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Total number of authors: 13

Published in: Proceedings of the National Academy of Sciences of the United States of America

Link to article, DOI: 10.1073/pnas.1909987116

Publication date: 2019

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Adaptive evolution reveals a tradeoff between growth rate and oxidative stress during naphthoquinone-based aerobic respiration

Amitesh Ananda, Ke Chen, Laurence Yang, Anand V. Sastry, Connor A. Olson, Saugat Poudel, Yara Seif, Ying Heffner, Patrick V. Phaneuf, Sibei Xu, Richard Szubin, Adam M. Feist, and Bernhard O. Palsson

Department of Bioengineering, University of California San Diego, La Jolla, CA 92039; Bioinformatics and Systems Biology Program, University of California San Diego, La Jolla, CA 92039; and Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, DK-2800 Kongens Lyngby, Denmark

To determine the consequences of substituting NQ for UQ, we deployed adaptive laboratory evolution (ALE) to evolve a genome-edited strain of E. coli, in which UQ biosynthesis was disabled, in an aerobic environment. To utilize oxygen, the evolved strains had to adapt to use NQ to carry out aerobic respiration. We found that: 1) NQ can support aerobic respiration, albeit at a lower growth rate than UQ; 2) key mutations that enabled the adaptation were associated with the pyruvate dehydrogenase complex transcriptional regulator (PdhR); 3) an adjustment in the activity of 3 transcriptional regulators (PdhR, RpoS, and Fur) supported efficient NQ use in the electron transport system (ETS); 4) the adaptation to NQ use comes with an elevated expression of RpoS-regulated periplasmic superoxide-quenching enzymes; and 5) genome-scale computational models of proteome allocation suggest that the use of NQ requires increased proteome allocation to reactive oxygen species (ROS) mitigation, resulting in lower growth rates. Taken together, these results provide a deep understanding of the adaptive rewiring, proteome cost for the NQ-dependent aerobic respiration, and oxidative stress.

Significance

A vectorial flow of electrons in the membrane generates proton-motive force, which is central to cellular respiration. Organisms, including the last universal common ancestor of living organisms (LUCA), have multiple electron transport systems to use diverse electron donor–acceptor pairs. Such respiratory flexibility enables survival in varying environments. The appearance of oxygen in Earth’s environment due to the Great Oxidation Event (GOE) caused a major transformation in microbial bioenergetics. Here we performed a systems-level analysis to examine the suitability of pre-GOE era respiratory quinone, naphthoquinone, in oxic environments and resource constraint requiring the advent of the high-redox-potential quinone.


The authors declare no competing interest.

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Data deposition: Resequencing and expression profiling data that support the findings of this study have been deposited to National Center for Biotechnology Information Sequence Read Archive (SRA accession no. PRJNA560068) and Gene Expression Omnibus (accession no. GSE135867), respectively.

1Present address: Department of Chemical Engineering, Queen’s University, Kingston, ON K7L 3N6, Canada.

2To whom correspondence may be addressed. Email: palsson@ucsd.edu.

www.pnas.org/cgi/doi/10.1073/pnas.1909987116

PNAS | December 10, 2019 | vol. 116 | no. 50 | 25287–25292
ETS and empirically establishes that the evolution of UQ conferred a growth advantage by resource conservation in the post-GOE environment.

Results and Discussion
Adaptive Laboratory Evolution of NQ-Dependent E. coli. The biosynthetic pathways of respiratory quinones diverge at the chorismate node. Chorismate lyase (UbiC) and isochorismate synthase (MenF) drive chorismate toward UQ and NQ biosynthesis, respectively (SI Appendix, Fig. S1A). We generated a UQ-deficient strain by knocking out the chorismate lyase ($\Delta$ubiC) to prevent chorismate flux toward UQ biosynthesis. The initial knockout strain was characterized, and ALE was subsequently used to probe the adaptive capabilities of this strain in an oxygen-rich environment (Fig. 1). As expected, the deletion of $\text{ubiC}$ resulted in diminished UQ levels, and an increased biosynthesis of NQ was observed (SI Appendix, Fig. S1B). Interestingly, all 3 replicates of the $\Delta$ubiC strains evolved to grow at a higher growth rate ($\sim 0.70 \text{ h}^{-1}$) (Fig. 1B and SI Appendix, Fig. S1C). However, none of the evolved strains could achieve a growth rate of the evolved WT strain ($\sim 0.95–1.0 \text{ h}^{-1}$) (8). Thus, despite the fitness gain of the $\Delta$ubiC strain during ALE, it was less than that of the wild type. We refer to all of the $\Delta$ubiC strains together as NQ-dependent strains, preevolved strain as $\Delta$ubiC, and evolved strains individually as ALE-1, ALE-2, and ALE-3 (Fig. 1A).

