2.1 Bacterial strains, plasmids, and plasmid preparations

The bacterial strains and plasmids used in this study are listed in Table 1. For plasmid maintenance, *E. coli* Top 10 was grown in Lennox broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) supplemented with a final concentration of 25 µg/mL

chloramphenicol, 20 µg/mL gentamicin, or 10 µg/mL tetracycline. Plasmids were purified using the Wizard Plus SV Miniprep DNA Purification System (Promega) with the manufacturer's protocol.

Table 1. Bacterial Strains and Plasmids

Bacterial strains and plasmids		
	Descriptions	Sources
Strains		
<i>Halomonas</i> sp. O-1	Wild-type <i>Halomonas</i> strain isolated from seawater collected from the Fukuoka Prefecture of Japan	Ilham et al., 2014
<i>E. coli</i> Top 10	<i>E. coli</i> strain used for cloning and plasmid maintenance purposes	Invitrogen
Plasmids		
pBBR1MCS	Broad-host range plasmid, chloramphenicol resistance (Cm ^R) marker	Kovach et al., 1994
pBBR1MCS-3	Broad-host range plasmid, tetracycline resistance (Tet ^R) marker	Kovach et al., 1995
pBBR1MCS-5	Broad-host range plasmid, gentamicin resistance (Gm ^R) marker	Kovach et al., 1995

2.2 Optimization of *Halomonas* Growth Conditions for Electroporation

Halomonas sp. O-1 was grown in 16 x 100 mm culture tubes containing 2 mL of yeast extract-tryptone (YT) medium (10 g/L tryptone, 5 g/L yeast extract) supplemented with variable concentrations of NaCl (1%, 3.5%, 5%, 10%, 15%, or 20% [w/v]). Cultures were grown at 30°C on a rotary shaker set to 250 RPM. The

cell densities (growth) of the cultures were determined by measuring their absorbances at 600 nm (OD₆₀₀) at 5 h post inoculation. All subsequent culturing of *Halomonas* sp. O-1 was performed using YT medium supplemented to a final concentration of 3.5% NaCl, pH of 7.5, and will be referred to as *Halomonas* Growth Medium [HGM].

Growth analysis of *Halomonas* sp. O-1 in HGM was performed by first creating seed cultures, which consisted of 16 x 100 mm culture tubes containing 2 mL of HGM inoculated with single colonies of *Halomonas* sp. O-1 plated on HGM. Seed cultures were incubated at 30°C on a rotary shaker set to 250 RPM for 16 h and used to inoculate 50 mL of media (in a 500 mL baffled shake flask) with 0.5% v/v (0.25 mL) cell culture followed by incubation at 30°C, 250 RPM for a total of 12 h. Growth was assayed by measuring cell densities at 600 nm (OD₆₀₀) every 1.5 h for the duration of the growth period.

2.3 Antibiotic Screening

Antibiotic screening was accomplished by seeding untransformed colonies of *Halomonas* sp. O-1 into 16 x 100 mm culture tubes containing 2 mL of HGM supplemented with the following antibiotics at variable concentrations: ampicillin (50–200 µg mL⁻¹), carbenicillin (50–200 µg mL⁻¹), chloramphenicol (2–10 µg mL⁻¹), gentamicin (5–20 µg mL⁻¹), kanamycin (25–100 µg mL⁻¹), and tetracycline (5–15 µg mL⁻¹). Inoculated cultures were grown at 30°C, 250 RPM and assayed for OD₆₀₀ at 24 and 48 h post inoculation.

2.4 Preparation of electrocompetent cells

Halomonas sp. O-1 cells were made electrocompetent by adapting a method originally devised for *Pseudomonas aeruginosa* (Smith and Iglewski, 1989). Cell cultures (50 mL) were grown in HGM at 30°C, 250 RPM to mid log phase ($OD_{600} = 0.500$), then harvested in 25 mL aliquots by centrifugation at 5,000 $\times g$ for 10 minutes at 23°C. Supernatant was decanted and the cells were gently washed using an equal volume (25 mL) of room temperature 300 mM sucrose. Note that all wash steps were performed at room temperature and resuspension of cell pellets was performed very delicately using wide-bore pipette tips to prevent cell shearing. Resuspended cells were collected by centrifugation at 5,000 $\times g$ for 10 minutes at 23°C and then washed using 0.5 culture volumes (12.5 mL) of 300 mM sucrose followed by a final centrifugation and resuspension using 0.01% (0.25 mL) culture volumes of 300 mM sucrose. Electrocompetent cells were then pooled and 100 μ L aliquots were immediately combined with vector DNA and applied to cuvettes for electroporation (see below).

