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New Target Genes Controlled by the *Bradyrhizobium japonicum* Two-Component Regulatory System RegSR▽†

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RegSR-like proteins, members of the family of two-component regulatory systems, are present in a large number of proteobacteria in which they globally control gene expression mostly in a redox-responsive manner. The controlled target genes feature an enormous functional diversity. In *Bradyrhizobium japonicum*, the facultative root nodule symbiont of soybean, RegSR activate the transcription of the nitrogen fixation regulatory gene *nifA*, thus forming a RegSR-NifA cascade which is part of a complex regulatory network for gene regulation in response to changing oxygen concentrations. Whole-genome transcription profiling was performed here in order to assess the full regulatory scope of RegSR. The comparative analysis of wild-type and Δ*regR* cells grown under oxic and microoxic conditions revealed that expression of almost 250 genes is dependent on RegR, a result that underscores the important contribution of RegR to oxygen- or redox-regulated gene expression in *B. japonicum*. Furthermore, transcription profiling of Δ*regR* bacteroids compared with wild-type bacteroids revealed expression changes for about 1,200 genes in young and mature bacteroids. Incidentally, many of these were found to be induced in symbiosis when wild-type bacteroids were compared with free-living, culture-grown wild-type cells, and they appeared to encode diverse functions possibly related to symbiosis and nitrogen fixation. We demonstrated direct RegR-mediated control at promoter regions of several selected target genes by means of DNA binding experiments and in vitro transcription assays, which revealed six novel direct RegR target promoters.

Rhizobia are soil bacteria able to establish a nitrogen-fixing symbiosis within the root nodule cells of legume host plants. The transition from the free-living to the symbiotic state is accompanied by drastic changes in bacterial metabolism, eventually leading to the formation of bacteroids specialized for nitrogen fixation and life in the microoxic environment within root nodules. Nitrogen fixation genes (*nif* and *fix*) are subject to tight regulation, and they are expressed only under the low-oxygen condition that prevails inside root nodules or is artificially maintained in culture (11).

In *Bradyrhizobium japonicum*, the root nodule symbiont of soybean, two hierarchically organized regulatory cascades, RegSR-NifA and FixLJ-$FixK_2$, control expression of nitrogen fixation genes and genes required for life under microoxic conditions, respectively (11). RegSR and FixLJ form typical bacterial two-component regulatory systems consisting of a sensory histidine protein kinase and a cytoplasmic response regulator (RegR and FixJ) (for reviews, see references 18, 35, and 53). While the activity of the FixL sensor kinase is controlled by oxygen via a prosthetic heme (see reference 11 and references therein), the nature of the signal sensed by RegS remains to be identified. Similarly, knowledge about the regulatory scope of *B. japonicum* RegR is limited. RegR was identified in the course of studying transcriptional regulation of the *B. japonicum fixR-nifA* operon, which is preceded by two overlapping promoters, P1 and P2 (3–5). RegR activates transcription originating from P2 under all oxygen conditions via binding to a DNA element located around position −67 upstream of the transcription start site. Upon a switch to low-oxygen or anoxic conditions, the redox-responsive NifA protein in concert with RNA polymerase containing RpoN (σ^{54}) enhances its own synthesis via activation of the −24/−12-type P1 promoter. This results in maximal expression not only of the *fixR-nifA* operon but also of other target genes (11, 51). Most recently, the NifA-RpoN regulon of *B. japonicum* was unraveled by a genome-wide transcriptome analysis, which identified numerous new NifA-RpoN-dependent genes (25).

Phenotypic analysis of the Δ*regR* strain revealed that although the strain is able to form nodules on soybean, it retains only residual nitrogen fixation activity (2%). Nodules elicited by the *regR* mutant showed a greenish interior harboring a decreased number of bacteroids, which is indicative of defects in proper nodule development (5). By contrast, mutants of the sensor kinase RegS differed only marginally in their symbiotic properties from the wild type on the same host plant.

Orthologs of the *B. japonicum* RegSR two-component regulatory proteins are widely distributed among proteobacteria, and they include the well-studied RegBA and PrrBA proteins of the purple nonsulfur bacteria *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, respectively. RegBA (PrrBA) was shown to control diverse cellular processes that either generate or utilize reducing equivalents and thus balance the cellular redox status, e.g., photosynthesis, CO_2 fixation, N_2 fixation,

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strains		
E.coli		
DH5α	λ$^-$ ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	Bethesda Research Laboratories, Inc., Gaithersburg, MD
S17-1	Sm^r Sp^r *hsdR* (RP4-2 *kan*::Tn*7 tet*::Mu; integrated in the chromosome)	52
BL21 (DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) (λ*c*Its857 *ind1 Sam7 nin5 lacUV5-T7 gene 1*)	54
B. japonicum		
110*spc*4	Sp^r wild type	49
2426	Sp^r Sm^r *regR*::Ω	5
9537	Sp^r Km^r bll2087::*aphII* (same orientation)	This work
9538	Sp^r Km^r bll2087::*aphII* (opposite orientation)	This work
9552	Sp^r Km^r bll2109::*aphII* (same orientation)	This work
9553	Sp^r Km^r bll2109::*aphII* (opposite orientation)	This work
Plasmids		
pBluescript SK+	Ap^r cloning vector	Stratagene, La Jolla, CA
pBSL15	Ap^r Km^r	1
pBSL86	Ap^r Km^r	1
pGEM-T Easy	Ap^r TA cloning vector	Promega, Madison, WI
pSUP202pol4	Tc^r (pSUP202) *oriT* of RP4	17
pSUP202pol6K	Tc^r (pSUP202pol4) KpnI linker inserted into SmaI site	58
pRJ2809	Ap^r (pRJ9519) *fixR* promoter on a 632-bp SacII-BamHI fragment	R. Emmerich, unpublished
pRJ8817	Ap^r (pRJ9519) *fixGHIS* promoter on a 524-bp XbaI-EcoRI fragment	38
pRJ9519	Ap^r (pBluescript SK+) 308-bp BstXI-KpnI fragment containing the *B. japonicum rrn* terminator cloned into the HincII and KpnI sites	6
pRJ9537	Km^r Tc^r (pSUP202pol6K) 2.3-kb XbaI-EcoRI fragment	This work
pRJ9538	Km^r Tc^r (pSUP202pol6K) 2.3-kb XbaI-EcoRI fragment	This work
pRJ9542	Ap^r (pRJ9519) bll2087 promoter on a 157-bp EcoRI-BamHI fragment	This work
pRJ9547	Ap^r (pRJ9519) blr2614 promoter on a 164-bp EcoRI-BamHI fragment	This work
pRJ9552	Km^r Tc^r (pSUP202pol4) 2.4-kb NotI-PstI fragment	This work
pRJ9553	Km^r Tc^r (pSUP202pol4) 2.4-kb NotI-PstI fragment	This work
pRJ9562	Ap^r (pGEM-T Easy) 434-bp PCR fragment comprising the blr1515 upstream region	This work
pRJ9564	Ap^r (pRJ9519) blr1515 promoter on a 394-bp BamHI-EcoRI fragment	This work
pRJ9567	Ap^r (pRJ9519) bll2109 promoter on a 251-bp BamHI-EcoRI fragment	This work
pRJ9569	Ap^r (pRJ9519) blr2501 promoter on a 349-bp NotI-PstI fragment	This work
pRJ9570	Ap^r (pRJ9519) blr1883 promoter on a 429-bp NotI-PstI fragment	This work
pRJ9571	Ap^r (pRJ9519) bll3193 promoter on a 322-bp BamHI-EcoRI fragment	This work
pRJ9573	Ap^r (pRJ9519) bll1285 promoter on a 443-bp BamHI-EcoRI fragment	This work
pRJ9574	Ap^r (pRJ9519) bll4833 promoter on a 435-bp BamHI-EcoRI fragment	This work
pRJ9576	Ap^r (pRJ9519) blr6267 promoter on a 388-bp BamHI-EcoRI fragment	This work
pRJ9577	Ap^r (pRJ9519) bll5807 promoter on a 316-bp BamHI-EcoRI fragment	This work
pRJ9578	Ap^r (pRJ9519) bll6633 promoter on a 277-bp BamHI-EcoRI fragment	This work
pRJ9601	Ap^r (pBluescript SK+) *B. japonicum rrn* promoter and *rrn* terminator on a 468-bp SacI-SmaI fragment	6

aerotaxis, and respiration (see references 12 and 31). In addition, RoxSR of *Pseudomonas aeruginosa* and RegSR of *Rhodopseudomonas palustris* were shown to regulate expression of a cyanide-insensitive oxidase and the uptake hydrogenase, respectively (9, 50). Finally, ActSR of *Sinorhizobium meliloti*, originally identified in the context of acid tolerance, also control genes involved in CO_2 fixation, nitrate assimilation, and N_2 fixation (16, 57). The response regulators of this family display an unusually high degree of conservation in their DNA binding domains. In fact, it was demonstrated for some members that they are functionally exchangeable both in vitro and in vivo, raising the question of whether they also control a similar set of target genes (13, 36).

In *B. japonicum*, no other direct RegR target genes have been studied in great detail apart from *fixR-nifA*. Here, we have assigned a large number of genes as novel members of the RegR regulon by comparing the transcriptome of the wild type with that of the Δ*regR* strain under free-living oxic and microoxic conditions and during symbiosis.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains used in this work are listed in Table 1. *Escherichia coli* was grown in Luria-Bertani medium (39) containing the following concentrations of antibiotics for plasmid selection (μg ml^{-1}): ampicillin, 200; kanamycin, 30; and tetracycline, 10. *B. japonicum* strains were cultivated in peptone salts-yeast extract medium (49) supplemented with 0.1% L-arabinose. Aerobic cultures (21% O_2) for microarray experiments were grown in 5-liter Erlenmeyer flasks containing 200 ml of medium with rigorous shaking (160 rpm) at 30°C. Microaerobic cultures (0.5% O_2 in the gas phase) and anaerobic cultures were grown as described previously (24, 25). When appropriate, antibiotics were used at the following concentrations (μg ml^{-1}): spectinomycin, 100; streptomycin, 50; kanamycin, 100; and tetracycline, 50 (solid medium) and 25 (liquid medium).

