

Regulation of bacterial photosynthesis genes by the small noncoding RNA PcrZ

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The small RNA PcrZ (photosynthesis control RNA Z) of the facultative phototrophic bacterium *Rhodobacter sphaeroides* is induced upon a drop of oxygen tension with similar kinetics to those of genes for components of photosynthetic complexes. High expression of PcrZ depends on PrrA, the response regulator of the PrrB/PrrA two-component system with a central role in redox regulation in *R. sphaeroides*. In addition the FnrL protein, an activator of some photosynthesis genes at low oxygen tension, is involved in redox-dependent expression of this small (s)RNA. Overexpression of full-length PcrZ in *R. sphaeroides* affects expression of a small subset of genes, most of them with a function in photosynthesis. Some mRNAs from the photosynthetic gene cluster were predicted to be putative PcrZ targets and results from an in vivo reporter system support these predictions. Our data reveal a negative effect of PcrZ on expression of its target mRNAs. Thus, PcrZ counteracts the redox-dependent induction of photosynthesis genes, which is mediated by protein regulators. Because PrrA directly activates photosynthesis genes and at the same time PcrZ, which negatively affects photosynthesis gene expression, this is one of the rare cases of an incoherent feed-forward loop including an sRNA. Our data identified PcrZ as a *trans* acting sRNA with a direct regulatory function in formation of photosynthetic complexes and provide a model for the control of photosynthesis gene expression by a regulatory network consisting of proteins and a small noncoding RNA.

α -proteobacteria | protochlorophyllide reductase

It has emerged over the last decade that small noncoding RNAs (sRNAs) have an important impact on gene regulation in bacteria. They affect gene expression by altering the rate of translation and/or the stability of their target mRNAs (1). sRNAs help the bacteria to adapt to various stresses and control metabolic functions, growth, toxin production, or virulence. Photosynthesis is an important process for ATP production in many bacteria. The simultaneous presence of pigments and oxygen can, however, cause the generation of reactive oxygen species, which makes a balanced control of the formation of photosynthetic complexes necessary. Our data reveal that in the anoxygenic phototrophic α -proteobacterium *Rhodobacter sphaeroides* the small RNA PcrZ (photosynthesis control RNA Z) affects formation of photosynthetic complexes by targeting mRNAs encoding pigment-binding proteins and enzymes for bacteriochlorophyll synthesis.

R. sphaeroides is a facultative photosynthetic bacterium, which forms photosynthetic complexes in response to environmental stimuli. Under high oxygen tension it performs aerobic respiration and expression of photosynthesis genes is repressed. The most important proteins involved in regulation of photosynthesis genes and their action in *R. sphaeroides* are shown in Fig. 1. The repressor PpsR binds to the upstream region of photosynthesis genes and prevents transcription (2–4). When oxygen tension decreases, the AppA antirepressor protein binds to PpsR and releases it from the DNA, allowing transcription (3, 5, 6). AppA can sense blue light through its N-terminal BLUF domain (5, 7) and redox signals through a heme that is bound to the SCHIC domain (2, 8). As long as oxygen is available, *Rhodobacter* performs aerobic respiration. However, at intermediate oxygen tension blue light, even at low intensities, prevents AppA from binding to PpsR and photosynthesis genes are repressed (8, 9), reducing the accumulation of the harmful singlet oxygen. The PrrB/PrrA two-component system senses the electron transport through the cbb3 oxidase

and induces transcription of photosynthesis genes at very low oxygen tension or in the absence of oxygen (5, 10–13). Furthermore, the FnrL protein activates some photosynthesis genes at low oxygen tension (13) and the PpaA regulator activates some photosynthesis genes under aerobic conditions (14). More recently CryB, a member of a newly described cryptochrome family (15), was shown to affect expression of photosynthesis genes in *R. sphaeroides* and to interact with AppA (16, 17). Remarkably, the different signaling pathways for control of photosynthesis genes are also interconnected, e.g., the *appA* gene is controlled by PrrA (18, 19) and a PpsR binding site is located in the *ppaA* promoter region (20). Thus, a complex network comprising several regulatory proteins controls the formation of the photosynthetic apparatus.

