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Survival of membrane-damaged *Escherichia coli* in a cytosol-mimicking solution

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Selective permeability of cell membrane is critically important for cell survival. The damage caused to cell membrane by pore-forming antimicrobial peptides may result in the loss of selective permeability and leakage of intracellular molecules, eventually leading to cell death. Here, we examined whether the membrane-damaged *Escherichia coli* cells survive in a cytosol-mimicking solution (CMS), which compensates for the lethal leakage of intracellular molecules. We prepared a CMS comprising 34 low molecular weight compounds from the cytosol and found that the cells were able to grow in CMS even in the presence of a pore-forming peptide, melittin. We confirmed that the melittin-treated cells lost selective membrane permeability by staining with membrane-impermeable dyes, propidium iodide and SYTOX green. Some stained cells maintained the colony formation ability in CMS. These results provide an evidence that *E. coli* cells can at least partially survive in the CMS even after the temporary impairment of membrane selective permeability. This study demonstrates a technique that allows temporal loss of the selective permeability of the cell membrane while maintaining the viability of cells that may be useful for the introduction of membrane-impermeable molecules into *E. coli* cells.

[**Key words:** *Escherichia coli*; Cell membrane; Membrane permeability; Membrane integrity; Melittin; Artificial cytosol]

Membrane integrity, the intactness of the selective permeability of a cell membrane, is critically important for cell survival. Defects in membrane integrity may cause the leakage of intracellular molecules and, consequently, cell death (1,2). Antimicrobial peptides such as melittin, temporin, and PGLa are common agents that impair membrane integrity, damage cell membrane, and induce leakage of internal molecules, leading to cell death (3–5). Considering the close relationship between membrane integrity and cell survival, some DNA staining dyes permeable only to damaged cell membranes, such as propidium iodide (PI) and SYTOX Green, are commonly used to distinguish between living and dead cells (6).

To date, microorganisms are used as whole-cell biocatalysts for the production of useful molecules, and the strategies have been improved in this field (7–14). Although important for cell survival, selective permeability of cell membrane limits the availability of substrates for intracellular metabolisms (7,11). In some cases, transporting systems are required for the use of hydrophilic compounds (15,16). To increase the availability of substrates, researchers have modified compounds by esterification with lipophilic groups (17,18) or peracetylation of sugars (17,19–21). Another possible strategy is the control of selective membrane permeability, which is not widely investigated. Any temporary increase in the membrane permeability of a living cell may allow access to additional substrates for metabolism and expand the possibility of bioproduction of useful molecules.

The increase in cell membrane permeability may possibly cause cell death, owing to the leakage of intracellular molecules. Various molecules such as low molecular weight compounds, proteins, and nucleic acids are present at high concentrations in the cells, and any damage to cell membrane may cause the lethal leakage of intracellular molecules. In particular, low molecular weight compounds easily leak owing to their small size. In the present study, we examined if a membrane-damaged cell may survive in the solution filled with low molecular weight compounds at concentrations similar to those observed in cell cytoplasm.

Here, we first prepared a cytosol-mimicking solution (CMS) comprising 34 low molecular weight compounds at concentrations similar to those in cell cytoplasm and found that the membrane-damaged *Escherichia coli* cells treated with an antimicrobial peptide could grow well in CMS. We also observed that the membrane-damaged *E. coli* cells were stained with membrane-impermeable dyes, PI and SYTOX green, and partially maintained their colony formation ability.

MATERIALS AND METHODS

Strains and plasmids Two *E. coli* strains with constitutive expression of green fluorescent protein (GFP) (DH5α/pUCcat-gfp) and its non-fluorescent mutant (DH5α/pUCcat-mgfp) were used in this study. The plasmid, pUCcat-gfp, was constructed based on pUC19 vector by replacing the ampicillin-resistant gene with chloramphenicol-resistant gene. The GFP gene was inserted under T7 promoter. The whole sequence is shown in Fig. S1. The mutant plasmid, pUCcat-

