Mem. Fac. Sci. Shimane Univ.,
20, pp. 77–85 Dec. 20, 1986

Autolysis induced by salts in a mutant of *Bacillus subtilis*

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(Received September 6, 1986)

Cells of *Bacillus subtilis* mutant 1508C were lysed by sodium ion concentrations above 0.1%. The wild strain was not affected by these conditions. Monovalent cations such as K^+, Li^+, and Cs^+ also were effective in lysis of the mutant cells. On the other hand, divalent cations such as Ca^{2+}, Mg^{2+}, and Mn^{2+} protected the cells from the lytic action of Na^+. Na-induced lysis was inhibited by the addition of antibiotics such as kanamycin or chloramphenicol, while antibiotic; D-cycloserine (an inhibitor of bacterial cell wall synthesis) did not inhibit the lytic process. Autolysis by NaCl occurred even in cells which were osmotically protected with 0.8 M sucrose, while the protoplasts derived from the mutant strain were not lysed by the addition of NaCl under same osmotic protection. These results suggest that the rapid lysis of mutant 1508C induced by NaCl is due to the action of autolysin(s).

Introduction

The lytic process of bacterial cells depends on mechanically resistant cell wall (peptidoglycan layer) that give osmotic protection (Epstein & Schultz, 1968; Ghuysen, 1977). Autolysis occurs when endogenous peptidoglycan hydrolases (autolytic enzymes) are activated. In autolysis, different factors, such as growth condition, osmotic environment and pH, have been recognized as being particularly important (Shockman, 1965). However, the conditions leading to autolysis remains obscure.

In a previous paper (Onoda *et al.*, 1985), we showed that growth of *Bacillus subtilis* mutant 1508C was inhibited when grown in nutrient medium containing NaCl (5% w/v). Very similar observations have been made in study of the sensitivity of *Bacillus subtilis* (Iijima *et al.*, 1969) and *Escherichia coli* (Sato *et al.*, 1971) to saline. In those saline-sensitive phenomena, bacteria cells lost their colony-forming ability in 0.15 M NaCl. Therefore, futher studies were undertaken to clarify the Na-sensitivity. We found that NaCl at a concentration of more than 0.1% rapidly lysed the mutant 1508C. Saline sensitive autolysis were also reported with *Staphylococcus aureus* (Gilpin *et al.*, 1972) and *Clostridium* species (Ogata & Hongo, 1973).

In this paper, we describe characters of cellular lysis of mutant 1508C of *Bacillus subtilis* which induced by NaCl. Our findings should be compared with reports of other instances of saline sensitivity.

Materials and Methods

Strains and growth conditions

Bacillus subtilus strain RIMD and mutant 1508C was used in this experiment. Strain 1508C, obtained from wild strain RIMD, have already been described (Onoda *et al.*, 1985). Cells were grown in nutrient broth at 37°C in a rotary shaker. Cell growth and decrease of turbidity by lysis were monitored by measuring the optical density at 600 nm (OD_{600}) by a Hitachi model 100–10 spectrophotometer.

Autolysis procedure

Cells were harvested at the exponential phase by centrifugation at 1,500 × g for 15 min. Washed twice with distilled water and then, resuspended in the appropriate autolysis solution to the optical density of 1.00−2.00 cm^{-1}. The cell suspension was incubated at 37°C with shaking and the samples (1 ml) were collected at 30 min. intervals. After 3-fold dilution with distilled water, OD_{600} was measured. The autolytic activity was calculated as the percent decrease of OD_{600} at the 0 time value.

Preparation of protoplast

The cells growing exponentially was harvested, washed and resuspended in the solution containing 10% of mannitol and 200 μg/ml lysozyme. After incubation at 37°C for 1.5 hr, the protoplasts were harvested by centrifugation at 9,000 × g for 10 min.

Binding of Fl-ConA to cell walls

The cells growing at mid-logarithmic phase were harvested and washed with cold buffer A containing 50 mM Tris-HCl (pH 7.4), 10 mM $CaCl_2$ and 1 mM $MgCl_2$. The cells were resuspended in the same buffer at the concentration of 0.3 (OD_{600}). 0.45 ml volume of Fl-ConA (ca. 0.45 mg) was added to 100 ul of buffer A or buffer A plus α-methyl-*D*-mannoside (final conc. 0.05 M), followed by the addition of 0.45 ml cell suspension (90 μg dry wt.). Mixture was incubated for 30 min. at 4°C, washed three times with buffer A, and resuspended in 2 ml of buffer A. Fluorescence was measured by Hitachi 850 fluorescence spectrophotometer. Exciting spectrum was 494.4 nm (slit range 5 nm) and emission spectrum was 524.8 nm (slit range 5 nm).

