Advanced Research in Microbial Metabolites and Technology
ARMMT

Controlled Turgidity; a simple and efficient method for transformation of DNA plasmids into *Halobacterium salinarum* R1.

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Article Info

Document Type:
Short Communication

Received 09/04/2022
Received in revised form 25/04/2022
Accepted 08/05/2022

Keywords:
Halobacterium salinarum,
Transformation,
DNA,
Hypo-osmotic shock

Abstract

Halobacterium salinarum is a model organism used for archaeal genomics studies in many investigations. This well-known microorganism loves hypersaline habitats. However, conventional methods of transformation of DNA for genetic manipulation of the halophile, like electroporation, are impossible, and the current procedure is time-consuming, sensitive, and difficult. Therefore, the development of easy and efficient methods for genetic manipulation, especially the transformation of this species, are important. In this study, we introduced a highly efficient, simple method for *H. salinarum* transformation. This method is based on the controlled short-time swelling-deswelling of this halobacterium. The halophile was exposed to hypo-osmotic shock by adding deionized water containing the desired plasmid to the cell suspension, which decreased the NaCl concentration to around 2.5M. After 60 seconds, the shock was removed by re-increasing the salt concentration of the suspension to 3.3M. During the osmotic shock, plasmids from the low salt concentrated fluid are sucked through the cell membrane. The transformation was confirmed by PCR and colony count. While the conventional PEG-mediated method is time-consuming and laborious due to the probability of DNA precipitation and cell aggregation by PEG, the present method eliminated PEG and its possible unwanted consequences. With the same amount of initial plasmid, the transformation frequency of the osmotic shock was more abundant in the proposed method compared with the PEG-mediated method. A growth of about 10^5 transformants/μg of DNA revealed osmotic shock is an efficient transformation method in *H. salinarum*. This fast and easy transformation method can be used to transform this microorganism and probably other halophiles instead of other current laborious methods.

1. Introduction

H. Salinarum is a slow-growing well-known species of Haloarchaea that lives in environments with high levels of salinity with a doubling time in the range of 1.2 ± 0.3 days. The most abundant protein of the microorganism, Bacteriorhodopsin, is a purple pigment that is important in dye-sensitized solar cells, optical switching, electronic sensors, etc. (Ashwini et al., 2017; Maslov et al.,

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DOI: 10.22104/ARMMT.2022.5591.1066

2018). *H. Salinarum* is rod-shaped and surrounded by S-layer. This S-layer is the result of the accumulation of cell surface glycoproteins with abundant sulfide residue that causes a negatively charged surface and, to some extent, maintains cell integrity in environments with a high concentration of salt (Eichler, 2003). Furthermore, to deal with high salinity osmotic stress, archaebacterium utilizes compatible solutes, such as potassium chloride, that are transferred into the cell in the opposite direction of the concentration gradient through specific transporters (Pérez-Fillol & Rodríguez-Valera, 1986). On the other hand, to prevent the precipitation of proteins in the high potassium concentration, they express acidic proteins with a negative charge that can tolerate salty solutions (Rajendrakumar Singh & Dar, 2017). These traits appear to cause difficult conditions for the transfer of DNA into halophilic bacteria.

Due to its easy-to-cultivate ability as well as the development of halobacterial genetic manipulation methods, this bacterium is considered a model for genomic and post-genomic research of archaea bacteria (Peck et al., 2000). Given that a convenient and efficient technique for transferring DNA into the cell is an important prerequisite for genetic manipulation, the availability of such a method could pave the way for research on this group of living organisms. The first multistep transformation method for *H. salinarum* was introduced in 1989 (Cline et al., 1989). However, other investigators have tried to improve this method because this archaebacterium grows slowly and is easily disrupted during handling, which influences transformation efficiency (Dyall-Smith, 2015). With regard to the recovery conditions of *H. salinarum,* we introduce an extremely simple and efficient method for transforming *H. salinarum* in this study.

Turgidity is the natural consequence of hypo-osmotic shock in some living cells' eukaryotes and prokaryotes that leads them to balance inside and outside osmotic pressure through the control of their membrane permeability (Márián et al., 1993). Turgidity may occur in microorganisms in some controlled laboratory conditions. During the production of compatible solutes via alternating osmotic shocks or *bacterial milking*, gram-negative bacterium Halomonas elongates, swells, and deswells repeatedly (Sauer & Galinski, 1998). Despite this, in some halophilic microbes, like *Halobacterium Salinarum*, uncontrolled movement of water into the cytoplasm terminates in cell bursting, probably due to denaturing of Halophilic proteins. Vauclare et al.'s study showed the recovery of *H. salinarum* sub-populations exposed to severe low salt conditions (Vauclare et al., 2020). In this study, accounting for the recovery conditions of *H. salinarum*, we introduce an extremely simple and efficient method for the transformation of *H. salinarum*.

