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ORIGINAL PAPER

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Design and validation of a partial-genome microarray for transcriptional profiling of the *Bradyrhizobium japonicum* symbiotic gene region

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Abstract The design and use of a pilot microarray for transcriptome analysis of the symbiotic, nitrogen-fixing *Bradyrhizobium japonicum* is reported here. The custom-synthesized chip (Affymetrix GeneChip®) features 738 genes, more than half of which belong to a 400-kb chromosomal segment strongly associated with symbiosis-related functions. RNA was isolated following an optimized protocol from wild-type cells grown aerobically and microaerobically, and from cells of aerobically grown *regR* mutant and microaerobically grown *nifA* mutant. Comparative microarray analyses thus revealed genes that are transcribed in either a RegR- or a NifA-dependent manner plus genes whose expression depends on the cellular oxygen status. Several genes were newly identified as members of the RegR and NifA regulons, beyond genes, which had been known from previous work. A comprehensive transcription analysis was performed with one of the new RegR-controlled genes (*id880*). Expression levels determined by microarray analysis of selected NifA- and RegR-controlled genes corresponded well with quantitative real-time PCR data, demonstrating the high complementarity of microarray analysis to classical methods of gene expression analysis in *B. japonicum*. Nevertheless, several previously established members of the NifA regulon were not detected as transcribed genes by microarray analysis, confirming the potential pitfalls of this approach also observed by other authors. By and large, this pilot study has paved the way towards the genome-wide transcriptome analysis of the 9.1-Mb *B. japonicum* genome.

Keywords Gene chip · NifA · Nitrogen fixation · Oxygen control · Symbiosis

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Introduction

When rhizobia convert from free-living bacteria to N_2-fixing root-nodule endosymbionts, they experience a 10,000–fold decrease in oxygen concentration (Kuzma et al. 1993). This adaptation is being studied in our laboratory with *Bradyrhizobium japonicum*, the soybean symbiont. A complex network of signal transduction pathways senses oxygen deprivation and prepares the bacterium for life in micro-oxia. Several sensor-regulator systems are known to respond either to the cellular redox status or to differences in O_2 concentration, which correlate with the temporal order of events during nodule formation. These regulatory systems are organized in cascades as follows. (1) The FixLJ–$FixK_2$ regulatory cascade is activated at or below 5% O_2 in the gas phase (Nellen-Anthamatten et al. 1998; Sciotti et al. 2003). Among the induced target genes is the *fixNOQP* operon that encodes the cbb_3-type cytochrome oxidase, a proton-pumping oxidase with high affinity for O_2, which enables *B. japonicum* to respire in micro-oxia (Preisig et al. 1996b; Arslan et al. 2000). For specific metabolic needs, the FixLJ–$FixK_2$ pathway is expanded at the downstream end by one additional transcription factor (NnrR, $FixK_1$, or $RpoN_1$), resulting—for example—in a FixLJ–$FixK_2$–NnrR cascade (Mesa et al. 2003). (2) The RegSR–NifA cascade induces nitrogen fixation (*nif*) genes encoding the

nitrogenase complex (Fischer 1994; Bauer et al. 1998). The RegSR system does not appear to respond directly to oxygen, but rather indirectly to redox-related physiological parameters that remain enigmatic in *B. japonicum*. Activation of *nif* genes by the NifA protein requires an O_2 concentration below 0.5% in the gas phase (Sciotti et al. 2003). It thus seems clear that *B. japonicum* uses specific check-points to monitor and control its transition from free-living aerobe to endosymbiotic micro-aerobe, thereby ensuring that micro-oxic respiratory energy metabolism is established prior to the production of the energy-consuming and oxygen-sensitive nitrogenase.

We have a continuing interest in identifying as many as possible of the target genes that are induced under low-oxygen conditions in order to fully understand the scope of the above regulatory pathways in the context of symbiosis. In previous work, we have applied different techniques in searches for new NifA-dependent genes, such as differential RNA–DNA hybridization (Nienaber et al. 2000; Fischer et al. 2001), proteome analyses (Fischer et al. 1993; Dainese-Hatt et al. 1999; Nienaber et al. 2001), −24/−12-type promoter trapping (Weidenhaupt et al. 1993) and bioinformatics tools to recognize such promoters and their associated genes in DNA sequences (Caldelari Baumberger et al. 2003). Each of these approaches have expanded the list of known NifA-regulated genes, half of which are nitrogen fixation-specific, with others associated with more general functions related to microaerobic or anaerobic metabolism. Moreover, several lines of evidence from mutant phenotypes have long suggested the existence of yet more NifA-controlled *B. japonicum* genes awaiting detection (Fischer et al. 1986).

Transcriptome analysis with microarrays is the method of choice to study bacterial gene expression on a genome-wide scale today. Successful applications but also potential pitfalls of the use of microarrays in studies on transcriptional regulation and the identification of new regulons are already well known and critically discussed in the literature (Conway and Schoolnik 2003; Rhodius and LaRossa 2003). Yet, a growing number of publications provides strong evidence for the usefulness of microbial whole-genome expression profiling (Wagner et al. 2003; Roh et al. 2004), including recent reports on two rhizobial species, *Sinorhizobium meliloti* and *Mesorhizobium loti* (Barnett et al. 2004; Becker et al. 2004; Uchiumi et al. 2004). We have recently begun to evaluate microarray technology for expression studies in *B. japonicum*. For this purpose, a pilot oligonucleotide-based microarray was produced that represented all known *B. japonicum* DNA sequences as of June 2002, i.e., before publication of the entire 9.1-Mb genome sequence (Kaneko et al. 2002a). The set of genes selected for chip design included the 400-kb symbiotic gene region (Göttfert et al. 2001) as well as a sizeable number of genes scattered over the chromosome and whose sequences had also been deposited in databases. We report here on the results of the ensuing microarray pilot study, as a prerequisite optimization and validation step towards the use of the still costly and work-intensive microarray technology in *B. japonicum* on a genome-wide scale.

Materials and methods

Bacterial strains, media and growth conditions

PSY medium (Regensburger and Hennecke 1983) supplemented with 0.1% L-arabinose was used for aerobic and microaerobic cultivation (21% and 0.5% oxygen in the gas phase, respectively) of the *B. japonicum* wild-type strain 110*spc*4 (Regensburger and Hennecke 1983) and mutant strains 2426 (*ΔregR*; Bauer et al. 1998) and A9 (*ΔnifA*; Fischer et al. 1986). Aerobic cultures were grown with rigorous shaking (160 rpm) in 500-ml Erlenmeyer flasks containing 20 ml medium. Microaerobic cultures of 20 ml were grown in 500-ml rubber-stoppered serum bottles. The gas atmosphere (0.5% O_2, 99.5% N_2) was exchanged every 8 to 14 h in a total growth period of 31 to 50 h. When appropriate, media for growth of *B. japonicum* contained the following concentrations of antibiotics (μg ml^{-1}): spectinomycin 100, streptomycin 50 and kanamycin 100.

Chip design

Sequence data for *B. japonicum* annotated open reading frames (ORFs) were taken from Swiss-Prot and TrEMBL databases (as of June 2002). The set of sequences included the 388 genes and ORFs located in the so-called symbiotic gene region (Göttfert et al. 2001) plus all of the 350 additional *B. japonicum* gene or gene fragment sequences that had been deposited in databases up to that date. The nomenclature of genes located in the symbiotic gene region corresponds to that adopted by Göttfert et al. (2001), whereas all other genes corresponded to the designations in the databases specified above. As controls, 15 additional bacterial genes (e.g. antibiotic resistance genes, reporter genes) and 10 soybean genes (e.g. leghemoglobin and actin cDNA sequences) were included in the chip design. Each reading frame was represented by 13 probe pairs, and each probe pair consisted of a perfectly matching 25mer oligonucleotide and a 25mer mismatching oligonucleotide that contained one nucleotide exchange in the middle of the oligonucleotide. Remaining space on the gene chip was used to probe apparent intergenic regions of ≥40 bp on both DNA strands with probe pairs that were uniformly distributed at an average spacing of 40 nucleotides (center to center). Oligonucleotide design and manufacturing of the inkBJAPETH1F gene chip were performed by Affymetrix Inc., Santa Clara, CA, USA.