Plant growth. Soybean seeds [*Glycine max* (L.) Merr. cv. Williams] were surface sterilized as previously described except that treatment with 30% H_2O_2 for 5 min was used for sterilization (21). Nodules for RNA isolation were

harvested 13 and 21 days postinoculation (dpi), immediately frozen in liquid nitrogen, and stored at −80°C until RNA isolation. The symbiotic phenotypes of the *B. japonicum* mutant strains 9537, 9538, 9552, and 9553 were determined in infection tests using soybean as the host plant, and nitrogenase activity was measured in an acetylene reduction assay (20, 23).

RNA isolation, cDNA synthesis, and microarray analysis. Cultures of *B. japonicum* were grown to mid-exponential phase (an optical density at 600 nm of 0.4 to 0.5). Cell harvest, RNA extraction, cDNA synthesis, fragmentation, and labeling were done as described previously (24, 25). Details of the custom-designed Affymetrix *B. japonicum* gene chip BJAPETHa520090 (Santa Clara, CA) and conditions for microarray hybridization have also been described previously (25). For bacteroid transcriptome analyses, nodules from five plants infected with the wild type or the *regR* mutant were collected for each hybridization experiment, and RNA was extracted as described previously (45). For each strain grown under free-living and symbiotic conditions, a minimum of five or three biological replicates was analyzed, respectively. Only the probe sets that were called "present" or "marginal" in ≥80% of the replicates of each experiment were considered for further analysis. Details on data processing, normalization, and further analysis including the identification of statistically overrepresented functional categories are described elsewhere (45). We considered genes passing the statistical tests as differentially expressed only if the relative change in expression (*n*-fold) was ≥2 or ≤−2 when different conditions were compared. Operon predictions were essentially done according to Mwangi and Siggia (40). An operon-like organization of genes (bicistronic or larger) was assumed if they were orientated in the same direction and separated by ≤32 nucleotides (nt). The allowed distance between genes was enlarged to 100 nt if the first three letters in the gene names were identical.

In silico search for RegR binding sites. Putative promoter regions (500 bp) located upstream of RegR-regulated genes or operons were searched for RegR binding motifs essentially as described previously for NifA and RpoN binding sites (25). A position-specific frequency matrix was generated on the basis of experimentally verified RegR binding sites (see Fig. 4). Predicted RegR binding sites (see Table S2 in the supplemental material) have a score which is higher than that of the motif with the lowest score in the set of known RegR binding sites that was used for generation of the position-specific frequency matrix.

Quantitative real-time PCR. The expression of genes blr3166, blr3167, and blr3168 was analyzed by quantitative reverse transcription-PCR using a QuantiTect Sybr Green PCR kit (Qiagen, Hilden, Germany) and a Rotor-Gene 3000 thermocycler (Corbett Research, Sydney, Australia). cDNA used as template in combination with primers 3166-5/3166-6 (blr3166), 3167-3/3167-4 (blr3167), and 3168-3/3168-4 (blr3168) corresponded to the cDNA prepared for the gene chip experiments. Sequences of these primers and of all other oligonucleotides used in this work are available from the authors on request. Each PCR contained 10 μl of 2× Sybr Green Master Mix, a 0.2 μM concentration of individual primers, and appropriate dilutions of cDNA in a total volume of 20 μl. Reactions were run in triplicates. Melting curves were generated for verifying the specificity of the amplification. Relative changes in gene expression were calculated as described elsewhere (46). Expression of the primary sigma factor *sigA* which, based on the microarray data, was found to be unchanged under different conditions was used as a reference for normalization (primers SigA-1069F and SigA-1155R).

Construction of bll2087 and bll2109 deletion mutants. Plasmids and strains used in this work are listed in Table 1. Deletion mutagenesis was done by marker exchange. Briefly, the 5′ and 3′ flanking regions of bll2087 and bll2109 were PCR amplified and cloned in the pBluescript SK+ vector. A kanamycin resistance cassette (*aphII*) was inserted between the *B. japonicum* DNA fragments. Constructs were cloned into the suicide vectors pSUP202pol6K (bll2087) and pSUP202pol4 (bll2109). The resulting plasmids pRJ9537, pRJ9538, pRJ9552, and pRJ9553 were then mobilized by conjugation into *B. japonicum* strain 110*spc*4 (wild type) yielding mutant strains 9537, 9538, 9552, and 9553. The correct integration of the *aphII* cassette in the chromosome by double crossover was verified by PCR.

Overproduction, purification, and phosphorylation of RegR. His-tagged RegR was overexpressed and purified from *E. coli* BL21(DE3) as described previously (14). For in vitro phosphorylation, RegR protein (20 μM final concentration) was incubated with 25 mM acetyl phosphate (Fluka, Buchs, Switzerland) in either DNA binding buffer (5) or in vitro transcription buffer (6) for 1 h at 30°C.

In vitro transcription assays. Plasmids used as templates for in vitro transcription are based on plasmid pRJ9519 which contains the *B. japonicum rrn* transcriptional terminator (6). They are listed in Table 1. Purification of *B. japonicum* RNA polymerase and multiple-round in vitro transcription assays were done as previously described (6, 38). Assays were performed in a 20-μl reaction mixture containing in vitro transcription buffer and increasing amounts of RegR protein (untreated or pretreated with acetyl phosphate). Radioactive transcription products were purified by phenol extraction and ethanol precipitation, separated on 6% denaturing polyacrylamide gels, and visualized with a phosphorimager using Quantity One software, version 4.6.1 (Bio-Rad, Reinach, Switzerland).

EMSAs. Binding of RegR to putative target promoters was initially tested using PCR-amplified DNA fragments. Acetyl phosphate-treated RegR (0 to 1 μM) was incubated with column-purified DNA fragments (3 to 95 nM) in DNA binding buffer in a total volume of 20 μl in the presence of 1 μg of poly(dI-dC) as a nonspecific competitor. After a 5-min incubation at room temperature, samples were mixed with loading dye and separated on 6% nondenaturing polyacrylamide gels in 1× Tris-borate EDTA electrophoresis buffer for 30 min at 180 V. Gels were stained with Sybr Green I according to the instructions of the provider (Invitrogen, Basel, Switzerland) and visualized on a UV transilluminator. Alternatively, electrophoretic mobility shift assays (EMSAs) were performed using radiolabeled PCR fragments or short oligonucleotides (30 to 35 bp). PCR fragments or single-stranded oligonucleotides (30 pmol) were end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (MBI Fermentas). The single-stranded oligonucleotides were then incubated with the complementary oligonucleotides (60 pmol) for 10 min at 95°C and slowly cooled down to room temperature to allow hybridization. Finally, both labeled oligonucleotides and PCR fragments were purified over NAP-10 Sephadex G-25 columns (Amersham Biosciences, Buckinghamshire, United Kingdom). EMSAs were done as described above using a 0.25 nM to 1 nM concentration of labeled PCR fragments or 2 nM oligonucleotide probes. Gels were dried, and radioactive bands were visualized with a phosphorimager.

Transcript mapping. The 5′ end of the blr1515 transcript synthesized in vivo was mapped in a primer extension experiment using oligonucleotide 1515-P1. RNA was isolated from aerobically grown wild-type and the *regR* mutant strains as described above. Approximately 10 μg of RNA and at least 100,000 cpm of ^{32}P-labeled primer were used per reaction, which was performed as previously described (2). Extension products were loaded on a 6% denaturing polyacrylamide gel adjacent to a sequencing ladder obtained with plasmid pRJ9562 and labeled primer 1515-P1. The 5′ ends of in vitro synthesized RNA from the *fixR* (P2), bll2087, and blr1515 promoters were mapped using oligonucleotide 9519-1 which hybridizes to a sequence located on vector pRJ9519 used for construction of the template plasmids (38). Sequencing ladders from plasmids pRJ2809, pRJ9542, and pRJ9564 were obtained with the same primer.

Microarray data accession numbers. The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) and are accessible via GEO Series accession numbers GSE9026 and GSE9100.

RESULTS AND DISCUSSION

Transcription profiling of the Δ*regR* strain grown under free-living conditions. Comparative analysis of the *B. japonicum* wild-type and the Δ*regR* strains grown under oxic conditions revealed 117 genes with differential expression (relative change of at least twofold). Seventy-four genes showed decreased expression in the mutant; i.e., RegR exerts direct or indirect positive control on these genes in the wild type. Of these genes, 25 are members of 19 putative bicistronic or larger operons (for definition of operons, see Materials and Methods). Increased expression in the mutant was observed for 43 genes (24 of them belonging to 13 operons). Under microoxic conditions, expression of 170 genes was altered between the two strains (126 genes with decreased expression [73 genes in 32 operons] and 44 genes with increased expression [18 genes in 11 operons]).

Differentially expressed genes in culture-grown cells were subdivided into three classes, reflecting their differential expression either under both free-living conditions (oxic and microoxic) or under one or the other condition only (Fig. 1A and Table 2). Figure 1A also illustrates the proportion of genes found to be differentially expressed in symbiosis based on a comparison of the transcriptomes of wild-type and Δ*regR* bacteroids (details given below).

