ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1990, p. 781–785
0066-4804/90/050781-05$02.00/0
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Vol. 34, No. 5

Lysis and Aberrant Morphology of *Bacillus subtilis* Cells Caused by Surfactants and Their Relation to Autolysin Activity

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Received 31 July 1989/Accepted 6 February 1990

The surfactants tested in this study lysed *Bacillus subtilis* 168 cells at the logarithmic growth phase. Results obtained with inhibitors and a mutant that had defective autolytic enzymes suggested that cell lysis resulted from the deregulation of autolysin activity. The addition of surfactants at sublytic concentrations produced twisted cells, filamented cells, or both. Autolysins extracted with 5 M LiCl from the cell wall fraction and lysozyme added to cells that were treated with surfactants restored the apparently normal cell rod morphology, suggesting that surfactants interfere with the role of autolysins in normal construction of the cell envelope. The rates of cellular autolysis and autolysin activity remaining in growing cells after exposure to a surfactant at a sublytic concentration decreased, although the rate of turnover of cell wall peptidoglycan was the same as that of control cells. Surfactants were suggested to interact with the regulatory system of autolysins and, thus, to affect the activities of autolysins in *B. subtilis* cells and to cause either morphological changes or cell autolysis, depending on the concentration of surfactants.

Surfactants have antimicrobial action and, thus, have been used for sanitation and decontamination in the food industry, homes, hospitals, and other environments. The mode of action of surfactants has not been explained in detail, although many reports have been presented (14, 22, 28). Deoxycholate (18), Triton X-100 (2), and cetyltrimethylammonium bromide (4) have been reported to have not only growth inhibitory actions but also lytic actions, in which cell autolysis seems to be involved. We have reported that fatty acids and their glycerol and sucrose esters cause autolysis of *Bacillus subtilis* cells (26, 27).

Autolysins have been reported to have roles in cell separation (10), flagellation (9), and cell wall turnover (3); and their activities are regulated during cell growth (3, 5, 25, 29). However, once bacterial cells are exposed to various treatments, such as low temperatures (19) and the addition of penicillin (25), monovalent cations at high concentrations (24), or some surfactants and fatty acids (2, 20, 26, 27), autolysins are deregulated to degrade the peptidoglycan structure. We have also observed that *B. subtilis* cells treated with fatty acids and their esters show a twisted shape, a filamentous shape, or both during subsequent growth (26, 27). It is likely that autolysin activity is related to the aberrant morphology caused by the compounds listed above, and, possibly, by other surfactants.

In this report, the results obtained with several surfactants on the lysis and altered morphology of cells are presented and the role of autolysins in these phenomena are discussed.

MATERIALS AND METHODS

Organisms and culture conditions. *B. subtilis* 168 *trp* cells were used in this study. In one experiment, a mutant of this strain, FJ2 *trp lyt*, which is defective in autolysin, *N*-acetylmuramyl-L-alanine amidase, and endo-β-*N*-acetylglucosaminidase activities (10), was also used. Cells were grown at 37°C in Spizizen salts supplemented with 0.5% glucose, 0.15% sodium glutamate, 5 μg of $MnCl_2$ per ml, and 20 μg of tryptophan per ml (26). When the optical density at 650 nm (OD_{650}) of the culture reached 0.25 in the logarithmic growth phase, a surfactant solution was added. The cells were cultivated in an incubator equipped with an automatic growth-recording apparatus (Ohtake Works, Ltd., Tokyo, Japan) (27), unless stated otherwise.

Microscopic observation and cellular autolysis. Cell morphologies were observed by phase-contrast microscopy. Cell autolysis was observed in 50 mM potassium phosphate buffer (pH 7.0) after the cells were harvested and washed, as described previously (27).