RNA isolation, synthesis of cDNA, and hybridization

Cultures of *B. japonicum* were grown in PSY-arabinose to an optical density (600 nm) of 0.35 to 0.5. Grown

cultures (10 ml) were immediately transferred into cold tubes containing one fifth of the culture volume of RNAprotect solution (Qiagen, Hilden, Germany). After centrifugation for 5 min (10,800*g*; 4°C) the supernatant was decanted and the cells were resuspended in 1 ml RNAprotect, centrifuged again and, after removing the supernatant, cells were frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated using the hot phenol extraction procedure described previously (Babst et al. 1996). RNA integrity was checked by agarose gel electrophoresis. RNA preparations from seven parallel cultures were pooled and treated with 10 units of DNase I (Amersham Biosciences Corp., Piscataway, NJ, USA) in 1 × One-Phor-All buffer (Amersham) for 30 min at 37°C. SUPERase-In™ (20 units; Ambion, Huntingdon, UK) was included in the reaction to inhibit potential RNase activity. The preparation was cleaned with RNeasy spin columns (Qiagen), and the eluted RNA was rechecked for integrity by agarose gel electrophoresis. cDNA was synthesized according to the Affymetrix antisense genome array protocol for *E. coli* (http://www.affymetrix.com), using the recommended reagents except that the amount of total RNA was adjusted to 20 µg. Spike-in control RNA was produced as described in the Affymetrix manuals. The resulting cDNA was quantified and fragmented according to the Affymetrix manual except that the time for fragmentation by DNase I was shortened to 4 min. For control, 200 ng of DNase I-treated cDNA were separated on a 4–20% acrylamide gradient gel and stained with SYBR green II (Molecular Probes, Inc., Eugene, OR, USA). Ideally, the fragmented cDNA migrated in a range that corresponded to 50 to 200 bp of the 50-bp ladder (Fermentas International Inc., Burlington, Canada) used as a size marker. The fragmented cDNA was then end-labeled using the Enzo®BioArray™ terminal labeling kit (Enzo Diagnostics Inc., Farmingdale, NY, USA) with biotin-ddUTP according to the manufacturer's instructions. Hybridisation, washing, staining, and scanning were done according to the Affymetrix manual using a gene chip fluidics station 400 (Affymetrix) and a gene chip Scanner 2500 (Affymetrix). For hybridization of individual gene chips, 2.5 to 3 µg of labeled cDNA were used in a total volume of 140 µl hybridization solution. Three independent replicas were analyzed in the comparison of microaerobically grown wild-type and *nifA* mutant cells (experiment A), and five replicas in the comparisons of aerobically grown wild-type and *regR*-mutant cells (experiment B).

Data analysis

Signal intensities were detected and analyzed with the Microarray Suite (MAS) Version 5.0 (Affymetrix) using the statistical algorithms described in the Affymetrix statistical algorithms description document. Data were globally scaled to a target intensity of 500, and default statistical parameters of MAS ($\alpha_1 = 0.05$, $\alpha_2 = 0.065$, $\tau = 0.015$, $\gamma_1 H = 0.0045$, $\gamma_1 L = 0.0045$, $\gamma_2 H = 0.006$, $\gamma_2 L = 0.006$) were used for comparative analysis of each pair of arrays. Data from the replicas were then processed using Microsoft Excel. Expression of genes whose probe sets showed an average fold-change value ≥2 (signal log ratio ≥1, and *P* values ≤ 0.01 in individual replicas) was considered to be increased, whereas that of genes with an average fold-change value ≤ −2 (signal log ratio ≤ −1, and change *P* values ≥0.98 in individual replicas) was defined as decreased. Raw signals of individual perfect-match (PM) and mismatch (MM) probe pairs retrieved from the MAS data set were used for calculation of position-specific hybridization signals for the *id880* 5′-end and its upstream intergenic region. Net signal intensities were calculated from all replicates of the wild-type versus *regR* mutant comparisons (experiment B) by substraction of the MM signal values from PM signals and graphically displayed alongside corresponding DNA regions.

Quantitative real-time PCR

cDNA used for chip experiments or freshly prepared cDNA was used as template for quantitative real-time PCR (qRT-PCR). Primers were designed using the Primer Express software (Applied Biosystems, Foster City, CA, USA). Primer sequences used were as follows: nifH-136F 5′-GGACTCGACTCGCCTTATTCTG-3′, nifH-195R 5′-GCGGCAAGACTCAAAATCG-3′ (for the *nifH* gene); exoU-549F 5′-GCTTCGACACCTCCCTCAGAT-3′, exoU-640R 5′-CAGAAGCTTTGGTGCGAACTC-3′ (for *exoU*); exoP-717F 5′-CCACCTACAAGGCCCAGAAC-3′, exoP-792R 5′-TTGCTGGCGGAGAGCTG-3′ (for *exoP*); exoT-567F 5′-CAGGCATTCGTGGAGCAATA-3′, exoT-626R 5′-CGATGTCTTGATAACGCCAACA-3′ (for *exoT*); sigA-1533F 5′-CTGATCCAGGAAGGCAACATC-3′, sigA-1617R 5′-TGGCGTAGGTCGAGAACTTGT-3′ (for *sigA*); 2087-685F 5′-CCCTTCCTCATGCTCTTACC-3′, 2087-746R 5′-TCCTTGTGGACATCCCAATA-3′ (for ORF *id880*); 2109-254F 5′-CAGCCGAGTACGTCTACCAG-3′, 2109-357R 5′-AATATCACCCGGCAAATGA (for ORF *id937*). Amplicon length was in the range of 60 to 100 bp. Primer concentrations were optimized to avoid non-specific products (e.g. primer dimers) in non-template controls. Each reaction contained 12.5 µl SYBR green PCR Mastermix (Applied Biosystems), 3.5 µl H_2O, forward and reverse primers in a total of 5 µl, and template in a total volume of 4 µl. Template amounts ranged from 0.1 to 100 ng cDNA per reaction, and primer concentrations from 50 to 400 nM. PCR reactions with *exoU*, *exoP*, *exoT* and *nifH* were run with the ABI Prism 7700 sequence detection system (Applied Biosystems) using the following steps: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. After amplification, dissociation

curve analysis was done using the corresponding ABI Prism software (Applied Biosystems). The dissociation protocol was 95°C for 15 s, 60°C for 20 s, followed by a ramp from 60°C to 95°C during 20 min. Real-time PCR reactions with ORFs *id880* and *id937* were performed with a RotorGene 3000 (Corbett Research, Sydney, Australia) in combination with the QuantiTect SYBR Green PCR Kit from Qiagen. The following steps were applied: 95°C for 15 min and 45 cycles of 95°C for 15 s, followed by 60°C for 1 min. The dissociation protocol was 60°C for 3 s, followed by a ramp from 60 to 95°C during 10 min. Calculation of fold-change values was done with the algorithm described by Pfaffl (2001) which corrects for potential differences in PCR efficiencies. The transcript of the primary sigma factor gene (*sigA*) showed a fold change of 1.2 ± 0.2 in all of the experiments with the pilot array. This transcript was therefore used as an internal (unregulated) reference for relative quantification.

