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Hydrogenases and pH Stress Response in E. coli

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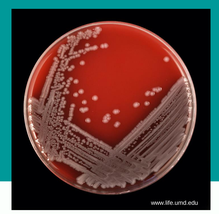

ABSTRACT

The role of hydrogenases in pH regulation and pH resistance in the enteric bacterium *E. coli* was studied. Mutants containing hydrogenase gene deletion were used to assess the importance of determined genes in pH regulation. The deletion of the gene encoding the hydrogenase maturation protein (*hypF*), resulted in a substantial decrease in the acid resistance of *E. coli* strain W3110, from a 50% survival in the wild type to a 1% survival in the *hypF* mutant. It was also determined, by methyl viologen assay, that the *hypF* deletion resulted in a complete loss of hydrogenase activity. However, hydrogenase activity was present in all the single hydrogenase deletion mutants, thus no single hydrogenase has an essential role in the regulation of pH. Analysis of the growth curves of the wild type strain versus the hydrogenase mutant, showed a point at which the generation time would significantly change. A difficulty catabolizing some compound was suggested for such a growth pattern. A new method was investigated for the assessment of the intracellular pH of *E. coli* by the use of the pH dependent fluorescence of EYFP (yellow fluorescent protein) using a fluorimeter to identify changes in emission due to changes in cytoplasmic pH. Preliminary results suggest the possibility to use this method to assess intracellular pH. Future study aim for the use of this technique in time courses following a pH challenge. The comparison of the intracellular pH recovery rate between the wild type strain and the hydrogenase mutants could give an important understanding of the role that these enzymes play in the recovery of the internal cytoplasmic pH.

INTRODUCTION

Hydrogenase enzymes catalyze the interconversion of hydrogen gas and hydronium ions: $H_2 \leftrightarrow 2H^+$. Hydrogenases enable certain pathogens to use molecular hydrogen as an energy source [1]. For example, in *Salmonella enterica* Serovar Typhimurium hydrogenases are a contributing factor to virulence [2]. Another reason for which hydrogenase is important is its role in bacterial production of molecular hydrogen. Molecular hydrogen is thought to be the clean fuel, and many researchers are working on the use of bioreactors for the production of hydrogen [3], [4]. For the purpose of this study *E. coli* will be used as a model organism.

E. coli has four different hydrogenase operons. Our lab recently showed by real-time PCR that the hydrogenases all are induced in acid anaerobically, but are induced aerobically in base (Hayes et al, submitted). This finding was a surprise, as hydrogenases were thought to be induced only anaerobically. We found that a *hypF* mutant that fails to assemble all hydrogenases shows impaired growth aerobically at high pH. However, there has been little study of hydrogenase function at high pH.

My purpose is to study the hydrogenase activity at low pH vs. high pH, and the role of hydrogenases in growth and in cytoplasmic pH regulation.

METHODS

Strain construction and survival assay

The strains were constructed using the P1 transducing phage [5]. Various genes containing a kanamycin gene insertion were transduced in the wild type strain W3110. This new strain constructs were tested for extreme low and high pH resistance (pH 2.0 and 10.0). Overnight cultures were exposed to pH 2.0 for 2 hours. The overnight cultures were diluted 200 fold in LBK (Luria Broth + KCl) adjusted to pH 2.0 or 10.0. Serial dilutions were plated on LBK and colonies were counted and compared to plated dilution of the overnight cultures not exposed to the extreme pH.

Growth curves

Overnight cultures grown at pH 5.0 or 8.5, were diluted 200 fold in 10 ml of LBK contained in 250 ml baffled flasks. The baffled flasks were incubated in a bath at 37 degrees and readings were taken every 15 minutes. The readings consisted in the withdrawal of 200 µl of culture for analysis. The O.D of these samples was taken and analyzed with excel. The generation rates were calculated and compared to those of the wild type strain.

Methyl viologen assay

This assay was based on a previously reported method [6]. Stationary-phase cultures in LBK medium were washed and resuspended in 5mM K2HPO4 pH 7, 5mM cysteine, 10mM benzylviologen, and sealed under hydrogen gas. Hydrogenase activity was detected as a blue color change in the samples tested, which was present for the wild type strain W3110.

Fluorimetry

The strain JLS0617, containing a plasmid encoding EYFP (J. Wilks) was used for these experiments. The strain was grown overnight in LBK and was resuspended in buffered minimal media pH adjusted to pH 7.5 and 5.0. Excitation spectra were taken in quartz cuvettes (10 mm path length) containing 3.5 ml of the cell suspension. A second reading was taken 30 seconds after the addition of 10µl benzoate. The spectra were taken with the following settings: emission λ = 500-620 nm (slit width 5 nm) and excitation λ = 480 nm (slit width 5 nm).

RESULTS AND DISCUSSION

The deletion of the gene encoding the hydrogenase maturation protein (*hypF*), resulted in a substantial decrease in the acid resistance of *E. coli* strain W3110. The overall survival of the *hypF* deletion mutant (strain JLS0611) after a two hour acid challenge at pH 2 was approximately 1%, while the percent survival of the wild type strain was approximately 50%. Mutants with single hydrogenase gene deletions (hydrogenases I, II and III), were tested for survival in extreme acid (pH 2) and base (pH 10), but no substantial decrease in survival was found [7] (Table 1).

Strain	Description	% Survival
W3110	wild type	54±6%
JLS0611	hydrogenases maturation protein mutant	1±0.2%
JLS0613	hydrogenase I mutant	49±8%
JLS0614	hydrogenase II mutant	44±8%
JLS0615	hydrogenase III mutant	58±12%

Table 1. % survival of overnight cultures of strains containing various hydrogenase deletions after acid challenge at to pH 2 for 2 hrs. Colonies were counted and compared to control plates. The % survival is expressed with its fractional error calculated from SEM (n=6).

