The transcriptional response to low temperature is weakly conserved across the *Enterobacteriaceae*

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Abstract

Bacteria respond to changes in their external environment like temperature by changing the transcription of their genes, but we know little about how these regulatory patterns evolve. We used RNA-seq to study the transcriptional response of a shift from 37°C to 15°C in wild-type *Escherichia coli, Salmonella enterica, Citrobacter rodentium, Enterobacter cloacae, Klebsiella pneumoniae,* and *Serratia marcescens*, as well as Δ*rpoS* strains of *E. coli* and *S. enterica.* We found that these species change the transcription of between 626 and 1057 genes in response to the temperature shift, but there are only 16 differentially expressed genes in common among the six species. GO enrichment of regulated genes suggests many species-specific phenotypic responses to temperature changes, but terms involved in iron metabolism, central metabolism, and response to osmotic stress are implicated in at least half of the species. The alternative sigma factor RpoS regulates about 200 genes between 37°C and 15°C in both *E. coli* and *S. enterica*, with only 83 genes in common between the two species. Divergence in the RpoS-regulon between the two species is due to both species-specific genes in each genome as well as differences in the regulation of shared genes.

25 Overall, there is limited conservation of the response to low temperature generally, or

26 the RpoS-regulated part of the response specifically, due both to some genes being

27 species-specific, as well as the species-specific regulation of shared genes. Regulatory

28 responses to a common stress evolve rapidly between closely related species.

29 Importance

We studied how different species of bacteria change the expression of their genes in

response to a change in temperature. We found that the six species in this study

change the level of expression of many of their genes in response to a shift from

human body temperature (37°C) to a temperature that might be found out of doors

(15°C). Surprisingly, there are very few genes that change expression in all six species.

This was due to each species possessing many unique genes that no other species

has and due to many genes that are present in the genome of each species changing

expression in only one species. This study is important to the field because it illustrates

that closely related species can share many genes but not use those genes in the

same way in response to the same environmental change.

40 Introduction

Bacteria change the levels of transcription of their genes to respond to changes

in their environments. These patterns of transcription, and the role of regulatory

proteins and other factors in determining these patterns have been measured

thoroughly in a handful of model species, like *Escherichia coli*. The degree to which our

understanding of *E. coli* gene regulation can inform our understanding of related

species will depend on how similar those species are. We know that the genomes of

bacterial species often differ dramatically in content due to horizontal gene transfer

(HGT), so it is possible that HGT may drive dramatic differences between related

species in what genes are differentially expressed in response to an environmental

change. Differences in transcriptome patterns between related species can also be due

to shared genes that have different patterns of regulation among species. In general,

we have little information about the relative roles of species-specific genes vs. species-

specific regulation of shared genes in shaping how regulons differ between relatives.

Changes in temperature are one challenge faced by many bacteria (1). Low

temperature can affect bacteria by rigidifying the lipid bilayer, by altering the folding of

proteins and RNAs, and by altering the assembly and activity of essential

macromolecular machinery like ribosomes (1). Low temperature may also serve as a

cue that bacteria have moved from one environment (such as a mammalian host) into

another environment (such as a surface at ambient temperature) (2).

A great deal is known about how *E. coli* responds to low temperature (1, 3). A

number of global regulatory factors have been implicated, including cold shock

proteins (4, 5), RNA degradation enzymes like RNase R and PNPase (5, 6), nucleoid

associated proteins like H-NS (7), global supercoiling levels (8), and the alternative

sigma factor RpoS (2, 9). Shifts between optimal temperature (37°C) and a lower

temperature have substantial impacts on the transcriptome. In a series of experiments

examining shifts between 37°C and 23°C (and vice versa), White-Ziegler and

coworkers documented that 423 genes, or about 10% of the *Escherichia coli* K-12

genome, are differentially expressed (DE) (2, 10). Further, they identified RpoS and H-

NS as two global regulators of these transcriptional patterns (2, 11).

How relatives of *E. coli* respond to changes in temperature is much less well

documented. The *Enterobacteriaceae* are a diverse group of bacteria (12) that exist in

many different thermal environments (13). This group includes a number of genera that

can be found in endothermic hosts either causing an infection or in the gastrointestinal

tract of healthy hosts (14). Many of these species can also be isolated from

ectothermic animals, plants, soil, water, plants (15–19). The presence of these

organisms outside of endothermic hosts suggests that the species need to cope with

broad changes in temperature.

Outside of *E. coli*, transcriptional responses to changing temperatures are much

less well understood across the *Enterobacteriaceae*. In general, we have little

understanding of how regulatory responses evolve in bacteria and at what rate these

regulons evolve. In addition to questions of rate, regulons could evolve because

different species lose different genes or gain different genes via horizontal gene

transfer (HGT). Regulons could also evolve because shared genes evolve different

regulatory controls in different species. We do not understand the relative importance

of these two modes of regulatory evolution. Investigating the tempo and mode of

regulatory evolution in the *Enterobacteriaceae* will both allow fundamental

understanding of bacterial evolution and allow researchers to better understand the

degree to which *E. coli* serves as an appropriate model system for related bacteria.

