Induction of Growth Phase-Specific Autolysis in *Bacillus subtilis* 168 by Growth Inhibitors

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Growth phase-specific autolysis of *Bacillus subtilis* by inhibitors of membrane permeability, inhibitors of macromolecule biosynthesis, inhibitors of cell wall biosynthesis and detergents were tested and characterized in glucose limited liquid medium. The minimum autolysin induction concentration (MAIC) of test compounds, which was at least 1/20th lower than the conventional autolysis induction concentration, induced autolysis only for cells at the glucose exhaustion point (diauxic point) of the growth phase, while it was not induced for cells at pre- and post-diauxic points. Inhibitors of macromolecule synthesis that are not known for inducing autolysis, such as chloramphenicol, rifampicin, nalidixic acid, and detergents, also induced specific autolysis. Two types of autolysis corresponding to the concentrations of compounds are distinguished: concentration-sensitive and concentration-insensitive types.

***Keywords*:** autolysis, *B. subtilis* 168, diauxic point

Bacillus subtilis is prone to autolysis due to environmental stressors, such as nutrient deficient conditions or growth inhibitors. Autolysis and the associated autolysins of *B. subtilis* have been investigated for many years, and major autolysins include LytA through LytF (Blackman *et al*., 1998; Margot *et al*., 1998; Margot *et al*., 1999; Smith *et al*., 2000; Sekiguchi *et al*., 2003). Nikolskaya and Galperin (2002) cloned and characterized the *lytR* gene that controls *lyt* gene expression. Autolysis occurs not only by the actions of cell wall digesting autolysins, but also by inhibition of peptidoglycan synthesizing enzymes (Chmara *et al*., 1998; Koch, 2000; Kiriukhin *et al*., 2001). Regarding the mechanisms of induced autolysis, Joliffe *et al*. (1981) reported that proton motive dissipaters such as ionophores, uncouplers or cytochrome oxidase inhibitors, induced autolysis in *B. subtilis* by changing its membrane potential.

We found that *B. subtilis* growing in liquid complex medium with a low concentration of chloramphenicol induced autolysis only during a narrow window of the mid-log phase; before and after that period, cells were not autolyzed by the same treatment with chloramphenicol. We found that the point of autolysis was the diauxic point of growth when the primary energy source, glucose, was exhausted. Even inhibitors of macromolecule biosynthesis, including chloramphenicol, rifampicin, and actinomycin, which are not known to induce autolysis, did induce autolysis at the diauxic point

of growth at relatively low concentrations. In this study, we analyzed the autolysis induction patterns of *B. subtilis* 168 by antibiotics, membrane potential disrupters, and detergents.

Materials and Methods

Bacterial strains, culture, and media
B. subtilis 168 strain was from the Bacillus Genetic Stock Center (BGSC) of The Ohio State University. Bacterial cells were grown in 1× LB medium or M9 minimal medium at 37°C. Yeast extract (YE) concentration in LB was reduced to 1/5th of normal 1× LB, and glucose concentrations in M9 were adjusted to 1/25th or 1/50th of normal (4 g/L, 0.4%), as needed.

Chemicals
Growth inhibitors used for induction of autolysis are shown in Table 1. Chemicals for SDS-PAGE, renaturing SDS-PAGE, buffers, ingredients of culture media and growth inhibitors used as autolysis inducers were purchased from Sigma Co., Difco Lab, and Merck Chemical Co.

Induction of autolysis
Overnight cultured bacterial cell suspension was inoculated into M9 or LB at a concentration of OD_{590nm}=0.1 and cultured. Aliquots of cell suspensions were removed at various points of the growth phase and treated with growth inhibitors. Continued growth or autolysis was monitored during continuous incubation using 'Spectronic 20' at 590 nm.

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Table 1. Growth inhibitors used for autolysis induction

Inhibitor	Mode of action
Chloramphenicol	Inhibits protein synthesis
Actinomycin D	Inhibits DNA synthesis
Erythromycin	Inhibits protein synthesis (50S RNA)
Nalidixic acid	Inhibits DNA synthesis and replication ; causes membrane damage
Rifampicin	Inhibits DNA-directed RNA polymerase
Sodium azide	Conducts H^+ ; inhibits cytochrome oxidase and ATPase
Potassium cyanide	Inhibits cytochrome oxidase
Carbonyl cyanide *m*-chloro-phenylhydrazone (CCCP)	Conducts H^+ ; uncouples oxidative phosphorylation
Gramicidine D	Conducts K^+, Na^+, H^+ (dissipates electrical and pH gradients)
Nigericin	Exchange K^+ for H^+ (dissipates pH gradients)
Monensin	Exchange Na^+ for H^+ (dissipates pH gradients)
Valinomycin	Conducts K^+ (dissipates electrical gradients)
Tween 80	Detergent
SDS	Detergent
Ampicillin	Inhibits cell wall synthesis

Determination of reducing sugar concentration

Reducing sugar concentration of liquid medium was determined by dinitrosalicylic acid (DNS) method (Miller, 1959). A 3 ml of reagent (1% DNS, 0.2% phenol, 0.05% Na_2SO_3, and 1% NaOH) was added to 3 ml of glucose containing solution. Mixture was heated for 5 min in a boiling water bath and then cooled under tap water until reached ambient temperature. OD was measured at 575 nm.

Protein preparation for SDS-PAGE

Protein samples for SDS-PAGE were extracted by boiling in SDS-PAGE sample buffer following Foster's (1992) method. Cells were harvested at 3,000×g for 10 min, washed once with dist. H_2O and re-pelleted at 27,000×g for 3 min. The cell mass was briefly dried under vacuum, suspended in SDS-PAGE sample buffer and boiled for 3 min. After centrifuging at 27,000×g for 5 min, the supernatants were removed and stored at -20°C. Proteins in culture supernatants were prepared by ammonium sulfate precipitation.

Cell wall preparation

Cell walls were extracted by the methods of Studer and Karamata (1988). Harvested *B. subtilis* 168 cells were suspended in dist. H_2O and disrupted with an ultrasonic cell disrupter (Heat systems-Ultrasonics, Inc., model W-380 with convertor C-3). From supernatants after 3,000×g spin, cell wall was pelleted by spinning at 27,000×g for 15 min. Washed and resuspended cell walls in 4% SDS were boiled for 10 min and pelleted at 27,000×g for 15 min. These preparations were imbedded into polyacrylamide gels for detecting autolysin activity.

