Role of the Global Transcriptional Regulator PrrA in *Rhodobacter sphaeroides* 2.4.1: Combined Transcriptome and Proteome Analysis^{∇†}

Jesus M. Eraso,¹ Jung Hyeob Roh,¹ Xiaohua Zeng,¹ Stephen J. Callister,²
Mary S. Lipton,² and Samuel Kaplan^{1*}

Department of Microbiology and Molecular Genetics, University of Texas Health Science Center, Houston, Texas 77030,¹ and
Biological Separations and Mass Spectrometry, Mail Stop K8-98, Pacific Northwest National Laboratory, Richland,
Washington 99352²

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The PrrBA two-component regulatory system is a major global regulator in *Rhodobacter sphaeroides* 2.4.1. Here we have compared the transcriptome and proteome profiles of the wild-type (WT) and mutant PrrA2 cells grown anaerobically in the dark with dimethyl sulfoxide as an electron acceptor. Approximately 25% of the genes present in the PrrA2 genome are regulated by PrrA at the transcriptional level, either directly or indirectly, by twofold or more relative to the WT. The genes affected are widespread throughout all COG (cluster of orthologous group) functional categories, with previously unsuspected “metabolic” genes affected in PrrA2 cells. PrrA was found to act as both an activator and a repressor of transcription, with more genes being repressed in the presence of PrrA (9:5 ratio). An analysis of the genes encoding the 1,536 peptides detected through our chromatographic study, which corresponds to 36% coverage of the genome, revealed that approximately 20% of the genes encoding these proteins were positively regulated, whereas approximately 32% were negatively regulated by PrrA, which is in excellent agreement with the percentages obtained for the whole-genome transcriptome profile. In addition, comparison of the transcriptome and proteome mean parameter values for WT and PrrA2 cells showed good qualitative agreement, indicating that transcript regulation paralleled the corresponding protein abundance, although not one for one. The microarray analysis was validated by direct mRNA measurement of randomly selected genes that were both positively and negatively regulated. *lacZ* transcriptional and *kan* translational fusions enabled us to map putative PrrA binding sites and revealed potential gene targets for indirect regulation by PrrA.

Rhodobacter sphaeroides 2.4.1 is a purple nonsulfur photosynthetic bacterium which is well studied for its remarkable metabolic versatility. It can grow aerobically, anaerobically in the presence of external electron acceptors, such as dimethyl sulfoxide (DMSO), photosynthetically in the light, and fermentatively and lithotrophically in the presence or absence of oxygen (72). As a reflection of its metabolic versatility, *R. sphaeroides* has a branched electron transport chain (ETC) that utilizes several terminal respiratory pathways. The expression of genes encoding components of these pathways is coordinately regulated, depending on the prevailing redox conditions and the terminal electron acceptor present.

The concentration of oxygen, when used as a terminal electron acceptor, regulates membrane biogenesis in *R. sphaeroides*; below approximately 3% oxygen, the intracytoplasmic membrane is synthesized (34). This organelle houses components necessary for the photosynthetic lifestyle, such as the various photosystems and electron carriers. In addition to O₂, incident light intensity also regulates intracytoplasmic membrane abundance and composition in the absence of oxygen.

Gene expression in *R. sphaeroides* is controlled by several well-defined regulatory elements. The expression of the photosynthesis (PS) genes is primarily controlled through the interplay of three major regulatory systems: the PrrBA two-component system (23, 38), the AppA/PpsR antirepressor/repressor system (25, 57), and FnrL (76). PrrBA are homologs of the RegBA two-component system in the related organism *Rhodobacter capsulatus* (21). PpsR is homologous to CrtJ, also found in *R. capsulatus*, and FnrL is homologous to Fnr from *Escherichia coli*. PpsR is a repressor of PS genes (50), and recent data from this laboratory suggest a much more extensive regulatory role for this protein (P. Bruscella et al., submitted for publication). Finally, FnrL has been shown to be selectively involved in the regulation of several tetrapyrrole biosynthetic genes, as well as genes encoding the *cbb*₃ oxidase and the *pucBA* operon, encoding the apoproteins of the light-harvesting complex II (LHII) spectral complex.

In the PrrBA two-component system, PrrA (RegA) serves as the response regulator and can bind DNA both in a specific and nonspecific manner (18, 23, 28, 36; J. M. Eraso and S. Kaplan, unpublished data). PrrB (RegB) is a membrane-localized histidine kinase/phosphatase.

Previous data from this and other laboratories have shown regulation by PrrA, and by RegA in *R. capsulatus*, of a considerable number of cellular functions, including PS, CO₂ fixation, N₂ fixation, H₂ uptake and oxidation, and the ETC (19, 21, 45). Because of the apparent importance of the Prr (Reg) system in

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, University of Texas Health Science Center, 6431 Fannin, Houston, TX 77030. Phone: (713) 500-5505. Fax: (713) 500-5499. E-mail: Samuel.Kaplan@uth.tmc.edu.

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selective aspects of anaerobic gene expression, we found it of general interest to assess the full measure of the Prr system in the metabolic profile of *R. sphaeroides*. In this work, we undertook an assessment of the totality of PrrA regulation in *R. sphaeroides*. To do this, we performed a microarray analysis of the transcriptome using the *R. sphaeroides* 2.4.1 GeneChip. Comparison of the wild type (WT) with an isogenic PrrA⁻ (PrrA2) (22) mutant strain grown anaerobically in the dark in the presence of DMSO as an electron acceptor revealed that approximately 25% of the genome was regulated by PrrA, directly or indirectly. These growth conditions were selected because they normally lead to PrrA activation compared to aerobic conditions as judged by PS gene expression, and cells with a mutated *prrA* are unable to grow photosynthetically (23). We observed that in addition to the numerous PrrA gene targets already known to exist, genes encoding proteins whose functions are involved in intermediary metabolism, repair of DNA and protein damage, cell motility and secretion, and translation constituted new targets for PrrA regulation, although regulated genes were highly represented in all COG (cluster of orthologous group) functional categories.

The microarray results presented here matched both our and others' earlier observations substantiating regulation of previously studied genes, such as genes residing in the PS gene cluster (PGC). A comparison of the proteome profiles of WT and PrrA2 cells revealed qualitatively remarkably similar results, even though only a fraction of the proteins, ~36%, were detected in our analysis, a value consistent with that previously observed for proteomic coverage (7, 8). The agreement between postscriptome and proteome data sets occurred despite likely posttranscriptional and posttranslational regulatory processes.

The present study unambiguously provides new evidence of a global role for PrrA as a transcriptional repressor, in addition to extending its role as a transcriptional activator. In addition, several target genes were randomly chosen for added study. A direct comparison of transcript specific signals, using Northern blot hybridization, confirmed the microarray results.

Finally, the use of genetic selection using translational *kan* fusions to genes negatively regulated by PrrA identified the probable locations of specific PrrA binding sequences involved in repression and revealed that these sequences can reside in the coding regions of some genes. Similarly, the use of transcriptional *lacZ* fusions to genes activated by PrrA identified DNA binding sequences involved in the direct regulation by PrrA and provided evidence for indirect regulation by PrrA through the likely regulation of genes encoding other transcriptional regulators.

(Preliminary results of this work were presented at the 105th General Meeting of the American Society for Microbiology, Atlanta, GA, 5 to 9 June 2005.)

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids are described in Table 1. *Escherichia coli* strains were grown at 37°C on LB medium (46), and *R. sphaeroides* 2.4.1 strains were grown at 30°C on Sistrom's medium A (SIS) (10) containing succinate as the carbon source. Tetracycline (Tet), ampicillin, kanamycin (Kan), streptomycin, spectinomycin (Sp), and trimethoprim (Tp) were used, when required, at concentrations previously described (23). *R. sphaeroides* cultures were grown aerobically, anaerobically in the dark and photosynthetically as previously described (14). Strain PrrA2 was made

previously (22), and it contains a substitution of 169 codons out of a total 185 within *prrA* with an Ω cassette, as shown in Table 1.

DNA manipulations and analysis. Standard protocols or manufacturer's instructions were followed to isolate plasmid DNA, as well as for restriction endonuclease, DNA ligase, PCR, and other enzymatic treatments of plasmids and DNA fragments. Enzymes were purchased from New England Biolabs, Inc. (Beverly, MA), Promega Corp. (Madison, WI), United States Biochemical Corp. (Cleveland, OH), Invitrogen (Carlsbad, CA), and Roche (Branchburg, NJ). Plasmid DNA was purified using the Wizard SV miniprep kit from Promega (Madison, WI). DNA fragments were purified using the QIAquick gel extraction kit (Qiagen Inc., Santa Clarita, CA). *Pfu* ultra DNA polymerase was used as the high-fidelity PCR enzyme (Stratagene-Agilent Technologies, La Jolla, CA). DNA sequencing was performed at the DNA sequencing core facility of the Department of Microbiology and Molecular Genetics (The University of Texas—Houston). The final versions of all relevant clones were sequenced to verify their construction.

Microarray experiments and GeneChip data analyses. The *R. sphaeroides* 2.4.1 GeneChip is custom designed and manufactured by Affymetrix Inc. (Santa Clara, CA). Quadruplicate cultures of PrrA2 cells were grown anaerobically in the dark in the presence of DMSO, and triplicate RNA samples were used for the analysis and compared to WT cells previously analyzed in our laboratory (in this study and in reference 45). The same standardized protocol (63) is always used in our laboratory to allow for comparison between different samples and reproducibility. cDNA synthesis, fragmentation, and labeling were performed according to the instructions for the *Pseudomonas aeruginosa* Genome Array by Affymetrix and the methods described previously (63). The scanned images were analyzed using the Affymetrix Microarray Suite 5.0 (MAS5) program to obtain signal values, detection of calls (present/absent/marginal), and *P* values. The changes in expression and hierarchical clustering of the hybridization intensity of the experimental probe sets were calculated using dChip software (41). The original filtering criterion between the group means was a 2-fold change, although a 1.5-fold change was also used, using the 90% confidence boundary for 0-fold change, which was calculated using the standard errors of the means from independent triplicate experiments. The Pearson correlation coefficient value (*r* value) was calculated using the Microsoft Excel program. The microarray mean signal value corresponding to a specific gene in either WT or PrrA2 cells was assigned as present when the present/absent/marginal call corresponding to at least one of the three cultures in the triplicate RNA set was assigned as present by the MAS5 software. The expression data were deposited in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/projects/geo), platform GPL162.

