Induction of Viable but Non-culturable (VBNC) State in Salmonella Cultured in M9 Minimal Medium Containing High Glucose

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An environmental isolate of Salmonella enterica serovar Enteritidis (SE) clone, SE Cl#15-1, loses its culturability during 72-h culture in M9 minimal medium containing 0.8% glucose, a concentration twice higher than that in normal M9 medium, whereas the bacterium retains its culturability in normal M9 medium. Live/dead analysis using the 5-cyano-2,3-di(p-tolyl) tetrazolium chloride (CTC)-reduction assay revealed that SE cells cultured in M9 medium containing 0.8% glucose died with time when in the “viable but non-culturable” (VBNC) state. Assay of the culturability of SE cells in the used supernatant (0.4 spent M9 or 0.8 spent M9) also indicated that 0.8 spent M9 soon showed a lethal effect on intact SE cells. These results suggest that large amounts of glucose metabolites might have been responsible for the toxicity. Analysis of the 0.8 spent M9 revealed that formate rapidly accumulated in the medium. The pH of the medium dropped to 4.7, leading to conversion of formate to formic acid, which might have damaged the bacterial cell membrane. These results suggest that the excessive amount of glucose in the M9 medium might have injured SE cells in the VBNC state by being metabolized to formic acid and other acidic compounds.

Key words viable but non-culturable (VBNC) induction; Salmonella; formate; glucose; prolonged culture

Salmonella is one of the most causative of food-borne diseases in the world; and it is detected not only in food but also in natural environments including river water, soil, manure, and air-borne dust.1–5) Among Salmonella spp., Salmonella enterica serovar Enteritidis (SE) is one of the dominant species, and has sometimes caused mass food poisoning in many countries.6–10) In consideration of the strong infectivity of Salmonella, a better understanding of the characteristics of environmental Salmonella isolates is necessary for the control of spreading and contamination of foods by Salmonella as well as for the risk management for food safety in general.

We previously reported that SEp22, 11–14) which is a novel pathogenicity-related factor of S. Enteritidis, and is identical to Salmonella Dps, 15) plays a very important role in the acquisition of the dry-resistance of S. Enteritidis.16) In the course of studying the characteristics of SEp22, we happened to find that the prolonged batch culturing of SE cells in M9 minimal medium containing 0.8% glucose (a concentration twice higher than that used in the standard culture) significantly reduced their culturability. Analysis of the result culture supernatant revealed that this two-fold higher dose of glucose induced a sudden decrease in pH and the accumulation of large amounts of acetate, formate, and pyruvate. It was also remarkable that pyruvate was produced to a much higher extent in the medium with 0.8% glucose than in that with 0.4% glucose in the same time-course studies.

Recent findings revealed that many pathogens could enter a viable but non-culturable (VBNC) state17,18) after exposure to adverse environmental conditions such as high/low temperature, osmotic stress, oxidative stress or nutritional starvation.19–22) In this present study, we showed that SE cells in M9 medium with 0.8% glucose were in this VBNC state and then proceeded to die, with the definition of “culturability” being colony-forming activity of the cells on LB-agar plates and that of “viability,” primarily metabolic activity for the reduction of 5-cyano-2,3-di(p-tolyl)tetrazolium chloride (CTC). These phenomena highlight a new condition under which intact SE cells can undergo transition to the VBNC state without the addition of any toxic agents. That is, the addition of twice the normal amount of the nutrient glucose to the medium would be bactericidal as a result of metabolism of SE cells. These findings should alert those individuals responsible for food safety management to this induction of VBNC in SE cells in the presence of high glucose.

MATERIALS AND METHODS

Bacterial Culture An environmental isolate of S. Enteritidis (SE) clone, SE Cl#15-1, obtained from CAF Laboratories12) (Fukuyama, Hiroshima, Japan), was used throughout this study. SE cells from the stock culture were inoculated into 10 mL of Luria–Bertani (LB) medium (DifcoTM, BD, Sparks, MD, U.S.A.) in a 50-mL conical tube, incubated at 37°C with shaking at 150 strokes/min, and then suspended in fresh LB medium at OD550 of 0.05. The density of the bacterial solution was also measured at 550nm (OD550) with a spectrophotometer (Shimadzu UV-150-02, Kyoto, Japan).