Chemicals

Peptone, Erlich meat extract were obtained from Kyokuto Pharmaceutical Industrial Co., Tokyo. Kanamycin was obtained from Meiji Seika Kaisha, Ltd. D-Cycloserine was obtained from Aldrich Chemical Company, Inc. ConA FITC was purchased from E.Y Laboratories, Inc. α-methyl-*D*-mannoside was obtained from Nakarai Chemicals, Ltd. Chroramphenicol was obtained from Wako Pure

Chemical Industries, Ltd. All other reagents used were of analytical grade.

Results

Effect of NaCl on autolysis

Autolysis of mutant 1508C cells was observed when suspended in nutrient medium containing NaCl at final concentration of 5%. To examine lytic effect of NaCl, following experiments were performed. The cells harvested in the mid-logarithmic phase were suspended in distilled water or a NaCl solution containing NaCl at various concentrations, and the cultures were reincubated at 37°C. Then, turbidity of the culture was monitored by optical density (OD_{600}). As shown in Fig. 1a, the turbidity did not decrease during incubation of 2 hr when the cells were suspended in distilled water alone. However, the remarkable reduction of the turbidity was observed when suspended in 0.4% NaCl solution. Optimal concentration of NaCl on autolysis was then examined. The mutant cells lysed rapidly when suspended in 0.1% NaCl solution (Fig. 2). Addition of 0.2% NaCl caused more rapid lysis of the mutant cells and then, the turbidity dropped to ca. 20% of the control for 2 hr incubation. On the other hand, the parent strain was not affected by these conditions (Fig. 1b and 2). The initial rate of autolysis exhibited similar value at temperature in the range between 27°C and 42°C. The amounts of UV-absorbing materials released from the NaCl-treated cells were examined. A portion (4 ml) of the lysis solution was removed at indicated intervals and absorbance of 260 and 280 nm in the supernatants were spectrophotometrically estimated. Fig. 3 shows that the relative absorbance of these materials increased with increasing time of incubation.

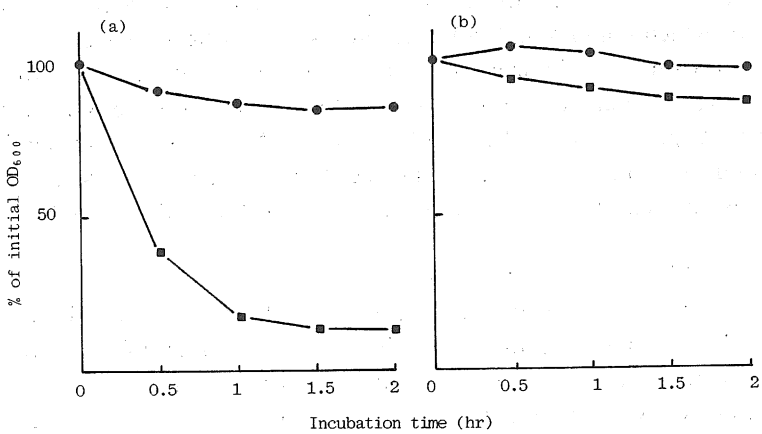

Fig. 1. Autolysis was assayed at 37°C as descrived in Materials and Methods. (a) mutant 1508C, (b) wild type. Autolysis was induced by: (●) distilled water, (■) 0.4% NaCl.

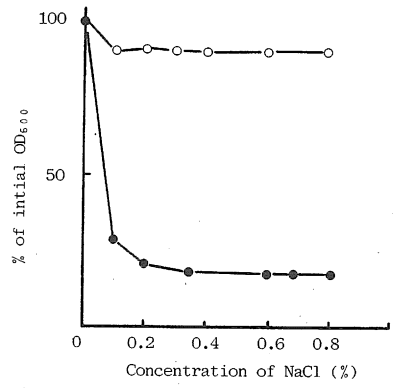

Fig. 2. Various concentration of NaCl was added to the cell suspension. After 2 hr incubation, autolysis was assayed. (○) wild type, (●) mutant 1508C.

