Hydrogenase-3 Contributes to Anaerobic Acid Resistance of \textit{Escherichia coli}

Ken Noguchi, Daniel P. Riggins, Khalid C. Eldahan, Ryan D. Kitko, Joan L. Slonczewski*

Department of Biology, Kenyon College, Gambier, Ohio, United States of America

Abstract

\textbf{Background:} Hydrogen production by fermenting bacteria such as \textit{Escherichia coli} offers a potential source of hydrogen biofuel. Because \textit{H}_2 production involves consumption of 2H\textsuperscript{+}, hydrogenase expression is likely to involve pH response and regulation. Hydrogenase consumption of protons in \textit{E. coli} has been implicated in acid resistance, the ability to survive exposure to acid levels (pH 2–2.5) that are three pH units lower than the pH limit of growth (pH 5–6). Enhanced survival in acid enables a larger infective inoculum to pass through the stomach and colonize the intestine. Most acid resistance mechanisms have been defined using aerobic cultures, but the use of anaerobic cultures will reveal novel acid resistance mechanisms.

\textbf{Methods and Principal Findings:} We analyzed the pH regulation of bacterial hydrogenases in live cultures of \textit{E. coli} K-12 W3110. During anaerobic growth in the range of pH 5 to 6.5, \textit{E. coli} expresses three hydrogenase isoenzymes that reversibly oxidize \textit{H}_2 to 2H\textsuperscript{+}. Anoxic conditions were used to determine which of the hydrogenase complexes contribute to acid resistance, measured as the survival of cultures grown at pH 5.5 without aeration and exposed for 2 hours at pH 2 or at pH 2.5. Survival of all strains in extreme acid was significantly lower in low oxygen than for aerated cultures. Deletion of \textit{hyc} (Hyd-3) decreased anoxic acid survival 3-fold at pH 2.5, and 20-fold at pH 2, but had no effect on acid survival with aeration. Deletion of \textit{hyb} (Hyd-2) did not significantly affect acid survival. The pH-dependence of \textit{H}_2 production and consumption was tested using a H\textsubscript{2}-specific Clark-type electrode. Hyd-3-dependent \textit{H}_2 production was increased 70-fold from pH 6.5 to 5.5, whereas Hyd-2-dependent \textit{H}_2 consumption was maximal at alkaline pH. \textit{H}_2 production, was unaffected by a shift in external or internal pH. \textit{H}_2 production was associated with \textit{hycE} expression levels as a function of external pH.

\textbf{Conclusions:} Anaerobic growing cultures of \textit{E. coli} generate \textit{H}_2 via Hyd-3 at low external pH, and consume \textit{H}_2 via Hyd-2 at high external pH. Hyd-3 proton conversion to \textit{H}_2 is required for acid resistance in anaerobic cultures of \textit{E. coli}.

Introduction

Bacterial hydrogen production by hydrogenase is studied as a promising source of clean alternative energy [1,2]. In the intestinal tract, \textit{H}_2 produced from bacteria fermentation enables methane production by methanogens [3] and contributes to the growth of pathogens such as \textit{Salmonella enterica} and \textit{Helicobacter pylori} [4,5]. Hydrogenase in \textit{Escherichia coli} has been suggested to decrease cytoplasmic acid stress and contribute to its acid resistance systems [6–9]. Because \textit{E. coli} need to survive the harshly acidic environment of the stomach to colonize the intestine, acid resistance systems enhance the infective ability of pathogenic \textit{E. coli} [10–12]. Several mechanisms have been characterized that enhance survival at pH 2.5 and below [13], such as the amino acid-dependent glutamate and arginine decarboxylases [14–16]. Genes encoding these enzymes and transporters are up-regulated during growth in moderate acid [7,17,18]. Most of the above studies of \textit{E. coli} acid resistance address aerated cultures. In natural environments such as the gastrointestinal tract, however, enteric bacteria experience low oxygen. Oxygen limitation and acid stress occur in the microaerobic environment of the stomach [19], which harbors many obligate and facultative anaerobic organisms such as \textit{Clostridium} and \textit{Veillonella} species [20,21]. In \textit{Salmonella typhimurium}, anoxic conditions are required for expression of the acid-resistance component arginine decarboxylase [22]. Hayes \textit{et al.} (2006) showed that all four hydrogenase isoenzymes are upregulated by acid under oxygen-limited conditions [7].

