Transcriptomics reveals a cross-modulatory effect between riboflavin and iron and outlines responses to riboflavin biosynthesis and uptake in *Vibrio cholerae*

Ignacio Sepúlveda-Cisternas1,2, Luis Lozano Aguirre3, Andrés Fuentes Flores1, Ignacio Vásquez Solis de Ovando1 & Víctor Antonio García-Angulo1

*Vibrio cholerae*, a pandemic diarrheagenic bacterium, is able to synthesize the essential vitamin riboflavin through the riboflavin biosynthetic pathway (RBP) and also to internalize it through the RibN importer. In bacteria, the way riboflavin biosynthesis and uptake functions correlate is unclear. To gain insights into the role of the riboflavin provision pathways in the physiology of *V. cholerae*, we analyzed the transcriptomics response to extracellular riboflavin and to deletions of *ribD* (RBP-deficient strain) or *ribN*. Many riboflavin-responsive genes were previously reported to belong to the iron regulon, including various iron uptake genes. Real time PCR analysis confirmed this effect and further documented that reciprocally, iron regulates RBP and *ribN* genes in a riboflavin-dependent way. A subset of genes were responding to both *ribD* and *ribN* deletions. However, in the subset of genes specifically affected in the ∆*ribD* strain, the functional terms protein folding and oxidation reduction process were enriched, as determined by a Gene Ontology analysis. In the gene subset specifically affected in the ∆*ribN* strain, the cytochrome complex assembly functional term was enriched. Results suggest that iron and riboflavin interrelate to regulate its respective provision genes and that both common and specific effects of biosynthesized and internalized riboflavin exist.

Redox reactions, consisting of electron transfers from an oxidizing molecule to a reducing one, lie at the core of many central physiological processes. These include oxidative phosphorylation, cell signaling, photosynthesis, DNA repair, carbohydrates metabolism, oxygen storage, photosensitization and protein folding among many others. In order to complete these reactions, enzymes usually require redox cofactor molecules which include nicotinamide-derived molecules, iron-sulfur clusters, thiamin, deazaflavin and transition metals like cooper, manganese, cobalt and zinc. However, iron is by far the most widespread metal redox cofactor, while molecules derived from riboflavin (also named vitamin B2), such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) constitute the main organic electron transfer cofactors, with an importance similar to that of iron. Genes encoding flavoproteins may comprise up to 3.5% of the genome of a species. Flavins are probably the most versatile cofactors, being able to catalyze one- and two-electron transfers, which allows their participation in electron bifurcation reactions. These molecules may also catalize non-redox reactions and are increasingly recognized as covalent catalysts, acting in the formation of flavin-substrate adduct intermediates.

There is evidence that flavins may act as signaling molecules in bacteria. Riboflavin and its breakage derivative lumichrome are able to mimic N-acyl homoserine lactone for activation of quorum sensing pathways in *Pseudomonas aeruginosa* and riboflavin is a chemoattractant to *S. oneidensis*. Riboflavin may as well be secreted by some bacteria to be used as electron shuttle to reduce Fe3+ into its more soluble Fe2+ form and to

---

1Programa de Microbiología y Micología, Instituto de Ciencias Biomédicas, Universidad de Chile, Santiago, Chile. 2Escuela de Biotecnología, Universidad Mayor, Campus Huechuraba, Santiago, Chile. 3Centro de Genómicas, Universidad Nacional Autónoma de México, campus Chamilpa Cuernavaca, Morelos, Mexico. Ignacio Sepúlveda-Cisternas and Luis Lozano Aguirre contributed equally to this work. Correspondence and requests for materials should be addressed to V.A.G.-A. (email: victorgarcia@med.uchile.cl)
complete the extracellular respiratory chain\textsuperscript{14–17}. In addition, this vitamin frequently represents a metabolic currency during bacteria-host or intermicrobial trade interactions\textsuperscript{18,19}.

Most bacteria are able to biosynthesize riboflavin through the riboflavin biosynthetic pathway (RBP). This pathway starts with guanosine triphosphate (GTP) and ribulose-5-phosphate to synthesize riboflavin using the RibA (GTP cyclohydrolase II), RibD (pyrimidine deaminase/reductase), RibH (lumazine synthase), RibB (3,4-dihydroxybutanone phosphate synthase) and RibE (riboflavin synthase) enzymes\textsuperscript{20,21}. The nomenclature of RBP enzymes varies among bacterial species and \textit{Escherichia coli} names\textsuperscript{22} are thoroughly used here. In bacterial genomes, RBP genes could form an operon or be positioned in different loci. In various species, some RBP genes are duplicated or multiplicated\textsuperscript{23}. In some cases, duplicated RBP gene orthologs appear to implement modularity to riboflavin production, where the RBP uses subsets of genes to provide riboflavin for specific purposes, such as secretion or interactions with the host\textsuperscript{24,25}. Bacteria may also use importer proteins to internalize riboflavin from the surroundings. Although many bacterial species rely exclusively on riboflavin uptake, many others possess both riboflavin biosynthesis and uptake. It is hypothesized that this overlay allows bacteria to take advantage of changing environments, turning on riboflavin uptake and stopping biosynthesis in nutrient rich niches, while granting autonomy when facing stringent conditions. It is also possible that riboflavin importers procure flavins for specific functions in riboflavin-prototrophic species\textsuperscript{23,26–28}.

\textit{Vibrio cholerae} are Gram negative proteobacteria responsible for cholera, a pandemic disease affecting mainly developing countries, characterized by acute, life-threatening diarrhea\textsuperscript{29}. Global cholera burden has recently been estimated in around 2.8 million cases with 95,000 deaths per year\textsuperscript{30}. Most \textit{V. cholerae} strains are innocuous indigenous members of estuarine and seawater microbiota, with a few strains from serotypes O1 and O139 causing almost all of cholera cases\textsuperscript{29–32}. In these bacteria, development of virulence is not only associated with the acquisition of virulence factors but also of specific alleles of virulence adaptive polymorphisms rotating in environmental species, which confer selective advantages like host colonization properties\textsuperscript{32}. Importantly, environmental water conditions such as temperature, salinity, pH and sunlight exposure have a major impact in the development of cholera epidemics and thus outbreaks are expected to increase due to global warming\textsuperscript{31,33}.

Cholera is mostly a waterborne disease, and after human consumption, \textit{V. cholerae} expresses several virulence factors. Cholera toxin is the main inducer of diarrhea. This toxin translocates into host enterial cells to promote constitutive activation of the adenylate cyclase, causing an increase in 

\[ \text{C} \]

expressed in order to favor host colonization\textsuperscript{33,34}. In the environment, as the flagellum, the HapA metalloprotease, Zot and RTX toxins and different iron acquisition systems are also virulence factors such as the flagellum, the HapA metalloprotease, Zot and RTX toxins and different iron acquisition systems are also virulence factors such as

\begin{itemize}
  \item Temperature
  \item Salinity
  \item pH
  \item Sunlight exposure
\end{itemize}

These conditions have a major impact in the development of cholera epidemics and thus outbreaks are expected to increase due to global warming\textsuperscript{31,33}.