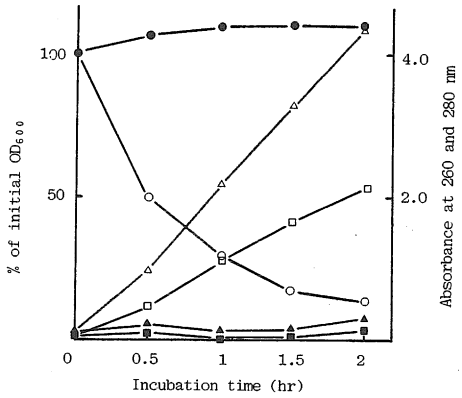

Fig. 3. Harvested cells were resuspended in 0.8 M sucrose with or without 0.6% NaCl. At indicated intervals, 10 ml of the cell suspension was collected and optical density of 600 nm was measured and then, a part of the suspension was centrefuged and the optical density, 260 and 280 nm, of the supernatant was measured, respectively. Symbols: (●) sucrose (OD_{600}); (■) sucrose-(OD_{280}); (▲) sucrose (OD_{260}); (○) sucrose+NaCl (OD_{600}); (□) sucrose+NaCl (OD_{280}); (△) sucrose +NaCl (OD_{260}).

Effect of NaCl to protoplasts

When *Staphylococcus aureus* mutant tar-1 was suspended in medium containing 1.0 M NaCl, the spheroplasts were extruded from hydrolyzed fragments of cell walls (Gilpin *et al.*, 1972). To test this possibility, 0.4% NaCl was added to the mutant cell suspension, which containing 0.8 M sucrose as a osmotic stabilizer. In spite of the presence of osmotic stabilizer, similar enhancement of autolysis by NaCl was observed (Fig. 4). Further, we tested the effect of NaCl on the lysis of protoplasts. Protoplasts prepared as described in Materials and Methods were suspended in 0.8 M sucrose solution which gave osmotic protection and then, 0.4% NaCl was added to the suspension. The protoplasts were not lysed in the addition of NaCl.

Effects of various salts on autolysis

There were specificity in the type of salt which induced lysis of the mutant (Table 1). Monovalent cations, such as NaCl, KCl, LiCl, and CsCl were effective to induction of the autolysis, whereas divalent cations, such as $CaCl_2$, $MgCl_2$ $FeCl_2$, $MnCl_2$ and $CuCl_2$ were ineffective. The relative effects of the salts were in the order: NaCl>CsCl>LiCl>KCl with autolysis of the mutant. Mg^{2+} or Ca^{2+} ion is known to protect bacteria from NaCl-induced lysis (De Voe & Oginsky, 1969; Gilpin *et al.*,

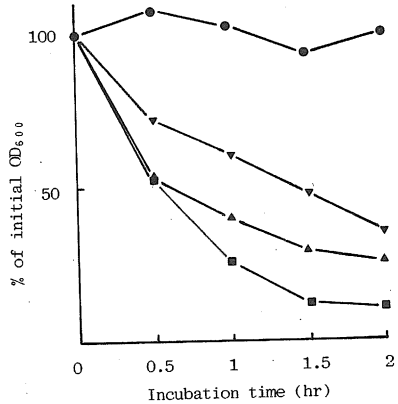

Fig. 4. Effect of NaCl on autolysis of mutant 1508C suspended in 0.8 M sucrose. Mutant cells were harvested and suspended in (●) 0.8 M sucrose alone, or 0.8 M sucrose containing (▼) 0.2%, (▲) 0.4%, (■) 0.6% NaCl, respectively. Autolyis was assayed at 37°C as descrived in Materials and Methods.

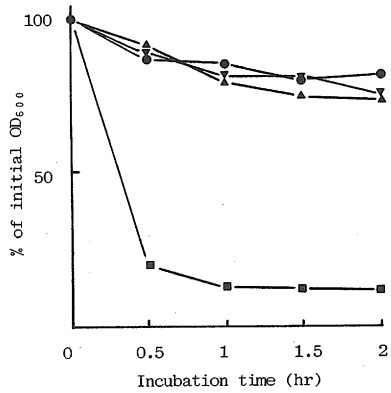

Fig. 5. Inhibition of autolysis of mutant 1508C by $MgCl_2$ and $CaCl_2$. Mutant cells were harvested and resuspended in (●) D. W., (▼) 0.4% NaCl+100 mM $MgCl_2$, (▲) 0.4% NaCl+100 mM $CaCl_2$, and (■) 0.4% NaCl alone. Autolysis was assayed at 37°C as descrived in Materials and Methods.