The four isoforms of hydrogenase catalyze the reversible oxidation of molecular hydrogen to 2H\textsuperscript{+}. However, each hydrogenase functions primarily in one direction. Hydrogenase-1 (Hyd-1, encoded by \textit{hya}) and hydrogenase-2 (Hyd-2, encoded by \textit{hyb}) are energy-conserving respiratory pathways consuming \textit{H}_2 with Hyd-2 acting as the primary consumption hydrogenase [23–25]. Hydrogenase-3 (Hyd-3) is the primary production hydrogenase [26]; along with formate dehydrogenase (FDH-II), Hyd-3 makes up the formate hydrogen lyase (FHL) complex, which breaks down formate to carbon dioxide and \textit{H}_2 [6,27–29]. The function of hydrogenase-4 (Hyd-4) is unclear; it may be a component of a second formate hydrogen lyase system (FHL-2)
Mutants deleted for hypF mutant lacks all hydrogenase activity [8,32]. A hypF mutant shows decreased acid resistance in partly aerated cultures [7]. Because the function of hydrogenases is intricately connected to metabolic pathways, the pH-dependence of H2 consumption must be measured in vivo. Previous studies of H2 production of hydrogenase mutants have been based on harvested cell concentrates, often with addition of 100 mM formate to increase FHL activity, although such high formate concentration is incompatible with growth [9,25,33]. Our electrode-based methods were applied to live, growing cultures. Since H2 production was hypothesized as a cellular mechanism for acid resistance, we observed the pH-dependent activity of hydrogenases under the conditions where acid resistance is induced (anaerobic growth at low external pH). We also characterized how pH regulates H2 production and consumption via each hydrogenase complex, determining the significance of each at low and high pH.

Results

H2 production as a function of pH

In order to test the role of hydrogenases at low pH, we observed E. coli H2 production across a range of pH values in strains W3110, JLS0920 (lacks Hyd-1), JLS0921 (lacks Hyd-2), and JLS0922 (lacks Hyd-3). H2 levels were measured using a Unisense electrode, as described under Materials & Methods. Our results using strain W3110 show that H2 production increased as pH decreased (Fig. 1). In strain JLS0921, lacking the primary consumption hydrogenase (Hyd-2), H2 gas was produced without consumption. In this strain a similar pattern of increasing H2 production with decreasing pH was observed (Fig. 1B), and at each pH H2 was produced faster than in the parental strain (Fig. 1A). By contrast, strain JLS0922, lacking the primary production hydrogenase Hyd-3, showed virtually no H2 production at any pH (Fig. 1C). Strain JLS0920, lacking Hyd-1, showed no difference in H2 production from the wild-type (data not shown).

The traces of H2 concentration (μM) over time were converted into production rates by taking the slope from 2–5 minutes (Fig. 2). In strain W3110, H2 production rate increased sharply at pH 5.5, showing a 70-fold increase from external pH 6.5 to 5.5. Meanwhile, above pH 6.5 H2 production was not detected (data not shown). Overall, conversion of protons to H2 by Hyd-3 was greatly increased at acidic pH.

In order to determine whether the change in H2 production was due to a change in enzymatic activity, W3110 was assayed for H2 production and then shifted to alternate pH values. Cells grown to mid-late log phase at pH 5.5 were assayed for 6 minutes before shifting the external pH to 6.5 and cells grown at pH 6.5 were shifted to external pH 5.5. Neither shift changed the H2

![Figure 1. Effect of pH on the H2 production by W3110, ΔhybC and ΔhyC. The lines represent traces of H2 concentration as a function of time. Before time 0, the cultures were sparged with 100% N2 in order to eliminate any residual H2 in the culture. Anaerobic cultures of W3110 (A), ΔhybC (B), and ΔhyC (C) were grown to log phase at pH 5.5, pH 6, and pH 6.5 and assayed for H2 production as stated in the Materials and Methods. Lines are representative samples of n = 3. doi:10.1371/journal.pone.0010132.g001](image1)