Mapping of the *id880* transcription start site

The transcriptional start site of *id880* was mapped in a primer extension experiment using oligonucleotide 2087-PE2 (5′-$_{-333}$GGCTCCAGGCAGAACATAATCCGTGTCA-TAC$_{-303}$-3′) [position numbers refer to those of *bll2087* (= *id880*) deposited in the *B. japonicum* genome sequence database (http://www.kazusa.or.jp/rhizobase/Bradyrhizobium/index.html)]. RNA was isolated from aerobically grown wild-type and *regR* mutant cells as described above. Approximately 25 µg of RNA and at least 100,000 cpm of [γ-^{32}P]ATP-labeled primer were used per extension reaction; this was carried out as described previously (Bauer et al. 1998). Extension products were purified by phenol extraction followed by ethanol precipitation before they were loaded on a denaturating 6% polyacrylamide gel next to a sequencing ladder obtained with the same primer and plasmid pRJ9540. This plasmid contains the promoter region of *id880* on a 589-bp *Xba*I-*Pst*I fragment cloned in vector pUC18.

Gel retardation assay for RegR binding to the *id880* upstream region

His-tagged RegR protein was purified as described previously (Emmerich et al. 1999). A 108-bp DNA fragment (2087-BS) comprising the region from −134 to −27 relative to the transcription start site of *id880* was obtained by PCR amplification with primers 2087-23 (5′-AGCAAGCTCTGGTGTCCAAG-3′) and 2087-24 (5′-TAAACGTCAACGCGACAAAG-3′). To test binding specificity of RegR to fragment 2087-BS, an arbitrarily chosen DNA fragment of comparable size (104 bp) was included as a control (2087-N). This fragment was amplified by PCR with primers 2087-11 (5′-TACGCTGCCTACACCCAAT-3′) and 2087-12 (5′-AGGAGGTAATGCCGTCTTGT-3′) comprising the region from +128 to +231 relative to the transcription start site of *id880*. Amplification of both fragments was carried out with *Pfu*-polymerase for 30 cycles (94°C for 30 s, 50°C for 30 s, 72°C for 1 min) in the presence of 10% DMSO. Amplification products were separated on 2% agarose gels and purified with a gel extraction kit (Macherey-Nagel, Düren, Germany). Approximately 1.5 µg of DNA fragments were end-labeled with 20 μCi [γ-^{32}P] ATP using 10U T4 polynucleotide kinase (MBI Fermentas, Vilnius, Lithuania) in a total volume of 50 µl followed by purification through Sepharose-NAP-10 columns (Amersham Biosciences). Gel retardation experiments were performed as described (Bauer et al. 1998). Briefly, about 28,000 cpm of labeled DNA fragments were incubated in DNA binding buffer (12 mM HEPES [pH 7.9], 6 mM KCl, 3 mM $MgCl_2$, 0.5 mM DTT, 4 mM Tris-HCl [pH 8.0], 6 mM EDTA) in a total volume of 20 µl with increasing concentrations of RegR in the presence of 1 µg poly(dI-dC) as non-specific competitor. After 5 min incubation at room temperature, loading dye was added to the samples which were then separated on a nondenaturating 6% polyacrylamide gel. The gel was vacuum-dried and visualized with a phosphoimager.

Results

Design of the gene chip

The inkBJAPETH1F gene chip (Table 1) includes 738 genes that make up about 8.9% of all annotated genes in the completed genomic sequence of *B. japonicum* (Kaneko et al. 2002a). In addition, 1,059 probe sets for 530 intergenic regions, and 25 control genes, including bacterial markers, are present on the microarray. All ORFs are probed by 13 probe pairs, each consisting of one matching and one mismatch-oligonucleotide. The intergenic regions are probed on both strands with 1 to 63 probe pairs, depending on their lengths.

Table 1 Design details of the inkBJAPETH1F gene chip

Genes	
Protein-coding ORFs	732
RNA genes (tRNA, rRNA, tmRNA)	6
Probe pairs per ORF	13
Intergenic regions (IGs) ≥40 bp	
Number of IGs	530
Total length of IGs (kb)	190
Average spacing of probe pairs (bp)	40
Probe pairs per IG (both strands)	1–63
Controls	
Antibiotic resistance genes, reporters	15
Host plant genes (soybean)	10
Affymetrix controls	45

Standardization of sample preparation and hybridization conditions

Total RNA was isolated from cultures and treated as described in Materials and methods. After final purification, 10 ml of culture yielded an average of 18 or 11 μg total RNA when cells had been grown aerobically or microaerobically, respectively. cDNA was synthesized with 20 μg total RNA as template. An average of 2.4 μg cDNA was obtained from each cDNA synthesis reaction. The cDNA was fragmented with DNase I. A time course showed that the incubation period for this reaction could be shortened from the ten minutes recommended in the original Affymetrix protocol for *E. coli* (http://www.affymetrix.com) down to four minutes in order to obtain cDNA fragments of the desired length. In experiment A (wild type vs. *nifA* mutant, both grown microaerobically), biotin-labeled cDNA from three biological replicas was hybridized to individual gene chips, whereas in experiment B (aerobically grown wild type vs. *regR* mutant, both grown aerobically) five replicas were obtained. Technical parameters such as scaling factor (1.1 ± 0.2) and background values (87 ± 14) were comparable in all eight gene chip experiments and within the working range recommended by Affymetrix. The fold change values of all regulated genes listed in Tables S1, S2 and S3 showed an average relative standard deviation of 29%. Experimental optimization showed that a maximum of 3 μg of cDNA should be used for a single chip hybridization experiment, as background values reached or—on occasions—exceeded the acceptable upper limit when higher amounts were used. The signals from five different in vitro synthesized control RNAs (spike-in controls) were called present in all eight individual hybridization experiments, and differed only by a factor of 1.1 ± 0.2 which probably reflects variations in sample preparation. An average of $54 \pm 5\%$ of all open reading frames were called present in all gene chip experiments.

Comparison of microaerobically grown wild-type and *nifA* mutant cells

Expression profile analysis of microaerobically grown *B. japonicum* wild-type and A9 (*ΔnifA*) mutant cells (experiment A) revealed 33 genes with decreased expression levels in the *nifA* mutant (see electronic supplementary material, Table S1). A selection of 20 of them is depicted in Fig. 1a and arranged in groups to reflect neighbouring locations on the chromosome. As a useful control, the kanamycin resistance gene (*aphII*) inserted in *nifA* led to the highest expression factor in the mutant strain A9 as expected, as this gene is of course absent in the wild type (fold change value 208 ± 58; Table S1).