Fitness gains result from the acquisition of beneficial mutations. We performed whole-genome sequencing of the NQ-dependent strains to identify the genetic basis of the increase in growth rate. All of the evolved NQ-dependent strains acquired only 1 common gene mutation (Fig. 1B and C). ALE-1 and ALE-3 showed a single-base substitution in the gene $\text{pdhR}$ (pyruvate dehydrogenase complex regulator), whereas ALE-2 had a base substitution upstream of the $\text{pdhR}$ gene (Fig. 1B, Inset and SI Appendix, Table S1). PdhR is negatively autoregulated, and the base substitution in ALE-2 was found to be located in the PdhR-binding region, also known as PdhR box, upstream of $\text{pdhR}$ gene (9). Remarkably, the base substitution in ALE-1 brought in a premature termination codon in the $\text{pdhR}$ ORF. This suggested an altered activity of PdhR responsible for the fitness changes of the evolved NQ-dependent strains and motivated us to examine the effect of the absence of this transcriptional regulator on the transcriptome of these strains. We thus generated a $\text{pdhR}$ knockout strain ($\Delta$pdhR) and performed transcriptome analysis of $\Delta$pdhR and the NQ-dependent strains under the same conditions.

We applied an effective signal deconvolution algorithm, independent component analysis (ICA), to perform an unbiased analysis of the transcriptional responses (Materials and Methods) (10). This analysis results in cohesive sets of independently modulated genes (called i-modulons), each of which often exhibits a high overlap with the gene targets of a specific transcriptional regulator (called regulators). In addition, ICA estimates an activity for each i-modulon in each expression profile, serving as a proxy for the activity of the linked regulator. We observed a differential activity of the pyruvate-related independently modulated set of genes (Fig. 1D). The activity of this i-modulon was high in the $\Delta$pdhR strain, suggesting a derepression of PdhR i-modulon. The activity of this component was higher in the evolved NQ-dependent strains than in preevolved $\Delta$ubiC strain, indicating a partial derepression of PdhR i-modulon due to the mutations in $\text{pdhR}$.

Respiratory Rewiring during NQ-Dependent ETS. To understand the respiratory changes in the NQ-dependent strains, we estimated the exchange rates of the respiratory metabolites in the WT, $\Delta$ubiC, and the 3 evolved $\Delta$ubiC strains. We observed a clear tradeoff between fermentative and oxidative respiration, shown by the change in oxygen uptake rate and lactate secretion rate (Table 1). As expected, the loss of UQ in the $\Delta$ubiC strain resulted in a significant lactate production (7) and a decreased oxygen uptake rate. However, after evolution, the oxygen consumption of the NQ-dependent strains improved by more than 60%, and lactate secretion was reduced by 80% on average.

We then explored the transcriptome shift underlying this altered respiratory behavior of the NQ-dependent strains. Since PdhR has been reported to have an impact on the ETS of E. coli (9), we examined expression changes in the ETS enzymes (Fig. 2A). The E. coli genome encodes 2 NADH dehydrogenases; the proton-translocating type 1 NADH dehydrogenase and nontranslocating

![Fig. 1. Experimental evolution of E. coli $\Delta$ubiC strain. (A) The schematic for generation of strains used in the study. (B) The growth rate evolution trajectories (smoothed data) and the convergent mutations (Inset) observed in $\Delta$ubiC replicates during laboratory evolution. SI Appendix, Fig. S1C shows the extended axis plot of these trajectories. (C) List of genes mutated during the evolution of the $\Delta$ubiC replicates. (D) Activity of the pyruvate i-modulon estimated by independent component analysis (ICA). $\Delta$pdhR has been used as the control strain. The bars with identical colors represent biological replicates of the corresponding strain.](image-url)
type II NADH dehydrogenase (Ndh). In the NO-dependent strains, there was an up-regulation in the expression of ndh. Interestingly, there appears to be an operational synergy between NO and Ndh (11). Earlier, an attempt to generate an Ndh-deficient strain of NO-producing bacterium was unsuccessful (12). ALE-1 strains with the frame-disruptive mutation in pddhR showed the highest up-regulation of ndh. There was also an increase in the expression of type I NADH dehydrogenase; however, deactivation of PddhR resulted in a constitutive expression of the type II NADH dehydrogenase in the evolved strains. Interestingly, there appears to be a subunit-level calibration in the expression of the proton-translocating units of the type I NADH dehydrogenase for the optimization of NO-based ETS (13, 14).