Thereafter the cells were incubated at 37°C with shaking at 150 strokes/min for 60 min. Next, a 1-mL aliquot of the cells was centrifuged at 2850×g for 20 min in a swing rotor-equipped KUBOTA 8920 centrifuge (Kubota, Tokyo, Japan) at 4°C, washed twice with 10 mL of ice-cold phosphate-buffered saline without calcium (PBS[−]), comprising NaCl (8.0g/L), KCl (0.20g/L), NaHPO4 (1.15g/L), and KH2PO4 (0.20g/L), pH 7.5, and resuspended in 10 mL of M9 minimal medium13) comprising Na2HPO4 (6.8g/L), KH2PO4 (3.0g/L), NaCl (0.5g/L), NH4Cl (1.0g/L), MgSO4 (2mm), CaCl2 (0.1mm), and 0.4% (w/v) glucose (DifcoTM). These cells were cultured at 37°C with shaking at 150 strokes/min for 4 h until they had reached the mid-logarithmic phase (OD550 of 0.6 to 0.7). The
cells were then harvested by centrifugation, washed twice with ice-cold PBS(−) as described above, and finally resuspended in ice-cold PBS(−) to OD550 of 6.30 (approximately $1 \times 10^9$ colony-forming unit (cfu)/mL).

**Effects of Glucose Concentration on the Growth of SE in M9 Medium** The mid-logarithmic SE cells (OD550 of 0.6 to 0.7) were suspended at OD550 of 0.05 in 10 mL of fresh M9 medium containing the normal (0.4%) or a high (0.8%) concentration of glucose in a 50-mL conical tube and cultured at 37°C with shaking at 150 strokes/min for various times up to 72 h. At each time point, the bacteria were chilled on ice, diluted serially with ice-cold PBS(−), and plated in 25-μL aliquots onto LB-agar plates, which were then incubated at 37°C overnight. The number of colonies was counted to estimate the number of viable bacterial cells as a measure of culturability.

**Effects of Successive Addition of Glucose on the Growth of SE in M9 Medium** Mid-logarithmic SE cells (OD550 of 0.6 to 0.7) were resuspended to OD550 of 0.05 in 10 mL of fresh M9 medium containing 0.4% glucose in a 50-mL conical tube and cultured at 37°C with shaking at 150 strokes/min for various times up to 4 d. Glucose was added to the medium every 24 h at the rate of 12 mg/mL/d, because our preliminary experiment showed that glucose was consumed by the cells at the rate of 0.5 mg/mL/h under these conditions (data not shown). Bacterial growth was monitored both by the density of the bacterial solution (OD550) and cfu’s at each time point, as described above.

**Quantification of Glucose Concentration of the Culture Medium** Bacterial culture supernatants were aseptically retrieved after centrifugation at 2850 × $g$ for 20 min at 4°C, and the resultant supernatants were filtered through a membrane filter with a pore size of 0.45 μm and assayed for their glucose concentration with Glucose Assay Reagent (Sigma-Aldrich, St. Louis, MO, U.S.A.).

**Preparation of Spent M9 Medium** Mid-logarithmic SE cells (OD550 of 0.6 to 0.7) were resuspended to OD550 of 0.05 in 10 mL of fresh M9 medium containing 0.4% or 0.8% glucose in a 50-mL conical tube and cultured at 37°C with shaking at 150 strokes/min, usually for 24 h. The culture supernatants were collected by centrifugation at 2850 × $g$ for 20 min at 4°C and then filtered through a membrane filter with a pore size of 0.45 μm. These supernatants from 0.4% and 0.8% glucose-containing cultures were designated as “0.4 spent M9” and “0.8 spent M9,” respectively.