2. Materials and methods

2.1. Materials

The chemicals used in this research, including NaCl, KCl, Tris, Sodium citrate, and MgSO4, were purchased from Merck, Germany. The Novobiocin was provided by Kayrosafe, Italy. Primers were synthesized by Sinaclon, Iran, and Agarose and Taq polymerase were purchased from KBC, Iran and Topazgen, Iran, respectively. The DNA size marker (100bp) was purchased from Fermentas.

2.2. Strains and growth conditions

The *Halobacterium salinarum R1* (DSMZ; DSM671) strain was cultured at 38ºC and 250rpm in a complex medium (CM) with 3.3M salt (192 g NaCl, 2g KCl, 3.418 g Tris Sodium citrate, 3g peptone, 5g yeast extract, and 20 g MgSO4 in 1 lit deionized water) (Cline & Doolittle, 1987). Novobiocin was added to the medium at a final concentration of 5 μg/ml as a selection component

to prevent the growth of untransformed *H. salinarum*. Simvastatin, mevinolin, and Novobiocine are GyrB inhibitors, and extra copies of the gyrB gene in the plasmid compensate inhibited enzymes from the genomic copy so the microorganism could grow.

The TG1 strain of E. coli, which was used to propagate the pAMEX::cdc48a shuttle vector, was grown in the LB medium.

2.3. Transformation of pAMEX::cdc48a into *H. salinarum*

The recombinant pAMEX::cdc48a plasmid (Fig.1), constructed by cloning of cdc48a fragment in the pAMEX shuttle vector to study the role of the cdc48a gene in the *H. salinarum R1* cell cycle (Mokari Bonabi Neda, 25-27 August 2015), was transformed into the archaea bacterium by two methods.

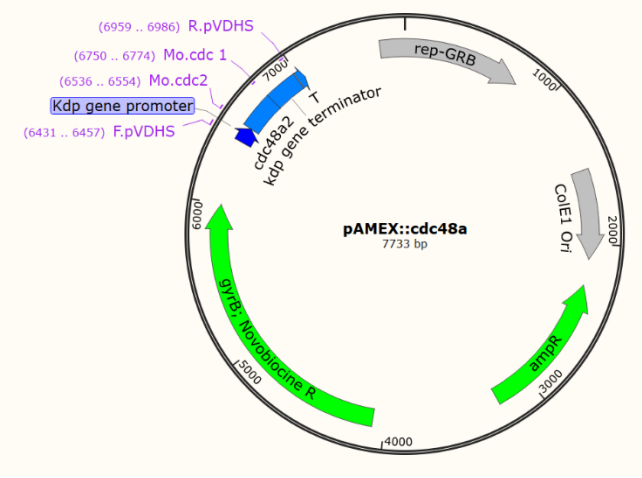

Figure 1. The pAMEX::cdc48a map and related primers positions.

This transformation was first done using the conventional method previously reported by Cline et al. (Cline et al., 1989), and the second method of transformation was designed and implemented based on the osmotic shock. Briefly, in the first step, the *H. Salinarum R1* strain was grown in complex medium to reach OD=0.8. Then, 5ml of this culture was centrifuged at 3000 rpm for 3 mins. Next,3ml of supernatant was discarded, and the cell pellet was resuspended in the remaining medium. In the next step, about 200 ng (Dyall-Smith, 2015) of pAMEX001::cdc48 recombinant plasmid was added to the suspension and incubated for 5 min at room temperature. Afterward, the cells were subjected to osmotic shock by adding 700 µl, 600 µl, 500 µl, and 400 µl of deionized water, which reduced the 3.3M initial molarity to 2.4M, 2.5M, 2.6M, and 2.7M, respectively. The cells were then left on the benchtop for 60 sec. To stop osmotic shock, 2ml of concentrated CM medium (1.2X with 4M salt) was added to the suspensions, gently mixed, and left for 20 min at room temperature. Then, the cell suspension was incubated at 38º C and shaken at 250 rpm for 20 hours. Next, Pellets from centrifugation of the bacteria at 3000 rpm for 5min were suspended in 1ml of CM. This centrifugation and resuspending may be ignored. Finally, 100-150µl of the cell suspension was spread on solid CM medium containing novobiocin (5µg/ml) three times and incubated at 38º C for 15 days.

H. salinarum transformed by the conventional PEG mediated method was considered as the control (Mirfeizollahi et al., 2019)

2.4. Colony count and PCR

Fifteen days after culturing, red colonies of *Halobacterium salinarum* appeared on a CM culture medium containing 5 micrograms per milliliter of novobiosin. The colonies grown on the plates were counted and compared with each other. In addition, some colonies were randomly selected and studied by PCR using specific primers, as shown in Table 1. Topaz Taq DNA Polymerase

(Topazgene, IR. IRAN) was used to confirm the transformations.

