Investigation of pH Stress Effects on Sporulation and Germination Efficiency in *Bacillus subtilis*

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ABSTRACT

When stressed, for example by nutrient starvation, the gram-positive *Bacillus subtilis* undergoes a process known as sporulation to form dormant endospores highly resistant to environmental extremes. pH dependency of sporulation was observed in *B. subtilis* AG174. Using a sporulation assay, the sporulation efficiency was quantified for cells cultured under different conditions. When grown in low pH, the sporulation efficiency was severely decreased when compared to that found at in high pH. This indicates that the formation of spores is inhibited at low pH. In order to determine whether the sporulation efficiency of *B. subtilis* was a result of sensing external or cytoplasmic pH, permeant weak acids were introduced to the growth medium. The permeant weak acid benzoate was able to collapse the ΔpH and greatly lower the sporulation efficiency. The inhibition of sporulation by benzoate is consistent with cells detecting internal pH. Another factor that we tested was the optical density to which the cells were allowed to grow to before acid shock in order to determine whether pH affects sporulation early or late in the cycle. However, cultures grown to any OD_{600} value up to 3.0 showed a decline in sporulation efficiency from the 55-80% (control) to 10-20% after the acid treatment. The decrease in sporulation after late acid treatment suggests that pH plays a role in late spore formation.

INTRODUCTION

• *B. subtilis* is a gram-positive species of soil bacterium. The *B. subtilis* species is non-pathogenic, unlike the related species, *B. anthracis*, and *B. cereus.*

• During sporulation, the vegetative cell differentiates into a sporangium composed of a larger mother cell compartment and a smaller forespore compartment. This forespore develops into a mature, dormant spore and is highly resistant to physicochemical stress. It has also been observed that the forespore drops by one pH unit in preparation of sporulation.

• If sporulation is decreased at high pH, then that would suggest that spore formation needs low pH. If sporulation is increased at low pH, then that would suggest that spore formation requires a pH gradient between the forespore and mother cell.

• Previous conducted experiments showed that cultures grown at neutral or acidic pH had a longer lag growth when shifted to alkaline conditions. Other experiments and microarray analysis revealed that in high pH, there is a greater genomic adaption. This suggests the potential effects varying pH has on sporulation.

MATERIALS AND METHODS

Sporulation with K^+ Benzoate treatment:
• *B. subtilis* were cultured in Difco sporulation medium for 30 hrs at 37 ˚C buffered with 100 mM 2-(*N*-morpholino)ethanesulfonic acid [MES] adjusted to pH 6; 100 mM 3-(N-morpholino)propanesulfonic acid [MOPS] adjusted to pH 7; and 100 mM 3-{[tris(hydroxymethyl)methyl]amino}propanesulfonic acid [TAPS] adjusted to pH 8. The treated cultures were heat-shocked at 80˚C for 10 min and serial dilutions were plated on potassium supplemented Luria broth (LBK).

Sporulation as a function of pH:
• *B. subtilis* were cultured in Difco sporulation medium overnight for 16 hrs at 37˚C buffered with 100 mM 3-(N-morpholino)propanesulfonic acid [MOPS] adjusted to pH 7.5. The overnights were diluted 100-fold and grown to mid-log phase. After reaching the appropriate OD, cultures were acid-shocked with 100 mM 2-(*N*-morpholino)ethanesulfonic acid [MES] to reach a pH of ~6 and then incubated for 24 hrs. The heat-treated cultures were exposed to 80˚C for 10 min. and serial dilutions were plated on potassium supplemented Luria broth (LBK).

RESULTS

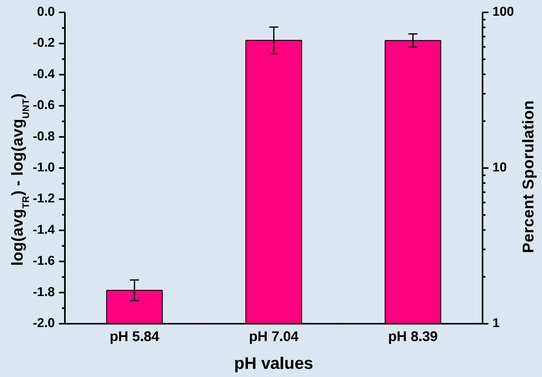

Figure 1.
Error bars represent SEM (n=6). Sporulation efficiency was inhibited at low pH. The starting pH values were 5.56, 6.51, and 7.50 respectively.