SDS-PAGE

A 12% SDS-polyacrylamide gel was used for analysis of proteins and stained with 0.25% Coomasie blue in methanol: acetic acid:H_2O=40:7:53. Fixing, staining, and destaining was performed by conventional procedures.

Renaturation SDS-PAGE

To detect autolysin activity, protein extracts were loaded on 12% SDS-polyacrylamide gels containing 0.05% (w/v) of *B. subtilis* 168 cell walls and electrophoresed (Foster, 1992). Proteins in the gel were renatured in renaturing solution (0.1% Triton X-100, 10 mM $MgCl_2$, 25 mM Tris-HCl, pH 7.5) in two steps: 1st at 30°C for 30 min and 2nd at 37°C for 16 h. Renatured gels were washed with dist. H_2O and stained with methylene blue (0.1% methylene blue in 0.01% KOH) for 3 h. During destaining with dist. H_2O, protein bands having autolysin activity became clear, while background that remained blue indicated methylene blue-stained cell walls.

Results

Growth phase-specific autolysis in *B. subtilis* induced by chloramphenicol

When *B. subtilis* cells at various growth phases were treated with a relatively low concentration (30 µg/ml) of chloram-
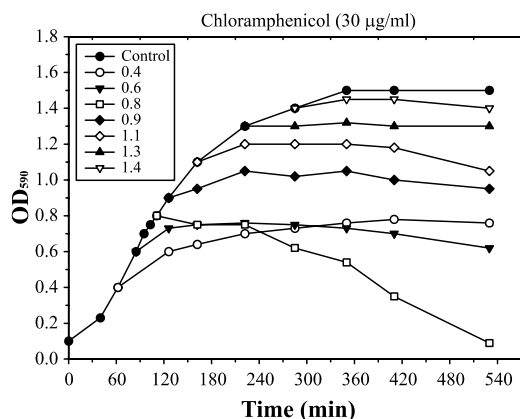

Fig. 1. Autolysis induction in *B. subtilis* 168 in liquid LB medium by chloramphenicol (CAM). Aliquots of *B. subtilis* 168 cell suspensions were taken at indicated OD_{590} and treated with 30 µg/ml CAM. OD_{590} of each CAM-treated cell suspension was measured during continued incubation. Numbers in inset indicate OD_{590} of cell culture at which CAM treated.

phenicol (CAM), only cells in a narrow range of log phase (OD_{590}=0.8~0.85 in 1× LB liquid medium) were specifically lysed, while cells before and after that point only showed bacteriostasis (Fig. 1). In follow-up tests, we found that the concentration of yeast extract (YE), more specifically the glucose left in LB medium, was related to CAM-induced cell lysis. In experiments using modified LB medium containing 1/5th strength of YE (1 g/L instead of 5 g/L),
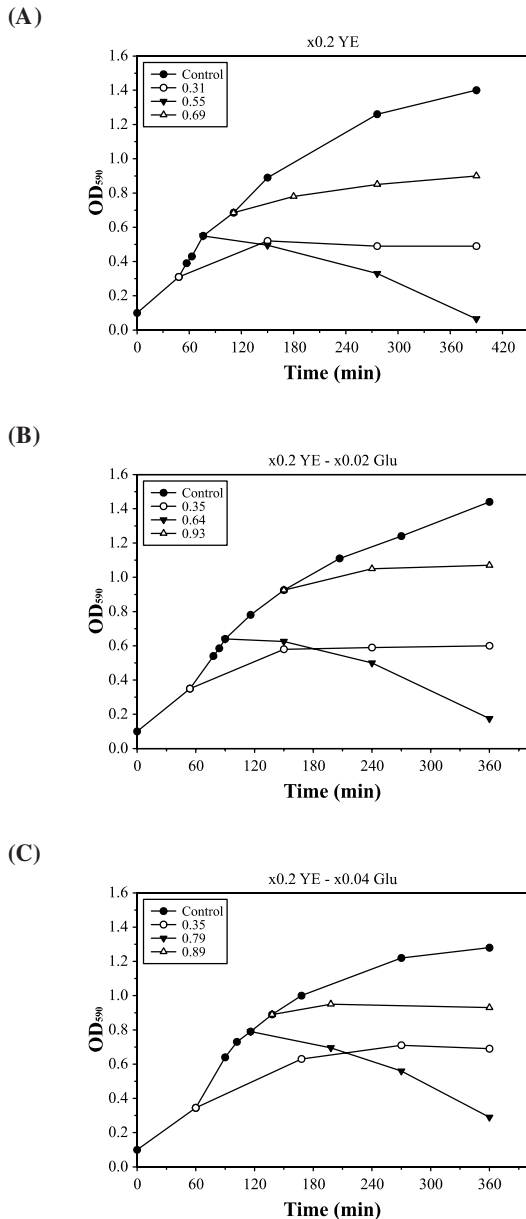
(A)

(B)

(C)

Fig. 2. CAM-induced autolysis in modified LB medium. For medium yeast extract (YE), contents were reduced to 1/5th (×0.2 YE) of 1× LB (A), for YE-LB medium supplemented with glucose they were reduced to 1/50th (×0.02) that of M9 minimal medium (B), and for YE-LB medium supplemented with glucose they were reduced to 1/25th (×0.04) times that of M9 minimal medium (C). Numbers in inset indicate OD_{590} of cell culture at which CAM treated.
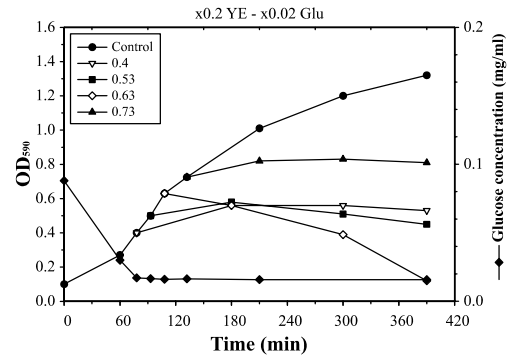