Recently, several sRNAs were identified in *R. sphaeroides*, some of which are specifically induced or processed in response to superoxide or singlet oxygen (21, 22). Among the sRNAs identified by RNAseq was PcrZ (RSs2430), which has homologs in all sequenced *R. sphaeroides* species, but not in other species with sequenced genomes. Here we show that PcrZ has an important role in balanced formation of the photosynthetic apparatus of *R. sphaeroides*.

Results

Oxygen-Dependent Expression of PcrZ Is Regulated by PrrA. PcrZ is transcribed from the intergenic region between RSP_0819 encoding a DEAD/DEAH box helicase and RSP_6134 encoding a hypothetical protein (Fig. 2A). The primary transcript is 136 nt in size (21). The levels of the PcrZ primary transcript increase after a shift from high to low oxygen tension by a factor of about 4 (Fig. 2B). A similar oxygen-dependent increase was previously observed for the polycistronic *puf* and *puc* transcripts in *Rhodobacter* species (8, 23). The primary *puf* transcript encodes proteins for the formation of the reaction center and light-harvesting I (LHI) complex, and the *puc* operon encodes proteins for the formation of the LHII complex. In addition to the primary PcrZ transcript, processing products were observed that accumulated with increasing incubation time at low oxygen tension (Fig. 2B). After 24 h (1,440 min) of incubation the level of the full-length transcript was even lower than before the transition to low oxygen and a small processing product of about 50 nt had strongly accumulated.

In *R. sphaeroides* transcription of photosynthesis genes at low oxygen tension is activated by the response regulator PrrA (24). A sequence with good similarity to the PrrA binding motif (20) is also present upstream of PcrZ (Fig. S1). To analyze the role of PrrA and other regulators of photosynthesis genes in PcrZ expression we performed Northern blots with RNA from strains

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Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE37381).