Materials and Methods

\textbf{Strains and growth conditions.} \textit{V. cholerae} N16961 strain and its \( \Delta \text{ribD} \) and \( \Delta \text{ribN} \) derivatives were grown overnight in LB plates at 37 °C. 5 ml of LB broth were inoculated with a colony of the plate cultures and incubated at 37 °C in an orbital shaker at 150 rpm until they reached an \( \text{OD}_{600\text{nm}} \) of 1.0. Next, cultures were centrifuged and pellet washed twice with \( T \) minimal medium\textsuperscript{19} and resuspended in 1 ml of fresh \textit{T}. 10 ml of \textit{T} medium or \textit{T} minimal medium were inoculated with 10 \( \mu \)l of the resuspensions and incubated at 37 °C and 180 rpm until an \( \text{OD}_{600\text{nm}} \) of 0.8. 1 ml of each culture was centrifuged and subjected to RNA extraction. When indicated, iron was omitted in \textit{T} media and 3 ml of cultures at \( \text{OD}_{600\text{nm}} = 0.3 \) were harvested for RNA extraction.

This growth protocol was performed three times independently for each condition and was similar for RNA subjected to transcriptomics and Real Time PCR (RT-PCR).
RNA extraction, retrotranscription, RNAseq and RT-PCR. RNA extraction was performed with the Thermo Scientific Genejet RNA purification kit according to manufacturer’s instructions. RNA extracts were digested with Turbo DNA-free DNase at 37 °C for 1 hour. For RNAseq, rRNA was removed using the Ribo-Zero removal kit and cDNA libraries were constructed using the TruSeq mRNA stranded kit, according to manufacturer’s instructions. Next, RNA was sequenced using the Illumina HiSeq platform to produce 100 bp paired-end reads, with ~40 million reads per sample. Sequencing raw data files, processed sequence data files and metadata information was deposited at the Gene Expression Omnibus database from NCBI (GSE107538). rRNA removal, cDNA libraries generation and RNAseq were performed at Genoma Mayor (Santiago, Chile).

For RT-PCR analysis, the AffinityScript QPCR cDNA Synthesis kit (Agilent Technologies) was used for cDNA synthesis according to manufacturer’s instructions. As a negative control, a reaction with no reverse transcriptase was included for each sample in each run. RT-PCR was performed using the Brilliant II SYBR Green QPCR Master Mix kit in a One-Step Applen Biosystems (Life Technologies) thermocycler. Relative expression in the indicated conditions was determined through the ΔΔCt method as developed before. The 16 s ribosomal RNA gene was used for normalization. For the assessment of the relative expression by RT-PCR of ribB, ribN, ribD and gyrB, the sets of primers used were ribB Fw/ribB Rv, ribN Fw/ribN Rv, ribD Fw/ribD Rv and gyrB Fw/gyrB Rv, respectively. Other RT-PCR primers are as follows: for tonB1, tonB1 Fw (5’-GGTGTGGCAGCATGCGCTTG-3’) and tonB1 Rv (5’-GGGGACTTCATCTGGATTAG-3’); for sodA, sodA Fw (5’-GCCAAGCGATATCCAAGG-3’) and sodA Rv (5’-GGTCAGTGCCGCTATCCTCATG-3’).

RNAseq data analysis. Quality control visualization and analysis (adapter and quality trimming) was performed using FastQC version 0.11.2 (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) and Trim_galore version 0.4.1 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/), respectively. Reads were mapped to the genome of *Vibrio cholerae* 01 biovar El Tor str. N16961 (RefSeq, NCBI) using Bowtie2 version 2.1.043. In all of the samples the alignment percentage of reads was above 98%. Differential expression analysis between samples was performed with the Bioconductor package edgeR version 3.18.144 using negative binomial model and exact test based on quantile-adjusted conditional maximum likelihood method (qCML). Genes with a statistically significant change in expression (P < 0.05) were selected for further analysis. Analyses of enrichment of Gene Ontology (GO) terms of biological processes in the indicated subsets of genes were performed on the online platform of the Gene Ontology Consortium (www.geneontology.org), and statistically significant (P < 0.05) functional terms were retrieved.

Results

Overview of the experiment. In *V. cholerae*, exogenous riboflavin downregulates the expression of the FMN riboswitch-containing gene *ribB*. In order to identify other genes whose expression is affected in response to riboflavin, we performed RNAseq in *V. cholerae* N16961 cultures growing in T minimal medium with or without riboflavin. Also, to start elucidating putative differential roles of the riboflavin provision pathways, we included in this analysis the ∆*ribD* and ∆*ribN* derivative strains. The *V. cholerae* ∆*ribD* is a riboflavin auxotroph unable to grow in T media without riboflavin, while the ∆*ribN* does not has an impairment to grow without riboflavin compared to the WT. A general overview of strains, growth conditions and transcriptomics comparisons is presented in Fig. 1. Four transcriptomics comparisons were performed as follows: WT growing without riboflavin versus WT with riboflavin (Comparison a in Fig. 1), WT versus ∆*ribD* both with riboflavin (b), WT versus ∆*ribN* both with riboflavin (c). The genes showing a difference of at least one fold in expression in any of these
comparisons were selected and are shown in Table 1. Additionally, a comparison of ∆ribN without riboflavin versus ∆ribN with riboflavin (d) was performed. Genes showing more than one fold change in this comparison and also found in any of the three previous comparisons are indicated in Table 1. In all cases, the genes selected presented a statistically significant change in expression (P < 0.05).

A total of 277 genes are differentially expressed in response to at least one of the three first conditions compared (Table 1). The results of the indicated comparisons is summarized as a Venn diagram in Fig. 2. 31 regulated genes were differentially expressed in the WT strain in response to extracellular riboflavin (Table 1). 177 genes were significantly affected by the mutation in ribD, of which 34 were also affected in the ribN mutant. A total of 108 genes were affected by the elimination of ribN growing in riboflavin, 74 of which were not affected by the ribD elimination. One gene was affected in the three comparisons, which corresponded to the FMN riboswitch-regulated ribB (VCA1060). These data are consistent with the notion that although the functions of riboflavin biosynthesis and transport through RibN overlap, there may also exist specific functions for each riboflavin provision pathway.

The riboflavin regulon of V. cholerae includes many iron regulated genes. The first transcriptomic comparison assessed the effect of riboflavin in the WT strain. The gene ribB was found at the top of the list of genes regulated by riboflavin, being highly repressed. This pattern is consistent with our previous report, although the degree of repression (roughly 12-fold) was higher than in our earlier determination (2-fold decrease)38. A previous RNA microarray study identified 84 genes regulated by iron in V. cholerae45. Most of the genes identified here as responding to riboflavin, are also members of such iron regulon (21 out of 31). V. cholerae possesses several transport systems dedicated to the uptake of various iron forms. These include the genes for synthesis and utilization of the vibriobactin siderophore, the ferric iron acquisition system FbpABC, the Hut heme transport and the VctPDGC system47–49. Likely, these systems are differentially required depending on the iron source available on each stage of the V. cholerae life cycle50. Genes related to most of these iron acquisition systems, except for the VctPDGC system, were found to be moderately regulated by riboflavin (Table 1), while all of such systems are known to be repressed by iron46,48. Other genes belonging to the iron regulon that were also detected responding to extracellular riboflavin included the bacterioferritin operon bjd-bjr and a few proteins with unknown function like the encoded by the VC1264, VC1266 and VCO143 open reading frames (ORFs). In addition, ybtA, coding for a member of the AraC family of transcriptional regulators involved in regulation of siderophore production in Yersinia50, was also repressed by riboflavin. Genes identified here which have not previously reported to be regulated by iron include hutC, coding for a transcriptional regulator of the histidine utilization operon, gca, coding for the autonomous glycol radical cofactor protein and two methyl-accepting chemotaxis protein genes. While most of the genes identified in this comparison were repressed by riboflavin, hutC and gca were activated. In our previous study, contrary to its effect on the WT strain, riboflavin induced the expression of ribB in a ∆ribN strain51. This suggested that riboflavin may induce changes in transcription in a manner independent of its internalization through RibN. For this reason, in order to globally identify effects of riboflavin independent of its uptake through RibN, we included a comparison of the transcriptomes in response to riboflavin in the ∆ribN strain. This analysis revealed that 16 of the genes affected by riboflavin in the WT are also affected in this strain (indicated by asterisks in Table 1 and full list in Table S1). This suggests that at least for these cases, the regulatory effect of riboflavin is independent of its internalization through RibN. In order to identify general functional relationships among the genes responsive to riboflavin, we performed analysis of enrichment of Gene Ontology (GO) terms of biological processes associated to this set. Such analysis seek to identify functional terms, as defined by the PANTHER classification system, overrepresented in a given group of genes51,52. Three GO biological processes were found statistically overrepresented (P < 0.05). These corresponded to iron ion transmembrane transport, cellular responses to iron ion and iron ion homeostasis.