Fig. 3. Relationship between autolysis induction by CAM (30 μg/ml) and glucose contents in modified LB [1/5th (×0.2) YE plus 1/50th (×0.02) glucose of M9]. Residual glucose was measured by the DNS (3,5-dinitrosalicylic acid) method. Reducing sugar in medium converted DNS to chromogenic 3-amino-5-nitrosalicylic acid and the amount of converted compound was measured by OD_{550}. Numbers in inset indicate OD_{590} of cell culture at which CAM treated.

cells lysed at OD_{590}=0.55 (Fig. 2A). And when glucose was supplied separately at 1/50th (×0.002) and 1/25th (×0.004) that of normal M9 medium, cell lysis was delayed to OD_{590} =0.64 and 0.79 (Fig. 2B and C), respectively. We reasoned that CAM sensitivity might be related to glucose starvation and, thus, we measured glucose concentration during cultivation. As shown in Fig. 3, at the point when glucose concentration dropped to basal level, cell lysis began; after that point, cells did not lyse. When we tested autolysis induction throughout the growth phase, another specific autolysis point could be distinguished at the late log phase. However, for this paper, we only concentrated on the first specific autolysis point.

We did the same autolysis induction tests in strains other than *B. subtilis* 168 with chloramphenicol. Similar levels of autolysis induction were observed for BD170, ATCC 19659, and ATCC 15245 strains.

Glucose consumption and growth curves in liquid culture

Measurement of a detailed growth curve for *B. subtilis* in minimal liquid culture showed that when glucose starvation occurred, a diauxic point could be seen as a retarded growth curve-shoulder. For the diauxic point to be seen, the amount of glucose in minimal medium had to be reduced (a secondary energy source in minimal medium could be casamino acid, a component of M9). When glucose was reduced to 1/25th (×0.04) of normal M9 medium, the diauxic point appeared around OD_{590}=0.4~0.45 (Fig. 4). CAM sensitive-cell lysis at the diauxic point in minimal medium is much more pronounced than in complex medium (Fig. 5).

Autolysin induced by CAM for different growth phase cells

Proteins were prepared from cells or from supernatants of CAM-treated or CAM-untreated cells at OD_{590}=0.7, 0.85, and 1.1-cell suspensions in LB medium. Prepared proteins
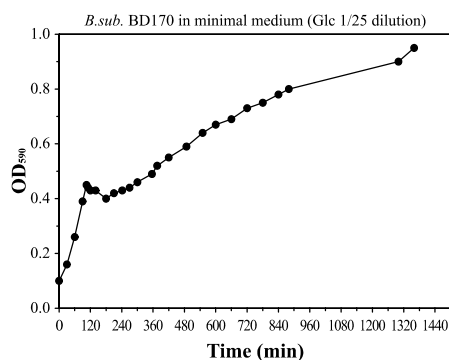
Fig. 4. Growth curve of *B. subtilis* in glucose limited (×0.04) M9 liquid medium. Diauxic point for growth appeared around OD_{590} =0.4~0.45.

were applied to both conventional and renaturation SDS-PAGE. Bands in Fig. 6A are total membrane proteins and bands in Fig. 6B are proteins with cell wall digestion activities. Proteins extracted from the pre-diauxic point are in lanes 1~4, from the diauxic point are in lanes 5~8, and from the post-diauxic point are in lanes 9~12. Proteins from cell walls are in odd-numbered lanes and from culture supernatants are in even-numbered lanes. Proteins from CAM-treated cell suspensions (proteins were extracted at 120 min after 50 μg/ml of CAM treatment) are in lanes 3, 4, 7, 8, 11, 12, and from CAM-untreated cell suspensions are in lanes 1, 2, 5, 6, 9, 10. At the diauxic point, many bands showing cell wall-digesting activities appeared in comparison with those obtained before and after the diauxic point. For cell suspensions treated with CAM, more bands appeared than for untreated.

Autolysis induction by growth inhibitors for *B. subtilis* 168

Autolysis induction for *B. subtilis* in liquid minimal medium culture was tested using ionophores, inhibitors of macromolecule synthesis, an uncoupler, inhibitors of cytochrome oxidase and detergents. At the MAIC (minimum autolysin induction concentration) of each compound, cells starved of
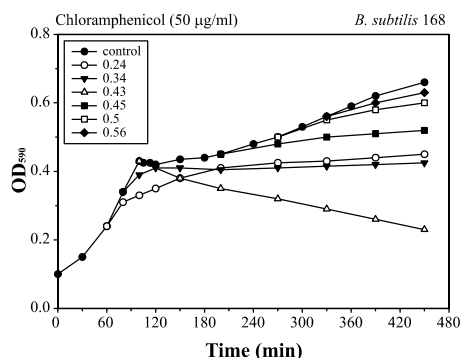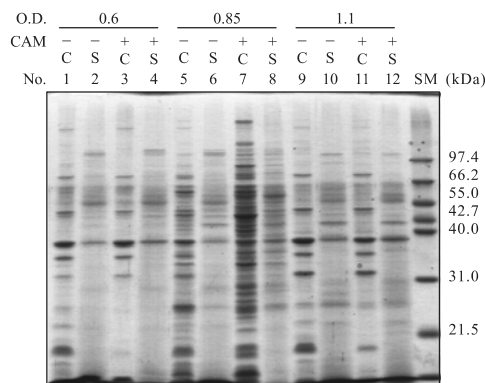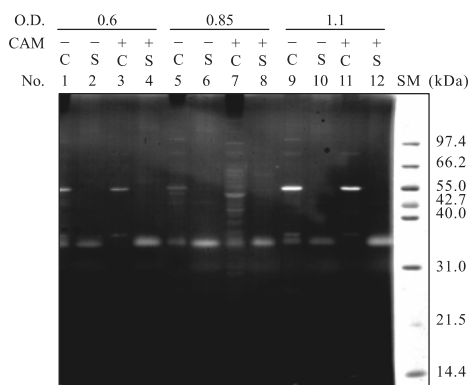

(A)

(B)