2.5 Electroporation of *Halomonas* sp. O-1

Electroporation was carried out using an ECM 399 Electroporation System (BTX Harvard Apparatus, voltage range of 60–2500V, fixed capacitance of 36 μ F, fixed resistance of 150 Ω). Aliquots (100 μ L) of electrocompetent cells were transformed with a variable amount of plasmid DNA in 2 mm gap parallel electrode cuvettes (Fisherbrand). Controls for all electroporation experiments consisted of cell aliquots without added DNA. Initial experiments were performed using a

variable voltage ranging from 1.5–2.3 kV and a constant concentration (100 ng) of plasmid DNA. Subsequent experiments were performed at a constant 2.1 kV with plasmid DNA ranging from 10–500 ng. Directly following electroporation, cells were quantitatively transferred from the electroporation cuvette to a sterile 16 x 100 mm culture tube containing 1 mL of HGM, which was then incubated for 1 h at 30°C, 250 RPM. Positive transformants were selected for by plating recovered cells (100–200 µL) directly onto HGM plates containing the appropriate antibiotic. Plated cells were incubated at 30°C for 24 h followed by colony formation unit (CFU) counts to quantify transformation efficiencies.

2.6 Cell Viability Assays

Cells were measured for viability during each wash step of the electroporation procedure. Cell suspensions were diluted (10⁶-fold) in sterile 5% saline solution to a level that would yield single colonies. Diluted cells were plated onto HGM plates and incubated at 30°C for 24 h followed by CFU counts. Aliquots of washed cells were taken throughout each step of the wash procedure and were diluted, plated, and incubated following the same methods described for cell culture. To ensure consistency, the reduced buffer volumes of subsequent wash steps were taken into consideration to maintain constant rates of dilution for all samples.

3. Results

3.1 Development of a low ionic strength growth medium

In order to make *Halomonas* sp. O-1 electrocompetent, we needed to develop a growth medium with a relatively low ionic strength that could still achieve adequate cell densities. Therefore, *Halomonas* sp. O-1 was grown in yeast extract-tryptone (YT) medium supplemented with NaCl to various concentrations (1, 3.5, 5, 10, 15 and 20% [w/v]). As shown in Figure 1, final NaCl concentrations of 3.5 and 5% yielded similar cell densities (OD_{600} of $\sim 1.2\text{--}1.4$) after 5 h of growth. These cell densities were significantly greater than those observed for cultures supplemented to 1, 10, 15 and 20% NaCl. Ultimately, the developed medium, termed *Halomonas* Growth Medium [HGM], consisted of the typical components found in YT medium supplemented to a final NaCl concentration of 3.5%, pH 7.5, and was capable of satisfying the need for a relatively low ionic strength media while maintaining adequate cell densities. A growth assay of *Halomonas* sp. O-1 in baffled shake flasks containing HGM was also performed to determine when early, mid, late, and stationary phase growth occurs (Fig. 2). Results of this experiment indicate that mid-phase growth occurs between 3 and 6 h post-inoculation with corresponding OD_{600} measurements of $\sim 0.1\text{--}0.5$, respectively.

Fig. 1. Growth of *Halomonas* sp. O-1 in YT medium supplemented to various concentrations of NaCl. *Halomonas* sp O-1 was grown in YT medium supplemented to 1, 3.5, 5, 10, 15 and 20% NaCl at 30°C in a rotary shaker at 250 RPM. At 5 h post inoculation, OD₆₀₀ was measured for each culture.. Experiments were performed in triplicate, and data points represent mean values with error bars representing standard deviation.

Fig. 2. Growth profile of *Halomonas* sp. O-1 in HGM. Baffled shake flasks containing 50 mL HGM were inoculated with 0.5% (v/v) seed culture and grown at 30°C in a rotary shaker at 250 RPM for a total of 12 h. Measurements of cell density at 600 nm were taken initially and every 1.5 h for entirety of growth period. Experiments were performed in triplicate, and data points represent mean values with error bars representing standard deviation.

3.2 Antibiotic screening

Untransformed *Halomonas* sp. O-1 was screened for antibiotic sensitivity to determine which resistance markers could be used for plasmid selection (Table 2). Growth was not observed for *Halomonas* sp. O-1 in HGM supplemented with

chloramphenicol (10 $\mu\text{g mL}^{-1}$), gentamicin (30 $\mu\text{g mL}^{-1}$), and tetracycline (15 $\mu\text{g mL}^{-1}$). However, cloudy or turbid growth was found for cultures grown in media supplemented with kanamycin (100 $\mu\text{g mL}^{-1}$), ampicillin (200 $\mu\text{g mL}^{-1}$), and carbenicillin (200 $\mu\text{g mL}^{-1}$), suggesting that *Halomonas* sp. O-1 is resistant to these antibiotics.