To validate the transcriptomic comparison, we determined the relative expression of ribB and tonB1 in T medium and T plus riboflavin by RT-PCR. The tonB1 gene encodes a component of one of the two TonB-ExbB-ExbD complexes that harness membrane proton motive force for its heterologous use in various iron transport systems in V. cholerae47. Thus, it seems to be an adequate gene to monitor the expression of iron acquisition systems. The expression of ribB and tonB1 was reduced 4-fold in response to added riboflavin (Fig. 3a). This is in agreement with the transcriptomics results although a higher effect of riboflavin was detected by RT-PCR. To assess if there may be additional genes known to be regulated by iron that are also regulated by riboflavin but missed in our transcriptomics analysis, we determined the expression of sodA. This gene is known to be repressed by iron48. Notably, the expression of sodA was reduced 4.13-fold by riboflavin. As controls, we determined the expression of the riboflavin biosynthetic gene ribD and of the ribN gene. We have previously demonstrated that the expression of these genes is not affected by riboflavin in standard T media48 and their expression did not change in response to riboflavin in our transcriptomics results. Accordingly, the expression of these two genes was not affected by riboflavin as determined by RT-PCR. One additional control was used, gyrB, which was not affected by the presence of exogenous riboflavin according to transcriptomics. The RNA of this gene was only slightly reduced by riboflavin (0.29-fold) as determined by RT-PCR.

Riboflavin and iron reciprocally regulate their provision genes. Thus far, results indicate that riboflavin regulates many genes that are also regulated by iron. The experiments were performed in standard T media. The recipe for this medium includes 20 μM FeCl and may be considered an iron-replete condition when compared to minimal media without added iron46,48. It is reported that in such conditions, the iron aquisition systems of V. cholerae are mainly repressed47. Thus, in the case of iron uptake genes, riboflavin seems to enhance the repression produced in high iron conditions. Along these lines, we aimed to determine the effect of riboflavin on the expression of iron regulated genes under iron-restrictive conditions. These conditions are known to induce the expression of iron uptake systems. For this, we grew V. cholerae in T media without any added iron.
| Gene ID | Gene Name | Gene Description | Fold Change (Log2) |
|--------|-----------|------------------|-------------------|
| VC0010 | ibpA      | amino acid ABC transporter periplasmic amino acid-binding portion | 1.809            |
| VC0018 | ibpA      | 16kDa heat shock protein A | -1.740           |
| VC0027 | threonine dehydratase | -1.226 | -1.191 |
| VC0028 | dihydroxy-acid dehydratase | 1.116 |
| VC0030 | ilvM      | acetolactate synthase II small subunit | 1.074 |
| VC0053 | hypothetical protein | -1.160 |
| VC0089 | hypothetical protein | -1.226 |
| VC0102 | hypothetical protein | -1.087 |
| VC0138 | hypothetical protein | -1.028 |
| VC0139 | hypothetical protein | -1.428 |
| VC0143 | hypothetical protein | -2.453 |
| VC0162 | ketol-acid reductoisomerase | -1.308 |
| VC0199 | hemolysin secretion ATP-binding protein\%2C putative | -1.428 |
| VC0200 | fhuA      | OMT ferrichrome | -4.538 |
| VC0201 | fhuC      | IMT ferrichrome | -1.349 |
| VC0202 | iron(III) ABC transporter\%2C periplasmic iron-compound-binding protein | -1.264 |
| VC0211 | prrE      | orotate phosphoribosyltransferase | 1.218 |
| VC0216 | methyl-accepting chemotaxis protein | 1.316 |
| VC0301 | hypothetical protein | -1.108 |
| VC0364 | bfd       | bacterioferritin-associated ferredoxin | -1.459 |
| VC0365 | tfr       | bacterioferritin | -1.124 |
| VC0366 | rpsF      | ribosomal protein S6 | 1.152 |
| VC0367 | rpsR      | primosomal replication protein N | 1.136 |
| VC0368 | rpsR      | ribosomal protein S18 | 1.099 |
| VC0373 | hypothetical protein | 1.060 |
| VC0382 | thioredoxin | 1.198 |
| VC0384 | sulfite reductase (NADPH) flavoprotein alpha-component | 1.078 |
| VC0385 | hypothetical protein | 1.170 |
| VC0386 | carbon storage regulator | 1.107 |
| VC0387 | hypothetical protein | 1.114 |
| VC0388 | extracellular solute-binding protein putative | 1.067 |
| VC0488 | hypothetical protein | 1.026 |
| VC0491 | hypothetical protein | 1.273 |
| VC0503 | conserved hypothetical protein | 1.050 |
| VC0515 | conserved hypothetical protein | 1.158 |
| VC0546 | hypothetical protein | 1.215 |
| VC0548 | cbrA      | carbon storage regulator | -1.264 |
| VC0549 | hypothetical protein | -1.109 |
| VC0550 | oxaloacetate decarboxylase alpha subunit | 1.054 |
| VC0589 | ABC transporter ATP-binding protein | 1.010 |
| VC0607 | pseudogene | 1.102 |
| VC0608 | ibpA      | Iron(III) ABC transporter | -1.439 |
| VC0625 | hypothetical protein | -1.140 |
| VC0633 | ompU      | outer membrane protein OmpU | 1.362 |
| VC0651 | conserved hypothetical protein | -1.261 |
| VC0652 | protease putative | -1.750 |
| VC0654 | conserved hypothetical protein | -2.054 |
| VC0655 | acetyltransferase putative | -1.471 |
| VC0706 | sigma-S4 modulation protein putative | -1.182 |
| VC0707 | hypothetical protein | -1.297 |
| VC0708 | conserved hypothetical protein | -1.043 |
| VC0711 | clpB      | clpB protein | -1.124 |
| VC0734 | malate synthase A | -2.092 |
| VC0735 | hypothetical protein | -1.022 |
| VC0736 | isocitrate lyase | 3.115 |
| VC0748 | aminotransferase NifS class V | 3.069 |
| VC0748 | hypothetical protein | 1.788 |
| VC0748 | aminotransferase NifS class V | -1.034 |