Among the genes showing decreased transcription in the *nifA* mutant, we recognized eight genes previously known to be under positive control by NifA (*nifD*, *nifK*, *nifH*, *nifS*, *nrgA*, *id91*, *id117*, *id525*; Fischer 1994; Nienaber et al. 2000; Caldelari Baumberger et al. 2003). In contrast, some prominent examples of known NifA-dependent genes were not reproducibly detected in this microarray experiment and included *nifE*, *nifN*, *nifX*, *nifB*, *fixA*, *fixB*, *fixC*, *nrgB*, *nrgC*, $groES_3$, $groEL_3$ and *fixR* (Fischer 1994; Nienaber et al. 2000). Apart from previously defined NifA targets, we also newly identified a number of other genes whose expression was significantly decreased in the *nifA* mutant. Notably, some of them are genetically and functionally linked, as exemplified by the *exoQ* (Q9RHC3), *exoU*, and *exoP* genes, or the *bioB* (*id897*), *panD* (*id899*), and *bioFDA* genes (*id901*, *id903*, *id904*) involved in the biosynthesis of exopolysaccharides and biotin, respectively (Fig. 1a). Strikingly, these genes are not associated with obvious regulatory sequences that resemble binding sites for NifA and the specialized RNA polymerase σ factor σ^{54} (Dixon and Kahn 2004). This clearly suggests an indirect NifA-dependent control of these genes (see Discussion). The apparent NifA dependence of expression of these genes was independently confirmed for three of them (*exoU*, *exoP* and *exoT*) by quantitative real-time PCR (qRT-PCR). Although the gene chip data indicated that *exoT* expression was only marginally decreased in the *nifA* mutant (1.8-fold-change compared with wild type), we included *exoT* in the qRT-PCR experiment because of its possible cotranscription with *exoP* as part of the same operon. The qRT-PCR approach fully confirmed the gene chip data and showed decreased *exoU*, *exoP* and *exoT* expression in the *nifA* mutant relative to the wild type (Table 2). It was also noted that the qRT-PCR-derived fold-change value for the *nifH* gene, which served as positive control, was about 5 times larger than in the gene chip experiment.

The up to 11-fold elevated expression of 24 other genes in the *nifA* mutant, 11 of which were known as heat-shock inducible genes (Table S1), came as a surprise. If this was the result of any kind of stress in the mutant, such a situation does apparently not affect growth because growth rates of wild type and *nifA* mutant were comparable. Many of these genes are clustered or organized in operons ($rpoH_1$-*hspBCdegP*, $groESL_1$, *hspD*, *hspEF*; Fig. 1b). This finding suggests that, in *B. japonicum*, the absence of a functional *nifA* gene induces a yet undefined stress response under the used growth conditions.

Comparison of aerobically grown wild-type and *regR* mutant cells

Comparative chip hybridization analysis of cDNA derived from aerobically grown wild-type and strain 2426 (*ΔregR*) cells led to the identification of 13 open reading frames with significantly decreased expression in the *regR* mutant (experiment B; ESM: Table S2). Unsurprisingly, the largest observed decrease of expression

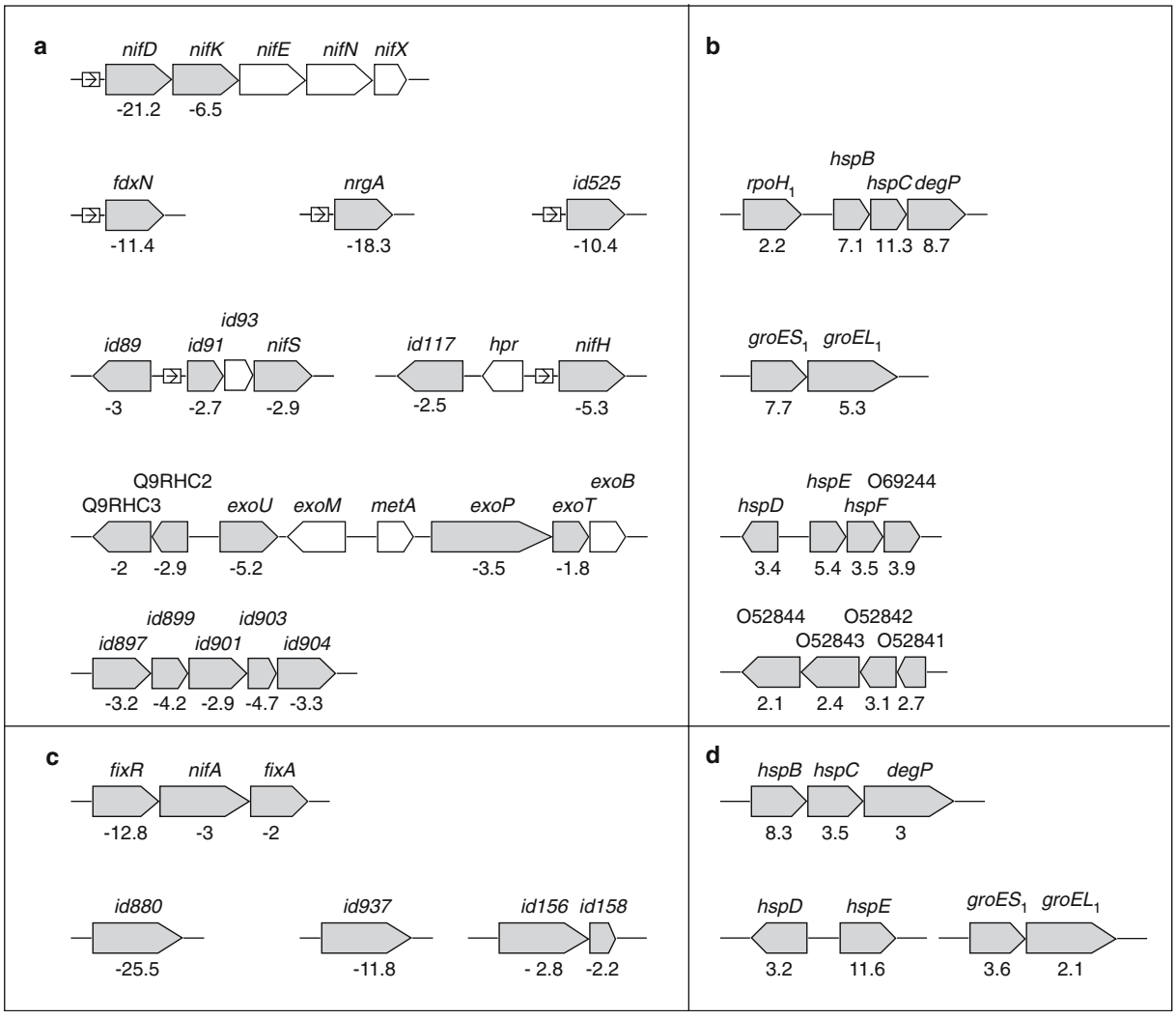

Fig. 1 A selection of *B. japonicum* genes and operons with altered expression levels in comparative gene chip experiments. **a**, impaired expression in the microaerobically grown *nifA* mutant. **b**, increased expression in the microaerobically grown *nifA* mutant. **c**, impaired expression in the aerobically grown *regR* mutant. **d**, increased expression in the aerobically grown *regR* mutant. Expression levels are relative to those of the wild-type strain grown under the same respective conditions (see Tables S1 and S2 of the Electronic Supplementary Material for a complete list of genes with altered levels of transcription). Fold-change values are indicated below the genes depicted in *gray* (*minus*, decreased expression; all others, increased expression). Genes shown in *white* within or adjacent to clusters and operons exhibited expression levels that were undistinguishable in the wild type and mutant strains. *Small horizontal arrows in boxes* shown upstream of genes in **a** symbolize the presence and productive orientation of both a σ^{54} consensus promoter and of a NifA-binding site. Genes located in the symbiotic gene region of *B. japonicum* are named as in Göttfert et al. (2001), whereas all other gene designations were taken from Swiss-Prot and TrEMBL databases (as of June 2002)

relative to wild-type (46-fold) was found for *regR* itself, and reflects the deletion of most of this gene in strain 2426. Similarly, expression of the known RegR target genes *fixR* and *nifA* (Bauer et al. 1998) was reduced by a factor of 12 and 3, respectively. Two other genes (*id880*, *id937*) showed a larger than 10-fold impaired expression. QRT-PCR analysis strongly confirmed the RegR-dependent expression of *id880* by revealing a 70-fold reduced gene expression in the absence of RegR (Table 2). Moreover, since this gene is located within the symbiotic region of the genome (Göttfert et al. 2001), *id880* was an interesting candidate for further investigations (see next section). Although a RegR-dependent expression was also found for *id937*, the fold-change value determined by qRT-PCR was lower than that in the gene chip experiment. Two other genes of unknown function (*id156*, *id158*) that showed >2-fold decreased expression in the *regR* mutant are located next to each other and thus might be organized in an operon (Fig. 1c).