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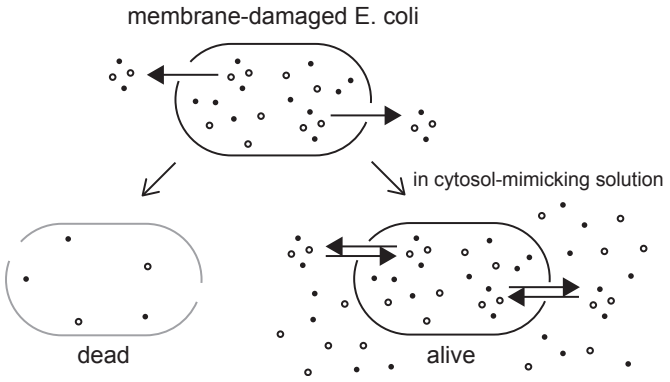

FIG. 1. Schematic diagram of the survival of a membrane-damaged *E. coli* cell in cytosol-mimicking solution (CMS). If the cell membrane is damaged and the selective permeability is impaired, low molecular weight compounds in the cytosol may leak out and cause cell death. The leakage may be compensated by an extracellular cytosol-mimicking solution containing essential low molecular weight compounds that are present in the cytosol.

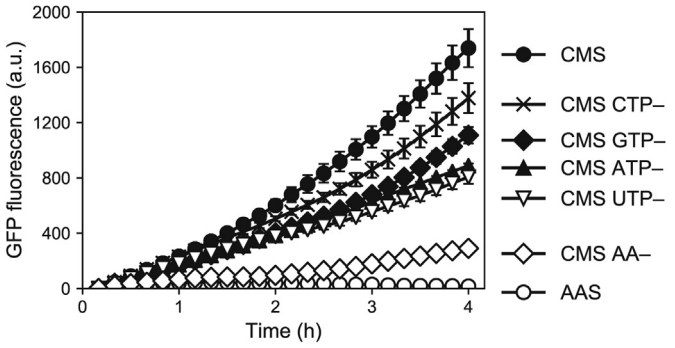

FIG. 3. Effect of CMS components on *E. coli* growth in the presence of melittin. *E. coli* growth was monitored as per the method described in Fig. 2 in the presence of various CMS derivatives such as complete CMS (CMS), CMS without each NTP (CMS CTP–, GTP–, ATP–, UTP–), CMS lacking the 20 amino acids (CMS AA–), and the solution containing 20 amino acids only (AAS) at same concentrations as in CMS. Error bars represent the standard deviations (N = 3).

mgfp, was constructed by deleting the last 21 bp of the GFP-coding region essential for GFP fluorescence (22) from pUCcat-gfp by polymerase chain reaction (PCR) using primers 5′-GCCTATTTCTCAAGAAGGACCATGTGGTC-3′ and 5′-TCTTGAGAAATAGGCTTGCGGCCG-3′, followed by self-ligation with In-Fusion Cloning kit (Takara, Shiga, Japan).

Culture and melittin treatment The GFP-expressing *E. coli* cells were precultured in 5 mL of Miller LB liquid media (Sigma–Aldrich Japan, Tokyo, Japan) containing 25 μg/mL of chloramphenicol at 160 rpm and 37 °C until the absorbance at 600 nm wavelength (OD_{660}) reached 0.5. After centrifugation at 8000 ×*g* and 4 °C for 5 min, the cells were suspended into an equal volume of 0.9% sodium chloride (NaCl) solution. About 0.8 μL of the suspended cells were mixed with 8.2 μL of the CMS or an amino acid solution (AAS) at an OD_{660} of 0.04. About 1 μL of 0.5 mM melittin, purchased from Sigma and dissolved in phosphate-buffered saline (PBS), was added and incubated at 37 °C and fluorescence measurements were performed after every 10 min with a real-time PCR machine (M x 3005P, Agilent Technologies Japan, Tokyo, Japan, ex: 492 nm, em: 516 nm). The background fluorescence at 0 h was subtracted from each measurement. For colony formation assay, the solution was spread onto 20 mL of Miller LB plate media and incubated at 30 °C for 48 h.