Fig. 6. SDS-PAGE profiles of proteins extracted from *B. subtilis* at OD_{590}=0.6 (lanes 1, 2, 3, 4), 0.85 (lanes 5, 6, 7, 8), and 1.1 (lanes 9, 10, 11, 12). Proteins from CAM-treated cells are in lanes 3, 4, 7, 8, 11, 12 and from CAM untreated cells are in lanes 1, 2, 5, 6, 9, 10. Coomasie blue stained SDS-PAGE profiles (A). Cell wall component-containing renaturation SDS-PAGE gel stained with methylene blue (B). SM, protein size marker; C, protein from cell; S, protein from culture supernatant.

the primary carbon source commenced lysis. That point was around OD_{590}=0.44 in our minimal medium system. Cells at points earlier or later than this point did not show autolysis or, if there was any, it was much slower than for cells at the starvation point. Even antibiotics, such as chloramphenicol, actinomycin D, and nalidixic acid, which are not known to induce autolysis, also induced autolysis at this point.

a) Autolysis induction by inhibitors of macromolecule biosynthesis

Chloramphenicol, rifampicin, actinomycin D, erythromycin, and nalidixic acid are inhibitors of macromolecular biosynthesis and are not known as autolysis inducing agents. However, all these compounds caused specific autolysis at the cells' diauxic point (Fig. 7). Treatments at the MAIC of the compounds at pre- and post-diauxic points did not lyse cells, although these conditions showed retarded cell growth or, rather, bacteriostasis. The energy source for cell growth after the diauxic point in minimal medium could be a casamino acid component.
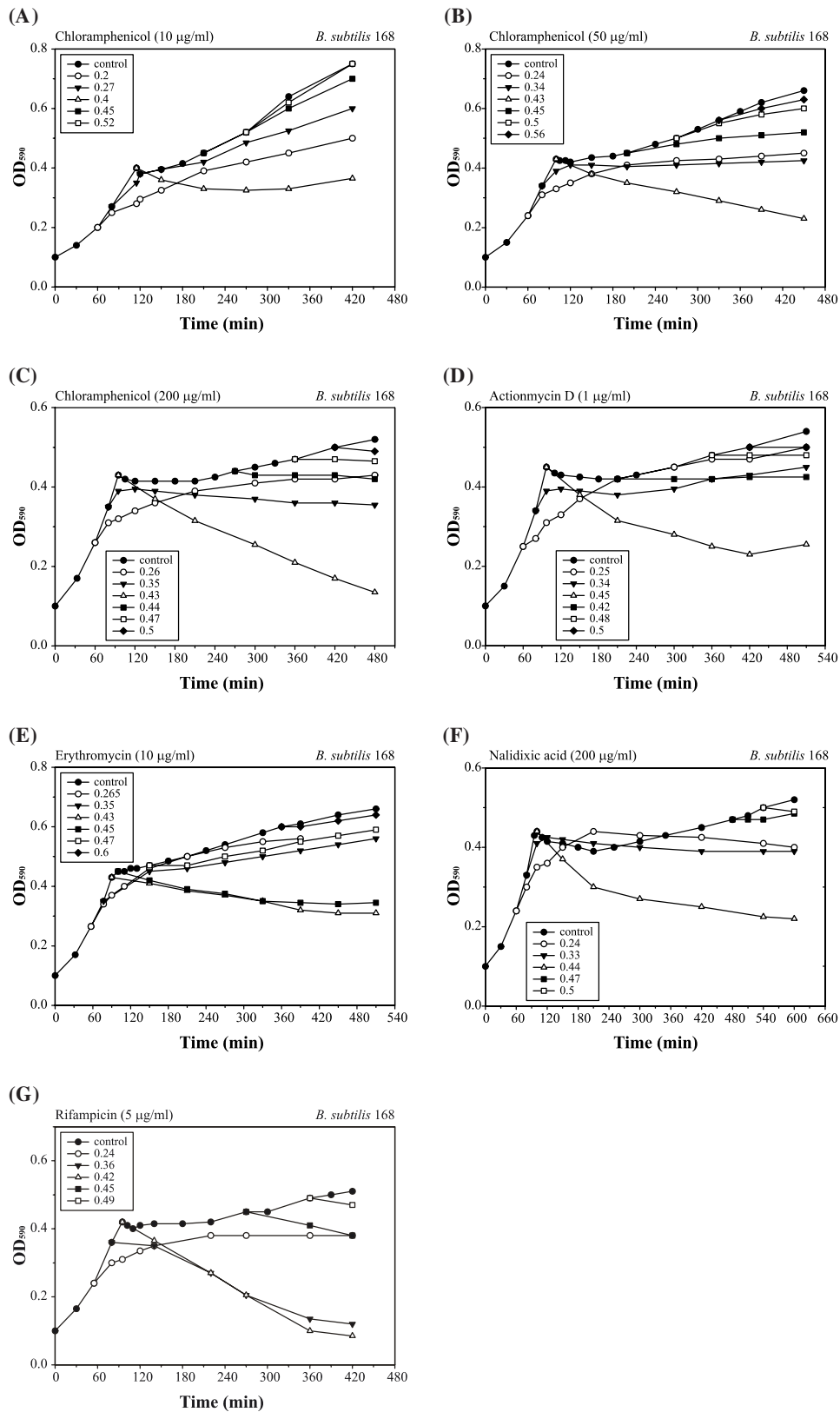

Fig. 5. Chloramphenicol (50 μg/ml)-induced autolysis at the diauxic point for growth in glucose-limited minimal medium. Numbers in inset indicate OD_{590} of cell culture at which CAM treated.