Extraction of autolysin and assay of autolysin activity. In the experiment with autolysin addition, cells were cultivated in a 2-liter flask containing 1 liter of the medium described above and were harvested at the late log phase. After washing with 50 mM Tris hydrochloride buffer (pH 8.0), the cells were broken with a French press. The cell wall fraction was obtained by repeated centrifugation (20,000 × *g*) for 20 min, three times with fresh buffer, and then with 0.5 M LiCl. Thereafter, the pellet was suspended in 5 M LiCl and kept at 0°C for 30 min to extract autolysins. After centrifugation (20,000 × *g*) for 20 min, the supernatant was dialyzed against 50 mM Tris buffer containing 0.5 M LiCl and concentrated by ultrafiltration through a UK10 membrane (Advantec; Toyo, Tokyo, Japan).

In the experiment in which we measured the autolysin activities of whole cells, the cells were grown to an OD_{650} of 0.25 after treatment with surfactant; and control cells were grown to the same OD_{650}, harvested, and washed by centrifugation (8,000 × *g*) for 5 min at 0°C. The resultant pellet was suspended in 5 M LiCl and kept at 0°C for 1 h. After centrifugation (8,000 × *g*) at 0°C, the supernatant was dialyzed and concentrated as described above. Autolysin activity was assayed as described elsewhere (24) by using [^{14}C]*N*-acetyl-D-glucosamine-labeled cell wall as a substrate. One unit of activity corresponded to the amount of enzyme that liberated 25% of the radioactivity in 30 min. Protein content was assayed by the method of Lowry et al. (15).

Turnover of cell wall peptidoglycan. Growing cells were pulse-labeled with *N*-acetyl-D-[^{3}H]glucosamine (0.7 μM, 5.25 GBq/mmol; Amersham Corp., Arlington Heights, Ill.) for 10 min at an OD_{650} of 0.235. Surfactant-treated cells were similarly labeled for 10 min after partial lysis and just before

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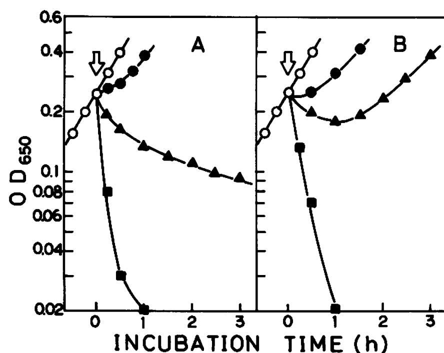

FIG. 1. Lysis of *B. subtilis* 168 cells induced by PTAI and Triton X-100. Surfactants were added at the times indicated by the arrows. (A) PTAI at 3 μM (●), 5 μM (▲), and 10 μM (■). (B) Triton X-100 at 70 μM (●), 105 μM (▲), and 150 μM (■). Control cells are also indicated (○).

subsequent regrowth to the same OD_{650}. Chasing was done by direct addition of nonradioactive *N*-acetyl-D-glucosamine at a final concentration of 10 mM without harvesting or washing of the cells. During chasing, samples (0.5-ml portions) were withdrawn at 20-min intervals and mixed with 0.5 ml of ice-cold 10% trichloroacetic acid containing 100 mM *N*-acetyl-D-glucosamine. After 30 min at 0°C, the mixture was filtered on a membrane filter (pore size, 0.45 μm; HA; Millipore Corp., Bedford, Mass.), and the filter was washed three times with 2 ml of 5% trichloroacetic acid containing 10 mM *N*-acetyl-D-glucosamine. The filters were placed in scintillation vials containing 10 ml of Scintisol EX-H (Wako Pure Chemical Industries, Osaka, Japan). The radioactivity was measured in a liquid scintillation counter (LS-7500; Beckman Instruments, Inc., Fullerton, Calif.).