Flow cytometry and cell sorting For flow cytometry and cell sorting, 0.8 μL of non-fluorescent *E. coli* strain (DH5α/pUCcat-mgfp) was mixed with 8.2 μL of CMS or AAS supplemented with PI (50 ng/μL; Dojindo, Kumamoto, Japan) or SYTOX Green (250 μM; Thermo Fisher Scientific K.K., Tokyo, Japan), followed by the addition of

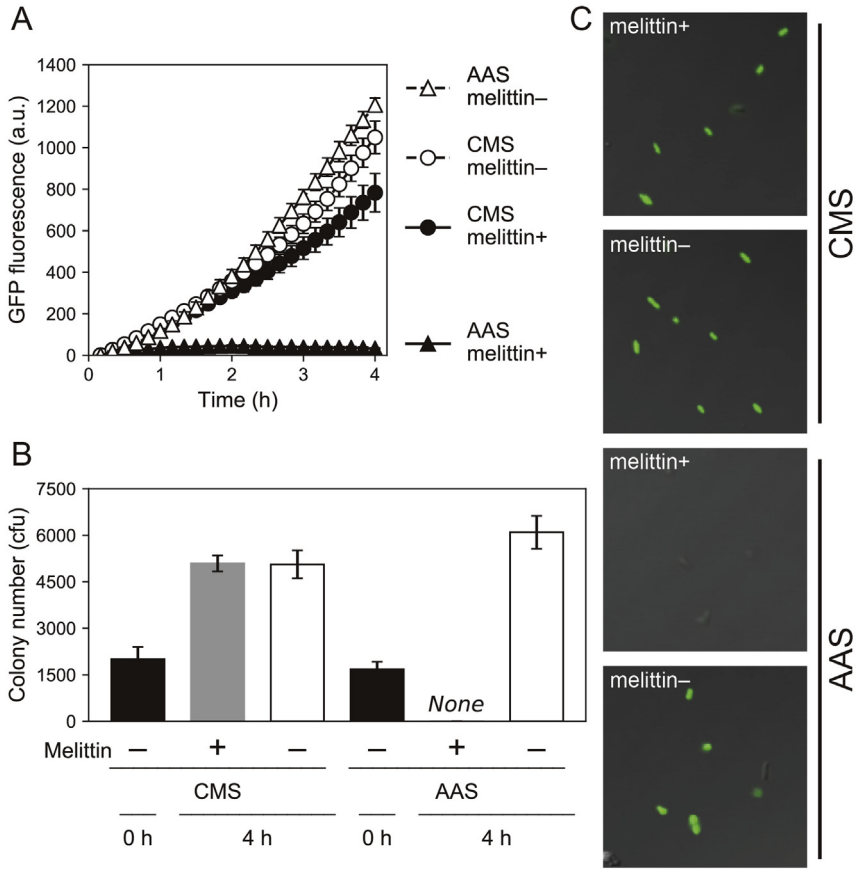

FIG. 2. Effects of CMS on *E. coli* growth in the presence of melittin. (A) *E. coli* growth in the presence of 50 μM melittin in CMS or AAS at 37 °C. The growth was evaluated by the fluorescence of the constitutively expressed GFP. (B) Colony formation ability of cells at 0 or 4 h under each condition. The cells at 0 and 4 h in the experiment shown in panel A were inoculated on LB plate, incubated at 30 °C for 48 h, and subjected to colony counting. Error bars represent standard deviation (N = 3). (C) Fluorescent microscopy of the cell at 4 h.