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A

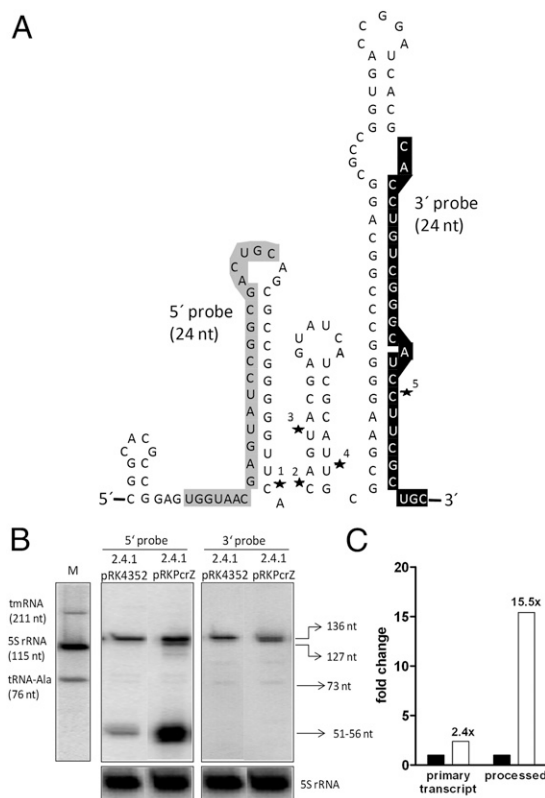


Fig. 3. The stable processing product of PcrZ stems from its 5' end. (A) Predicted secondary structure of PcrZ by the Mfold program (25). Solid stars indicate the five different 3' ends, identified by 3' RACE. Hybridization sites of different oligonucleotide probes used for Northern blot analysis are displayed (5' probe in gray and 3' probe in black). (B) Northern blot analysis of total RNA, isolated from *R. sphaeroides* 2.4.1pRK4352 and 2.4.1pRKPrZ. Cultures were grown under low-oxygen conditions to an OD₆₆₀ of 0.8. Two different oligonucleotides, binding near the 5' and 3' ends of PcrZ, were used for hybridization. Detection of PcrZ primary transcript (136 nt) and processed fragments (127 nt, 73 nt, and 51–56 nt) is indicated. tmRNA (coding piece), 5S rRNA, and tRNA-Ala oligonucleotides served as internal size markers. (C) Quantification of Northern blot signals from strains 2.4.1pRK4352 (solid bars) and 2.4.1pRKPrZ (open bars). RNA levels were calculated after normalizing PcrZ signal intensities to 5S rRNA signal intensities (B, 5' probe). 2.4.1pRK4352 intensities were set to 1 and fold changes of 2.4.1pRKPrZ were calculated relative to 2.4.1pRK4352.

(217 genes with an expression change of ≤ 0.7 -fold). Only 7 genes showed a higher expression (≥ 1.75), when PcrZ was overexpressed. This was the case for RSP_4246, a SinR-like protein of unknown function in *R. sphaeroides*, for three transporters (RSP_1613, RSP_3297, and RSP_3386) and for RSP_7386 encoding asparagine synthase. For most of the affected genes the change in expression was quite small (0.5- to 2-fold). Many of the down-regulated genes are involved in the formation of the photosynthetic apparatus (Table 1). These genes include the *puf* and *puc* operons, several genes for bacteriochlorophyll synthesis, the *crtA* and *crtD* genes for carotenoid synthesis, and *appA*. Because AppA functions as an antagonist of PpsR, which represses photosynthesis genes, a lower expression of the *appA* gene could indirectly cause a stronger repression of other photosynthesis genes by PpsR. Additional genes with lower expression in the strain with increased PcrZ levels encode cytochrome c2; cytochrome b562; and transcriptional regulators of the LuxR, TetR, and LysR families. Both cytochromes are involved in cyclic photosynthetic electron transport.

Surprisingly, many genes for tRNAs or sRNAs seemed to be less expressed in the overexpression strain. This was also true for PcrZ itself, which we demonstrated to be overexpressed by Northern blots. We conclude that the microarray data did not give re-

liable results for small structured RNAs, maybe due to different efficiencies of the two labels (Cy3/Cy5). This assumption was also supported by an analysis of total RNA on urea gels. Despite the lower expression levels as indicated in the microarray, there was no significant change in the level of tRNAs (Fig. S5).

We applied real-time RT-PCR for selected genes to verify the microarray data. The lower expression levels of *appA*, *ppaA*, *puc24*, *bchO*, *bchY*, *bchN*, RSP_1574, and RSP_3324 as well as the higher expression level of RSP_4246 in strain 2.4.1pRKPrZ were confirmed. The fold change was mostly larger in the real-time RT-PCR than in the microarray dataset (Table 1).

Target Prediction and Confirmation. We assumed that PcrZ may directly target mRNAs for photosynthesis genes. An IntaRNA search (27, 28) suggested a base pairing between PcrZ and several mRNAs that showed altered expression levels in the microarray analysis (Table S1). When only putative targets with predicted energy values of less than -16 kcal/mol were considered, the predicted interacting nucleotides mapped preferentially to the three stem-loop structures in the 5' part of PcrZ. For one predicted target (*bchN*) the region of base pairing to PcrZ included the translational start site, and for others the predicted site of hybridization was in the coding region (e.g., *puc24*). Targeting of the coding sequence was previously demonstrated for MicC in *Salmonella* (29). To test the putative interaction of PcrZ with some of these mRNAs we established an in vivo reporter system for *R. sphaeroides*, which is also applicable to other α -proteobacteria. In this two-plasmid system potential target mRNAs are translationally fused to the *lacZ* gene on plasmid pPHU4352, thereby expressing the target constitutively from a 16S rRNA promoter. The sRNA counterpart is expressed from the identical promoter on plasmid pBBR4352. We used this system to investigate sRNA–mRNA interaction more directly and to prove that altered mRNA levels, as monitored by microarray, impact translation.