Table 2. Antibiotic sensitivity of *Halomonas* sp. O-1.

Antibiotic	Growth Phenotype		
	Concentration ($\mu\text{g mL}^{-1}$)	24 h	48 h
Chloramphenicol	2	+	+
	5	-	+
	10	-	-
Kanamycin	25	+	+
	50	-	+
	100	-	+
Tetracycline	5	-	+
	10	-	+
	15	-	-
Ampicillin	50	+	+
	100	+	+
	200	+	+
Carbenicillin	50	+	+

	100	+	+
	200	+	+
Gentamicin	5	+	+
	15	-	+
	30	-	-

All experiments were performed in duplicate in liquid HGM containing antibiotic at the indicated concentrations. Cultures were grown at 30°C, 250 RPM shaking for the duration of the assay. Cultures were recorded as having either turbid growth (+) or zero growth (-).

3.3 Preparation and viability of electrocompetent cells

Based on a procedure devised for *Pseudomonas aeruginosa*, we prepared electrocompetent cells of *Halomonas* sp. O-1 using a 300 mM sucrose solution as the sole wash buffer (Smith and Iglewski, 1989). To determine the effect of the wash procedure on cell viability, colony formation unit (CFU) plate assays were performed on initial cell cultures and throughout the wash procedure (Fig. 3). Results revealed only a 20% decrease in overall cell viability when the non-ionic 300 mM sucrose buffer was used as a wash solution, therefore, it was concluded that the relatively low loss of cell viability was a worthwhile sacrifice for creating a concentrated cell solution with high resistivity capable of being electroporated without a high potential for arcing. Additionally, centrifugation and wash steps performed at room temperature (23°C) were found to enhance the homogeneity of cell resuspensions compared to procedures performed between 4°C and 10°C.

Fig. 3. Effect of wash procedure on viability of *Halomonas* sp. O-1. Viability was calculated as percentages of surviving cells (CFUs) relative to the initial CFUs (set at 100%). Experiments were performed in triplicate, and data points represent mean values with error bars representing standard deviation.

3.4 Electroporation of *Halomonas* sp. O-1

Table 1 lists the vectors that were used for electroporation of *Halomonas* sp. O-1, all of which are pBBR1MCS derivatives; a relatively small (<5.3 kb), broad-host, and mobilizable suite of plasmids containing multiple cloning sites within the *lacZα* gene and several antibiotic resistant variants (Kovach et al., 1994; Kovach et al.,

1995). The antibiotics chosen for selection were chloramphenicol, tetracycline, and gentamicin (Table 2) and the corresponding vector notations are pBBR1MCS, pBBR1MCS-3, and pBBR1MCS-5, respectively (Table 1).

Optimal settings for electroporation were determined through a series of preliminary experiments that tested key parameters such as parallel electrode cuvettes with 1 and 2 mm gaps, variable volumes of electrocompetent cells, and applied voltage. Electroporations of *Halomonas* sp. O-1 with a 1 mm gap cuvette (40 μ L cell volume) or a 2 mm gap cuvette (100 μ L of cell volume) with a constant plasmid amount (100 ng) successfully resulted in positive transformants, although the latter resulted in enhanced transformation efficiencies. Experiments using 2 mm gap cuvettes and a range of cell volumes (40–200 μ L) resulted in arcing for volumes <100 μ L whereas for volumes >100 μ L there was a reduction in the time constant in which charge was delivered and led to lower overall transformation efficiencies. Lastly, it was found that when using a 2 mm gap cuvette and 100 μ L cell aliquots, voltages below 1.5 kV failed to deliver a charge and voltages above 2.3 kV almost invariably resulted in arcing. It should also be noted that all electroporations were optimized for and performed using a ECM 399 Electroporation System (BTX Harvard Apparatus, voltage range of 60– 500V, fixed capacitance of 36 μ F, fixed resistance of 150 Ω) and use of this methodology with a different device should take into consideration the capacitance and resistance of that device in addition to the voltage settings and cuvette size. Based on results from these trial and error experiments, cuvette gap size and washed cell volume were kept constant at 2 mm and 100 μ L, respectively, for all subsequent experiments. Although variations in

wash buffer, cuvette size, and cell volume may still produce viable transformants, the chosen parameters were found to be optimal for the device and *Halomonas* strain being tested.