(A)

Chloramphenicol (10 μg/ml) *B. subtilis* 168

(B)

Chloramphenicol (50 μg/ml) *B. subtilis* 168

(C)

Chloramphenicol (200 μg/ml) *B. subtilis* 168

(D)

Actionmycin D (1 μg/ml) *B. subtilis* 168

(E)

Erythromycin (10 μg/ml) *B. subtilis* 168

(F)

Nalidixic acid (200 μg/ml) *B. subtilis* 168

(G)

Rifampicin (5 μg/ml) *B. subtilis* 168

Fig. 7. Autolysis induction in growth phase *Bacillus subtilis* 168 cells in glucose-limited M9 minimal medium by five antibiotics. Aliquots of *B. subtilis* 168 cell suspensions were taken out at indicated OD_{590} and treated with 10, 50, and 200 μg/ml of CAM (A, B, C), 1 μg/ml of actinomycin (D), 10 μg/ml of erythromycin (E), 200 μg/ml of nalidixic acid (F), and 10 μg/ml of rifampicin (G). OD_{590} of antibiotic treated cell suspension was measured during continued incubation. Numbers in inset are OD_{590} of cell culture at which antibiotic treated.
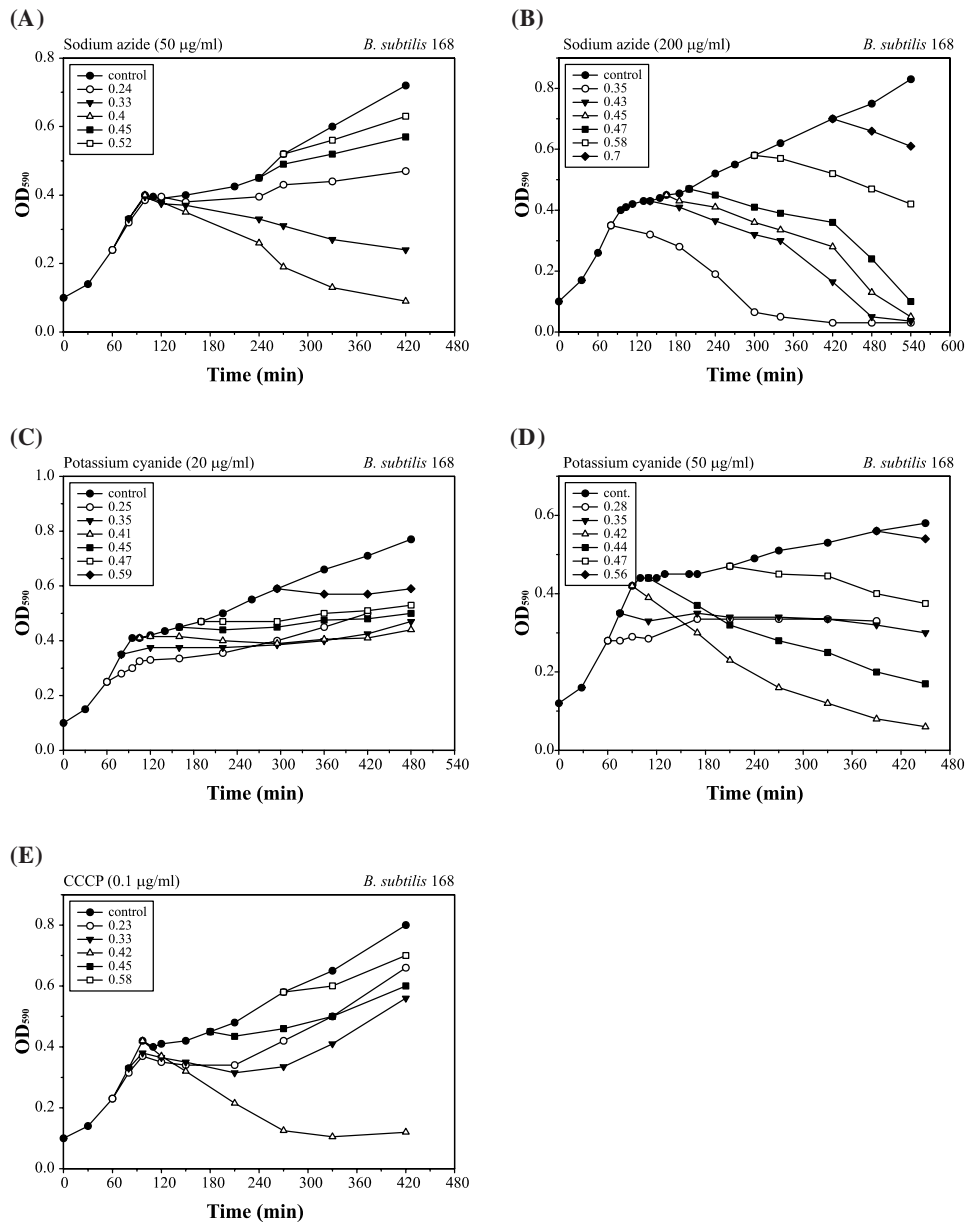
Fig. 8. Autolysis induction by oxidative phosphorylation uncoupler (CCCP), cytochrome oxidase inhibitor (sodium azide), and ATPase inhibitor (potassium cyanide). A 50 μg/ml and 200 μg/ml of sodium azide (A, B), 20 μg/ml and 50 μg/ml of potassium cyanide (C, D), and 0.1 μg/ml of CCCP (E) were added to each cell suspensions of which OD_{590} are indicate in inset.
Continued growth or autolysis was monitored during continuous incubation.

b) Autolysis induction by oxidative phosphorylation uncoupler, cytochrome oxidase inhibitor and ATPase inhibitor

Carbonyl cyanide m-chrolophenylhydrazone (CCCP), potassium cyanide (oxidative phosphorylation uncoupler), and sodium azide (ATPase inhibitor) are known to be powerful, non-specific autolysis inducers regardless of the growth period (Joliffe *et al.*, 1981). A 50 μg/ml concentration of sodium azide induced diauxic point (OD_{590}=0.4)-specific autolysis in glucose limited minimal medium, while at 200 μg/ml it induced non-specific autolysis in all growth phases (Fig. 8A

and B). Cells treated with 50 μg/ml of sodium azide at the pre- (OD_{590}=0.24) and post-diauxic points (OD_{590}=0.52) were not lysed, but showed retarded growth or bacteriostasis.