We chose the mRNA *bchN* for the *in vivo* assay with a predicted interaction site from -3 to +29 with respect to its start codon (Fig. 5A). *bchN* is part of the *bchFNBHL* operon and encodes the N-subunit of the light-independent protochlorophyllide oxidoreductase (DPOR). This DPOR complex is responsible for protochlorophyllide reduction to chlorophyllide (30, 31). Fig. 5D clearly shows that β -galactosidase activity from the *bchN::lacZ* reporter plasmid decreases when PcrZ is overexpressed on plasmid

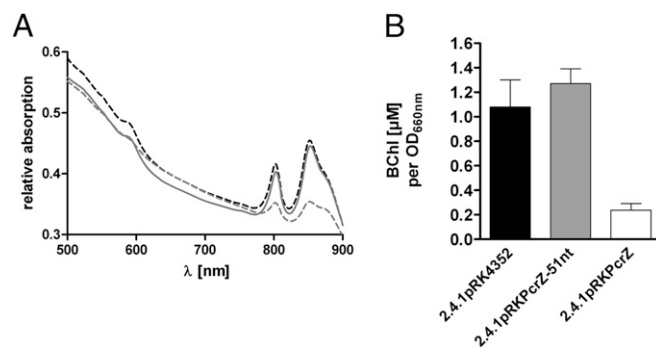


Fig. 4. PcrZ reduces levels of photosynthetic complexes. (A) Absorption spectra of *R. sphaeroides* whole-cell extracts. Three independent cultures of each strain were grown under low-oxygen conditions to an OD₆₆₀ of 0.8. The absorbance was measured from 500 nm to 900 nm. One representative spectrum of 2.4.1pRK4352 (solid dashed line), 2.4.1pRKpCrZ (shaded dashed line), and 2.4.1pRKpCrZ-51nt (shaded line) is shown. Peaks at 800 and 850 nm correspond to the light-harvesting complex II (B800–850) and the light-harvesting complex I (B870) of the photosynthetic apparatus. (B) Relative bacteriochlorophyll content of *R. sphaeroides* strains 2.4.1pRK4352 (solid bar), 2.4.1pRKpCrZ-51nt (shaded bar), and 2.4.1pRKpCrZ (open bar) grown under low-oxygen conditions to an OD₆₆₀ of 0.8. The relative bacteriochlorophyll content was calculated on the basis of the absorbance at 770 nm after acetone-methanol (7:2) extraction of 4 mL cells, normalized to the OD₆₆₀. Results from three independent experiments are shown with error bars depicting the SE of mean.