To determine the efficacy of electroporation, experiments were performed using a range of voltages (1.5–2.3 kV) and a range of plasmid concentrations (10–500 ng). It was found that the pBBR1MCS-5 (Gen^R) plasmid yielded the most consistent and highest numbers of transformants (10⁴ CFUs ug⁻¹ DNA). The pBBR1MCS (Cm^R) plasmid was also suitable for electroporation in *Halomonas* sp. O-1, but with lower yields (~10³ CFUs ug⁻¹ DNA). Non-DNA electroporated controls were also plated onto media containing these antibiotics and consistently yielded no transformants. Despite the high sensitivity of *Halomonas* sp. O-1 to chloramphenicol (Table 2), transformants harboring pBBR1MCS (Cm^R) were capable of persisting on selection plates containing Cm at concentrations of 25 µg mL⁻¹, albeit at greatly reduced transformation efficiencies compared to 10 µg mL⁻¹. Lastly, we were unable to successfully transform the pBBR1MCS-3 (Tet^R) [5228 bp] vector in *Halomonas* sp. O-1 and all electroporations using this vector yielded no transformants on *HGM* selection plates containing Tet at 15 µg mL⁻¹. Because the pBBR1MCS-5 (Gen^R) showed the most promising results, all additional assays for voltage optimization and the effect of plasmid concentration on electroporation efficiency were performed with this plasmid.

Using the previously defined parameters, electroporation assays were first performed over a range of voltages at a constant vector DNA amount of 100 ng (Fig. 4). When plotted logarithmically, there is a relatively linear increase in

transformation efficiency from 10^3 CFUs μg^{-1} DNA at 1.5 kV to 10^4 CFUs μg^{-1} DNA at 2.1 kV followed by a slight decrease in efficiency at voltages above 2.1 kV. These results indicate the optimal voltage for electroporation of *Halomonas* sp. O-1 using this device was to be 2.1 kV. Transformations at voltages of 2.3 kV produced similar results to 2.1 kV, but arcing became much more common at this high potential and required electroporations to be repeated on multiple occasions. Controls containing electroporated cells without added DNA were also performed at each voltage and yielded no transformants.

Fig. 4. Optimization of electroporation conditions for vector DNA uptake by *Halomonas* sp. O-1. Electrocompetent cells prepared using the described wash procedure (See Materials and Methods) were transformed with constant 100 ng of pBBR1MCS-5 (4768 bp) [Gen^R] empty vector at a range of

voltages (1.5–2.3 kV). Experiments were performed in triplicate, with data points representing mean values and error bars representing standard deviations.

Following the determination of optimal voltage, the final set of assays performed in this study sought to establish a relationship between DNA amount and the resulting number of transformants. Using a variable amount of vector DNA (pBBR1MCS-5) ranging from 10–500 ng per electroporation, all electroporations for this series were performed at the previously optimized voltage of 2.1 kV. Results show a positive, linear relationship between DNA amount and transformation efficiency with a resulting slope of ~ 10 transformants ng^{-1} DNA (Fig. 5). Furthermore, non-DNA electroporated controls yielded no transformants when screened on *HGM* supplemented with gentamicin at $30 \mu\text{g mL}^{-1}$.

Fig. 5. Efficiency of *Halomonas* sp. O-1 electroporation based on plasmid amount. Cells were washed using the previously described methods, then transformed with a variable amount of DNA (10–500 ng) at a constant 2.1 kV. Experiments were performed in triplicate, with data points representing mean values and error bars representing standard deviations.

4. Discussion

Halomonas sp. O-1 was a recently discovered seawater isolate shown to naturally produce PHAs (Ilham et al., 2014) and, similar to other members of this genus, has enormous potential for industrial scale applications (Quillaguamán et al., 2005; Tan et al., 2014; Yue et al., 2014). However, this potential for exploiting *Halomonas* spp. for industrial use has been hindered by the lack of genetic tools and

techniques for manipulating these organisms (Chen, 2012). The most common method for transforming this genus has been bacterial conjugation, a somewhat tedious and time-consuming method compared to electroporation-based methods. The study presented here has provided the first detailed evidence that a *Halomonas* spp. can be made electrocompetent and is capable of being modified using plasmid DNA via electroporation.

Bacteria-based electroporation procedures typically require a non-ionic osmotic stabilizer with high resistivity as the wash buffer in order to sustain an adequate pulse duration (~5 ms) at a high enough voltage (>1.5 kV) to permeate the cell membrane while maintaining a relatively low current to avoid excessive cell mortality (Shigekawa and Dower, 1988). A recent review has stated that the creation of viable, electrocompetent cells is the major hurdle preventing electroporation-based transformations of halophilic bacteria (Yin et al. 2015). Due to the halophilic nature of *Halomonas* sp. O-1, in which a generally high ionic strength media is preferred for optimal growth, several obstacles had to be overcome to generate electrocompetent cells without greatly diminishing cell viability.