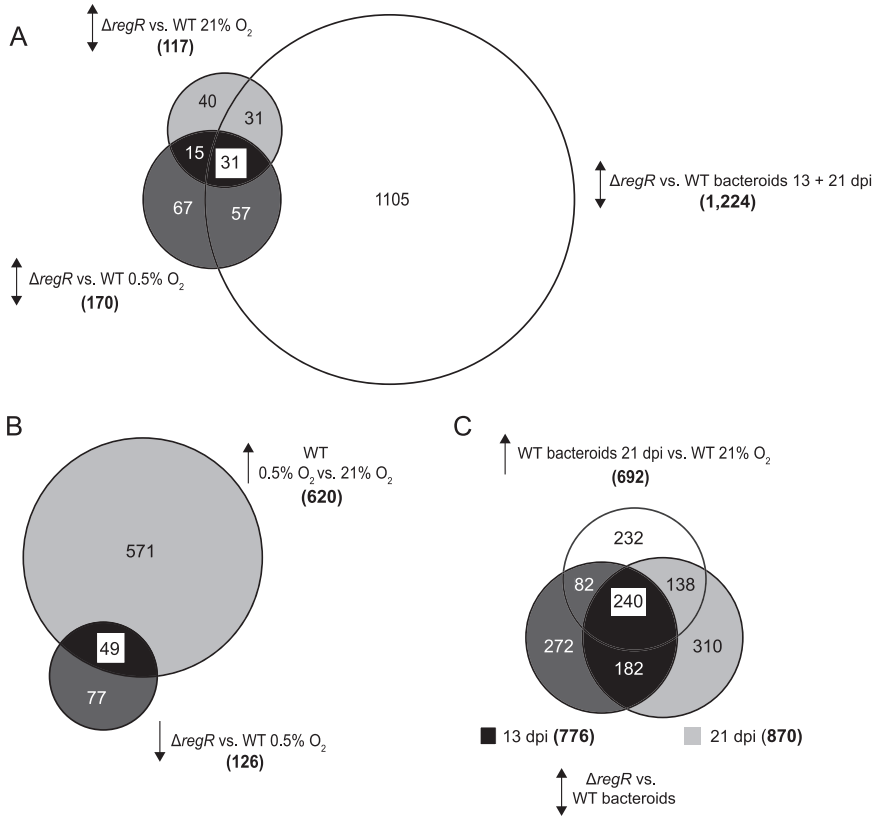

FIG. 1. Venn diagrams representing the number of differentially expressed genes in transcriptome comparisons of the *B. japonicum* wild type (WT) and the Δ*regR* strain grown under free-living and symbiotic conditions. Strains and conditions are indicated alongside the circles. Overlapping sectors are highlighted in different gray tones. Up and down arrows reflect increased and decreased gene expression in microarray analyses, respectively. Numbers in parentheses indicate the total number of differentially expressed genes. (A) Number of genes differentially expressed in the Δ*regR* strain under oxic (21% O_2), microoxic (0.5% O_2), and symbiotic conditions (bacteroids at 13 and 21 dpi). See text for a definition of the genes in the overlaps (classes 1, 2, and 3). (B) Number of genes induced upon a switch from oxic to microoxic conditions in the wild type and genes showing a decreased expression in the Δ*regR* strain under microoxic conditions. The overlap shows the proportion of oxygen-responsive genes activated by RegR. (C) Number of genes differentially expressed in a comparison between wild-type and Δ*regR* bacteroids at 13 and 21 dpi. Genes induced in wild-type bacteroids compared to free-living, aerobically grown cells are in the circle on top.

Class 1 is composed of 46 genes showing qualitatively similar differential expression behavior under oxic and microoxic conditions (Table 2). As expected, the previously known RegR-controlled gene *fixR* is a class 1 member. Likewise, expression levels of the promoter-distal *nifA* gene of the *fixR-nifA* operon decreased in *regR*-deficient cells under both conditions; however, the relative change in expression was above our stringent cutoff value only under oxic conditions. Class 1 also includes the gene bll2087 that was recently discovered as a new RegR target in a pilot microarray study from our group (24). Examples among the novel RegR targets grouped in class 1 are genes coding for putative RND (resistance-nodulation-cell division)-type efflux proteins (Blr1515 and Blr1516) whose amino acid sequences are similar to those of *P. aeruginosa* MexCD (40% and 50% identity) and *E. coli* AcrAB (38% and 48% identity) (30, 48). RND-type transporters are generally known for conferring antibiotic resistance, but they are also important for successful colonization by, and persistence of, human pathogenic bacteria (47). The functional characterization of such transport systems in plant-associated bacteria has suggested a role in tolerance against plant-derived secondary metabolites (e.g., flavonoids and isoprenoids), which may facilitate better colonization of the host (7, 19, 43).

Expression of 12 of the 46 class 1 genes was increased in *regR* mutant cells, e.g., blr7905 (*cit*) encoding a putative citrate-proton symporter (relative change of more than eightfold). This suggests some kind of direct or indirect negative control of RegR on certain genes in the wild type.

Class 2 contains 124 genes that are RegR controlled only under microoxic but not oxic conditions, including several *nif* and *fix* genes (Table 2) which are known to be induced in the wild type by oxygen deprivation and controlled by the RegR-dependent *nifA* gene product (25, 45).

Class 3 is made up of 71 genes that are differentially expressed under oxic but not microoxic conditions. In class 3, a subset of 20 genes was identified whose expression is downregulated in wild-type cells grown under microoxic conditions (45). Among these are blr3166 (*glc* coding for glyoxylate carboligase), blr3167 (*hyi*, hydroxypyruvate isomerase), and blr3168 (tartronate semialdehyde reductase), which also belong to the genes with the highest decrease in expression in the mutant. These genes are known to encode signature enzymes

TABLE 2. *B. japonicum* genes differentially expressed by a factor of ≥2 in the Δ*regR* strain compared with the wild type[a]

Class and gene no.[b]	Putative operon member (gene no.)[b,c]	Gene name[d]	Description	Relative change in expression (*n*-fold) under the indicated condition[e]	
				21% O_2	0.5% O_2
Class 1 (regulated by RegR under both conditions)					
bll0693			Unknown protein	2.3	2.3
bll0904		*regR*	Two-component response regulator	−111.6	−84.0
bll1285			Unknown protein	−11.4	−11.2
bll1322			Hypothetical protein	−2.5	−2.6
blr1429			Unknown protein	−3.5	−3.2
blr1515		*acrA*	RND multidrug efflux membrane permease	−22.2	−21.2
	blr1516	*acrB*	RND multidrug efflux transporter	−29.5	−21.6
blr1783			Putative transposase	−2.5	−2.2
blr2036*		*fixR*	Oxidoreductase	−13.0	−5.7
bll2087		id880	Unknown protein	−24.3	−11.2
bll2268			Putative xylose operon repressor	−3.1	−2.8
blr2501			Hypothetical protein	−12.2	−13.1
bsl2596			Unknown protein	3.9	4.0
blr2614			Hypothetical protein	−4.3	−3.0
blr3161			Hypothetical protein	−2.4	−3.5
bll3363			Unknown protein	−2.4	−2.2
bll3753			Hypothetical protein	4.3	4.4
blr3769			Hypothetical protein	−2.5	−2.5
blr3771			Hypothetical protein	−3.3	−4.1
blr4031			Hypothetical glutathione *S*-transferase-like protein	2.0	2.2
bll4130			Transcriptional regulatory protein LysR family	−2.5	−2.7
bsl4167			Putative glutamine synthetase translation inhibitor	−3.1	−2.8
blr4182			Transcriptional regulator	−3.3	−4.7
blr4238			Hypothetical protein	−3.4	−2.7
blr4257			Putative hydrolase	2.4	2.3
	bsr4258		Hypothetical protein	2.6	3.3
	blr4259		Hypothetical protein	2.4	2.0
	blr4260		Hypothetical protein	2.4	2.4
bll4725			Unknown protein	3.8	4.8
bll4833			Unknown protein	−3.7	−3.6
blr5220		*hspE*	Small heat shock protein	2.9	2.7
bll5477			Similar to formate dehydrogenase	−2.5	−5.2
bll5478			Similar to formate dehydrogenase	−2.2	−4.6
blr5693			Probable substrate-binding protein	−2.3	−2.9
bll5807			Hypothetical protein	−8.2	−5.7
blr6210			Hypothetical protein	−6.5	−6.1
blr6267			Transcriptional regulator	−3.1	−2.8
bll6513			Hypothetical protein	−30.2	−8.1
bsl6653			Unknown protein	−2.8	−3.0
bll6844			Unknown protein	−2.2	−5.3
bll6850		*fliR*	Probable flagellar biosynthetic protein	−2.1	−2.2
bll6855		*fliC*	Probable flagellar protein	−2.1	−6.7
bll6880			Unknown protein	−2.0	−3.2
blr6918			Probable substrate-binding protein	−2.8	−2.2
bll7514		*lpxK*	Tetraacyldisaccharide 4-kinase	2.2	2.8
blr7905		*cit*	Citrate-proton symporter	11.9	8.9
Class 2 (regulated by RegR only under microoxic conditions)					
bll0243			Hypothetical protein		−2.3
blr0305			Unknown protein		3.2
blr0306			Hypothetical protein		3.0
blr0366			Unknown protein		2.5
bll0718			Putative transporter		2.4
bll0818			Unknown protein		−5.3
blr0907			Hypothetical protein		2.2
bll1026			Hypothetical protein		2.0