Probe sets for 11 ORFs showed significantly increased expression levels in the *regR* mutant (Table S2). Not surprisingly, the streptomycin resistance gene inserted in *regR* led to the most prominent apparent increase of expression (computed as a fold-change value of $1{,}016 \pm 354$) due to its absence in the wild type. Similar

Table 2 Comparison of fold-change values of selected NifA- and RegR-regulated genes deduced from gene chip and qRT-PCR experiments

Gene	Known or putative function	Fold-change values calculated from	
		Gene chip	qRT-PCR
NifA-regulated			
exoU	UDP-hexose transferase	5.2 ± 0.9	3.8 ± 0.7
exoP	Succinoglycan biosynthesis transport protein	3.5 ± 1.7	3.2 ± 0.7
exoT	UDP-hexose transferase	1.8 ± 0.3	3.3 ± 0.5
nifH	Nitrogenase component II	5.3 ± 2.4	28.8 ± 6.0
RegR-regulated			
id880	Unknown protein	25.5 ± 7.6	73.3 ± 6.3
id937	Transcriptional regulatory protein (Crp family)	11.8 ± 4.7	4.4 ± 0.6

to the situation in the *nifA* mutant, a number of heat-shock genes also displayed elevated expression in the *ΔregR* strain, indicating again that the absence of the RegR–NifA regulatory cascade provokes a general stress response. Also here, however, the growth rates of wild type and *regR* mutant were comparable.

Transcription analysis of *id880*

The finding that *id880* transcription strongly depended on *regR* made this gene an attractive candidate for studies on RegR-mediated transcriptional activation, even though the predicted product of *id880* showed no similarity to a functionally characterized protein in the database. As a first step, we mapped its transcription start site. RNA isolated from aerobically grown *B. japonicum* wild-type and *regR* mutant cells was used as template for primer extension with oligonucleotide 2087-PE2. A very prominent cDNA product was detected with wild-type RNA but not with RNA extracted from the *regR* mutant strain (Fig. 2a). Surprisingly, this corresponded to a transcript that starts at a position located 53 bp downstream of the annotated translation start of *id880* (ATG*; Fig. 2b). The presence of a putative −35 promoter box further supported the result of the transcript mapping experiment. Thus, it seems as if the beginning of the *id880* reading frame had been annotated incorrectly. Indeed, inspection of the sequence downstream of the transcription start revealed the presence of four or five alternative ATG start codons in the same reading frame (Fig. 2b).

To further evaluate this finding, we made use of the special gene chip design which includes probe pairs corresponding both to inter- and intragenic regions. Signal intensities of individual probe pairs derived from the *id880* region of interest were compared between the wild type and the *regR* mutant (Fig. 2c). As expected, signals from intragenic probes were consistently higher in the wild-type strain than in the *regR* mutant in all five individual hybridization experiments. However, no difference in signal intensities between the two strains was observed when four probe pairs derived from the intergenic region upstream of *id880* were compared. The intergenic probe pair located closest to *id880* ends only 8 bp upstream of the originally assigned ATG* start codon. According to the previous annotation, it would most likely cover transcribed, non-coding 5′ region of *id880* and thus should yield a differential signal in the comparison between wild type and the *regR* mutant. The fact that this was not the case indicates that this region is not transcribed, which perfectly agrees with our transcription start site mapping.

Given the RegR-dependency of *id880*, we tested RegR binding to the *id880* promoter region in gel retardation assays. A 108-bp DNA fragment located upstream of the experimentally determined transcription start was used as a target (2087-BS; Fig. 2b). Binding of RegR was indeed detected as indicated by the retarded band whose intensity depended on protein amount (Fig. 3). By contrast, no RegR binding was observed to a comparable control fragment (2087-N; cf. Fig. 2b) even when high protein amounts were applied (Fig. 3, lane 10).

Comparison of microaerobically and aerobically grown wild-type cells

Experiments A and B have also enabled us to directly compare the transcription profile of wild-type cells grown under oxic and micro-oxic conditions. This allowed us to identify a total of 42 microaerobically induced genes or ORFs (ESM: Table S3), corresponding to 5.7% of all ORFs represented on the inkBJAP-ETH1F gene chip. Microaerobically up-regulated expression was observed for a large number of genes that contribute to various functions related to *B. japonicum* metabolism under restricted oxygen supply or anoxia: genes encoding alternative respiratory enzymes (*fixNOQP*, *fixGHIS*, *napEDABC*, *nirK*), proteins involved in heme biosynthesis (*hemA*, *hemB*, $hemN_1$, $hemN_2$), and transcription factors ($rpoN_1$, *nosR*, *nnrR*, $fixK_2$, $fixK_1$). Amongst the genes with highest or very high induction factors of 151, 48, 22, and 13 were the *fixO*, *fixN*, *fixQ*, and *fixP* genes, respectively, which code for the subunits of the cbb_3-type cytochrome oxidase (Preisig et al. 1993). Likewise, the *fixGHIS* genes required for maturation of the cbb_3-type oxidase (Preisig