**Estimation of the Effects of Spent M9 Medium on the Growth of SE** SE cells from the stock culture were inoculated into 10 mL of LB medium and incubated at 37°C with shaking at 150 strokes/min for 60 min, and then a 1-mL aliquot was washed twice with ice-cold PBS(−). The cells were resuspended in 10 mL of fresh M9 medium, incubated at 37°C with shaking at 150 strokes/min overnight for adaptation of the cells to this medium. Thereafter, the cells were centrifuged, resuspended in an appropriate amount of fresh M9 medium to achieve OD550 of 0.05, and grown to the mid-logarithmic phase (OD550 of 0.6 to 0.7), as described above. Next, the cells were washed with ice-cold PBS(−) at 4°C and resuspended to approximately $1 \times 10^9$ cells/mL (OD550 of 6.30) in either fresh M9 medium or the spent M9 medium (0.4 spent M9 or 0.8 spent M9). Then 1 mL of the cells was cultured at 37°C with shaking at 150 strokes/min for various times up to 72 h, and growth was monitored in terms of cfu’s as described above.

**CTC-Reduction Assay and Viability Count of SE Cells** To examine the effects of M9 containing 0.8% glucose and the spent M9 medium on the respiratory activity of SE, we harvested approximately $5 \times 10^8$ cells by centrifugation at 18,000 × $g$ for 5 min at 4°C in an angle rotor-equipped TOMY MX-160 centrifuge (TOMY SEIKO, Tokyo, Japan) and resuspended them in 500 μL of fresh M9 medium containing 0.4% glucose. CTC staining of the cells was performed with the reagents of a Bacstain CTC Rapid Staining Kit (DOJINDO, Kumamoto, Japan) according to the instructions provided with the kit. CTC reduction is an indicator of bacterial aerobic respiration.23,24) The following procedures were performed essentially in the dark throughout the experiment: the bacteria were stood still for 60 min in an incubator at 37°C with the reagents of a Bacstain CTC Rapid Staining Kit. They were then washed with 500 μL of PBS(−) by centrifugation at 18,000 × $g$ for 5 min at 4°C. Finally, the resultant cell pellet was resuspended in 500 μL of PBS(−), which was then divided into 40-μL aliquots; and each aliquot was placed in each well of a sterile, poly-L-lysine-coated 8-well glass slide. The cells were allowed to adhere to the slide glass by standing for 30 min on ice, and then they were fixed with 40 μL of 4% (w/v) paraformaldehyde/PBS(−) on ice for 30 min. Cellular DNA was stained with 5 μL of 20 μg/mL DAPI (4',6-diamino-2-phenylindole lactate; Sigma-Aldrich), after which the slide was rinsed twice with PBS(−). Perma Fluor Mountant Medium (Thermo Scientific, Rockford, IL, U.S.A.) was then applied to the slide, which was sealed with a cover glass, and thereafter examined under a confocal laser-scanning microscope (LSM510; CARL ZEISS, Oberkochen, Germany).

The viability of the bacterial cells was determined from the following formula:

$$\text{Viability} \% = \left( \frac{\{\text{Live cells (CTC/DAPI-positive cells)}\}}{\text{Whole cells (DAPI-positive cells)}} \right) \times 100$$

**HPLC Analysis of the Glucose Metabolites in the Spent M9 Medium** Analyses for the presence of pyruvic acid and other organic acids in the culture medium were carried out by using a Waters high-performance liquid chromatograph system (model 510 pump, M680 gradient controller, and 486 tunable absorbance detector [Waters, Milford, MA, U.S.A.]) equipped with 2 stainless-steel columns, one being a Cosmosil® C18-PAQ (4.6 mm i.d.×25 cm; Nacalai Tesque, Inc., Kyoto, Japan). The mobile phase was 20 m M H₃PO₄ (pH 2.1); and the flow rate, 0.5 mL/min. A 24-μL aliquot of sample solution was injected into the chromatograph after adjusting the pH of medium supernatant to 2.1 with H₂PO₄. The wavelength for detection of organic acids was set at 210 nm.