![Figure 2. Effect of pH on the H2 production rate of W3110, ΔhybC and ΔhyC. Anaerobic cultures were grown to log phase at pH 5.5 (black bars), 6 (hatched bars), and 6.5 (white bars) and assayed for H2 production as described in the Materials and Methods. H2 production rate was calculated as stated in the Materials and Methods. Error bars represent SEM, n = 3; those that were too small to see clearly were omitted. The experiment was conducted twice. doi:10.1371/journal.pone.0010132.g002](image2)
production (data not shown). Additionally, to assess whether a change in internal pH would affect Hyd-3 activity, benzoate was added to depress cytoplasmic pH. Benzoate is a permeant weak acid that partly equilibrates internal and external pH by dissociating to release protons in the cytoplasm. At external pH 5.5, 5 mM benzoate lowers the cytoplasmic pH from approximately pH 7.5 to pH 6 [34]. Benzoate did not affect H₂ production at pH 5.5 or pH 6.5 during the 6 min observation after addition (data not shown). Neither changes in external pH nor cytoplasmic pH affected H₂ production, so the change in H₂ production was not caused by a change in enzymatic activity.

**hycE expression as a function of pH**

In order to determine whether the change in H₂ production was associated with a change in hyc expression, the mRNA levels of hycE, encoding the large subunit of Hyd-3, were measured in W3110 from external pH 7 to pH 5.5 (Fig. 3). hycE expression increased as the external pH decreased, whereas the activity of Hyd-3 was unchanged with a shift in external or internal pH. Thus the change in H₂ production more likely arises from the increased hyc expression rather than a change in enzyme activity.

**H₂ consumption as a function of pH**

The pH-dependence of H₂ consumption has yet to be defined. We observed H₂ consumption in the strains W3110, JLS0920, JLS0921, and JLS0922, cultured at both low and high pH. H₂ levels were measured as described. Our results using W3110 showed that H₂ is produced at acidic pH, but at neutral and alkaline pH, H₂ is consumed (Fig. 4). JLS0920, lacking Hyd-1, showed no difference in H₂ consumption when compared with the wild-type (data not shown). In JLS0922, lacking Hyd-3, H₂ was consumed without production. At neutral and alkaline pH, H₂ production in JLS0922 (Fig. 4B) resembled that observed in the wild-type strain (Fig. 4A), but at pH 5.5 the strain showed only H₂ consumption. Even in a solution saturated with 20% H₂, only H₂ production was observed in JLS0921, lacking Hyd-2 (data not shown). The traces of H₂ concentration (mV) over time were converted into rates taking the slope from 2–5 minutes (Fig. 5). JLS0922 shows less than a 2-fold difference in consumption from pH 8 to 5.5, suggesting that the regulation of H₂ consumption is not as strongly pH-dependent as H₂ production.

**Extreme acid survival of hydrogenase mutants**

We investigated whether _E. coli_ strains grown under conditions of high hydrogenase activity would show hydrogenase-dependent acid resistance. For control comparison, bacteria were cultured...
and difficult cultures. At acidic pH, H₂ production was dependent only on production and consumption were measured in vivo, in growing which was more important under acidic conditions. Hydrogen production in various hydrogenase mutants in order to determine Materials and Methods. A negative value for H₂ consumption means H₂ (white bars) and assayed for H₂ consumption as described in the Materials and Methods. Error bars represent SEM, n = 3. The experiment was conducted twice.

Discussion

E. coli use several mechanisms to resist the harshly acidic conditions of the stomach. A previous report revealed the contribution of hydrogenase to acid resistance in partly aerated cultures [7]. Here we clarify that finding to show that specifically Hyd-3, which is a part of the FHL complex, consumes protons to contribute to acid resistance of anaerobic cultures.

We first defined the pH regulation of both H₂ consumption and production in various hydrogenase mutants in order to determine which was more important under acidic conditions. Hydrogen production and consumption were measured in vivo, in growing cultures. At acidic pH, H₂ production was dependent only on Hyd-3, and was increased by the deletion of Hyd-2, whereas H₂ consumption was only dependent on Hyd-2.

Both H₂ production and consumption rates are commonly measured under conditions such as cells cultured at pH 6.5 and assayed in the presence of 100 mM sodium formate [25]. Under physiological conditions, intracellular formate concentration reaches only as high as 20 mM [35]. Our results are consistent with the previous finding that Hyd-3 is the main production hydrogenase. We further show that hydrogen production is induced at low pH in the absence of exogenous formate.