Table 1. Primer sequences and related PCR product length used to confirm transformed colonies.

Primers name	**Sequence (5′-3′)**	**PCR Product Length bp**
Mo.cdc1	GCGGTTTCTAGACAGTACTTCTGTTGGGCCG	239
Mo.cdc2	GTCCGGGATCCAGTCTTGGCGGTC	
$F._{pVDHS}$	TTGGCGTAGAATTCAGGGATGTACACCACCGTGGCT	558
$R._{pVDHS}$	ACAGTTGAGCTCAGATCCGCGATTACGAACCGACC	

For this purpose, the colonies were removed from the plate surface, suspended in 15 microliters of sterile distilled water, and then incubated at 65°C for 15 min. After centrifugation at 10,000 rpm for 5 minutes, the supernatant was used to perform PCR.

3. Results and discussion

3.1. Results

After transforming *Halobacterium salinarum* with both the previously reported method and osmotic shock, the grown colonies were counted on the selective CM culture medium. The results are summarized in Table 2.

Table 2. Comparison of transformation by osmotic shock in different concentrations of salt and the conventional PEG media ted method as a control.

Colony count	**NaCl percent for osmotic shock**	**Salt con. for osmotic shock**	**Added H_2O**	**Initial con. of Salt**
6	15.77%	2.7mol/lit	400µl	3.3mol/lit
400	15.1%	2.6mol/lit	500 µl	3.3mol/lit
700	14.6%	2.5mol/lit	600 µl	3.3mol/lit
100	14%	2.4mol/lit	700 µl	3.3mol/lit
2	Control: Conventional PEG mediated method			

Amplification by three different pairs of the four primers presented in Table 1 were used to confirm the transformants (Figure 2). PCR reactions were prepared in three different combinations of primers as follows to ensure that the cells received the plasmid containing the cloned fragment in the correct direction. The pairs of primers used together were MO-cdc1+F.pVDHS, MO-cdc2+R.pVDHS, and F.pVDHS+R.pVDHS.

It was assumed that the colonies obtained by the conventional transformation method received the vector, so PCR was not performed for them.

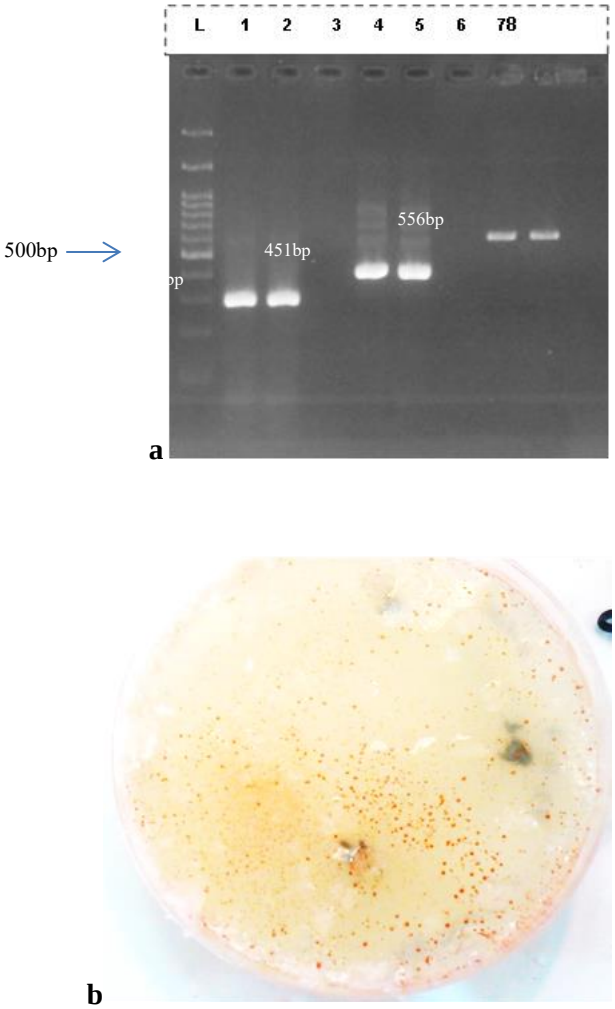

Figure 2. **a**: Polymerase Chain Reaction to confirm transformed H. salinarum by osmotic shock. Lane L: Thermo Scientific™ GeneRuler 100 bp DNA Ladder, Lanes 1 &2: PCR product of two colonies by primersMO-cdc1, F.pVDHS, Lanes 4 & 5: PCR product of primers MO-cdc2, R.pVDHS, and Lanes 7 & 8: PCR product of primers F.pVDHS, R.pVDHS. **b**: H. salinarum transformed colonies on the solid CM medium containing novobiocin (5µg/ml) at 38º C after 15 days.