**Effects of Medium pH and Glucose Metabolites on the Growth of SE** Mid-logarithmic SE cells (OD550 of 0.6 to 0.7) were resuspended in fresh M9 medium to OD550 of 0.10, whose pH had been adjusted to 4.7 or 5.9 by changing the NaHPO₄/KH₂PO₄ ratio. Formate or pyruvate was added to the culture media as candidates of the responsible glucose metabolites, and the cultures were then stood at 37°C for 24 h. After incubation, the bacterial growth and viability were estimated by OD550 and cfu’s, respectively. The addition of formate or...
Pyruvate was performed by using a 1 M stock solution of them, the pH of which had been adjusted to 4.7 or 5.9 by changing the acid/salt ratio of formate or pyruvate, respectively. Formic acid, pyruvic acid, and their sodium salts were obtained from Sigma-Aldrich.

**Incorporation of Formate by the Cells**

Mid-logarithmic SE cells (OD 550 of 0.6 to 0.7) were resuspended in fresh M9 medium to OD 550 of 0.10, whose pH had been adjusted to 4.7 as described above (final volume: 500 µL), and then incubated at 37°C for up to 20 min with [14C] formate (2.1 GBq/mmol, Moravek Biochemicals Inc., Brea, CA, U.S.A.). After incubation, 750 µL of ice-chilled PBS(−) was added to the culture, which was then chilled on ice to stop incorporation of the formate. Then the cells were washed 3 times with ice-chilled PBS(−) by centrifugation at 16100 × g for 5 min at 4°C in an angle rotor-equipped Eppendorf 5415R centrifuge (Eppendorf, Hamburg, Germany). The resultant cell pellets were dissolved in 0.1 M NaOH, neutralized with HCl, and then resuspended in ACS II liquid scintillation cocktail (Amersham, Piscataway, NJ, U.S.A.). Radioactivity of [14C] was measured using a TRI-CARB 1600TR liquid scintillation counter (Packard Instrument Company, Meridian, CT, U.S.A.).

**Statistical Analysis**

Significance of differences between 2 groups were analyzed by performing Student’s t-test. For comparison of more than 2 groups with comparable variances, one-way ANOVA and the Tukey–Kramer’s post hoc test was carried out.

**RESULTS**

**High Concentration of Glucose in M9 Medium Induced the VBNC State in SE Cells**

In this study, we examined the effect of the amount of glucose on the growth of SE cells in M9 medium. As shown in Fig. 1A, the SE cells cultured with 0.4% glucose showed the normal growth curve; i.e., 7.2 logs of SE cells grew to 9.3 logs by 18 h, reaching a plateau at 24 h that remained until 72 h. On the other hand, the addition of 0.8% glucose resulted in a unique growth curve, showing an increase in the cfu value up to 8.8 logs by 18 h and then a rapid decrease, which suggested rapid cell damage in this latter period. The bacterial cell density at OD 550 did not show any significant difference between the 0.4% and 0.8% glucose cultures (Fig. 1B). The residual glucose concentration in these media rapidly decreased by 18 h; however, a larger amount of glucose was utilized by the cells cultured with 0.8% glucose.

![Fig. 1. Effect of High Glucose on SE Cell Cultures](image-url)
glucose remained in the high-glucose medium than in the normal one, suggesting that the SE cells had ceased to metabolize glucose under this high-glucose condition (Fig. 1C).

We also examined the respiratory activity of the cells by assessing CTC reduction\(^\text{23, 24}\) for estimation of cell viability, to determine whether the SE cells survive by entering VBNC state or not. Figure 1D shows confocal laser scanning micrographs of SE cells that had been incubated in M9 medium containing either 0.4% or 0.8% glucose, and the number of CTC-positive cells is shown in Fig. 1E. As shown in Figs. 1D(i—iii) and E, almost all of the cells in M9 containing 0.4% glucose were respiratory active until 48 h, with good accordance with their culturability (Fig. 1A). However, cells in M9 containing 0.8% glucose showed quite different results; i.e., they maintained their respiratory activity at an even higher level at 24 h and 38 h (81% at 24 h, 62% at 38 h: Figs. 1D(v, vi), E) than would have been expected from their culturability, which was drastically reduced (0.0024% at 42 h: Fig. 1A). At 48 h, although they had lost their culturability almost entirely (Fig. 1A), 11% of them were still active in respiration (Figs. 1D(vii), E). These results suggest that the twice higher amount of glucose in M9 medium induced majority of the SE cells to enter the VBNC state at 38 and 48 h (indicated by arrows in Fig. 1E) and subsequent cell death. We also examined this effect on another species of Salmonella, S. Typhimurium, and obtained similar survival curves concerning CTC-reduction and cfu (data not shown). Therefore, the induction of VBNC by 0.8% glucose in M9 medium was not specific to S. Enteritidis Cl#15-1. Thus, further experiments in this study were performed by using S. Enteritidis Cl#15-1.