Chemicals. The surfactants used throughout this study were Triton X-100 (Wako Pure Chemical Industries) and palmityltrimethylammonium iodide (PTAI; a gift of Hiroki Kourai, Tokushima University, Tokushima, Japan). For comparison, sodium dodecanoate (Nakarai Chemicals, Ltd., Kyoto, Japan), glycerol monododecanoate (99% purity; Riken Vitamin Co., Ltd., Tokyo, Japan), and sucrose monohexadecanoate (99%) purity, Mitsubishi Kasei Corp., Tokyo, Japan), which were used in previous studies (26, 27), were also tested. In some experiments, Tego 51 (a mixed preparation of dodecyl- and tetradecyl-diaminoethylglycines, a product of T. Goldschmidt AG, Essen, Federal Republic of Germany, obtained from Nippon Shoji, Japan), *N*-dodecylbetaine (prepared by H. Kourai), sodium dodecyl sulfate (Nakarai Chemicals), dodecylpyridinium chloride (Tokyo Chemical Industry, Tokyo, Japan), and dodecylglucoside (Boehringer GmbH, Mannheim, Federal Republic of Germany) were also used. As inhibitors, glutaraldehyde (Ohken Shoji Co., Ltd., Tokyo, Japan) and *p*-chloromercuribenzoic acid (Wako Pure Chemical Industries) were used. Bovine serum albumin and lysozyme were purchased from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Cell lysis caused by surfactants. Logarithmically growing cells of *B. subtilis* 168 were lysed by the addition of a variety of the surfactants that were tested. As representative examples, the results obtained with PTAI and Triton X-100 are shown (Fig. 1). At concentrations of 10 μM PTAI and 150 μM Triton X-100, the cells were rapidly lysed. To compare the lytic activities of various surfactants, we plotted the OD_{650}s of the cultures 1 h after exposure to a surfactant at various concentrations, which were expressed as a percentage of the initial OD_{650}, and determined the concentration required for 50% lysis. The lytic activity decreased in the following order (numbers in parentheses indicate the concentrations required for 50% lysis): PTAI (5.4 μM) > sucrose monohexadecanoate (12 μM) ≃ dodecylpyridinium chloride (15 μM) > Tego 51 (31 μM) > *N*-dodecylbetaine (72 μM) ≃ glycerol monododecanoate (78 μM) > Triton X-100 (101 μM) > sodium dodecyl sulfate (190 μM) > sodium dodecanoate (270 μM). This was roughly in the order of cationic, amphoteric, nonionic, and anionic surfactants, although there was a difference in the lengths of alkyl chains of the molecules. Sucrose monohexadecanoate had a stronger lytic action than was expected, given that it is nonionic.

Role of autolysins in surfactant-induced lysis. We investigated whether cell lysis induced with surfactants resulted from autolysis rather than the membrane-solubilizing effect, as occurs with dodecanoic acid (27), its glycerol ester, and sucrose monohexadecanoate (26). We examined the effects of 0.0025% glutaraldehyde and 1 mM *p*-chloromercuribenzoic acid on the lysis of *B. subtilis* cells caused by PTAI at 7.5 μM. Both agents inhibited cell lysis, with the extent of inhibition at 1 h after addition being 100 and 57%, respectively. Cell lysis of the autolysin-defective mutant FJ2 caused by PTAI at 6 μM was only 24% compared with 85% for the wild-type 168 strain after incubation for 1 h, indicating that this mutant is much less susceptible than the wild type. The same results for the inhibitors and the mutant were obtained with the other surfactants that were tested. These findings suggest that the surfactant-induced lysis of *B. subtilis* cells arises from autolysin activity rather than solubilization of the membrane.

Effects of sodium and potassium ions on lysis. Cell lysis caused by surfactants seemed to be enhanced by the presence of sodium and potassium ions in the growth medium, as was expected from our previous results that a high concentration of monovalent cations induces cell lysis (24). We therefore compared the level of lysis by surfactants in media containing various concentrations of sodium and potassium ions by reducing or adding these ions. As an example of the results that we obtained, PTAI at 6 μM caused only 68% lysis 1 h after its addition in a modified Spizizen salts medium, in which the total concentration of sodium plus potassium ions was decreased to 18 μM. However, 84 and 92% lysis occurred in ordinary Spizizen salts medium (240 μM as sodium plus potassium ions) and in medium containing 450 μM sodium plus potassium ions, respectively. The same results were also obtained with Triton X-100, dodecanoic acid, and glycerol monododecanoate (data not shown).

