Regulation of riboflavin biosynthesis and transport genes in bacteria by transcriptional and translational attenuation

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ABSTRACT

The riboflavin biosynthesis in bacteria was analyzed using comparative analysis of genes, operons and regulatory elements. A model for regulation based on formation of alternative RNA structures involving the RFN elements is suggested. In Gram-positive bacteria including actinomycetes, Thermotoga, Thermus and Deinococcus, the riboflavin metabolism and transport genes are predicted to be regulated by transcriptional attenuation, whereas in most Gram-negative bacteria, the riboflavin biosynthesis genes seem to be regulated on the level of translation initiation. Several new candidate ribotransporters were identified (impX in flavin Desulfitobacterium halfniense and Fusobacterium nucleatum; pnuX in several actinomycetes, including some Corynebacterium species and Streptomyces coelicolor; rfnT in Rhizobiaceae). Traces of a number of likely horizontal transfer events were found: the complete riboflavin operon with the upstream regulatory element was transferred to Haemophilus influenzae and Actinobacillus pleuropneumoniae from some Gram-positive bacterium; non-regulated riboflavin operon in Pyrococcus furiousus was likely transferred from Thermotoga; and the RFN element was inserted into the riboflavin operon of Pseudomonas aeruginosa from some other Pseudomonas species, where it had regulated the ribH2 gene.

INTRODUCTION

Riboflavin (vitamin B2) is an essential component of the basic metabolism, being a precursor of coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). Many microorganisms as well as plants and fungi synthesize riboflavin, but it is not produced by vertebrates.

The best studied system of the riboflavin biosynthesis in bacteria is the rib operon of Bacillus subtilis encoding a pyrimidine deaminase/reductase, α -subunit of riboflavin synthase, GTP cyclohydrolase/3,4-dihydroxy 2-butanone 4-phosphate (3,4-DHBP) synthase, and β -subunit of riboflavin synthase (1). These enzymes form a pathway that creates one riboflavin molecule from one molecule of GTP and two molecules of ribulose 5-phosphate (Fig. 1). At the next stage, bifunctional flavokinase/FAD-synthase converts riboflavin to FMN and FAD, which serve as prosthetic groups for many oxidoreductases (1). Riboflavin operons were also studied in Bacillus amyloliquefaciens (2), Actinobacillus pleuropneumoniae (3) and Bartonella species (4). In Photobacterium phosphoreum and Photobacterium leiognathi, the riboflavin genes reside within the lux operon (5,6), whereas in Vibrio fisheri, the pyrimidine deaminase/reductase genes are convergent to the *lux* operon (7). In contrast to these genomes, the riboflavin biosynthesis genes of Escherichia coli do not form a single operon, but are scattered on the chromosome (8). The operon structures in other genomes were not studied experimentally.

The traditional gene names are different in *E.coli* and *B.subtilis* (Fig. 1). The bifunctional enzyme pyrimidine deaminase/reductase RibG and the α -subunit of riboflavin synthase RibB from *B.subtilis* have their counterparts in *E.coli* named RibD and RibE, respectively. Moreover, *E.coli* has two separate genes, *ribB* and *ribA*, that encode 3,4-DHBP synthase and GTP cyclohydrolase, respectively, whereas in *B.subtilis* these functions are encoded by one gene *ribA*. For consistency, we use the *E.coli* gene names throughout. Thus, the *B.subtilis ribG*, *ribB* and *ribA* genes are renamed here to *ribD*, *ribE*, *ribB/A*, respectively.

Little is known about the mechanisms of regulation of the bacterial riboflavin genes. Metabolic studies gave no evidence for any regulation of the riboflavin biosynthesis genes in *E.coli* (8). Based on genetic studies, the regulatory role in *B.subtilis* had been initially ascribed to the *ribC* and *ribR* loci (9,10) and

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Figure 1. The riboflavin biosynthesis pathway in bacteria. *Bacillus* gene names are underlined.

the *ribO* region located between the promoter and the coding region of the *ribGBAH* operon (11). Later it has been shown that *ribC* and *ribR* encode flavokinase/FAD-synthase and monofunctional flavokinase, respectively (12–14). The ribo-flavin production is repressed by FMN, but not riboflavin (13,15), which explains why inactivation of *ribC* and *ribR* leads to overproduction of riboflavin.

Mutations in the regulatory region *ribO* release the repression in *B.subtilis* and *B.amyloliquefaciens*, and a hypothetical trancription terminator has been observed between this region and the translation start of the first gene in the operon (2,11). A short transcript corresponding to the leader region of the *rib* operon was identified by northern hybridization analysis (16). It has been suggested that the regulation involves a termination–anti-termination mechanism (2,15). Indeed, this locus is conserved in several bacteria from diverse taxonomic groups (2), and it can fold into a conserved RNA secondary structure with a base stem and four hairpins, named the *RFN* element (17).

In addition to the riboflavin biosynthesis genes, the *RFN* element was observed upstream of *ypaA* genes in several Gram-positive genomes. The product of this gene, YpaA, has five predicted transmebrane segments, which has lead us to the prediction that it is a transporter of riboflavin or related compounds, co-regulated with other riboflavin genes (17). Both these predictions have been verified in experiments. YpaA was shown to transport flavins (18). FMN was shown in a microarray-based experiment to decrease the level of the full-length transcripts of the riboflavin operon and *ypaA*, and to cause appearance of short attenuator transcripts (15).

The current availability of many complete genomes gives an opportunity to compare genes encoding one metabolic pathway and their regulation in a variety of bacteria. The comparative analysis is a powerful approach to the prediction of the DNA and RNA regulation in bacterial genomes (19). In particular, it has been used to analyze attenuators of transcription of the aromatic amino acid operons in γ -proteobacteria (20), to predict the secondary structure of RNA (21), and to find candidate iron-responsive elements in *E.coli* (22). In such studies, analysis of complementary substitutions in aligned sequences is used to construct a single conserved structure. Another comparative technique for analysis of gene functions is based on the assumption that functionally coupled genes are often clustered on the chromosome (23). Simultaneous analysis of probable operon structures and regulatory elements is the most effective theoretical method of functional annotation when the standard homology-based methods are insufficient.

In this study we applied the comparative genomics techniques to identify the riboflavin biosynthetic genes in almost all available bacterial genomes. Analysis of the candidate *RFN* elements was used to predict the mechanism of regulation on the level of transcription in Gram-negative bacteria, and on the level of translation in most Gram-negative bacteria. Analysis of regulation and positional clustering of genes resulted in identification of a number of new riboflavin-related transporters. Finally, the evolutionary history of the riboflavin operons, involving a number of horizontal transfer events, was elucidated.

MATERIALS AND METHODS

The complete and partial sequences of eubacterial genomes were downloaded from GenBank (24). Preliminary sequence data were obtained also from the WWW sites of The Institute for Genomic Research (http://www.tigr.org), University of Oklahoma's Advanced Center for Genome Technology (http://www.genome.ou.edu), the Sanger Centre (http:// www.sanger.ac.uk), the DOE Joint Genome Institute (http:// www.jgi.doe.gov), and the ERGO Database, Integrated Genomics, Inc. (25).

The RNA-PATTERN program (Alexey G. Vitreschak, unpublished data) was used to search for *RFN* elements. The input RNA pattern described the RNA secondary structure and sequence consensus motifs. The RNA secondary structure was described as a set of the following parameters: the number of helices, lengths of helices, loop lengths, and description of topology of helix pairs. The RNA pattern of the *RFN* element was constructed using the training set of 20 *RFN* elements from our previous paper (17). Each genome was scanned with the *RFN* pattern. The RNA secondary structures of anti-terminators and anti-sequestors were predicted using Zuker's algorithm of free energy minimization (26) implemented in the Mfold program (http://bioinfo.math.rpi.edu/~mfold/rna).

The similarity search was done using BLAST (27) and GenomeExplorer (28). Transmembrane segments (TMSs) were predicted using the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html). Multiple sequence alignments were constructed using CLUSTAL X (29). Phylogenetic trees were constructed by the maximum likelihood algorithm implemented in PHYLIP (30) and plotted using the GeneMaster program (A.A.Mironov, unpublished data). Candidate operons were defined as chains of genes transcribed in the same direction such that distance between adjacent genes did not exceed 100 nt.

RESULTS

RFN elements and genes of riboflavin biosynthesis and transport

Scanning of the genomic sequences by RNA-PATTERN trained at known *RFN* elements identified 61 elements in 49 genomes. Then, a similarity search was used to identify the riboflavin biosynthesis (RB) genes. It showed that riboflavin biosynthesis is a widely distributed metabolic pathway in eubacteria. Only spirochetes, mycoplasmas and rickettsia have neither RB genes nor *RFN* elements (Table 1). At that, note that the absence of genes can be reliably claimed only for complete genomes. *RFN* elements were found only upstream of the RB and riboflavin transport genes (Table 1).

The RB genes form a single *ribDE(B/A)H* operon in all complete genomes of the *Bacillus/Clostridium* group except both *Listeria*, *Enterococcus faecalis* and *Streptococcus pyogenes*. The absence of the riboflavin biosynthetic pathway in the latter bacteria is compensated by the existence of the riboflavin transporters YpaA found in all complete genomes of this group except *Bacillus halodurans*. The *Bacillus/Clostridium* group has the most tightly regulated pathway among all considered bacteria, since all RB operons from this group, as well as the transporter genes *ypaA*, have upstream *RFN* elements.

A different structure of the RB operon was observed in actinomycetes. In Thermomonospora fusca, this operon consists of ribE, RTFU01116 (named here pnuX, see below), ribB/A and ribH. The upstream region of this operon contains a candidate RFN element. Streptomyces coelicolor has a similar organization of the riboflavin operon and RFN. The pnuX gene is homologous to the nicotinamide mononucleotide transporter pnuC from enterobacteria and encodes a protein with six predicted TMSs. Orthologs of the pnuX gene, RDI02242 and RCGL00070, were detected in two other actinomycetes, Corynebacterium diphtheriae and Coryne*bacterium glutamicum*. In these genomes *pnuX* is not clustered with RB genes, but an RFN element was found upstream of pnuX in C.glutamicum. The genome of Atopobium minutum does not contain *pnuX*; however, it has another transporter gene, ypaA, preceded by an RFN element. Notably, all four RFN elements detected in actinomycetes occur upstream of transporters: *pnuX*, or a *pnuX*-containing operon, or *ypaA*. We propose that *pnuX* encodes a new type of riboflavin transporter not homologous to *vpaA*.