Continued on facing page

TABLE 2—*Continued*

Class and gene no.[b]	Putative operon member (gene no.)[b,c]	Gene name[d]	Description	Relative change in expression (*n*-fold) under the indicated condition[e]	
				21% O_2	0.5% O_2
bll1070			Probable dioxygenase		−2.0
bll1110			Hypothetical protein		2.1
bll1207			Hypothetical protein		2.0
bsl1208			Hypothetical protein		3.2
blr1402			Hypothetical protein		2.1
bsr1739*		*fdxN*	Ferredoxin		−6.2
blr1743*		*nifD*	Nitrogenase molybdenum-iron protein alpha chain		−6.6
	blr1744*	*nifK*	Nitrogenase molybdenum-iron protein beta chain		−6.4
	blr1745*	*nifE*	Nitrogenase molybdenum-cofactor synthesis protein		−4.9
	blr1746*	*nifN*	Nitrogenase molybdenum-cofactor synthesis protein		−3.2
	blr1748*	id79	Hypothetical protein		−2.3
bsr1749*		id80	Hypothetical protein		−2.0
bll1754*		id89	Hypothetical protein		−3.2
blr1755*			*Rhizobium etli iscN* homolog		−4.5
blr1756*		*nifS*	Nitrogenase metalloclusters biosynthesis protein		−2.8
	bsr1757*	*fixU*	Nitrogen fixation protein		−3.2
blr1759*		*nifB*	FeMo cofactor biosynthesis protein		−2.2
	bsr1760*	*frxA*	Ferredoxin-like protein		−2.0
bll1767*		id121	Hypothetical protein		−2.4
blr1769*		*nifH*	Dinitrogenase reductase protein		−3.9
blr1770*		*nifQ*	Molybdenum processing protein		−2.6
bll1777*		*ahpC*	Alkyl hydroperoxide reductase		−2.5
bll1791		id172	Hypothetical protein		2.2
blr1850*			Unknown protein		−2.9
bll1872*			Hypothetical protein		−2.5
bll1906*			*N*-acetyltransferase NrgA homolog		−4.7
bll1944*			Hypothetical protein		−2.9
blr1964*		id568	Putative sugar hydrolase		−2.1
bsr2010*			Unknown protein		−2.4
blr2038*		*fixA*	Electron transfer flavoprotein beta chain		−2.7
bll2060*		*groES*$_3$	GroES3 chaperonin		−3.1
	bll2059*	*groEL*$_3$	GroEL3 chaperonin		−2.1
bll2063*		*nrgC*	Phenolhydroxylase homolog		−2.5
blr2071		id841	Similar to inosamine-phosphate amidinotransferase		2.8
blr2106*		*ectC*	L-Ectoine synthase		−10.0
blr2131*			Probable oxygenase		−5.6
	blr2132*		Unknown protein		−4.1
	blr2133*		Hypothetical protein		−3.5
	blr2134*		Hypothetical protein		−2.8
	blr2135*		Hypothetical protein		−2.4
	blr2136*		Putative aminotransferase		−2.0
blr2144*		*cyp112*	Cytochrome P-450 BJ-1		−5.0
	blr2145*	*cyp114*	Cytochrome P-450 BJ-3		−2.3
blr2147*		*cyp117*	Cytochrome P-450 BJ-4		−2.2
blr2148*			Farnesyl diphosphate synthase		−3.0
	blr2149*		Hypothetical protein		−2.8
blr2429			Unknown protein		−2.0
bll2446			Hypothetical protein		2.1
blr2474			Hypothetical protein		2.9
blr2475		*tspO*	Tryptophan-rich sensory protein		2.4
	blr2476		Hypothetical protein		2.1
blr2696			Cytochrome *c* peroxidase		2.8
	blr2697	*virA*	VirA-like protein		3.9
blr2721		*htrA*	Probable serine protease do-like precursor		2.2
bll3807		*msbB*	Lipid A biosynthesis lauroyl acyltransferase		2.1
blr3848			Hypothetical protein		−2.1
blr4046			Unknown protein		2.4

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TABLE 2—*Continued*

Class and gene no.[b]	Putative operon member (gene no.)[b,c]	Gene name[d]	Description	Relative change in expression (*n*-fold) under the indicated condition[e]	
				21% O_2	0.5% O_2
bll4166			Unknown protein		−2.0
bll4282			Probable acetoacetyl-coenzyme A synthetase		−2.1
bll4326			Putative methyl-accepting chemotaxis protein		−2.5
bll4391			Unknown protein		−2.0
blr4450			Hypothetical protein		−2.3
bll4816			Unknown protein		2.1
blr5025			Hypothetical protein		−2.0
blr5221		*hspF*	Small heat shock protein		2.0
blr5346			Putative hydrolase		2.2
bll5475			Putative formate dehydrogenase		−3.4
bll5476			Formate dehydrogenase iron-sulfur subunit		−5.3
bll5480			Putative chaperone		−4.1
	bsl5479		Hypothetical protein		−4.7
blr5556			Hypothetical protein		−2.4
blr5637			Unknown protein		2.2
blr5712			Hypothetical protein		2.2
blr5768			Unknown protein		2.3
blr6103			Putative oxygenase		3.0
blr6578			ABC transporter permease protein		2.7
	blr6579		ABC transporter ATP-binding protein		2.6
bsl6617			Unknown protein		2.9
bll6747			Unknown protein		−2.5
blr6846			Two-component response regulator		−3.2
bll6848			Hypothetical protein		−2.6
	bll6847		Hypothetical protein		−2.4
bll6854		*flbT2*	Flagellin synthesis repressor protein		−2.6
	bll6853	*flgD*	Hook formation protein		−3.4
	bsl6852	*fliQ*	Flagellar biosynthetic protein		−2.2
bll6858		*flgE*	Flagellar hook protein		−3.8
	bll6857	*flgK*	Hook associated protein I homolog		−3.4
	bll6856	*flgL*	Probable flagellar hook-associated protein		−3.7
bll6864		*fliF*	Flagellar M-ring protein		−2.7
	bll6863		Unknown protein		−2.4
	bll6862	*motB*	Probable flagellar motor protein		−3.3
	bll6861	*motC*	Probable chemotaxis protein precursor		−2.7
	bll6860		Unknown protein		−2.6
	bll6859		Hypothetical protein		−2.7
bll6865		*fla*	Flagellin		−3.3
bll6866		*fla*	Flagellin		−2.5
bll6867		*fliP*	Flagellar biosynthetic protein		−2.1
bll6876		*flgB*	Flagellar basal-body rod protein		−3.4
	bll6875	*flgC*	Flagellar basal-body rod protein		−4.4
	bll6874	*fliE2*	Flagellar hook-basal body complex protein		−3.3
	bll6873	*flgG*	Flagellar basal-body rod protein		−3.3
	bll6872	*flgA*	Probable flagellar protein		−2.5
	bll6871	*flgI2*	Flagellar P-ring protein precursor		−4.5
	bll6870		Hypothetical protein		−2.1
	bll6869	*flgH2*	Flagellar L-ring protein precursor		−2.8
bll6879		*fliN*	Probable flagellar motor switch protein		−4.0
	bll6878	*fliG*	Probable flagellar motor switch protein		−2.5
	bll6877	*flhB*	Flagellar biosynthetic protein		−2.5
bll6882		*motA*	Chemotaxis protein		−3.0
	bll6881		Hypothetical protein		−3.2
blr6883			Hypothetical protein		−3.2
blr6951*		*modA*	Molybdenum ABC transporter; Molybdate-binding protein		−8.9
	blr6952*	*modB*	Molybdenum ABC transporter permease protein		−4.9
bll7861			Putative rhizopine catabolism protein		2.0
bll7938			Hypothetical protein		−3.8
bll7952			Probable selenium-binding protein		−2.5

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TABLE 2—*Continued*

Class and gene no.[b]	Putative operon member (gene no.)[b,c]	Gene name[d]	Description	Relative change in expression (*n*-fold) under the indicated condition[e]	
				21% O_2	0.5% O_2
Class 3 (regulated by RegR only under oxic conditions)					
bll0246		*bam*	Indoleacetamide hydrolase	−2.4	
bsr0247			Unknown protein	−2.0	
blr0257			Two-component sensor histidine kinase	−2.3	
bll0375			Probable alginate *O*-acetyltransferase	−2.1	
bll0597			Cytochrome b561 family protein	−2.6	
bll0805			Hypothetical protein	−2.2	
bsr1232			Hypothetical protein	2.5	
blr1307			Hypothetical protein	−2.0	
bll1476		*cysD*	Sulfate adenylate transferase subunit 2	2.8	
bsr1514			Unknown protein	−2.3	
blr2037		*nifA*	*nif*-specific regulatory protein	−3.6	
blr2074		id855	NoeE homolog	2.1	
bsl2086			Unknown protein	−2.7	
bll2109			Transcriptional regulatory protein Crp family	−3.1	
bll2158			Unknown protein	−2.0	
blr2815			Putative transketolase family protein	−2.4	
blr2891			Putative phenylacetic acid degradation protein	2.8	
bsr2892		*paaB*	Phenylacetic acid degradation protein	3.0	
blr2893		*paaC*	Putative phenylacetic acid degradation protein	2.9	
	blr2895	*paaE*	Putative ferredoxin reductase electron transfer component protein	2.2	
	blr2896	*paaI*	Phenylacetic acid degradation protein	2.3	
	blr2897	*paaK*	phenylacetate-coenzyme A ligase	2.6	
blr2976			Putative methyl accepting chemotaxis protein	−2.2	
bll3049			Hypothetical protein	−2.1	
bll3148			Hypothetical protein	−2.0	
bll3150			Putative Oxalate:formate antiporter	−3.8	
blr3159			Hypothetical protein	−2.7	
blr3166		*gcl*	Putative glyoxylate carboligase protein	−12.7	
	blr3167	*hyi*	Putative hydroxypyruvate isomerase protein	−36.4	
	blr3168		Oxidoredutase	−12.0	
blr3169			Hypothetical protein	−5.8	
blr3188			Unknown protein	2.3	
bsr3237			Unknown protein	2.6	
bll3759			Hypothetical protein	−3.4	
bll3872			HlyD family secretion protein	2.0	
bll3937			Hypothetical protein	−2.0	
blr4028			Putative RNA polymerase	2.7	
blr4176			Unknown protein	−2.2	
bll4201			Unknown protein	3.1	
blr4261			Hypothetical protein	2.2	
bll4394			Hypothetical protein	2.0	
blr4695		*kup3*	Potassium uptake protein	2.3	
bll4784			Aldehyde dehydrogenase	−2.2	
bll5217			Probable glycosyl transferase	2.5	
bll5218			Unknown protein	2.5	
bll5219			Small heat shock protein	2.5	
bsl5225			Unknown protein	2.4	
blr5226			Heat shock protein	2.7	
	blr5227	*groEL*$_1$	Heat shock protein	2.5	
blr5228			Unknown protein	2.4	
	blr5229		Unknown protein	5.4	
blr5231		*rpoH*$_1$	sigma32-like factor	2.2	
blr5233		*hspB*	Small heat shock protein	2.9	
	blr5234	*hspC*	Small heat shock protein	2.2	
blr5730			Hypothetical protein	−2.3	

