HHS Public Access

Author manuscript

Eng Rep. Author manuscript; available in PMC 2024 April 05.

Published in final edited form as:

Eng Rep. 2020 May ; 2(5): . doi:10.1002/eng2.12161.

Prolonged culture in aerobic environments alters *Escherichia coli* H_2 production capacity

Nash D. Rochman[1], **David Raciti**[2], **Felipe Takaesu**[3], **Sean X. Sun**[4]

[1]Institute for NanoBioTechnology, Johns Hopkins University, Baltimore, Maryland

[2]Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, Maryland

[3]Department of Biology, Johns Hopkins University, Baltimore, Maryland

[4]Departments of Mechanical Engineering and Biomedical Engineering, Johns Hopkins University, Baltimore, Maryland

Abstract

Growing interest in renewable energy continues to motivate new work on microbial biohydrogen production and in particular utilizing *Escherichia coli* a well-studied, facultative anaerobe. Here we characterize, for the first time the H_2 production rate and capacity, of *E coli* isolates from the 50 000th generation of the Long-Term Evolution Experiment. Under these reaction conditions, peak production rates near or above 5 mL per hour for 100 mL of lysogeny broth (LB media) was established for the ancestral strains and batch efficiencies between 0.15 and 0.22 mL H_2 produced per 1 mL LB media were achieved. All 11 isolates studied, which had been aerobically cultured in minimal media since 1988, exhibited a decreased H_2 production rate or capacity with many strains unable to grow under anaerobic conditions at all. The genomes of these strains have been sequenced and a preliminary analysis of the correlations between genotype and phenotype shows that mutations in gene *ydjO* are exclusively observed in the two isolates which produce H_2, potentially suggesting a role for this gene in the maintenance of wild type metabolic pathways in the context of diverse mutational backgrounds. These results provide hints towards uncovering new genetic targets for the pursuit of bacterial strains with increased capacity for H_2 production as well as a case study in speciation and the control of phenotypic switching.

Correspondence: Nash D. Rochman, Institute for NanoBioTechnology, Johns Hopkins University, Baltimore, MD. nashdeltarochman@gmail.com, David Raciti, Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD. draciti1@jhu.edu, Sean X. Sun, Departments of Mechanical Engineering and Biomedical Engineering, Johns Hopkins University, Baltimore, MD. ssun@jhu.edu.

Nash D. Rochman and David Raciti contributed equally to this publication.

AUTHOR CONTRIBUTIONS

Nash D. Rochman, David Raciti, Felipe Takaesu, and Sean X. Sun designed experiments; Nash D. Rochman, David Raciti, and Felipe Takaesu conducted experiments and data analysis; Nash D. Rochman, David Raciti, Felipe Takaesu, and Sean X. Sun wrote the article.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

Keywords

biohydrogen; *E coli* ; evolution; genetics; *ydjO* ; phenotypic switching

1 | INTRODUCTION

Growing interest in renewable energy and sustainable manufacturing continues to put a spotlight on hydrogen production. While a range of renewable resources are in a mature state of development including wind and solar, hydrogen may be utilized not only as a fuel source but also as an energy carrier.[1] Biological means of hydrogen production may be less energy intensive than chemical methods, through the capitalization of organic waste products and reaction conditions at less demanding temperatures and pressures.[2]

These economic considerations motivate the mapping of cellular metabolic pathways and the identification of efficient routes towards products of interest. The near-ubiquitous capacity for anaerobic fermentation among microbes utilizing a broad array of substrates,[3] has afforded the identification of a host of bacterial and, more recently algal,[4,5] candidates. Owing to its easy cultivation, fast growth rate, and mature position as a classic model organism, many studies have focused, and continue to focus, on the use of *Escherichia coli*:[2] a facultative anaerobe commonly found in the lower intestine of warm-blooded animals, a largely anaerobic environment.[6] Many strategies have been employed to improve hydrogen production rate and yield. In addition to optimizing reaction conditions for a given microbe, it is possible to genetically engineer novel strains for improved production in a given environment. A range of target genes have been identified and successful mutants have been generated.[7,8] On the other hand, finding mutants with decreased or cessated hydrogen production[9] is also valuable. Knowledge of these mutants expands the list of target genes known to be important or essential for anaerobic metabolism.

Extended culture in an aerobic environment may be expected to select for adaptations in a strain of *E coli* which could adversely affect its fitness or otherwise alter its growth when returned to an anaerobic environment. The identification of such adaptations and the genomes of such mutants would not only shed light on basic questions regarding evolutionary phenotypic selection and metabolic regulation, but potentially establish a set of genes as targets for engineering strains with higher H_2 yield or better efficiency.