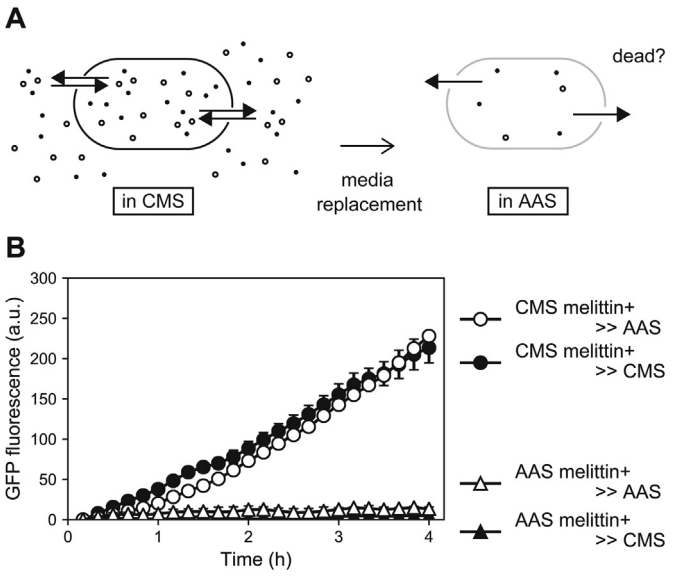

FIG. 4. Effects of media replacement on the growth of melittin-treated *E. coli*. (A) Schematic procedure of the experiment. *E. coli* cells were treated with melittin in CMS for 5 min, followed by the replacement of the medium with AAS. If melittin-induced membrane damage in CMS persists after cell wash, the washed cells may not grow in AAS. However, any repair in membrane damage in CMS within 5 min may allow the growth of washed cells in AAS. (B) The growth of the cells after medium replacement. Legend indicates conditions before and after medium replacement. *E. coli* cells were incubated with 50 μM melittin in CMS (CMS melittin+) or AAS (AAS melittin+) at 37 °C for 5 min, and the media were replaced with CMS (>> CMS) or AAS (>> AAS) after cell wash. The cells were incubated at 37 °C and GFP fluorescence was monitored. The error bars represent standard deviation (N = 3).

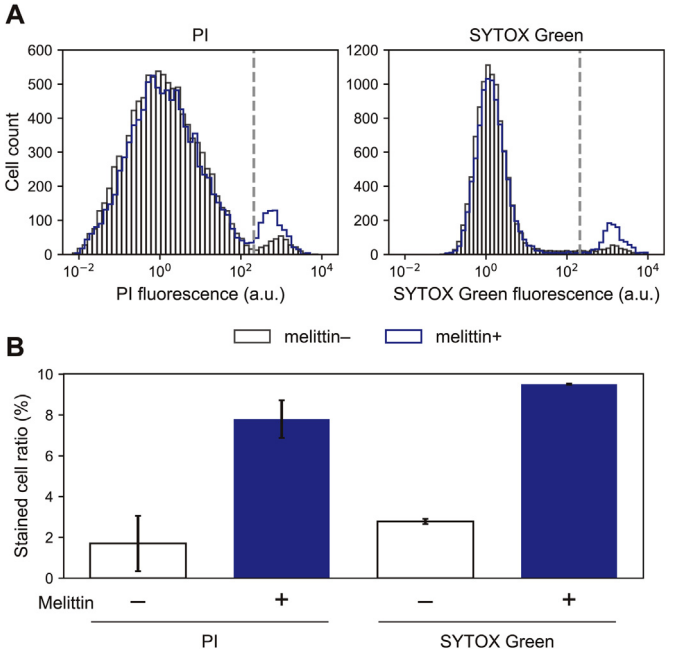

FIG. 5. Flow cytometry analysis of melittin-treated *E. coli* cells stained with membrane-impermeable dyes. After incubating *E. coli* cells with or without 50 μM melittin in the presence of membrane-impermeable dyes, PI or SYTOX Green, in the CMS at 37 °C for 5 min, the fluorescence intensity of 10,000 cells was analyzed with a flow cytometer. (A) Histogram of fluorescence intensity. The dotted lines indicate the threshold that distinguish stained and unstained cells. (B) The ratios of the stained cells to the total cells. Error bars represent standard deviations (N = 6 for PI, N = 3 for SYTOX Green).

1 μL of 0.5 mM melittin solution. The cell mixture was incubated at 37 °C for 5 min, and 10 μL of each sample was diluted with 100 μL PBS and subjected to flow cytometry analysis (FACS Aria II, Becton Dickinson, Franklin Lakes, NJ, USA). For PI staining analysis, 488 and 616 nm wavelengths were used for excitation and emission, respectively. For SYTOX Green analysis, 488 and 530 nm wavelengths were used for excitation and emission, respectively. For the collection of PI-stained cells, 10 μL of each sample was mixed 100 μL of CMS and subjected to follow cytometry analysis. The cells with high fluorescence were collected into 100 μL of CMS. All the collected cells were spread onto 20 mL of Miller LB plate media and incubated at 30 °C for 48 h for colony formation assay.