Morphology of cells growing after treatment with surfactants. PTAI, Triton X-100, and dodecylpyridinium chloride caused an aberrant morphology of *B. subtilis* 168 cells at sublytic concentrations, as do dodecanoic acid and glycerol monododecanoate (26). Similar results were also observed with 38 μM Tego 51, 50 μM dodecylbetaine, 135 μM sodium dodecyl sulfate, and 80 μM dodecylglucoside (data not shown). The concentrations that caused lysis of some cells and that allowed subsequent growth were used in further experiments. In other words, at these concentrations, the OD_{650} of the culture decreased and thereafter returned to the original OD_{650}, 0.25, which took 1 to 2 h after the addition of a surfactant. We observed microscopically samples taken from the culture that were incubated with a surfactant for 3 h, except for dodecylpyridinium chloride, in which cells

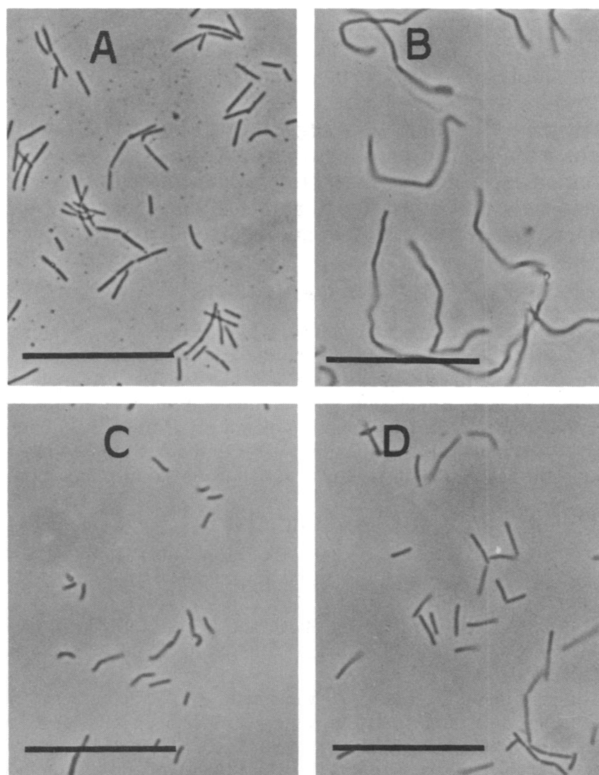

FIG. 2. Reversal effects of autolysin and lysozyme on the morphological change of *B. subtilis* 168 cells treated with Triton X-100 at 75 μM. (A) Control cells; (B) cells treated with Triton X-100 for 3.5 h; (C) cells incubated with 5 M LiCl extract for 1.5 h after treatment with Triton X-100 for 2 h; (D) cells incubated with 5 μg of lysozyme per ml for 1.5 h after treatment with Triton X-100 for 2 h. Bars, 40 μm.

were incubated for 5 h because there were no marked alterations in the cells for the first 3 h.

Among the surfactants tested, Triton X-100 (75 μM) and Tego 51 (38 μM) caused the most change in cell morphology, as glycerol monododecanoate does (26), and produced twisted, distorted, and elongated cells (Fig. 2B). Other surfactants produced short filaments of cells with little or no distortion; the degree of change varied with the concentration of the surfactant and the exposure time. Prolonged incubation allowed cells to regain their normal rod shape, probably because of dilution of the surfactant molecules which interacted with cells during cell growth.