COG functional group analysis. The COG functional groups (71) for the whole genome of *R. sphaeroides* were assigned by the Genome Analysis and System Modeling Group of the Life Sciences Division of Oak Ridge National Laboratory. They can be found at <http://www.rhodobacter.org>. In each category denoted by a capital letter, all genes were counted and subsequently subdivided into three groups: positively regulated, having a WT/PrrA2 mean microarray value of ≥ 1.5 -fold; negatively regulated, exhibiting a PrrA2/WT mean microarray value of ≥ 1.5 -fold; and not regulated, showing a WT/PrrA2 or PrrA2/WT ratio of < 1.5 -fold. Percentages were calculated for each individual category in terms of the total number of genes in a group belonging to a category compared to the total number of genes in the specific category.

Proteome analysis. Peptide-centric proteomic measurements on WT and PrrA2 cultures were made using the accurate mass and time tag approach (68). Briefly, measured accurate masses and liquid chromatography (LC) elution times obtained from a LC-coupled Fourier transform ion cyclotron resonance mass spectrometer (LC-FTICR-MS) (11 T) were matched (49) to peptide-specific information contained within a previously generated reference database (8). Quadruplicate LC-FTICR-MS measurements were made with the order of analysis established by randomized blocking. Sample preparation protocols (global, soluble, and insoluble protein extracts), conditions for the LC separation of peptides, and FTICR-MS instrument operation parameters have been described elsewhere (8).

The reference database contains protein information, elution times, and calculated masses of peptides previously identified from LC tandem mass spectrometry analyses of WT aerobic and photosynthetic *R. sphaeroides* 2.4.1 cell cultures (8). This database was stringently filtered prior to the matching of LC-FTICR-derived data to eliminate peptides that had a low probability of correct sequence assignment (assigned using the SEQUEST algorithm) (8, 60). Additionally, the set of peptides identified from the matching process was further filtered to reduce those peptides with ambiguous matches (51) (i.e., instances where measured LC-FTICR-MS peptide mass and elution time could match to more than one peptide in the database) and peptides with sequences linked to more than one protein. A protein was considered positively identified in the WT and PrrA2

TABLE 1. Bacteria and plasmids used in this study

Bacterial strain or plasmid	Genotype or phenotype ^a	Reference or source
<i>E. coli</i> strains		
DH5 α Phe	F ⁻ ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44</i> λ ⁻ <i>thi-1 gyrA</i>	23
S17-1	<i>relA1 phe::Tn10dCm</i> C600::RP-4 2-(Tc::Mu)(Kan::Tn7) <i>thi pro hsdR hsdM⁺ recA</i>	66
<i>R. sphaeroides</i> strains		
2.4.1 (ATCC BAA-808)	Wild type	W. R. Sistrom
PrrA2	2.4.1 Δ <i>prrA</i> :: Ω Sp ^r ; St ^r	22
PRRBCA2	2.4.1 <i>prrBCA</i> Δ BspEII-Tth111I ^b :: Ω Tp; Tp ^r	52
Plasmids		
pBluescript II	Ap ^r ; with T3 and T7 promoters	Stratagene
pCF1010	Promoterless <i>lacZ</i> transcriptional fusion vector; Tet ^r Sp ^r St ^r	39
pJE3153	pBSISK ⁺ containing a 0.75-kb SmaI fragment with the <i>fmrL-hemZ</i> regulatory region; Ap ^r	52
pJE3169	pCF1010 derivative containing <i>fmrL::lacZ</i> transcriptional fusion; Tet ^r Sp ^r St ^r	This study
pJE4445	pBSII XbaI-HindIII containing a 3,375-bp PCR fragment with <i>pqqEDCBA</i> ; Ap ^r	This study
pJE4448	pBSII XbaI-HindIII containing a 3,439-bp PCR fragment with RSP3162-RSP3163-RSP3164; Ap ^r	This study
pJE4449	pBSII XbaI-HindIII containing a 1,303-bp PCR fragment with RSP3361; Ap ^r	This study
pJE4451	pBSII XbaI-HindIII containing a 1,299-bp PCR fragment with RSP0474-RSP0475; Ap ^r	This study
pJE4452	pBSII XbaI-HindIII containing a 758-bp PCR fragment with RSP2389; Ap ^r	This study
pJE4708	pRK415::368-bp PCR fragment from RSP2389 (codon 20 ^c):Kan (codon 12 ^d) translational fusion divergently transcribed from the <i>tet</i> gene; Tet ^r	This study
pJE4740	pML5::430-bp PCR fragment from RSP3361 (335 bp upstream and 95 bp within the gene); RSP3361 Δ 2::lacZ transcriptional fusion (16 bp ^e); Tet ^r	This study
pJE4742	pML5::414-bp PCR fragment from RSP3361 (319 bp upstream and 95 bp within the gene); RSP3361 Δ 1 Δ 2::lacZ transcriptional fusion (32 bp ^e); Tet ^r	This study
pJE4935	pML5::446-bp PCR fragment from RSP3361 (351 bp upstream and 95 bp within the gene); WTRSP3361::lacZ transcriptional fusion; Tet ^r	This study
pJE4936	pML5::430-bp PCR fragment from RSP3361 (335 bp upstream and 95 bp within the gene); RSP3361 Δ 1::lacZ transcriptional fusion (16 bp ^e); Tet ^r	This study
pJE4957	pML5 derivative containing (P ^{up}) RSP2389::lacZ transcriptional fusion with a T-to-C substitution immediately before -35; Tet ^r	This study
pJE4958	pML5::368-bp PCR fragment from RSP2389 (309 bp upstream and 60 bp within the gene); WT RSP2389::lacZ transcriptional fusion; Tet ^r	This study
pJE5090	pSL301 derivative containing an approximately 4.0-kb EcoRI insert from pML5P ^{down} <i>rrnB</i> harboring the MCS, most of <i>lacZ</i> , and an upstream divergently transcribed 336-bp fragment containing the <i>rrnB</i> P ^{down} promoter; Ap ^r	This study
pJE5404	pML5P ^{down} <i>rrnB</i> ::769-bp PCR fragment from <i>ppaA-ppsR</i> within pLX41 (715 bp upstream to and including codon 47 of <i>ppaA</i> , and 54 bp within the <i>ppsR</i> gene); <i>ppsR</i> ::lacZ transcriptional fusion; Tet ^r	This study
pLX41	IncQ <i>ppsR</i> ::lacZ; Sp ^r St ^r	26
pML5	Promoterless <i>lacZ</i> transcriptional fusion vector; Tet ^r	35
pRK415	IncP; Tet ^r	32
pRK2013	ColE1 replicon, Tra ⁺ of RK2; Kan ^r	16
pSL301	3.2-kb superlinker vector containing an extended polylinker; Ap ^r	Invitrogen
puc4K	Source of the <i>kan</i> gene from Tn903 used for Kan translational fusions; Ap ^r Kan ^r	73

^a Drug resistance phenotypes: Ap^r, ampicillin resistant; Kan^r, kanamycin resistant; Sp^r, spectinomycin resistant; St^r, streptomycin resistant; Tet^r, tetracycline resistant; Tp^r, trimethoprim resistant. Abbreviations: pBSISK⁺, pBluescript II SK⁺; pBSII, pBluescript II; MCS, multiple cloning site.

^b The 5' overhangs were made blunt with Klenow fragment of DNA polymerase I before cloning.

^c Codon within the structural part of the *R. sphaeroides* gene at which the translational fusion was made.

^d Codon within the structural part of the *kan* gene at which the translational fusion was made.

^e Size of the deletion encompassing the PrrA site.

cultures if two or more peptides were detected in at least one of the four replicates.

Relative quantitative comparisons between proteomes associated with each cell culture were based upon two "label-free" proteomic approaches: (i) the total number of peptides detected for a protein ("mass tag") (77), and (ii) the sum of abundances of peptides (determined by integrating the areas under each peak of the LC-FTICR-MS spectra for a detected peptide) for a protein ("abundance") (8) in four replicates emanating from WT and mutant PrrA2 cultures and expressed as either a "mass tag" or an "abundance" ratio between WT and PrrA2 cells (1). By comparing these abundance metrics for each specific protein only to itself in WT cells, as opposed to PrrA2 cells, we eliminated the bias inherent to the proportionality of protein mass to its abundance in terms of the number of specific peptides detected. In addition, proteins assigned as present only in one

strain, namely, WT or PrrA2, and absent in the other, were also scored. The total number of predicted proteins was based on JGI annotation models (http://genome.jgi-psf.org/finished_microbes/rhosp/rhosp.home.html).

Computer programs. Software analyses were performed using the computer programs ARTEMIS (Sanger Institute of the Wellcome Foundation), DNA Strider (Institute de Recherche Fondamentale, Commissariat à l'Energie Atomique, France), MAS5 and GCOS (Affymetrix), Microsoft Excel (Microsoft), and Oligo 4.0 (National Biosciences Inc., Plymouth, MN). The Microsoft Excel program was used to assign changes in gene expression found when comparing WT and PrrA2 mutant cells, to the different COG-categorized genes, as well as to the 409 genes whose expression levels had been found to significantly shift in the transition from PS to aerobic conditions of growth in a previous study from this laboratory (2).

Conjugation techniques. Plasmids were mobilized using di- and triparental matings from *E. coli* S17-1 (66) and DH5 α Phe (23) strains, respectively, into *R. sphaeroides* strains as described elsewhere (14).