We next tested the effect of the successive addition of glucose to the cultures. Since the rate of glucose consumption by the logarithmic SE cells in M9 medium containing 0.4% glucose was 0.5 mg/mL/h (data not shown), the amount of glucose to be added was set at 12 mg/mL daily. As shown in Figs. 2A and B, the successive addition of glucose showed similar effect on the growth of SE cells as was observed for the prolonged culturing with 0.8% glucose; i.e., this excessive amount of glucose resulted in the almost complete loss of the

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**Fig. 2. Effect of Successive Addition of Glucose on the Culturability and Glucose Concentration in SE Cell Cultures**

Culturability (A), bacterial cell density (B), and glucose concentration in the medium (C) are shown. Incubation was performed by either the batch (○) or fed-batch (□) method, as described in the text. Results are shown as the means±S.D. for 3 independent experiments (*\(p<0.005\) and **\(p<0.001\) vs. batch method, Student’s \(t\)-test). N.D. means “not detected.”

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**Fig. 3. Effect of Spent M9 Media on the Culturability and the Respiratory Activity of SE Cells**

Confocal laser-scanning micrographs of the cells stained with CTC/DAPI (A), summary of the number of CTC-positive cells (B), and culturability (C) are shown. Incubation was performed either in the 0.4 spent M9 (□), the 0.8 spent M9 (△), or normal M9 medium (■) as the control. The viable cells with CTC-reducing activity were stained in red (arrows in i, iv, vii); and total cells, incubated with DAPI, are stained in blue (arrowhead in ix). Dead cells correspond to the cells stained with DAPI but without CTC reduction; namely, they appear in blue alone. Arrows in (B) represent that cells seem to be in the VBNC state. Results are shown as the means±S.D. for 3 independent experiments (*\(p<0.001\) vs. either M9 or 0.4 spent M9, Tukey–Krammer’s post hoc test). N.D. means “not detected.”
culturability of SE cells after the cells had grown transiently to 9.3 logs. The residual amount of glucose in the medium was increased by the daily supplementation with glucose, also suggesting again the cessation of glucose metabolism by SE cells (Fig. 2C). To ascertain that the cytotoxicity of glucose was mediated by the accumulation of glucose metabolites in the medium but not in the cells, we also performed supplementation with glucose to SE cells grown to 9.3 logs after overnight culture, not by the addition of 12 mg/mL glucose to the culture, but by replacement of the culture medium with fresh M9 containing 0.4% glucose. After separation of the cells from the culture medium by centrifugation, the cells in these cultures showed no cell damage (data not shown), suggesting that the accumulation of glucose metabolites in the medium per se, but not that in the cells, seemed to be responsible for the induction of the cytotoxicity.

Induction of SE to the VBNC State and Subsequent Cell Death by Glucose Metabolites

The results in Figs. 1 and 2 suggest that the addition of excessive glucose might have induced the VBNC state (Fig. 1) and subsequent cell death through mal-metabolism of glucose (Figs. 1, 2). To validate this possibility, we examined the effects of the spent M9 media, i.e., the culture supernatant from SE cells grown for 24 h in M9 containing 0.4% glucose (0.4 spent M9) or 0.8% glucose (0.8 spent M9).

Figure 3 shows the effect of the spent M9 medium on the culturability of the SE cells. Fresh M9 medium and the 0.4 spent M9 showed no significant effect on the CFU of the cells. However, the 0.8 spent M9 decreased it immediately and significantly; i.e., the fresh, intact SE cells rapidly lost the mass of their culturable population, which dropped from 9.2 logs to 2.0 logs during incubation with the 0.8 spent M9 medium for 24 h. This decrease was not observed at 4°C (data not shown), suggesting that the uptake and subsequent cellular metabolism of certain glucose metabolites in the 0.8 spent M9 might have compelled the intact SE cells to lose cell integrity rapidly and thus to enter the non-culturable state.