Single species studies of the transcriptomic response to low temperature exist

for several species of *Enterobacteriaceae*, including *E. coli* (2, 10), *Salmonella enterica*

(20), *Klebsiella pneumoniae* (21) and *Enterobacter sp.* (22). Unfortunately, it is difficult

to directly compare existing studies of individual species because of the large

differences in the culture conditions, the experimental approaches to RNA

quantification, and the bioinformatic and statistical analyses in these studies. To

directly compare the transcriptional response, the experimental and analytical

approaches should be the same. In addition, batch effects in both bacterial growth and

RNA-seq steps can be avoided by performing all of the bacterial growth, RNA

processing, library construction, and sequencing in parallel with a balanced

experimental design.

In this study, we studied how five species of bacteria in the *Enterobacteriaceae*

plus one outgroup species responded to the shift from 37°C to 15°C, as might occur

when bacteria leave a mammalian host and find themselves in a temperate external

environment. We used comparative genomic tools to identify homologous genes in

each species, and then used RNA-seq to examine the patterns of transcription in each

species across this temperature transition. In addition, we studied the role of the

alternative sigma factor RpoS in this transition in *E. coli* and *S. enterica.* We performed

these studies in parallel for all strains in the study, avoiding possible confounding

differences due to experimental or analytical approaches or due to batch effects.

Results

Most genes are either shared across all species or are species-specific

This study worked with five species of *Enterobacteriaceae*, (*E. coli, S. enterica, C. rodentium, E. cloacae, K. pneumoniae*) and one outgroup species in the *Yersiniaceae* (*S. marcescens*) (12). Their genomes contained between 3968 and 4709 protein coding sequences (Figure 1A), which were the basis of all of the analyses in this paper. We used xenoGI (23) to understand the evolutionary histories of the six genomes. xenoGI uses both synteny and sequence similarity to define groups of genes called locus families. We defined all genes in a locus family as orthologues.

Figure 1: Patterns of shared and unique genome content. (A) The total number of protein coding genes found in the genome of each of the six species. (B) Upset plot of shared and unique genes in these species. Bar heights represent the number of genes found only in this set of species, as indicated by the dots below the bars. For example, the first bar on the left shows the number of genes that are found only in *S. marcescens,* while the second bar represents genes found in all six genomes, the core genome. Only sets with more than 50 genes are shown.

There are 1838 genes found in every genome, a set of genes denoted the core

genome. The core genome accounts for 39% to 46% of all genes in a genome. The

number of species-specific genes varies from 981 to 1872 (Figure 1B), which accounts

for 24% to 47% of all genes in the genome. Most genes in a species are either

species-specific or ancestral to the group in this analysis. There are also small subsets

of genes that are found in an individual clade, such as the 124 genes found in only *E.*

coli and *S. enterica.*

All species grow much slower at 15°C than 37°C

We investigated the effect of temperature on growth of the six species in this

study in LB medium. We measured the growth rate at 37°C, human body temperature,

and the lag phase and growth rate after a shift to 15°C, as might occur when moving

from a host into the external environment. All species in this study grow much faster at

37°C than at 15°C (Table 1). All species had doubling times under 30 minutes when

growing at 37°C, but doubling times were 5 to 14 times as long at 15°C. Cultures all

underwent a lag phase of one to two hours before resuming growth. For subsequent

studies on gene expression in response to low temperatures, we worked with cultures

that had been at 15°C for three hours, as all strains had resumed growth after the

temperature shift at this point. After three hours at 15°C, strains should be past the

short-term response to cold (involving changes in supercoiling) and into the longer-

term response, involving transcriptional changes driven by changing activity of

transcription factors (8).

144   **Table 1:** Growth parameters at 15°C and 37°C in LB[†]

Strain	Doubling time at 37°C (mins)	Lag time after shift to 15 °C (mins)	Doubling time at 15°C (mins)
E. coli wt	28.5 ± 0.3	63.7 ± 12.2	328.0 ± 15.2
E. coli ΔrpoS	25.5 ± 0.2	98.1 ± 3.6	303.5 ± 6.4
S. enterica wt	29.6 ± 1.7	96.2 ± 6.7	175.5 ± 3.8
S. enterica ΔrpoS	27.5 ± 1.2	97.8 ± 10.1	188.8 ± 7.4
C. rodentium wt	28.1 ± 0.6	104.0 ± 23.1	342.2 ± 30.9
E. cloacae wt	28.0 ± 3.6	90.3 ± 29.7	394.2 ± 44.6
K. pneumoniae wt	26.8 ± 0.7	119.4 ± 7.7	268.9 ± 19.8
S. marcescens wt	29.9 ± 1.3	123.8 ± 9.8	160.9 ± 7.1

145   [†] Values are the mean ± standard error of the mean. N = 3 for all measurements.

Limited conservation of patterns of differential expression in response to temperature shift

To examine how these species alter their transcriptome in response to temperature change, we performed a RNA-seq experiment on samples growing exponentially at 37°C (immediately before a shift to 15°C), and then after three hours growth at 15°C. In the three hours between growth at 37°C and 15°C, patterns of gene expression changed substantially. 626 to 1057 genes were significantly differentially expressed (defined as an FDR-adjusted p-value < 0.01 and an absolute log_2-fold change > 2) between the two temperatures across the different species (Figure 2A). This corresponds to between 14 and 26% of all protein coding genes in the genome being DE. We found little conservation of the set of DE genes in each species (Figure 2B,C). For example, each species has between 274 and 505 genes that are uniquely DE in that species alone. In contrast, there are only 16 genes that are DE in every species studied. The transcriptional response to low temperature varies strongly among these species.