Motivated by this hypothesis we set out to characterize, to our knowledge for the first time, the H_2 production of strains produced during the Long-Term Evolution Experiment (LTEE) from the lab of Richard Lenski.[10] The genomes of the strains utilized have been sequenced[11] which enabled us to perform an analysis correlating genotype to H_2 output phenotype. We observed a subset of evolved strains to grow slowly, in comparison to their ancestral strain, or not at all when cultured anaerobically in LB media despite observing robust growth during aerobic culture for all strains. We further observed another subset to grow robustly when anaerobically cultured but fail to produce H_2. We hope these observations highlight the potential interest of these strains and their genomes to the bioH_2 community as a negative example towards the goal of engineering more productive or more efficient strains. These results further serve as a case study in speciation where adaptation to a novel environment

over a reasonably short period of time, on an evolutionary timescale, brought about the complete loss of access to the native metabolic phenotype for a subset of the population. We hope this work brings to light yet another facet of the LTEE which may prove useful towards understanding the control of phenotypic switching and the evolutionary balancing act of remaining adaptable while yet adapting to a specific environment.

2 | METHODS

We sought to conduct a simple, comparative analysis among isolates grown in batch reaction for (1) volumetric batch efficiency (liters H_2 produced per experiment), (2) H_2 concentration as a function of time, and (3) H_2 volumetric production rate as a function of time. Our use of LB media which consists of multiple, complex carbon sources made it impractical to explicitly measure mass efficiency; however, under these unoptimized growth conditions we expect to be far from the theoretical maximum yield of (2.0 mol/mol glucose) which has been approached in previous work[8] utilizing genetically modified *E coli*. Despite these drawbacks, we believe this simple approach has value because it (1) is easy to reproduce requiring minimal specialized equipment and reagents and (2) is likely to generalize to a wider array of media conditions and cell densities than an experimental design optimized for high H_2 yield and efficiency.

2.1 | Reactor design

A simple bioreactor, 4-port 170 mL round bottom flask with a thermal heating jacket (Ace Glass Inc.), continuously magnetically stirred (rpm unspecified), and an in-line gas chromatograph equipped with a thermal conductivity detector (GC-TCD, SRI Instruments, Model no. 8610–0070, Multiple Gas Analyzer no. 1) with N_2 as the internal carrier gas (N_2 carrier gas was utilized to enable detection of H_2) was assembled (Figure 1A). Gas was supplied by Airgas Inc. The calibration gas was a mixture of H_2 in N_2 (forming gas) and was diluted with N_2 to obtain multiple points to establish a calibration curve that experimental data were referenced against. All gases utilized were ultra-high purity grade. The sum of the calibration gas flow was equal to the experimental flow rate of N_2 during experiments to ensure the same pressure profiles were present throughout the reactor. Mass flow controllers (MFCs) were acquired from MKS Instruments and had maximum flow rates at least double of what was utilized during experiments. Temperature (set to 37°C as measured by an RTD probe, Ace glass Inc.) and continuous nitrogen flow (initially purging at a flow rate of 50 mL/min and then set to 5 mL/min) were controlled. O_2 and H_2 concentration in the gaseous phase of the reactor were measured.

2.2 | Inoculation

Prior to reactor prep, *E coli* were cultured for approximately 9 hours until an optical density (OD) of between 0.3 and 0.5 was reached. The reactor was then filled with 100 mL of Lysogeny broth (LB media), purged (50 mL/min flow-rate for 30 minutes, O_2 concentration fell below detectable levels within 10 minutes), and inoculated with 5 mL of cellular culture via syringe (unwashed). Nitrogen flow-rate was set to 5 mL/min and GC-TCD measurements for H_2 and O_2 were taken every 10 minutes. Experiments were also conducted where the reactor was inoculated with a much smaller initial cell number

(from a standard inoculating loop) and while this increased incubation time and the duration preceding the first nonzero H_2 reading from the GC-TCD, no difference was observed in the subsequent H_2 production curve. LB was chosen as the culture medium over the minimal media (Davis minimal broth supplemented with 25 mg/L glucose[10]) in which the LTEE strains were originally produced, to support a higher OD in stationary-phase. OD was periodically measured using standard equipment (600 nm lightsource, Beckman Coulter spectrophotometer).

2.3 | GC-TCD signal processing

The GC-TCD outputs the H_2 concentration of the reactor's gaseous phase. For all GC-TCD data, two experiments (biological repeats) are shown for every strain. In applicable panels, each experiment timecourse is displayed as a thin gray line and the mean timecourse for that strain is bolded and colored. Most experiments produced tolerably smooth GC-TCD output; however, when outliers were observed they were manually removed. Due to the presence of noise in a subset of the data, smooth curves approximating GC-TCD output were constructed using the MATLAB loess method, a locally weighted linear regression, with a span adjusted to suit the duration of each experiment (See Figure 1A.Inset). All GC-TCD output displayed is smoothed unless otherwise noted.