Continued
| Gene ID | Gene Name       | Gene Description                                       | Fold Change (Log2) |
|---------|-----------------|--------------------------------------------------------|--------------------|
| VC0749  | NifU-related protein |                                                        | −1.254             |
| VC0750  | hesB            | hesB family protein                                     | −1.166             |
| VC0753  | ferredoxin      |                                                        | −1.008             |
| VC0754  | conserving hypothetical protein |                                                | −1.079             |
| VC0765  | conserving hypothetical protein |                                            | −1.475             |
| VC0771  | vibB            | vibriobactin-specific isochorismatase                   | −1.315             |
| VC0824  | tpx             | tagD protein                                            | 1.636              |
| VC0855  | dnaK            | dnaK protein                                            | −1.560             |
| VC0856  | dnaI            | dnaI protein                                            | −1.504             |
| VC0863  | conserving hypothetical protein |                                        | 1.115              |
| VC0878  | rpmE2           | ribosomal protein L31P family                           | −1.276             |
| VC0879  | rpmI            | ribosomal protein L36 putative                          | −1.121             |
| VC0895  | hypothetical protein |                                            | −1.190             |
| VC0905  | metQ            | D-methionine transport system substrate-binding protein | 1.230              |
| VC1049  | aphB            | transcriptional regulator LysR family                   | −1.111             |
| VC1075  | conserving hypothetical protein |                                        | −1.086             |
| VC1077  | hypothetical protein |                                            | −1.136             |
| VC1091  | oligopeptide ABC transporter periplasmic oligopeptide-binding protein | 2.133             |
| VC1114  | bioC            | biotin synthesis protein BioC                           | −1.556             |
| VC1115  | bioD            | dethiobiotin synthetase                                 | −1.750             |
| VC1117  | htxX            | heat shock protein HtxX                                  | −1.069             |
| VC1139  | phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphohydrolase | 1.072             |
| VC1147  | iron-containing alcohol dehydrogenase                   | 1.203              |
| VC1157  | response regulator                                       | 1.183              |
| VC1169  | trpA            | tryptophan synthase alpha subunit                        | 1.028              |
| VC1175  | hypothetical protein                                     | 1.153              |
| VC1206  | gtrR            | histidine utilization repressor                         | 1.631◊             |
| VC1217  | conserving hypothetical protein                           | −1.070             |
| VC1224  | hypothetical protein                                     | −1.101             |
| VC1286  | thiopurine methyltransferase                               | −1.344             |
| VC1227  | hypothetical protein                                     | −1.250             |
| VC1235  | sodium/dicarboxylate symporter                            | 1.325              |
| VC1248  | methyl-accepting chemotaxis protein                       | 1.355              |
| VC1264  | irpA            | fuction unknown, COG3487                                | −1.406◊             |
| VC1266  | hypothetical periplasmic lipoprotein, like to irpA, COG3488 | −1.086             |
| VC1278  | transcriptional regulator MarR family                     | 2.100              |
| VC1279  | transporter BCCT family                                   | 4.896              |
| VC1280  | hypothetical protein                                      | 1.144              |
| VC1314  | transporter putative                                      | 1.487              |
| VC1315  | sensor histidine kinase                                   | 1.179              |
| VC1324  | hypothetical protein                                      | 1.104              |
| VC1343  | peptidase M20A family                                     | −1.335             |
| VC1373  | DnaK-related protein                                      | −1.039             |
| VC1386  | chaperone                                                 | −1.079             |
| VC1414  | taq             | thermostable carboxypeptidase 1                          | 1.145              |
| VC1489  | hypothetical protein                                      | −1.609             |
| VC1510  | hypothetical protein                                      | −1.454             |
| VC1511  | formate dehydrogenase cytochrome B556 subunit             | 1.521              |
| VC1512  | formate dehydrogenase iron-sulfur subunit                 | 1.604              |
| VC1513  | pseudogene                                                | 2.147              |
| VC1514  | hypothetical protein                                      | 2.306              |
| VC1515  | chaperone formate dehydrogenase-specific putative         | 2.761              |
| VC1516  | iron-sulfur cluster-binding protein                        | 2.750              |
| VC1517  | hypothetical protein                                      | 1.484              |
| VC1518  | hypothetical protein                                      | 1.735              |
| VC1523  | conserving hypothetical protein                           | 1.852              |

Continued
| Gene ID | Gene Name | Gene Description                                                                 | Fold Change (Log2) |       |       |
|---------|-----------|----------------------------------------------------------------------------------|-------------------|-------|-------|
| VC1524  | ABC transporter permease protein                                                 |                    |       |       |
| VC1547  | exbB      | exbB related linked to tonB2                                                     | −1.006            |       |       |
| VC1548  |           | hypothetical, linked to tonB2                                                   | −1.083            |       |       |
| VC1551  |           | glycerol-3-phosphate ABC transporter permease protein                            | −1.055            |       |       |
| VC1559  |           | hypothetical protein                                                             | −1.371            |       |       |
| VC1560  |           | catalase/peroxidase                                                              | −1.450            |       |       |
| VC1563  |           | conserved hypothetical protein                                                   | 1.068             |       |       |
| VC1564  |           | hypothetical protein                                                             | 1.155             |       |       |
| VC1565  | tolC      | outer membrane protein TolC putative                                            | 1.202             |       |       |
| VC1581  | msoL      | NADH dehydrogenase putative                                                      | 2.736             |       |       |
| VC1582  |           | conserved hypothetical protein                                                   | 1.969             |       |       |
| VC1588  |           | hypothetical protein                                                             | −1.127            |       |       |
| VC1704  | metE      | 5-methyltetrahydropteroylglutamate–homocysteine methyltransferase                | 3.435             |       |       |
| VC1719  | torR      | DNA-binding response regulator TorR                                              | −1.718            |       |       |
| VC1731  |           | conserved hypothetical protein                                                   | −1.084            |       |       |
| VC1808  |           | hypothetical protein                                                             | 1.396             |       |       |
| VC1823  | fruA      | PTS system fructose-specific IIB component                                        | 1.385             |       |       |
| VC1865  |           | hypothetical protein                                                             | −1.376            |       |       |
| VC1871  |           | conserved hypothetical protein                                                   | −1.034            |       |       |
| VC1949  | pvcA      | pvcA protein                                                                     | 1.021             |       |       |
| VC1950  |           | biotin sulfoxide reductase                                                       | −1.785            |       |       |
| VC1951  | yecK      | cytochrome c-type protein YecK                                                   | −1.854            |       |       |
| VC1956  | mltB      | lytic murein transglycosylase putative                                            | −1.242            |       |       |
| VC1957  |           | conserved hypothetical protein                                                   | −1.314            |       |       |
| VC1958  |           | hypothetical protein                                                             | −1.144            |       |       |
| VC1962  |           | lipoprotein                                                                      | −1.070            | −1.215|       |
| VC1971  | menE      | o-succinylbenzoic acid–CoA ligase                                                | 1.181             |       |       |
| VC1972  | menA      | o-succinylbenzoate–CoA synthase                                                  | −1.587            |       |       |
| VC1973  | menB      | naphthoate synthase                                                              | −2.445            |       |       |
| VC1974  | menH      | conserved hypothetical protein                                                   | −2.129            |       |       |
| VC2001  | yeaD      | conserved hypothetical protein                                                   | 1.019             |       |       |
| VC2007  |           | transcriptional regulator ROK family                                             | 1.118             |       |       |
| VC2013  | ptsG      | PTS system glucose-specific IIBC component                                        | 1.038             |       |       |
| VC2036  | asd       | aspartate-semialdehyde dehydrogenase                                             | 1.069             |       |       |
| VC2045  | sobA      | superoxide dismutase Fe                                                          | −1.249            | −1.328| −1.574|
| VC2051  | icmG      | cytochrome c biogenesis protein                                                  | −1.131            |       |       |
| VC2052  | icmF      | cytochrome c-type biogenesis protein CcmF                                          | −1.306            |       |       |
| VC2053  | icmE      | cytochrome c-type biogenesis protein CcmE                                          | −1.828            |       |       |
| VC2054  | icmD      | heme exporter protein D                                                          | −1.708            |       |       |
| VC2055  | icmC      | heme exporter protein C                                                           | −1.490            |       |       |
| VC2076  | feoC      | putative ferrous iron transport protein C                                         | −1.421            |       |       |
| VC2077  | feoB      | ferrous iron transport protein B                                                  | −1.489            |       |       |
| VC2078  | feoA      | ferrous iron transport protein A                                                  | −1.172            |       |       |
| VC2149  |           | hypothetical protein                                                             | −1.007            |       |       |
| VC2174  | ushA      | UDP-sugar hydrolase                                                              | 1.318             |       |       |
| VC2221  |           | hypothetical protein                                                             | 1.443             |       |       |
| VC2271  | ribD      | riboflavin-specific deaminase                                                     | −1.385            |       |       |
| VC2272  | mdrR      | conserved hypothetical protein                                                   | 1.858             |       |       |
| VC2323  |           | conserved hypothetical protein                                                   | −1.227            |       |       |
| VC2352  |           | NapC family protein                                                              | 1.381             | 1.164 |       |
| VC2357  |           | hypothetical protein                                                             | 1.362             |       |       |
| VC2361  | gvcA      | formate acetyl transferase-related protein                                        | 1.163             | 1.092 |       |
| VC2363  | thrB      | homoserine kinase                                                                | 1.009             |       |       |
| VC2364  | thrA      | aspartokinase I/homoserine dehydrogenase threonine-sensitive                      | 1.391             |       |       |
| VC2367  |           | hypothetical protein                                                             | −1.123            |       |       |
| VC2368  | arcA      | aerobic respiration control protein FexA                                          | −1.409            |       |       |
| VC2371  |           | conserved hypothetical protein                                                   | −1.303            |       |       |