The media developed in this study, termed *Halomonas* Growth Media or HGM, is made from the core components tryptone, yeast extract, and NaCl with a final salt concentration of 3.5%, and was brought to a pH of 7.5. The salinity and pH of this media is based upon empirical evidence presented in this study (Fig. 1) and also took into consideration the natural conditions of this organism and mimicked the salinity (~3.5%) and pH (7.5–8) of ocean water (Fofonoff, 1985). For the

electrocompetency procedure that was devised, cells were harvested at mid-log phase growth (Fig. 2) and used 300 mM sucrose as a non-ionic wash buffer. Despite the lack of ionic strength in the wash buffer, results show that 80% cell viability was maintained throughout the wash procedure (Fig. 3). Taken together, it is believed that the relatively low-ionic strength of HGM compared to media containing the preferred salt concentration for this microorganism (5–15%) (Ilham et al., 2014) encouraged a phenotype that was capable of enduring the non-ionic conditions of the sucrose wash and ultimately allowed for the creation of viable, electrocompetent cells.

Other electroporation procedures concerning bacteria are often performed at low temperatures (0–4°C) and may involve a low temperature incubation of washed cells prior to electroporation to enhance plasmid uptake efficiency (Prasanna and Panda, 1997; Shigekawa and Dower, 1988). However, transformational efficiency was found to be best when electrocompetent cells were created at room temperature (23°C) and electroporations were performed immediately after the final wash step, which is similar to contemporary and rapid methods for creating electrocompetent cells of *Pseudomonas aeruginosa* (Choi et al., 2006).

A vital component for successfully screening positive transformants for the uptake of plasmid DNA is the use of antibiotic resistance markers (Schweizer, 2008). This requires the organism of interest to have susceptibility to the chosen antibiotic in order to ensure the efficacy of selection and subsequent plasmid stability in the host. Based on the results of antibiotic screening assays (Table 2), *Halomonas* sp. O-1 was found to be sensitive to the antibiotics chloramphenicol, tetracycline, and

gentamicin; therefore, vectors containing resistance cassettes for the listed antibiotics were chosen for electroporation trials (Table 1). Of these vectors, pBBR1MCS (Cm^R) and pBBR1MCS-5 (Gen^R) were found to be compatible in *Halomonas* sp. O-1 and represent viable resistant cassettes for future applications in this organism and possibly other members of the genus.

Based on the success of preliminary electroporation assays using the pBBR1MCS-5 vector, experiments to optimize voltage and plasmid concentration were performed and resulted in consistent and reproducible results (Fig. 4, 5) in which transformants on the scale of 10⁴ µg⁻¹ of plasmid DNA were obtained. Efficiency on this scale is relatively low compared to methodologies utilized in bacteria such as *E. coli* and *P. aeruginosa* where transformants are often obtained at levels higher than 10⁹ µg⁻¹ DNA (Calvin and Hanawalt 1988; Choi et al., 2006), however, in one of the first successful applications of electroporating plasmid DNA into intact bacterial cells performed by Powell et. al. (1988), obtaining transformants of *Streptococci* spp. on the scale of 10⁴ µg⁻¹ DNA was considered a success and set the stage for rapid improvement of this methodology. Additionally, based on the linear trend seen in Fig. 5, a further increase in DNA amount to a level of 1 µg or higher may result in an increased number of transformants, but may not increase electroporation efficiency as expressed as transformants per µg of DNA.

In conclusion, this study represents another step in expanding the repertoire of molecular methods available to genetically engineer *Halomonas* spp. The pBBR1MCS broad host vectors were shown to be capable of uptake and replication in *Halomonas* sp. O-1 and produced transformants on the magnitude of 10⁴ µg⁻¹

DNA. Additionally, optimized parameters for growth, electrocompetency, and electroporation conditions were established. Recently, a constitutive promoter library with an inducible promoter system has been developed in *Halomonas* TD01 (Li et al., 2016). Using this study as a benchmark, it may be possible to engineer novel vectors for use in *Halomonas* sp. O-1 containing inducible promoter systems to allow for precise control of recombinant gene expression. Additionally, rapid, plasmid-based gene knockout methods originally developed for use in *Pseudomonas aeruginosa* may now be applied to *Halomonas* sp. O-1 (Choi et al., 2006). Lastly, this method can be tested in other members of the genus, particularly those already being engineered for industrial use such as *Halomonas* TD01 and *H. campaniensis* (Tan et al., 2014; Yue et al., 2014).

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