2.4 | Data analysis

Despite regulating the inoculation volume and OD, considerable variability in the duration of the lag phase (data not shown) among and between strains was observed. To amend this, GC-TCD data are not displayed in lab time but rather adjusted time where time 0 is set to be the first GC-TCD reading to exceed 500 ppm (parts per million by volume) for each experiment. (see Figure 1B). In addition, due to the disparate duration of H_2 production across strains (See Figure 2B), it is useful to examine production in units of scaled time where time 0 is set to be the time of the first GC-TCD reading to exceed 500 ppm and time 1 is set to be the time of the maximum observed GC-TCD reading.

In addition to the measured H_2 concentration in the gaseous phase of the reactor, we are interested in calculating the total volume of H_2 produced by each time-point and the volumetric rate of H_2 production. Given the H_2 concentration in the reactor's gaseous phase $h(t)$ (as directly measured by GC-TCD), the time step between measurements Δt (10 minutes), the volume of the gaseous phase of the reactor V (70 mL), and the flow-rate r (5 mL/min), we may express the change in the total volume of H_2 (the sum of H_2 currently in the reactor and that which has been previously produced): $\Delta H_2(t+\Delta t) = [h(t+\Delta t) - h(t)]\ V + h(t+\Delta t)\ r\Delta t$. Dividing by Δt and taking the limit as Δt tends to zero yields an expression for the H_2 production rate:

$$\frac{dH_2}{dt} = \frac{dh(t)}{dt}V + h(t)r\,. \tag{1}$$

Noting that $h(t)$ is reported in units of ppm, to get the volumetric production rate in units of mL/min and mL/hour, a conversion constant of 10^6 and 60×10^6 respectively must be used.

This expression may be integrated to yield the total volume of H_2 produced as a function of time:

$$H_2(t) = h(t)V + r\int_0^t h(t')dt' \tag{2}$$

where $h(0) \equiv 0$ and, as with the rate, units of mL may be attained through a conversion constant of 10^6.

3 | GENETIC INFERENCE

Given the genomes of the isolates (available here https://github.com/barricklab/LTEE-Ecoli[12]), we were able to explore genotype-phenotype correlations. For simplicity, in the case of large scale mutations which extend across multiple genes (a minority of cases), we only record the gene containing the first nucleotide. Five groups were considered: (1) grows robustly and produces H_2; (2) grows robustly and does not produce H_2; (3) does not grow; (4) the union of 1 and 2, grows; and (5) the union of 2 and 3, does not produce H_2. For each group, we constructed the exclusive intersection of all mutated genes relative to the ancestor, that is we made a list of genes which were mutated in every isolate from the group and only isolates from that group. We additionally examined the nonexclusive intersections of these groups, that is genes mutated in every isolate from the group and additionally isolates outside the group.

4 | RESULTS

There are two sets of strains (LTEE clonal isolates) used in this study, descendant from ancestral strains 606 (Ara−, unable to grow on arabinose) and 607 (Ara+, able to grow on arabinose).[13] We found our 607 isolate to consistently achieve higher peak H_2 concentrations and to produce a larger volume of total H_2 per experiment than our 606 isolate (see Figure 1B,C) which may be attributed to variability among or between the two ancestral populations, though this observation is not further investigated in the present work. The remaining isolates are all products of the LTEE, cultured aerobically in minimal media for 22 years and approximately 50,000 generations (see Figure 2A). Clonal isolates from all six descendant populations of 606 were studied (11389, 11330, 11339, 11364, 11333, 11336) as well as five descendant populations of 607 (11342, 11345, 11348, 11367, 11370), with no isolate from the Ara+1 population investigated.

A detailed study of one pair of isolates, evolved strain 11330 and its ancestral strain 606, was performed. Strain 11330 was observed to achieve a much lower peak H_2 concentration than 606 (See Figure 2B). Despite far reduced H_2 concentration, the duration of production was much greater for strain 11330 and the total volume of H_2 produced per experiment was comparable (see Figure 2B.Inset, C). The low concentration and long time course of H_2 production for strain 11330 indicated a slowed growth rate under anaerobic conditions relative to strain 606 while growth in aerobic conditions (in LB liquid culture) was observed to be comparable (See Figure 2D.Top). OD correlated well with H_2 concentration for strain

11330 (see Figure 2D.Bottom) and overnight anaerobic growth resulted in comparable stationary phase OD (between 0.35 and 0.4) for both strains. Finally, as expected from the widely differing peak H_2 concentrations, the maximum H_2 production rate was observed to be approximately 10-fold higher in the ancestor, 606, and occurred near or shortly before peak H_2 concentration was observed for both strains (see Figure 2E). Note that the rates displayed in Figure 2E are in units of mL H_2 per hour for the reaction chamber containing 100 mL of LB media. In summary, while the batch efficiency may be estimated to be comparable for both strains (approximately 0.16 mL H_2 produced per 1 mL LB media) and roughly the same cell number was produced (though cell size differs between the strains which alters the OD[14]), the duration of the experiment was substantially (more than 5-fold) longer for strain 11330.