RESULTS

Preparation of CMS Melittin may cause the leakage of intracellular low molecular weight compounds through cell membrane damage. These lethal effects may be compensated through the supply of components from an outer solution, termed as CMS, that provides all the essential compounds in the cytosol at sufficient concentrations (Fig. 1). The concentrations of low molecular weight compounds in *E. coli* cytosol have been previously reported (23–27). Based on these data, we chose 34 compounds that are considered essential for cell growth, including amino acids, nucleotide triphosphates (NTPs), deoxynucleotide triphosphates (dNTPs), magnesium, and polyamines, and determined the concentration of each compound based on the values reported in the cytosol (Table 1). It should be noted that the CMS lacks metabolic intermediates such as components of glycolysis; these intermediates could be omitted as long as the final product, ATP, is supplied.

Growth of melittin-treated *E. coli* in CMS As a strategy to impair the membrane integrity of *E. coli* cells, we used an antimicrobial peptide, melittin, a component of honey bee venom. Melittin is reported to form pores of around 3 nm diameter on cell membranes at specific concentrations (4,28–30). This pore size primarily allows permeation of low molecular weight compounds in the cytosol but not proteins.

To evaluate whether *E. coli* cells survive in CMS even in the presence of melittin, the cells were incubated with 50 μM melittin in CMS at 37 °C for 4 h. As a control experiment, the same setup was performed in another solution containing similar concentrations of 20 amino acids, termed as AAS which also contains 100 mM potassium glutamate to keep phylogenic osmotic pressure. The *E. coli* strain used in this study constitutively expressed GFP and the fluorescence was monitored as an indicator of cell growth. GFP fluorescence increased only slightly in the presence of AAS with melittin (AAS melittin+), but significantly increased in CMS with melittin (CMS melittin+) to the similar levels as those without melittin (AAS melittin− and CMS melittin−) (Fig. 2A). Similar result was observed with another pore-forming peptide, PGLa (Fig. S2). To confirm cell growth in CMS, we evaluated the colony formation ability of cells before and after 4 h of incubation. We inoculated 10 μL of each sample at 0 or 4 h onto LB plate and counted the number of colonies after incubation at 30 °C for 48 h. For the cells incubated in CMS, approximately 2.5 times more colonies were observed at 4 h than at 0 h both with and without melittin, while no colony was observed for the cells treated with melittin in AAS at 4 h (Fig. 2B). These results indicate that *E. coli* cells could grow in CMS even in the presence of melittin. Fluorescent microscopy of the cells after 4 h incubation revealed that the cells in CMS maintained the normal shape and GFP fluorescence even in the presence of melittin (Fig. 2C).

We tested the effects of some components in CMS on the ability of *E. coli* cells to grow in the presence of melittin. *E. coli* cells were incubated at 37 °C with 50 μM melittin in various solutions lacking some components of CMS and the GFP fluorescence was monitored.