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TABLE 2—*Continued*

Class and gene no.[b]	Putative operon member (gene no.)[b,c]	Gene name[d]	Description	Relative change in expression (*n*-fold) under the indicated condition[e]	
				21% O_2	0.5% O_2
bll5890			Monocarboxylic acid permease	−3.3	
bsl5891			Hypothetical protein	−4.9	
blr6211			Unknown protein	−2.3	
bll6262		*osmC*	Probable osmotically inducible protein	−2.2	
	bll6261		Hypothetical protein	−2.5	
bll6851		*flhA*	Flagellar biosynthesis protein	−2.0	
blr6885		*fliI*	Flagellum-specific ATP synthase	−2.2	
bll6890			Unknown protein	−3.2	
bsl6958			Hypothetical protein	2.6	
blr7064			Probable ABC transporter substrate-binding protein	−2.2	
blr7102			Unknown protein	−2.5	
blr7589			Putative oxidoreductase	3.7	
blr7780			Hypothetical protein	−3.6	
blr7813			Transcriptional regulatory protein GntR family	2.1	
blr7848			Probable substrate-binding protein	−2.3	
bll7969			Putative dihydrodipicolinate synthase	−2.3	

[a] Both the Δ*regR* and wild-type strains were grown under under free-living, oxic (21% O_2) or microoxic (0.5% O_2) conditions.
[b] Genes in boldface are induced in the *B. japonicum* wild-type strain upon a switch from oxic to microoxic conditions; genes in italics are down-regulated (45). Genes labeled with asterisks are known or putative NifA targets (25).
[c] Operon predictions were done as described in Materials and Methods.
[d] Gene names are according to the EMBL-EBI database.
[e] Gene expression changes (*n*-fold) are average values retrieved by microarray analysis of five biological replicates of the *B. japonicum* wild type and Δ*regR* mutant grown under oxic (21% O_2) and microoxic (0.5% O_2) conditions.

for the metabolism of C_2/C_3 carbon compounds in *E. coli* (8). The observed regulatory pattern suggests that RegR strongly activates expression of these genes only under oxic conditions. Diminished mRNA levels in *B. japonicum* were confirmed by quantitative reverse transcription-PCR with relative change values of 25.5-fold (blr3166), 31.7-fold (blr3167), and 35.2-fold (blr3168) between wild type and the *regR* mutant.

Thirty-one genes of class 3 are more highly expressed in Δ*regR* cells, e.g., the *paa* genes (blr2891 to blr2897) encoding enzymes involved in phenylacetic acid degradation. This pathway serves to degrade aromatic compounds in several gram-negative bacteria (26) and might play a role in the catabolism of plant-derived flavonoids in *B. japonicum*, preferably under low-oxygen conditions where the apparent negative regulatory effect of RegR is abrogated (45).

Figure 1B shows that the 126 genes with decreased expression in Δ*regR* cells under microoxic conditions include 49 genes that are induced in culture-grown *B. japonicum* wild-type cells under the same conditions (45), which corresponds to 8% (49/620) of all low-oxygen-responsive genes identified. Notably, 39 of these 49 genes are known or putative targets of the RegR subsidiary NifA protein which activates transcription of σ^{54} (RpoN)-dependent promoters (marked with asterisks in Table 2). The remaining 10 genes are candidate targets for a redox control mediated either directly via RegR or via regulatory proteins other than NifA, e.g., $FixK_2$.

The identification of RegR-dependent genes that were upregulated in wild-type *B. japonicum* cells exposed to either an oxic or a microoxic environment suggests that the RegSR system is somehow involved in sensing different ambient oxygen conditions. Yet this finding does not indicate whether the upregulation is a response to the cellular redox status or to oxygen per se. By analogy with the well-elaborated sensing mechanism of the orthologous two-component regulatory systems RegBA in *R. capsulatus* or the ArcBA system in *E. coli*, it seems attractive to speculate that the redox state of the membrane-localized quinone pool is an important cue also for *B. japonicum* RegSR (32, 33, 55). Alternatively, electron flow through the electron transport chain might play a role in modulating the activity of RegSR similar to the proposed model for control of the PrrBA system in *R. sphaeroides* by cytochrome cbb_3 oxidase (29, 42).

Transcription profiling of the Δ*regR* strain in symbiosis. Transcriptome data retrieved from the comparison between Δ*regR* bacteroids and wild-type bacteroids at 13 and 21 dpi revealed 1,224 genes (511 genes belonging to 311 putative operons) with differential expression at both time points as compiled in Table S1 in the supplemental material and illustrated in Fig. 1A. For comparison, Table S1 in the supplemental material also contains information on (i) whether these genes are induced or repressed in wild-type bacteroids at 21 dpi compared with free-living aerobically grown wild-type cells (45) and (ii) whether these genes were also differentially expressed in the comparison between wild-type and Δ*regR* cells grown in culture.

The large majority (84%) of the differentially expressed genes in bacteroids are activated by RegR (decreased expression in the mutant). This meets the expectation that response regulators of this global family act predominantly as activators (12). By contrast, a recent microarray analysis of an *R. sphaeroides prrA* mutant grown under anoxic conditions revealed that 60% of the differentially expressed genes were subject to

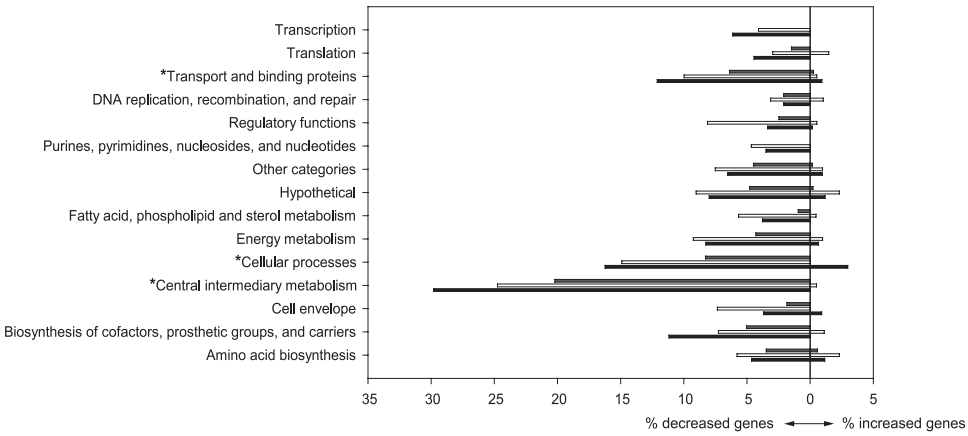

FIG. 2. Functional categories of differentially expressed genes between wild-type and *regR* mutant bacteroids according to the Kazusa annotation (27). Percentages of genes with decreased or increased expression in comparisons between wild-type and Δ*regR* bacteroids at 13 dpi (black columns), 21 dpi (white columns), or both time points during symbiosis (gray columns) were calculated by dividing the number of differentially expressed genes in each category by the total number of genes in each category. Overrepresented categories are labeled with asterisks.

negative control by PrrA (34). This indicates that there are substantial differences in the regulatory mode of these regulators in *B. japonicum* and *R. sphaeroides*.

Only 7% of the 1,224 genes were also controlled by RegR under low-oxygen conditions (Fig. 1A), suggesting that a much wider spectrum of functions is affected by RegR in symbiosis than in free-living conditions. A group of 31 genes (12 genes belonging to 9 putative operons) revealed the same regulatory pattern under all of the conditions investigated in this work (Fig. 1A; see also Table S1 in the supplemental material). As an example of this group, bll2087 (unknown function) was further analyzed by mutagenesis. The bll2087 deletion strains 9537 and 9538 showed a wild-type phenotype with regard to the number and dry weight of nodules and nitrogen fixation (acetylene reduction) activity in symbiosis with soybean (data not shown).

Only about half of the RegR-dependent genes in young (13 dpi) and mature (21 dpi) bacteroids were affected in their expression at both time points (Fig. 1C). Remarkably, 29% of the genes in this overlap map to the so-called 681-kb symbiotic island which comprises 7% of the *B. japonicum* genome (27). A large number of genes from this region are also found among those genes which are RegR-controlled only in young bacteroids (83/354).

From the set of 692 genes induced in wild-type bacteroids compared to aerobically grown cells (45), 54% (378/692) were identified as members of the RegR regulon at 21 dpi (Fig. 1C). This confirms that RegR is an important regulator of genes related to the symbiotic lifestyle. About one-third of these genes (113/378) were known from previous work to be regulated by NifA and RpoN under anoxic conditions, 51 of which are likely direct targets (25). Assuming that the full regulatory scope of NifA was uncovered in the previous study (25), the remaining 265 (378 minus 113) RegR-dependent bacteroid-induced genes (21 dpi) are controlled directly or indirectly by RegR but independently of NifA. Interestingly, 45% (118/265) of these RegR-dependent genes are at the same time controlled by RpoN in mature bacteroids (45). This coregulation is most likely indirect through RegR-dependent transcription factors other than NifA that interact with the RNA polymerase-σ^{54} complex in symbiosis. A candidate is the Fis-type transcriptional regulator encoded by blr5735, which harbors a predicted RpoN-interacting domain and is induced in bacteroids.