Table 1. Effect of salts on autolysis of *B. subtilis* mutant and wild strains.

Addition of salts	% of initial turbitity at 2 hr	
	Mutant	Wild
NaCl	14.6	84.8
KCl	31.5	86.6
LiCl	16.9	86.1
CsCl	16.3	86.0
$CaCl_2$	92.1	98.7
$MgCl_2$	84.7	98.5
$FeCl_2$	100.0	100.0
$MnCl_2$	100.0	100.0
$CuCl_2$	100.0	100.0

The mutant and wild strains in mid-logarithmic phase were harvested and suspended in distilled water containing salt at final concentration of 0.4%, respectively. After 2 hr incubation, % of initial OD_{600} was assayed.

1972; Ogata & Hongo, 1973). A similar protective effect was noticed in autolysis of the mutant when $MgCl_2$ or $CaCl_2$ was added to 0.4% NaCl solution at final concentration of 100 mM (Fig. 5).

To determine whether this lytic inhibition by Mg^{2+} was reversible, $MgCl_2$ was added to the cell culture for 60 min. at 37°C before NaCl treatment, washed twice

with 0.8 M sucrose, and incubated at 37°C in lysis solution. A 50% decrease in turbidity was noted during 2 hr of incubation. A similar protective effect was observed with Ca^{2+}.

Effect of antibiotics on autolysis

The effect of various antibiotics on the rate of autolysis was examined. The cells were grown to mid-logarithmic phase in nutrient broth medium, and antibiotics (kanamycin: 10 μg/ml; chloramphenicol: 50 μg/ml; D-cycloserine: 160 μg/ml) were added at two fold concentration of minimal inhibitory concentration (MIC), for 30 min. before harvesting, respectively. The harvested cells were then suspended in standard lysis solution, and the rate of autolysis was determined. D-cycloserine-treated cells rapidly lysed in the presence of NaCl, as well as untreated cells, whereas kanamycin-treated cells lysed slower than untreated cells (Fig. 6). Chloramphenicol also gave similar result to kanamycin.

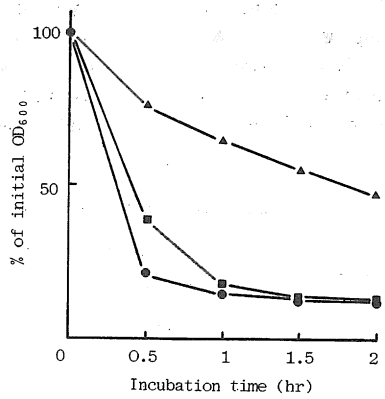

Fig. 6. Effect of antibiotics on autolysis of mutant 1508C. Antibiotics; kanamycin (10 μg/ml), and D-cychloserine (160 μg/ml) was added respectively to culture for 30 min before harvested. Then, harvested cells were resuspended in 0.4% NaCl solution. Autolysis was assayed at 37°C as descrived in Materials and Methods. Symbols; (▲) kanamycin, (●) D-cychloserine, and (■) not treatment.

Interaction between Fl-ConA and cell wall

Since lipoteichoic acids are known to inhibit autolysis of isolated walls of *Streptococcus faecalis* (Cleveland *et al.*, 1975, 1976), quantative distribution of teichoic acid on cell walls of the mutant was compared with that of the wild strain. Fluorescein-conjugated concanavaline A (Fl-ConA) which interacts specifically with the teichoic acid (Doyle, R. J, *et al.*, 1972, 1975), was used to determine the distribution of α-*D*-glucosylated teichoic acid on the surface of growing cells. Fl-ConA was added at a final concentration of 0.45 mg/ml to cell suspension in the

presence or absence of α-methyl-*D*-mannoside, a potent inhibitor of ConA-polysaccharide complexes, and fluorescences of Fl-ConA binding to cell walls were determined in wild and mutant 1508C. A fluorescence efficiency in Fl-ConA-wall complexes were approximately equal in both the strains (Table 2). On the other hand, when the inhibitor was added to Fl-ConA before formation of the Fl-ConA-wall complex, similar decrease of the fluorescence efficiency were observed in both the strains. In addition, when Fl-ConA-wall complexes were washed with α-methyl-*D*-mannoside, both the strains lost ca. 30% of the fluorescence by washing with the inhibitor.