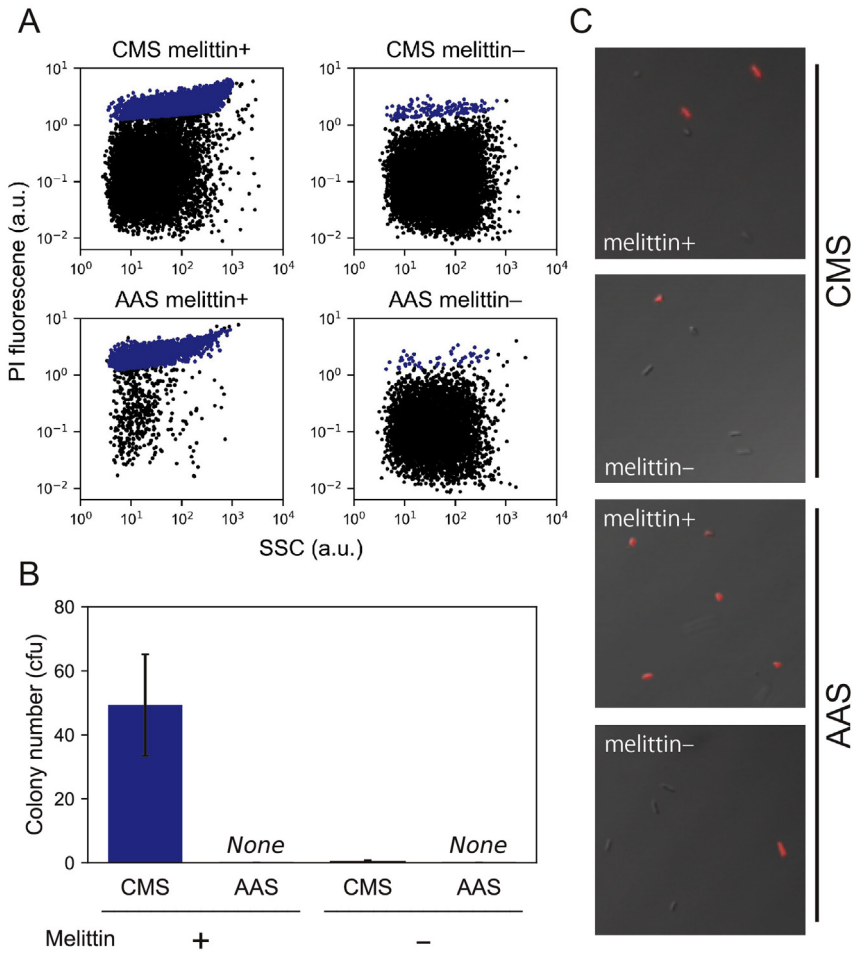

FIG. 6. Colony formation ability of *E. coli* cells stained with PI. (A) The scatter plot of 10,000 cells at the axes of the side-scattered light intensity (SSC) versus PI fluorescence. After incubation with or without 50 μM melittin in the presence of membrane-impermeable dyes, PI or SYTOX Green, in the CMS at 37 °C for 5 min, the *E. coli* cells were subjected to flow cytometry analysis. The cells showing high PI fluorescence (shown in blue) were collected by a cell sorter and incubated on LB plate at 37 °C for 48 h for the evaluation of colony formation ability (B). For melittin-treated cells in CMS or AAS, approximately 70,000 or 5000 cells were analyzed, respectively, to collect 5000 stained cells. All the collected cells were used for colony formation assay. For the cells incubated without melittin in CMS or AAS, the number of cells similar to that evaluated after melittin treatment were analyzed. All stained cells were collected and used for colony formation assay. (B) Result of colony formation assay. Error bars represent standard deviation (N = 3). (C) Fluorescent microscopy of PI-stained cells before flow cytometry analysis.

The fluorescence of solutions lacking one of the CMS components was lower than that of the complete CMS (Fig. 3, CMS), indicating that all the components (20 amino acids and each NTP) contributed to the growth of melittin-treated cells (Fig. 3). The fluorescence intensity was the lowest for the cells incubated in CMS lacking 20 amino acids (CMS AA−) but was higher for CMS derivatives lacking each NTP. The fluorescence intensity of all CMS derivatives lacking NTPs was similar until 2 h but varied thereafter; the fluorescence intensity at 4 h was lower for the solutions lacking ATP and UTP (CMS ATP- and UTP-) but was higher for those without GTP (CMS GTP−) and CTP (CMS CTP−). These results show that each component contributes to the growth of *E. coli* cells in the presence of melittin to a different extent.

We next investigated the time span of cell membrane damage in CMS. *E. coli* cells treated with 50 μM melittin in CMS or AAS at 37 °C for 5 min were washed and resuspended in new CMS or AAS without melittin. The growth of the resuspended cells was monitored by fluorescence analysis at 37 °C for 4 h. The melittin-treated cells may not grow in AAS after the removal of melittin if cell membrane damage was retained in CMS for more than 5 min; however, the repair of the damage in CMS within 5 min may allow the washed cells to flourish even in AAS (Fig. 4A). We found that the washed cells treated with melittin in CMS grew in both CMS and AAS (Fig. 4B, CMS melittin+ >> AAS or >> CMS), while the washed cells pre-treated with melittin in AAS as a control failed to thrive in both CMS and AAS (Fig. 4B, AAS melittin+ >> AAS or >> CMS). These results show that the membrane damage caused by melittin was repairable in CMS within 5 min.