There are 3 cytochrome oxidases in the E. coli genome that can transfer electrons to oxygen. There was no significant differential regulation of the cytochrome bo3 oxidase (cyoABCDE). Cytochrome bd-I oxidase (cydBD) expression was higher in the NO-based strains, specifically in the preevolved strain. The evolved strains showed an increased expression of cytochrome bd-II oxidase (appBC). Notably, the specific activity of cytochrome bd-II oxidase is reported to be higher for naphthoquinone than benzoquinone (15), and these alternate oxidases are more ROS-protective as well (16). The ETS of evolved strains thus appeared to be tailored to a more efficient route of electron flow for NO utilization. A similar transcriptional rewiring was observed in the glycolytic cycle where the evolved strains, despite retaining high activity of pyruvate dehydrogenase complex, up-regulated the expression of pyruvate oxidase (poxB), which is reported to relieve oxidative stress (17).

In addition to the changes in the ETS enzyme usage, the shift in cellular respiration should also be apparent by comparing fluxes through biochemical reactions in central metabolism. Accordingly, we incorporated the experimentally measured metabolite exchange rates and transcriptomics data into a genome-scale model of metabolism and protein expression enhanced by a protein-folding network, FoldME (18) and simulated the flux map for each experimental strain at their specific growth rate (Materials and Methods). The resulting metabolic flux map shows a clear alteration in use of the glycolysis pathway, mixed fermentation pathway, and the tricarboxylic acid (TCA) cycle (Fig. 2B). The preevolved ΔubiC strain, which exhibits a fermentative phenotype determined by experimental measurement, is characterized by heavy lactate secretion and glycolysis flux supported solely by the Embden–Meyerhof–Parnas pathway. After evolution,
glucose metabolism becomes heavily dependent on the Entner–Doudoroff pathway and further diversifies to different levels of complexity of using the oxidative pentose phosphate pathway. Our simulations show that the evolved strains reduce the high lactate production level by rebalancing their glycolytic proteome to mimic the WT pathway usage and increase aerobic respiration through the TCA cycle.

**Oxidative Stress Mitigation.** Increase in aerobic respiration results in higher oxidative stress. The respiratory electron carriers are a major source of periplasmic superoxide production and, in the absence of UQ, the respiratory chain becomes more autooxidizable (19). Therefore, an increased NQ-based electron flow will require an extensive oxidative defense system.

The *E. coli* genome has elaborate oxidative stress response machinery that is mostly regulated by 3 transcriptional regulators: OxyR, SoxS, and RpoS. We examined the activity of the 3 i-modulons regulated by these transcriptional regulators. There was no significant change in the activity of the canonical peroxide and superoxide stress regulons, OxyR and SoxS, respectively (*SI Appendix*, Fig. S2). However, we observed a high activity of the alternative sigma factor RpoS i-modulon in the evolved strains (*SI Appendix*, Fig. S2). There are 3 superoxide dismutases responsible for the conversion of superoxide to less damaging peroxyde (SodA, SodB, and SodC). SodA and SodB are the cytoplasmic dismutases, whereas SodC catalyzes the dismutation reaction in the periplasm. Although there was no significant up-regulation in the expression of cytoplasmic superoxide dismutases, the periplasmic superoxide was invariably up-regulated in all 3 of the evolved strains (Fig. 3A).

The peroxide is further converted to water and oxygen by 2 catalases, KatG and KatE. The expression of katE showed a pattern similar to that of the superoxide dismutase sodC (Fig. 3A). Notably, KatE has a higher turnover rate than KatG (20). WrbA, NAD(P)H:quinone oxidoreductase, is responsible for the reduction of quinones to hydroquinones which prevent interaction of semiquinone to oxygen, thereby preventing the generation of superoxide. This enzyme is active during aerobic growth of *E. coli* and is proposed to function in a highly oxidizing environment (21). The expression of wrbA was similar to superoxide dismutase and catalase (Fig. 3A). All these up-regulated ROS-mitigating enzymes belong to RpoS regulon. A comprehensive examination of all stress-related i-modulons showed a response similar to RpoS i-modulon in case of EvgA and GapWX/EWX (*SI Appendix*, Fig. S3). These i-modulons are related to acid stress, and their induction could be either adaptive or an indirect consequence of RpoS i-modulon activation (22). Taken together, these results show that a subset of the *E. coli* ROS defense system, regulated by RpoS, was up-regulated to quench the ROS generated due to the electron leakage from ETS.