Of note, a large proportion (55%) of the differentially expressed genes in bacteroids displayed only a moderate regulation by RegR (relative change of two- to threefold) while retaining significant expression in the absence of RegR. This might reflect either constitutive expression or coactivation by other regulators. Given the considerable size and diversity of the RegR regulon, it is not unexpected to encounter coregulation of RegR with other, more specific regulators that function in individual branches of the RegR regulon. Such coregulators also may account for the different expression levels of individual RegR-dependent genes in young and mature bacteroids. In fact, the involvement of additional regulators that integrate signals other than the redox status sensed by RegB seems to be common at RegA-dependent promoters in *R. capsulatus* (12, 22).

Overrepresented gene categories in the symbiotic RegR regulon. The RegR-controlled genes that were differentially expressed at 13 and 21 dpi were grouped into 15 categories as defined by Kaneko et al. (31) (Fig. 2). Fifty percent of the genes (611/1,224) have no assigned function or encode hypothetical proteins, which does not allow us to draw conclusions about their physiological roles. Among the 613 remaining genes, three functional categories were significantly overrepresented at 13 dpi and/or 21 dpi: (i) cellular processes, (ii) transport and binding proteins, and (iii) central intermediary metabolism.

(i) Cellular processes. Genes belonging to the cellular processes category were overrepresented in young bacteroids. For example, genes encoding heat shock proteins (*hspABC*) or enzymes involved in detoxification of reactive oxygen species (*ahpCD*) were expressed at reduced levels in Δ*regR* bacteroids. These genes are strongly induced in wild-type bacteroids compared to free-living cells, possibly in response to oxidative stress generated in nodules (44, 45).

TABLE 3. RegR-controlled *B. japonicum* genes whose promoter region was tested for RegR binding in electrophoretic mobility shift assays

Gene no.[a]	Gene name	Description	Genomic region[b]	Shift[c]	Putative operon members[d]
bll0597		Cytochrome *b561* family protein	−150 to +179	+	
bll1285*		Unknown protein	−211 to −59	++	
bll1858		Hypothetical protein	−217 to +14	−	
bll2109		Transcriptional regulator CRP family	−55 to +179	++	
bll2125		Taurine dioxygenase	−313 to −15	−	
bll2268		Putative xylose operon repressor	−168 to +32	++	
bll3193		Transcriptional regulator CRP family	−323 to −8	+	
bll3363		Unknown protein	−146 to +60	+	
bll4130		Transcriptional regulator LysR family	−323 to +20	+	
bll4833*		Unknown protein	−186 to +92	++	
bll5480		Putative chaperone	−216 to +109	−	bsl5479, **bll5478**, **bll5477**, bll5476, bll5475
bll5805		Transcriptional regulator CRP family	−278 to +66	−	
bll5807*		Hypothetical protein	−191 to +235	++	
bll6513		Hypothetical protein	−184 to −24	++	
bll6633		Unknown protein	−212 to +67	++	
blr1515*	*acrA*	RND multidrug efflux membrane permease	−167 to +17	++	**blr1516**
blr1853		Cytochrome P450 family protein	−264 to +21	−	
blr1883	*rpoN*$_1$	RNA polymerase sigma factor	−231 to −17	++	
blr2501*		Unknown protein	−235 to +9	++	
blr2614		Sodium/hydrogen exchanger	−172 to +15	++	
blr3166	*gcl*	Glyoxylate carboligase	−463 to +2	−	blr3167, blr3168
blr3769		Hypothetical protein	−212 to +8	−	blr3770, **blr3771**
blr4182		Transcriptional regulator	−230 to +34	++	
blr5693		Probable substrate-binding protein	−163 to +36	++	
blr6267		Transcriptional regulator	−233 to +48	++	
blr6918		Probable substrate-binding protein	−230 to +60	+	
blr7589		Indolepyruvate ferredoxin oxidoreductase	−354 to −18	+	
blr7905	*cit*	Citrate-proton symporter	−163 to +11	++	
bsl4167		Putative glutamine synthetase translation inhibitor	−601 to +27	++	
bll2087*[e]		Unknown protein	−82 to +23	++	

[a] Genes whose promoter regions were tested for RegR binding. Genes in boldface are differentially expressed by a factor ≥2 in the comparison of the wild type with the Δ*regR* strain under all conditions analyzed in this work (free-living and symbiotic growth). All other genes were differentially expressed in the comparison under either free-living or symbiotic conditions. Genes for which in vitro transcription was shown using purified *B. japonicum* RNAP and RegR protein (Fig. 6) are marked with asterisks.

[b] Genomic region included in the PCR fragment used for EMSAs. Coordinates refer to the first nucleotide position of the annotated translation start site of the gene listed in column 1.

[c] Indicates qualitatively whether RegR binding was strong (++), weak (+), or absent (−).

[d] Operon predictions were done as described in Materials and Methods. Genes in boldface are differentially expressed under all conditions analyzed in this work (free-living and symbiotic), and the promoters of the genes listed in column 1 were tested for RegR binding.

[e] RegR binding to the promoter region of bll2087 was shown in a previous work (24).

(ii) Transport and binding proteins. Transport and binding proteins are overrepresented only in the data set retrieved from young bacteroids. Seventy-five percent of the RegR-dependent transport and binding proteins encode components of ABC-type transport systems, several of which are strongly induced in the wild type during symbiosis.

(iii) Central intermediary metabolism. The category of central intermediary metabolism genes includes nitrogen fixation genes and hydrogenase genes which showed reduced expression in Δ*regR* bacteroids. Expression of the genes encoding the high-affinity cbb_3-type terminal oxidase (*fixNOQP*) and *rpoN*$_1$, both known targets of FixLJ-FixK$_2$ (41), was RegR dependent at 13 dpi, pointing to a hitherto undescribed link between the RegSR-NifA and FixLJ-FixK$_2$ regulatory cascades. Notably, also in *S. meliloti*, where the nitrogen fixation regulatory genes *nifA* and *fixK* are under control of the FixLJ proteins (11), ActR sets an additional level of control over these genes in response to low pH and low-oxygen conditions (57).

More than 60 genes which are RegR-dependently expressed in bacteroids are involved in transcription regulation, >30 of which are specific for the RegR regulon in mature bacteroids. This points toward an extensive expansion of the RegR regulon concept. New hierarchical cascades, similar to the RegSR-NifA cascade, in which additional environmental stimuli are sensed and transduced to specific subgroups of target genes are highly conceivable. For example, the Crp-like protein encoded by bll2109 might be a member of such a cascade. Interestingly, knockout mutations in the bll2109 gene (strains 9552 and 9553) caused a two-day delay in anaerobic growth of *B. japonicum* with nitrate as the terminal electron acceptor (data not shown). Since denitrification genes in *B. japonicum* are governed by the FixLJ-FixK$_2$-NnrR cascade (37), the peculiar bll2109 mutant phenotype could be interpreted as a cross-pathway coregulation of the latter cascade via the RegR-dependent control of bll2109.

RegR binds directly to the promoter regions of new target genes. Because microarray analysis does not allow differentiation between directly and indirectly controlled genes, we performed DNA binding studies to identify direct RegR target genes. In addition to 14 other promoters, we selected for further studies 16 promoters (belonging either to single genes or operons) from the group of 31 RegR-controlled genes which

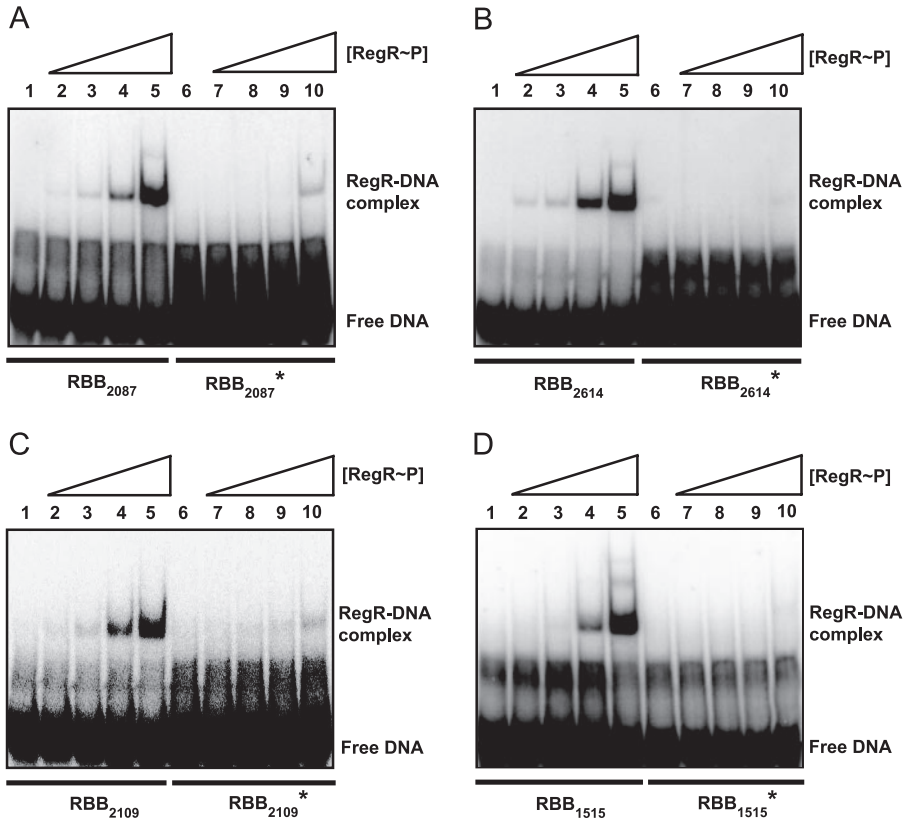

FIG. 3. Analysis of RegR binding to RBBs in promoter regions of RegR-controlled genes using EMSAs. Increasing amounts of purified RegR and RegR~P were incubated with constant amounts of double-stranded ^{32}P-labeled oligonucleotides carrying RBBs derived from promoter regions of genes bll2087 (A), blr2614 (B), bll2109 (C), and blr1515 (D). RBBs with wild-type sequences (RBB_x, lanes 1 to 5) and RBBs with a G-to-T exchange (RBB_x*, lanes 6 to 10) (compare with Fig. 4 and 5) were applied each at a concentration of approximately 1 nM. RegR protein concentrations were 0.07 μM (lanes 2 and 7), 0.2 μM (lanes 3 and 8), 0.6 μM (lanes 5 and 9), and 1.8 μM (lanes 6 and 10). No RegR protein was added to the control reactions in lanes 1 and 6. Samples were run on 6% nondenaturing polyacrylamide gels and visualized with a phosphorimager.

are differentially expressed under all conditions (Fig. 1A; see also Table S1 in the supplemental material). The latter group was assumed to contain a maximal number of direct RegR targets.