Continued
| Gene ID | Gene Name | Gene Description | Fold Change (Log2) |
|---------|-----------|------------------|-------------------|
| VC2372  | hypothetical protein | WT → WT RF+ | -1.395 |
| VC2373  | glutamate synthase large subunit | WT RF+ → ΔribD RF+ | 1.126 |
| VC2417  | single-stranded-DNA-specific exonuclease RecJ | WT RF+ → ΔribN RF+ | -1.098 |
| VC2418  | thioldisulfide interchange protein DsbC | ΔribD RF+ | -1.200 |
| VC2419  | integrase/recombinase XerD | ΔribN RF+ | -1.173 |
| VC2466  | sigma-E factor negative regulatory protein RseA | ΔribD RF+ | -1.130 |
| VC2486  | hypothetical protein | ΔribN RF+ | -1.035 |
| VC2490  | 2-isopropylmalate synthase | ΔribD RF+ | 1.135 |
| VC2508  | ornithine carbamoyltransferase | ΔribN RF+ | -1.487 |
| VC2509  | hypothetical protein | ΔribD RF+ | -1.032 |
| VC2510  | aspartate carbamoyltransferase catalytic subunit | ΔribN RF+ | 1.319 |
| VC2524  | single-stranded-DNA-specific exonuclease RecJ | ΔribD RF+ | -1.199 |
| VC2534  | conserved hypothetical protein | ΔribN RF+ | 1.076 |
| VC2544  | fructose-16-bisphosphatase | ΔribD RF+ | 1.614 |
| VC2550  | sulfate adenylate transferase subunit 2 | ΔribN RF+ | 1.463 |
| VC2562  | 2′3′-cyclic-nucleotide 2′-phosphodiesterase | ΔribD RF+ | 1.206 |
| VC2565  | elaA protein | ΔribN RF+ | -1.108 |
| VC2568  | peptidyl-prolyl cis-trans isomerase FKBP-type | ΔribD RF+ | 1.042 |
| VC2637  | peroxiredoxin family protein/glutaredoxin | ΔribN RF+ | -1.378 |
| VC2644  | N-acetyl-gamma-glutamyl-phosphate reductase | ΔribD RF+ | -1.289 |
| VC2645  | acetylornithine deacetylase | ΔribN RF+ | -1.080 |
| VC2656  | fumarate reductase flavoprotein subunit | ΔribD RF+ | 1.103 |
| VC2657  | fumarate reductase iron-sulfur protein | ΔribN RF+ | 1.360 |
| VC2658  | fumarate reductase 15 kDa hydrophobic protein | ΔribD RF+ | 1.708 |
| VC2659  | fumarate reductase 13 kDa hydrophobic protein | ΔribN RF+ | 1.699 |
| VC2674  | protease HslVU ATPase subunit HslU | ΔribD RF+ | -1.330 |
| VC2675  | protease HslVU subunit HslV | ΔribN RF+ | -1.258 |
| VC2689  | 6-phosphofructokinase isozyme I | ΔribD RF+ | -1.076 |
| VC2699  | C4-dicarboxylate transporter anaerobic | ΔribN RF+ | 1.040 |
| VC2706  | conserved hypothetical protein | ΔribD RF+ | 1.577 |
| VC2720  | conserved hypothetical protein | ΔribN RF+ | 1.529 |
| VC2728  | phosphoenolpyruvate carboxykinase | ΔribD RF+ | -1.197 |
| VC2738  | phosphoenolpyruvate carboxykinase | ΔribN RF+ | -1.084 |
| VCA0011 | maltT regulatory protein | ΔribD RF+ | 1.086 |
| VCA0013 | maltP maltoextrin phosphorylase | ΔribN RF+ | 1.713 |
| VCA0014 | maltQ maltose phosphorylase | ΔribD RF+ | 1.698 |
| VCA0015 | hypothetical protein | ΔribN RF+ | 1.630 |
| VCA0016 | 14-alpha-glucan branching enzyme | ΔribD RF+ | 1.642 |
| VCA0025 | transporters NaC family | ΔribN RF+ | 1.244 |
| VCA0053 | purine nucleoside phosphorylase | ΔribD RF+ | 1.062 |
| VCA0087 | hypothetical protein | ΔribN RF+ | -1.004 |
| VCA0129 | hypothetical protein | ΔribD RF+ | -1.146 |
| VCA0180 | peptidase T | ΔribN RF+ | -1.236 |
| VCA0205 | C4-dicarboxylate transporter anaerobic | ΔribD RF+ | -1.364 |
| VCA0216 | hypothetical membrane, linked to VCA0215 and VCA0217 | ΔribN RF+ | 1.170 |
| VCA0231 | linked to vcaA, function unknown | ΔribD RF+ | 1.136 |
| VCA0245 | PTS system IIA component | ΔribN RF+ | 1.105 |
| VCA0246 | SpoT protein | ΔribD RF+ | 1.073 |
| VCA0268 | methyl-accepting chemotaxis protein | ΔribN RF+ | -1.056 |
| VCA0269 | decarboxylase group II | ΔribD RF+ | 1.152 |
| VCA0344 | hypothetical protein | ΔribN RF+ | 1.218 |
| VCA0511 | anaerobic ribonucleoside-triphosphate reductase | ΔribD RF+ | 1.012 |
| VCA0516 | PTS system fructose-specific IIIC component | ΔribN RF+ | 1.175 |
| VCA0517 | 1-phosphofructokinase | ΔribD RF+ | 1.948 |
| VCA0518 | PTS system fructose-specific IIA/FPR component | ΔribN RF+ | -1.919 |
| VCA0523 | aminotransferase class II | ΔribD RF+ | 1.778 |
| VCA0540 | formate transporter 1 putative | ΔribN RF+ | -1.113 |