Figures 3A(i–ix) are confocal laser scanning micrographs of SE cells that had been incubated in fresh M9, the 0.4 spent M9 or the 0.8 spent M9 medium and the number of CTC-positive cells is shown in Fig. 3B. The results in Figs. 3A (i–vi) are in good accordance with those of the unchanged CFU (Fig. 3C), showing that these SE cells maintained both respiratory activity and colony-forming activity. In contrast, SE cells cultured in the 0.8 spent M9 showed a gradual reduction in, but yet high level of, the respiratory activity up to 6 h from the start of the culture (92.6±2.1% to 71.3±6.4%, Figs. 3A(vii, viii), B); although their culturable population declined more rapidly with time (9.2 logs to 5.9 logs by 6 h, Fig. 3C).

These results suggest that intact SE cells rapidly lost a greater number of their culturable population than their respiration-active population when exposed to the 0.8 spent M9 medium, probably due to some toxic substances derived from glucose metabolism in the 0.8 spent M9 medium that had much more severe effects on cell growth and/or cell division than on respiration, thus resulting in the induction of the VBNC state in the cells (indicated by arrows in Fig. 3B).

As for the difference between the results from the colony-formation assay and CTC-reduction assay at the time point of 24 h, it seems worthy of note that the numbers of the bacterial cells observed were different between the plating method (10⁹ cells with no dilution) and the direct counting method (10⁷ cells serially diluted from 10⁹ cells on the slide and directly counted by microscopy, at least 100 cells per field) and that there might be a population of CTC-negative (“non-viable”) and colony-formation-slightly-positive (“culturable”) cells.

Analysis of Glucose Metabolites in 0.8 Spent M9 Medium

Before analysis of glucose metabolites in both the 0.4 and the 0.8 spent M9 media, time-dependent changes in the pH of the SE cultures were assessed.

The pH values of each spent M9 medium decreased from the start of the culture, with the pH of the 0.4 spent M9 decreasing to pH 5.8–5.9 and that of the 0.8 one dropping to pH 4.7–4.8 by 72 h (Fig. 4A). Therefore, the extent of the decrease in pH seemed to depend on the amount of glucose in the culture medium at the beginning, suggesting that the generation of a large amount of organic acids from glucose might have been involved in the toxicity toward SE cells.

Analyses of glucose metabolites in the spent M9 medium were performed by HPLC. The results showed remarkable changes in formate and pyruvate (identified by LC/MS; Supplementary Fig. S1) in these spent media; and the changes in the amounts of formate, pyruvate, and acetate in both 0.4 and 0.8 spent M9 showed different kinetics (Figs. 4B to D). The 0.8 spent M9 contained large amounts of pyruvate (15–17 mM), whereas the 0.4 spent M9 did not (Fig. 4B). Acetate in the 0.4 spent M9 increased time-dependently (13 mM at 18 h and 20 mM at 72 h), which degree of increase was not seen in the...
whereas it increased rapidly in the 0.8 spent M9 up to 9 mM by 24 h, and decreased to 4 mM by 72 h (Fig. 4D). These results imply that after glucose metabolism to pyruvate, acetate was formed normally in M9 containing 0.4% glucose by 72 h, which formation was impaired in M9 containing 0.8% glucose. Instead, formate as well as pyruvate was formed at higher levels. At the 18-h time point, culturability was not so much different between M9 containing 0.4% and 0.8% glucose (Fig. 1A). However, after 24 h, formate was produced rapidly in M9 containing 0.8% glucose, and it remained at high levels up to 48 h.

The rapid and high-level production of pyruvate in M9 containing 0.8% glucose was remarkable throughout the incubation up to 72 h. These results imply that mal-metabolism of pyruvate in SE cells exposed to 0.8% glucose might have been involved in the induction of loss of cell culturability. Besides, the pH decrease, as well as the release of formate in the early stage of incubation of SE cells in M9 containing 0.8% glucose, might have caused the loss of cell integrity. To examine this possibility, we examined the effect of pyruvate/formate and medium pH on the biological activity of SE cells. Since the pH of the 0.8 spent M9 decreased to 4.7 by 24 h, whereas that of the 0.4 spent M9 dropped to 5.9 (Fig. 4A), the effects of formate and pyruvate on SE cells were tested at these pH's.