Figure 2: Patterns of differential expression in response to temperature shift of all genes in the genome. (A) The number of genes significantly DE genes (FDR-adjusted $p < 0.01$, absolute $\log_2$-fold change > 2) as a proportion of all genes in the genome for each species. Upregulated genes are expressed more highly at 15°C than 37°C. (B) Upset plot of shared and unique patterns of DE genes in these species. Bar heights represent the number of genes, while the dots below represent the species involved in that group.

One factor that could contribute to the limited overlap in DE genes among

species is considerable variation in genome content, with each genome containing

between 981 to 1872 species specific genes (Figure 1B). Each species-specific gene

that is DE is an automatic contribution to the set of unique DE genes of that species.

However, these species-specific genes are actually less likely to be DE between

temperatures than expected if all genes had equal probability of being DE (Figure 3A;

chi-square test, $p < 5 \times 10^{-4}$ for all six species.) Each species' set of unique genes does

not disproportionately contribute to the divergence in patterns of transcription.

What about the conserved genes? Genes in the core genome are particularly

likely to be DE (Figure 3B; chi-square test, $p < 2 \times 10^{-3}$ for all six species). However,

while core genes are particularly likely to be DE, which genes are DE in each genome is

not well-conserved (Figure 4). For example, of the 561 core genes that are DE in *E. coli*,

231 of these genes are not DE in any other species, even though all species in that

study have those genes. There is not a total lack of conservation of expression pattern,

however. For example, there are 60 core genes that are DE in both *E. coli* and *S.*

enterica and in no other species. Species-specific expression of core genes plays a

major part in giving each species a distinctive transcriptional response to temperature

shift.

Figure 3: Observed vs. expected number of DE genes. The number of DE genes among the (A) species-specific genes and (B) the core genes. Observed counts are shown as gray bars, while the expected counts are shown with black dashed lines. Expected values were calculated as the number of genes in that category multiplied by the proportion of all genes that were DE. In every case, the observed count is significantly different from the expected count (chi-square test, $p < 5 \times 10^{-4}$ for each comparison in (A), $p < 2 \times 10^{-3}$ for each comparison in (B)).

Figure 4: Patterns of differential expression of core genes in response to temperature shift. (A) The number of significantly DE core genes that are (A) upregulated or (B) downregulated in response to temperature shift. Upregulated genes are expressed more highly at 15°C than 37°C. Bar heights represent the number of genes, while the dots below represent the species in that group.

We extended our analysis to the other internal nodes on the phylogeny and

examined not just the core genes but all those genes that show some level of sharing

between species. At each node on the phylogeny, we considered those genes that

have an orthologue in each descendent of that node, but in none of the other species,

and are DE in at least one of the species. In every case, we see that if a gene is DE in

at least one genome, the most likely outcome is that it is DE in only that one genome

(Figure 5) with decreasing frequencies as the number of species increases. Thus, even

when genes are shared between multiple species, patterns of differential expression

are primarily not conserved.

Figure 5: Distribution of differentially expressed genes among members of a clade. (A) Phylogeny of the species in this study, with nodes labeled. (B) For each gene present in all species descended from a given node, total bar length represents the number of genes that are DE in at least one species. Length of colored segments of the bar represent the number of genes for which that many species are DE.

Another way to visualize the overall similarity of the transcriptional response or lack thereof is by principal component analysis (PCA). We calculated the PCA using the 1838 core genes. The first two principal components explain 40% of the variance in normalized gene expression levels. PC2, which accounts for 15% of the variance in gene expression, separates 15°C samples from 37°C samples, as all 37°C samples have larger values of PC2 than the 15°C samples from the same species (Figure 6). (Other principal components not visualized here do not clearly separate samples based on temperature.) Thus, there is some conserved difference in the expression of core genes between 15°C and 37°, but it is a small proportion of all variation in gene expression among the species.

Figure 6 Principal component analysis of expression of the 1838 core genes.

Functions of genes that are DE between warm and cold environments

To better understand the functional consequences of the lack of conservation of DE genes, we used Gene Ontology (GO) enrichment to understand what groups of genes were differentially expressed. We used eggNOG-mapper (24) to assign GO terms to each gene in each genome. (This was done even for genomes like *E. coli* K-12 that had pre-existing GO annotations so that all species had comparable annotations in our analysis.) As a check on the quality of the annotation, we examined the GO terms assigned to each orthologue of the core genome. We found that 1668 of 1838

(91%) of core genes had identical GO annotations in all six genomes, suggesting that

the annotation worked consistently for most genes. Most of the remaining 118 core

genes had identical annotations for 5 of 6 genomes, suggesting that again the

annotation worked well but was not perfectly consistent with what we defined as the

core genome.

We then performed GO enrichment analysis using topGO (25) on the RNA-seq

data from each species. Consistent with the lack of conservation of differentially

expressed genes, there was overall little conservation of enriched GO terms across the

genomes (Figure 7). Of the 151 GO terms that were enriched in at least one genome,

only 10 were enriched in at least three genomes (Table S2). 107 of the 151 terms were

enriched in only a single genome.