Two *RFN* elements were found in *Fusobacterium nucleatum*. The first one is located upstream of the *ribHDE(B/A)* operon, whereas the second one precedes a new gene encoding a hypothetical protein with nine candidate TMSs. This gene, named *impX*, is not similar to any known protein and has only one ortholog in a Gram-positive bacterium from the *Bacillus/ Clostridium* group, *Desulfitobacterium halfniense*. This ortholog is also *RFN*-regulated. Thus, we predict that ImpX is one more new riboflavin transporter.

Genomes of all cyanobacteria and chlamydia as well as the genome of Aquifex aeolicus have a complete set of RB genes but no RFN elements. Thermotoga maritima, Chloroflexus aurantiacus, Deinococcus radiodurans and Thermus thermophilus have a single RFN element upstream of the ribDE(B/A)H operon, the structure of the operon is similar to that in B.subtilis. The only exception is T.thermophilus where ribH is a separate gene without an RFN element. Thermotoga maritima has ypaA which is not preceded by an RFN element.

Most proteobacteria have some redundancy of the RB genes due to paralogs of the *ribH*, *ribB/A* and *ribE* genes. Moreover, some genomes contain not only the fused *ribB/A* gene, but also additional single *ribB* or *ribA* genes. The genomes of all proteobacteria, except rickettsia, have several single RB genes as well as at most one probable RB operon which usually is preceded by *ybaD* and followed by *nusB* genes.

The most tightly RFN-regulated RB genes in proteobacteria are *ribB* and *ribH2*. *ribB* is always a single gene and in all cases it has an upstream RFN element. The ribH2 gene, which is paralogous to *ribH*, was found in some α -proteobacteria and Pseudomonas species. ribH2 as a single gene is always regulated by an RFN element with only one exception in Rhodopseudomonas palustris. Phylogenetic analysis of the RB protein sequences reveals two examples of possible horizontal transfer of the ribDE(B/A)H operon from the Bacillus/Clostridium group to two genomes of Pasteurellaceae, Haemophilus ducrevi and A.pleuropneumoniae (see below). In both cases the RFN element preceding the RB operon is also well conserved. In general, the RFN elements were found in the genomes of almost all proteobacteria. The exceptions are Xylella fastidiosa, both Neisseria, Caulobacter crescentus, ɛ-proteobacteria (Helicobacter pylori and Campylobacter jejuni) and some unfinished genomes from the α -proteobacteria group.

The last gene of the hypothetical RB operon *ybaD-ribDEHnusB-mlr8412* in *Mesorhizobium loti* encodes a hypothetical transmembrane protein with 11 predicted TMSs. This gene is similar to transporters from the MFS family and has orthologs with the same operon structure in two other rhizobium genomes, *Sinorhizobium meliloti* and *Agrobacterium tumefaciens*. Possibly, *mlr8412* encodes a new type of riboflavin transporter in Rhizobiaceae, and we tentatively name it *rfnT*.

Possible attenuation mechanism for the *RFN*-mediated regulation

The alignment of 61 *RFN* elements confirms a high degree of conservation of the *RFN* primary and secondary structure (Fig. 2). The improved secondary structure of the *RFN* element is shown in Figure 3. The *RFN* element consists of five conserved helices, one variable stem–loop, and one facultative additional stem–loop. The lengths of the latter two hairpins are very variable and depend on the taxonomy. The maximal observed length of additional stem–loops exceeds

Table 1. The operon structures of the fibonavin biosynthesis (KD) and transport genes in eubacte	Table 1.	The operon structur	es of the riboflavin	biosynthesis (RB)) and transport	genes in eubacter
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Genome	AB	RBS operons	Single RBS	Riboflavin			
							transporters
α-Proteobacteria							
Rhodobacter sphaeroides #	RS	ybaD-ribD/ribE2-X-ribBA-ribH-nusB					
Magnetospirillum magnetotacticum #	MMA	ybaD-ribD-ribE2-ribBA-ribH-nusB					
Rhodopseudomonas palustris #	RPA	ybaD-ribE2-ribD-ribH-nusB	ribBA			ribH2	
Mesorhizobium loti	MLO	ybaD-ribD-ribE2-ribH-nusB-rfnT	ribBA		&^	ribH2	
Sinorhizobium meliloti	SM	ybaD-ribD-ribE2/ribH-nusB-rfnT	ribBA		&^	ribH2	
Agrobacterium tumefaciens	AT	ybaD-ribD-ribE2/ribH-nusB-rfnT	ribBA		&^	ribB	
Brucella melitensis #	BME	ybaD-ribD-ribE2-ribH-nusB	ribBA		&^	ribH2	
Caulobacter crescentus #	CC	ybaD-ribH1-nusB/ribD-ribE-ribBA-ribH2					
β-Proteobacteria							
(Neisseria)	(NM, NG)	ybaD-ribD/ribA=ribBA/ribH-nusB		ribE			
(Bordetella)	(BP, BPA)	ribBA-ribH-nusB	ribD	ribE	&^	ribB	
(Burkholderia), (Ralstonia)	(BU, BPS);	ribD-ribE2=ribBA-ribH-nusB	ribA		&^	ribB	
	(REU, RSO)						
γ-Proteobacteria					^		
(Enterobacteriaceae)	(EC, TY, KP, YP)	ybaD-ribD-ribH-nusB	ribA	ribE	&	ribB	
(Pasteurellaceae)	(HI, VK, AB)	ybaD-ribD/ribH-nusB	ribA	ribE	&	ribB	
~ Haemophilus ducreyi #, Actinobacillus	DU, AO	&* ribD-ribE-ribBA-ribH					
pleuropneumoniae #		â					
Pseudomonas aeruginosa	PA	ybaD-ribD=& ribE2-ribBA-ribH-nusB	ribA		^		
Pseudomonas fluorescens #, P.syringiae #	PU, Psy	ybaD-ribD-ribE2-ribBA=ribH-nusB	ribA/ribBA		&	ribH2	
Pseudomonas putida #	Ppu	ybaD-ribD-ribE2-ribBA=ribH-nusB	ribA/ribBA	ribE	&	ribB	
Shewanella putrefaciens #	Spu	ybaD-ribD-ribE2-ribBA-ribH-nusB	ribA	ribE	&	ribB	
Vibrio cholerae	VC	ybaD-ribD-ribE2-ribBA=ribH-nusB	ribA		&	ribB	
Xylella fastidiosa	XFA	ybaD=ribD=ribE2-ribBA-ribH-nusB	ribA		^		
Acinetobacter calcoaceticus #	AC	ybaD-ribD-X-ribE2/ribBA-ribH]	ribA		&	ribB	
Buchnera sp. APS	BUC	mltA-ribH-thiL-ribD-nusB	ribA	ribE			
ε-Proteobacteria	HP, CJ	ribBA-X-ribA/ribH-nusB	ribD	ribE			^
The Bacillus/Clostridium group	BS, BA, ZC, BE;	&* ribD-ribE-ribBA-ribH					& ypaA
	SA; LLX; PN; CA, DF						
~ Bacillus halodurans	HD	&* ribD-ribE-ribBA-ribH					None
~ Bacillus amyloliquefaciens	Bam	&* ribD-ribE-ribBA-ribH					?
~ (Listeria), Streptococcus pyogenes	(LO, LI); ST	None					&* ypaA
~ Enterococcus faecalis #, Streptococcus	EF, MN	None?					& ypaA
<i>mutans</i> #							
~ Desulfitobacterium halfniense #	DHA	ribD]/[ribE-ribBA-ribH					&* impX
Actinomycetes							
(Mycobacterium)	(MT, ML)	ribE-X-ribBA-ribH	ribD				
Corynebacterium diphtheriae #	DI	ribD-ribE-ribBA-ribH					X-pnuX
Corynebacterium glutamicum #	GLU	ribD-ribE-ribBA-ribH					& pnuX
Streptomyces coelicolor #	SX	& ribE-pnuX-ribBA-ribH/ribA-ribD					
Thermomonospora fusca #	TFU	& ribE-pnuX-ribBA-ribH					<u>^</u>
Atopobium minutum #	AMI	None?					& ypaA
The Thermus/Deinococcus group		· · · · · · · · · · · · · · · · · · ·					
Deinococcus radiodurans	DR	& ribD-ribE-ribBA-ribH				_	
Thermus thermophilus #	TQ	& ribD-ribE-ribBA			ribH		
Cyanobacteria			ribBA/ribD	ribE	ribH		
Other groups of eubacteria							
Thermotoga maritima	TM	&* ribD-ribE-ribBA-ribH					ypaA
Fusobacterium nucleatum #	FN	&* ribH-ribD-ribE-ribBA					&* impX
Chloroflexus aurantiacus #	CAU	& ribD-ribE-ribBA-ribH	ribA				
Aquifex aeolicus	AA (OD OT)	ribF-ribD/ribH-nusB	ribBA	ribE			
(Chlamydia)	(QP, QT)	ybaD/ribE/ribD-ribBA=ribH					
Arcnaea	DE						
ryrococcus furiosus	РГ	ridbA-ribH-ribD-ribE					

The standard *E.coli* names of the RB genes are used throughout (see the text for explanation and Fig. 1 for the *B.subtilis* equivalents). *ribBA* denotes the fusion gene encoding the protein consisting of two domains, RibB and RibA. Genes forming one candidate operon (with spacers <100 bp) are separated by '-'. Larger spacers between genes are marked by '='. Operons from different loci, if shown in one column, are separated by slashes '/'. Non-RB genes are shown as *X*. The predicted *RFN* elements and possible terminators and sequestors are denoted by '&', '*' and ','', respectively. The contig ends are marked by square brackets.

The genome abbreviations are given in column 'AB' with unfinished genomes marked by '#'. The names of taxonomic groups given in parentheses indicate similar operon structures of the RB genes in all available genomes from the group, the exclusions are listed in the table and marked '~'. Additional genome abbreviations are: *Neisseria meningitidis* (NG), *Neisseria gonorrhoeae* (#, NG); *Bordetella pertussis* (#, BP), *Bordetella bronchiseptica* (#, BPA); *Burkholderia pseudomallei* (#, BPS); *Ralstonia eutropha* (#, REU), *Ralstonia solanacearum* (RSO); *Escherichia coli* (EC), *Salmonella typhi* (TY), *Klebsiella pneumoniae* (#, KP), *Yersinia pestis* (YP); *Haemophilus influenzae* (HI), *Pasteurella multocida* (VK), *Actinobacillus actinomycetemcomitans* (#, AB); *Helicobacter pylori* (HP), *Campylobacter jejuni* (CJ); *Bacillus subtilis* (BS), *Bacillus anthracis* (#, BA), *Bacillus cereus* (#, ZC), *Bacillus stearothermophilus* (#, BE), *Staphylococcus aureus* (SA), *Lactococcus lactis* (LLX), *Streptococcus pneumoniae* (PN), *Clostridium acetobutylicum* (CA), *Clostridium difficile* (#, DF); *Listeria monocytogenes* (LO), *Listeria innocua* (LI); *Mycobacterium tuberculosis* (MT), *Mycobacterium leprae* (ML); *Chlamydia pneumoniae* (QP), *Chlamydia trachomatis* (QT).