We went on to probe for changes in H_2 production rates and capacities in the remaining strains. Two biological repeats were conducted for each strain with the exception of 11345 and 11342 for which only one biological repeat was conducted. All 11 LTEE strains studied were observed to grow robustly (qualitative turbidity) in LB media under aerobic conditions. All 11 LTEE strains were observed to have a reduced H_2 production rate, 82% produced no H_2 at all, and 36% were observed to be incapable of anaerobic growth in LB media. Peak concentrations were within 1%−2.2% for all strains which produced any H_2. In addition to strain 11330, another 606 descendant strain 11389, was observed to have a reduced peak H_2 concentration and extended duration of production (See Figure 3A) with a total volume of H_2 produced comparable to strains 606 and 11330 (See Figure 3A.Inset).

These two strains present the first of three phenotypes observed among the LTEE isolates: robust growth, albeit slowed, with comparable batch efficiency (approximately 0.16 mL H_2 produced per 1 mL LB media) to the ancestral strain 606. Only this phenotype had a nonempty exclusive intersection of all mutated genes relative to the ancestor consisting of two genes, *ydjO*, and *ptsA*. The variations observed in *ydjO* were in many ways comparable, one single nucleotide polymorphism (SNP) for both strains 11389 and 11330, 25 nucleotides apart. Those observed in *ptsA* were not, with two relatively distant SNPs observed in 11330 and only a large scale amplification extending beyond *ptsA* in 11389.

Two more strain 606 descendants, strains 11333 and 11336, as well as three strain 607 descendants, strains 11342, 11345, and 11348, were observed to produce no H_2 despite growing robustly (Note: occasional sporadic H_2 readings below 10 ppm and rarely spikes up to 500 ppm were observed for strain 11348 indicating the potential presence of a minority phenotype though technical error in the integration parameters of the GC-TCD cannot be wholly excluded). These five strains present the second phenotype observed: robust growth with no H_2 production.

The remaining two strain 606 descendants, strains 11333 and 11336, and two strain 607 descendants, strains 11367 and 11370, failed to grow in LB media under anaerobic culture conditions and present the third phenotype observed. A summary of this categorization is displayed in Figure 3B. It is notable that half of the isolates studied which descend from strain 606 produced a total volume of H_2 comparable to their ancestor while none of the strain 607 descendants produced any; however, this may be unrelated to any differences

between the ancestral strains and simply be one possible sampling of a large number of evolutionary outcomes.

Figure 3C displays the nonexclusive intersections for the three phenotypes. We sought to establish differences in the mutational signatures between isolates inside and outside of groups 4 (grows) and 5 (does not produce hydrogen); however, we found no distinguishing characteristics in group 4 and the nonexclusive intersection of group 5 contains just three genes, *pykF*, *iclR*, and *nadR*. These three genes are mutated in every isolate and were not further considered. Thus no monogenic explanation for the establishment of these phenotypes is apparent.

5 | DISCUSSION

Our observation that all 11 of the LTEE isolates tested presented with a reduced H_2 production rate or capacity when cultured in LB highlights the potential interest of these strains and their genomes[11] to the bioH_2 community. These results may be utilized similarly to those of previous studies demonstrating genetic variants which ceased hydrogen production.[9] While these negative examples lack the potential for immediate application that mutants with improved yields possess,[7,8] they provide insight into the larger signaling framework responsible for maintaining access to anaerobic growth strategies. In fact, no single gene or simple mutational signature was found to be suggestive of the loss of H_2 production or the capacity for anaerobic growth (see Figure 3C) in these strains indicating these strains likely leverage different pathways to reach the same phenotype.

On the other hand, the only two strains which maintain H_2 production display mutations in gene *ydjO*, one mutation for each strain, both SNPs, 25 nucleotides apart. It is possible that these variations in *ydjO* are somehow stabilizing access to the wild type metabolic pathways in the context of diverse mutational backgrounds. While believed to be protein-coding, the function of this gene is not well understood[15,16] and we hope this work will motivate further investigation into the potential role of *ydjO* in anaerobic respiration.

Multiple growth strategies for anaerobic respiration have been established for *E coli*. Modulating the carbon source,[17] and the removal of metabolic intermediates[18] prove effective methods for optimizing the yield of desired byproducts. Even doping with oxygen,[19] usually a “poison” for dark fermentation, has been shown capable of improving H_2 yield showcasing the multiple roles every chemical actor may play in the regulation of anaerobic metabolism. With the design of increasingly sophisticated reaction conditions, including the introduction of novel nanomaterials[20] we edge closer to a complete picture of the possible suite of responses to external stimulus. Inclusion of even more exotic nanostructures could serve to enhance the desired output or the incorporation of additional functionality into the reactor including sensors or fuel cells that feed off the product stream of the microbial reactor.[21,22]

It is tempting to speculate given the data presented in this study how growth strategies differ among these isolates. The striking balance between the reduction in peak H_2 concentration and increased duration of production in the first phenotype resulting in a well conserved

range of batch efficiencies and comparable cell numbers suggests that strains exhibiting this phenotype follow the same or similar pathways as their ancestor but with reduced rate constants. Strains exhibiting the second phenotype appear to access different pathways than their ancestors which do not produce H_2 as a byproduct and strains exhibiting the third phenotype appear to have lost either the ability to access any pathways affording anaerobic respiration or the necessary environmental sensing and phenotypic switching to recognize and meet the need.

