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Mechanosensitive channel functions to alleviate the cell lysis of marine bacterium, *Vibrio alginolyticus*, by osmotic downshock

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Abstract The mechanosensitive channel with large conductance of *Escherichia coli* is the first to be cloned among stretch-activated channels. Although its activity was characterized by a patch clamp method, a physiological role of the channel has not been proved. The marine bacterium, *Vibrio alginolyticus*, is sensitive to osmotic stress and cell lysis occurs under osmotic downshock. We introduced an *mscL* gene into *Vibrio alginolyticus*, and the mechanosensitive channel with large conductance functions was found to alleviate cell lysis by osmotic downshock. This is the first report to show a physiological role of the mechanosensitive channel with large conductance.

Key words: Mechanosensitive channel with large conductance; Osmotic lysis; Marine bacterium; *Vibrio alginolyticus*

1. Introduction

The mechanosensitive channel with large conductance (MscL) of *Escherichia coli* is the first MS channel that has been cloned [1,2]. The activities of MscL were detected by a patch clamp method using giant spheroplasts [3] or giant liposomes fused with *E. coli* membranes [4]. MscL was purified from *E. coli* membranes [1] and reconstituted into proteoliposomes [1,5]. It is a membrane-bound protein with two membrane-spanning domains and with N- and C-termini located in the cytoplasmic side of the cell membrane [6]. It comprises 136 amino acid residues with a mass of 15 kDa [1,7]. In the native membrane, MscL was presumed to be present as homohexamers [8]. *E. coli* has other mechanosensitive channels of smaller conductance such as MscS and MscM [4]. Although the loss of MscL does not lead to a detectable effect on the growth of *E. coli* [9,10], the ubiquitous presence of MS channels in plasma membranes suggests their importance. One possible role of these MS channels was shown to be to act as emergency valves for the release of turgor upon sudden osmotic downshock [2].

It is well known that halophilic bacteria lyse when suspended in a hypotonic medium and that NaCl is much more effective than KCl in preventing cell lysis [11]. We previously reported that the minimum concentration of KCl for preventing cell lysis (lysis point) of the slightly halophilic marine bacterium, *Vibrio alginolyticus*, increases with the increase in the osmotic pressure of the cells [12]. Apparently, KCl prevented cell lysis by balancing the internal osmotic pressure of the cells. Therefore, we considered that the lytic response of *V. alginolyticus* to KCl medium must be an excellent system for testing the physiological response of MscL. The *mscL* gene from *E. coli* was introduced into *V. alginolyticus* and was expressed in the cell membranes.

This paper reports that MscL really functions to prevent the cell lysis of *V. alginolyticus* by osmotic downshock.

2. Materials and methods

2.1. Cell growth, lysis experiments and preparation of spheroplasts

V. alginolyticus was grown aerobically in a rich medium containing 0.5% yeast extract, 0.5% polypeptone, 0.2% glucose, 23 mM (0.4%) K_2HPO_4 and 0.5 M NaCl at 37°C. The cells were harvested in the late exponential phase of growth by centrifugation at 4°C, washed once with 1 M NaCl containing 50 mM Tris-HCl (pH 7.5) and finally suspended in the washing medium at a cell density corresponding to about 50 optical density units (ODU) at 550 nm.

To prepare spheroplasts, harvested and washed cells were suspended in 50 mM Tris-HCl (pH 8.5), 2 mM EDTA, 100 μg/ml of lysozyme and 0.95 M sucrose at a cell density of 70 ODU. To the mixture, 0.66 volume of 50 mM Tris-HCl (pH 8.5) containing 60 μg/ml of lysozyme was added, and incubated for 20 min at 30°C. We confirmed by phase differential microscopy that more than 99% of cells were converted to round shape.

To determine the effect of salts on the lysis of the cells, 0.1 ml aliquots of the final cell suspension were added to 4.9 ml of a solution containing various concentrations of the different salts and quickly mixed. The turbidity of the resulting suspension was measured at 550 nm after 15 min at room temperature. The results are expressed as a percentage of the OD of the suspension in 1 M NaCl.

2.2. Construction of mscL-containing plasmid

Plasmid pYT4 (2.89 kbp) was prepared by cutting pYMM2 [13] with *Pst*I, followed by ligation, where the kanamycin resistance gene was deleted. A 3.3 kbp DNA *Pst*I fragment containing the *mscL* gene was purified from pBS936 (obtained from Dr. J.-M. Guillon [14]), and it was ligated into the *Pst*I site of pYT4 to construct pYT5 (6.2 kbp). Plasmid pYT6 (5.7 kbp) was prepared by cutting pYT5 with *Eco*RV, followed by ligation, where the *mscL* gene was deleted. *V. alginolyticus* bacteria containing pYMM2 or pYT plasmids were grown in the presence of 2.0 μg/ml of chloramphenicol.