	1	2,		2' 3	3	Add		3'	Va	riabl	e	4	4'	5	5'	1 I'
BS	TTGTATCT	CGGGG-CAGG	TOGAAAT	CCCGACCG	CIGGT	21	AGCC	GTGAC		4	8	TGGATTCAGTTTAA-G	GAAGCCGA	CAGTGAA-AGT	TGGAT-GGGA	AGGATGAT
BA	AGCATCCT	CGGGG-TCGG	GTGAAATT	CCCAACCGO	CGGT	19	AGTC	GTGAC	8	5	8	TGGATCTAGTGAAACT	TAGGCCCGA	CAGT-AT-AGT	TGGAT-GGGA	GAAGGATATG
BE	TGCATCCT	CGGGG-CAGG	GTGAAATT	CCCGACCGG	CGGT	20	AGCC	GCGA	3	4	3	AGGATCCGGTGCGATT	COGGAGCOGA	CAGT-AT-AGT	TGGAT-GGGA	GAAGGATGCC
HD	TTTATCCT	CGGGG-CTGG	GTOGAAAT	CCCGACCGG	CGGT	19	AGTC	GTGAC	10	4 1	0	TGGACCTGCTGAAAAT	COGGACOGA	CAGTGAA-AGT	TEGAT-OGGA	GRAGGAAACG
Bam	TOTATCOTT	CGGGG-CTGG	GTGARAAT	CCCGACCCG	CGGT	23	AGCO	GTGAC	8	4	8	TGGATTCACTGAAAAA	TGAAGCCGI	CAGTGAA-AGT	TGGAT-GGGA	AGGATGAG
CA	CATCTTCT	CACCC-ATCC	TCABAT	CCCARTCCC	CCCT	2	ACCO	GCAA	3	4	3	ACATCCCCTTABACT	COCCCCCCC	CACTTAN-ACT	TCCAT-CAAA	CARCANATAC
DF	CTTAATCT	CCCCC-TACC	GTGABATT	CCCARTCO	caar	2	ACCO	GCG	7	6	7		CCARACCOCK	CACT-AA-BCT	TGGAT-OCAA	CALGATATTT
SA	TAATTCTT	CGGGG-CAGG	GTGARATT	CCCARCCCC	CAGT	6	AGCC	TGCGAC	11	3 1	11	CTGATCTAGTGAGATT	TAGAGCCGA	CAGTTAA-AGTY	TGGAT-GGGA	GAAGAATGT
LLX	ATAAATCT	CACCC-CACC	CTCTART	CCCTACCCC	Ceer	2	ACCO	GCCA	4	4	4	ATCATTCGTCARACT	COCACCOCCE	CACT-AT-ACT	TCCAT-CAAA	CAACATAATA
PN	AACTATCT	CAGGG-CAGG	GTGALLTT	CCCTACCG	Taat	2	AGCO	ACGA	3	4	9	ATCATTTCCTCALATT	CANAGCOGI	CAGT-AT-AGT	TGGAT-GAAA	GAAGATAAAA
TM	AAACGCTCT	CCCCC-CACC	CTCCART	CCCGACCCG	CGGT		AGCO	CCCAC	5	4	5	TTGACCCCCCCCCAATT	COCCCCCC	CGCTCAN-ACTY	CGGAT-GGGA	CACACCETCA
DR	GACCTOTT	CCCCC-CCCC	CCABBT	CCCCACCCC	Cach	15	ACCO	GCGAA	8	12	0	CCGATGCCGCGCAACT	CACCACCOGE	CGCTCAC-ACT	CGGBC-GAAA	CARCACCAC
TO	CACCTCCT	CGGGGG-CGGG	TOGALGT	CCCCACCGO	CGGT	3	AGCC	GCGAA	5	4	5	CCGACCCGGTGGAAT	COGGGGCCGA	CGCTGAA-AGT	CGGAT-GGGA	AGGAGGGC
AO	AATAATCT	CACCC-CACC	GTCABBTT	CCCGATCCC	Cast	2	ACTO	GCGA	7	7	7	AGGA ACCONTGAGAT	COCCTACCCA	CACT-AT-ACT	TGGAT-CCAR	CARCATCANA
DU	TTTATCT	CAGGG-CAGG	GTGARATT	CCCGATCCC	Teet	2	ACTO	GCGA	13	4 1	12	BGGAACTAGTGAAATT	TAGTACCCH	CAGT-AT-ACT	TGGAT-GGAA	CAAGAGCAGA
CAU	GAAGACCT	CGGGG-CARG	CTCALAT	COTGATCO	CGGT	20	AGCO	GCGA	3	4	3	AGGACCCG TGTGAT	COCCCCCC	CGGT-AT-AGT	CGGAT-GGGA	ACCTCCCC
FN	TAAAGTCT	CACCC-CACC	CTCARATT	CCCGACCCG	TCCT	2	ACTO	ACG	5	4	5	CATTTCCTCABATT	CCARACCCC	CAGT-AG-ACT	TGGAT-OCCA	CAACAATTAC
TEU	ACGCGTGC	CCGGG-GTCG	GTGALAGT	CCGARCCCC	CGGT	3	AGTO	GCGAC	8	5	8	TGGAACCGGTGAAACT	COGGTACCGA	CGCTGAA-AGT	CGGAT-GGGA	GTAGTACGTO
SX	-AGCGCACT	CCGGG-CTCG	TGALAGT	CCCAACCCC	CCC	3	ACTO	GCGAC	8	5	8	TTGACCAGGTGAAATT	CCTCCACCCA	CONTAN-ACT	CGGAT-GGGA	CACTGCGCG
BIT	GTGCGTCT	CAGGG-CGGG	CTCABATT	CCCCACCOC	caar	30	ACCO	GCCAGCG		137	~	GTCAGCAGA TCTGCTGAGA AG	CCAGAGCCGA	COCTTAG-ACT	COGAT-OGAL	CARCATCTOC
BDC	CTCCCTCT	CAGGG-CCGG	CCALLT	CCCCACCCC	caa	21	ACCO	CCARCO		4		CTCACCA CATCTCC TCCCATC	CCACACCCCC	COCTCAT-LOT	CCCBT-CAAA	AGATOTOC
DEU	TTACGTOT	CAGGG-CGGG	CTOCARTT	CCCCACCOG	cae	31	AGCO	GCGAGCG	7	5	7	GTCAGCAGATCTGACAGAGAG	CCACCCCCC	CONTAN-BOT	CCCAT-CAAA	CARCATOCOC
RSO	GTACGTCT	CAGGG-CGGG	GTOGAATT	CCCCACCOC	caar	21	AGCO	GCAGCG	11	3 1	11	GTCAGCAGATCAGATGAGATGAGATG	CCRGGGGCCGI	CGGTCAG-AGT	CGGAT-GGAA	GAAGATGTGC
EC	CCTTATTC	CAGGG-CGGG	CCANATT	CCCCACCGG	caa	17	AGCC	GCGAGCG	8	4	8	GACAGCAGETCCGCTGTAATT	COGGGGCCGA	CGGTTAG-AGT	CGGAT-GGGA	GAGTAACG
TY	COTTATTC	CACCC-CCCC	CCARPT	CCCCACCOC	Caar	67	ACCO	GCGAGCG	8	3	8	CTCACCACA TCCCCTCTAAT	COCCOCC	COCTTAN-ACT	CGGAT-GGGA	CACCETAACC
KP	GCTTATTC	CAGGG-CGGG	CCALLT	CCCCACCGG	CGGT	20	AGCC	GCGAGCG	8	4	8	GTCAGCAGATCCGGTGTAAT	COGGGGCCCGI	CGCTTAN-ACTY	CGGAT-GGGA	GAGAGTAACG
HT	TCCCATTC	CACCG-CACC	CTCABBT	CCCTACCCC	Teen	2	ACCO	ACCACCC	26	0 3	20	CTCACCACA TTTCCTCALAT	CCABBCCCC	CACT-AB-BCT	TCCBT-CAAB	CASTABAS
VK	GCGCATTC	CAGGG-CAGG	GTGADATT	CCCTACCG	TOOT	14	AGCO	ACCAGOG	11	9 1	11	GTCAGCAGA TTTGGTGAGA AT	CARACCOGA	CAGT-AT-AGT	TGGAT-GAAA	GAGAATAAGC
VC	CAATATTC	CAGGG-CGGG	CCANATT	CCCCACCGG	Teen	13	AGCO	ACGAGCG	5	4	5	GTCAGCAGATCTGGTGAGAA	CAGGCCGI	CGCTTAC-AGT	CGGAT-GAGA	GAGAATGACA
VP	COTTATTC	CACCO-CGCC	CTCARACT	CCCCACCOC	coor	40	ACCO	GCGAGCG	16	6 1	6	GTCAGCAGACCCGGTGTAAT	CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	COUTTAT-ACT	COGAT-OCCA	CACACTAACC
AB	GCGCATTC	CAGGG-CAGG	GTGALLGT	CCCTACCCC	TCCT	25	AGCC	ACGAGCG	16	4 2	7	GTCAGCAGA TTTGGTGCGAAT	CCARGCCCGI	CAGTGAC-AGT	TGGAT-GAAA	GAGAATAAAA
BP	GTACGTCT	CAGGG-CGGG	GTOCAATT	CCCCACCG	CGGT	18	AGCO	GCGAGCG	10	4 1	0	GTCAGCAGACCTGAGATG	CCAGGCCGA	CGCTCAT-ACT	CGGAT-GAGA	AGATGTOC
AC	ACATCOCT	CAGGG-CGGG	GCGTAATT	CCCCACCGO	CGGT	16	AGCO	GCGAGCA	10	3 1	11	CGCAGATCTGGTGTALAT	CCAGAGCCGA	CGGT-AT-AGT	CGGAT-GAAA	CARGACGACG
Spu	AACAATTC	CAGGG-CGGG	GTGAAACT	CCCCACCG	CGGT	34	AGCC	GCGAGCG	6	6	6	GTCAGCAGATCTGGTG 52 1	CCAGAGCCGA	CGGT 31 AGT	CEGAT-CEAA	GAGAATGTAA
Pou	GTCGGTCT	CAGGG-CGGG	GTGTAAGT	CCCCACCG	caar	13	AGCO	GCGAGCG	7	3	7	GTCAGCAGATCTGCAACT	CAGAGOCCA	CGGTCAT-AGT	CGGAT-GAAA	AGGCGTCA
AT	GGTTGTTC	CAGGG-CGGG	GTGCAATT	CCCCACCG	CGGT	17	AGCC	GCGAGCG	7	9	7	GTCAGCAGATCCGGTGAGAG	COGGAGCOGA	CGGT-AT-AGT	CGGAT-GGAA	GAGGACAAGG
PU	AAACGTTC	CAGGG-CGGG	GTOCAATT	CCCCACCGG	CGGT	19	AGCC	GCGAGCG	19	4 1	8	GTCAGCAGACCCCCCTCTCATT	COGGGGCCGA	CGGTCAC-AGT	CEGATEAAGA	GAGAACGGGA
Psv	TAACGTTC	CAGGG-CGGG	GTOCAACT	CCCCACCGC	CGGT	19	AGCC	GCGAGCG	15	4 1	16	GTCAGCAGACCCGGTGTGAT	CCGGGGGCCGA	CGGTCAT-AGT	CGGATGAAGA	GAGAGCGGGA
PA	TAACGTTC	CAGGG-CGGG	GTGAAAGT	CCCCACCG	CGGT	19	AGCO	GCGAGCG	14	4 1	13	GTCAGCAGACCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	COGGGGCCGA	CGGTCAT-AGT	CGGATAAAGA	GAGAACGCGA
MLO	TAAAGTTC	CAGGG-CGGG	GTGAAAGT	COCCACCO	caar	16	AGCO	GCGAGCG	8	5	8	GTCAGCAGA TCCGGTGTGATT	COGGAGCOGA	CGGTTAG-AGT	CEGAT-GAAA	GAGGACGAAA
SM	AAGCGTTC	CAGGG-CGGG	GTGAAATT	CCCCACCGO	CGGT	34	AGCC	GCGAGCG	8	3	8	GTCAGCAGATCCGGTCGAATT	COGGAGCOGA	CGGTTAT-AGT	CGGAT-GGAA	GAGAGCAAGC
BME	GCTTGTTCT	CGGGG-CGGG	GTGAAACT	CCCCACCG	CGGT	17	AGCC	GCGAGCG	10	15 1	LO	GTCAGCAGATCCGGTGAGATC	COGGAGCOGA	CGGTTAA-AGT	CGGAT-GGAA	GAGAGCGAAT
BS	ATCAATCT	CGGGG-CAGG	GTGAAATT	CCCTACCG	CGGT	18	AGCC	GCGA	5	4	5	AGGATTCGGTGAGATT	COGGAGCOGA	CAGT-AC-AGT	TGGAT-GGGA	AGATGGAG
BA	GTCTATCT	CGGGG-CAGG	GTGAAAAT	CCCGACCGO	CGGT	27	AGCC	GCGA	3	5	3	AGGATTTGGTGAT	CCAAAGCCGA	CAGT-AT-AGT	TGGAT-GGGA	GAAGATGGAG
BE	ATTCATCT	CGGGG-CAGG	GTGAAATT	CCCGACCG	CGGT	20	AGCC	GCGA	3	4	3	AGGATCCGGTGCGAGT	COGGAGCOGA	CAGT-AT-AGT	TGGAT-GGGA	GAAGATGAAG
CA	AATGATCT	CAGGG-CAGG	GTGAAATT	CCCTACCG	CGGT	2	AGCC	GCGAG	3	4	3	TATGATCCGGTTTGATT	CCGGAGCCGA	CAGT-AA-AGT	TGGAT-GAAA	GAAGATATAT
DF	GAAGATCTT	CGGGG-CAGG	GTGAAATT	CCCTACCG	CGGT	2	AGCC	GCG	6	4	6	GATTTGGTGAGATT	CCAAAGCCGA	CAGT-AA-AGT	TGGAT-GAGA	GAAGATATTT
EF	GTTCGTCT	CAGGGGCAGG	GTGTAATT	CCCGACCG	TGGT	3	AGTC	ACGAC	5	3	5	ATTGAATTGGTGTAATT	CCAATACCGA	CAGT-AT-AGT	TGGATAAA	GAAGATAGGG
LLX	AAATATCT	CAGGG-CACC	GTGTAATT	CGGGACCGG	CGGT	21	ACTO	GCGAT	4	4	4	TTGAAGCAGTGAGAA	CTGCTAGCGA	CAGT-AA-AGT	TGGAT-GGAA	GAAGATGAAC
LO	GTTCATCT	CGGGG-CAGG	GTGCAATT	CCCGACCO	TGGT	3	AGTO	CACGAT	3	10	3	TTGACTCTGGTGTAATT	CCAGGACCGA	CAGT-AT-AGT	TGGAT-GGGA	GAAGATGTTG
PN	AAGAGTCT	CAGGG-CAGG	GTGAAATT	CCCGACCG	CGGT	125	AGTO	GTG	3	4	3	GATGTGGTGAGAT	CCACAACCGA	CAGT-AT-AGT	TGGAT-GGGA	GAAGACGAAA
ST	AAGTGTCT	CAGGG-CAGG	GTGTGATT	CCCGACCG	CGGT	14	AGTC	GCG	3	4	3	GATGTGGTGTAACT	CCACAACCGA	CAGT-AT-AGT	TGGAT-GAGA	GAAGACCGGG
MN	AAGTGTCT	CAGGG-CAGG	GTGAGATT	CCCGACCO	CGGT	104	AGTO	CGCG	3	4	3	GATGTGCTGAAAT	CCACAACCGA	CAGT-AA-AGTY	TGGAT-GGGA	GAAGACTGAG
SA	ATTCATCT	CGGGG-TCGG	GTGTAATT	CCCAACCG	CAGT	6	AGCC	TGCGAC	11	3 1	11	CTGATCTAGTGAGATT	TAGAGCOGA	CAGT-AT-AGT	TGGAT-GGGA	GAAGATGGAG
AMI	TCACAGTT	CAGGG-CGGG	GTGCAATT	CCCCACTO	CGGT	14	AGCO	GCGC	5	5	5	TGATCTGGTGCAAAT	CCAGAGCCAA	CGGT-AT-AGT	CGGAT-GGAA	GAAACGGAGC
DHA	ACGAACCT	CGAGG-TAGG	GTGAAATT	CCCGACCG	CGG	20	AGCC	GCAAC	11	4 1	11	CGACTGACTTGGTGAGACT	CCAAGGCCGA	CGGT-AT-AGT	CGGAT-GGGA	GAAGGTACAA
FN	AATAATCT	CGGGG-CAGG	GTGAAATT	CCCGACCG	TGGT	2	AGTC	CACG	4	6	4	GATTTGGTGAAATT	CCAAAACCGA	CAGT-AG-AGT	TGGAT-GAGA	GAAGAAAAGA
CTIT		CACCO-COCC	COART	COCCACCOC	Coon	20	ACCO	00000000	10	4 1	0	CTCACCACA TCCCCTTAA	0000000000	COCCAR_BOT	COCAT-CCAR	CLOBRCC

