Investigation of *roc* genes in pH regulation of *Bacillus subtilis*

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Abstract

Cytoplasimic alkaline pH-stress in *Bacillus subtilis* was observed. Internal pH was observed using a pH-sensitive green fluorescent protein (GFP). The GFP in *B. subtilis* revealed that internal pH was maintained in an alkaline environment. Previous research has established that the *roc* operon is up-regulated at high pH and may help maintain cytoplasmic pH homeostasis at high external pH. The significance of the *rocF* mutant, which is deficient for arginase, was investigated using growth curves and survival assays in alkaline conditions. The mutant and its parent strain, 168 were grown at pH 6.0, 7.0, 8.7, and 9.1. Growth was measured using optical density at 600 nm (OD_{600}). Initially, the mutant grew faster than the parent strain at pH 9.1, but after 4-5 hours OD_{600} in Δ*rocF* dropped, while 168 entered stationary phase. The survival assay demonstrated that 168 had 75% survival when exposed to pH 9.8 for 2 hours compared to the mutant that had 24% in the same conditions. The growth curves and survival assays identified a phenotypical difference in pH regulation between the *rocF* mutant and parent strain, 168. This evidence supports the hypothesis that *roc* genes help pH regulation during base stress. Further research could include analyzing sporulation in strains that lack *roc* genes and dependence on certain amino acids, such as arginine, which is metabolized by the *roc* pathway in alkaline conditions.

Introduction

•*Bacillus subtilis* is a model system for gram-positive bacteria, for instance the food pathogen *Bacillus cereus* and others that encounter environments in which the pH varies (Wilks et al., 2009). When bacteria enters a new environment, they need to regulate their internal pH in order to continue normal functions.

•A group of operons that are up-regulated at high pH is the *roc* operons. These genes could give new insights into pH regulation of the cell (Wilks et al., 2009)

•One gene of particular interest in the *roc* operons is *rocF. rocF* codes for an arginase, which is the first step in arginine catabolism. Its hypothesized that the catabolism of arginine imports a proton and generates acids, which could help counteract high pH-stress (Wei et al., 2006).

Materials and Methods

•**Fluorescence:** Cells were grown overnight in Luria broth with KCl (LBK) buffered at pH 8.5, 37°C, and rotating for 16-20 hrs. Cultures were then diluted 1:50 into baffled flasks with M63 minimal media (7.45 g/liter KCl, 2 g/liter casein hydrolysate, 2 g/liter $(NH_4)_2SO_4$, 0.4 g/liter KH_2PO_4, and 0.4 g/liter K_2HPO_4) (Miller, 1972) buffered at pH 8.5, 37°C. Cells were grown up to OD_{600} of 0.4. Fluorescence of the cells were recorded for wavelengths between 480nm and 510nm by a Fluoromax-3 spectrofluorimeter (Horbia Jobin Yvon). The fluorescence of the cells were correlated with internal pH with two known pH points. Each experiment was done in triplicate.

•**Growth Curve:** Strains were grown overnight in modified LBK buffered at pH 6.0, 7.0, or 8.7, 37°C, and rotating, for 16-20 hrs. Cultures were then diluted 1:100 into baffled flasks at pH 6.0, 7.0, 8.7, or 9.1. Growth of cells were recorded every hour using OD_{600}. Each experiment was done in triplicate.

•**Survival Assay:** Strains were grown overnight in LBK buffered at pH 8.7, 37°C, rotating, for 16-20 hrs. Cultures were diluted 1:500 into baffle flasks at pH 8.7. The cells were grown up to around OD_{600} 0.3. The cells were then exposed to pH 9.8 in differently supplemented media at 37°C, rotating for 2 hrs; then they were serially diluted and plated. The control was serially diluted and plated immediately. Plates were kept at 37°C for 2-3 hrs and then transferred to 30°C till the next day. Each colony was counted as a viable cell. In the experiment, each condition was replicated six times.