Figure 7. Shared patterns of GO enrichment. Bar heights represent the number of GO terms
enriched, while the dots below represent the species involved in that comparison.

Iron metabolism genes are affected by temperature in multiple species

One group of GO terms that are enriched in multiple genomes are those

associated with iron acquisition and use. Enterobactin is a siderophore that is

synthesized by many species of Enterobacteraceae (26). This molecule is synthesized

inside the cell and secreted into the environment, where it can bind to ferric iron. The

iron-enterobactin complex is then actively transported into the cell where the

enterobactin is cleaved, freeing the iron (26). Iron is scarce inside of mammalian hosts

and genes that allow for iron scavenging are important virulence factors in many of the

species in this study (27). The enterobactin biosynthesis term (GO:0009239) is enriched

in *E. coli, S. enterica, C. rodentium,* and *K. pneumoniae*, with the associated genes

downregulated at 15°C in these species (Figure 8A). Genes for enterobactin transport

(GO:0042930) are enriched in *S. enterica, C. rodentium, E. cloacae* and *K. pneumoniae*,

with the associated genes also downregulated (Figure 8B). The *E. coli* results are

consistent with previous findings that 37°C increases the expression of iron utilization

genes in that organism relative to lower temperatures (10). The overall picture is that *S.*

enterica, C. rodentium, and *K. pneumoniae*, like *E. coli,* do less to acquire iron at 15°C

than at 37°C. That said, this is not a completely conserved regulatory pattern, as

enterobactin biosynthesis levels are largely unchanged in response to temperature in

E. cloacae and *S. marcescens.*

Figure 8: Genes involved in iron metabolism change expression in the cold. Log-2
fold change of gene expression between 15°C and 37°C for genes annotated as
involved in several aspects of iron metabolism (A) enterobactin biosynthesis, (B)
enterobactin transport, (C) iron-sulfur cluster assembly, and (D) iron-sulfur cluster
transfer. Orange dots represent a single DE gene, while green dots are a gene that is
not DE. (E,F) The expression of genes coding for two global regulators previously
identified as associated with these phenotypes, *fur* and *hns*. Black dots represent the
mean, error bars are the SEM, and gray dots are individual measurements.

One notable use for iron in enteric bacteria is the production of iron-sulfur

clusters. Genes associated with iron-sulfur cluster assembly (GO:0016226) that are

significantly DE are all upregulated in 15°C relative to 37°C in most species (Figure 8C).

However, there are multiple genes expressed at lower levels in *C. rodentium* at 15°C

and one such in *E. coli.* Genes associated with the transfer of mature iron-sulfur

clusters into proteins (GO:0097428) that are significantly DE are primarily upregulated

(Figure 8D), although the number of DE genes varies among the species. One notable

contrast between iron-sulfur cluster synthesis and transfer and enterobactin synthesis

and transport is that *E. cloacae* and *S. marcescens* do not have marked responses to

temperature in their enterobactin genes but do have similar transcriptional patterns of

iron-sulfur cluster metabolism genes as other species.

One key regulator of genes associated with iron metabolism in many species is

the protein Fur. Fur is primarily a transcriptional repressor (28), but it can serve as a

positive regulator in cases where it disrupts repression by another protein, such as its

action as an anti-silencer of H-NS at the *ftnA* promoter (29). Fur mRNA levels are

significantly higher at 15°C than 37°C only for *C. rodentium* and *E. cloacae* (Figure 8E).

E. coli and *S. enterica* showed clear repression of enterobactin biosynthesis with non-

significant (and very modest) changes in *fur* levels, suggesting that temperature

regulation of Fur cannot easily explain the observed patterns in the transcription of

enterobactin biosynthesis genes in response to temperature. Similarly, *hns* mRNA

levels are only significantly higher at 15°C for *K. pneumoniae* and *S. marcescens*

(Figure 8F), suggesting that changes in H-NS abundance do not account for changes

in enterobactin biosynthesis genes in response to temperature. While the direction of

change in *fur* and *hns* levels are consistent with the changes in enterobactin levels, the

modest and non-significant changes in mRNA abundance suggest that other

regulatory factors beyond Fur and H-NS regulate this change in iron utilization in some

species.

Other enriched GO terms

Genes involved in the TCA cycle and with glycine metabolism were significantly

enriched in four genomes each (Table S2). (TCA cycle genes are also annotated as

involved in citrate metabolism, GO:0006101). These genes were largely upregulated in

response to cold (Figure 9A, B). LB medium contains no fermentable sugars, so the

TCA cycle is presumably being fed by metabolizable amino acids (30). This result is

consistent with a previous proteomic study that identified several TCA cycle enzymes

as upregulated following downshift from 37°C to 20°C (31). Other GO terms enriched

in multiple species include the response to osmotic stress (Figure 9C) and single-

species biofilm formation (Figure 9D). The overlap between osmotic stress resistance

and low temperature response has been previously noted (1). Low temperature has

previously been shown to increase biofilm production and the expression of biofilm

associated genes in *E. coli* (2), and these results suggest that this response may be

conserved in some other members of this group of organisms.

Figure 9: Groups of genes that change in multiple species. Log-2 fold change of gene expression between 15°C and 37°C for genes annotated as involved in (A) the tricarboxylic acid cycle, (B) glycine catabolism, (C) responding to osmotic stress, and (D) single-species biofilm formation on an inanimate surface. Orange dots represent a single DE gene, while green dots are a gene that is not DE.