The in vitro pH-dependent activity of the consumption hydrogenase, Hyd-2, is maximal at high pH [23]. In the current report, we saturated an anaerobic culture with H₂ to directly measure Hyd-2-dependent H₂ consumption. This revealed that Hyd-2-dependent consumption increased under alkaline conditions, reaching a maximum at pH 8. Additionally, at pH 5.5 the wild-type strain showed no H₂ production despite being in a H₂ saturated environment. Because alkaline conditions appear to enhance H₂ consumption, it was not expected to contribute to extreme acid survival.

A previous study using E. coli MC4100 finds less than 2-fold increase in H₂ production from pH 7.5 to pH 5.5 [26], whereas we found a 70-fold increase from pH 6.5 to pH 5.5. It is possible that this discrepancy can be attributed to the previous use of 0.2% glucose in the growth media used by Ref. [26], since hyc expression is repressed by glucose [36].

In order to maximize H₂ production yield, it is of importance to understand whether conditions used to induce H₂ production, such as decreasing pH, increase hyc expression or enhance the enzymatic activity. Our results show that a shift from pH 6.5 to 5.5, a shift from pH 6.5 to 6.5, or the addition of 5 mM benzoate did not affect H₂ production, whereas expression of the large
subunit of Hyd-3 increased as pH decreased from pH 7 to pH 5.5 (Fig. 3). Thus, the observed increase in H$_2$ production is likely due to an increase in hyc expression [7], rather than a direct effect of external or internal pH on Hyd-3 activity.

Extreme-acid survival assays using aerobic cultures showed no hydrogenase-dependent acid resistance (Fig. 6). However, anaerobic cultures required Hyd-3 for survival at or below pH 2.5. The requirement for Hyd-3 increased as the pH decreased, showing a greater effect at pH 2 when compared to pH 2.5. Acid resistance systems are generally defined using aerobic cultures [7,12,15,37] and Hyd-3 is the first reported mechanism that is necessary for anaerobic but not aerobic cultures.

The low-oxygen requirement for the acid resistance phenotype makes sense because Hyd-3 is only expressed anaerobically, controlled by the transcriptional activator FhlA [36,38]; and under aerobic conditions Hyd-3 is inactive [28]. Nevertheless, other acid resistance systems are known to be co-induced by acid and anaerobiosis yet still show an acid resistance phenotype with aerobic cultures. For instance, arginine decarboxylase, anaerobiosis yet still show an acid resistance phenotype with resistance systems are known to be co-induced by acid and anaerobiosis yet still show an acid resistance phenotype with aerobic cultures.

Materials and Methods

Bacterial strains, media, and growth conditions

E. coli K-12 strain W3110 [40] was used as the wild-type strain in all experiments. Deletion alleles containing a kanamycin resistance insertion (Km$^R$) were transduced from the Keio collection [41] into the wild-type strain by P1 phage transduction (Table 1).

Cells were grown in LBK medium (10 g/L tryptone, 5 g/L yeast extract, and 7.45 g/L KCl) [7,42]. Overnight growth medium was supplemented with kanamycin (25 μg/ml) for mutants. Media were buffered with 100 mM Homopiperazine-N,N’-bis-2-(ethanesulfonic acid) (HOMOPIPES), pH 7 and plated, representing the unexposed controls. Plates were also serially diluted into LBK 100 mM MOPS, pH 7 and plated, representing the unexposed controls.

RNA isolation and real-time quantitative PCR

E. coli W3110 cultures were grown to mid-late log phase (OD$_{600}$ = 0.4–0.5) as stated above in closed-cap anaerobic tubes at pH values from pH 5.5 to pH 7.0. Bacterial RNA from three independent cultures at each condition was stabilized using the cold 10% phenol-ethanol stop solution as previously described [7,43] and isolated using the RNeasy Kit (Qiagen) followed by DNase treatment (Ambion).

Expression of hycE was quantified using real-time PCR using an ABI Prism7500 DNA analyzer (Applied Biosystems) as described previously [7]. The forward primer sequence was 5'-GAA-AACGCGATGCGTATCCAG - 3' and the reverse primer sequence was 5' - CAGAATGCGCGGATCAT - 3'. The SYBR Green PCR One-Step Protocol (Applied Biosystems) was used, in which cDNA reverse transcription and PCR amplification occur in the same well. Nucleic acid concentrations were as follows: 0.1 nM forward primer, 0.1 nM reverse primer, and 50 ng target RNA. PCR cycling conditions were as follows: reverse transcription at 48°C for 30 min and 95°C for 10 min, 40 cycles of denaturation at 92°C for 15 s, and extension at 60°C for 1 min. The total RNA in each sample amplified was used as the basis to normalize individual gene expression profiles. Expression levels of the average of three technical replicates of each biological replicate are presented relative to the expression in the pH 7 anaerobic control.