c) Autolysis induction by ionophores

Monensin, valinomycin, gramicidin, and nigericin are ionophores that are potent specific autolysis inducers (Fig. 9). Gramicidin and nigericin induced specific autolysis only at 0.005 μg/ml, and cells before and after the diauxic point were not inhibited. One interesting result is that the growth of gramicidin D and valinomycin treated pre-diauxic point
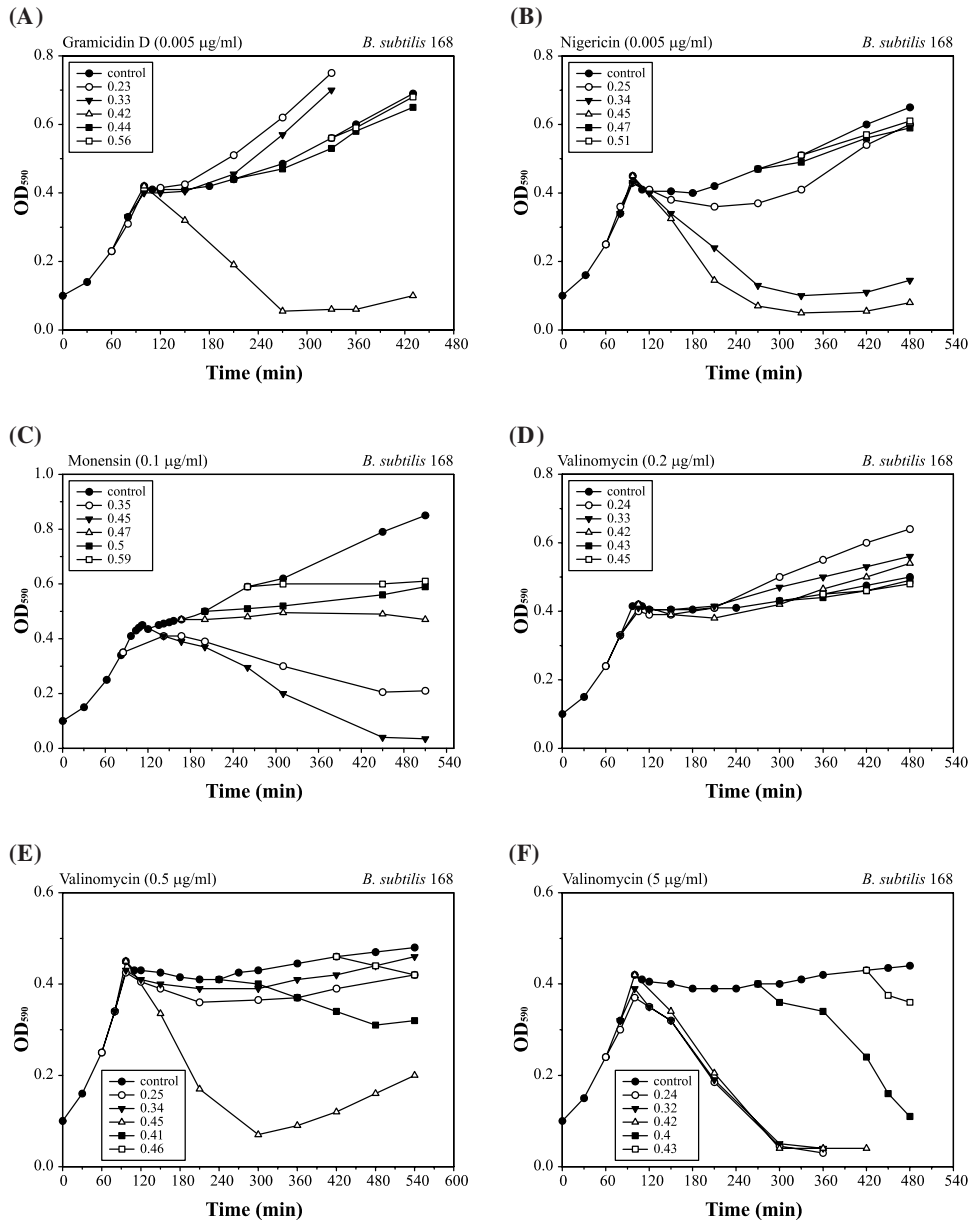
Fig. 9. Autolysis induction by four ionophores, gramicidin D, nigericin, monensin, and valinomycin. A 0.005 µg/ml of gramicidin D (A), 0.005 ug/ml of nigericin (B), 0.1 ug/ml of monensin (C), and 0.2, 0.5, and 5 µg/ml of valinomycin (D, E, F) were added to each cell suspensions of which OD$_{590}$ are indicate in inset. Continued growth or autolysis was monitored during continuous incubation.

cells exceeded that of untreated controls (Fig. 9A and D).

d) Autolysis induction by detergents
Detergent SDS also induced typical specific autolysis at 20 µg/ml. However, Tween 80 did not induce up to 0.1% (1 mg/ml w/v) (Fig. 10). While SDS-treated diauxic point cells showed specific autolysis, SDS-treated pre-diauxic point cells showed an overgrowth compared to control cells, as was the case with gramicidin D or valinomycin (Fig. 9A and 9D~F). Tween 80 had no significant effects on the whole growth phase cells at the tested concentrations (Fig. 10).

e) Autolysis induction by ampicillin
The autolysis induction pattern by ampicillin was somewhat different from those described above. Ampicillin-treated di-auxic point cells did not commence lysis. Rather, after a short period of continued growth, ampicillin-treated pre-di-auxic point cells began to lyse (Fig. 11).

This result is consistent with the action mechanism of ampicillin which is being applied to enrichment of mutant that non-dividing (diauxic point cells) are not affected and only dividing (growing) cells are killed by β-lactam antibiotics.
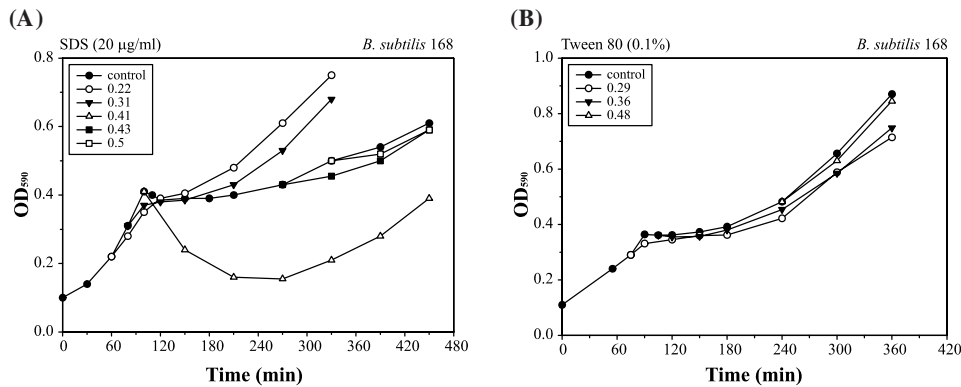
(A)

SDS (20 μg/ml)　　　　　　　　　　*B. subtilis* 168

●	control
○	0.22
▼	0.31
△	0.41
■	0.43
□	0.5

(B)

Tween 80 (0.1%)　　　　　　　　　　*B. subtilis* 168

●	control
○	0.29
▼	0.36
△	0.48

Fig. 10. Autolysis induction by two detergents, SDS and Tween 80. A 20 μg/ml of SDS (A), and 0.1% (1 mg/ml) of Tween 80 (B) were added to each cell suspensions of which OD_{590} are indicate in inset. Continued growth or autolysis was monitored during continuous incubation.