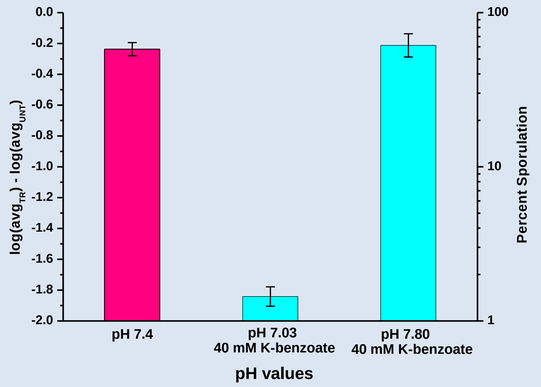

Figure 2.
K-benzoate collapsed the ΔpH thus preventing sporulation at pH 7.0, but had no effect at pH 7.8. The control started at pH 6.83. The experimental conditions were started at pH 6.82 and 7.32. K^+ benzoate (40 mM) was added before the 30 hr. incubation period. Pink represents control and cyan represent K^+ benzoate addition. Error bars represent SEM (n=6).

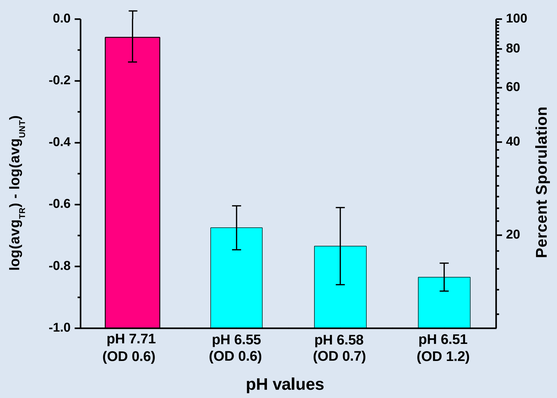

Figure 3.
Cells grown to any OD value before acid treatment, could not sporulate. The control was grown to OD 0.6 and then stabilized with the addition of 100 mM MOPS. The experimental conditions were shocked with 100 mM MES in hopes to have the final pH at ~pH 6.0. Pink represents control and cyan represent experimental groups. Error bars represent SEM (n=6).

CONCLUSION

• **pH and sporulation efficiency.** Through the first series of experiments, we discovered that *B. subtilis* sporulates anywhere up to 80% at pH 7-9 but only sporulates with a 1% efficiency at pH 6. Our data refutes our initial hypothesis, where we expected that the cells grown in alkaline conditions would have shown decreased sporulation, because sporulation is the reaction to acidic stress, not basic. This hypothesis was supported by the knowledge that the internal pH of the forespore in *Bacillus megaterium* decreases by approximately 1 pH unit during sporulation and rises 1 pH unit at the commencement of germination.

• **Permeant weak acids and sporulation efficiency.** The second set of experiments was set into motion in order to determine whether the sporulation efficiency of *B. subtilis* was a result of the sensing external pH or cytoplasmic pH. This was accomplished by adding 40 mM Potassium benzoate (the same results were observed with DMO (5,5-dimethyloxazolidine-2,4-dione) and sorbate) to the Difco sporulation medium. When one overnight was adjusted to approximately pH 7.0, the permeant weak acids collapsed the ΔpH causing the internal pH to drop from the regulated 7.5 to 7.0. When this occurred, the cell was no longer able to sporulate (1% sporulation efficiency for benzoate and sorbate; 10% sporulation efficiency for DMO). However, when the other overnight was buffered at pH 7.5, the benzoate was ineffective because there was not a transmembrane pH difference. The sporulation efficiency was not affected but rather did as well as the control (60% sporulation efficiency). This result is consistent with a requirement for a proton potential between the forespore and the mother cell. The potential could be needed to transport materials into the forespore for spore development.

• **Growth time and sporulation efficiency.** The third set of experiments was performed to determine if the amount of time the cells had to grow before an acid treatment (to pH 6) would affect the sporulation efficiency. The growth was quantified through optical density measurements at 600 nm. Our data showed that no matter what optical density the cells were grown to, the sporulation efficiency would decrease from the 55-80% found in the controls to around 10-20% after the acid treatment. Therefore, acid treatment affects a late stage of sporulation.

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