Construction of riboprobe vectors. Internal fragments from the RSP3361 gene, RSP0793 gene, RSP3163 gene, RSP0474 gene, and RSP2389 gene were cloned from pJE4449, pJE4445, pJE4448, pJE4451, and pJE4452, respectively. pJE4460 contains a 158-bp SalI-SacII fragment, pJE4461 contains a 178-bp NotI-SacII fragment, pJE4463 contains a 283-bp EcoRI-SmaI fragment, pJE4464 contains a 152-bp SfiI-EcoRI fragment, and pJE4465 contains a 193-bp SacII-XmnI fragment from the RSP3361 gene, RSP0793 gene, RSP3163 gene, RSP0474 gene, and RSP2389 gene, respectively. Radioactive probes were made using these highly purified plasmid DNAs in *in vitro* transcription reactions. The MAXIscript T7/T3 kit and ULTRAhyb hybridization buffer were purchased from Ambion, Inc., Austin, TX.

RNA isolation and Southern and Northern blot hybridization techniques. RNA was isolated from cells grown anaerobically in the dark and assayed as described previously (40, 79). Signals were detected and quantitated using a Storm phosphorimager (Amersham). The relative RNA concentrations in the blots were normalized for differences in concentration during loading using [α -³²P]dCTP-labeled DNA probes encoding *R. sphaeroides* rRNA (17).

Construction of transcriptional *lacZ* fusions. pJE4935 harbors the RSP3361:*lacZ* transcriptional fusion which contains a 446-bp PCR fragment from the RSP3361 gene (351 bp upstream and 95 bp within the gene) fused to *lacZ* in pML5. pJE4449 was used as a template for PCR. Combinatorial PCR, as described previously (24), was used to delete the PrrA sites in the regulatory region of the gene to construct fusions Δ 1, Δ 2, and Δ 1 Δ 2.

pJE4958 harbors the RSP2389:*lacZ* transcriptional fusion which contains a 368-bp PCR fragment from the RSP2389 gene (309 bp upstream and 60 bp within the gene) fused to *lacZ* in pML5. pJE4452 was used as template for the PCR. The genetic selection for Kan resistance (Kan^r) described later gave rise to the P^{pp} and Δ 2 derivatives of this fusion (Table 1).

pJE5404 is a pML5P^{down}*mmB* (Table 1) derivative which harbors a *ppsR*:*lacZ* transcriptional fusion. It contains a 769-bp PCR fragment obtained from the *ppaA*-*ppsR* region within pLX41 (26). Unlike the *ppsR*:*lacZ* fusion in pLX41, the fusion in pJE5404 starts at codon 47 of *ppaA*, 715 bp upstream of *ppsR* and extends 54 bp into the *ppsR* gene. It therefore contains only a promoter upstream of *ppsR*, located within *ppaA*, and it lacks the *ppaA* promoter and first 46 codons.

The *furL*:*lacZ* transcriptional fusion used in this study is contained in pJE3169. This plasmid was constructed by inserting the approximately 0.75-kb SmaI fragment harboring the *furL*-*hemZ* regulatory region present in pJE3153 (52) into pCF1010 in the orientation opposite to that in pJE3170 (52).

Construction of translational *kan* fusions. pJE4708 is a pRK415 derivative which harbors the RSP2389:*kan* translational fusion as a 368-bp PCR fragment from pJE4452 (309 bp upstream and 60 bp within the gene) fused to *kan* from puc4K in frame at codon 12. Transcription orientation is opposite that of the resident *tet* gene to avoid runaway transcription from the *tet* promoter (Table 1).

Genetic selection for loss of PrrA repression. WT and PrrA2 cells containing pJE4708 harboring the RSP2389:*kan* fusions were spread on SIS plates containing Tet or Tet/Kan, with Kan concentrations ranging from 5 to 50 μ g/ml. The efficiencies of plating were recorded. The presence of 15 μ g/ml Kan was sufficient as a selective agent in terms of decreased plating efficiency. In all cases, PrrA2 cells harboring the fusions showed higher resistance to Kan than WT cells did.

β -Galactosidase assays. *R. sphaeroides* cultures used for the determination of β -galactosidase activity were grown as described previously (23), and assays were performed as described elsewhere (69). The data provided are the averages of at least two separate experiments each performed in duplicate. Standard deviations were always $\leq$ 15%. Protein concentration of cell extracts was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as a standard.

Materials. 5-Bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal) was purchased from United States Biochemical Corp., Cleveland, OH. *o*-Nitrophenyl- β -D-galactopyranoside (ONPG) was purchased from Sigma Chemical Co., St. Louis, MO. GenePure LE agarose was purchased from ISC BioExpress, Kaysville, UT. [α -³²P]dCTP (3,000 Ci/mmol) and [α -³²P]CTP (800 Ci/mmol) were purchased from Amersham Corp., Arlington Heights, IL. All other chemicals used in this work were reagent grade.

RESULTS

Quantitation of PrrA-regulated genes in *R. sphaeroides* 2.4.1 by microarray analysis. To determine the extent of PrrA regulation in *R. sphaeroides*, we performed microarray analysis

using the *R. sphaeroides* DNA GeneChip, as described in Materials and Methods, on three independent PrrA[−] (PrrA2) cultures grown anaerobically in the dark in the presence of DMSO as an electron acceptor, since PrrA[−] cells are not able to grow photosynthetically (23). An analysis of three independent WT cultures had been performed previously (in this study and in reference 45), and these data were used as a control for the PrrA2 mutant. The two strains have the same growth rate when growing anaerobically in the dark.

Pairwise comparisons of the data sets from any two PrrA[−] cultures derived from the triplicate set showed an *r* (Pearson coefficient) value very close to 1, for example, between PrrA2-3 and PrrA2-4 cultures, the *r* value was 0.993. This value was similar for those calculated previously for the WT cultures, 0.992 for WT-1 and WT-3 cultures, used for comparison. In contrast, when comparing a WT to a PrrA[−] data set, as in WT3 and PrrA2-4 cultures, the *r* value was 0.743, indicating extensive changes in the gene expression values between the mutant and WT. This difference in gene expression values was also observed by analyzing the hybridization intensities of WT and PrrA2 mutant by hierarchical clustering (data not shown).

Details of PrrA regulation: activation and repression. We chose both a 1.5-fold and a more conservative 2-fold change in expression when comparing WT to PrrA2 cells in our analysis. Whereas in 381 (~9%) genes, expression was higher in the WT, 677 (~16%) genes exhibited expression values at least twofold higher in the mutant compared to the WT, suggesting negative regulation by PrrA; this was an unexpected result based upon previous studies of the role of PrrA in gene regulation in *R. sphaeroides*. This was also confirmed by hierarchical clustering analysis (data not shown). Thus, ~25% of genes in the genome, as represented by the construction of the GeneChip (55), show expression values differing by twofold or more when comparing WT and PrrA2 cells and are therefore regulated by PrrA, either directly or indirectly. A total of 3,224 genes, or ~75%, were not regulated in this manner.

When choosing a less-stringent, but statistically significant, 1.5-fold difference in expression, gene regulation increased; 606 (~14%) genes are positively regulated, 1,249 (~29%) are negatively regulated, and 1,855 do not change their expression by $\geq$ 1.5-fold. Thus, choosing a 1.5-fold cutoff in gene expression levels suggests that PrrA is involved in the regulation of over 43% of the genes in the genome, either in a direct or indirect manner. All seven *R. sphaeroides* linkage groups, i.e., the two chromosomes (chromosomes I and II), as well as the five endogenous plasmids, contain genes regulated by PrrA by the above criteria, and the number of these correlates with the size of the replicon.

The PrrA gene targets were divided into four classes, depending on whether their expression levels changed between 2- to 5-fold (class I), 5- to 10-fold (class II), 10- to 20-fold (class III), or >20-fold (class IV), whether positively or negatively. The results are shown in Fig. 1. A total of 298, 40, 13, and 7 genes were downregulated in the mutant with respect to the WT in classes I, II, III, and IV, respectively (black bars). These genes show positive regulation by PrrA. In contrast, 438 genes, 20 genes, and 1 gene were upregulated in the mutant in classes I, II, and III, respectively (white bars). They show negative regulation by PrrA. In addition, the mean signals corresponding to 23 genes were assigned as "present" in the WT and

FIG. 1. Comparison of global gene expression in the WT and PrrA2 cells. Increasing gene expression changes are represented by white bars (upregulated genes in PrrA2 cells with respect to expression in the WT), and decreasing gene expression changes are represented by black bars (downregulated genes). The genes were divided into four classes, according to the changes in gene expression by comparing the ratio of microarray mean values in the WT and the PrrA2 mutant as follows: class I (2- to 5-fold), class II (5- to 10-fold), class III (10- to 20-fold), and class IV (>20-fold). The number of genes present in each category is indicated above or below each bar. A total of 218 genes were expressed in the PrrA2 mutant but not in the WT (A-WT). A total of 23 genes were expressed in the WT but not in the PrrA2 mutant (A-PrrA2).

“absent” in the mutant, whereas, conversely, those from 218 genes were “present” in the mutant and “absent” in the WT, approximately 10-fold higher than those in the previous category. In general, the levels of expression of the activated genes were much higher than those for the repressed genes, and this could explain this 10-fold difference. Alternatively, this could also be due to contributions from regulators other than PrrA, which might be more prevalent in the case of the activated genes, and therefore provide a certain basal level of expression, so their mean expression signals would not be found as “absent” when PrrA is deleted. Also, activated genes could be “leakier,” and repressed genes could be more tightly regulated. Interestingly, this difference could point toward different modes of action for PrrA when acting as an activator of gene expression versus as a repressor. In summary, PrrA regulated 1,058 genes (677 upregulated and 381 downregulated in the mutant) by twofold or more, either directly or indirectly, out of 4,284 genes as represented on the GeneChip of *R. sphaeroides* 2.4.1.