Continued
and determined the effect of riboflavin by RT-PCR. Notably, the expression of *tonB1* and *sodA*, as well as that of the controls *ribD* and *gyrB*, remained around the same with or without riboflavin in such iron-restrictive conditions. The data are presented in Table 1, which lists the genes whose expression was affected in response to exogenous riboflavin or deletions of *ribD* or *ribN*. The genes with at least one fold change in expression and statistical significance (P < 0.05) are shown. Bold gene IDs indicate genes regulated by iron as reported in ref. 46. RF, riboflavin. ◊ Genes with expression affected by riboflavin also in the Δ*ribN* strain (comparison d in Fig. 1).

| Gene ID  | Gene Name         | Gene Description                                      | Fold Change (Log2)                  |
|---------|-------------------|-------------------------------------------------------|-------------------------------------|
| VCA0550 | hypothetical protein | -1.096                                                |
| VCA0551 | hypothetical protein | -1.394                                                |
| VCA0592 | *malG*            | MutT/nudix family protein                             | 1.661                               |
| VCA0621 | *trxB*            | shioredoxin 2                                          | -2.122                              |
| VCA0819 | *groES*           | chaperonin 10Kd subunit                                | -1.222                              |
| VCA0820 | *groEL*           | chaperonin 60Kd subunit                                | -1.119                              |
| VCA0823 | *ectC*            | ectoine synthase                                        | -1.116                              |
| VCA0824 | *ectB*            | diaminobutyrate–pyruvate aminotransferase             | 1.820                               |
| VCA0835 | *ectA*            | L-24-diaminobutyric acid acetyltransferase            | 1.691                               |
| VCA0867 | *ompW*            | outer membrane protein OmpW                            | 1.639                               |
| VCA0897 | *devB*            | devB protein                                           | -1.127                              |
| VCA0908 | *futX*            | unknown, linked to *futZ*                              | -1.626                              |
| VCA0909 | *futW*            | unknown, linked to *futZ*                              | -1.091                              |
| VCA0910 | *tonB1*           | TonB1 protein                                          | -3.208◊                             |
| VCA0911 | *exbB1*           | TonB system transport protein ExbB1                    | -3.328◊                             |
| VCA0912 | *exbD1*           | TonB system transport protein ExbD1                    | -2.996◊                             |
| VCA0913 | *futB1*           | hemin ABC transporter%2C periplasmic hemin-binding protein HutB | -2.383◊                             |
| VCA0914 | *futB2*           | hemin ABC transporter%2C permease protein%2C putative | -1.868◊                             |
| VCA0944 | *malF*            | maltose ABC transporter permease protein               | 1.853                               |
| VCA0945 | *malE*            | maltose ABC transporter periplasmic maltose-binding protein | 1.986                               |
| VCA0954 | *cheV*            | chemotaxis protein CheV putative                       | -1.029                              |
| VCA0965 | *GGDEF* family protein | -1.396                                                |
| VCA0966 | hypothetical protein | -1.335                                                |
| VCA0967 | hypothetical protein | -1.507                                                |
| VCA0968 | hypothetical protein | -1.527                                                |
| VCA0979 | methyl-accepting chemotaxis protein | 1.006                                                |
| VCA0981 | hypothetical protein | 1.008                                                |
| VCA0985 | oxidoreductase/iron-sulfur cluster-binding protein | -1.381                                                |
| VCA0988 | methyl-accepting chemotaxis protein | -1.119                                                |
| VCA1006 | organic hydroperoxide resistance protein putative | -1.130                                                |
| VCA1007 | hypothetical protein | -1.064                                                |
| VCA1009 | hypothetical protein | -1.260                                                |
| VCA1010 | conserved hypothetical protein | -3.403                                                |
| VCA1014 | hypothetical protein | 1.080                                                |
| VCA1027 | *malM*            | maltose operon periplasmic protein putative            | 1.060                               |
| VCA1028 | *lamB*            | maltoporin                                             | 2.485                               |
| VCA1060 | *ribB*            | 34-dihydroxy-2-butanone 4-phosphate synthase           | -3.58                               |
| VCA1063 | *speC*            | ornithine decarboxylase inducible                      | 1.067                               |
| VCA1064 | hypothetical protein | 1.366                                                |
| VCA1069 | methyl-accepting chemotaxis protein | 1.383                                                |
| VCA1099 | ABC transporter permease protein | 1.081                                                |

Table 1. List of genes whose expression is affected in response to exogenous riboflavin or deletions of *ribD* or *ribN*. The genes with at least one fold change in expression and statistical significance (P < 0.05) are shown. Bold gene IDs indicates genes regulated by iron as reported in ref. 46. RF, riboflavin. ◊ Genes with expression affected by riboflavin also in the Δ*ribN* strain (comparison d in Fig. 1).
conditions (Fig. 3b). This may indicate that the negative modulatory effect of riboflavin is surpassed under the highly inducing conditions triggered by iron restriction. Strikingly, the expression of ribN was diminished by half by riboflavin in this condition, in contrast with the results obtained in iron repleted media, were riboflavin has no effect. This suggests that riboflavin modulates the expression of ribN but only during iron restriction. To corroborate that tonB1 is being induced in response to iron restriction in our experiments, we compared the expression of this gene when growing without iron versus standard T media. We assessed this in media with and without riboflavin. Irrespective of the presence of riboflavin, the expression of tonB1 is highly increased (more than 10-fold) in low iron media, although the induction effect was higher without riboflavin (Fig. 4). Remarkably, although iron has no effect in the expression of ribD when riboflavin is present, in riboflavin-free media this gene was highly repressed in iron-restrictive conditions. The same occurred for the ribN gene. Nonetheless, a different effect applied for the ribB gene. In the absence of riboflavin, iron had no effect, while iron restriction increased the expression of this gene 3-fold in the presence of exogenous riboflavin. Collectively, results indicate that riboflavin and iron interplay affects the expression of iron and riboflavin provision genes in a gene-specific manner.

Genes affected both in the ribD and ribN mutants. We have recently hypothesized that riboflavin transport, instead of merely replacing for the RBP, may afford riboflavin for specific physiological functions. The results of the transcriptomics comparisons performed here show that 34 of the genes whose expression is affected by the elimination of riboflavin biosynthesis are also affected by the elimination of the RibN importer (Table S2). This clearly suggests that functional overlap between riboflavin biosynthesis and internalization occurs. Five of these genes belong to the iron regulon. These are the VC1515, VC1514, VCA0908 and VCA0907 ORFs. VC1514 encodes a protein of unknown function putatively encoded in the same operon as VC1515 and these genes belong to the iron regulon. These are the VC1515, VCA1516, VC1514, VCA0908 and VCA0907 ORFs. VC2045, coding for a superoxide dismutase and VC0753, encoding a ferredoxin, were also included in this subgroup. An analysis of enrichment of GO terms of biological processes associated to this set rendered no significant overrepresentation.

Most of the genes within this group followed the same pattern of regulation irrespective of whether the elimination was on ribD or ribN. However, three genes were differentially affected by the mutations. VCA0517 and VCA0518, encoded in an operon of a phosphotransferase system for fructose transport, were upregulated roughly 3.5 times in the ΔribD strain but downregulated 3.7 and 2.1 times respectively in the ΔribN strain. Likewise, the gene for OmpU, one of the major porins in this species, was upregulated 2.5 times in the ΔribD strain but downregulated 2.3 times in the ΔribN strain. These represent an intriguing group, as the transcription of these genes seems to be reciprocally regulated by riboflavin biosynthesis and riboflavin uptake through RibN.