Species-specific gene expression changes in response to temperature

While there are a few GO terms that are enriched across multiple species and suggest a common phenotypic response to low temperatures, the vast majority of enriched GO terms are species-specific. Here we highlight several species-specific responses. Similar plots of genes associated with a GO term enriched in at least one species are available in the Zenodo archive for interested readers.

Ribosomes and electron coupled proton transport are enriched only in *E.*

coli

Several GO terms that were enriched only in *E. coli* are involved with protein

production and metabolism. Genes annotated as involved in ribosome assembly

(GO:0000027 and GO:0000028, for ribosomal large and small subunit assembly,

respectively) are enriched in the DE genes of *E. coli* but not in other species (Figure

10A). (This GO term includes the structural components of the ribosome as well as

factors that modulate ribosome assembly.) The mechanistic basis for this difference in

ribosomal protein mRNAs is hard to predict because the levels of regulation of

ribosomal proteins are so complicated. Ribosomal protein operons are directly

regulated by ppGpp + dksA in *E. coli* (32). Ribosomal protein transcripts have very

short half-lifes when translationally repressed (32), and both changes in RNA structure

and a set of regulatory proteins modulate translation rate in response to lower

temperature (1).

Genes coding for proteins that produce the proton motive force via electron

transport (GO:0015990) are DE in *E. coli* more than other species (Figure 10B) . These

genes, *cyoABCD*, and *nuoLMN* encode for the cytochrome bo3 quinol:oxygen

oxidoreductase and NADH:ubiquinone oxidoreductase I respectively. The EcoCyc

database (33) lists at least 12 regulatory proteins that may regulate the transcription of

one or more of these genes in *E. coli*, so substantial work may be required to

determine the basis of this species-specific downregulation.

Figure 10: Responses to low temperature unique to *E. coli*. Log-2 fold change of gene expression between 15°C and 37°C for genes annotated as involved in (A) production of the ribosome, and (B) electron transport coupled proton transport. Orange dots represent a single DE gene, while green dots are a gene that is not DE.

Colanic acid biosynthesis is enriched only in *C. rodentium*

Colanic acid is the capsular polysaccharide in the *E. coli* and some relatives (34–

36). In our data the genes annotated with the GO term for colanic acid biosynthesis

("GO:0009242") were enriched in *C. rodentium* but not in other species. Because some

genes in the *wca* cluster, which are biosynthetic enzymes for colanic acid (35), were

not annotated with this GO term, we created a custom gene set that included all genes

annotated with GO:0009242 plus all genes from the *wca* cluster that were missing that

annotation. (The strains of *K. pneumoniae* and *S. marcescens* we worked with do not

have orthologues to the *wca* genes found in the other four species.) 70% genes in the

combined set are significantly upregulated in *C. rodentium*, while many fewer (if any)

genes are upregulated in each of the other species (Fig 11A).

In *E. coli*, the Rcs phosphorelay system is an important regulator of colanic acid

biosynthesis (37). The response regulator RcsB forms a heterodimer with the RcsA

protein, and these two proteins are required for maximal capsule expression (38). The

gene *rcsA* has an expression pattern that closely matches the pattern of its target

genes, being significantly upregulated 10-fold in *C. rodentium,* while being upregulated

at most 2-fold in the other five species (Fig 11B). In contrast, RcsB shows a similar

pattern of expression across five of the six species, rather than being upregulated only

in *C. rodentium* (Fig 11C). The evolution of species-specific regulation of RcsA in *C.*

rodentium is a promising candidate for the evolutionary change that brought about the

species-specific pattern of a large group of genes.

Figure 11: Upregulation of colanic acid related genes only in *C. rodentium*. (A) Log 2-fold change of gene expression between 15°C and 37°C for genes associated with production and use of colanic acid. Orange dots represent a single DE gene, while green dots are a gene that is not DE. (B, C) Expression of two known regulators of this process, *rcsA* and *rcsB*. Black dots represent the mean, error bars are the SEM, and gray dots are individual measurements.

RpoS regulation of the response to temperature shift

The alternative sigma factor RpoS is known to be activated at low temperature

in *E. coli* where it regulates the expression of several hundred genes (39). We

compared the role of RpoS in *E. coli* and its close relative *S. enterica* during growth at

15°C. We measured RpoS protein levels using western blotting and found that RpoS

has a similar pattern of expression in both species, with RpoS accumulating during

exponential growth at 15°C to a level like stationary phase at 37°C, but not during

exponential growth at 37°C (Figure S1). As expected, the Δ*rpoS* mutants did not

produce measurable RpoS.