Acid resistance assays

The conditions for testing acid resistance (survival in extreme acid) were based on those described previously, with modifications [7,12]. The percent of surviving cells was assessed using aerobic and anaerobic cultures. To test aerobic cultures, cells were grown to stationary phase without antibiotics (16–18 h, 37°C) in LBK 100 mM HOMOPIPES, pH 5 in culture tubes with a capacity 7.5 times the culture volume. The tubes were rotated vertically to ensure aeration (40 rpm). The overnight cultures were diluted 200-fold into unbuffered LBK at pH 2. Exposure tubes were incubated for 2 h with vertical rotary aeration (40 rpm) at 37°C. The initial cell density during extreme acid exposure was approximately 1×10$^8$ CFU per ml. Following the 2 h exposure, the cultures were serially diluted and streaked onto LBK-agar plates. The overnight cultures were also serially diluted into LBK 100 mM MOPS, pH 7 and plated, representing the unexposed controls. Plates were incubated overnight at 37°C.

To measure acid survival of anaerobic cultures, cells were grown to stationary phase (16–18 h, 37°C) in LBK 100 mM MES, pH 5.5 in closed screw cap tubes. The tubes were rotated slowly end over end (8 rpm) to ensure that the cells distributed evenly throughout the medium [7,44,45]. The overnight cultures were diluted 200-fold into closed screw cap tubes with unbuffered LBK at pH 2.5 or pH 2. Exposure tubes were incubated for 2 h rotating end over end (8 rpm) at 37°C. The initial cell density during extreme acid exposure was approximately 3×10$^8$ CFU per ml. Following the 2 h exposure, the cultures were serially diluted and plated. The overnight cultures were also serially diluted into LBK 100 mM MOPS, pH 7 and plated, representing the unexposed controls. Plates were incubated overnight at 37°C.

Survival rates were calculated as follows: the raw data were log$_{10}$-transformed before taking the mean. The means were subtracted to get a log$_{10}$ ratio, which roughly correlates to percent survival. The ratios are log$_{10}$-transformed because survival data represent data points on an exponential death curve, and is thus expected to follow a log-normal distribution. All errors stated are standard error of the mean (SEM). Each experimental condition included six biological replicates from the same overnight culture.

| Strain     | Genotype          | Source          |
|------------|-------------------|-----------------|
| W3110      | K-12 (F’ λ+)      | [40]            |
| JLS0920    | W3110 hysB::Km    | This work       |
| JLS0921    | W3110 hycC::Km    | This work       |
| JLS0922    | W3110 hycE::Km    | This work       |
| JLS0925    | W3110 hycF::Km    | This work       |

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For the external pH shift assays, the conditions were the same as the H₂ production assays except that the assay was conducted for 12 minutes, and KOH or HCl was added 6 minutes after the start of the assay to change the external pH from 5.5 to 6.5 or from 6.5 to 5.5. For the internal pH shift assays, the cells were again assayed for 12 minutes, and potassium benzoate was added 6 minutes after the start of the assay to expose the cells to a 5 mM final concentration.

The rate of H₂ production and consumption was calculated based on the slope of H₂ concentration (mV) versus time from 2–5 minutes and normalized to OD₆₀₀. The first 2 minutes were used for sensor equilibration. The mV reading was converted to μM of H₂ using a conversion factor of 0.0837 μM/mV. This factor was determined using the calibration procedure stated in the Unisense manual: a 2-ml sample of LBK was sparged with 20% H₂/N₂ and assayed at 37°C with a stir bar (200 rpm). The maximum mV reading was correlated to known values of saturated H₂ concentration. All errors stated are standard error of the mean.

**Author Contributions**

Designed the study: KN. Conducted experiments: KN. Drafed the manuscript: KN. Conducted hydrogenase assays: DPR KCE RDK. Revised the manuscript: RDK JLS. Supervised the entire study: JLS.

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