Genes specifically affected in response to ribD elimination. The transcription of 139 genes was significantly affected by the elimination of ribD but not by the elimination of ribN. This comprised
the largest set of genes defined by any of our comparisons (Table S3). The VC1279 ORF, encoding a putative member of the Betaine/Carnitine/Choline Transporters (BCCT) family, was the highest regulated gene, being induced 29.6 times in response to ribD elimination. Also atop of the list were the genes VC1704, encoding a 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase required for cystein and methionin biosynthesis and VC0734, coding for a malate synthase. The list included some iron regulated genes, such as exbD1, related to the TonB1 system and many genes related to the PTS system for fructose and glucose uptake.

In the GO terms enrichment analysis for this subset two terms were overrepresented: protein folding and oxidation-reductions process.

Genes specifically affected in response to ribN mutation. We identified genes whose expression changed in the ribN mutant but not in the ribD mutant. 73 genes corresponded to this pattern of regulation (Table S4). In this list two genes involved in heme export, VC2054 and VC2055 were downregulated, which could also be related to the riboflavin-iron metabolic interplay highlighted across the transcriptomics results. Notably, many ribosome assembly genes also appeared in this set of genes. Among the most regulated genes are menB and menF, both involved in menaquinone (vitamin K) biosynthesis, bioD, required for biotin biosynthesis, VC1950, which encodes a biotin sulfoxide reductase that allows biotin salvatage and yecK and ccmE, two genes involved in cytochrome c-type biogenesis. The list included other genes also involved in biotin biosynthesis and cytochrome c-type biogenesis such as bioC and ccmF, respectively. All of these proteins were downregulated in the ∆ribN strain. Thus it seems that the lack of transport of riboflavin through RibN downregulates menaquinone, biotin and cytochrome c biosynthesis. Accordingly, the GO term cytochrome complex assembly was significantly enriched in this subset of genes. Menaquinone and cytochromes participate in electron transfer chains. Notably, the ArcA response regulator that control aerobic respiration was also downregulated. Thus, these results suggest that internalized riboflavin may be involved in respiratory chain processes in V. cholerae.

Figure 3. Effect of riboflavin on the expression of genes under different iron conditions. Relative expression of the indicated genes with and without riboflavin in T media (a) or T without added iron (b), as determined by RT-PCR. WT V. cholerae was grown until medium exponential phase at 37 °C, RNA extracted and RT-PCR assayed as described in Materials and Methods. Results shown are the average and standard deviation of three independent experiments.

Figure 4. Effect of iron in the expression of genes under different riboflavin conditions. The relative expression of the genes in T without iron versus complete T, with and without riboflavin as indicated. Growth conditions were similar as those described in Fig. 3. Results shown are the average and standard deviation of three independent experiments.
Discussion

This study assessed the effect of riboflavin on gene expression in *V. cholerae*. Many of the genes affected by riboflavin are known to be regulated by the iron levels in the media. The determination of the expression of genes by RT-PCR added *sodA* to the list of genes downregulated by riboflavin. Thus, our transcriptomics analysis may be underestimating the number of genes regulated by riboflavin and the overlapping of iron and riboflavin regulons could be more extensive. Genes belonging to five out of six iron acquisition systems present in this species were negatively modulated by the presence of riboflavin in T media. These systems are known to be repressed in iron-rich environments and induced under iron deprivation. When assessed its effect in low iron, riboflavin no longer repressed iron regulated genes. Thus, riboflavin seems to accent a high iron condition in the expression of iron uptake systems and possibly other iron regulated genes, while having no repressive effect during iron starvation. Contrarily, the riboflavin transport gene *ribN*, which is expressed independently of riboflavin in T media with iron, was negatively modulated by exogenous riboflavin during iron starvation. Reciprocally, iron repressed the expression of *ribD* and *ribN* but only in the absence of exogenous riboflavin, while inducing the expression of *ribB* in the presence of riboflavin. These three genes are encoded in separated transcriptional elements. Noteworthy, *ribB* is the only one conserving a FMN riboswitch, likely rendering the expression of this gene coupled to the levels of intracellular riboflavin. This may be responsible for its differential regulation. The increase in expression of *ribB* in low iron may reflex a decrease in intracellular riboflavin levels. This may seem paradoxical given that this effect only occurs in the presence of extracellular riboflavin. However, we have previously observed an increase in *ribB* transcription in the presence of riboflavin in a *ribN* mutant, and such result replicated in this transcriptomics analysis. This suggested that the presence of extracellular riboflavin increases intracellular riboflavin requirements. Thus, this increase may be exacerbated in low iron conditions, which may explain this result. In general, the expression of iron regulated genes was found to be modulated by the presence of both iron and riboflavin in the media in a coordinated fashion. At least in the case of riboflavin provision genes, this regulation is gene-specific. Altogether, this may reflex a paramount regulatory crosstalk between the two most important redox cofactors in nature. The iron-riboflavin interregulatory effect may be common also in other bacteria. RBP genes have been found upregulated under iron-deficiency conditions by high throughput approaches in different bacteria such as *Caulobacter crescentus*<sup>44</sup>, *Methylocystis*<sup>45</sup> and *Clostidium acetobutylicum*<sup>46</sup>. The physiological relevance of this is unknown. One probable explanation is that in these species the lack of iron could be compensated by enhancing the biosynthesis of riboflavin, another important redox cofactor. Indeed, flavodoxins seem to substitute for ferredoxins in electron transfer reactions under iron starving conditions in different organisms across kingdoms<sup>47–46</sup>. Nonetheless in *V. vulnificus*, a bacterial species philogenetically related to *V. cholerae*, the RBP genes are downregulated under iron restriction<sup>61</sup>, which is a similar effect to what we observed in this study for *ribD* and *ribN*. Our work provided evidence of the reciprocal phenomenon for the first time, in which the availability of riboflavin alters iron metabolism in bacteria. Altogether, the overlay between riboflavin and iron regulons suggests the existence of a network interconnecting riboflavin and iron homeostasis and probably a common regulatory mechanism. This seems an important feature that grants further study.

The way riboflavin biosynthesis and uptake correlate to fulfill the flavin needs in riboflavin opportunistic species is still unclear. Nonetheless, some studies shed light into the role of riboflavin transporters in bacterial physiology. The RibM riboflavin importer is able to provide flavins to a RBP-deficient mutant of *Corynebacterium glutanicum* when growing with extracellular riboflavin, albeit the levels of the intracellular riboflavin pools are lower than those in the WT strain<sup>52</sup>. In *Staphylococcus aureus*, the Energy coupling factor (ECF)-RibU riboflavin uptake system is able to fully substitute for the RBP during *in vitro* growth with riboflavin traces and also during mouse infection<sup>63</sup>. Overexpression of RibU, the substrate-binding component of this system, helps overcome heat stress in *Lactococcus lactis*. The RfuABCD riboflavin uptake system in *Borrelia burgdorferi* is required to set an efficient oxidative stress response and for colonization in the murine model<sup>66</sup>. In the case of RibN, it is required for full colonization of pea plant roots at early stages by the riboflavin prototroph *Rhizobium leguminosarum*. In *V. cholerae*, riboflavin biosynthesis is sufficient to grow in river water but RibN provides a competitive advantage<sup>45</sup>. Here, transcriptome comparisons suggest that riboflavin biosynthesis and uptake have common and specific effects in gene transcription, which may be related to functions performed by these two riboflavin provision pathways. Remarkably, GO functional terms were distinctively defined in the subsets affected by each deletion. While *protein folding* and *oxidation-reductions process* were enriched in the genes specifically affected by the lack of riboflavin biosynthesis, *cytochrome complex assembly* was enriched in the set of genes pointedly affected by the *ribN* mutation. Other genes involved in electron transport chain were also affected in the Δ*ribN* specific set. Hence, this study may serve as a start point to characterize cellular functions requiring exogenous riboflavin in this species. Notably, the number of genes affected by the elimination of riboflavin biosynthesis was significantly higher than those affected by the presence of external riboflavin or abrogation of RibN. This may pose that biosynthesized riboflavin is engaged in more physiological functions than exogenous riboflavin. The fact that extracellular riboflavin downregulates the monocistronically encoded *ribB* but does not affect the expression of the main RBP operon on which other *ribB* homolog may be encoded also supports this view<sup>49</sup>. This effect could allow to retain the capacity to fully biosynthesize riboflavin in the presence of exogenous riboflavin. Importantly, the elimination of RibN does not necessarily abolish riboflavin uptake, as the presence of additional riboflavin transport systems has not been experimentally determined in this strain. This could be accomplished by the determination of the levels of riboflavin needed to support growth in a double Δ*ribD*/Δ*ribN* strain. However, our attempts to obtain such strain have failed even in the presence of high riboflavin concentrations in the media. Nonetheless, the increase in expression of *ribB* induced by exogenous riboflavin in the *ribN* mutant may suggest that riboflavin is not entering the cell by a different transporter.