RNA-seq experiments with Δ*rpoS* strains of *E. coli* and *S. enterica* allowed us to

define the RpoS regulon at 15°C*.* During exponential growth at 15°C, 232 genes are DE

between wt and Δ*rpoS* in *E. coli.* This represents 22% of the number of genes that are

DE between 37°C and 15°C in the wild-type strain. This implies that while RpoS

regulates many genes in the cold, it is only a fraction of the genes that are cold-

regulated overall. Reassuringly, of the 232 DE genes in *E. coli* in this study, 189 (81%)

were previously identified as RpoS-regulated at 15°C by Adams *et al.* (39). In *S.*

enterica, 189 genes are DE between wt and Δ*rpoS* strain, 80% of the number of genes

that are RpoS-regulated in *E. coli.*

83 genes are DE in both *E. coli* and *S. enterica* (Figure 12). This includes genes

previously reported to be RpoS-dependent (40–42) such as *dps, katE, mlrA, osmC,*

osmY, otsA, otsB, sodC, and *wrbA.* Consequently, GO terms involved in response to

osmotic stress or response to hydrogen peroxide are enriched in both species. While

there are 83 genes RpoS-regulated in both species, the majority of the RpoS-regulon in

E. coli or *S. enterica* is not RpoS-regulated in the other species (Figure 12). For

example, of the 232 RpoS-regulated genes in *E. coli,* 149 of those genes (64%) are not

RpoS-regulated in *S. enterica.* Those 149 genes are composed of 50 genes that are

not present in *S. enterica,* and 99 genes that have an ortholog in *S. enterica*, but that

orthlog is not RpoS-regulated in *S. enterica.* Like our earlier analysis with the entire

cold-response regulon, divergence of the RpoS-regulon between *E. coli* and *S.*

enterica is due to a mixture of both different regulation of genes shared between the

two species and the presence of species-specific genes.

Figure 12: Partial overlap in the RpoS regulon of *E. coli* and *S. enterica*. Bar
lengths are the number of RpoS-regulated genes in that species, with colors
denoting the status of those genes in the other species. For example, the green part
of the *E. coli* bar represents the 99 genes that are RpoS-regulated in *E. coli* that are
also present in *S. enterica,* but are not RpoS-regulated in *S. enterica.*

Genes that are RpoS-regulated only in *E. coli* include *astABCDE,* responsible for

arginine catabolism (GO:0006527), *dppABCDF*, involved in di-peptide transport, *gabD*,

astC, and *ldcC*, involved in lysine metabolism (GO:0006553). These genes have

orthologs in *S. enterica* but are not significantly DE in that organism, pointing to a role

for RpoS in amino-acid catabolism in *E. coli* that is not present in *S. enterica.* RpoS

also has an *E. coli*-specific role in regulation of genes *gadE, gadW, gadX, hdeA, hdeB,*

and *slp,* which are part of the acid fitness island not present in related species like *S.*

enterica (43).

Genes that are RpoS-regulated only in *S. enterica* include *glgB, glgC, glgP,* and

glgX, responsible for the biosynthesis and catabolism of glycogen. (These genes are

expressed more highly at 15°C than 37°C in both *E. coli* and *S. enterica*, but are only

RpoS-regulated in *S. enterica*.) There are also genes that are RpoS-dependent and

present only in *S. enterica*, like *invA*, *invE*, *prgI, sipD*, and *sprB*. These genes are all

located on the Salmonella Pathogenicity Island 1 and are not present in *E. coli* (44)*.

Much like the broader response to low temperature, the RpoS-regulon at 15°C is

mostly not conserved between *E. coli* and *Salmonella* due a mix of species-specific

genes and different regulation of conserved genes.

Discussion

All of the species studied respond to a shift in growth temperature from 37°C to

15°C by changing the expression of a large proportion of their genes; the number

ranges from 626 to 1057 genes, or 14 to 26% of all genes in the genome. For *E. coli*,

this is more than twice the number of genes than were previously reported in

microarray studies of a shift from 37°C to 23°C (2, 10). The difference can probably be

attributed to the combination of the more extreme temperature shift in this study, and

the difference in technologies used to measure mRNA abundance.

A major finding of this study is the lack of conservation of the transcriptional

response to low temperature across the *Enterobacteriaceae*. All the species grow

markedly slower at 15°C than at 37°C and all changed expression of hundreds of

genes in response to the temperature shift, the identity of these genes is mostly not

conserved among species. The lack of conservation of which genes are DE is due to

both the fact that each species has unique genome content, and that even when genes

are shared, they may be DE in only a subset of species. While species-specific genes

do contribute to the diversity of differentially expressed genes in this group, these

species-specific genes are actually less likely to be DE than is the core genome.

Species respond to a common stress by changing the expression of a different set of

their shared genes.

This lack of conservation of which genes are differentially expressed extends to

a lack of conservation of phenotypes as predicted by enriched GO terms. Most of the

enriched GO terms for a species are not shared with other species, which implies that

bacteria in this group of species may have different phenotypic responses to the same

stress. For example, *E. coli* responds to the lower temperature by down-regulating the

mRNAs of ribosomal proteins and proteins involved in electron transport to generate

the proton motive force. Surprisingly, this effect is unique to *E. coli*. It is well known

that the abundance of ribosomal proteins in *E. coli* is lower at slower growth rates (45),

and our data on the abundance of ribosomal protein mRNAs are consistent with it. All

of the other species in this study all grow slower at 15°C than at 37°C, and some grow

as slowly as *E. coli* at 15°C, but none show the same down-regulation of ribosomal

protein mRNA. It is possible that these other species do not achieve down-regulation

of ribosomal activity at lower growth rate via the same mechanism, or do not down-

regulate ribosomal activity at all.