Under our experimental conditions, RegR bound to 23 of 30 investigated promoter regions that were amplified by PCR, albeit with different apparent affinities (Table 3). Notably, except for one gene (blr3769), the genes that are RegR controlled under all environmental conditions displayed consistent RegR binding to their upstream DNA regions, including the promoter region of blr7905 whose expression is negatively affected by RegR. To narrow the regions of RegR-DNA interaction, we tested RegR binding to ^{32}P-labeled double-stranded oligonucleotides (30 to 35 bp) derived from 13 promoters (out of 23 RegR binders), comprising DNA sequences potentially recognized by RegR (RegR binding boxes [RBB]) (16). Binding was observed to DNA probes originating from promoter regions of the positively controlled genes: blr1515, blr2614, bll2109, blr1883 ($rpoN_1$), blr4182, blr2501, and bll2087 (data not shown). RegR binding to the latter gene (bll2087) confirms a previous, preliminary result (24). Two RegR binding sites were mapped upstream of blr2501 (RBB sites 1 and 2 of blr2501, designated $RBB_{2501\text{-}1}$ and $RBB_{2501\text{-}2}$, respectively).

In-depth analyses were subsequently performed for four genes in order to determine RegR binding specificity to the respective DNA targets (Fig. 3). While a shifted band was already detectable for RBB_{2087}, RBB_{2614}, and RBB_{2109} upon addition of a 67- to 200-fold molar excess (0.07 to 0.2 μM) of phosphorylated RegR (RegR~P) (Fig. 3A, B, and C, lanes 2 and 3), a shift for RBB_{1515} was observed only upon addition of 600-fold excess (0.6 μM) of protein (Fig. 3D, lane 4). By analogy with a previous study (15), the implication of a conserved guanine residue in these RBBs (Fig. 4) for RegR binding was investigated with point mutations (G-to-T exchange). When mutant derivatives RBB_{2087}*, RBB_{2614}*, RBB_{2109}*, and RBB_{1515}* were used as targets, RegR binding was strongly diminished (Fig. 3A to D, lanes 6 to 10), demonstrating that this G residue is indeed critical for RegR-DNA interaction.