Table 1. Selection of PcrZ-responsive genes in *R. sphaeroides*

Category and RSP no.*	Gene	Ratio [†]	Description
Photosynthesis			
RSP_0294		0.53	Magnesium-protoporphyrin IX monomethyl ester cyclase
RSP_1574		0.61 (0.47)	Cytochrome b562
RSP_1565	<i>appA</i>	0.65 (0.37)	AppA, antirepressor of PpsR, sensor of blue light
RSP_0261	<i>bchY</i>	<u>0.74 (0.28)</u>	Chlorophyllide reductase
RSP_0280	<i>bchJ</i>	0.49	Bacteriochlorophyll synthase, 23-kDa subunit
RSP_0272	<i>crtA</i>	0.59	Spheroidene monooxygenase
RSP_0266	<i>crtD</i>	0.58	Methoxyneurosporene dehydrogenase
RSP_0296	<i>cycA</i>	0.65	Cytochrome c2
RSP_0283	<i>ppaA</i>	0.67 (0.46)	Regulatory protein, PpaA
RSP_6256	<i>pucA</i>	0.58	LHII α , light-harvesting B800/850 protein
RSP_6158	<i>puc2A</i>	0.68 (0.52)	Light-harvesting complex α -subunit
RSP_0273	<i>bchI</i>	0.57	Magnesium chelatase, ChlI subunit
RSP_0275	<i>bchO</i>	0.52 (0.52)	Magnesium chelatase
RSP_0291	<i>puhA</i>	<u>0.75</u>	Reaction center H protein
RSP_0290		0.61	Light-harvesting 1 (B870) complex assembly
RSP_0289	<i>bchM</i>	0.62	Mg-protoporphyrin IX methyl transferase
RSP_0288	<i>bchL</i>	0.68	Light-independent protochlorophyllide reductase iron protein
RSP_0286	<i>bchB</i>	0.63	Light-independent protochlorophyllide reductase subunit B
RSP_0285	<i>bchN</i>	<u>0.78 (0.43)</u>	Light-independent protochlorophyllide reductase subunit N
RSP_0314	<i>pucB</i>	0.62	LHII β , light-harvesting B800/850 protein
RSP_0315	<i>pucC</i>	0.49	Light-harvesting 1 (B870) complex assembly
RSP_0258	<i>pufA</i>	0.55	LHI α , light-harvesting B875 protein
RSP_6108	<i>pufB</i>	0.49	LHI β , light-harvesting B875 subunit
Transcriptional regulators			
RSP_1435		0.62	Regulatory protein TetR family
RSP_2027		0.68	Transcriptional regulator LysR family
RSP_3324		0.53 (0.49)	Transcriptional regulator LuxR family
RSP_4246		2.49 (5.71)	Putative SinR-like protein
Transporter			
RSP_1613		1.76	TRAP-T family transporter, DctP subunit
RSP_3297		<u>1.73</u>	ABC branched-chain amino acid transporter
RSP_3386		1.88	TRAP-T family transporter, periplasmic binding protein
Replication			
RSP_0674		0.62	DNA polymerase III subunit- δ
Metabolism			
RSP_7386		1.98	Asparagine synthase (glutamine hydrolyzing)

*Genes with RSP numbers in boldface type are a possible PcrZ target as predicted by IntaRNA (27). RSP_0273-0275, RSP_0285-0291, RSP_0314-0315, and RSP_6108-0258 respectively belong to same operons.

[†]Expression level of selected genes (2.4.1pRKPrZ vs. 2.4.1pRK4352) is shown. Numbers defined by underline did not pass our selection criteria (>1.75 or <0.7). Numbers in parentheses depict the results from real-time RT-PCR validations.

pBBR_{PcrZ}. Insertion of single (M1) or triple (M3) base exchanges into the predicted *bchN* binding region of PcrZ at positions 47 or 46–48, respectively, led to β -galactosidase activities comparable to those in the pBBR4352 control. This result indicates a hindrance of PcrZ-*bchN* interaction. When compensatory M1 or M3 mutations are inserted into *bchN* at positions +20 or +19–21, respectively, binding to the corresponding mutated PcrZ counterparts is restored. This restoration is reflected by decreased β -galactosidase activity. When the reporter constructs harboring the M1 or M3 mutations were present together with wild-type PcrZ, the β -galactosidase level was not significantly lower than in the control. To further elucidate the role of Hfq in PcrZ function we also tested the effect of PcrZ on expression of the *bchN::lacZ* reporter in an *hfq*⁻ background. The presence of PcrZ still resulted in reduced β -galactosidase activity, but the reduction was not as pronounced as in the wild-type strain.

Furthermore, we tested the *puc2A* target, which was predicted to interact from position +279 to +295 (relative to the start codon) with PcrZ (Fig. 5B). *puc2A* is part of the *puc2BA* operon that encodes α - and β -subunits of the light-harvesting complex II (32). The *puc2A::lacZ* reporter plasmid showed a strong reduction of β -galactosidase activity in the presence of elevated PcrZ levels (Fig. 5E). This reduction identifies *puc2A* mRNA as a direct target of PcrZ. AppA was another putative direct interaction partner of PcrZ as predicted by IntaRNA (Table S1). However, in our reporter system PcrZ had no significant effect on expression of

the *appA::lacZ* reporter, indicating that the effect on *appA* mRNA levels is indirect.