Evaluation of membrane permeability by flow cytometry We demonstrated the growth of melittin-treated cells in CMS, but whether these cells actually lost their selective permeability in CMS was unclear. To distinguish the cells that lost their selective membrane permeability from the normal cells, we stained *E. coli* cells with PI or SYTOX Green, DNA fluorescent dyes that are impermeable to normal cell membrane. *E. coli* cells were treated with 50 μM melittin in CMS supplemented with PI or SYTOX Green at 37 °C for 5 min, and the fluorescence intensity of cells was monitored by flow cytometry. The histogram of fluorescence intensity is shown in Fig. 5A. The stained cells were defined as those in the right of the broken line, and the stained cell ratio (the number of stained cells to the total number of cells) was calculated (Fig. 5B). The stained cell ratio increased following melittin treatment from 1.7% or 2.8%—7.8% or 9.5% for PI or SYTOX Green, respectively, indicating that some of the melittin-treated *E. coli* cells became permeable to these membrane-impermeable dyes in CMS. However, the ratio of the stained cells was small,

TABLE 1. Composition of cytosol-mimicking solution.

Components	Concentration (mM)	Reference
Asp, Val, Gln, Ala	1.6[a]	23
Arg, Asn, Lys, Pro	0.4[a]	23
Ile, Leu, Thr, Met	0.4[a]	23
Set, His, Tyr, Phe, Trp	0.05[a]	23
Cys	0.4[a]	ECMDB00574[b]
Gly	0.4[a]	24
Potassium glutamate	100[a]	23
dATP, dCTP	0.06	23
dGTP	0.06[c]	
dTTP	5	23
ATP	10	23
GTP	5	23
CTP	3	23
UTP	8	23
Spermidine	2.7	25
Putrescine	2	25
Cadaverine	1.75	25
Magnesium acetate	26.5	CyberCell Database: CCDB[d]
10-Formyl-tetrahydrofolate (μg/μL)	0.01[c]	34

[a] These components are included in AAS.
[b] *E. coli* Metabolome Database (http://ecmdb.ca/compounds/ECMDB00574).
[c] As the concentrations in the cytosol were not determined in previous reports for two compounds, 10-formyl-tetrahydrofolate (10FD) and deoxyguanosine triphosphate (dGTP), we used the optimum value for in vitro translation for 10FD and the same concentrations as dATP for dGTP.
[d] CyberCell Database (http://ccdb.wishartlab.com/cgi-bin/STAT_NEW.cgi).

indicating that the majority of the cells were impermeable to these dyes even in CMS in the presence of melittin. This observation is clearly contrasting to the 97% and 81% of stained cells observed in AAS with 50 μM melittin for PI and SYTOX Green, respectively (Fig. S3). The decrease in the number of stained cells in CMS may be associated with the quick repair of the damaged membrane in CMS, as observed in the previous experiment (Fig. 4).

Growth experiment for PI-permeable cells To obtain a direct evidence that the membrane-damaged cell is viable in CMS, we examined the colony formation ability of PI-stained cells. After the treatment of *E. coli* cells with 50 μM melittin in CMS or AAS in the presence of PI at 37 °C for 5 min, the cells were subjected to flow cytometry analysis. In this experiment, we analyzed approximately 70,000 and 5000 cells for the samples in CMS and AAS, respectively, and collected 5000 stained cells from both solutions using a fluorescence-activated cell sorter. The scatter plot is shown in Fig. 6A. The stained cells with high fluorescence shown in blue were collected and inoculated on LB plate at 30 °C for 48 h for colony formation assay. For the melittin-treated cells in CMS, 50 colonies were formed that corresponded to 1% of the collected cells, while no colonies were observed for the melittin-treated cells in AAS (Fig. 6B, melittin+). Thus, a part of the cells that lost selective permeability maintained the colony formation ability in CMS. We also performed fluorescent microscopy before applying to flow cytometry and found that the similar frequency of the stained cells, 16% (24/155 cells, melittin+ in CMS), 3.6% (6/143 cells, melittin− in CMS, 92% (94/102 cells, melittin+ in AAS), and 1.7% (2/108 cells, melittin− in AAS).