Reversal effects of autolysins and lysozyme on cell morphology. To check whether either autolysins extracted from isolated *B. subtilis* cell wall preparations with 5 M LiCl or lysozyme added externally could restore cells to their normal morphology, we added these to the culture 1.5 h after the addition of 75 μM Triton X-100, at the time when the OD_{650} of the treated culture started to increase again. Since the concentration of proteins in the 5 M LiCl autolysin extract was 180 μg/ml, we also examined the nonspecific effects of protein using bovine serum albumin. Both the autolysin extract (37 U of *N*-acetylmuramyl-L-alanine amidase activity, 12 μg of protein in a total culture volume of 10 ml) and lysozyme (5 μg/ml) were found to reverse to normal the morphologies of cell that were treated with Triton X-100 (Fig. 2), although bovine serum albumin had no detectable effect when it was used at the same concentration as that of

TABLE 1. Autolysin activities of *B. subtilis* cells that regrew after treatment with surfactants[a]

Surfactant (concn [μM])	Sp act (U/mg of protein)	Total activity (%)[b]
None	12	100
PTAI (4.0)	137	97
Triton X-100 (75)	43	45
Dodecanoic acid (210)	49	74
Glycerol monododecanoate (63)	64	67
Sucrose monohexadecanoate (12)	45	40

[a] After the addition of surfactants, regrowing cells were harvested at an OD_{650} of 0.25. Autolysin was extracted with 5 ml of 5 M LiCl from the resultant cell pellet, and the extract was dialyzed against 50 mM Tris buffer (pH 7.0) containing 0.5 M LiCl, as described in the text.

[b] Untreated cells had a total activity of 22 units at an OD_{650} of 0.25, when cultivated in 200 ml of Spizizen salts medium.

protein (data not shown). Similar results were also observed with PTAI, dodecanoic acid, and glycerol monododecanoate (data not shown).

Cell autolysis, autolysin activity, and cell wall turnover of surfactant-treated cells. Since the results presented above suggest that the activity of autolysins is inhibited directly or indirectly by surfactants or, alternatively, that some of functional autolysin molecules may be released from cells by surfactants, we examined the ability of cells to autolyse in a buffer and autolysin activity on cells that grew after treatment with surfactants at sublytic concentrations. Exponentially growing cells harvested at an OD_{650} of 0.25 autolysed 69 and 81% after incubation in 50 mM potassium phosphate buffer (pH 7.0) for 30 and 60 min, respectively. On the other hand, cells that regrew after partial lysis caused by addition of the surfactants PTAI (4 μM), Triton X-100 (75 μM), glycerol monododecanoate (65 μM), or dodecanoic acid (210 μM) lysed much less in the buffer. In the latter, the degree of autolysis was 36, 34, 37, and 29% in 30 min and 54, 53, 45, and 48% in 60 min, respectively, for the four detergents.

Also, the total activities of autolysin in surfactant-treated cells generally decreased, although little reduction was observed with PTAI-treated cells (Table 1). On the other hand, the specific activities of autolysin extracted by 5 M LiCl from cells that regrew after treatment with surfactants greatly increased compared with the specific activity of autolysin obtained from untreated exponentially growing cells (Table 1). These results suggest that some autolysin still remained in cells even after treatment with a surfactant, although at least some was released from the cells, as indicated by the decrease in the total activity. However, autolysin release was relatively less than that of other envelope-associated proteins.

The rate of turnover of peptidoglycan in cells treated with the surfactants PTAI, Triton X-100, or glycerol monododecanoate was substantially identical to that of untreated cells, despite a slight increase in the apparent doubling time during subsequent regrowth.

DISCUSSION

The results obtained with *B. subtilis* cells treated with various surfactants in this study confirm that the cell lysis caused by surfactants is due to the deregulation of autolytic enzymes, as has also been suggested for fatty acids and their glycerol and sucrose esters (26, 27). The data obtained from experiments with the inhibitors glutaraldehyde, *p*-chloromercuribenzoate, and erythromycin and an autolysin-defective mutant support the above hypothesis. The result

that the concentration of sodium and potassium ions affected the extent of cell lysis caused by surfactants also is consistent with this hypothesis, in that autolysis is induced and that autolysin activity in vitro and whole-cell autolysis are stimulated by these cations (1, 13, 21, 24, 31).