The RSP_0557::lacZ reporter plasmid served as a nontarget control and accordingly is not affected by PcrZ levels. We also tested a *sinR*::lacZ reporter, but β -galactosidase activity was too low to give reliable data.

To test whether the amount of target mRNAs influences PcrZ processing, we also performed Northern blot analyses with RNA isolated from *R. sphaeroides* strain App11 and *Rhodobacter capsulatus* strain 37b4 expressing PcrZ from a plasmid. App11 has the *appaA* gene deleted and due to the strong repression by PpsR expresses photosynthesis genes at strongly reduced levels (6). *R. capsulatus* wild-type strains do not harbor a gene for PcrZ. In both strains we observed the identical processing products and the same extent of PcrZ processing as in *R. sphaeroides* strain 2.4.1 (Fig. S3).

Discussion

Facultative photosynthetic bacteria like *R. sphaeroides* show a high metabolic versatility and respond to environmental impacts by adapting their metabolism. Formation of photosynthetic complexes is induced by decreasing oxygen tension and is inhibited by light at intermediate oxygen tension. The regulation of photosynthesis genes in *Rhodobacter* has been intensively studied in the past and a regulatory network involving several regulatory proteins and parts of the electron transport chain emerged (10).

oxygen tension *puf* and *puc* genes show a transient strong increase in expression. Fig. 1 integrates PcrZ into the regulatory network controlling photosynthesis genes in *R. sphaeroides*. Remarkably, PcrZ is exclusively found in species of *R. sphaeroides* and even *R. capsulatus*, which shares many of the regulatory proteins to control photosynthesis genes, lacks a homolog of this sRNA. It appears that early in evolution facultative photosynthetic bacteria acquired proteins for a major control of photosynthesis genes but that at least some mechanisms for fine-tuning developed later in evolution and may be species specific. Despite the fact that PcrZ is most likely restricted to *R. sphaeroides*, it can be expected that sRNAs play an important role in controlling the formation of the photosynthetic apparatus also in other bacteria.

Materials and Methods

Bacterial strains used in this study are listed in Table S2. Details on their construction are given in SI Materials and Methods. *R. sphaeroides* and *R. capsulatus* strains were cultivated at 32 °C in a malate minimal-salt medium (41) under continuous shaking at 140 rpm. For phototrophic growth, strains were cultured without agitation in sealed flat glass bottles filled to the top with medium and illuminated with 60 W·m⁻² of white light. Conditions of high oxygen tension (8 mg/L soluble O₂) were applied by cultivation of strains in beaked flasks. For oxygen-shift experiments, *R. sphaeroides* precultures were grown under high-oxygen conditions overnight to an OD₆₆₀ of 0.8–0.9. Precultures were diluted to an OD₆₆₀ of 0.4 and grown under low

oxygen tension (0.5 mg/L soluble O₂). For the *lacZ*-based in vivo reporter system, all cultures were grown under high oxygen tension. *E. coli* strains (Table S2) were cultured in Luria–Bertani broth at 37 °C with continuous shaking at 180 rpm.

Microarray analysis was performed as described before (26). In brief, total RNA of strains 2.4.1pRKpCrZ and 2.4.1pRK4352 was chemically labeled with Cy3 and Cy5, respectively. Multiarray analysis was performed with the Bioconductor package Limma for R. On the basis of calculated MA plots, genes were considered reliable if the average signal intensity [A-value: 1/2 log₂ (Cy3 × Cy5)] was ≥ 12. To filter out potentially insignificant changes among genes that passed the reliability criterion, a cutoff value was applied; i.e., those genes were retained whose average expression value of 2.4.1pRKpCrZ (a) compared with the average value of the control treatment 2.4.1pRK4352 (b) was either a ≥ 1.75b or a ≤ 0.7b. Microarray data are deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>; accession no. GSE37381).

For description of additional procedures and information on strains, oligonucleotides, and plasmids see SI Materials and Methods.

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