Discussion

Chemical concentration- and cell growth phase-dependent specific autolysis

Autolysis induction by growth inhibitors, including cytochrome oxidase inhibitors, oxidative phosphorylation uncouplers, and ionophores, results from membrane potential dissipation (Joliffe *et al.*, 1981). In that report, autolysis was induced by adding these compounds either directly to log phase *B. subtilis* culture or to antibiotics-treated, growth inhibited culture. They used 40 mM (2.6 mg/ml) or 75 mM (4.9 mg/ml) of sodium azide and non-specific autolysis was induced regardless of cells' conditions: log phase cells or growth inhibited cells. In our experiments, using a 1/20th lower concentration than the conventional autolysis induction concentration (50 μg/ml sodium azide), autolysis was induced at the diauxic point of growth phase cells. The lowest concentration that induced growth specific autolysis,
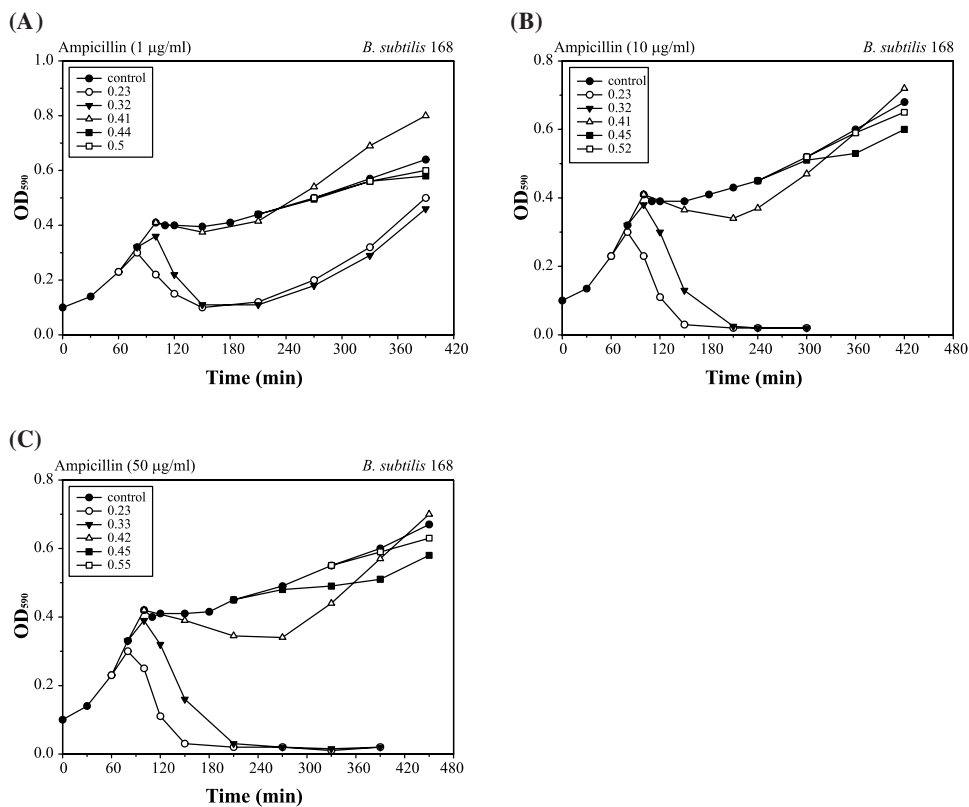
(A)

Ampicillin (1 μg/ml)　　　　　　　　*B. subtilis* 168

●	control
○	0.23
▼	0.32
△	0.41
■	0.44
□	0.5

(B)

Ampicillin (10 μg/ml)　　　　　　　*B. subtilis* 168

●	control
○	0.23
▼	0.32
△	0.41
■	0.45
□	0.52

(C)

Ampicillin (50 μg/ml)　　　　　　　*B. subtilis* 168

●	control
○	0.23
▼	0.33
△	0.42
■	0.45
□	0.55

Fig. 11. Autolysis induction by ampicillin. A 1 μg/ml (A), 10 μg/ml (B), and 50 μg/ml of ampicillin (C), were added to each cell suspensions of which OD_{590} are indicate in inset. Continued growth or autolysis was monitored during continuous incubation.

Table 2. Autolysis induction concentrations

Inhibitor	Autolysis induction concentration	
	Ref[a]	MAIC[b] in this experiment
Chloramphenicol	NT[c]	1.5×10^{-4} M (50 µg/ml)
Actinomycin D	NT	7.96×10^{-7} M (1 µg/ml)
Erythromycin	NT	1.36×10^{-5} M (10 µg/ml)
Nalidixic acid	NT	7.86×10^{-4} M (200 µg/ml)
Rifampicin	NT	6.07×10^{-6} M (5 µg/ml)
Sodium azide	7.5×10^{-2} M	7.7×10^{-4} M (50 µg/ml)
Potassium cyanide	4.0×10^{-2} M	7.68×10^{-4} M (50 µg/ml)
Carbonyl cyanide m-chloro-phenlyhydrazone	4.0×10^{-2} M	4.89×10^{-4} M (0.1 µg/ml)
Gramicidine D	1 µg/ml	0.005 µg/ml
Nigericin	1×10^{-6} M	6.7×10^{-9} M (0.005 µg/ml)
Monensin	1.44×10^{-5} M	1.44×10^{-7} M (0.1 µg/ml)
Valinonycin	8.98×10^{-5} M	4.49×10^{-7} M (0.5 µg/ml)
SDS	NT	20 µg/ml
Tween 80	NT	> 1 mg/ml
Ampicillin	NT	1 µg/ml

[a] Joliffe *et al.* (1981)
[b] minimum autolysis induction concentration
NT, not tested

the minimum autolysin-induction concentration (MAIC), differed from compound to compound in accordance with their toxicities. The higher toxicity groups were ionophores and a phosphorylation uncoupler (CCCP) for which MAIC ranged from 0.005 µg/ml~0.5 µg/ml. At the MAIC for all test compounds, only cells at the glucose exhaustion point (diauxic point) were autolysed, while cells at the pre- and post-diauxic points were not lysed. Changing the energy sources appears to make *B. subtilis* cells very susceptible to various kinds of growth inhibitors and leads to autolysis.