2.3. Expression of His-tagged MscL in V. alginolyticus

We prepared His-tagged MscL by PCR using two primers: *mscL*-*Pst*I primer is ATCTGCAGGATCCCTTTCCTGGCAGGAAAATGGC, which starts 67 bp upstream from *mscL* start codon and *mscL*-His primer is ATCTGCAGCTAGTGGTGGTGGTGGTGGTGAGAGCGGTTATTC, which adds six His after MscL. Purified 0.5 kbp DNA fragments were cut by *Pst*I, then ligated into the *Pst*I site of pYT4 to construct pYT9.

pYT9 was electroporated into *V. alginolyticus*. Cells were grown in the rich medium containing 2.0 μg/ml of chloramphenicol, and harvested cells were washed with 1 M NaCl containing 10 mM Tris-HCl (pH 7.5). The cells were lysed by suspending into a lysis medium containing 10 mM NaCl, 5 mM EDTA and 10 mM Tris-HCl (pH 7.5), and then the lysed cells were washed with the lysis medium. The lysed cells containing 20 mg of membrane proteins were solubilized with an extraction buffer containing 3% β-octylglucoside and 1 mM PMSF. MscL-6His in the extracts was purified using His-bind resin (Novagen) according to the procedure described in the protocol. Pu-

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rified MscL-6His was concentrated by 10% TCA. Proteins were applied on SDS-PAGE and then visualized by Coomassie staining.

3. Results and discussion

3.1. Effect of KCl and NaCl on the lysis of intact cells and spheroplasts

Fig. 1 shows the responses of intact cells and spheroplasts of *V. alginolyticus* exposed to various concentrations of NaCl or KCl. Identical to the results of our previous report [12], the minimum concentrations of salt required for preventing lysis of the intact cells were 0.2 M and 0.6 M for NaCl and KCl, respectively. In the present experiments, the lytic responses of spheroplasts were also determined to estimate the contribution of outer membranes and peptidoglycan layers in preventing the cell lysis. Surprisingly, the lysis curves of spheroplasts for NaCl and KCl were essentially the same as those of the intact cells. A slight decrease in the lysis point for KCl could be understood by considering a slight loss of the intracellular solutes from spheroplasts during preparation. Thus, in the marine bacterium *V. alginolyticus*, the outer membranes and peptidoglycan layers of the cell envelope played no positive role in preventing cell lysis. Peptidoglycan layers, however, were required to maintain the rod shape of the intact cells. These results clearly indicate that differences in the lytic response of the cells to NaCl and KCl should be attributed to the different responses of the inner cytoplasmic membranes to osmotic stress. Since KCl prevents cell lysis by balancing the internal osmotic pressure of the cells [12], we considered that the presence of a mechanosensitive channel like MscL will prevent the cell lysis in KCl medium.

3.2. Effect of MscL on the cell lysis of V. alginolyticus

The *MscL* gene from *E. coli* was introduced into the cells of *V. alginolyticus* by electroporation. As shown in Fig. 2, the cells of *V. alginolyticus*/pYT5 having the *mscL* gene became much more tolerant to osmotic downshock in KCl medium. The plasmid pYT9 having a His-tagged *mscL* gene was also effective in preventing the cell lysis in KCl medium. As shown in Fig. 3, His-tagged MscL was recovered in the membrane fraction, indicating the expression of MscL protein in the membranes. The membrane fraction from the cells carrying pYT9 (lane 3) also had a protein band identical to MscL. On

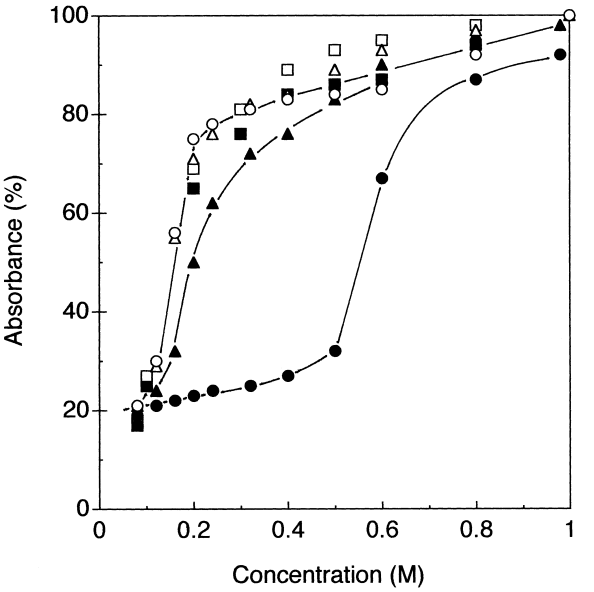

Fig. 2. Effects of MscL on the lysis of intact cells of *V. alginolyticus*. Intact cells carrying pYT4 (circles), pYT5 (triangles) or pYT9 (squares) were suspended in NaCl (open symbols) or KCl (closed symbols). The average values of at least three separate experiments are presented.