An alignment of the eight experimentally determined RBBs together with the previously identified *fixR* upstream activation sequence (15, 56) is shown in Fig. 4. A common element in the DNA sequences is an imperfect inverted repeat consisting of (T/C)G(C/T)GNC and GNCNC, which is separated by an AT-rich spacing of 3 to 5 nt. This element differs from the RegR box [NGNG(A/G)C(A/G)TTNNGNCGC] that had previously been elaborated with a SELEX binding-site selection assay in our group (15) because a variable spacing and a more degenerate right-half site (GNCNC) had to be taken into account. It

```
bll2087          AACGTATGTCGC-ttt-GTCGCGTTGA
blr2501_1        TTGACTCGCGCA-tattGTCGCAGCCC
                          *
bll2109          GCGCACTGCGTCacaatGACACGACTT
blr1515          AGGTCATGTGACattggGCCGCCCGCG
blr1883 (rpoN1)  TGCCCTCGCGTCacagtGCCGCATCCG
blr2036 (fixR)   CGCGTGCGCGACattagGACGCAAAAC
blr2501_2        GTGACTTGCGGCaaattGACCAATTCA
blr2614          TCCGCATGTGACgaattGCTCGCTTGC
blr4182          CCAAGATGTGACtgactGTCACATGGA
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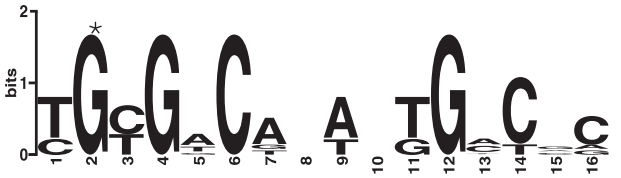

FIG. 4. Comparison of DNA sequences included in the oligonucleotides used for EMSAs in this work (Fig. 3). The RegR binding site present in the *fixR* promoter region is included (15, 56). Shown is the minimal overlap of the oligonucleotides upon alignment with ClustalW plus manual refinements. Nucleotides in reverse capital letters represent the GC-rich putative half-sites of the RegR binding motifs; nucleotides in lowercase letters indicate the AT-rich spacer (variable in number) between the left and right half-sites. Note that gaps were introduced manually in the central portion of the two motifs shown at the top. In the lower part of this figure, the consensus sequence for the RegR binding site is shown based on the underlined positions of the seven ungapped DNA sequences provided in the upper part. The sequence logo was created using WebLogo (10). The conserved G that was mutated to a T in the experiment shown in Fig. 3 is marked with an asterisk in both parts of the figure.

is, however, rather similar to the (C/T)(G/C)CGG(C/G)-N_{0-10}-G(T/A)C(G/A)(C/A) motif that has been proposed for the PrrA transcriptional regulator based on a bioinformatics approach (34).

The similarly high G+C content of the RegR binding site and the *B. japonicum* genome sequence (64.1%) plus the variably spaced half-sites of RBBs impair in silico motif searches in the *B. japonicum* genome. Nevertheless, we have used a matrix based on the seven experimentally verified RBBs which share the 5-nt spacing between the half-sites (Fig. 4) to search for similar motifs in putative promoter regions of the entire *B. japonicum* genome (see Materials and Methods). The cutoff threshold was defined by RBB_{2614} which showed the lowest score of all RBBs used for generating the matrix. Using this strategy, putative RegR binding motifs were identified within 500 bp upstream of 226 individual genes or operons. Remarkably, 47 of them (41 new plus 6 previously known promoters) are associated with genes or operons whose expression is positively regulated by RegR at least under one of the conditions tested in this study (see Table S2 in the supplemental material). The fact that we identified only a minor fraction of RegR-regulated promoters can be explained by (i) our stringent definition of the cutoff value, (ii) the restriction to motifs with a 5-nt spacer, and (iii) indirect control via RegR-dependent regulators (e.g., NifA).

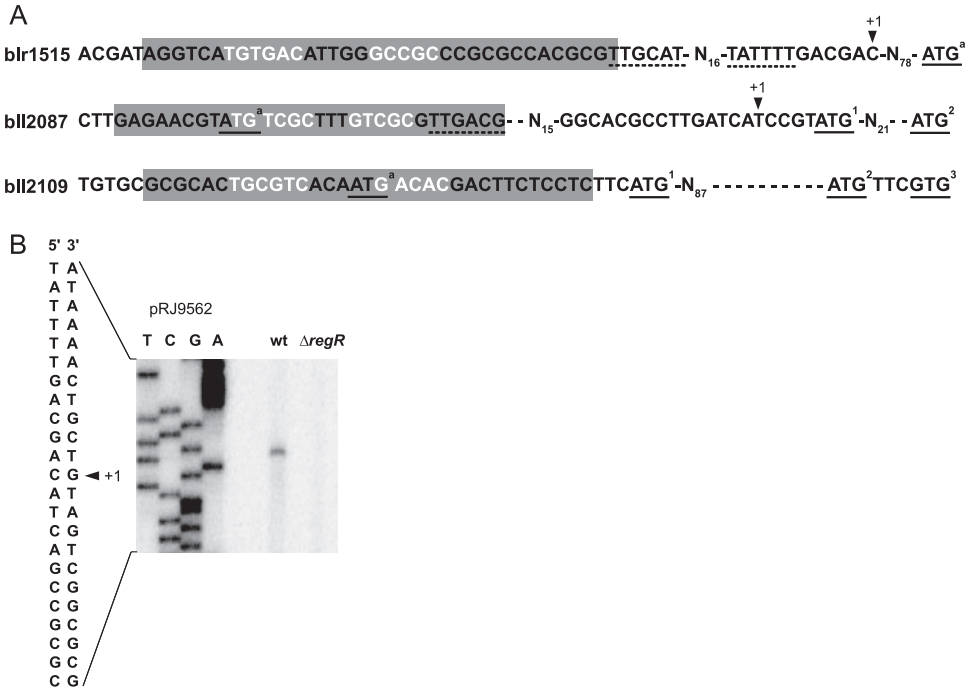

FIG. 5. Analysis of the promoter regions of selected RegR-dependent genes. (A) DNA sequence comparison of the blr1515, bll2087, and bll2109 promoter regions. Gray boxes mark the oligonucleotide sequences used in EMSAs (Fig. 3), and nucleotides that form the putative RegR binding sites are shown in white letters. Transcriptional start sites (+1) are indicated by an arrowhead above the blr1515 and bll2087 promoter sequences. Putative −10 and −35 promoter elements are underscored by dashed lines. Start codons annotated in the *B. japonicum* genome database (http://www.kazusa.or.jp/rhizobase) are shown as ATG[a], whereas underlined codons with superscripts 1, 2, or 3 denote putative alternative translation start sites. (B) Transcription start site mapping of blr1515. RNA for primer extension was isolated from aerobically grown *B. japonicum* wild-type (wt) and Δ*regR* cells. Extension products obtained with the ^{32}P-labeled primer 1515-P1 were separated on a 6% denaturing polyacrylamide gel loaded next to a sequencing ladder generated with plasmid pRJ9562 and the same primer. The relevant sequence of the blr1515 promoter region is shown on the left with the transcription start site emphasized (+1, arrowhead).

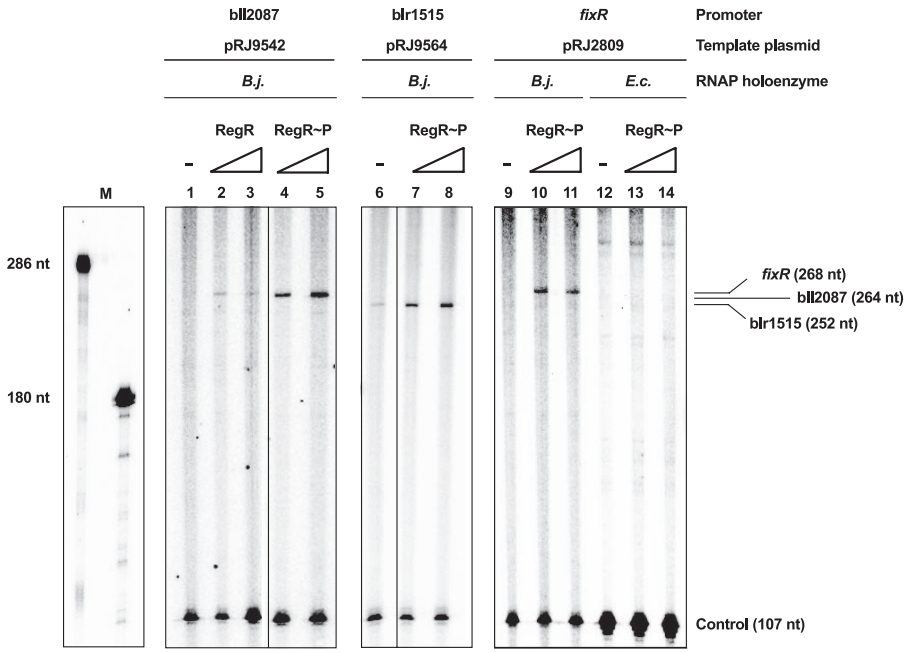

FIG. 6. In vitro transcription activated by RegR. Transcripts from the template plasmids pRJ9542, pRJ9564, and pR2809 comprising the bll2087, blr1515, and *fixR* promoter regions, respectively, were synthesized by multiple-round in vitro transcription using purified *B. japonicum* (*B.j.*) or *E. coli* (*E.c.*) RNAP holoenzyme and either untreated RegR or RegR~P, as indicated. RegR(~P) concentrations were as follows: no protein (lanes 1, 6, 9, and 12), 1 μM (lanes 2, 4, 7, 10, and 13), and 3 μM (lanes 3, 5, 8, 11, and 14). Transcripts were separated on 6% polyacrylamide gels and visualized with a phosphorimager. RNA size markers (M) were generated as described in Materials and Methods. The expected sizes of specific transcripts and of the control transcript are indicated on the right. The transcription products generated from the individual template plasmids were run on separate gels.

Location of RegR binding sites in individual promoter regions. The new RegR binders determined above were examined more closely with respect to the distance between RBB and the annotated translation start site. In six of the eight cases (RBB_{1515}, RBB_{1883}, RBB_{2614}, RBB_{4182}, $RBB_{2501\text{-}1}$, and $RBB_{2501\text{-}2}$), the RBBs are located in a reasonable distance relative to the putative start codon (−45 to −223 bp) so that a promoter sequence can be accommodated in between and that the RBB can function as an upstream activating sequence for transcriptional regulation of the downstream gene. We sought for confirmation of this inference in at least one case by mapping the transcription start site of blr1515 (Fig. 5A and B). A prominent cDNA as the primer extension product was obtained with wild-type RNA but not with RNA extracted from Δ*regR* cells (Fig. 5B), thus validating the RegR-dependent expression seen in microarrays. The mapped blr1515 transcription start site is positioned 53 nucleotides downstream of the center of RBB_{1515}. Putative −35/−10 promoter elements were also identified (Fig. 5A).

The location of RegR binding sites was exceptional in two cases (RBB_{2087} and RBB_{2109}) where they overlapped with the annotated translation start sites of bll2087 and bll2109 (Fig. 5A). In fact, we had previously mapped the transcription start site of bll2087 downstream of the annotated start codon (24), which then placed RBB_{2087} at position −44 relative to the transcription start site and helped identify an alternative start codon for bll2087 (Fig. 5A).

Unfortunately, we did not succeed in mapping the bll2109 transcription start site. Yet from β-galactosidase activity assays with a set of translational bll2109′-′*lacZ* fusions, we obtained strong evidence that translation of bll2109 is initiated at one of the two alternative start codons, ATG^2 and GTG^3 (superscripts indicate alternative start codons) (Fig. 5A), located >100 bp downstream of the originally annotated start codon (data not shown). Hence, the distant location of RBB_{2109} to either ATG^2 or GTG^3 is more likely of functional relevance.

In vitro transcription of RegR target genes. To test direct target gene activation by RegR, we performed in vitro transcription assays with purified *B. japonicum* RNA polymerase (RNAP) and RegR protein. When a promoter-containing bll2087 fragment was used as a template, RNAP alone was unable to synthesize detectable amounts of specific transcript from this promoter (Fig. 6, lane 1), whereas the addition of purified, His-tagged RegR (Fig. 6, lanes 2 [1 μM RegR] and 3 [3 μM RegR]) resulted in detectable transcription, yielding a transcript of the expected 264-nt length. The use of equal amounts of RegR that had been phosphorylated with acetyl phosphate (RegR~P) led to a noticeable enhancement of the bll2087 transcript level (lanes 4 and 5), demonstrating that phosphorylation stimulates transcription activation activity of RegR. In all cases, transcript formation from the RNA I control promoter (107 nt) was synthesized independently of RegR.

In contrast to the bll2087 template, the blr1515 template was already transcribed at a basal level by RNAP in the absence of RegR (Fig. 6, lane 6); however, transcription was stimulated upon addition of RegR, and it was maximal when RegR~P was used, demonstrating that RegR acts as a direct transcriptional activator also at the blr1515 promoter (Fig. 6, lanes 7 and 8; also data not shown).

The third example shown in Fig. 6 is the well-known RegR target *fixR*: no in vitro transcript was detectable without RegR, whereas a transcript of the expected length (268 nt) was synthesized in the presence of RegR~P (Fig. 6, lanes 9 to 11). This demonstrates for the first time that RegR is sufficient to activate transcription from the *fixR* (P2) promoter. Applying primer extension, the transcription start points of the in vitro synthesized transcripts from the bll2087, blr1515, and *fixR* promoters were the same as determined in vivo for all three genes (data not shown).

Not shown in Fig. 6 is the equally efficient RegR-dependent in vitro activation of 4 out of 10 additionally tested promoters (those of bll1285, blr2501, bll4833, and bll5807). The absence of detectable transcripts synthesized from the blr1883 ($rpoN_1$), bll2109, blr2614, bll3193, blr6267, and bll6633 promoters might be explained by the requirement for either an alternative transcription factor, whose synthesis in vivo would then depend on RegR, or another factor not present in the in vitro reactions. Also, it cannot be ruled out that in these six cases the promoters are too weak to result in efficient transcription. Indeed, microarray signal intensities for transcripts originating from these promoters are lower than those for which in vitro transcription was easily detectable. Alternatively, the (cryptic) promoters might not be entirely present on the fragment cloned in the template plasmids.

The presence of putative −35/−10 promoter elements in the *fixR* promoter (3) and the bll2087 and blr1515 promoters (Fig. 5A) suggests that they are recognized by the *B. japonicum* primary sigma factor (σ^{80}) which is dominant in the RNAP preparation. In a previous work it was shown that promoter elements recognized by the *B. japonicum* RNAP-σ^{80} complex are similar to those recognized by *E. coli* RNAP-σ^{70} (6). Accordingly, *E. coli* RNAP-σ^{70} was able to transcribe from the *B. japonicum rrn* housekeeping promoter. However, as exemplified by the *fixR* promoter (Fig. 6, lanes 12 to 14), no transcripts corresponding to the transcripts produced by the *B. japonicum* RNAP were retrieved from all of the seven RegR-dependent promoters when *E. coli* RNAP was used in the in vitro assays. *E. coli* RNAP may not, therefore, be able to recognize these promoters and/or is unable to interact with RegR. In contrast, Karls and coworkers (28) have reported that a constitutively active mutant variant of RegA (RegA*) was able to activate in vitro transcription from the *cycA* P2 promoter, using either the *R. capsulatus* or the *E. coli* RNAP containing σ^{70}. The mutation is supposed to change RegA* conformation such that it mimics the phosphorylated state of wild-type RegA; however, it cannot be ruled out that the mutation facilitates RegA* interaction with *E. coli* RNAP, which may not work productively with RegA~P.

Concluding remarks. The results reported here have put forth a number of important facets of the global regulatory role played by the RegSR system in *B. japonicum*. We identified several genes encoding proteins involved in oxidative and reductive pathways as members of the RegR regulon. Yet there are RegR targets like the large group of transporters, which cannot be placed easily into the context of redox-related functions. Apparently, functional diversity of target genes is a common attribute of RegR-type regulators in the different proteobacterial species. Our data also demonstrate that RegSR is an important regulatory system for the *B. japonicum*-soybean symbiosis. Moreover, numerous genes were identified which are not at the same time dependent on the subordinate NifA protein, which strongly suggests that the symbiotic phenotype of the *regR* mutant cannot solely be attributed to its control of *nifA* expression. Such genes will be attractive targets for deletion-insertion mutagenesis in future work. Given the high number of regulatory genes that were identified as members of the RegR regulon, we now have to envisage a much greater complexity of regulatory networks in which RegR is integrated. Finally, a significant advancement was made in this study by the demonstration of direct activation of the *fixR-nifA* promoter as well as several novel promoters by RegR in vitro. The systematic application of these techniques to other candidates found by microarray analysis to be RegR dependent will presumably expand the bona fide RegR regulon even further.

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