Induction of the Cytotoxicity by Combination of Low pH and Formate

As shown in Figs. 5A, B, the cfu's of the SE cells dropped dose-dependently in the presence of formate at pH 4.7, whereas no decrease was observed at pH 5.9; but they increased slightly at both low and high pH's in the presence of pyruvate up to 10 mM. These results suggest that some of the pyruvate had been consumed as a nutrient, since the \( \text{OD}_{590} \) value increased during the incubation at both low and high pH's (data not shown); although the pyruvate appeared to be toxic for the SE cells at excessive concentration (30 mM; much higher amount than actually detected in 0.8 spent M9) at pH 4.7. These results suggest that the combination of both low pH and a high concentration of formate led to the toxicity toward SE cells, probably through formation of non-ionic form of formate.

We also assessed the respiratory activity of the cells that had been treated with 1–10 mM formate, and found that few cells were positive for CTC reduction after the addition of 1 mM formate and that almost all cells were CTC reduction negative at concentrations of formate higher than 3 mM after incubation for 24 h at pH 4.7 (data not shown).

In general, non-ionic substances easily cross the cell membrane by simple diffusion and ionic substances do not.\(^ {25} \) Since the \( pK_a \) value of formic acid is 3.75,\(^ {26} \) we expected that much more formate might be incorporated into the SE cells under low pH condition, leading to the bactericidal effect. To ascertain the hypothesis for the mechanism of the loss of culturability, we examined incorporation of \([^{14}C]\) formate together with changes in culturability during short term incubation. The culturability decreased rapidly by 5 min (Fig. 5C), while \([^{14}C]\) formate was not significantly incorporated into cells by these incubations at pH 4.7 (Fig. 5D), contrary to our expectation. These results show that formate exerts rapid cytolytic effects at pH 4.7, but also imply that the amount of formate incorporation itself into the cells does not substantially correlate with the cytolytic effects.

DISCUSSION

In this study, we showed that an excessive amount of glucose, 0.8%, in M9 medium induced the VBNC state and subsequent cell death in SE cells caused by the accumulation of large amount of glucose metabolites. Although SE cells grew in M9 medium containing 0.8% glucose similarly as in M9 medium containing 0.4% glucose, by 18 h they rapidly underwent a decrease in their culturability by entering the VBNC state and then died (Fig. 1). This decrease does not seem to have been simply due to overactive metabolism of glucose in the cells at the stationary phase but rather to the accumulation of glucose metabolites in the high-glucose medium. Supporting this idea, successive supplementation with glucose by the fed-batch method had an effect on cultures grown in M9 con-
taining 0.4% glucose similar to that of the high-glucose medium (Fig. 2). In addition, the 0.8 spent M9 also had similar VBNC-inducing as well as cytotoxic effects as the M9 containing 0.8% glucose (Fig. 3). These results support the idea that excessive amounts of glucose in M9 cultures leads to the accumulation of glucose metabolites that would be responsible for these phenomena.

Focusing on the induction of the VBNC state, it should be noted that we defined the VBNC state as a “respiratory active but non-culturable” state in this study, which could be estimated quantitatively in terms of CTC-reducing activity and colony-forming activity, respectively. The CTC-reduction assay is based on the activity of the electron-transport system of bacterial cells, which activity would be critical to supply energy to the viable cells. We showed that the SE cells treated with M9 containing 0.8% glucose (Fig. 1) or with the 0.8 spent M9 (Fig. 3) showed remarkably decreased culturability but little loss of respiratory activity at early incubation times. This difference between culturability and respiratory activity might have been due to differential susceptibility to the stresses, as we showed in the preceding paper, which reported that macromolecule (DNA, protein) synthesis is more sensitive than enzyme activity concerning energy metabolism. Although we did not examine macromolecule synthesis in this present study, incubation in M9 containing 0.8% glucose or in the 0.8 spent M9 might have resulted in lower activity of macromolecule synthesis than in lower respiratory activity.

