Note

Decrease in Cytoplasmic pH-Homeostatic Activity of the Alkaliphile *Bacillus Lentus* C-125 by a Cell Wall Defect

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Cytoplasmic pH homeostatic activities of cell wall-defective derivatives of the alkaliphile *Bacillus lentus* C-125 were assessed using a pH-sensitive fluorescent probe, BCECF. It was shown that the acidic cell wall components took part in maintenance of the cytoplasmic pH neutrality at alkaline pH.

Key words: alkaliphile; alkaliphilic *Bacillus lentus*; cell wall; cytoplasmic pH; pH homeostasis

The facultative alkaliphile, *Bacillus lentus* C-125, grows between pH 6.8 and 10.8.1) It was proposed recently that this strain belonged to *Bacillus harodulans*.2) A Δψ-dependent Na+/H+ antiporter directs maintenance of the cytoplasmic pH around neutrality,3) as reported for another alkaliphile, *Bacillus pseudo*firmus OF4.4) However, it is likely that the cell wall is also important for development and maintenance of the alkaliphily.5) The protoplasts are unstable at high alkaline pH.6) The cell walls of Gram-positive bacteria may act as reservoirs of H+ ions generated intracellularly.7,8) The cell walls of C-125 are composed of A1g peptidoglycan, teichuronic acid (TUA), and teichuronopeptide (TUP). The TUA consists of galacturonic acid, glucuronic acid, and N-acetyl-D-2-amino-2,6-dideoxygalactose.9) The TUP is a polymer in which poly-L-glutamic acid binds covalently to polyglucuronic acid.10) Quantities of these acidic polymers in the cell walls of strain C-125 increase with increasing culture pH.11) In this study, we examined cytoplasmic pH homeostasis of the cell-wall-component-defective derivatives using the pH-sensitive fluorescent probe 2',7'-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein (BCECF). BCECF has been used to measure cytoplasmic pH of eucaryotic or bacterial cells.12,13) BCECF was used to measure cytoplasmic pH of C-125.14)

Estimation of cytoplasmic pH by fluorescence intensity of intracellular BCECF

All the strains used are derivatives of C-125. Strain C-125-11 is a mutant lacking TUA. Strain C-125-90 derived from C-125-11 lacks TUA and the polyglutamic acid moiety of TUP.15) Strain C-125-F19, which was constructed by protoplast fusion between C-125-90 and wild type, contains TUA and lacks the polyglutamic acid chain of TUP.16) The parent C-125 grows between pHs 6.8 and 10.8 with optimum growth at pH 9. Mutant C-125-11 grows between pHs 6.8 and 10.7 with a growth optimum pH at 8. Strains C-125-F19 and C-125-90 grow between pHs 6.8 and 10.5, and both show growth optima at pH 7.17) C-125-90 restores the alkaliphily and TUP synthesis by introduction of gene *tupA* cloned from the parent.18) These bacteria were aerobically grown at 37°C in a medium consisting of K2HPO4, 13.7 g; KH2PO4, 5.9 g; citric acid, 0.34 g; MgSO4・7H2O, 0.05 g; glucose, 10 g; peptone, 5 g; yeast extract, 1 g, and Na2CO3, 10.6 g per liter of deionized water.19) The culture pH was kept at 10.0 during growth of the bacteria with a pH-stat apparatus.17) The cells in the stationary phase of growth (OD660 2) were loaded with BCECF using its membrane-permeable derivative, acetoxymethyl ester of BCECF (Sigma Chem. St. Louis, MO, USA), as described.14)

The BCECF-loaded cells were suspended in 0.1 M NaCl–0.1 M KCl–0.1% glucose–0.1 M TES–NaOH (pHs 6.0 to 8.6) or 0.1 M NaCl–0.1 M KCl–0.1% glucose–0.1 M glycine–NaOH (pHs 8.0 to 11.5). After incubation at 37°C for 30 min, the fluorescence intensity of the intracellular BCECF was measured. BCECF was excited at the wavelength 450 or 510 nm, and the emission wavelength was set at 535 nm. Samples were stirred at 37°C during the fluorescence measurement. Two parameters, the fluorescence intensity of BCECF at 510 nm (F510) and the ratio of the intensities at 510 and 450 nm (Ratio510/450), were used as measures of cytoplasmic pH.13,18) Ionophore