Results

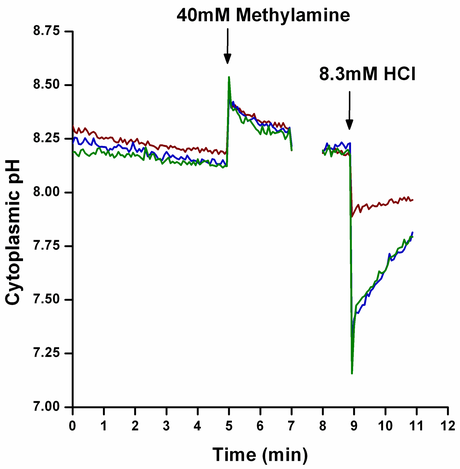

Figure 1
Effect of methylamine on cytoplasmic pH of *B. subtilis*. The change in cytoplasmic pH of *B. subtilis* after methylamine addition was observed. Cells were grown up in supplemented M63 minimal media at pH 8.5 to OD_{600} 0.4. After 5 minutes, 40 mM methylamine was added to equilibrate external and internal pH of the cell. At 9 minutes, 8.3 mM HCl was added to shift the pH down. Fluorescence intensity was converted to pH units using the internal standard curves.

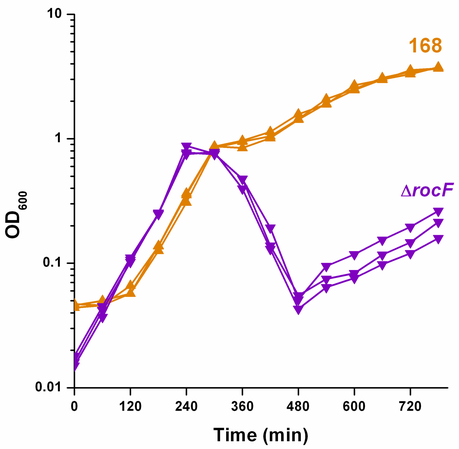

Figure 2
Growth of mutant Δ*rocF* and parent strain, 168 at pH 9.1 over 13 hours. Both strains were grown overnight at pH 8.7. The cells were diluted into LBK at pH 9.1. The OD_{600}—a measure of cell growth—was recorded for each flask, every hour for 13 hours. Each strain had three replicates.

Results cont.

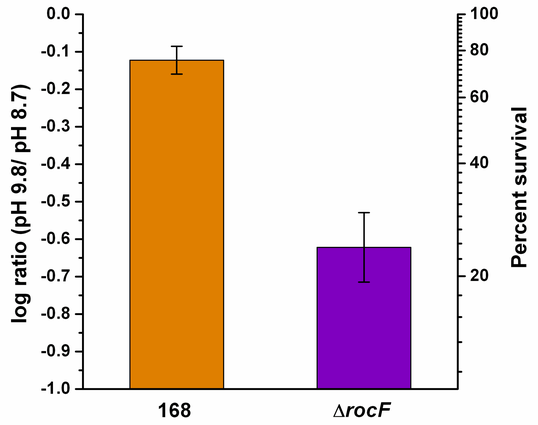

Figure 3
Survival of 168 and Δ*rocF* when exposed to pH 9.8. Both strains were grown overnight at pH 8.7. The cells were diluted to pH 8.7 and grown to mid-log phase (OD_{600} ~0.3). Control cells were diluted and plated. Experimental cells were exposed to pH 9.8 for 2 hours, then diluted and plated. Cells were allowed to grow overnight. Colonies were counted as viable cells. The experimental cells were compared to the control cells by subtracting the log of number of viable cells from the control from the log of number of viable cells from the experimental. (Error bars = standard error of the mean).

Discussion

•*Bacillus subtilis* regulates its cytoplasmic pH in mild base (fig. 1).

•A difference in growth between Δ*rocF* and its parent strain 168 was observed (fig. 2). Initially the mutant grew faster than the parent but growth for the mutant decreased after 4-5 hours. An increase in growth of the mutant was observed after 9 hours, which might be due to a decrease in external pH because of cell fermentation products.

•The survival assay demonstrated that 168 survived exposure to alkaline conditions better than Δ*rocF*, 75% survival compared to 24% survival, respectively (fig. 3).

•The only genetic difference between the parent and mutant is the deletion of *rocF*; therefore, *rocF* plays a part in alkaline-stress survival within *Bacillus subtilis*.

•**Future experiments** should include: reliance on amino acids that are utilized at high pH, such as arginine, which is metabolized by the *roc* pathway in alkaline conditions and analyzing sporulation of different *roc* mutants.

References

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