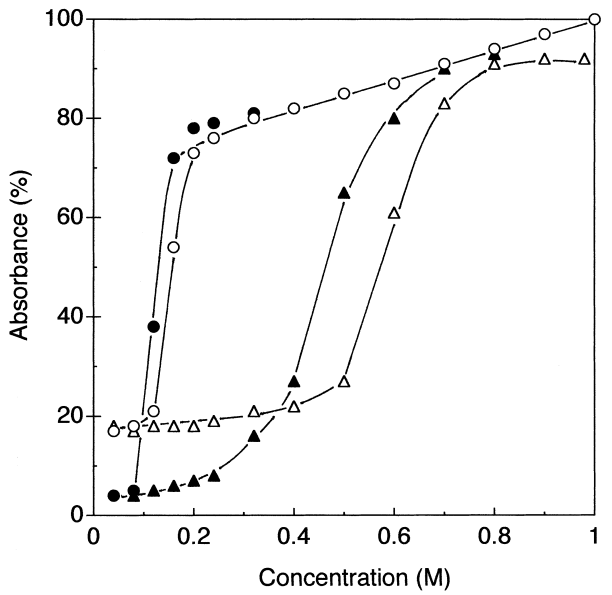

Fig. 1. Effects of NaCl and KCl on the lysis of intact cells and sheroplasts of *V. alginolyticus*. Responses of intact cells (open symbols) or spheroplasts (closed symbols) to different concentrations of NaCl (circles) and KCl (triangles) were measured as described in Section 2. The average values of at least three separate experiments are presented.

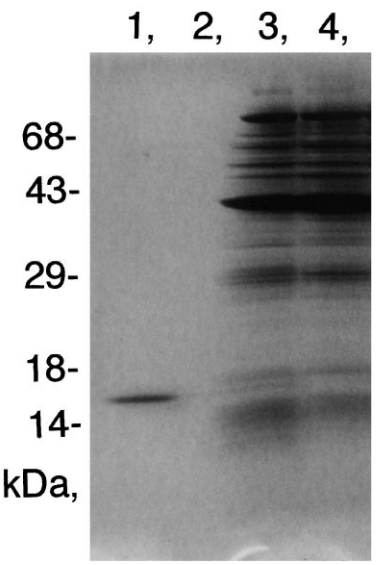

Fig. 3. Expression of His-tagged MscL in the membranes of *V. alginolyticus*. Lane 1, purified MscL-6His protein from the cells carrying pYT9; lane 2, as the control for pYT9, the cells carrying pYT4 were treated as described in Section 2 and the final fraction was applied to SDS-PAGE; lane 3, extracted membrane proteins from the cells carrying pYT9 (20 μg proteins); lane 4, extracted membrane proteins from the cells carrying pYT4 (20 μg proteins).

the other hand, *V. alginolyticus*/pYT4 having no *mscL* gene showed no changes in the lysis curve for KCl, and its membrane fraction (lane 4) had no protein band corresponding to MscL. These results clearly indicate that MscL functions as a mechanosensitive channel under osmotic stress, where the intracellular solutes are quickly released from the channel to relieve the turgor of the cell membranes. These responses will lead to the protection against cell lysis caused by osmotic downshock.

With respect to the lysis response to NaCl, 0.2 M NaCl was sufficient to effectively prevent the cell lysis of *V. alginolyticus* and this concentration was unaffected by the introduction of MscL. Since the lysis point for NaCl was not influenced by the increase in the osmotic pressure of the cells, we previously considered that Na^+ prevented cell lysis by providing a sufficient mechanical strength of the envelope against osmotic stress. However, since the spheroplasts have the same lytic response to NaCl (see Fig. 1), our previous assumption is unlikely due to the absence of intact outer membranes and peptidoglycan layers. Rather we need to explain the lytic difference between NaCl and KCl as a property of cytoplasmic membranes. It is clear that the cytoplasmic membranes of *V. alginolyticus* do not have a MscL-like mechanosensitive channel. To explain the specific protective effect of Na^+, it is necessary to postulate the possible existence of a mechanosensitive channel in the cytoplasmic membranes, which is specifically activated by Na^+. Further studies are required to make this problem clear.

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