These results further motivate a discussion of the relative fitness of these strains when anaerobically cultured in LB media. It is a far reaching result that the LTEE strains continue to exhibit fitness gains relative to their ancestral strains when measured under culture conditions similar to those in which they are evolving.[23] It may be expected that due to environmental specialization over time, LTEE isolates may exhibit reduced fitness relative to their ancestors when placed in a very different environment even when the ancestral strains are well adapted to that environment. This is the case for strains exhibiting phenotype 3 and while direct competition assays were not completed, it may be expected to be the case for strains exhibiting phenotype 1. On the other hand, strains exhibiting phenotype 2 grew robustly when cultured anaerobically and without investigating direct competition, it is impossible to predict fitness gains or losses. The mutational spectra of a descendant of strain 606 evolving under anaerobic conditions was observed to differ dramatically from that obtained evolving under aerobic conditions[24,25] indicating that adaptations beneficial in an anaerobic environment are likely to bear little resemblance, taken at face value, to those acquired during periods of aerobic evolution.

It is still possible, however, that adaptations acquired in one environment, even one both constant and tightly regulated, confer a fitness advantage in unrelated environments. Features which regulate the adaptability of an organism and not a specific adaptation, likely related to the control of nongenetic heterogeneity within the population, would generalize well to many environments eliciting a stress response.[26,27] It would be interesting to see how the convergence of the LTEE strains to the three distinct phenotypes under the culture conditions described here may relate to the strains' aerobic phenotypes,[28] the discrete nature of the metabolic pathways occupied, and the underlying control of phenotypic switching.

6 | CONCLUSIONS

We have demonstrated, through the use of LTEE isolates graciously provided by the Lenski lab, that prolonged culture in an aerobic environment is capable of altering the growth strategy and fitness of *E coli* when reintroduced to an anaerobic environment. We expect these results to extend to other facultative anaerobes evolved under similar conditions. Such populations are of great value not only to the evolutionary biology community but also to the biohydrogen community as their genomes contain mutations in regions important for the regulation of anaerobic metabolism. The identification of these mutations, as negative examples, may reveal strategies for the design of novel microbes with improved hydrogen production rate and yield. To this end, we believe future work focused on the anaerobic culture of the LTEE strains on defined media and the subsequent analysis of spent media for metabolic byproducts and intermediates for pathway identification is motivated.

ACKNOWLEDGEMENTS

We thank Prof. Richard Lenski and Neerja Hajelan for providing us with these strains as well as Shahnur Ahmed for assisting in the preliminary work leading to this publication.