The ability of surfactants to induce autolysis corresponded to the generally accepted order of their antimicrobial activity (28), except for sucrose monohexadecanoate, suggesting that polar group as well as electrostatic properties of surfactants may be important in their interaction with the autolysin-regulating system in the cell envelope. In cell lysis of *Escherichia coli* caused by ethanol and chaotropic agents, hydrophobic interaction has been suggested to have a role in the regulation of autolysin activity (12). Other investigators have also reported surfactant-induced autolysis with *Streptococcus faecalis* (2), *Staphylococcus aureus* (4, 20), and *Streptococcus pneumoniae* (18).

Interestingly, surfactants at sublytic concentrations caused morphological changes of *B. subtilis* cells similar to those caused by dodecanoic acid, glycerol monododecanoate, and, to a lesser extent, sucrose monohexadecanoate, as described previously (26). It is well-known that penicillin and other β-lactam antibiotics inhibit septum formation of bacterial cells and cause aberrant morphology (11, 16). Similar effects have also been observed with *B. subtilis* mutants that are defective in penicillin-binding proteins 2a, 2b, and 3 (23).

As a model, it seems likely that the observed changes in cell morphology may be due to the release of a level of autolysins or the inhibition of autolysins, which have a role in the normal morphogenesis of the cell envelope and cell division. The facts that the normal morphology of surfactant-treated cells was restored by the addition of autolysins and lysozyme and that the total activity of autolysin decreased by surfactant treatment may support the hypothesis presented above. Fan and Beckman (6) reported the restorative effects of those enzymes on cells of a temperature-sensitive, autolysin-defective mutant of *B. subtilis* growing at a high temperature without any surfactant. Similar restorationlike effects of added lysozyme and autolysin extract have also been observed with filaments of *B. subtilis* mutants that are defective in autolysins (8, 17).

Surfactants added at low concentrations cause morphological changes and do not affect the in vitro activity of autolysin extracted with 5 M LiCl from the cell wall (unpublished data).

Although autolysins have been suggested to have a role in cell wall turnover, the turnover rates of surfactant-treated cells were identical to those of untreated cells. Recently, Vitkovíc et al. (30) have indicated, by using a variety of autolysin-defective mutants, that there is no correlation between the rate of wall turnover and the autolysin level. Results of the present study may also indicate that a decreased level of autolysin in surfactant-treated cells does not directly reduce the turnover rate.

Surfactants at low concentrations may possibly interfere through a disorganizing effect on the cytoplasmic membrane with the autolysin-regulating system or release some autolysin and, possibly, other molecules that are associated with them, such as membrane lipids, proteins, and lipoteichoic acid. Therefore, they may disturb the well-controlled autolysin activity that is necessary for normal cell division. As a result, surfactants may result in unbalanced growth of the cell envelope and thus cause morphological changes.

At higher concentrations, however, surfactants cause a dissociation between the autolysin-regulating system and autolysin or deenergization of the cell membrane, which Jolliffe et al. (13) have suggested is a regulatory factor, to allow autolysin to attack the peptidoglycan structure of the cell wall, causing cell lysis.

The results obtained here suggest that autolysins may play a role in the formation of the normal rod shape of *B. subtilis* cells and, possibly, in the normal balanced growth of cell envelopes, supporting the hypothesis suggested for some mutants that are defective in autolysin activity (3, 7).

ACKNOWLEDGMENTS

This work was supported in part by the Chemical Materials Research and Development Foundation, Kanagawa, Japan.

We are grateful to Mitsubishi Kasei Corp., Tokyo, Japan, for providing us with a high-purity preparation of sucrose monohexadecanoate, and to Hiroki Kourai, Tokushima University, for preparing PTAI and *N*-dodecylbetaine. We also thank Yung-Hoon Ahn and Kazuhisa Sawada for technical assistance in some experiments.

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