The alternative sigma factor RpoS is one regulatory protein that plays a role in

the transcriptional response to temperature shift. Much like the overall cold response,

the RpoS-dependent part of the cold response is only partially conserved between *E.*

coli and *S. enterica.* The divergence between the two species is due to a mix of

divergent expression of genes shared between the two species and species-specific

genes that are part of the RpoS-regulon. The divergence in RpoS-dependence of

genes shared by *E. coli* and *S. enterica* was somewhat surprising given that both

species produce similar amounts of RpoS at 15°C. Divergence in RpoS-dependence of

shared genes could be due to changes in the RpoS protein itself, in the promoters of

these divergently expressed genes, or could be due to changes in the many trans-

acting factors that interact with RpoS to determine the transcriptional output of a

promoter (42) . Understanding this molecular basis of these changes is an important

future area of research.

Other authors have also noted that species-specific genes are a substantial part

of the divergence of regulons between species pairs. For example, the OmpR protein

regulates transcription of a group of genes in response to physical stresses like

osmolarity and pH. The OmpR regulon in *E. coli* and *S. enterica* is only weakly

conserved, and the authors noted that many species-specific genes were in the

regulon (41). The RovA protein of *Yersinia* is involved in transcribing important virulence

genes in those organisms. The RovA-regulon of *Yersinia enterocolitica* and *Y. pestis* is

also only weakly conserved due at least in part to the species-specific genes regulated

by RovA in each species (47). This paper extends this idea to the RpoS-regulon, and

shows that while species-specific genes are indeed important for driving divergence of

the regulon, differential expression of shared genes is at least as important for this

regulon.

In conclusion, we have studied the transcriptional response to a change in

temperature from 37°C to 15°C in five species of the *Enterobacteriaceae* and one

outgroup. We have found that there is limited conservation of this response, with each

species changing the transcription of hundreds of genes that are unchanged in the

other species. This divergence is not due primarily to species-specific genes in each

genome, as core genes are more likely than expected to be differentially expressed in

response to temperature change. This pattern of evolutionary divergence is also true of

RpoS-regulon, where divergence is due more to shared genes being RpoS-regulated in

only a single species than it is due to species-specific genes being part of the regulon.

These results highlight the importance of the evolution of transcriptional patterns of

shared genes in the evolution of bacterial responses to stress.

513   Materials and Methods

514   *Strains and strain construction*

Six species were used in this study: *E. coli, Salmonella enterica, Citrobacter*

rodentium, Enterobacter cloacae, Klebsiella pneumoniae, and *Serratia marcescens.* We

used strains with wild-type alleles for *rpoS* for each species, along with Δ*rpoS* strains of

E. coli and *S. enterica* that we had access to (Table S1). To construct the isogenic

Δ*rpoS* strain of *S. enterica*, the Δ*rpoS::cm* allele of a *S. enterica* 14028s strain provided

by the McClelland Lab (48) was moved by P22 transduction (49) into our lab isolate of

S. enterica 14028s.

Growth conditions

Strains were inoculated from -80°C frozen cultures and grown aerobically

overnight in 5 mL of Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1%

NaCl) at 37°C with shaking at 225 rpm. The next morning, 250µL of each culture was

diluted into 25 mL of LB in a 250 mL borosilicate flask and grown at 37°C in an

incubator shaking at 225 rpm. When cultures reached OD_{600} of 0.25 - 0.35, flasks were

transferred to a water bath at 15°C shaking at 225 rpm. (OD_{600} of 0.3 is in the middle of

exponential growth in these cultures.) When cultures were grown for RNA and protein

isolation, no measurements of OD_{600} were performed at intermediate time points

between the shift and sampling of RNA and protein. When cultures were grown to

measure growth rate, OD_{600} measurements were taken every 20 to 30 minutes after the

shift to 15°C.

To minimize confounding factors, we always grew all 8 strains in parallel for

each experimental replicate.

RNA Isolation

RNA was isolated from the same cultures and at the same time as protein

samples. 1.5 mL of bacterial culture was pelleted and resuspended in 500 µL of Trizol

pre-heated to 65°C and then stored at -80°C until use. Samples were purified on a

column using the Zymo Direct-Zol RNA Miniprep kit. They were then treated twice with

DNase I using Turbo DNA-free (Invitrogen) for 30 minutes each at 37℃ to ensure that

all DNA was degraded. The samples were further purified on a column using the Zymo

RNA Clean and Concentrator Kit and again stored at -80℃ until sequencing. The

concentration of the samples was checked using a NanoDrop spectrophotometer and

degradation was checked for by electrophoresing samples on an ethidium bromide

agarose gel.

N = 4 biological replicate samples were collected for each strain at each

temperature.

Generation of RNA-Seq data

Illumina cDNA libraries were generated using a modified version of the RNAtag-

seq protocol (50). Briefly, 500 ng to 1 µg of total RNA was fragmented by heating to

94°C in NEB Alkaline Phosphatase Buffer, depleted of any residual genomic DNA,

dephosphorylated, and ligated to DNA adapters carrying 5'-AN_8-3' barcodes of known

sequence with a 5' phosphate and a 3' blocking group. Barcoded RNAs were pooled

and depleted of rRNA using the RiboZero rRNA depletion kit (Epicentre). Pools of

barcoded RNAs were converted to Illumina cDNA libraries in 2 main steps: (i) reverse

transcription of the RNA using a primer designed to the constant region of the

barcoded adaptor with addition of an adapter to the 3' end of the cDNA by template

switching using SMARTScribe (Clontech) as described (51); (ii) PCR amplification using

primers whose 5' ends target the constant regions of the 3' or 5' adaptors and whose

3' ends contain the full Illumina P5 or P7 sequences. cDNA libraries were sequenced

on the Illumina [NextSeq 500] platform at the Broad Institute to generate paired end

reads.