In a recent study from this laboratory, we have followed changes in gene expression following a transition of *R. sphaeroides* from anaerobic photosynthetic growth to fully aerobic

growth at 30% O₂ concentration (2). In this study, a total of 409 genes were found to significantly change their expression levels during the course of the shift and were placed into three major classes, according to the kinetics of their expression profiles. We scored these same genes for possible PrrA regulation in the PrrA2 mutant using a twofold cutoff. Out of the 409 genes showing changes in the Arai et al. study (2), 91 were positively regulated by PrrA, and 80 were negatively regulated, and the complete analysis is shown in Table S1 in the supplemental material. The remaining 238 genes of the 409 genes found to undergo change during the course of the growth shift were not observed to change in the present study. When combining the results of these two independent studies, approximately 42% of the gene changes observed when shifting cells from photosynthetic to aerobic growth occur in genes regulated by PrrA. This represents a significant fraction of the observed changes and attests to the importance of PrrA in *R. sphaeroides*. In addition, out of the 1,058 PrrA-regulated genes found in the present study, 171, approximately 16%, change their expression values during a shift from photosynthetic to aerobic conditions of growth.

Validation of microarray results. Because of the large numbers of new genes affected by deletion of *prrA*, we considered it essential to validate our results by looking at some representative genes whose expression pattern was already well studied. These genes had been found to be PrrA regulated by use of direct mRNA quantitation, as well as by use of reporter fusion analysis, performed in this and other laboratories. For example, numerous studies had concentrated on genes located in the PGC, an approximately 67-kb region located on chromosome I (9). Our previous (30) and present results agree with the results of earlier studies and significantly extend these earlier observations. PrrA regulated most genes positively to various degrees. In fact, this study provided the first opportunity to analyze expression levels of all genes comprising the PGC in the same experiment. From here on we refer to changes in microarray gene expression using the RSP (*R. sphaeroides*) number, followed by the gene designation, if assigned, and the gene expression change value (*n*-fold), with either a minus sign (for genes downregulated in the mutant), or no sign (for genes upregulated in the mutant).

The genes showing the greatest change encode the apoproteins of the photosynthetic apparatus and were as follows: (i) the RSP0314 gene (*pucB*) (−145-fold), (ii) the RSP6108 gene-RSP0258 gene (*pufBA*) (−12.1-fold), (iii) the RSP0291 gene (*puhA*) (−19.8-fold), and (iv) the RSP0257 gene-RSP0256 gene (*pufLM*) (−46.4- and −44.6-fold) (this study and in reference 30). The RSP0314 gene, RSP6108 gene-RSP0258 gene, RSP0291 gene, and RSP0257 gene-RSP0256 gene are part of the LHII, LHI, and reaction center spectral complexes. The genes involved in photopigment biosynthesis were also similarly regulated, although to a lesser extent. In addition, new gene targets for PrrA regulation were also uncovered, such as the RSP0317 gene (*hemN*) (−2.3-fold), encoding one of several coproporphyrinogen III oxidases, involved in tetrapyrrole biosynthesis. This gene is also regulated by FnrL (RSP0698 protein) (52) and is therefore expressed in the PrrA2 mutant strain.

Similarly, genes encoding proteins of the ETC are also targets for PrrA regulation. The *ccoNOQP* operon (RSP0696

gene-RSP0695 gene-RSP0694 gene-RSP0693 gene) was positively regulated by PrrA (-2.9 -, -2.9 -, -2.8 -, and -2.4 -fold). These genes encode subunits of the *cbb3* terminal oxidase. The *fbcCBF* operon (RSP1396 gene-RSP1395 gene-RSP1394 gene) and *fbcQ* (RSP2687 gene) encode subunits of the cytochrome *bc₁* complex and were positively regulated by PrrA by -4.0 -, -3.7 -, -2.9 -, and -4.0 -fold, respectively (30). Conversely, the RSP1877 gene, RSP1826 gene, and RSP1829 gene, encoding subunits I, II, and III of the *aa3* terminal oxidase (1.0 -, -2.1 -, and -1.6 -fold, respectively), as well as *qxtAB* (RSP3212 gene-RSP3210 gene), encoding a quinol oxidase (-1.2 - and -1.2 -fold, respectively) were barely sensitive to mutation of *prfA* (for a list containing these genes ordered by COGs, see Table S2A in the supplemental material).

Direct analysis of mRNA levels. To extend our validation of the microarray results, we chose genes at random which were PrrA regulated both positively and negatively. In all cases, Northern blot hybridizations were performed to determine mRNA levels directly, using riboprobes specific for each gene. The RSP0474 gene (*cycP*), which encodes cytochrome *c'* (Fig. 2A), and the RSP3361 gene (Fig. 2B), which is either suggested to encode a type I restriction enzyme, according to a PFam model comparison, or which is assigned to COG4748, and annotated as an uncharacterized conserved protein, were chosen as representatives for strong PrrA positive regulation. In both cases, the MAS5 software program had assigned "absent" calls to the signal from the mutant, which are described as A-PrrA, referring to absent in the PrrA2 mutant, while present in WT. Two transcripts were detected for each gene from the WT, and the changes in the signal ratios matched those in the microarray experiment, as shown in Fig. 2A and B. Analysis of the upstream regulatory sequences of these genes for the presence of putative PrrA binding sites (47) revealed the presence of two consensus sites in the RSP3361 gene (shown later), and one site containing one mismatch in the case of *cycP*, located from -107 to -92 , with respect to nucleotide 1 within the gene.

The RSP0793 gene (*pqqB*), which encodes the pyrroloquinoline quinone (PQQ) biosynthetic protein B (Fig. 2C), the RSP2389 gene (*gpx*), which encodes a glutathione peroxidase (Fig. 2D), and the RSP3163 gene (*coxL*), which encodes a probable oxidoreductase (and has otherwise been assigned to COG1529 and has been annotated as an aerobic-type carbon monoxide dehydrogenase large subunit [CoxL]) (Fig. 2E), were chosen as representatives of PrrA negative regulation. Compared to WT cells, the RSP0793 gene, the RSP2389 gene, and the RSP3163 gene had large increases in PrrA2 cells, with increases of 5.6 -, 12.2 -, and 11.2 -fold, respectively. In this case, the MAS5 software program had assigned "absent" calls to the signal from the WT while present in the PrrA2 mutant for the RSP2389 gene and RSP3163 gene. Similar to those genes analyzed which showed positive regulation by PrrA, the changes in the signal ratios for these genes also matched those in the microarray experiment. From the diversity of genes selected for analysis and the fact that they map throughout the genome, we believe that we have, to a substantial extent, validated the microarray analysis.

PrrA control of biological processes. Next we determined the PrrA genomic target distribution in terms of the likely biological function(s) encoded by PrrA-regulated genes given

the limitations of gene annotation. For this purpose, genes corresponding to all functional categories based on COGs (71) and cited at <http://www.rhodobacter.org>, were analyzed systematically for PrrA regulation, as described in Materials and Methods. The results are shown schematically in Fig. 3 and in detail in Tables S2A and S2B in the supplemental material. In this classification, a specific gene can be assigned to more than one category; thus, there is a certain amount of redundancy associated with this analysis. Except for 3 functional categories, out of a total of 18, quantitatively and quantitatively (percentage-wise), the general distribution of genes in the classes was that the number of genes downregulated in the PrrA2 mutant (activated by PrrA) was less than the number of genes upregulated in the PrrA2 mutant (repressed by PrrA), which was less than the number of genes not regulated by PrrA (no changes noted). We have chosen negative gene expression values for genes downregulated in the PrrA2 mutant compared to the WT, and, conversely, positive gene expression values for upregulated genes.

The three exceptions were found in categories J (translation, ribosomal structure and biogenesis), N (cell motility and secretion), and C (energy production and conversion). In category N (cell motility and secretion) and category C (energy production and conversion), the number of genes downregulated in the mutant was larger than those upregulated, denoting a bias toward positive PrrA regulation, as shown in Fig. 3 and in Table S2A in the supplemental material.

Category J (translation, ribosomal structure, and biogenesis) was the class with the greatest number of genes regulated by PrrA ($\sim 65\%$) and the greatest number of genes positively regulated by PrrA ($\sim 28\%$) (see Table S2B in the supplemental material). Conversely, categories L (DNA replication, recombination and repair), M (cell envelope biogenesis, outer membrane), N (cell motility and secretion), C (energy production and conversion), I (lipid metabolism), and R (general function prediction) contained the highest percentages of genes not regulated by PrrA (approximately 60%). In addition, the class with the lowest number of genes downregulated (6%) was category M (cell envelope biogenesis, outer membrane). In the case of upregulated genes, category F (nucleotide transport and metabolism) contained the highest number of genes. Conversely, category N (cell motility and secretion) contained the lowest number. Thus, certain biases, in terms of general biological processes, exist in the PrrA regulatory network in *R. sphaeroides*. Here we present data for some individual genes newly found to be PrrA regulated, but we refrain from presenting a systematic analysis and instead refer the reader to Tables S2A and S2B in the supplemental material, where the results for all genes showing a change in expression of ≥ 1.5 -fold in WT and PrrA2 mutant cells are shown.

Genes encoding numerous ribosomal proteins were downregulated in the PrrA2 mutant, together with genes encoding other translation factors (see Table S2A in the supplemental material). Conversely, tRNA genes were upregulated in the PrrA2 mutant (Fig. 4). Nearly 80% of the total number of tRNAs in *R. sphaeroides*, as represented in the GeneChip, are negatively regulated by PrrA, and the effect is shared by tRNAs carrying all 20 amino acids. In some representative cases, such as those involving the amino acids Ser, Pro, and Ala, there is a direct correlation between codon abundance and the change in

FIG. 2. Northern blot hybridization analysis of expression of the RSP0474 gene (*cycP*), RSP3361 gene, RSP0793 gene (*pqqB*), RSP2389 gene (*gpx*) and RSP3163 gene (*coxL*) in *R. sphaeroides* 2.4.1. The RNAs were prepared from WT and PrrA2 cells grown anaerobically in the dark with DMSO as an electron acceptor, as described in the text. The riboprobes used are described in Materials and Methods. The letter A in parentheses means absent, and it refers to the microarray signal value for a particular open reading frame being scored as absent by the MAS5 program. cyt. c', cytochrome c'.

upregulation for the specific tRNA carrying the complementary anticodon, whereas in the case of Val and Leu, this correlation is inverse. Consistent with tRNA gene regulation, *mpA* (RSP1060 gene), encoding the protein component of RNase P, responsible for maturation of tRNA precursors, was scored as absent in WT, denoting either no expression or, more likely, a level too low for detection. Similarly, several genes encoding aminoacyl-tRNA synthetases were also upregulated in PrrA2 cells. Interestingly, the regulatory protein Fis, which

is lacking in *R. sphaeroides* (45) and which regulates rRNA and tRNA genes (64) in *E. coli*, also performs coordinate regulation of expression of tRNA genes (5).