Figure 2. Multiple alignment of 58 *RFN* elements from eubacteria. The first column contains the genome abbreviations (see Table 1). The riboflavin operons, single *ribH2* genes, single *ribB* genes and possible riboflavin transporters are marked in blue, red, magenta and green, respectively. The complementary stems of the RNA secondary structure are shown by arrows in the upper line. Base-paired positions are highlighted by the yellow background. Conserved positions and non-consensus nucleotides are shown in red and blue, respectively. Black indicates non-conserved positions. The lengths of additional (Add.) and variable stem–loops are given.

100 nt. The length of the variable stem–loop varies from 10 to 137 nt. All other stem–loops and internal loops in the *RFN* secondary structure are highly conserved, the only exception being the long loops in the fourth and fifth helices in *Shewanella putrefaciens*. These loops of 55 and 35 nt, respectively, can form additional stem–loops.

The *RFN* elements can be classified into two major types based on the existence of two conserved fragments, AGCG and GTCAGCA, located in the branching loops adjacent to the variable helix. The *RFN* elements occurring in all proteobacteria (excluding *H.ducreyi* and *A.pleuropneumoniae*, see below) have these conserved sequences, whereas *RFN* elements without sequences are observed in the Gram-positive bacteria and other taxonomic groups, the only exception being the *RFN* element upstream of the *pnuX* gene in *C.glutamicum*, that belongs to the proteobacterial type. Recently, it was shown that FMNs regulate expression of the RB operon and *ypaA* in *B.subtilis* (15). We propose here a possible mechanism of the FMN-mediated regulation via the *RFN* element (Fig. 4).

Downstream of all RFN elements, there are potential hairpins that are either followed by runs of thymidines (and thus are candidate terminators) or overlap the translation start region of the first gene in the operon (and thus are candidate sequestors). Moreover, all RFN elements are capable of forming alternative structures, in which the base stem of RFN interacts with the regulatory hairpin (terminator or sequestor). This leads to formation of a structure alternative to the regulatory hairpin, similar to transcriptional and translational attenuation by competing RNA structures. Thus, two different types of regulation are suggested, attenuation of transcription via an anti-termination mechanism and



Figure 3. The conserved structure of the *RFN* element. Upper case letters, invariant (absolutely conserved) positions; lower case letters, strongly conserved positions. Dashes and asterisks indicate obligatory and facultative base pairs, respectively. Degenerate positions: R = A or G; Y = C or U; K = G or U; B = not A; V = not U. N, any nucleotide; X, any nucleotide or deletion.

attenuation of translation by sequestering of the Shine– Dalgarno (SD) box.

In Gram-positive bacteria, *T.maritima* and *C.aurantiacus*, terminator-like RNA structures are located between the predicted *RFN* element and the start of translation of RB genes. We found complementary fragments of RNA sequences that partially overlap both the first helix of *RFN* and the left stem of the terminator (Figs 4A and 5A). Furthermore, these complementary fragments always form the main helix of a new, more stable alternative secondary structure with ΔG smaller than ΔG of the *RFN* element. We predict that this

structure functions as an anti-terminator, alternative to both the *RFN* element and the terminator. Thus, the *RFN* element is the predicted anti-antiterminator that in the repressing conditions of excess FMN prevents formation of the anti-terminator hairpin. Then, the terminator forms and transcription is preliminarily terminated. Without FMN, non-stabilized *RFN* is replaced by the more energy-favorable anti-termination conformation that allows the transcription read-through. In this model *RFN* acts as an anti-antiterminator.