Table 2. Binding of Fl-ConA to cell walls of *Bacillus subtilis* mutant and wild strains.

compounds supplemented	Fluorescence efficiency (unit)*			
	mutant	% of control	wild	% of control
Fl-ConA	1.18	100	1.13	100
Fl-ConA + α-methyl-*D*-mannoside	0.77	65	0.64	57

* 1 unit = Fl-ConA 1 μg/ml = 0.56

Discussion

In the present paper, induction of autolysis by NaCl on the mutant 1508C of *Bacillus subtilis* were studied. Conditions leading to cell lysis have been investigated in a number of gram-positive and negative bacteria. Lysis induced by sodium ion has been reported in *Clostridium species* (Ogata & Hongo, 1973 and 1974), and *Staphylococcus aureus* (Gilpin *et al.*, 1972). Minimal NaCl concentration which was required for lysis of *Bacillus subtilis* mutant 1508C were much lower than those of *Clostridium* species and *Staphylococcus aureus*. Sodium chloride could be replaced by potassium, cesium, or lithium chloride on induction of the cell lysis of mutant 1508C. This is similar to the result shown by Ogata and Hongo with *Clostridium*. Presence of divarent cations such as Mg^{2+} Ca^{2+}, Cu^{2+} Mn^{2+}, and Fe^{2+} has a protective action on NaCl-induced lysis, resembling to the result shown by Ogata and Hongo. However, the order of protective effect by salts was not concident. Leduc and Heijenoort (1980) have suggested that the release of Mg^{2+} or Ca^{2+} from cell walls may play a key role in the triggering of autolysis. Our result that inhibition of lysis by Mg^{2+} were remained ever after washing seem to support a possibility that Mg^{2+} united to its binding sites on cell envelope and could not remove entirely by washing.

The cells of *Escherichia coli* (Sato *et al.*, 1971) at the logarithmic phase of growth lost their colony-forming ability during incubation in saline, while the colony-forming ability was recovered rapidly when NaCl-treated cells were incubated in Tris buffer

containing $MgCl_2$. Lytic reaction of mutant 1508C resemble this saline-sensitive phenomena in some respects that were induced by monovalent cations and inhibited by divalent cations. In those phenomena however, injured cells were capable of reverting. In the case of salt-induced autolysis of *Staphylococcus aureus* shown by Gilpin *et al.*, (1972), in the presence of osmotic protection of NaCl, the spheroplasts were extruded from hydrolysed fragments of cell walls and autolysis was induced by dilution in distilled water. In our study, amounts of protein and nucleic acid released from cells exposed to NaCl increased with increasing time of incubation. During this procedure, lysed cells were observed by phase contrast microscopy. Futher, addition of NaCl lead to complete lysis of intact cells in the mutant even when the mutant cells were protect osmotically in the presence of sucrose (0.8 M). However, no lysis of protoplasts was induced by addition of NaCl, suggesting that autolysin(s) involved in cell walls was activated by salts and may act on not only walls but cell membrane.

The cell walls of *Bacillus subtilis* containes two major macromolecular species, peptidoglycan and polyglycerol phosphate teichoic acid. Teichoic acid, specific cell envelope components, have been found to play a critical role in the regulation of autolysis (Tomasz & Holtje, 1977). If the contents of teichoic acid in mutant 1508C were more less than that in wild strain, induction of autolysis might be explained by these factors. However, fluorescence of Fl-ConA which bound to cell wall were approximately equal in both the strains. This result suggest that the distribution of teichoic acid on the cell surface in mutant 1508C was similar to that in wild strain.

Cell lysis induced by sodium ion in *Clostridium saccharoperbutylacetonicum* and *Staphylococcus aureus* were inhibited by treatment with antibiotics such as chloramphenicol. Cell lysis of mutant 1508C was inhibited by antibiotics known to impare autolysin; kanamycin and chloramphenicol, thus, suggesting that an autolysin(s) is concerned in autolysis of the mutant cells. However, the role of Na ion remains obscure and is being further investigated in our laboratory.

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