In general, the expression of genes involved in DNA repair and recombination and in protein repair and folding, as well as genes encoding proteins produced in response to certain kinds of stress, were upregulated in the PrrA2 mutant. For example, *gpx* (RSP2389 gene), the gene encoding glutathione peroxidase, was scored as absent in the WT while present in PrrA2,

FIG. 3. Quantitation of the number of genes regulated by PrrA in the COG functional categories. The percentages were calculated with respect to the total number of genes in each category, which is 100%. Percentages corresponding to upregulated genes in PrrA2 cells compared to WT cells (above the line) are represented by white bars, and those corresponding to downregulated genes (below the line) are represented by black bars. A 1.5-fold value was used as cutoff. The categories represented can be found at <http://www.rhodobacter.org> and are as follows: category J, translation, ribosomal structure and biogenesis; category K, transcription; category L, DNA replication, recombination and repair; category D, cell division and chromosome partitioning; category O, posttranslational modification, protein turnover, chaperones; category M, cell envelope biogenesis, outer membrane; category N, cell motility and secretion; category P, inorganic ion transport and metabolism; category T, signal transduction mechanisms; category C, energy production and conversion; category G, carbohydrate transport and metabolism; category E, amino acid transport and metabolism; category F, nucleotide transport and metabolism; category H, coenzyme metabolism; category I, lipid metabolism; category Q, secondary metabolites biosynthesis, transport and catabolism; category R, general function prediction only; and category S, function unknown. Additional data for this figure are presented in Tables S2A and S2B in the supplemental material.

and *trxA* (RSP1529 gene), which encodes thioredoxin, a protein associated with oxidative stress (43), was upregulated by 7.5-fold in the mutant. Other genes in this category are included in Table S2A in the supplemental material.

The RSP1467 gene, which encodes a fatty acid (FA) desaturase (alkane 1-monooxygenase), was downregulated substantially in the mutant (by 8.9-fold) (−8.9). This gene product decreases membrane fluidity through membrane lipid modulation. In contrast, *cfaS* (RSP2144 gene), encoding a cyclopropane fatty acid (CFA) synthase, was upregulated in the mutant (2.4-fold). This enzyme performs cyclopropanation of the double bonds of unsaturated FAs to make CFAs, which pack in the membrane less densely than saturated FAs but more densely than unsaturated FAs. These possible changes in membrane fluidity could explain some secondary effects observed in PrrA2 cells.

The *exbB* (RSP0920 gene), *exbD* (RSP0921 gene), and *tonB* (RSP0922 gene) genes that most likely form an operon and regulate outer membrane transport were upregulated in the PrrA2 mutant by 2.9-, 4.2-, and 3.9-fold, respectively. A recent study from this laboratory revealed an increase in transcription for these same genes during a shift from photosynthetic to aerobic growth conditions (2).

PrrA control over intermediary metabolism was found to be extensive, as shown in Fig. 3 and in Table S2A in the supplemental material. To illustrate this point, examine the differential regulation of gluconeogenesis and the Entner-Doudoroff

pathway and the biosynthesis of coenzyme PQQ. According to these data, PrrA stimulates gluconeogenesis, directly or indirectly, while repressing the Entner-Doudoroff pathway. In addition, the coenzyme PQQ is encoded by genes in the *pqqAB-CDE* operon. *pqqB* (RSP0793 gene) (confirmed with direct mRNA measurement) and *pqqC* (RSP0792 gene) were upregulated in the mutant by 5.6- and 3.3-fold, respectively, whereas *pqqD* (RSP0791 gene) and *pqqE* (RSP0790 gene) were scored as absent in WT. This coenzyme has multiple functions in intermediary metabolism, and in addition, it has recently been reported to be involved in preventing oxidative damage (70).

Proteome analysis of the WT and PrrA2 mutant. For the relative quantitative comparison of WT and PrrA2 proteomes, a general qualitative agreement was observed between protein abundance, estimated as a ratio between the total abundances of peptides (determined by integrating the areas under each peak of the LC-FTICR-MS spectra for a detected peptide), and the number of unique peptides (mass tags) detected for a specific protein, also expressed as a ratio (see Tables S3A and S3B in the supplemental material). For some proteins, discrepancies between both quantitative approaches were observed, which was not surprising because of the variability associated with “label-free” quantitative proteomic approaches in general (6). In light of this variability, both approaches were used for comparison with transcriptome measurements, as has been previously described (77).

FIG. 4. PrrA regulation of tRNA genes. The tRNA genes are indicated at the top of the figure, with the microarray mean signal values represented by black bars. All genes were upregulated in the PrrA2 mutant with respect to the WT. The corresponding codon for each tRNA gene is represented at the bottom, with the least abundant, Gln_CAA, on the left, and the most abundant, Ala_GCC, on the right. Out of 55 tRNA genes, 41 are represented in the *R. sphaeroides* GeneChip. In the cases when more than one codon is shared by the same amino acid, the relative abundance is represented by the height of the black triangle.

The protein encoded by *cycP* (RSP0474 gene), whose microarray mean signal value had been scored as absent in the PrrA2 mutant but present in the WT, was scored as absent (not detected as present) in the PrrA2 mutant, as shown in Table 2. Similarly, the protein encoded by the RSP3361 gene, with the exact same microarray mean signal value as *cycP*, showed higher abundance and total mass tag in the WT than in PrrA2 cells (Table 2). Thus, for these two genes activated by PrrA, protein levels, as measured by the ratios of abundance and mass tag count, correlate with the trend observed for their mRNAs.

For the three genes repressed by PrrA and tested in this study by Northern blot hybridization, *pqqB* (RSP0793 gene), *gpx* (RSP2389 gene), and *coxL* (RSP3163 gene), their signal values were scored as absent in the WT but present in the PrrA2 mutant in the microarray analysis. Consistent with the microarray data, the proteins encoded by *pqqD* (RSP0791 gene) and *coxL* (RSP3163 gene) were scored as absent in the WT and present in PrrA2 cells. Although the gene product for *pqqB* was not detected, we followed *pqqD*, which is most likely

part of the same operon. In the case of *gpx* (RSP2389 gene), its protein product was not detected in this study (see below). Thus, there is also a correlation between protein and mRNA levels in the case of genes upregulated in the PrrA2 mutant compared to the WT.

We extended this study to the complete *R. sphaeroides* proteome by comparing the WT and PrrA2 mutant data sets. For each gene/protein pair, we calculated the ratio of the microarray mean signal value for the gene and the ratio for the corresponding protein abundance and total number of protein-specific peptides detected (total mass tag) for the WT and the PrrA2 mutant. Notwithstanding the limitations inherent to this type of protein analysis, where approximately 30% to 40% of the total number of proteins are customarily detected (8, 77), the object of this analysis was to determine to what extent relative mRNA levels are reflected both qualitatively and quantitatively in their cognate proteins.

The results are shown in Fig. 5 and in Tables S3A and S3B in the supplemental material. Detected peptides were matched to 1,536 annotated proteins, which correspond to approxi-

TABLE 2. Proteome and transcriptome analysis for selected genes

RSP no.	Gene	Presence of protein in WT and PrrA2 cells ^a	Abundance ratio ^b	Mass tag ratio ^c	mRNA comparison (fold change) ^{a,d}
RSP0258	<i>pufB</i>	A-PrrA			−12.1
RSP0314	<i>pucB</i>	Present in both	−56.8	−6.0	−145.0
RSP0474	<i>cycP</i>	A-PrrA			A-PrrA
RSP0791	<i>pqqD</i>	A-WT			A-WT
RSP1517	<i>spbA</i>	Present in both	−3.7	−1.6	−4.0
RSP1518	<i>prrA</i>	A-PrrA			NA
RSP2389	<i>gpx</i>	Not detected			A-WT
RSP3163	<i>coxL</i>	A-WT			A-WT
RSP3361		Present in both	−13.9	−4.0	A-PrrA

^a A-PrrA refers to a protein being scored as absent in PrrA2 cells but present in WT cells. A-WT refers to a protein being scored as absent in WT cells but present in PrrA2 cells. Not detected refers to a protein being scored as absent in WT and PrrA2 cells.

^b Ratio of protein abundance between WT and PrrA2 cells. Negative values refer to higher abundance in WT cells than in PrrA2 cells.

^c Ratio of total mass tag between WT and PrrA2 cells. Negative values refer to higher abundance in WT cells than in PrrA2 cells.

^d Comparison of microarray mean signal values in WT and PrrA2 cells. NA, not available (since *prrA* is mutated in PrrA2 cells, no value could be ascribed to the ratio of the mean signal values in the microarray experiment).

mately 36% coverage of predicted proteins based on JGI annotation models (http://genome.jgi-psf.org/finished_microbes/rhosp/rhosp.home.html), due to technical limitations in the detection method, partly due to the use of stringent filtering to increase reliability seen here and in other studies (8, 77). The proteins were separated into three classes according to their present/absent assignment. A total of 1,202 proteins were assigned as present in both WT and PrrA2 cells, whereas 117 were present in the WT but absent in the mutant, and conversely, 217 were present in the mutant and absent in WT. Of the genes encoding the 1,202 proteins scored as present in both WT and PrrA2 cells, 251 were downregulated, whereas 364 were upregulated in the PrrA2 mutant compared to the WT, and the remaining 587 genes were not regulated by PrrA, as determined by using a ≥ 1.5 -fold cutoff (see Table S3B in the supplemental material).