In other cases, mostly in Gram-negative bacteria, the RNA hairpins downstream of the RFN element sequester the ribosome-binding site (the SD-box). In most cases we have found a highly conserved sequence, GCCCTGA, which overlaps the proposed sequestor hairpin and is complementary to the base stem of the RFN element (Figs 4B and 5B). These two complementary sequences always form the stem of the RNA secondary structure, anti-sequestor, which is more stable than the RFN element. The proposed mechanism of translational regulation of the RB operons is similar to the termination-anti-termination mechanism described above, but includes the SD-sequestor instead of the terminator. In the repressing conditions, RFN prevents formation of the antisequestor, and the SD-sequestor structure represses the initiation of translation. In the de-repressing conditions, RFN is replaced by the anti-sequestor that releases the SD-box and allows for initiation of translation.

We have observed two main arrangements of sequestors with respect to the SD-box and the start codon. The highly conserved sequestors in *ribB* in γ -proteobacteria overlap both the start codons and the SD-boxes, whereas in *ribB* of β -proteobacteria, sequestors overlap only the SD-boxes.

Analysis of the 5'-non-coding RNA regions of the *ypaA* genes reveals two possibilities for the regulation. In most cases the predicted terminator hairpin overlaps the SD-box of the *ypaA* gene. Therefore, this hairpin can function both as a terminator and a sequestor. The *RFN* elements of *ribD* of *T.thermophilus* and *ypaA* of *A.minutum* overlaps the SD-boxes



Figure 4. The predicted mechanism of the RFN-mediated regulation of riboflavin genes: (A) transcription attenuation; (B) translation attenuation.

A			A still som instant		
	1	RFN	Antiterminato	1'	Terminator
Bam	GACAAAAAAATATTGATTGTATCCTTCGGGGGCTGGGTG		TCTGGATGGGAGAAGGATGA	59	GTAAAGCCCCCGAATGTGTAAACATTCGGGGCTTTTTGACGCCCAAAT
BS	GGACAAATGAATAAAGATTGTATCTTCGGGGCAGGGTG		TCTGGATGGGAGAAGGATGA	59	CTAAAGCCCCGAATTTTTTATAAATTCGGGGGCTTTTTTGACGGTAAA
BA	CTATAATTTGAGCAAACAGCATCCTTC		TCTGGATGGGAGAAGGATAT	250	CCAAACCCCCAAGGATATTAAAATCCTTGGGGTTTTTTGTTTTTTTT
BE	ACATAACGATATAGTGATGCATCCTTCGGGGGCAGGGTG		TCTGGATGGGAGAAGGATGC	155	CCCGGGGGTTTCATTTTATTG
HD	AAATTGAATAATTAATTTTTTTTTTCCTTCGGGGGCTGGGTG		TCTGGATGGGAGAAGGAAAC	148	TCTCTGGGGCCTTTTTTGCGCGC
CA	TAATGGTAATTTAATAGGATGTTCTTCAGGGATGGGTG		TCTGGATGAAAGAAGAAATA	34	AATCTOCGAAGGATTACCTTTCTTTGGAGATTTTTTATTTG
DF	TAAATATAAATTTAATACTTAATCTTCGGGGTAGGGTG		TCTGGATGGAAGAAGATATT	63	CTCAGGGTTTTTTGTTTAAAAA
LLX	ACTTTAGCTACAATTGAATAAATCTTCAGGGCAGGGTG		TCTGGATGAAAGAAGATAAT	127	AAAAGACCCTGAAATTTTATTTTAGGGTCTTATTTTTATTAG
PN*	ATCATCTGTAATTGAATAACTATCTTCAGGGCAGGGTG		TCTGGATGAAAGAAGATAAA	81	TGTATGCCTTGAGTAGTCCCCTATTCAAGGTATATTTTTTGGAGG
PN*	ATCATCTGTAATTGAATAACTATCTTCAGGGCAGGGTG		TCTGGATGAAAGAAGATAAA	19	CGTGCTCTGAAATGATTACTTGTCATTTCAGAGCATTTTTGTTAATC
TM	AAAACTGAATACAAAAGAAACGCTCTCGGGGGCAGGGTG		TCCGGATGGGAGAGAGCGTG	13	GGGTCCCTTTTCTTTACA
AO	ATTTGCAACAATTTTTTTAATAATCTTCAGGGCAGGGTG		TCTGGATGGAAGAAGATGAA	33	TTTACAAGCCTTGAGATCGAAAGATTTCAAGGCTTTTTTCATCATTA
DU	AATTTTTTTAATACTATTTTAATCTTCAGGGCAGGGTG		TCTGGATGGAAGAAGAAGAAGAG	47	TGCATAAGCCTTGAGATCTTAGGATTTCAAGGCTTTTTCATTAGTTA
FN	TAATCGAATATGTAAAATAAAGTCTTCAGGGCAGGGTG		TCTGGATGGGAGAAGAATTA	18	GTTTGAGCATTTTTTTTTTATTAA
SA	TATAACAATTTCATATATAATTCTTTCGGGGGCAGGGTG		TCTGGATGGGAGAAAGAATG	74	GATGTGAGGATTTTGTTTATA
DHA	ACTCTTTTTAGATGAATACGAACCTTCGAGGTAGGGTG		TCCGGATGGGAGAAGGTACA	43	GTTTATGCCTCGAGGAACACCATTTCCTCGAGGCATTTTTGTTCTTTC
FN	GAAAAATAAATATTAAAAATAATCTTCGGGGCAGGGTG		TCTGGATGAGAGAAGAAAAG	40	AATTCGGTTTTTTATTTT
CA	AATATAAAAAAAAAAAAAGAATGATCTTCAGGGCAGGGTG		TCTGGATGAAAGAAGATATA	19	CGTTGGGGCCTTTTTAATGCT
DF	AAAATTAAAAAATCAAAGAAGATCTTCGGGGGCAGGGTG		TCTGGATGAGAGAAGATATT	45	ATAAAAACTCGAAGATAGGGTCTTCGAGTTTTTTGTTTTTCCTAA
BS	TAATTAAATTTCATATGATCAATCTTCGGGGGCAGGGTG		TCTGGATGGGAGAAGATGGA	103	AAAGAACCTTTCCGTTTTCGAGTAAGATGTGATCGAAAAGGAGAAAGGAGAATGAAGTGAAA
BA	GGGAAAATAGAATATCGGTCTATCTTCGGGGGCAGGGTG		TCTGGATGGGAGAAGATGGA	54	ATTCTCCCTTTGTGTAAAACACAAAGGGTTTTTTCGTTCTATG
BE	ATAAAAATGTATAAGCGATTCATCTTCGGGGGCAGGGTG		TCTGGATGGGAGAAGATGAA	114	GGCAGCCTTCTTCTTGTGAGGATGAATCACGAGAAGGGGAGGAGAACAAGCATG
PN	GTTTTTTGTTATGATAAAAGAGTCTTCAGGGCAGGGTG		TCTGGATGGGAGAAGACGAA	137	AACTTCTTCTGATTTTATAGAAAATTGGAGGAACCTGTTATGACA
ST	TAAATCTGCTATGCTAGAAGTGTCTTCAGGGCAGGGTG		TCTGGATGAGAGAGACCGG	130	GGAACTTCTTTCAATTTGAAAAAATTGGAGGAATTTTTTAATGTC
MN	ATTTTTTGATATGCTATAAGTGTCTTCAGGGCAGGGTG		TCTGGATGGGAGAAGACTGA	138	GGCCTTCTTTCGATTTGTAAAAATTGGAGGAATTTTTTTATGAA
SA	AAATTTAATAATGTAAAATTCATCTTCGGGGGTCGGGTG		TCTGGATGGGAGAAGATGGA	17	TCCTCCTATTCTTACGAGATGAATGGAAGGAGAAAATTGAATATG
EF	AAAAAATATAATACAAGGTTCGTCTTCAGGGGCAGGGT		GTCTGGATAAAGAAGATAGG	33	CTACTCTATTTTTCCCTGCAGAAAAATAGGGTTTTTTTGTATGA
LLX	TTTTTGTGCTATAATAAAAATATCTTCAGGGCACCGTG		TCTGGATGGAAGAAGATGAA	66	TCAACTTCCTCGAAATTTGAAGAAT-TATTTTCTCATATTTGGAGGTTTTTTTATGT
LO	ATTGTAAGAAAATATTCGTTCATCTTCGGGGGCAGGGTG		TCTGGATGGGAGAGATGTTC	3 79	ATGCACAAACTCTCCCTCAACTTTTTTAGTTGAGGTTTTTTATTTGC