REFERENCES

1. Züttel A, Remhof A, Borgschulte A, Friedrichs O. Hydrogen: the future energy carrier. Philos Trans R Soc A Math Phys Eng Sci. 2010;368(1923):3329–3342. 10.1098/rsta.2010.0113.
2. Rosales-Colunga LM, Rodríguez ADL. *Escherichia coli* and its application to biohydrogen production. Rev Environ Sci Biotechnol. 2015;14(1):123–135. 10.1007/s11157-014-9354-2.
3. Sivagurunathan P, Kumar G, Bakonyi P, et al. A critical review on issues and overcoming strategies for the enhancement of dark fermentative hydrogen production in continuous systems. Int J Hydrogen Energy. 2016;41(6):3820–3836. 10.1016/j.ijhydene.2015.12.081.
4. Shobana S, Saratale GD, Pugazhendhi A, et al. Fermentative hydrogen production from mixed and pure microalgae biomass: key challenges and possible opportunities. Int J Hydrogen Energy. 2017;42(42):26440–26453. 10.1016/j.ijhydene.2017.07.050.
5. Show KY, Yan Y, Zong C, Guo N, Chang JS, Lee DJ. State of the art and challenges of biohydrogen from microalgae. Bioresour Technol. 2019;121747. 10.1016/j.biortech.2019.121747. [PubMed: 31285100]
6. Nicholson JK, Holmes E, Kinross J, et al. Host-gut microbiota metabolic interactions. Science. 2012;336(6086):1262–1267. 10.1126/science.1223813. [PubMed: 22674330]
7. Mirzoyan S, Trchounian A, Trchounian K. Hydrogen production by *Escherichia coli* during anaerobic utilization of mixture of lactose and glycerol: enhanced rate and yield, prolonged production. Int J Hydrogen Energy. 2019;44(18):9272–9281. 10.1016/j.ijhydene.2019.02.114.
8. Yoshida A, Nishimura T, Kawaguchi H, Inui M, Yukawa H. Enhanced hydrogen production from glucose using ldh-and frd-inactivated *Escherichia coli* strains. Appl Microbiol Biotechnol. 2006;73(1):67–72. 10.1007/s00253-006-0456-9. [PubMed: 16683133]
9. Mokhtar M, Yusoff MZM, Ali MSM, Mustapha NA, Wood TK, Maeda T. Pseudogene YdfW in *Escherichia coli* decreases hydrogen production through nitrate respiration pathways. Int J Hydrogen Energy. 2019;44(31):16212–16223. 10.1016/j.ijhydene.2019.04.228.
10. Wiser MJ, Ribeck N, Lenski RE. Long-term dynamics of adaptation in asexual populations. Science. 2013;342(6164):1364–1367. 10.1126/science.1243357. [PubMed: 24231808]
11. Tenaillon O, Barrick JE, Ribeck N, et al. Tempo and mode of genome evolution in a 50,000-generation experiment. Nature. 2016;536(7615):165. 10.1038/nature18959. [PubMed: 27479321]
12. Deatherage DE, Barrick JE. Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using breseq. Engineering and Analyzing Multicellular Systems. New York, NY: Humana Press; 2014:165–188. 10.1007/978-1-4939-0554-6_12.
13. Lenski RE.Source of founding strain; 2000. http://myxo.css.msu.edu/ecoli/strainsource.html.
14. Lenski RE.Cell size data through generation 10,000; 2000. http://myxo.css.msu.edu/ecoli/cellsize.html.
15. Justino MC, Vicente JB, Teixeira M, Saraiva LM. New genes implicated in the protection of anaerobically grown *Escherichia coli* against nitric oxide. J Biol Chem. 2005;280(4):2636–2643. 10.1074/jbc.M411070200. [PubMed: 15546870]
16. Rojas TCG, Lobo FP, Hongo JA, et al. Genome-wide survey of genes under positive selection in avian pathogenic *Escherichia coli* strains. Foodborne Pathog Dis. 2017;14(5):245–252. 10.1089/fpd.2016.2219. [PubMed: 28398866]
17. Valle A, Cantero D, Bolívara J. Metabolic engineering for the optimization of hydrogen production in *Escherichia coli*: a review. Biotechnol Adv. 2019. 10.1016/j.biotechadv.2019.03.006.
18. Hassan GK, Massanet-Nicolau J, Dinsdale R, et al. A novel method for increasing biohydrogen production from food waste using electrodialysis. Int J Hydrogen Energy. 2019;44(29):14715–14720. 10.1016/j.ijhydene.2019.04.176.

19. Paillet F, Marone A, Moscoviz R, et al. Improvement of biohydrogen production from glycerol in micro-oxidative environment. Int J Hydrogen Energy. 2019;44(33):17802–17812. 10.1016/j.ijhydene.2019.05.082.
20. Pugazhendhi A, Shobana S, Nguyen DD, et al. Application of nanotechnology (nanoparticles) in dark fermentative hydrogen production. Int J Hydrogen Energy. 2019;44(3):1431–1440. 10.1016/j.ijhydene.2018.11.114.
21. Huang Z, Raciti D, Yu S, et al. Synthesis of platinum nanotubes and nanorings via simultaneous metal alloying and etching. J Am Chem Soc. 2016;138(20):6332–6335. 10.1021/jacs.6b01328. [PubMed: 27090384]
22. Wang L, Zeng Z, Gao W, et al. Tunable intrinsic strain in two-dimensional transition metal electrocatalysts. Science. 2019;363(6429):870–874. 10.1126/science.aat8051. [PubMed: 30792302]
23. Lenski RE, Wiser MJ, Ribeck N, et al. Sustained fitness gains and variability in fitness trajectories in the long-term evolution experiment with *Escherichia coli*. Proc R Soc B Biol Sci. 2015;282(1821):20152292. 10.1098/rspb.2015.2292.
24. Finn TJ, Shewaramani S, Leahy SC, Janssen PH, Moon CD. Dynamics and genetic diversification of *Escherichia coli* during experimental adaptation to an anaerobic environment. Peer J. 2017;5:e3244. 10.7717/peerj.3244. [PubMed: 28480139]
25. Shewaramani S, Finn TJ, Leahy SC, Kassen R, Rainey PB, Moon CD. Anaerobically grown *Escherichia coli* has an enhanced mutation rate and distinct mutational spectra. PLoS Genet. 2017;13(1):e1006570. 10.1371/journal.pgen.1006570. [PubMed: 28103245]
26. Rochman ND, Popescu DM, Sun SX. Ergodicity, hidden bias and the growth rate gain. Phys Biol. 2018;15(3):036006. 10.1088/1478-3975/aab0e6. [PubMed: 29461250]
27. Rochman N, Si F, Sun SX. To grow is not enough: impact of noise on cell environmental response and fitness. Integr Biol. 2016;8(10):1030–1039. 10.1039/c6ib00119j.
28. Lenski RE. Convergence and divergence in a long-term experiment with bacteria. Am Nat. 2017;190(S1):S57–S68. 10.1086/691209. [PubMed: 28731830]