It is known that digestible sugars can cause metabolic inhibition by acidification, such as mixed-acid fermentation (normally observed under anaerobic conditions) and glucose overflow metabolism. Cerca et al. reported that Staphylococcus epidermidis enters the VBNC state in biofilms as a consequence of the accumulation of acidic metabolites due to excessive glucose metabolism; and several other reports showed that acetate accumulates in aerobic cultures due to the overflow of glucose metabolism. Our study also obtained similar results: excessive glucose metabolism in 0.8% M9 medium led to the accumulation of large amounts of acidic compounds such as acetic acid, resulting in a sudden decrease in pH, probably due to the overwhelming of the buffering effect of M9 medium (Fig. 4). Contrary to the reports from other laboratories, our results suggest that formate, not acetate, would be the most feasible to induce the VBNC state as well as the cytotoxic effects at an acidic pH of around 4.7. However, it still remains unknown at the molecular level as to how formate and pyruvate were generated in the SE cells in our experiments, although there are some reports showing that formate is normally produced under anaerobic conditions. Formate can cause reactive oxygen species in yeast and mammalian cells leading to cell death. In Gram-negative bacteria, it is generated by pyruvate-formate lyase (PFL), a radical-containing enzyme in its active form, during anaerobiosis or under poor aeration in production-scale aerobic bioreactors. In contrast, our preliminary experiment showed that this bactericidal effect was observed even in flask cultures (Erlenmeyer flasks) under aerobic conditions obtained by shaking, suggesting that formate seems to have been generated even under aerobic conditions in our study (data not shown). Under some aerobic condition, it might be due to the functional PFL, being repaired by YfID protein under low pH and functions as a substitute glycyl radical domain to repair oxygen-induced damage to the PFL glycyl radical.

However, the precise concentration of dissolved oxygen in the culture system is under investigation in our laboratory.

Focusing on pyruvate accumulation, Ojima et al. reported that an imbalance in the redox status could trigger the accumulation of pyruvate in Escherichia coli by the overexpression of formate dehydrogenase H (FdhH), regenerating NAD⁺ from NADH. The results in their report seem to provide a clue to a better understanding of our present findings, by implying that excessive metabolism of glucose leads to such an imbalance in the redox status.

On the other hand, it has been reported that the secretion of pyruvate occurs in mammalian cells to protect them from oxidative stress. We previously showed that pyruvate has an important role in the resuscitation of SE cells from the H₂O₂-induced VBNC state, probably through reactivation of macromolecule synthesis. It might be feasible that SE cells, when cultured with high glucose, might secrete pyruvate as a protective agent against such acidic stress. However, our preliminary experiment did not seem to support a protective effect of pyruvate against cytotoxic effects of formate under low pH conditions (data not shown). It might be due to the difference in stress response between oxidative (H₂O₂) and metabolic (low pH and formate) stress. Further study is needed to reveal the metabolic pathways responsible for pyruvate accumulation in M9 medium containing 0.8% glucose.

Besides, the toxicity of acetate seems to have contributed far less to the lethality of the 0.8 spent M9 than that of formate, since our preliminary experiment indicated that the addition of acetate at the same amount as formate resulted in only scarce toxicity at pH 4.7 (data not shown).

Taken together, our findings indicate that a glucose concentration in M9 medium twice as high as the normal one (0.4%) would be enough to induce mal-metabolism of glucose in SE cells, causing the bacteria to proceed from the VBNC state to cell death, probably through the generation of formic acid under acidic conditions due to the overwhelming of the buffering effect of M9 medium. Formic acid might rapidly damage the cell membrane by its aldehyde group, probably by making conjugate with amino residue of phosphatidylethanolamine although the precise mechanisms remain to be elucidated. Further studies are required to understand the precise mechanism of the induction of the VBNC state by glucose metabolism in order to lessen the risk of a threat to food safety by pathogenic VBCN cells, because VBCN Salmonella has the possibility to resuscitate and convert into viable, reproductive and pathogenic one under certain conditions.

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