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1.1		1000	Antisequestor			
	1	RFN	← _1'		SD-sequestor	
EC	AATCCGCTTATTCTCAGGGCGGGGGGGGGGGGGGGGGGG		TCCGGATGGGAGAGAGTAACG	59	TACCATGATTCTGGTAACCATAATTTTAGTGAGGTTTTTTACCATGAATCAGACGCTA	1
TY	AACCCGCTTATTCTCAGGGCGGGGGGGGGGGGGGGGGGG		TCCGGATGGGAGAGGGTAACG	61	CTGCCCTGATTCTGGTAACCATAATGTTAATGAGGTTTTTTTACCATGAATCAGACGCTA	
KP	ATCTCGCTTATTCTCAGGGCGGGGGGGGGGGGGGGGGGG		TCCGGATGGGAGAGAGTAACG	61	CTGCCCTGATTCTGGTAACCATAATTTTAATGAGGTTTTTTTACCATGAATCAGACGCTC	
HI	TTAGCTCGCATTCTCAGGGCAGGGTG		TCTGGATGAAAGAGAATAAAA	41	CAGCCCTGATTCTGGTATTTAATTGAAATCTCAAAT-TAGGAAATTACTATGAATCAGTCAATT	
VK	TATTTGCGCATTCTCAGGGCAGGGTG		TCTGGATGAAAGAGAATAAGC	76	CAGCCCTGATTCTGGTATCTAAATATCTTTATATTTCAAGGAATTTACTATGAATCAGTCTATT	YX
AB	TAGGCGCGCATTCTCAGGGCAGGGTG	;	TCTGGATGAAAGAGAATAAAA	54	CCCCCCTGATTCTGGTATAAATTCATCTTATTAAA-ARGCATTTACTATGAATCAGTCATTA	
YP	ATGGGGCTTATTCTCAGGGCGGGGGGG		TCCGGATGGGAGAGAGTAACG	194	CCGCCCTGATTCTGGTAATCCATAATTTTTTAATGAGGTTTCTTTACCATGAATCAGACGCTT	
VC	CACAACAATATTCTCAGGGCGGGGGGGGGGGGGGGGGGG	;	TCCGGATGAGAGAGAATGACA	83	GGAGTATTACCATGATTCTGGTCATTTTTTGGAGTATTACCATGAATCAGTCCTCA	
Spu	CTATCAACAATTCTCAGGGCGGGGGGGGGGGGGGGGGGG		TCCGGATGGAAGAGAATGTAA	145	ACGCCCTGATTCTGGATATTCCCATGTCGTATTTTTGAAGGATATTAA-CCATGAATCAGTCTTTA	
MLO	GACGTTAAAGTTCTCAGGGCGGGGGGG		TCCGGATGAAAGAGGACGAAA	44	CGTGCGTCCTGATTCTGGTTCGAAACGGAAGGATGGACCCATGAATCAGCATTCC	
AC	AAGCGACATCGCTTCAGGGCGGGGGGGGGGGGGGGGGGG		TCCGGATGAAAGAAGACGACG	51	TACGAGAATGTTTAACCGTAATTTACGAGAGCATTTCATATGTC	
BP	AAGCAGTACGTCTTCAGGGCGGGGGGG		TCCGGATGAGAGAAGATGTGC	62	GCGAGAGCGTTTTCGCCATTTCCTTTTTTGCGAGAGCGTTTCAATGTCC	£
BPS	AGTCAGTGCGTCTTCAGGGCGGGGGGG		TCCGGATGAAAGAAGATGTGC	86	GASCCCTGARACGTTTTTCGCCCATTCATGTTTCGCGAGGAGCGTTTCACATG	0
BU	AATCAGTGCGTCTTCAGGGCGGGGGGG	;	GCCGGATGGAAGAAGATGTGC	99	ATGCCCTGARACGTTTTTCGCCCAACTTTTGCGATGAGCGTTTCAACTATGT	۲Ŋ
REU	CATCGTTACGTCTTCAGGGCGGGGGGGGGGGGGGGGGGG		TCCGGATGAAAGAAGATGGGC	77	ATCCCCTGANACGCCCATCCATGGANATCCACGCACGGAGCGTTTCAATGCTG	r
RSO	GCTTGGTACGTCTTCAGGGCGGGGGGG		TCCGGATGGAAGAAGATGTGC	80	CGTGCCCTGGAACGTCTTGTCGCCCATTTCAGCGAGGAGCGTTTCCATGTTG	1
PP	GGTCGGTCGGTCTTCAGGGCGGGGGGG		TCCGGATGAAAGAAGGCGTCA	50	CGAGGACGTTCATCGATCATTCACGAGGACCGTTCATGTTCA	
PY	GCCGGTAACGTTCTCAGGGCGGGGGGG	;	CCGGATGAAGAGAGAGCGGGA	91	ATGCCCTGTTTTTTCATTAAATTAAACAGGAGTCAGAACACGTGC	
PU	CGGCGAAACGTTCTCAGGGCGGGGGGG		CCGGATGAAGAGAGAACGGGA	68	ACGCCCTGTTTTTCACAC	
PA	GGCCGTAACGTTCTCAGGGCGGGGGGG		CCGGATAAAGAGAGAACGGG	53	GAAAGCCCTGTTTTTCACGAAAGCAGGAGTTCGTCATATGTC	
BME	CGCGGGCTTGTTCTCGGGGCGGGGGGGGGGGGGGGGGGG	;	TCCGGATGGAAGAGAGCGAAT	54	GCGCCCTGATTCTAGTTTCGTG	
CAU	AATCCGAAGACCTTCGGGGGCAAGGTG		TCCGGATGGGAGAAGGTCGGC	116	CGCGATGCCCCGAAGGTGTGTTCAGGGGTGTCGCGATGAAC	
TFU	GTACACACGCGTGCTCCGGGGTCGGT	2	GGATGGGAGGTAGTACGTGGT	58	GCCTTACCCCGGAGCCTGACCTGCCTAGGGGGAAGGCTTCTCGC	
GLU	TGAGTTTTGTTCTCAGGGCGGGGGGG		TCCGGATGCAAGAGAACCG	32	AAGGCCCCGAGGATTACATGCTTTTAAATCCTTTGAAAAGGCGACAAGATCATGAATCCTATAACCG	
DR	GAACCGACCTCTTTCGGGGGGGGGGGGGGGGGGGGGGGG		TCCGGACGAAAGAAGGAGGA	-GA	CGCTCAGCTTGCCCCCCAGCAGGCCGCCGCGTATG	
SM	GTCGCAAGCGTTCTCAGGGCGGGGGGGGGGGGGGGGGGG		TCCGGATGGAAGAGAGCAAGC	45	ATCATTGGAAAAATGCCAACCCTGAAAGGCTTGAGACCCATGACCATGACCATGACCATGACCATGACCATGACCATGACCATGACCATGACCATGACCATGACCATGACCATGACCATGACCATGACCATGACGACCATGACGACGACGACGACGACGACGACGACGACGACGACGACG	
TQ	TTCGGCACCTCCTTCGGGGGGGGGGGGGGGGGGGGGGGG		TCCGGATGGGAGAAGGAGGGCC	ACT	recec	
AMI	CTTACTCACAGTTTCAGGGCGGGGGGGGGGGGGGGGGGG		TCCGGATGGAAGAAACGGAGCG	CCT	FATGG	

Figure 5. The conserved RNA elements in the upstream regions of the *RFN*-regulated genes: (A) the *RFN* elements and potential terminators; (B) the *RFN* elements and potential SD-sequestors. The yellow background indicates the first stem of the *RFN* element. The blue background indicates the proposed terminator/SD-sequestor. Mangenta text indicates the main stem of the anti-terminator or anti-sequestor. Arrows in the upper line show the complementary stems of these RNA secondary structures. The SD-box and the start codon are shown in green and blue, respectively. The pink background indicates additional helices in the loop of the SD-sequestor. The color code of the genome abbreviations in the first column is as in Figure 3. The presence of two terminators in the upstream region of the *S.pneumoniae* riboflavin operon is marked by an asterisk. The distinct groups of conserved sequestors from γ and β -proteobacteria are marked.

directly. In these cases we predict that *RFN* regulates translation without additional RNA elements. In the presence of FMN, the stabilized *RFN* element represses initiation of translation. Conversely, *RFN* is not stable in the absence of FMN which results in opening of the SD-box and releasing from repression.

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The left stem of the main helix of the predicted antiterminator overlaps either the left or the right part of the base stem of the *RFN* element (Fig. 5A). In the first case, the antiterminator is formed by the spacer between *RFN* and the terminator with intact *RFN* hairpins. At that, the spacer potentially folds into an additional secondary structure. In the second case, which is predominant for the *ypaA* genes, the alternative secondary structure of the anti-terminator only partially overlaps the *RFN* element and the terminator.

Paralogs and horizontal transfer

Comparison of RB protein phylogenetic trees with the standard trees for ribosomal proteins reveals some unusual branches. The most interesting observation is the likely



Figure 6. The maximum likelihood phylogenetic tree of bacterial riboflavin synthases encoded by the *ribH* gene. The genome abbreviations are listed in Table 1. The separate branch including the *ribH2* genes from some α -proteobacteria and *Pseudomonas* species is shown by bold lines. The *ribH* genes likely to be horizontally transferred are boxed.

horizontal transfer of the RB operon from the *Bacillus/ Clostridium* group to two proteobacterial genomes. For instance, the RibH proteins from *H.ducreyi* and *A.pleuropneumoniae* cluster with RibH from the *Bacillus/ Clostridium* group (Fig. 6). The same holds for other phylogenetic trees (data not shown). Moreover, the RB operon structure in these two proteobacteria differs from that of other proteobacteria, and is similar to the operon structure observed in the *Bacillus/Clostridium* group. Finally, the *RFN* elements upstream of these operons are of the Gram-positive type.

Another example of possible horizontal transfer is the RB operon of *F.nucleatum*. Again, the RB proteins of this bacterium cluster with the *Bacillus/Clostridium* group (e.g. in the RibH tree, Fig. 6). Furthermore, the new *RFN*-regulated transporter ImpX from *F.nucleatum* has only one ortholog in *D.halfniense*, the latter belonging to the *Bacillus/Clostridium* group.

Finally, the RB operon observed in *Pyrococcus furiosus* also seems to be transferred, probably from *T.maritima*, as the *P.furiosus* RB proteins consistently cluster with those of *T.maritima* (e.g. in the RibH tree, Fig. 6), whereas closely related *Pyrococcus horicoshii* and *Pyrococcus abyssi* have no RB genes.

Some α -proteobacteria and *Pseudomonas* species have two *ribH* paralogs, *ribH* in the RB operon and a single gene *ribH2* (Table 1). Interestingly, most single *ribH2* genes, in contrast to *ribH*, are regulated by the *RFN* element. The RibH2 proteins form a separate group in the RibH phylogenetic tree. The same was observed for the *ribE* gene in proteobacteria. Some genomes, in particular *E.coli*, have a single *ribE* gene. Other genomes either have a related, non-orthologous gene in the RB operon, named *ribE2*, or both *ribE2* and *ribE* (Table 1). Unlike single *ribH2* genes, single *ribE* genes are not regulated by *RFN*. Finally, most proteobacteria contain single *ribB* and *ribA* genes as well as a fused *ribB/A* gene which is usually located in the RB operon. The single *ribB* gene in all cases is *RFN*-regulated.

As mentioned above, the riboflavin operons in proteobacteria are not regulated by *RFN*, with the only exception being *P.aeruginosa*. In this genome we have observed a possible insertion of the *RFN* element inside the *ybaD-ribD-ribE2*- rib(B/A)-ribH-nusB operon upstream of the ribE2 gene. Strikingly, the alignment of the upstream sequences of the ribE2 gene from P.aeruginosa and the ribH2 genes from other Pseudomonas species shows that both RFN and the predicted sequestor are highly conserved (Fig. 7). This highly conserved region overlaps with the ribH2 start codon. However, the sequestor does not overlap the SD-box of the ribE2 gene in P.aeruginosa, as the ribE2 start codon lies ~100 nt downstream. A possible explanation of this mysterious case of the RFN-mediated regulation is that regulation is carried out via translation of the leader peptide. Such a mechanism was described for regulation of the erythromycin resistance genes in B.subtilis (31). Another possibility is that we observe the result of a recent horizontal transfer, which will eventually lead to formation of a functional regulation cassette or will be eliminated. The latter possibility seems to be corroborated by the phylogenetic tree of RFN elements (Fig. 8). Indeed, the RFN element of ribE from P.aeruginosa tightly clusters with the RFN elements of ribH genes from other Pseudomonas species.