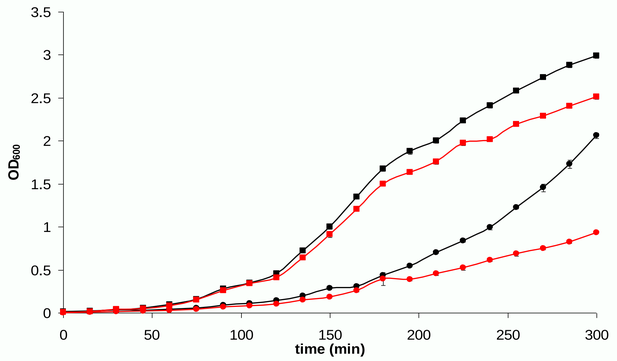

Figure 1. Growth curves of wild type strain W3110 (—) and the Δ*hypF* mutant JLS0611 (—) in LBK (Luria Broth + KCl) adjusted to pH 5.0 (●) and pH 8.5 (■). The strains were grown aerobically at 37°C. Overnight cultures were diluted 200-fold. Error bars: SEM (N=3).

By using the methyl viologen assay, it was determined that the *hypF* deletion resulted in a complete loss of hydrogenase activity. Hydrogenase activity was present in all the single hydrogenase deletion mutants. These results would suggest that no hydrogenase has a crucial role in the interconversion of hydrogen into protons and vice versa.

The growth curves carried out under both acidic (pH 5.0) and basic (pH 8.5) conditions did not show any significant difference in growth rate between the single hydrogenase deletion mutants and the wild type strain. When analyzing growth curves of the wild type strain and the *hypF* deletion mutant, a difference can be detected in their growth rates**.** The two strains presented the same growth rate to a certain point at which the rates would change, with the *hypF* mutant growing at a lower rate (Fig.1). For the growth at pH 5.0 the generation time of the *hypF* mutant would almost double compared to that of W3110 while there was a small difference for growth at pH 8.5. This difference may be explained by the depletion of a main metabolite. The cells would need to change the current metabolic mechanism in order to metabolize this other source of carbon or nitrogen.

The comparison between the emission spectra before and after the benzoate addition would suggest the possibility of the use of such a method to assess intracellular pH. When comparing the emission spectrum of the culture resuspended in pH 7.5 medium, only a slight drop in the signal was noticeable, since it is known that the internal pH of *E. coli* is around pH ~7.6 (Fig. 2A). However, when benzoate is add to the culture resuspended in pH 5.0, the signal drops dramatically because the internal pH would change from ~7.6 to 5.0 because benzoate uncouples the membrane allowing for a free flow of protons between the interior and exterior of the cell (Fig. 2B). This technique could be used to assess the difference between the internal pH of the wild type and mutant strains and time assays could be performed to asses the rate of recovery of the internal pH after an initial rapid change in the extracellular pH.

Future studies will be geared towards developing a further understanding of the role played by the hydrogenases in the latter portion of the log phase growth of *E. coli* strain W3110. Also the fluorimetry technique should be used to assess the internal pH of the wild type and the hydrogenase mutants and to see if there is any difference in the rate of intracellular pH recovery. Preliminary studies suggest a role of hydrogenases in the metabolism of the amino acid glutamine. We are going to conduct further studies in order to get a better understanding of this metabolic mechanism.

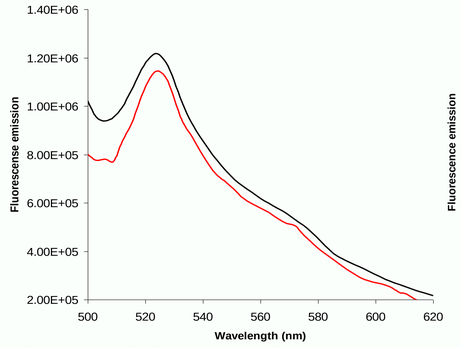

Figure 2A. Emission spectrum of JLS0617 resuspended in minimal media buffered to pH 7.5 (—) and emission spectrum of the same sample taken after 30 seconds of addition of benzoate (—). Emission λ = 500-620 nm and Excitation λ = 480 nm.

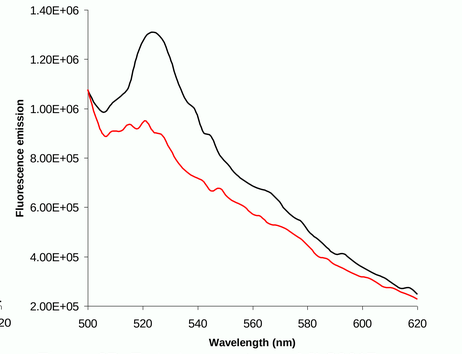

Figure 2B. Emission spectrum of JLS0617 resuspended in minimal media buffered to pH 5.0 (—) and emission spectrum of the same sample taken after 30 seconds of addition of benzoate (—). Emission λ = 500-620 nm and Excitation λ = 480 nm.

CONCLUSIONS

- Deletion of the gene encoding the hydrogenase maturation protein (*hypF*), resulted in a substantial decrease in the acid resistance of *E. coli* strain W3110.
- The *hypF* deletion resulted in a complete loss of hydrogenase activity while no single hydrogenase has a specific role.
- Hydrogenases seem to be used for the catabolism of some compound during late log phase.
- The use of the yellow fluorescent protein (EYFP) to determine cytoplasmic pH with the use of a fluorimeter seems to have great potential. The use of such a method could be of great use for the understanding of the role of *HypF* in internal pH recovery.

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