In Fig. 5 we provide data showing that, for specific gene/protein pairs on a global scale, the microarray values for gene expression are qualitatively consistent with the corresponding protein abundance and mass tag (number of specific tryptic peptides detected) value in more cases than not, although disagreements were found. For example, out of the 251 proteins whose genes were downregulated in the PrrA2 mutant, 63 proteins as opposed to 30 proteins showed higher mass tag values in the WT by ≥ 1.5 -fold compared to the PrrA2 mutant (Fig. 5A; also see Table S3B in the supplemental material). In terms of abundance, 98 proteins showed higher values in the WT, compared to 90 proteins showing higher values in the mutant. As an example, for *puc2B* (RSP1556 gene), which encodes a β polypeptide homologous to that encoded by *puc1B*, the microarray value was -44.7 -fold, indicating strong positive regulation by PrrA. Concomitantly, mass tag and abundance ratios for the corresponding RSP1556 gene were both -2.3 , indicating that the values for both parameters were

higher in the WT than in PrrA2 cells by 2.3-fold (see Table S3A in the supplemental material). Thus, the levels of RSP1556 were in qualitative agreement with the corresponding mRNA levels for *puc2B*, but certainly not in quantitative agreement. This numerical disparity between mRNA and protein values has been observed previously in our laboratory (77) and might be due in part to mRNA half-life. In conclusion, proteins encoded by genes positively regulated by PrrA were detected with higher frequency in the WT than in the PrrA2 mutant, as indicated by the observed mass tag and abundance ratios.

Similarly, of the 364 proteins encoded by genes upregulated in the PrrA2 mutant, 121 proteins showed higher (≥ 1.5 -fold) mass tag values in the mutant than in the WT, while only 50 showed higher values in the WT (Fig. 5B; also see Table S3B in the supplemental material). In terms of abundance, 203 proteins showed higher abundance values in the mutant versus 54 showing higher values in the WT (see Table S3B in the supplemental material). Thus, in general, proteins encoded by genes negatively regulated by PrrA are more abundant and detected by more tryptic-specific peptides in the PrrA2 mutant than in the WT. For example, the RSP0150 gene, which encodes a histidine kinase involved in signal transduction and which had been scored as absent in the WT but present in PrrA2 cells from the transcriptome analysis, had corresponding mass tag and abundance values of 4.0 and 18.6, indicative of its presence in the mutant, compared to the WT (see Table S3A in the supplemental material).

We also scored proteins that were either detected as present in the WT but absent in PrrA2 cells or vice versa for regulation by PrrA cells. A total of 117 proteins (present in the WT but absent in PrrA2 cells) and 217 proteins (expressed in the PrrA2

FIG. 5. Protein detection as a function of PrrA gene regulation. The data represent the total number of proteins detected in both the WT and the PrrA2 mutant and encoded by 615 genes regulated by PrrA by ≥ 1.5 -fold, as a function of both their mass tag and abundance. The mass tag number of specific peptides detected per protein and the abundance for each protein were calculated, independent of each other, as a ratio (≥ 1.5 -fold) of the total value in WT and PrrA2 cells. Black bars represent WT/PrrA2 ratios of ≥ 1.5 -fold, whereas white bars represent PrrA2/WT ratios of ≥ 1.5 -fold, for both mass tag as well as abundance. (A) Proteins encoded by 251 genes downregulated in PrrA2 cells compared to the WT. (B) Proteins encoded by 364 genes upregulated in PrrA2 cells. Additional data for this figure are presented in Tables S3A and S3B in the supplemental material.

FIG. 6. Microarray and proteome analysis for gene/protein pairs of the photosynthesis gene cluster. The PGC is shown at the bottom of the figure. Only genes whose encoded proteins are detected in our proteomic study are indicated. White bars refer to values of the proteomic parameter mass tag number of specific peptides detected for each protein, expressed as a WT/PrrA2 ratio (left ordinate). When a protein is scored as absent in either WT or PrrA2 cells, the bar was omitted and the letter A (for absent) was substituted for the bar. Black bars represent the ratio of mean microarray signal values for the corresponding gene (right ordinate). Genes whose encoded proteins are absent in the PrrA2 mutant are indicated with one asterisk, and genes whose encoded proteins are absent in the WT are indicated with two asterisks. The gene/protein pairs represented are as follows: *dxxA*/RSP0254, *pufX*/RSP0255, *pufM*/RSP0256, *pufL*/RSP0257, *pufA*/RSP0258, *bchZ*/RSP0260, *bchY*/RSP0261, *bchX*/RSP0262, *bchC*/RSP0263, *crtE*/RSP0265, *crtI*/RSP0271, *crtA*/RSP0272, *bchl*/RSP0273, *bchD*/RSP0274, *bchP*/RSP0277, *bchJ*/RSP0280, *bchE*/RSP0281, *bchN*/RSP0285, *bchB*/RSP0286, *bchH*/RSP0287, *bchL*/RSP0288, *bchM*/RSP0289, *pufA*/RSP0291, RSP0293 gene/RSP0293, *cycA*/RSP0296, RSP0307 gene/RSP0307, *ureD*/RSP0310, RSP0311 gene/RSP0311, RSP0312 gene/RSP0312, *pucB*/RSP0314, and *hemN*/RSP0317.

mutant but not in the WT) (see Tables S3A and S3B in the supplemental material) were found. The majority of proteins detected in the PrrA2 mutant but not in the WT were found to be encoded preferentially by genes upregulated in PrrA2 (94 genes were upregulated, whereas 22 were downregulated) (see Table S3B in the supplemental material), showing relatively good agreement between mRNA and protein levels.

As a specific example to illustrate the correspondence between the mRNAs and corresponding proteins, we present data for the gene/protein pairs of the PGC. Those data are depicted in Fig. 6. Data pertinent to *pufB* (RSP0258 gene), encoding the LHI β polypeptide, and *pucB* (RSP0314 gene), encoding the LHII β polypeptide, are also included in Table 2. Changes in the transcriptome values (in this study and in reference 30) calculated from WT and PrrA2 cells are plotted together with the corresponding “mass tag” ratios for each specific protein derived from WT and PrrA2 cells. PrrA positively regulates most genes residing in the PGC (30). Here we show that 31 genes, out of 63 residing in the PGC, encode proteins scored as present by our chromatographic experimental approach. Eighteen polypeptides were detected in both WT and PrrA2 mutant cells, and in all cases, the corresponding “mass tag” ratio (white bars) was greater in the WT than in the PrrA2 mutant, reflecting significantly higher amounts of those polypeptides in the WT. This was consistent with the microarray mean signal values (black bars), which showed most of

these genes being downregulated in the PrrA2 mutant, with respect to the WT, as indicated by the microarray mean signal values represented in the figure. In nine cases (designated with an asterisk), a specific protein was scored as absent in PrrA2 cells by the chromatographic methods, also indicating downregulation in the mutant. Conversely, four proteins were scored as absent in the WT while present in PrrA2 cells (designated with two asterisks). These proteins are encoded by genes known not to be regulated by PrrA, except for *bchH* (RSP0287 gene), which encodes magnesium-protoporphyrin methyltransferase. Thus, in the specific example of the PGC, there is excellent correlation between transcriptome and proteome data sets.

Use of transcriptional *lacZ* fusions to detect direct positive regulation by PrrA. Considering the large number of genes regulated by PrrA and the fact that many of these genes encode regulators themselves, such as *appA* (RSP1565 gene) (see Table S2A in the supplemental material), PrrA is likely to exercise its regulatory role both directly and indirectly, that is, by both directly binding to the genes which it regulates or by regulating the expression of other genes encoding regulatory proteins which in turn regulate these genes. In an effort to discover new genes that are directly regulated by PrrA, we constructed transcriptional fusions to several target genes. These genes were selected by scanning their regulatory regions for the presence of the putative consensus PrrA binding site

FIG. 7. Expression of the RSP3361 gene. (A) Regulatory region of the RSP3361 gene. The two PrrA sites are indicated with boxes, with the consensus PrrA binding sequence depicted above. The putative σ^{70} promoter is indicated by the large black letter P. (B) Mean signal values representing expression from the RSP3361 gene from different microarray experiments. Growth conditions are indicated at the bottom of the graph as follows: 30% +O₂ and 2% +O₂, aerobic growth with 30% and 2% oxygen, respectively; Dark/DMSO -O₂, anaerobic growth in the dark with DMSO as an electron acceptor; PS 3W -O₂, PS 10W -O₂, and PS 100W -O₂, anaerobic photosynthetic growth with incident light intensity of 3, 10, and 100 W/m², respectively. (C) β -Galactosidase activities of cultures of wild-type (black bars) and PrrA2 (white bars) *R. sphaeroides* with RSP3361::lacZ under anaerobic growth conditions in the dark and with DMSO. At the bottom of the graph, WT represents the wild-type fusion, and $\Delta 1$, $\Delta 2$, and $\Delta 1+2$ indicate fusions with the upstream PrrA site, downstream PrrA site, or both PrrA sites deleted, respectively.

(47). The DNA sequence of this site is (C/T)-(G/C)-C-G-G-(C/G)-N-G-(T/A)-C-(G/A)-(C/A). In this degenerate sequence, two boxes (underlined) containing six and five nucleotides, respectively, are separated by a variable spacing region (N) with a length ranging from 0 to 10 nucleotides.

The RSP3361 gene, which is positively regulated by PrrA (Table 2 and Fig. 2B), contains this consensus sequence twice in its regulatory region, upstream of the putative promoter, as shown in Fig. 7A. We designated these PrrA consensus sites, PrrA site 1 and PrrA site 2. PrrA site 1 is upstream and PrrA site 2 is downstream, 190 bp and 133 bp, respectively, with respect to the coding sequence of the RSP3361 gene. The expression of this gene is lowest under aerobic conditions and is maximal under anaerobic conditions, especially when cells grow photosynthetically at medium light (10-W/m² incident light intensity), as shown in Fig. 7B.