Each RNA pool consisted of 16 samples, a 15°C and a 37°C sample from each

of the 8 strains. N = 4 total pools were constructed and sequenced.

RNA-seq Data Analysis

Sequencing reads from each sample in a pool were demultiplexed based on

their associated barcode sequence using custom scripts. Up to 1 mismatch in the

barcode was allowed provided it did not make assignment of the read to a different

barcode possible. Barcode sequences were removed from the first read as were

terminal G's from the second read that may have been added by SMARTScribe during

template switching.

Reads were aligned to the reference genomes (Table S1) using BWA v0.7.17

(52). Reads were counted with htseq-count v2.0.1 (53). Further analysis was done in R

v4.4.0 (54).

Before conducting the analysis reported in this paper, we performed initial

quality control to ensure that each sample had at least 1,000,000 read-pairs that

mapped to protein coding sequences. One sample was below this threshold and

removed. In addition, three samples did not cluster with any of the other samples in

PCA or a heatmap and were also removed. The samples removed were one from *C.*

rodentium at 15°C, two from *K. pneumoniae* at 37°C, and one from *S. marcescens* at

37°C.

Differential expression analysis was performed using the package DESeq2

v1.40.2 (55). Genes were considered significant if they had a FDR-adjusted p-value of

less than 0.01 and an absolute value of fold-change greater than 2.

eggNOG-mapper (24) was used to assign GO terms to each gene in each

genome. GO enrichment for each species was performed with the R package topGO

(25). The GO tree for each species was pruned to remove terms with fewer than 5

genes. The weight01 algorithm was used with Fisher's exact test to calculate p-values

on enrichment of GO terms. GO terms were considered significantly enriched if they

had a p-value of less than 0.05.

Protein Isolation & Western Blotting

Total protein was collected immediately before the shift to 15°C and 3 hours

after initial cold exposure. Samples with cell density equivalent to 1mL of OD_{600} = 0.3

were pelleted at at least 20,000 x g for 30 s, resuspended in 100µL of 2x Laemmli

sample buffer (LiCor), heated at 95°C for 5 minutes, and then stored at -20°C until they

were used.

A quantitative western blot was used to measure the levels of RpoS in the cell at

each time point, in a procedure slightly modified from (15). 10µL of each protein

sample was electrophoresed on a 4 - 20% gradient polyacrylamide gel (Bio-Rad) in

Tris-glycine running buffer (25 mM Tris base, 250 mM glycine, 0.5% SDS) at 200V for

35 minutes at room temperature. Electrophoresis was used to transfer proteins to a

Immobilon-FL PVDF membrane in transfer buffer (48 mM Tris base, 39 mM glycine,

0.0375% SDS, 20% methanol) at 100V for 45 minutes at 4°C. In order to quantify the

total amount of protein in the samples, the membranes were stained with 5 mL of

REVERT Total Protein Stain solution and imaged using the LiCor Odyssey CLx imager.

The membranes were blocked with 10mL of Odyssey Blocking Buffer for 1 hour at

room temperature.

RpoS was detected using an anti-RpoS monoclonal antibody (clone 1RS1,

BioLegend). The blocked membranes were labeled with primary antibody (0.4 µg/mL

mouse anti-RpoS, 10mL Odyssey Blocking Buffer, 0.2% Tween 20) at 4°C with

shaking at 55 rpm overnight. Once labeled, the membrane was washed four times with

10 - 15mL of 1x Tris-buffered saline with Tween 20 for 5 minutes each wash. The

membranes were probed with a fluorescent secondary antibody (IRDye 800CW goat

anti-mouse, LiCor) that was diluted in 10mL of Odyssey Blocking Buffer with 0.2%

Tween 20 and 0.01% SDS. The membranes were incubated with the diluted secondary

antibody for 1 hour at room temperature and were washed as described previously,

with an additional wash at the end with 1x Tris-buffered saline without Tween 20.

Membranes were dried for 2-3 hours between 2 sheets of Whatmann 3MM blotting

paper before imaging on a Li-Cor Odyssey CLx imager.

RpoS levels were quantified by analyzing the amount of fluorescence of each band
with Image Studio v 2.1 software.
Data analysis was performed in R v4.4.0 (54). RpoS levels were normalized by
dividing them by the total amount of protein at each time point, as quantified by the
total protein stain.
632

xenoGI analysis

634   The program xenoGI v3.1.1 (23) was used to reconstruct the evolutionary history
635 of the genomes used in this study. The phylogeny used by xenoGI was inferred using a
636 concatenation of the amino acid sequences of all of the core genes as identified by
637 xenoGI. The phylogeny was inferred by Astral v5.6.3 (56) using the built-in helper
638 function of xenoGI.
639

Data Availability

641   The RNA-seq FASTQ files are deposited in the Gene Expression Omnibus with
642 ascension number GSE267531.
643   All other data and scripts used for data analysis are in a Zenodo archive at DOI
644 10.5281/zenodo.11460468
645
646
647

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