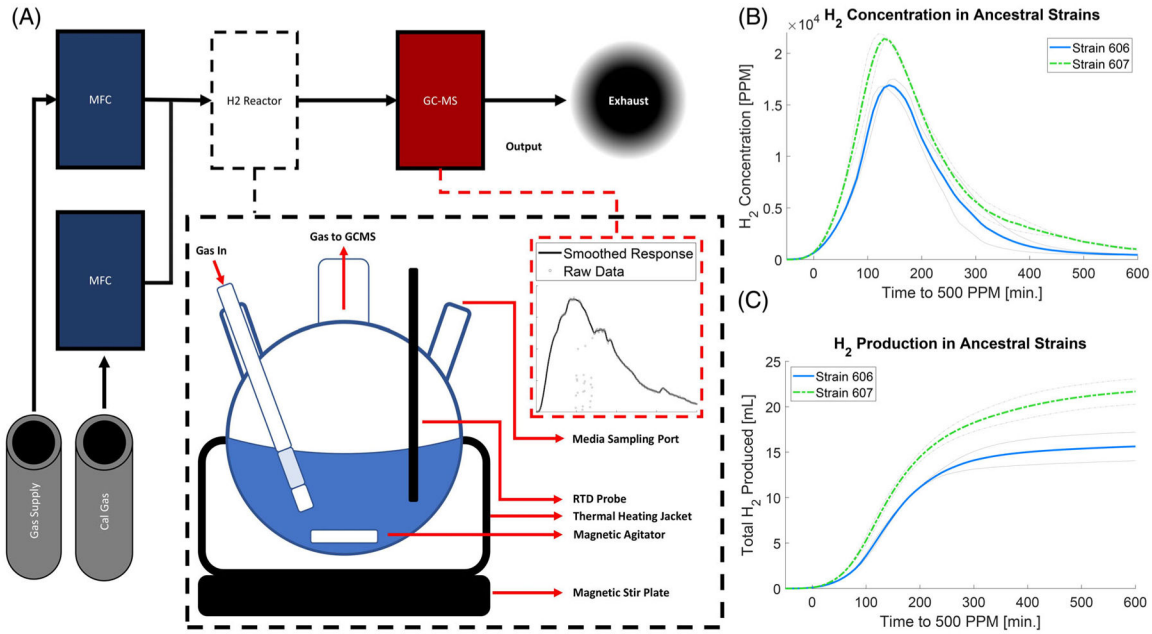

FIGURE 1. Reactor design and validation.

(A) Bioreactor schematic. Primary N_2 supply and calibration gas (cal gas) pass through one of two MFCs before reaching the reactor. (*Inset*) Treatment of detector noise. **(B)** GC-TCD output, H_2 concentration. Strains 606, 607 shown. Mean of two experiments shown in bold, color, and individual experiments shown in gray. **(C)** Total H_2 production. The total amount of H_2 produced may be estimated from the GC-TCD readings (see Methods). Strain 607 consistently produced more H_2 than strain 606