Consistent with the structural analysis (above), the tree of *RFN* elements can be roughly divided into two main branches corresponding to Gram-positive and Gram-negative types. Another noteworthy feature of this tree is that in five of nine genomes containing two *RFN* elements, these elements form a single branch. On the other hand, no consistent clustering of functionally similar *RFN* elements (those upstream of the *rib* operons in the *Bacillus/Clostridium* genomes and those upstream of the *ypaA* genes) was observed. Thus, it is likely that the evolution of the *RFN* elements involved several independent genome-specific duplications.

DISCUSSION

Using the global analysis of RFN elements in available bacterial genomes, we have found that this conserved RNA regulatory element is widely distributed in eubacteria. Analysis of the operon structure shows that RFN predominantly regulates single RB genes (ribB or ribH2) in proteobacteria and the RB operon in most Gram-positive bacteria. Thus, in contrast to only one regulated step of riboflavin biosynthesis in the former taxonomic group, the complete riboflavin biosynthetic pathway is under FMN-mediated regulation in the latter group of bacteria. Another phylogenetic observation is that all observed RFN elements are divided into two major groups based on conservation of the fragment close to the variable stem-loop. Moreover, single RB genes seem to be regulated on the level of translation, whereas the RB operons are predicted to be regulated on the level of transcription. As a result, Gram-negative and Gram-positive bacteria significantly differ in the RFN element structure, the target of RFN-mediated regulation, and the predicted mechanism of the regulation.

The exact mechanism of regulation is not clear. It is known that FMNs can specifically bind to RNA aptamers (32). The FMN-mediated regulation of the RB genes apparently requires high conservation of the sequence and the structure of *RFN* due to possible FMN binding to this site. Preliminary experimental data seem to confirm involvement of the transcriptional and translational attenuation in the regulation of RB gene expression in *B.subtilis* and *E.coli*, respectively,

PU ribH2 Psy ribH2 PA ribE	AAGTCGTTTTTCACGCTTCGAATAATCCGATGGCCAATCACAAAAGGCGCAATGCACAGC TACTCCGCGTCCTTATAAGAAAAAGGCAGGTGCACTGTACG-AAAGTGTCCATGCATGAT GCCCCATCTGCGGGACGCCTGAACTTCGCTGCGCGGCGACG-CGGCGGAGGCGATGCACAAG * * * * * * * * * * * * * * * * * * *
PU ribH2 Psy ribH2 PA ribE	CCGGCAATCTGTGTTAAAAACGCTGCGCCGTCCGATAAAGGGCGGCGAAACGTTCT CAGG CGAACAAACTGTGATAAAAATCCCGCACTGGACACGTAATGTGCCGGTAACGTTCT CAGG CGGCGCAACTGTGGTAAAACGCCACACGGCCATCTCGGTGGCCGTAACGTTCT CAGG * * ***** ***** * * * * * * * * * * *
PU ribH2 Psy ribH2 PA ribE	GCGGGGTGCAATTCCCCACCGGCGGTAATTGCGCGCAATGTGCATAGCCCGCGAGCGCTT GCGGGGTGCAACTCCCCACCGGCGGTAATGGCGCGCAATGCGTCTAGCCCGCGAGCGCTT GCGGGGTGAAAGTCCCCACCGGCGGTAATGGCGCCCAAGGCGCCTAGCCCGCGAGCGCTT ******** ** ************************
PU ribH2 Psy ribH2 PA ribE	GGTGACGGACACGGCATAAGCCGTGAGCGGCAGCAAGGTCAGCAGACCCGGTGTGATTCC GGGGGTTTCAGCTTTGGCTGACGACCTGCAAGGTCAGCAGACCCGGTGTGATTCC GCCGGAC-CGGCCACCGCCGGACGACAAGGTCAGCAGACCCGGTGCGATTCC * ** *** *** *** *** **************
PU ribH2 Psy ribH2 PA ribE	GGGGCCGACGGTCACAGTCCGGATGAAGAGAGAGACGGCCGGGGGCCGACGGTCATAGTCCGGATGAAGAGAGAGGGGATTGGTGTCTGCAAGAACGTCT GGGGCCGACGGTCATAGTCCGGATAAAGAGAGAACGGGAT
PU ribH2 Psy ribH2 PA ribE	CGTTCGCGCGCGAGCGTGCGTACCCTTGAATCCCTT GCAAAACCTCATCATGAACGACATCGTTCAGGAGGCTTTCCTACGCGACTTGAATCCCTT TGCCTGCCTGGGCGTACCCTGTGCGCCCGCAAGATCCCCT
PU ribH2 Psy ribH2 PA ribE	TCGATTCATAA CGCCTG TTTTTCACACAAAC <u>AGGAG</u> TCAGAAC <u>ATG</u> CAACC TCGATCCAAAATGCCCTGTTTTTTCATTAAATTAA
PU ribH2 Psy ribH2 PA ribE	CACCGCTATCGACAGCAAAAGCAAACACACCCACGGCGAGCGCGTCGCGTTCATCCAGGC TACTGCAATCGACAGCAAAAGCCAATCCGAACGAACGCGTCGCTTTCATCCAGGC ATCTTGGAATACCCGCGCTATGGCACGCAGGCCGCTCGGCGTCGCGGCCGGC
PU ribH2 Psy ribH2 PA ribE	CTGCTGGCACAAGGAAATCGTCGACCAGAGCCGTAAAGGCTTCCTCGCCGAAATGATC CTGCTGGCATAAGGATATCGTCGATCAGAGCCGTAAAGGTTTTGTCGCCCGAAATGGCC CGCCTGTGCGGCGGAAAAACG <u>GGAGG</u> TCGC <u>ATG</u> TCACCGGCA <u>TAA</u> TCGAGCGATCGGC * *** *** *** * ** * * * * * * * * * *

Figure 7. The multiple alignment of the *ribH2* upstream regions from *P.fluorescens* (PU) and *P.syringiae* (Psy) and the *ribE2* upstream region from *P.aeruginosa* (PA). The highly conserved *RFN* elements and the predicted SD-sequestors are boxed. The main stems of the predicted anti-sequestors are shown in bold-face. The predicted SD-boxes and start codons of *ribH2* and *ribE* are underlined. The predicted SD-box, start and stop codons of a possible short leader ORF upstream of *ribE* are set in bold-face and underlined.



Figure 8. The maximum likelihood phylogenetic tree of the *RFN* elements. The names of the first genes of the *RFN*-regulated operons are given. The genome abbreviations are listed in Table 1.

although this model seems to be insufficient to explain all observations (A. S. Mironov, personal communication). Indeed, a number of other factors are known to be involved in the riboflavin regulation in a variety of bacteria. In particular, the ribA gene of E.coli is regulated by the soxRS locus, which is responsible for the superoxide stress response (33), and ribBA of H.pylori is regulated by the iron utilization repressor FUR (34,35). Together with observations of Furregulation of the superoxide stress-related genes sodA in E.coli (36), fumC and sodA in P.aeruginosa (37), sodA and sodB in Pseudomonas putida (38) and, vice versa, the regulation of the fur gene by SoxRS in E.coli (39) and regulation of siderophore biosynthesis by oxidative stress in Azotobacter vinelandii (40), this establishes an interconnection between response to the superoxide stress, iron metabolism and flavinogenesis, supported also by the observed coregulation of the latter two systems in the yeast Pichia guilliermondii (41).

One of the remaining open problems is the meaning of positional clustering of *ybaD* and *nusB* genes with riboflavin operons in proteobacteria. The hypothetical protein YbaD contains a Zn-ribbon domain and is highly conserved in bacteria. Zn-ribbons participate in various functions, in particular DNA or RNA binding and redox reactions (42). The RNA-binding protein NusB is involved in anti-termination of *E.coli* ribosomal RNA operons and lambdoid phage genes (43,44). Their functional relationship with the riboflavin biosynthesis is not clear, although is has been suggested that YbaD is the riboflavin repressor (45). At that, it might be relevant that the *ybaD*- and *nusB*-containing RB operons are not regulated by *RFN* with only one exception in *P.aeruginosa* resulting from horizontal transfer. However, other genes in the same genomes are often regulated by *RFN*.

There are four riboflavin-related transporters. One of them is ypaA in Gram-positive bacteria from the Bacillus/Clostridium group, A.minutum (an actinomycete) and T.maritima. It is always a single gene preceded by RFN elements in all genomes excluding T.maritima. Its specificity for riboflavin and co-regulation with the RB genes was predicted in our previous paper (17) and confirmed in experiments (15,18). Two newly identified transporters, pnuX from actinomycetes and *impX* from *F.nucleatum* and Gram-positive bacterium D.halfniense are always regulated by RFN, the former as a single gene or within an operon, and the latter in both cases as a single gene. The PnuX protein is homologous to the mononucleotide transporter PnuC from enterobacteria. One more candidate transporter is rfnT from Rhizobiaceae. However, this prediction is less certain than that for other transporters, as it is based solely on positional clustering.

Finally, this study has demonstrated that the evolutionary history of the RB genes involves a number of horizontal transfer events both of the structural genes and the regulatory *RFN* element. These events manifest in protein phylogenetic trees, operon structures and the *RFN* element architecture.