Collectively, this study comprises an integral analysis of the response induced by availability of riboflavin in *V. cholerae* on what constitutes, to the best of our knowledge, the first approach to a riboflavin regulon in bacteria.
45. Fuentes Flores, A., Sepúlveda Cisternas, I., Vásquez Solís de Ovando, J. I., Torres, A. & García-Angulo, V. A. Contribution of riboflavin supply pathways to Vibrio cholerae in different environments. *Pathol. Biol.* **9**, 64 (2017).

46. Mey, A. R., Wyckoff, E. E., Kanukurthy, V., Fisher, C. R. & Payne, S. M. Iron and fur regulation in *Vibrio cholerae* and the role of fur in virulence. *Infect. Immun.* **73**, 8167–8178 (2005).

47. Wyckoff, E. E., Mey, A. R. & Payne, S. M. Iron acquisition in *Vibrio cholerae*. *Biometals Int. J. Role Met. Ions Biol. Biochem. Med.* **20**, 405–416 (2007).

48. Payne, S. M., Mey, A. R. & Wyckoff, E. E. *Vibrio* Iron Transport: Evolutionary Adaptation to Life in Multiple Environments. *Microbiol. Mol. Biol. Rev.* **80**, 69–90 (2016).

49. Peng, E. D., Wyckoff, E. E., Mey, A. R., Fisher, C. R. & Payne, S. M. Nonredundant Roles of Iron Acquisition Systems in *Vibrio cholerae*. *Infect. Immun.* **84**, 511–523 (2016).

50. Anisimov, R., Brem, D., Heesemann, J. & Rakin, A. Transcriptional regulation of high pathogenicity island iron uptake genes by YbtA. *Int. J. Med. Microbiol.* **295**, 19–28 (2005).

51. Ashburner, M. et al. Gene Ontology: tool for the unification of biology. *Nat. Genet.* **25**, 25–29 (2000).

52. Craig, S. A., Carpenter, C. D., Mey, A. R., Wyckoff, E. E. & Payne, S. M. Positive regulation of the *Vibrio cholerae* porin OmpT by iron and fur. *J. Bacteriol.* **193**, 6505–6511 (2011).

53. Craig, S. A., Carpenter, C. D., Mey, A. R., Wyckoff, E. E. & Payne, S. M. Iron acquisition in *Vibrio cholerae*. *Appl. Environ. Microbiol.* **76**, 7356–7358 (2010).

54. da Silva Neto, J. F., Lourenço, R. F. & Marques, M. V. Global transcriptional response of *Caulobacter crescentus* to iron availability. *Microbiol. Mol. Biol. Rev.* **80**, 69–90 (2016).

55. Balasubramanian, R., Levinson, B. T. & Rosenzweig, A. C. Secretion of flavins by three species of methanotrophic bacteria. *Environ. Microbiol.* **11**, 1918–1929 (2009).

56. Mackey, K. R. M. et al. Divergent responses of *Atlantic coastal and oceanic Synechococcus* to iron limitation. *Proc. Natl. Acad. Sci. USA* **112**, 9944–9949 (2015).

57. Vasileva, D., Janssen, H., Hönicker, D., Ehrenreich, A. & Bahl, H. Effect of iron limitation and fur gene inactivation on the transcriptional profile of the strict anaerobe *Clostridium acetobutylicum*. *Microbiol. Res. Engl.* **158**, 1918–1929 (2012).

58. Thamer, W. et al. A two [4Fe-4S]-cluster-containing ferredoxin as an alternative electron donor for 2-hydroxyglutaryl-CoA dehydratase from *Acidaminococcus fermentans*. *Arch. Microbiol.* **179**, 197–204 (2003).

59. Zhang, Y. et al. Functional pyruvate formate lyase pathway expressed with two different electron donors in *Saccharomyces cerevisiae* at aerobic growth. *FEBS Lett.* **59**, 28024 (2015).

60. Chowdhury, N. P., Klomann, K., Seubert, A. & Buckel, W. Reduction of Flavodoxin by Electron Bifurcation and Sodium Ion-Dependent Reoxidation by NAD + Catalyzed by Ferredoxin-NAD + Reductase (Fnr). *J. Biol. Chem.* **291**, 11993–12002 (2016).

61. Fujise, D. et al. Iron and Fur in the life cycle of the zoonotic pathogen *Vibrio vulnificus*. *Environ. Microbiol.* **18**, 4005–4022 (2016).

62. Takemoto, N., Tanaka, Y., Inui, M. & Yokawa, H. The physiological role of riboflavin transporter and involvement of FMN-ribosib in its gene expression in *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* **98**, 4159–4168 (2014).

63. Wang, H. et al. Dual-Targeting Small-Molecule Inhibitors of the Staphylococcal aureus FMN Ribosib Disrupt Riboflavin Homeostasis in an Infectious Setting. *Cell Chem. Biol.* **24**, 576–588.e6 (2017).

64. Chen, J., Shen, J., Solem, C. & Jensen, P. R. Oxidative stress at high temperatures in Lactococcus lactis due to an insufficient supply of riboflavin. *Appl. Environ. Microbiol.* **79**, 6140–6147 (2013).

65. Chen, J., Shen, J., Ingvar Hellgren, L., Ruhald Jensen, P. & Solem, C. Adaptation of Lactococcus lactis to high growth temperature leads to a dramatic increase in acidification rate. *Sci. Rep.* **5**, 14199 (2015).

66. Showman, A. C., Aranjuez, G., Adams, P. P. & Jewett, M. W. Gene bbo318 is Critical for the Oxidative Stress Response and Infectivity of *Borrelia burgdorferi*. *Infect. Immun.* **84**, 3141–3151 (2016).

Acknowledgements

We thank Daniela Gutiérrez for logistic support for transcriptomics analysis. This work was funded by CONICYT-FONDECYT (Chile) Grant Number 1150818.

Author Contributions

I.S.C. performed cultures, RNA extractions, RT-PCR, conceived experiments and analyzed results. L.L.A. performed transcriptomics analysis, analyzed results and contributed to paper writing. A.F.F. discussed results and provided technical support in experiments. I.V.S.D.O. analyzed results and prepared tables. V.A.G.A. conceived the study, analyzed results and wrote the paper.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-21302-3.

Competing Interests: The authors declare no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2018