The effect of changes in the ROS mitigation ability was validated by challenging the cells to higher oxidative stress. We examined the response of these strains toward 2 distinct ROS-generating agents: hydrogen peroxide and paraquat (23) (Fig. 3B and *SI Appendix*, Fig. S4). There was no significant difference in

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**Fig. 3.** Oxidative stress mitigation. (A) Expression changes in ROS-quenching enzymes. Fold-changes have been calculated with respect to the wild-type strain [periplasmic superoxide dismutase, sodC; cytosolic superoxide dismutase, sodB and sodA; bifunctional hydroperoxidase, katG; monofunctional catalase, katE; alkyl hydroperoxide reductase, ahpC and ahpF; NAD(P)H:quinone oxidoreductase, wrbA]. (B) Estimation of the growth retardation caused due to addition of peroxide (1 mM H$_2$O$_2$) and superoxide (1 mM paraquat) generating chemicals by calculating the relative increase in the lag phase. For each strain, the mean of the estimated lag phase across untreated replicates (control) was calculated. The lag-phase estimates of the conditions with treatment was then divided by the corresponding control condition (yielding the relative lag phase). The error bars represent the SD for the lag estimates across replicates (n ≥ 3). The effect of adaptive evolution was significant in the paraquat treatment (P value < 0.001) but not in peroxide treatment (P value = 0.13) (*Materials and Methods*). (C) Adaptive changes in the activity of Fur i-modulon in the evolved strains. Iron-starved, iron-replete, and paraquat-treated WT conditions serve as controls. The bars with identical colors represent biological replicates of the corresponding strain. (D) Genome-scale model-based calculation of the impact of periplasmic nonproductive electron leak on the growth rate of *E. coli*. *SI Appendix*, Fig. S5 shows the extended axis plot of these calculations.
of which is believed to produce superoxide from ETS (31). The electron reduction to the semiquinone radical, the autooxidation
The 2-electron reduction of these quinones proceeds via a single-electron transfer to enable the electronic circuit of the ETS.
para positions and undergo a keto-hydroxy cycling by a 2-
phenazines, contain a quinone scaffold with 2 carbonyl groups at

couples (2). These electron carriers, with the exception of
biosynthesis to be more costly than NQ; however, the major
strain (Fig. 3C), suggesting a decreased repressor activity of Fur.
Superoxide stress is reported to negatively impact Fur functioning,
as it is a mononuclear iron protein (25). We observed a similar
increase in the activity of this i-modulon in parquat-treated cells.
Thus, increased oxidative stress could be responsible for the ac-
activity change in the ΔubiC strain. An inverse relation between
D. This i-modulon is associated with the Fur (ferric uptake regulator)
regulon, which is responsible for cellular iron homeostasis (24).
The Fur-i-modulon was found to be highly active in the ΔubiC
strain (Fig. 3C), suggesting a decreased repressor activity of Fur.

Genome-Scale Calculation of Tradeoff between Growth and ROS Defense. The knowledge of growth rate limitation and ROS de-
defense rewiring encouraged us to compute the cost of respiratory
dissipation. We used the genome-scale model of metab-
olism, macromolecule expression, and oxidative stress, OxidizeME
(27), to estimate the impact of periplasmic superoxide genera-
and spontaneous autoxidation of NQ on the growth rate. First, we found that the cost (metabolic and protein expression)
of detoxifying superoxide had a strong negative impact on the
growth rate (Fig. 3D and SI Appendix, Fig. S5). The chemistry of
NQ makes it more prone to autoxidation (28). Upon account-
for the decrease in ETS efficiency by diverting electrons from
NQ toward superoxide generation, we computed a further de-
crease in the growth rate (Fig. 3D). Thus, despite an elevated
ROS defense capability, the growth capability of NQ-dependent
strains is certainly restricted. The need for ROS mitigation thus
explains the limited fitness improvement of the NQ-dependent
strains through the need to reallocate the proteome from growth
to stress response functions.

The biosynthetic pathways for both respiratory quinones, UQ and
NQ, are very similar. Both are derived from the shikimate
pathway and undergo prenylation. However, there are some
distinct metabolic requirements for the synthesis of these qui-
nones. NQ biosynthesis requires 2-ketoglutarate, thiamine PPI,
CoA, and ATP as cofactors, whereas UQ biosynthesis requires
oxygen, flavoprotein, and NADH (29). We performed a com-
parative cost analysis of the 2 biosynthetic pathways using the
genome-scale reconstruction of the metabolic network in E. coli
K-12 MG1655 (iML1515) (30). The computation showed UQ
biosynthesis to be more costly than NQ; however, the major
contributor to this difference was a higher oxygen requirement
(SI Appendix, Table S2).