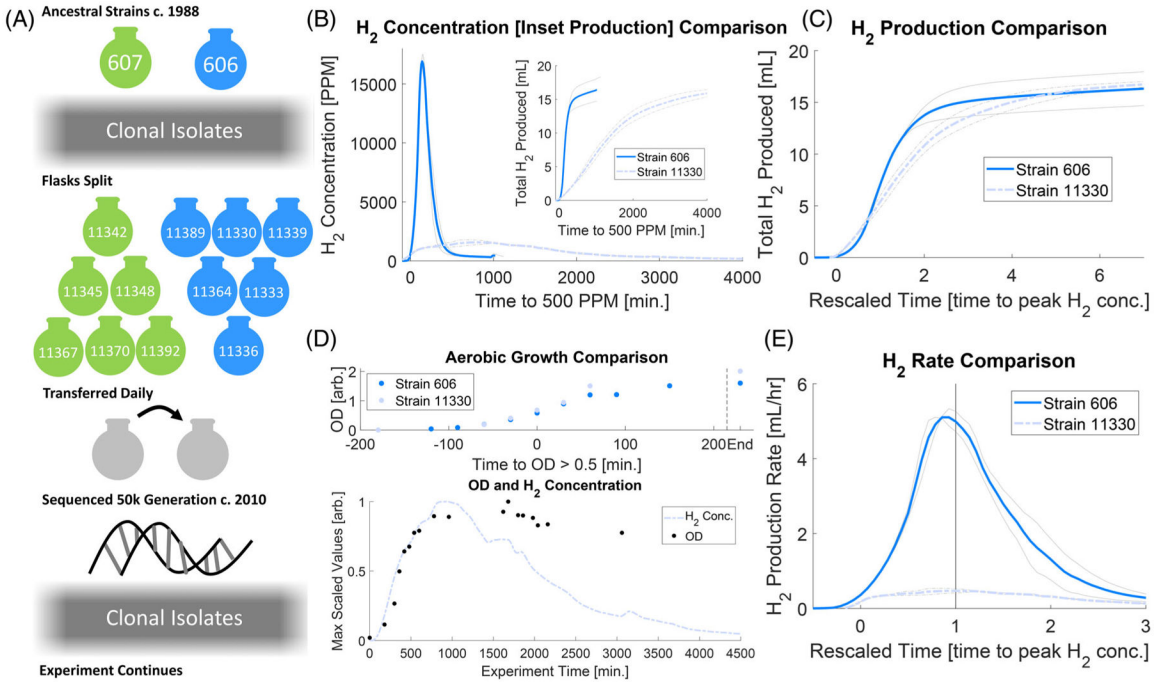

FIGURE 2. Observed reduction in H_2 production rates.

(A) Schematic of LTEE experiment. The strains used in this study were a gift from the Lenski lab Michican State University. **(B)** Reduced peak H_2 concentration observed for strain 11330. (*Inset*) Despite the lower concentration, longer duration of production resulted in comparable total volumes produced. **(C)** Total H_2 production in scaled time. When scaled by the time to peak H_2 concentration, the total production curves overlap significantly. **(D)** Correspondence between H_2 concentration and cell number. (*Top*) Under aerobic conditions, both strains 606 and 11330 display similar growth curves (time "End" specifies the final OD observed after overnight growth). (*Bottom*) H_2 concentration correlates well with OD during exponential phase for strain 11330. **(E)** The rate of H_2 production may be estimated from the GC-TCD readings (see Methods). Mean of two experiments shown in bold, color for each strain, and individual experiments shown in gray for (B), (C), and (E)

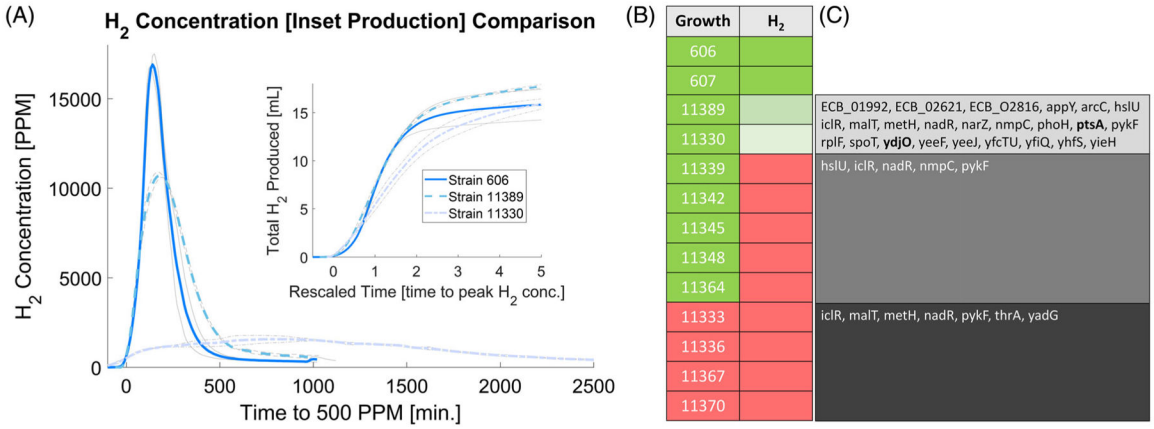

FIGURE 3. Three distinct phenotypes observed.

Strains were observed (1) to grow robustly (qualitative turbidity) and produce H_2, (2) to grow robustly with negligible H_2 production, or (3) fail to grow when cultured anaerobically in LB media. Furthermore, three degrees of the first phenotype were observed with strains 11389 and 11330 exhibiting progressively decreased production rates relative to their ancestral strain 606 despite producing comparable total volumes of H_2. **(A)** H_2 concentration for strains 606, 11389, and 11330. (*Inset*) Total H_2 produced by strains 606, 11389, and 11330. **(B)** Cartoon classifying each strain by its characteristic growth (green-robust/red-null) and observed vigor of H_2 production green (vigorous production) to red (negligible/no production). **(C)** Table containing the list of genes mutated in every isolate from each phenotype (nonexclusive intersection). Genes *ptsA* and *ydjO* are bolded to indicate they are exclusively mutated in phenotype 1. Mean of two experiments shown in bold, color for each strain, and individual experiments shown in gray for (A)