To deny the possibility that the contaminated unstained cells contributed to colony formation as a consequence of miss-sorting during the cell sorting process, we performed the same experiment without melittin in both CMS and AAS. We analyzed the same number of cells as those with melittin treatment (approximately 70,000 and 5000 for CMS and AAS, respectively) and collected the cells that exhibited high fluorescence (Fig. 6A, blue dots in right panels). These cells were plated on LB plate, as performed in the experiment with melittin. In both solutions, almost no bacterial colony was observed (Fig. 6B, melittin−), indicating that the contamination of intact cells was negligible.

DISCUSSION

In this study, we aimed to establish a method to retain the viability of *E. coli* cells upon temporary impairment of membrane permeability. We found that *E. coli* cells grew in a CMS containing some major intracellular low molecular weight compounds at concentrations similar to those in the cytosol even in the presence of 50 μM melittin, which almost completely inhibited cell growth in a solution containing only amino acids (Fig. 2). Some of the melittin-treated cells in CMS were permeable to membrane-impermeable PI and SYTOX green (Fig. 3), and a few stained cells maintained their colony formation ability (Fig. 5). These results provide an evidence that *E. coli* with impaired selective permeability can survive in CMS.

We found that only a small fraction of the melittin-treated *E. coli* cells in CMS was stained with PI or SYTOX green (Fig. 5), while almost all the cells in AAS were stained (Fig. S3). This partial staining in CMS may be attributed to the quick repair of the damaged membrane in CMS, as shown in Fig. 4. Alternatively, certain components in CMS may directly inhibit the activity of melittin, consistent with the growth of melittin-treated cells (Fig. 2) and reduced staining of cells in CMS (Fig. 5). However, this interpretation may be contradicted by some observations. As shown in Fig. 3, the removal of NTP or amino acids from CMS resulted in a decrease in the growth of melittin-treated cells to different levels, suggesting that no component in CMS inhibits melittin activity; however, many components may contribute to the compensation of the effect of melittin at different levels. This phenomenon is consistent with our model shown in Fig. 1, wherein each component in CMS may differently act on melittin-treated cells depending on its role in cellular metabolism. It should be noted that although we obtained results that support the growth of the membrane-damaged (i.e., PI-stained) cell in CMS, we cannot deny the possibility that some factors in CMS could weaken the effect of melittin to some extent.

We found that the cells stained with membrane-impermeable dyes in CMS formed colonies (Fig. 5), but the colony number was only 1% of all the collected cells. This small ratio is probably attributed to several reasons. First, the melittin-treated cells may be unable to tolerate the sorting process, wherein the cells are exposed to laser beams. Second, PI staining may be toxic because PI is a DNA intercalating dye. Third, the CMS used herein may not be optimal for bacterial growth. CMS contained only 34 low-molecular-weight compounds that we thought were essential (Table 1) but lacked most of the intermediate metabolites such as glucose phosphate and glycerol phosphate. Fourth, the membrane-damaged cells can incorporate toxic compounds from outside, such as NaCl, included in the cell suspension. Such incorporation also possibly impairs the growth of the cells. Hence, optimization of experimental conditions focusing on these points may increase the colony formation rate in the future.

The method we developed herein facilitates the temporal loss of selective membrane permeability and facilitates the entry of membrane-impermeable substances for intercellular metabolism. Microorganisms are utilized for the production of useful molecules such as medicines and fuel, but the production of such molecules often demands complicated genetic modification of the metabolic pathways (31). If the cells incorporate precursor compounds that are naturally impermeable to cell membrane, it may significantly improve bioproduction. Furthermore, the increase in membrane permeability may be useful for the incorporation of different types of unnatural amino acids, thereby contributing to the production of

unnatural proteins useful for various research applications (32). However, membrane permeability lasts only for a short period of time, and only a small fraction of the cells incorporate membrane-impermeable compounds. An important challenge is to increase the membrane permeability period. A possible strategy includes the production of melittin inside the cell in a controllable manner and to continuously maintain membrane damage (33). Although further studies are warranted, the method presented here serves as the first step for the development of a new technique to control cell membrane permeability.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2019.05.005.

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