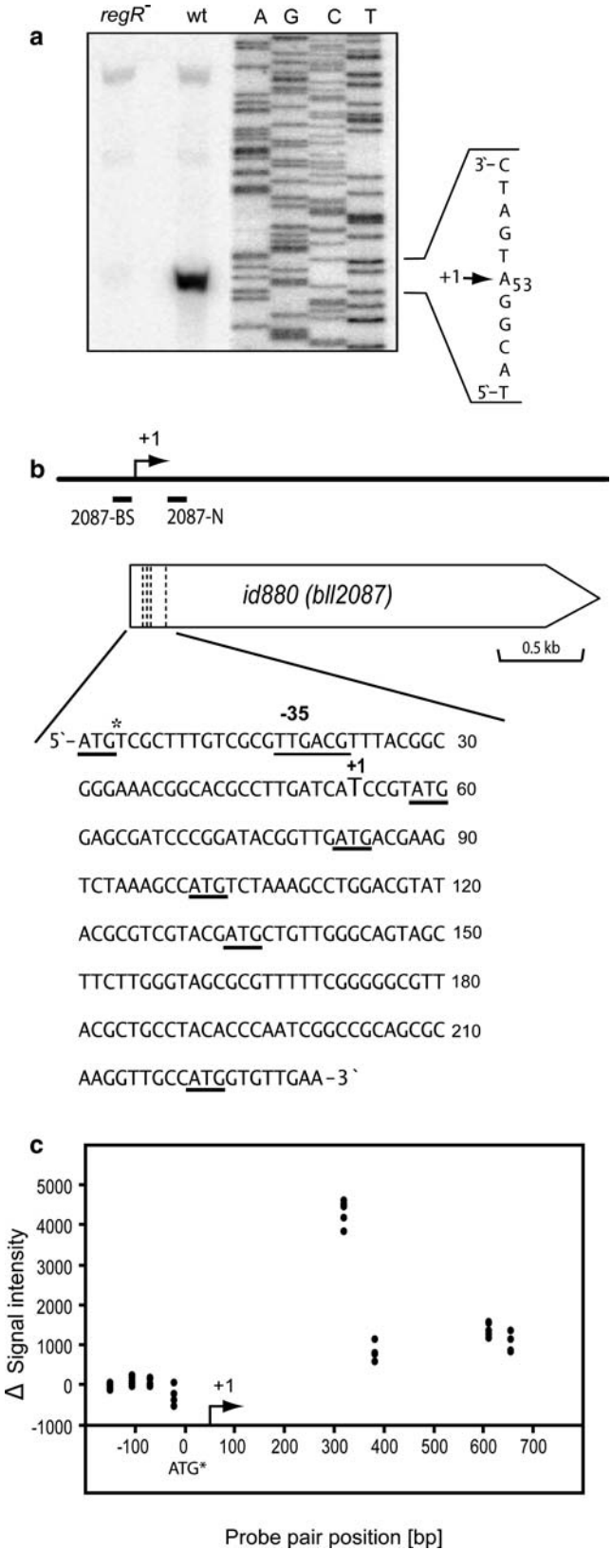

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Fig. 2 Transcription analysis of the *id880* gene. **a**, transcription start site determination by primer extension. *Left two lanes* show cDNA products obtained with primer 2087-PE2 and RNA isolated from aerobically grown *B. japonicum* wild-type (wt) and *regR* mutant cells (*regR*⁻). The sequence ladder (A,G,C,T) was created with the same primer and plasmid pRJ9540. The transcription start is emphasized by "+1" (*arrow*) in the sequence of the non-coding strand shown on the right. Numbering of the +1 nucleotide position refers to the *id880* partial sequence shown in **b**. **b**, genetic map of the *id880* locus including 230 bp of DNA sequence from the *id880* 5′ end. The sequence starts with ATG* which corresponds to the *id880* start codon as annotated in the *B. japonicum* genome sequence database (http://www.kazusa.or.jp/rhizobase/Bradyrhizobium/index.html). The T residue marked "+1" corresponds to the transcription start site (A_{53}) shown in **a**. Based on the original annotation, A_{53} is located within coding sequence of *id880*. A putative –35 promoter box and alternative ATG start codons of *id880* (symbolized by *vertical dashed lines* in the *id880* gene symbol) are *underlined*. *Short horizontal black bars* (2087-BS, 2087-N) indicate positions of PCR amplification products that were used in the gel retardation experiment shown in Fig. 3. **c**, graphical representation of hybridization signal intensities derived from individual probe pairs present on the gene chip. Signal intensities were calculated as described in Materials and methods. The location of individual probe pairs is indicated relative to the original *id880* annotation (ATG* at position 0). Four probe pairs on the left are located in the *id880* nontranscribed upstream region whereas the four pairs on the right represent regions within the 5′ coding region of *id880*. Individual data points at identical nucleotide positions reflect the differential signal intensities (Δ) observed in each of the five hybridization replicates, when wild type and *regR* mutant 2426 were compared

et al. 1996a) were induced by factors between 7 and 21 under micro-oxic conditions. The finding that genes predicted to encode a protocatechuate-3,4-dioxygenase (*pcdAB*) and alkyl hydroperoxide reductase (*ahpC*) were microaerobically up-regulated 3- to 6-fold (Table S3) is intriguing. Indeed, their functions are generally thought to be related to aerobic rather than to microaerobic metabolism (Antelmann et al. 1996; Harwood and Parales 1996).

The group of 41 microaerobically down-regulated genes was functionally quite diverse. Many of these genes do not have an obvious role in aerobic metabolism (Table S3). Expression of the *atpB* gene, which encodes subunit B of the F_0F_1-type ATPase, was decreased about two-fold under micro-oxic conditions. This may reflect diminished overall energy generation under these conditions. Conversely, the *coxABC* genes coding for the aa_3-type aerobic terminal oxidase were not found among the genes that are down-regulated under microaerobic conditions even though one may expect expression of this enzyme to be genetically repressed in microoxia (Gabel and Maier 1993).

Discussion

We have applied a gene chip-based strategy to monitor the transcription profile of a selection of *B. japonicum* genes in the wild type and in two different regulatory mutants which had already been intensively analyzed by means of traditional methods such as reporter gene fusions and direct transcript measurements. The selection of 738 genes was dictated by the availability of *B. japonicum* gene sequences as of June 2002. This group

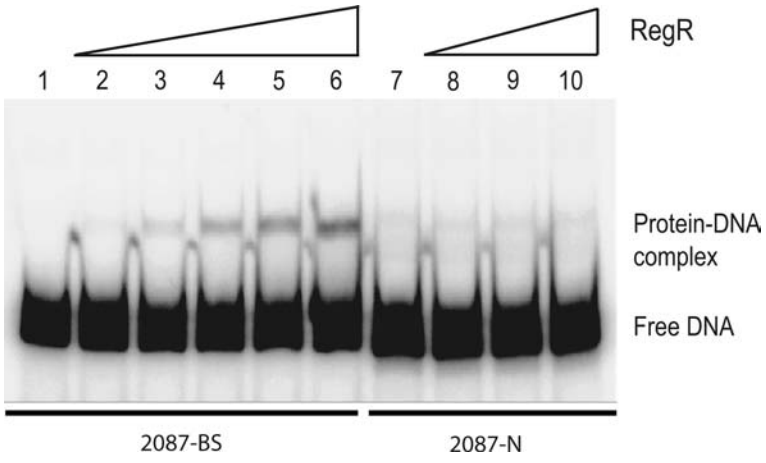

Fig. 3 Electrophoretic mobility shift experiment with RegR protein and *id880* promoter region. Radiolabeled DNA fragments 2087-BS (lanes *1–6*) or 2087-N (lanes *7–10*) were incubated with these concentrations of purified His_6-tagged RegR protein: no protein (lane *1*, *7*), 0.04 μM (*2*), 0.11 μM (*3*), 0.22 μM (*4*, *8*) 0.44 μM (*5*, *9*), 0.9 μM (*6*, *10*). After 5 min incubation at room temperature, samples were run on a non-denaturing 6% polyacrylamide gel and visualized with a phosphoimager

of genes represents 8.9% of the 8,317 potential protein-coding genes annotated in the whole genome sequence, which was published while our work was in progress (Kaneko et al. 2002a). The rationale behind this project was to establish gene chip technology in our laboratory and to validate the obtained gene chip data by comparing them in the context of previously determined regulatory circuits (see Sciotti et al. 2003, and references therein). The obvious step following this pilot study will be the use of a whole-genome chip for genome-wide transcriptome analysis.

Targets of the inkBJAPETH1F gene chip were not restricted to coding regions, but featured intergenic regions of ≥40 bp as well, which were covered on both DNA strands by uniformly distributed probe pairs. Intergenic regions were included to evaluate their usefulness in low-resolution transcript mapping of genes and operons or in identifying yet unrecognized ORFs or untranslated (regulatory?) RNAs. Although no systematic analysis of hybridization patterns of intergenic regions was undertaken here, our preliminary evaluation revealed that many probe pairs located immediately adjacent to the 5′ and 3′ ends of ORFs show similar expression patterns as ORF-internal probe pairs. Hence, such probe pairs probably detect 5′ and 3′ transcribed but untranslated regions. On the other hand, we also obtained evidence pointing to the existence of transcripts synthesized from hitherto unassigned DNA regions (data not shown).

One example for the usefulness of probe pairs for intra- and intergenic regions has been demonstrated in the transcription analysis of gene *id880* (Fig. 2c). They turned out to be valuable tools in a fine-structure mapping of the transcribed region, which eventually led to a revised annotation for *id880*.