We tested induction of autolysis in *E. coli* and *Staphylococcus aureus*, however, no such phenomenon was observed (data not shown).

Types of growth inhibition and autolysis responding to stepwise increments of chemical concentrations
Two types of response to growth inhibitor concentration increments were distinguished. 1) Concentration-sensitive type showed rapid changes of dose response from a low, no-effect concentration to a high, growth phase-nonspecific induction of autolysis within a comparatively narrow concentration range. Oxidative phosphorylation uncoupler, cytochrome oxidase inhibitors, ATPase inhibitor and ionophores appear to belong to this type (Fig. 9D, E, and F). 2) Concentration-insensitive type chemicals induced only specific autolysis over a broad range of concentrations, and did not induce non-specific autolysis even at relatively high concentration. Inhibitors of macromolecule biosynthesis, ampicillin, and detergents induced this type of autolysis (Fig. 7A, B, and C).

Rapid growth for low concentration ionophore-treated pre-diauxic point cells
Most pre-diauxic cells exposed to the MAIC of compounds did not commence lysis, even if they consumed the primary energy source: glucose. They simply continued slow growth or maintained bacteriostasis. However, the growth of pre-diauxic point cells treated with valinomycin, gramicidin D or SDS exceeded the growth of untreated control cells (Fig. 9A, D, and Fig. 10A). We do not know how a low concentration of these compounds influenced the pre-diauxic point cells to grow rapidly. We presume that pre-diauxic point cells might degrade these compounds and then utilize the products as efficient energy sources or changes in permeability caused by these chemicals might facilitate to uptake carbon sources. Further experiment is needed.

Autolysis induction capability of growth inhibitors
At the diauxic point of the growth phase, all test compounds except Tween 80 induced specific autolysis, even though the MAIC was different from compound to compound. At this point, we do not know if the membrane potential of a diauxic point cell might be dissipated by any growth inhibiting agents and then lead to autolysis. Alternatively, the mechanisms and related autolysins for this diauxic point-specific autolysis are different from non-specific, membrane disturbed autolysis. Different MAIC for these compounds and different response curves to the compounds might indicate that target molecules for each inhibitor and the route leading to eventual autolysis might be different from those for non-specific autolysis.

Autolysin bands on renaturation polyacrylamide gel
During the period of specific autolysis, numerous protein bands with autolysis activities were revealed for cell wall components by renaturation polyacrylamide gels. Analysis to determine if these bands are actual autolysins and if they are different from known major autolysins is needed. We are attempting to develop 2D renaturation gel electrophoresis for this purpose.

Acknowledgements

References

Blackman, S.A., T.J. Smith, and S.J. Foster. 1998. The role of autolysins during vegetative growth of *Bacillus subtilis* 168. *Microbiology* 144, 74-82.

Chmara, H., S. Milewski, R. Andruszkiewicz, F. Mignini, and E. Borowski. 1998. Antibacterial action of dipeptidases containing an inhibitor of glucosamine-6-phosphate isomerase. *Microbiology* 144, 1349-1358.

Foster, S.J. 1992. Analysis of autolysin of *Bacillus subtilis* 168 during vegetative growth and differentiation by using renaturing polyacrylamide gel electrophoresis. *J. Bacteriol.* 174, 464-470.

Joliffe, K., R.J. Doyle, and U.N. Steips. 1981. The energized membrane and cellular autolysis in *Bacillus subtilis*. *Cell* 25, 753-763.

Kiriukhin, M.Y., D.V. Debabov, D.L. Shinabarger, and F.C. Neuhaus.

2001. Biosynthesis of the glycolipid anchor in lipoteichoic acid of *Staphylococcus aureus* RN4220: role of YpfP, the diglucosyldiacylglycerol synthase. *J. Bacteriol*. 183, 3506-3514.

Koch, A.L. 2000. Simulation of the conformation of the murein fabric: the oligoglycan, penta-muropeptide, and cross-linked nona-muropeptide. *Arch. Microbiol*. 174, 429-439.

Margot, P., M. Pagni, and D. Karamata. 1999. *Bacillus subtilis* 168 gene *lytF* encodes a gamma-D-glutamate-meso-diaminopimelate muropeptidase expressed by the alternative vegetative sigma factor, sigmaD. *Microbiology* 145, 57-65.

Margot, P., M. Wahlen, A. Gholamhusenian, P. Piggot, and D. Karamata. 1998. The *lytE* gene of *Bacillus subtilis* 168 encodes a cell wall hydrolase. *J. Bacteriol*. 180, 749-752.

Miller, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31, 426-428.

Nikolskaya, A.N. and M.Y. Galperin. 2002. A novel type of conserved DNA-binding domain in the transcriptional regulators of the AlgR/AgrA/LytR family. *Nucleic Acids Res*. 30, 2453-2459.

Sekiguchi, J., Y. Hiroki, and K. Shinichirou. 2003. Localization of the vegetative cell wall hydrolases LytC, LytE, and LytF on the *Bacillus subtilis* cell surface and stability of these enzymes to cell wall-bound or extracellular proteases. *J. Bacteriol*. 22, 6666-6677.

Smith, T.J., S.A. Blackman, and S.J. Foster. 2000. Autolysins of *Bacillus subtilis*: multiple enzymes with multiple function. *Microbiology* 146, 249-262.

Studer, R.E. and D. Karamata. 1988. Cell wall proteins in *Bacillus subtilis*, p. 146-150. Antibiotic inhibition of bacterial cell surface assembly and function. *In* P. Actor (ed). American Society for Microbiology, Washington, D.C., USA.