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Abbreviations: BCECF, 2',7'-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein; TES, N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; TUA, teichuronic acid; TUP, teichuronopeptide
Cytoplasmic pH maintenance of the cell wall component-defective mutants in alkaline environments

The significance of the pH homeostatic activity of C-125 depends on the culture pH. The difference in the activity would interfere with evaluating the contribution of the cell walls to cytoplasmic pH maintenance. Therefore, the cytoplasmic pH-homeostatic activities were compared using the bacteria grown at the same constant pH. We measured cytoplasmic pH of the derivatives grown at pH 10. At this pH, C-125 grows well and develops the homeostatic activity well of the derivatives grown at pH 10. At this pH, C-125 depends on the culture pH. The diﬀerence in the activity would interfere with evaluating the contribution of the cell walls to cytoplasmic pH maintenance. Therefore, the cytoplasmic pH-homeostatic activities were compared using the bacteria grown at the same constant pH. We measured cytoplasmic pH of the derivatives grown at pH 10. At this pH, C-125 grows well and develops the homeostatic activity well although C-125-90 grows poorly. The cytoplasmic pH of each derivative exposed to pH lower than 9 was almost the same as that of the parent (Fig. 1). When the derivatives were exposed to pH 9 or 10 and above, their cytoplasms were alkalized more severely than that of the parent. Traces of cytoplasmic pH of the two TUP-defective derivatives (C-125-90 and C-125-F19) were intermediate between those of the parent grown at pH 7 and 8.5. This fact indicated that pH homeostatic activity levels of the two derivatives, despite the growth at pH 10, were lower than that of the parent grown at pH 8.5. The activity of a TUA-less derivative (C-125-11) was intermediate between those of the TUP-derivatives and the parent.

Thus, it was suggested that the cytoplasmic pH maintenance was aided by high anionic polymers present in the cell walls of C-125 in highly alkaline environments with pH 9–10.7. TUP seems to contribute to lowering the cytoplasmic pH by cancelling the alkalization rate is likely due to diﬀerences in rates at which OH− ions pass through the anionic cell wall matrixes of the two organisms. The discrepancy in the contents of the anionic charges ﬁxed in the cell walls, as described above. The discrepancy in the alkalization rate is likely due to diﬀerences in rates at which OH− ions pass through the anionic cell wall matrixes of the two organisms.

Intracellular alkalization caused by sudden upward shift of the extracellular pH

The pH homeostasis of C-125 is lost by the gramicidin treatment. The cells of C-125 and C-125-90 were treated with the ionophore at pH 7.8. After cytoplasmic pHs of these cells were equalized to the extracellular pH, the extracellular pH was increased by three units by the addition of NaOH. Cytoplasms of both the organisms were alkalized to pH 8.6 immediately and thereafter gradually, after an upward shift of the extracellular pH (Fig. 2). Alkalization of the cytoplasm was more rapid in the C-125-90 cells than the C-125 cells. The alkalization depends on eﬄux of cytoplasmic H+ ions and inﬂux of extracellular OH− ions across the cell wall matrix and cytoplasmic membrane. It is presumed that the gramicidin treatment permitted H+ ion transfer across the membrane. The two organisms are diﬀerent in contents of the anionic charges ﬁxed in the cell walls, as described above. The discrepancy in the alkalization rate is likely due to diﬀerences in rates at which OH− ions pass through the anionic cell wall matrixes of the two organisms.

Thus, it is likely that penetration of OH− ions is suppressed by high anionic charges present in the cell wall matrix of the alkaliphile, as previously predicted. Recently, an S-layer composed of a comparatively acidic protein was discovered on the cell surface of alkaliphile B. pseudofermentum OF4. In the case that environmental concentration of Na+ ions is low, this layer contributes to alkaliphily of the bacterium at alkaline pH higher than 10.5. It is likely that the S-layer suppresses inﬂux of OH− ions, as the TUP of C-125.
Fig. 2. Alkalization in the Cells Treated with Gramicidin by
Upward Shift of Extracellular pH.

C-125 wild type (○) and C125-90 (△) were grown at pH 10
and loaded with BCECF. The cells were incubated in 20 μM
gramicidin-0.1 M NaCl-0.1 M KCl-0.1 M glycine-HCl (pH 7.8) at 37°C for 30 min. The extracellular pH
was shifted to pH 10.9 by a sudden addition of a small volume
of 8 M NaOH at 0 min. Increase in the cytoplasmic pH was fol-
lowed by monitoring F340.

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