An average of 54% of all coding regions represented on the gene chip was called present, which is comparable to values reported for other prokaryotic microarray experiments (52–75%; Rosenow et al. 2001; Ochsner et al. 2002; Lobner-Olesen et al. 2003). The somewhat low proportion of expressed genes detected with the inkBJAPETH1F microarray may be due to the fact that our chip is biased for genes expressed during symbiosis, a physiological state that was not addressed in this study. The issue of the reliability of current sequence annotations is also relevant in this context. The chip comprises 388 genes of the symbiotic gene region as annotated by Göttfert et al. (2001). Fifty-eight of those genes were not annotated in the same way in the whole-genome sequence (Kaneko et al. 2002b). Conversely, in the latter annotation of the symbiotic gene region, we note 65 open reading frames that are absent in the annotation by Göttfert et al. (2001). Thus, our chip may comprise probe sets designed for incorrectly assigned genes. This could reduce the number of genes called present.

Gene chip data were validated by comparison with the current knowledge of the FixLJ–$FixK_2$ and RegSR–NifA regulons that was previously established through gene expression studies by traditional methods (Fig. 4; Sciotti et al. 2003, and references therein). Based on chip data derived from aerobically and microaerobically grown wild-type cells, almost all of the known target genes of the oxygen-responsive FixLJ–$FixK_2$ cascade (except for the *hupSL* and *norCBQD* genes) were found among the microaerobically induced genes. In addition, the *napEDABC* genes, which are organized in an operon preceded by a putative FixK box, also showed increased expression levels under micro-oxic conditions. Recent results obtained in the group of M. Delgado (Granada, Spain) have indeed confirmed that these genes are part of the FixLJ–$FixK_2$ regulon (M. Delgado, personal communication). The *napEDABC* operon encodes a periplasmic nitrate reductase which is required for anaerobic nitrate respiration (Delgado et al. 2003). Our results suggest that micro-oxia leads to a basal induction of the *napEDABC* operon; yet, it is likely that maximal induction requires in addition the presence of nitrate. The same regulatory pattern was observed previously with the *nirK* and *norCBQD* denitrification genes (Velasco et al. 2001; Mesa et al. 2002). The reason why gene chip

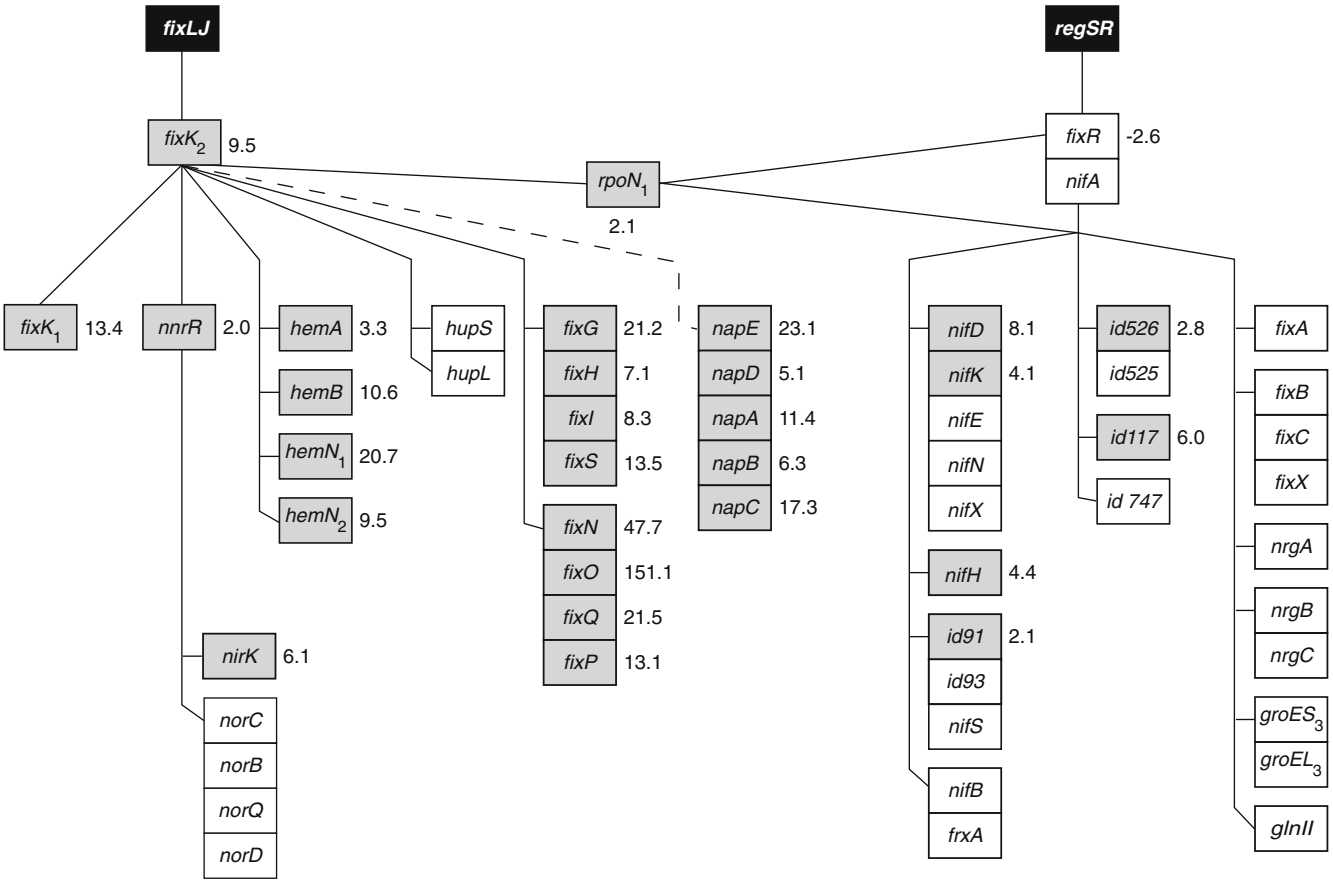

Fig. 4 Integration of microarray data into the previously established FixLJ–FixK$_2$ and RegSR–NifA regulatory network. *Single boxes* and *vertically stacked boxes* symbolize monocistronic and polycistronic genes, respectively. Results from gene chip experiment A (microaerobically grown wild-type vs. *nifA* mutant cells) and from experiment B (aerobically grown wild-type vs. *regR* mutant cells) are indicated for genes known from previous work to belong to either of the cascades with the respective fold-change values (microaerobically vs. aerobically grown wild-type cells) when statistically significant differences were found (numbers adjacent to *grey boxes*). Genes in *white boxes* indicate genes whose expression did not significantly differ in aerobic and microaerobic cells. *Solid lines* refer to experimentally demonstrated regulatory connections; the control circuit between *fixK*$_2$ and the *napEDABC* operon (*dashed line*) is still tentative

experiments detected *nirK* but failed to reproducibly detect microaerobic induction of the *norCBQD* genes is not known.

In contrast to the situation in the FixLJ–FixK$_2$ regulon, only a fraction (six out of 25) of the previously described members of the RegSR–NifA regulon was reproducibly found among the microaerobically induced genes in the gene chip experiments (Fig. 4). Genes exhibiting the expected expression pattern comprise the structural genes for the nitrogenase complex (*nifDK*, *nifH*) and three additional genes of unknown function (*id91*, *id117*, *id525*; Ebeling 1990; Caldelari Baumberger et al. 2003).