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REFERENCES

- Perkins, J.B. and Pero, J.G. (2001) Vitamin biosynthesis. In Sonenshein, A.L., Hoch, J.A. and Losick, R. (eds), *Bacillus subtilis and Its Relatives: From Genes to Cells*. American Society for Microbiology, Washington, DC, pp. 279–293.
- Gusarov,I.I., Kreneva,R.A., Podcharniaev,D.A., Iomantas,I.V., Abalakina,E.G., Stoinova,N.V., Perumov,D.A. and Kozlov,I.I. (1997) Riboflavin biosynthetic genes in *Bacillus amyloliquefaciens*: primary structure, organization and regulation of activity. *Mol. Biol.*, **31**, 446–453.
- Fuller, T.E. and Mulks, M.H. (1995) Characterization of Actinobacillus pleuropneumoniae riboflavin biosynthesis genes. J. Bacteriol., 177, 7265–7270.
- Bereswill,S., Hinkelmann,S., Kist,M. and Sander,A. (1999) Molecular analysis of riboflavin synthesis genes in *Bartonella henselae* and use of the *ribC* gene for differentiation of *Bartonella* species by PCR. J. Clin. Microbiol., 37, 3159–3166.
- Lee, C.Y., O'Kane, D.J. and Meighen, E.A. (1994) Riboflavin synthesis genes are linked with the *lux* operon of *Photobacterium phosphoreum*. *J. Bacteriol.*, **176**, 2100–2104.
- Lin,J.W., Chao,Y.F. and Weng,S.F. (2001) Riboflavin synthesis genes ribE, ribB, ribH, ribA reside in the lux operon of *Photobacterium* leiognathi. Biochem. Biophys. Res. Commun., 284, 587–595.
- Lee,C.Y., Szittner,R.B., Miyamoto,C.M. and Meighen,E.A. (1993) The gene convergent to *luxG* in *Vibrio fischeri* codes for a protein related in sequence to RibG and deoxycytidylate deaminase. *Biochim. Biophys. Acta*, **1143**, 337–339.
- Bacher, A., Eberhardt, S. and Richter, G. (1994) Biosynthesis of riboflavin. In Neidhardt, F.C. (ed.), *Escherichia coli and Salmonella. Cellular and Molecular Biology*. American Society for Microbiology, Washington, DC, pp. 657–664.
- Kreneva,R.A. and Perumov,D.A. (1990) Genetic mapping of regulatory mutations of *Bacillus subtilis* riboflavin operon. *Mol. Gen. Genet.*, 222, 467–469.
- Solov'eva,I.M., Iomantas,Iu.A., Kreneva,R.A., Kozlov,Iu.I. and Perumov,D.A. (1997) Cloning of *ribR*, an additional regulatory gene of the *Bacillus subtilis* riboflavin operon. *Genetika*, **33**, 739–743.
- Kil,Y.V., Mironov,V.N., Gorishin,I.Yu., Kreneva,R.A. and Perumov,D.A. (1992) Riboflavin operon of *Bacillus subtilis*: unusual symmetric arrangement of the regulatory region. *Mol. Gen. Genet.*, 233, 483–486.
- Gusarov, I.I., Kreneva, R.A., Rybak, K.V., Podcherniaev, D.A., Iomantas, Iu.V., Kolibaba, L.G., Polanuer, B.M., Kozlov, Iu.I. and Perumov, D.A. (1997) Primary structure and functional activity of the *Bacillus subtilis ribC* gene. *Mol. Biol.*, **31**, 820–825.
- Mack,M., van Loon,A.P., Hohmann,H.P. (1998) Regulation of riboflavin biosynthesis in *Bacillus subtilis* is affected by the activity of the flavokinase/flavin adenine dinucleotide synthetase encoded by *ribC*. *J. Bacteriol.*, **180**, 950–955.
- Solovieva,I.M., Kreneva,R.A., Leak,D.J. and Perumov,D.A. (1999) The *ribR* gene encodes a monofunctional riboflavin kinase which is involved in regulation of the *Bacillus subtilis* riboflavin operon. *Microbiology*, **145**, 67–73.
- Lee, J.M., Zhang, S., Saha, S., Santa Anna, S., Jiang, C. and Perkins, J. (2001) RNA expression analysis using an antisense *Bacillus subtilis* genome array. *J. Bacteriol.*, **183**, 7371–7380.
- Azevedo, V., Sorokin, A., Ehrlich, S.D. and Serror, P. (1993) The transcriptional organization of the *Bacillus subtilis* 168 chromosome region between the *spoVAF* and *serA* genetic loci. *Mol. Microbiol.*, 10, 397–405.
- Gelfand,M.S., Mironov,A.A., Jomantas,J., Kozlov,Y.I. and Perumov,D.A. (1999) A conserved RNA structure element involved in the regulation of bacterial riboflavin synthesis genes. *Trends Genet.*, 15, 439–442.
- Kreneva,R.A., Gelfand,M.S., Mironov,A.A., Iomantas,I.A., Kozlov,I.I., Mironov,A.S. and Perumov,D.A. (2000) Study of the phenotypic occurrence of *ypaA* gene inactivation in *Bacillus subtilis. Genetika*, 36, 1166–1168.

- Gelfand,M.S., Novichkov,P.S., Novichkova,E.S. and Mironov,A.A. (2000) Comparative analysis of regulatory patterns in bacterial genomes. *Brief Bioinform.*, 1, 357–371.
- Panina,E.M., Vitreschak,A.G., Mironov,A.A. and Gelfand,M.S. (2001) Regulation of aromatic amino acid biosynthesis in gammaproteobacteria. J. Mol. Microbiol. Biotechnol., 3, 529–543.
- Eddy,S.R. and Durbin,R. (1994) RNA sequence analysis using covariance models. *Nucleic Acids Res.*, 22, 2079–2088.
- Dandekar, T., Beyer, K., Bork, P., Kenealy, M.R., Pantopoulos, K., Hentze, M., Sonntag-Buck, V., Flouriot, G., Gannon, F. and Schreiber, S. (1998) Systematic genomic screening and analysis of mRNA in untranslated regions and mRNA precursors: combining experimental and computational approaches. *Bioinformatics*, 14, 271–278.
- Overbeek, R., Fonstein, M., D'Souza, M., Pusch, G.D. and Maltsev, N. (1999) The use of gene clusters to infer functional coupling. *Proc. Natl Acad. Sci. USA*, 96, 2896–2901.
- Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Rapp, B.A. and Wheeler, D.L. (2000) GenBank. *Nucleic Acids Res.*, 28, 15–18.
- Overbeek, R., Larsen, N., Pusch, G.D., D'Souza, M., Selkov, E.Jr, Kyrpides, N., Fonstein, M., Maltsev, N. and Selkov, E. (2000) WIT: integrated system for high-throughput genome sequence analysis and metabolic reconstruction. *Nucleic Acids Res.*, 28, 123–125.
- Lyngso,R.B., Zuker,M. and Pedersen,C.N. (1999) Fast evaluation of internal loops in RNA secondary structure prediction. *Bioinformatics*, 15, 440–445.
- Altschul,S., Madden,T., Schaffer,A., Zhang,J., Zhang,Z., Miller,W. and Lipman,D. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.*, 25, 3389–3402.
- Mironov,A.A., Vinokurova,N.P. and Gelfand,M.S. (2000) GenomeExplorer: software for analysis of complete bacterial genomes. *Mol. Biol.*, 34, 222–231.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*, 25, 4876–4882.
- 30. Felsenstein, J. (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. *J. Mol. Evol.*, **17**, 368–376.
- Hue,K.K. and Bechholer,D.H. (1991) Effect of *ermC* leader region mutations on induced mRNA stability. J. Bacteriol., 173, 3732–3740.
- Hermann, T. and Patel, D.J. (2000) Adaptive recognition by nucleic acid aptamers. *Science*, 287, 820–825.
- Koh, Y.S., Choih, J., Lee, J.H. and Roe, J.H. (1996) Regulation of the *ribA* gene encoding GTP cyclohydrolase II by the *soxRS* locus in *Escherichia coli. Mol. Gen. Genet.*, 251, 591–598.

- Fassbinder,F., van Vliet,A.H., Gimmel,V., Kusters,J.G., Kist,M. and Bereswill,S. (2000) Identification of iron-regulated genes of *Helicobacter pylori* by a modified fur titration assay (FURTA-Hp). *FEMS Microbiol. Lett.*, 184, 225–229.
- Worst,D.J., Gerrits,M.M., Vandenbroucke-Grauls,C.M. and Kusters,J.G. (1998) *Helicobacter pylori ribBA*-mediated riboflavin production is involved in iron acquisition. *J. Bacteriol.*, **180**, 1473–1479.
- Hassan,H.M. and Schrum,L.W. (1994) Roles of manganese and iron in the regulation of the biosynthesis of manganese-superoxide dismutase in *Escherichia coli. FEMS Microbiol. Rev.*, 14, 3153–3123.
- 37. Hassett,D.J., Howell,M.L., Ochsner,U.A., Vasil,M.L., Johnson,Z. and Dean,G.E. (1997) An operon containing *fumC* and *sodA* encoding fumarase C and manganese superoxide dismutase is controlled by the ferric uptake regulator in *Pseudomonas aeruginosa: fur* mutants produce elevated alginate levels. J. Bacteriol., **179**, 1452–1459.
- Kim,Y.C., Miller,C.D. and Anderson,A.J. (1999) Transcriptional regulation by iron of genes encoding iron- and manganese-superoxide dismutases from *Pseudomonas putida*. *Gene*, 239, 129–135.
- Zheng, M., Doan, B., Schneider, T.D. and Storz, G. (1999) OxyR and SoxRS regulation of *fur. J. Bacteriol.*, 181, 4639–4643.
- Tindale,A.E., Mehrotra,M., Ottem,D. and Page,W.J. (2000) Dual regulation of catecholate siderophore biosynthesis in *Azotobacter vinelandii* by iron and oxidative stress. *Microbiology*, **146**, 1617–1626.
- Fedorovich, D., Protchenko, O. and Lesuisse, E. (1999) Iron uptake by the yeast *Pichia guilliermondii*. Flavinogenesis and reductive iron assimilation are co-regulated processes. *Biometals*, 12, 295–300.
- Aravind,L. and Koonin,E.V. (1999) DNA-binding proteins and evolution of transcription regulation in the archaea. *Nucleic Acids Res.*, 27, 4658–4670.
- Nodwell,J.R. and Greenblatt,J. (1993) Recognition of boxA antiterminator RNA by the *E. coli* antitermination factors NusB and ribosomal protein S10. *Cell*, **72**, 261–268.
- 44. Luttgen,H., Robelek,R., Muhlberger,R., Diercks,T., Schuster,S.C., Kohler,P., Kessler,H., Bacher,A. and Richter,G. (2002) Transcriptional regulation by antitermination. Interaction of RNA with NusB protein and NusB/NusE protein complex of *Escherichia coli*. J. Mol. Biol., **316**, 875–885.
- Wolf,Y.I., Rogozin,I.B., Kondrashov,A.S. and Koonin,E.V. (2001) Genome alignment, evolution of prokaryotic genome organization and prediction of gene function using genomic context. *Genome Res.*, 11, 356–372.