To test whether the RSP3361 gene is directly regulated by PrrA, we constructed an RSP3361::lacZ fusion as described in Materials and Methods and containing 351 bp of upstream sequence to the start codon of the gene (WT fusion). In addition, we constructed similar fusions but with removal of either PrrA site 1 ($\Delta 1$ fusion) or PrrA site 2 ($\Delta 2$ fusion) or a combination of both ($\Delta 1+\Delta 2$ fusion), as described in Materials and Methods. The results are shown in Fig. 7C. The WT fusion, when assayed in WT and PrrA2 cells grown anaerobi-

cally in the dark with DMSO showed an approximately nine-fold-decrease in lacZ expression in the mutant PrrA2 cells compared to the WT, indicating that, as suggested by microarray, Northern hybridization, and proteomic experiments, PrrA positively regulates the RSP3361 gene. Furthermore, deletion of PrrA site 1 ($\Delta 1$) brought expression down by approximately 37% in the WT, while the deletion of PrrA site 2 ($\Delta 2$) completely abolished induction by PrrA. Thus, the implication is that PrrA binds directly to the regulatory region of the RSP3361 gene at PrrA site 2 and possibly at PrrA site 1.

PrrA as a repressor of gene expression. In order to study the repressor function of PrrA, we chose the RSP2389 gene (*gpx*) and RSP0793 gene (*pqqB*), encoding glutathione peroxidase and the B subunit of coenzyme PQQ, respectively, as candidate genes, since PrrA repression of these genes had been shown by microarray analysis and direct mRNA measurement, as shown in Fig. 2D and C, respectively. In the proteomic study, the corresponding proteins had been scored as absent in both the WT and the PrrA2 mutant. In the case of a lacZ transcriptional fusion to the WT RSP2389 allele, there was 5.2-fold higher expression in PrrA2 cells than in the WT cells, as shown in Fig. 8A. This result is in agreement with data obtained from microarray (Table 2), as well as direct Northern hybridization (Fig. 2D) experiments and is consistent with repression of the RSP2389 gene by PrrA.

FIG. 8. PrrA as a repressor of gene expression. (A) β -Galactosidase activities of cultures of wild-type (black bars) and PrrA2 (white bars) *R. sphaeroides* with RSP2389::lacZ under anaerobic growth conditions in the dark and with DMSO. At the bottom of the graph, WT represents the wild-type fusion, and P^{up} indicates the fusion containing the T-to-C substitution immediately before the -35 promoter element. (B) Regulatory region of the RSP2389 gene. The putative PrrA site (site 2) located in the coding region of the RSP2389 gene is boxed, with the consensus PrrA binding sequence shown above. A second site (site 1) is also indicated. $\Delta 1$ and $\Delta 2$ represent the two in-frame deletions. The two mutations in the promoter region are also shown, with P^{up} indicating the mutation in the P^{up} RSP2389::lacZ fusion.

Since we were certain that this gene is repressed by PrrA, we constructed a translational fusion of *gpx* (RSP2389 gene) to a gene from Tn903 encoding a protein imparting resistance to Kan (Table 1). The fusion was introduced into WT and PrrA2 cells, and the cells were plated at different Kan concentrations to select for Kan^r mutants. The mutations in *trans* were not further analyzed for this study. Four *cis* mutations allowing WT cells to grow on Kan at high concentrations (25 and 50 μ g/ml) were examined. Assuming the presence of a σ^{70} -type promoter, as indicated in Fig. 8B, two were P^{up} mutations, and two were in-frame deletions ($\Delta 1$ and $\Delta 2$) within the structural portion of the RSP2389 gene contained in the fusion. The P^{up} mutations were a T-to-C substitution immediately before the putative -35 promoter element, and there was another substitution, a G-to-A substitution, which changed a putative -10 promoter element from the original TATGCT sequence to the TATACT sequence, which is closer to the optimal consensus TATAAT sequence for σ^{70} promoters (48). The in-frame deletions removed codons 9 to 16 in $\Delta 1$ and codons 11 to 20 in $\Delta 2$ both within the structural portion of the RSP2389 gene, as annotated, and contained within the translational fusion. Interestingly, a putative PrrA binding site overlapped codons 15 to 20. This putative site contained two mismatches (*) with respect to the consensus. We designate it site 2 to differentiate it from another putative site (site 1), located 19 bp upstream of the likely start codon in the RSP2389 gene (Fig. 8B). Since site 2 was actually removed in the in-frame deletion, partially in $\Delta 1$,

but completely in $\Delta 2$, we reasoned that PrrA might bind to this site and repress expression of the RSP2389 gene. As a proof of principle to show that the selection had worked as expected, the P^{up} mutant allele gave higher expression values (13.1-fold in the WT and 6.1-fold in PrrA2 cells but still exhibiting PrrA repression [2.4-fold]). Work is in progress to analyze gene expression from an RSP2389 allele in which the PrrA site 2 has been mutated so as to abolish putative PrrA binding (Eraso and Kaplan, unpublished).

PrrA as an indirect regulator of gene expression. Our microarray results (Fig. 3; also see Table S2B in the supplemental material) suggest that approximately 48% of the genes assigned to the transcription category (category K in Fig. 3) and 46% of genes in the signal transduction category (category T), many of which have regulatory functions, are regulated by PrrA. If we were to envision that some of these regulators might themselves not be direct targets for PrrA but, instead, be targets for additional PrrA-dependent regulators in what has been designated a “distributive cascade” of gene expression (4), this would suggest that there is potential for indirect regulation of gene expression by PrrA in *R. sphaeroides*. Some of the PrrA gene targets found in this study are also targets for other global regulators, like the PpsR repressor, in the case of genes in the PGC, and FnrL, the transcriptional regulator of photosystem formation and tetrapyrrole biosynthesis (75). Thus, we decided to investigate whether PrrA regulates the

expression of genes encoding either of these two global regulators and therefore can act in an indirect fashion.

For this purpose, we tested the effects of PrrA interruptions, either PrrA2 (streptomycin resistant [St^r]/spectinomycin resistant [Sp^r]) (in the case of *ppsR*) or PrrBCA (trimethoprim resistant [Tp^r]) (in the case of *fnrL*), on the expression of *lacZ* transcriptional fusions to *fnrL* (RSP0698 gene) and *ppsR* (RSP0282 gene). We used two different host strains due to drug marker compatibility. In the case when the entire Prr system, encompassing *prpA*, *prpB*, and *prpC*, was deleted, the strain is designated PRRBCA2. Previous experiments had shown that the phenotypes of PrrA2 and PRRBCA are similar (22). *fnrL::lacZ* expression was down ~ 3.1 -fold in the PRRBCA2 mutant compared to the WT, when the cells were grown anaerobically (data not shown). Thus, since FnrL is itself a global regulator affecting genes involved in respiration, photopigment biosynthesis, and PS (52, 54, 67), the Prr system might also regulate FnrL targets indirectly by controlling, in part, *fnrL* expression.

In addition, we used a *ppsR::lacZ* fusion, which starts at codon 47 of *ppaA*, 715 bp upstream of nucleotide 1 in *ppsR*, and extends 54 bp into the *ppsR* gene, therefore containing exclusively promoter sequences located upstream of *ppsR* and not including a promoter upstream of *ppaA* as in pLX41 (26). Expression of this fusion was 6.5-fold higher in PrrA2 cells than in the WT (data not shown). Interestingly, one putative PrrA binding site, according to the above consensus, was found 5 nucleotides upstream of the last nucleotide within *ppaA*. Considering that the intergenic region between *ppaA* and *ppsR* is 57 bp, the location of this binding sequence is consistent with it being able to lead to repression upon PrrA binding, although further proof is necessary, especially in terms of the elucidation of the promoter elements driving expression of *ppsR*.

Taken together, PrrA might regulate global gene expression levels indirectly by affecting the expression of genes regulated by FnrL and by the extensive PpsR regulon (50; Bruscella et al., submitted). Further investigation to prove this point is required, since in terms of microarray results, the expression of *fnrL* and *ppsR* was indistinguishable in WT and PrrA2 cells with values of ~ 1.1 -fold for both (see Discussion). In addition, two other regulatory genes, *appA* (RSP1565 gene) and *ppaA* (RSP0283 gene) were downregulated in PrrA2 cells, showing gene expression changes of -2.8 - and -8.1 -fold, respectively. In the case of AppA, by positively regulating the gene encoding the antirepressor for PpsR, PrrA might indirectly control expression of the PpsR regulon.

DISCUSSION

Previous reports from this and other laboratories have established the PrrBA two-component system in *R. sphaeroides*, and its homolog in *R. capsulatus*, the RegBA system, as a master regulator of gene expression in response to changes in cellular redox (23, 38, 65). With the availability of a genomic sequence and the *R. sphaeroides* 2.4.1 GeneChip (2, 50, 63), and in conjunction with high-throughput proteomic technology (7, 8, 77), determination of the fullest extent of PrrA regulation, at the mRNA and protein levels, has progressed to an operational level, as described here.

In numerical terms, the transcriptome analysis revealed that

PrrA regulates, depending on the cutoff value used, from approximately 25% (2-fold cutoff) to 43% (1.5-fold cutoff) of the total genomic content in *R. sphaeroides*, both directly and indirectly, with a higher number of genes upregulated in the PrrA2 mutant. Proteome results in terms of the percentage of expressed proteins (36%) for *R. sphaeroides* were in accordance with previously reported percentages (7, 8). In terms of the genes encoding these proteins, approximately 20% of them were downregulated in the mutant and 32% were upregulated, which is in excellent agreement with the percentages obtained for the whole genome. In addition, there was good qualitative correlation between mRNA and protein levels. Thus, in the case of genes encoding the proteins detected in our proteomic study, their expression values paralleled the detected levels of their protein products for a substantial number of them, although exceptions, as revealed in our study, were significant. Whether the same is true in other organisms remains to be seen, since we are not aware of similar studies having been performed thus far, but an increasing body of evidence emphasizes the importance of posttranscriptional regulation in both prokaryotes (12) and eukaryotes (27).