3.2. Discussion

Industrial and biotechnological applications of *H. Salinarum* and other halophilic Archaea and their products, like bacteriorhodopsin and ectoine, have been previously proposed and discussed (Ashwini et al., 2017; Buenger & Driller, 2004). The potential importance of lipase, protease, amylase, liposome, and other useful products of these halophiles that were explored in 2011 (Litchfield, 2011) suggests that genetic manipulation in this field can provide future approaches to their applied studies. Additionally, the study of the microorganisms' environmental interactions may be used as a base to introduce new products and applications in this field.

Environmental factors like salt concentration play a very important role in halophiles' life, e.g., at a salt concentration as low as 15% NaCl, H. salinarum cells become fragile (Mei et al., 2017); however, the organism cannot tolerate concentrations lower than 15% without disruption (Oren, 2008). Reducing the salt concentration near this borderline does not appear to affect cell viability, but cells will become turgid or puffy (Sauer & Galinski, 1998). Consequently, the surrounding salt solution could be concentrated and deconcentrated several times in a controlled manner, a bioprocess called *bacterial milking*. During this process, water leaves the cytoplasm in cells exposed to the highly concentrated solution and carries molecules, such as ectoine, out of the cells (Sauer & Galinski, 1998). This makes us wonder, could macromolecules, like DNA, be taken up if the cells are suspended in a low concentration salt solution that pulls water in? The development of a simple, fast, and efficient method for transforming *H. salinarum* gave us the answer, DNA can be pulled into the cells under these conditions, a solution that may be important for genetic engineering and post-genomic research in archaea.

Other studies have been conducted on similar concepts. For example, van Wolferen (van Wolferen et al., 2016; Wagner et al., 2017) considered the negatively charged surface layer

(S-layer) in archaea, specially halo-archaea, as a foreign DNA absorption barrier. Suppose we expand the conclusions of the above paragraph. In that case, this idea of a bacteria barrier could be adapted by changing the conditions that govern their living environment, the salt-saturated environment. Moreover, Xie et al. found that, despite the negative charge, in conditions that are very similar to the above extraction of DNA but based on silica and saturated salt, the surfaces of silica adsorb DNA due to the formation of cationic bridges (Xie et al., 2019). Using this reasoning, a negatively charged s-layer in the presence of high concentrations of salt and the formation of the cationic bridge should adsorb DNA.

We ascertained that similar conditions could be provided in the laboratory and then used those conditions to explore the transfer of DNA plasmids into *Halobacterium salinarum* R1. Our results showed that DNA uptake by haloarchaea during osmotic shock may be a kind of nutritional behavior in estuaries where the freshwater of rivers meeting a salty lake provides vigorous turbulence, which mixes the salt and fresh water. This circulation provides nutrients and dissolved oxygen, and the osmotic shock is like a pumping system that feeds the halophiles.

As mentioned above, during a PEG-mediated transformation, plasmids may precipitate, and cell aggregation could become a major problem in high concentrations of PEG, making this method both time-consuming and laborious. However, PEG and its potentially unwanted interferences have been eliminated in our osmotic shock method. The transformation frequencies obtained using our simple method are approximately 10^5 transformants/µg of DNA when salinity is reduced to 2.5M for the hypo-osmotic shock. This is comparable with the *Escherichia coli* transformation results using the heat shock method with approximately 10^5-10^7 transformants/µg of DNA and the conventional PEG-mediated *Haloferax volcanii* transformation (Dyall-Smith, 2015; Froger & Hall, 2007; Rahimzadeh et al., 2016). Compared with the PEG-mediated method, the transformation frequency of osmotic shock was abundant, and the resulting approximately 10^5 transformants/µg of DNA revealed osmotic shock is an efficient approach for transforming *H. salinarum*. As a result, this easy and fast transformation method could be used instead of other laborious methods to transform this microorganism and possibly other halophiles.

4. Conclusion

Compared with the previously reported time-consuming PEG-mediated method for transforming *H. salinarum* with 120 transformants/µg of plasmid DNA, our hypo-osmotic shock method is a highly efficient and very simple method for transforming this halophile (Cline et al., 1989; Zibat, 2001) and is most likely extensible to other halophilic microorganisms.

Conflict of Interest

The authors declare that there is no conflict of interests.

Acknowledgements

The authors would like to thank the Malek Ashtar University of Technology for the financial supports of this

research.

Ethical approval

This article does not contain any studies with human participants or animals performed by any

of the authors.

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