Conclusions
Except for acetato- and methanogens (except Methanosarcinales),
the bioenergetic chains of all organisms exploit redox-active
electron carriers for optimal use of higher-redox-span substrate
couples (2). These electron carriers, with the exception of
phenazines, contain a quinone scaffold with 2 carbonyl groups at
para positions and undergo a keto-hydroxy cycling by a 2-
electron transfer to enable the electronic circuit of the ETS.
The 2-electron reduction of these quinones proceeds via a single-
electron reduction to the semiquinone radical, the autoxidation
of which is believed to produce superoxide from ETS (31). The
modern-age microbial respiratory systems consist of diverse res-
piratory quinones. This quinone diversification is believed to be
an adaptive consequence of the oxygenation of Earth’s envi-
ronment. Interestingly, quinone evolution has resulted in the
emergence of both high- and low-redox-potential molecules from
the ancestral NQ (2). While high-redox-potential quinone, UQ,
is believed to be a crucial adaptive feature in post-GOE bioen-
ergies, owing to its lesser propensity to noncatalyzed oxidation,
low-potential quinone, rhodoquinone (RQ), appears to have
emerged to optimize the biosynthetic pathways (2, 3, 32). A
comprehensive profiling of quinone distribution in the bacterial
world indicates an association between quinone composition and
respiratory behavior in the Gram-negative species, with obligate
anaerobes and aerobes exclusively consisting of NQ and UQ, re-
spectively, whereas facultative aerobes have a mix of the 2
quione types (33). However, NQ is the only quinone identified
in Gram-positive bacterial species (33). The majority of bacterial
species, including the most prevalent phyla (actinobacteria and
proteobacteria), retained NQ as either exclusive or substitutable
respiratory quinone (2). In facultative NQ-dependent genera like
Escherichia, Klebsiella, and Proteus, UQ and NQ are associated
with aerobic and anaerobic lifestyles, respectively (33–35). The
maintenance of efficient aerobic respiratory chains in obligate
NQ-dependent species inspired us to examine the cost of ox-
dative respiration in UQ-deficient E. coli.

We addressed the paradox of the different nature of NQ-
based respiration in obligate and facultative NQ-dependent
species using adaptive laboratory evolution of engineered NQ-
dependent strains. Systems-level examination of the evolved NQ-
dependent strains showed an extensive physiological rewiring
orchestrated by the activity of 3 transcriptional regulators
resulting in proteome reallocation from growth to ROS stress-
mitigating functions. Notably, the evolved NQ-dependent strains
shifted from fermentative to oxidative energetics. However, the
resource partitioning to periplasmic ROS defense system pre-
vented the strain from achieving full growth capacity. In-
terestingly, a relatively smaller molar ratio of NQ is reported to
be required for the functioning of ETS as compared to UQ,
which may be due to the need for efficient redox recycling to
reduce nonproductive electron dissipation (36–38). In an oxy-
gen-type condition, the additional cost of UQ biosynthesis may not
have a substantial impact, and the resource conservation due to
lesser ROS production might have a growth-promoting effect.
This reverse tracing of the natural course of quinone evolution
thus provided a fundamental understanding of the environment-
specific cost of the electron carriers. Extrapolation of this study,
especially in pathogenic bacteria with NQ as the sole electron
carrier, may enable therapeutic targeting of microbial energetics.
RQ, a respiratory quinone with redox potential similar to NQ,
enables hypoxic survival of several eukaryotes including para-
sitic helminths (39). Thus, understanding the physiological
properties and vulnerabilities associated with the functioning of
low-redox-potential quinone holds translatable potential across
life forms.

Materials and Methods
Genome-edited E. coli strain was evolved in the laboratory using an au-
mated system. Detailed materials and methods used for strain generation,
adaptive laboratory evolution, DNA sequencing, RNA sequencing, i-modulon
decomposition, phenotype characterization, respiratory quinone quantitation,
proteome-constrained simulation, and genome-scale computations are pro-
vided as SI Appendix.

Data Availability. Resequencing and expression profiling data that support
the findings of this study have been deposited to NCBI Sequence Read Archive
(SRA accession: PRJNA560068) and Gene Expression Omnibus (GSE135867),
respectively.
ACKNOWLEDGMENTS. This work was funded by Nova Nordisk Foundation Grant NNF10CC1016517 and NIH Grants R01GM057089 and U01AI124316. We thank Marc Abrams (Systems Biology Research Group, University of California San Diego) for assistance with manuscript editing. The support of University of California San Diego, Chemistry and Biochemistry Molecular Mass Spectrometry Facility is duly acknowledged.


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