In terms of abundance ratios for the WT and PrrA2 proteomes, we found that the induction ratios tended to be smaller than the corresponding microarray ratios. A similar phenomenon had been observed in previous studies when comparing transcriptome data to two-dimensional gel quantitation data (13), and it could, in part, be explained by the different detection sensitivities pertinent to each experimental approach or by a manifestation of posttranslational processes.

We subsequently scored the COG functional categories for PrrA regulation, using transcriptome comparisons between the WT and PrrA2 mutant. For this purpose, we also used a less-stringent 1.5-fold cutoff value to allow for inclusion of all possible targets, since it has been reported that arrays often underestimate induction ratios (13). We found that the percentage values for positively, negatively, and not regulated genes, remained constant, within ranges, among most categories, and in all cases were higher for negatively regulated genes. Certain deviations from this observation were found.

Genes encoding ribosomal proteins tended to be, in general, downregulated in the mutant, whereas other genes whose encoded proteins have functions in translation, like genes encoding tRNA synthetases as well as tRNA genes, were upregulated. In fact, PrrA regulation of tRNA genes is remarkable in terms of consistency and is reminiscent of regulation by Fis of rRNA and tRNA genes in *E. coli* (64). In this respect, we have suggested in the past that PrrA might act as a "substitutive" regulator for Fis in *R. sphaeroides*, since this regulator is not present in *R. sphaeroides* (45). It is tempting to speculate that since we observe genes encoding ribosomal proteins being downregulated in the PrrA2 mutant, while concomitantly, genes encoding other components of the translation machinery are upregulated, like those encoding tRNAs and tRNA synthetases, cells compensate for the decline in the number of ribosomes by increasing their protein synthesis capacity through amino acid availability. In addition to translation, cell motility, and secretion, genes were also found to be downregulated in the PrrA2 mutant.

Genes encoding proteins involved in DNA metabolism, specifically recombination and repair, as well as those involved in

protein folding and repair of protein damage, were upregulated in the PrrA2 mutant. We envision that when cells transit from an aerobic to an anaerobic environment, oxidative damage decreases, therefore providing the need to downregulate genes encoding proteins involved in repair processes. In *E. coli*, the response regulator ArcA, which is phosphorylated under similar growth conditions to PrrA, also represses genes involved in oxidative damage repair (44). Genes encoding proteins whose functions are involved in iron internalization were also upregulated in the PrrA2 mutant. This might suggest either that less iron is needed under anaerobic redox conditions when PrrA is activated and/or that since Fe^{2+} is much more stable anaerobically, cells transport the metal in that redox state, in which it is already soluble. Similar results had been found when analyzing gene expression changes during transit from photosynthetic to aerobic growth (2).

Both the RSP0946 gene, which encodes an uncharacterized homolog of topoisomerase I, and *gyrB* (RSP0772 gene), encoding the B subunit of DNA gyrase, were upregulated in the PrrA2 mutant. Even though the possible extent of DNA topological change due to these changes in gene expression has not been investigated, it is noteworthy that DNA gyrase, and its effect on DNA topology, is an effector of gene expression in *R. sphaeroides* (42) and *E. coli* (58, 62), and it is tempting to speculate on possible effects of PrrA on DNA conformation.

In terms of regulation of metabolic genes, we have presented the case for gluconeogenic genes being downregulated in the PrrA2 mutant, whereas Entner-Doudoroff genes are upregulated, reminiscent of the opposing control of gluconeogenesis and glycolysis by glucagon, which favors gluconeogenesis, and insulin, which favors glycolysis (59). This suggests that the Entner-Doudoroff pathway, which is an oxidative pathway, must be favored when *R. sphaeroides* cells grow in the presence of oxygen as electron acceptor, when the ETC is in a more oxidized state, which would also, to a certain extent, be influenced by the redox state of the carbon source. Alternatively, in the absence of oxygen, the potential for oxidative phosphorylation is clearly decreased, and thus, the need for reductant going down the glycolytic pathway decreases, shifting the equilibrium toward gluconeogenesis. Since PrrA coordinately regulates genes encoding proteins used in both pathways, it can act as a switch and, most importantly, serve as a link between the redox state of the ETC and cellular metabolic responses. In addition, we found a large number of poorly characterized genes that are also regulated by PrrA, and this is reminiscent of Fnr in *E. coli* (29), as well as for other global regulators (5), and future research on some of these genes with unknown function might contribute to our understanding of the regulation of gene expression by PrrA.

Positive and negative regulation by PrrA. The definition of transcriptional regulators as uniquely possessing either an activator, or a repressor function, is undergoing revision. More and more often transcriptional regulators are found in which both functions are assigned to the same protein, especially in the case of global regulators, which have a larger number of gene targets. As examples, Fnr (29), ArcA (44), Fis (33), OxyR (78) and RpoS (56, 61, 74), in *E. coli*, and CtrA (37), in *Caulobacter*, have all been assigned activator and repressor functions. Thus, even though the global repressor function for

PrrA was unknown until this study, it seems logical that an *R. sphaeroides* master regulator can also perform both roles.

Our PrrA data indicate that, when acting as an activator, PrrA binds to sites localized in the regulatory regions of target genes (this study; Eraso and Kaplan, unpublished). In addition, as a repressor, PrrA might act indirectly by negatively affecting the levels of another repressor, as in the case of PpsR, as shown by use of the *ppsR::lacZ* fusion, but it might also bind to sites localized within the coding regions of genes, as in the RSP2389 gene. It is not uncommon for repressors to bind within the structural portion of a gene, as has been shown in *Bordetella pertussis*, *Helicobacter pylori*, and *E. coli* (3, 15, 78). In the case of PrrA, this mode of action would not necessarily preclude repression by binding to more canonical sites in the regulatory regions of repressed genes, as has been shown for repression by RegA* of the *hupSLC* operon in *R. capsulatus* (20), encoding the membrane-bound [NiFe] hydrogenase.

Approximately 43% of the *R. sphaeroides* genes were directly or indirectly regulated by PrrA, when using a 1.5-fold cutoff. Even considering the possibility that a large number of these genes are targets for indirect regulation by PrrA, this implies that redox regulation dictated by electron flow through the ETC, which ultimately determines the activation state of PrrA, is the single most important factor that actually regulates the expression of these many genes. Since AppA senses the redox state of the ETC (53) to activate or inhibit the function of the PpsR master regulator (50; Bruscella et al., submitted), the extent of genes whose expression is partly or totally governed by the redox state of the ETC is actually even larger.

We found in this study that PrrA represses the expression of a *ppsR::lacZ* transcriptional fusion containing only *ppsR*, and not the upstream gene *ppaA*, as in case of fusions used in previous studies (26). Paradoxically, the microarray results for *ppsR* were indistinguishable when the levels of expression of this gene in WT and PrrA2 cells are compared. Whereas the cause for this disparity is not obvious, and factors like mRNA turnover, as well as the very low expression levels exhibited by *ppsR*—approximately fourfold lower than that for *ppaA*—might be responsible, the transcriptional fusion data are consistent with the fact that the cellular levels of the PpsR protein, which is also responsive to redox control, had been found to be inversely proportional to *pprA* gene expression in a previous study (50). Interestingly, the expression of *ppsR* is 2.5-fold higher in WT cells growing aerobically than in cells grown anaerobically with DMSO (J. H. Roh and S. Kaplan, unpublished). Furthermore, we found that PrrA activates the expression of *appA*, encoding the PpsR antirepressor, by -2.8 -fold. Taken together, PrrA and PpsR exhibit bimodal regulation, with respect to each other, as the inverse proportionality in their expression levels suggests. Thus, the idea that PpsR is the “master regulator” of PS gene expression may actually be a conclusion based on the dominant role that PrrA plays in its expression and the functional state of its gene product through the effect of PrrA on *appA* expression. Thus, the so-called dominant role of PpsR is in reality the result of the functional state of PrrA.

In the case of FnrL, which is also sensitive to the prevailing cellular redox conditions, we found that expression of an *fnrL::lacZ* transcriptional fusion was also found to be positively regulated by PrrA by approximately threefold. Interestingly, a

comparison of the expression of *furL* under seven different conditions of growth has revealed that it is maximal (2,161 microarray expression units) under anaerobic conditions, with 3-W/m² incident light intensity, and lowest (708 microarray expression units) under anaerobic conditions in the dark with DMSO.

Global regulation in *R. sphaeroides*. We have observed here that the large majority of genes which changed their expression levels significantly upon a shift from anaerobic photosynthetic to aerobic conditions of growth (2) are PrrA regulated. In addition, most of the genes found to be regulated by PrrA in this study were not scored as changing their expression levels during the shift conducted in this previous study (2) (see Table S1 in the supplemental material). Since during such a shift the cellular levels of phospho-PrrA decrease, unphosphorylated PrrA might also have a relevant regulatory role, as suggested previously (11, 22, 23, 31). Additionally, other regulators might be involved in controlling the expression levels of those PrrA-regulated genes whose expression does not change significantly during the shift. For example, in the case of genes that are repressed by PrrA under anaerobic conditions of growth, PpsR might, in some cases, act as the repressor when oxygen is present. In this respect, the PpsR regulon is much more extensive than previously anticipated, and PrrA and PpsR act in conjunction (Bruscella et al., submitted).

The results of this study suggest that in the transition from aerobic to anaerobic growth, when PrrA is activated, *R. sphaeroides* cells must be undergoing an extensive metabolic adjustment, which requires PrrA to not only regulate, directly and indirectly, the expression of metabolic genes per se, but in addition, as described above, to fine tune the expression of newly described genes whose encoded proteins have functions in translation, general transcription, energy production and conversion, repair to DNA and protein damage, as well as genes spread in all the remaining COG categories.

In conclusion, this study provides new evidence for PrrA as a global regulator of gene expression in *R. sphaeroides*, and by determining the total extent of its gene regulation, qualitatively confirmed by proteomic analysis, it sets a foundation for future studies in terms of gene activation and repression as well as the exact identity of the PrrA DNA recognition sequence.

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