The decreased expression of *fixR* and the lack of detectable *nifA* expression under microaerobic conditions (Fig. 4, Table S3) were completely unexpected results that do not agree with the previously established model of a NifA-dependent, positive autoregulation of the *fixR–nifA* operon (Thöny et al. 1989; Barrios et al. 1995; Sciotti et al. 2003). To address this issue, we performed additional microarray experiments with *nifA* and *regR* mutants. The strong RegR dependency of aerobic *fixR* expression was clearly confirmed (Table S2). In contrast, the deletion of *nifA* was not followed by a change of the *fixR–nifA* transcript level. This might in part be explained by the peculiarity of the *nifA* mutant construct (Fischer et al. 1986), in which only a central portion of *nifA* is deleted and replaced by a kanamycin resistance cassette. This gives rise to the possibility that a partial and possibly artefactual *nifA*-specific transcript could be detected in the gene chip experiment. The observed decreased expression of the *fixR–nifA* operon in the microaerobic wild type, however, cannot readily be explained by current knowledge.

NifA is an activator of genes that are transcribed in a σ^{54}-RNA polymerase-dependent manner (Morett and Buck 1989; Morett et al. 1991) under microaerobic conditions (Fischer and Hennecke 1987; Fischer et al. 1988), whereas the protein is inactive under aerobic conditions. Therefore, NifA-dependent genes are ex-

pected to exhibit (1) increased expression levels in the wild type grown microaerobically as compared with the wild type grown aerobically, and (2) decreased expression levels in the *nifA* mutant compared to the wild type when both are grown microaerobically. Only six genes fulfilled both of these two criteria. Five of them (*nifDK*, *id91*, *id117*, *nifH*) belong to transcription units that were previously shown to be NifA-dependent (Nienaber et al. 2000; Caldelari Baumberger et al. 2003, and references therein). The sixth member of this group (*ahpC*) is unlikely to be a direct target of NifA because it lacks the promoter elements that are characteristic for NifA-dependent genes (see below).

In general, gene chip data were in good agreement with previous measurements of reporter gene fusions. However, the fold-change values derived from β-galactosidase activities with translational *lacZ* fusions in wild-type and *nifA* mutant backgrounds differed by at least a factor of ten from those determined by gene chip experiments, except in the case of *nifD*. A good example for big quantitative variations is provided by *nifH*, a NifA-dependent gene with the highest induction level in the wild-type compared with a *nifA* mutant, based on β-galactosidase assays (fold-change: 640; Gubler and Hennecke 1988). The observed fold-change in the gene chip experiment was 5.3, and the validation of this induction with quantitative real-time PCR showed a fold-change value of 29 (Table 2). Thus, it appears that, in this case, the gene chip underestimates the true fold-change. Such differences between qRT-PCR and gene chip are not unusual and have been reported for other microarrays as well (Roh et al. 2004). As opposed to microarray data, β-galactosidase assays seem to overestimate fold-change values in comparison with qRT-PCR. This may result either from accumulation of β-galactosidase, or from an overall lower rate of protein synthesis in the *nifA* mutant than in the wild type.

Surprisingly, although 27 gene probe sets did show a decreased expression in the *nifA* mutant, the same sets did not show increased expression levels in the microaerobically grown wild type. Interestingly, a recent study of global gene expression in *S. meliloti* under various conditions (Becker et al. 2004) also showed induction of known NifA-dependent *nif* genes in bacteroids but not in free-living cultures grown under micro-oxic conditions. A possible explanation for such findings would be that the appropriate oxygen concentration for full NifA-dependent expression was only met in bacteroids. Indeed, we observed previously that, in *B. japonicum*, NifA-dependent genes can be induced to higher levels under anaerobic conditions (YEM medium plus nitrate) as compared with microaerobic conditions (Thöny et al. 1989). Nevertheless, we used micro-oxic growth conditions (PSY medium, 0.5% oxygen in the gas phase) in the present study because it was critical to use the same medium for both aerobic and micro-aerobic cultures. Furthermore, the same conditions proved to be suitable for induction of *nifH* and *fixB* in a recent study by Sciotti et al. (2003); yet, this may not hold true for all other NifA-dependent genes. Despite the aforementioned problem with the change of the medium, we have recently made first exploratory tests for gene expression with microarrays in anaerobic cultures grown with nitrate as the terminal electron acceptor. Many more of the previously recognized NifA-dependent genes were shown to be expressed, as compared with microaerobic cultures (F. Hauser, unpublished observations). Therefore, in future genome-wide transcript analyses, it is mandatory to compare gene expression in cells grown in all three culture conditions (aerobic, microaerobic, anaerobic/NO_3^-).

Apart from the possibility of limited NifA activity caused by insufficient microaerobiosis, the observed lack of detection of some of the known NifA-dependent genes may have been due to interfering crosshybridization with transcripts originating from homologous genes present elsewhere in the *B. japonicum* genome. Indeed, the choice of oligonucleotides featured on the inkB-JAPETH1F gene chip could not be validated against the entire genome sequence which became available only after the chip was designed (Kaneko et al. 2002a, b).

This work has uncovered a number of genes that so far were not known to be under direct or indirect control by NifA. The DNA sequence upstream of these putative new NifA targets was examined for the possible presence of a NifA binding site and a σ^{54} promoter (Fischer 1994; Dixon and Kahn 2004), but neither of them were identified. This makes it unlikely that such genes are direct NifA targets. However, the NifA dependency of three candidate genes (*exoP*, *exoT*, *exoU*) was validated by qRT-PCR (Table 2). These results can be explained in two ways: (1) NifA activates transcription of a regulatory gene, whose product in turn controls transcription of those *exo* genes for which we detected a decreased expression in the *nifA* mutant. (2) Up-regulation of *bona fide* NifA-dependent functions might influence the overall physiological state of the cell and, hence, lead to increased expression of *exo* genes which are, therefore, regarded as being NifA-dependent, too. It is worth noting here that mutations in *exoP* and *exoT*, whose expression is influenced by NifA, were shown to affect exopolysaccharide composition and nodulation capacity (Becker et al. 1998). Most likely, the *bioB* (*id897*), *panD* (*id899*), and *bioFDA* genes (*id901*, *id903*, *id904*) involved in biotin biosynthesis represent additional examples of such indirectly NifA-controlled genes. This suggests a role for biotin in the *B. japonicum*-soybean symbiosis, similarly to what was described previously for the *S. meliloti*–alfalfa interaction (Streit et al. 1996).

The RegR regulon is much less defined than the NifA regulon so far. In fact, only one characterised operon (*fixR–nifA*) was known to be expressed in a RegR-dependent manner (Bauer et al. 1998), and the gene chip results are in complete agreement with this previous finding, i.e., there was a decreased *fixR–nifA* expression in the *regR* deletion mutant as compared with the wild type. While the precise signal for activation of RegSR remains unclear (Sciotti et al. 2003), in the present study

we identified two new RegR-dependent target genes (*id880*, *id937*) in the symbiotic gene region by the gene chip approach and confirmed this finding by qRT-PCR. The rather weak consensus sequence elaborated previously for the RegR binding site on DNA (Emmerich et al. 2000; Laguri et al. 2003) made it difficult to find putative RegR binding boxes upstream of *id880* and *id937*. Nevertheless, we were able to experimentally demonstrate binding of purified RegR protein to the *id880*-upstream DNA region (Fig. 3), after having precisely located the site where RegR-dependent *id880* transcription starts. This will allow us to narrow down the RegR–DNA contact site more precisely in future work.

In conclusion, the data reported here show that—despite some limitations—microarray technology is able to detect changes in transcript levels with total cDNA samples from *B. japonicum*. In particular, the results obtained for the FixLJ–$FixK_2$ regulon have demonstrated nicely how consistent gene chip data and previous results can be. On the other hand, the current design, sample preparation and evaluation method led to underestimation of the number of NifA-dependent genes. Finally, a major disadvantage of this gene chip remains: since the sequence information does not represent the full genome, complex regulatory processes might be obscured. The design of a whole-genome microarray for *B. japonicum* and its experimental validation will hopefully alleviate this limitation.

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