INTRODUCTION

Since bacteria are exposed to frequent changes in their environment, they have developed mechanisms to sense and respond to external stimuli. Many bacteria are exposed to light, which can be beneficial as an energy source but can also be harmful by damaging cells through UV radiation or the generation of reactive oxygen species. Different classes of photoreceptors have been identified in bacteria over recent decades, but for only few bacterial photoreceptors is a biological function known (Braatsch & Klug, 2004; Spudich, 2006; Losi, 2007; Losi & Gärtner, 2008; Purcell & Crosson, 2008).

A single bacterial species, such as *Rhodobacter sphaeroides* 2.4.1, can even harbour a variety of photoreceptor proteins (Klug & Masuda, 2009). This species contains two red-light-absorbing bacteriophytochromes of high similarity (97% identity), BphG1 (RSP_4191) and BphG2 (RSP_4111). In both proteins the photosensing PAS-GAF-PHY module is linked to a GGDEF-EAL output module for cyclic di-GMP (c-di-GMP) synthesis and degradation (Tarutina et al., 2006). In addition, *R. sphaeroides* 2.4.1 contains several flavin-based blue light photoreceptors. The sensors of blue light using FAD (BLUF) domain (Gomelsky & Klug, 2002) was detected as the photoreceptor module of the AppA protein, which regulates photosynthesis gene expression in response to blue light and redox signals (Gomelsky & Kaplan, 1997; Braatsch et al., 2002; Masuda & Bauer, 2002). CryB, a cryptochrome-like protein, was identified more recently as another blue light photoreceptor affecting expression of photosynthesis genes in *R. sphaeroides* (Hendrichsk et al., 2009a). Interestingly, the *cryB* gene itself is under the control of an RpoHII promoter, linking the CryB-dependent response to the RpoE/RpoHII-mediated response to photooxidative stress (Anthony et al., 2005; Glaeser et al., 2007; Nuss et al., 2009). In addition, the presence of a LOV (light, oxygen, voltage) domain protein in *R. sphaeroides* binds a flavin chromophore and undergoes a photocycle has been described (Hendrichsk et al., 2009b). LOV domains form a subset of the PAS (PER–ARNT–SIM) family that is an important module in diverse signalling processes (Taylor & Zhulin, 1999) in all domains of life. In a unique photocycle reaction, the non-covalently bound flavin chromophore forms a covalent adduct with a conserved cysteine residue upon blue light illumination, initiating signal transmission to a variety of output domains (Crosson et al., 2003; Losi, 2007). The *R. sphaeroides* LOV domain protein, RsLOV, lacks, however, any output domain, but has an unusual extended C-terminal β-helix of almost 50 aa and shows very slow dark state recovery (Hendrichsk et al., 2009b). Its biological function was not known.
The physiological role of LOV domain proteins is best understood in the plant phototropins. The phot1 and phot2 proteins of Arabidopsis thaliana are coupled to serine/threonine kinase domains and regulate, for example, phototropism, chloroplast movement and stomatal opening (Briggs & Christie, 2002; Crosson et al., 2003). The LOV-containing White Collar 1 (WC1) protein of Neurospora crassa is involved in circadian clock regulation (Briggs, 2007). LOV domains are also widely distributed in bacteria (Losi & Gartner, 2008). The LOV domain of the Bacillus subtilis YtvA protein is coupled to a sulphate transporter/anti-sigma-factor antagonist (STAS) domain and acts as an inhibitor of an anti-sigma factor (Losi et al., 2002). In Brucella abortus, a LOV-histidine kinase mediates light-dependent virulence of the bacterium in mammalian macrophages (Swartz et al., 2007). Cell attachment in Caulobacter crescentus is regulated by the LOV-histidine kinase LovK, which interacts with the single-domain response regulator LovR (Purcell et al., 2007). The effect of blue light on LovK–LovR-mediated attachment is, however, weak, unless LovK is overexpressed.

Only a few LOV domain proteins lack an output domain. Among these, the VIVID protein of Neurospora crassa undergoes a conformational change upon illumination and is involved in photoadaptation (Schwerdtfeger & Linden, 2003; Zolowsk & Crane, 2008). A bacterial protein consisting of the LOV domain alone has been characterized in Pseudomonas putida wild-type 2.4.1 and 2.4.1Δlov were cultivated under the following conditions: micro-aerobic growth in the dark was performed at 32 °C in 50 ml Erlemeyer flasks containing 40 ml maleate minimal medium (Drews, 1983) with continuous shaking at 140 r.p.m. For blue light experiments, cultures were grown in 500 ml beaked Erlemeyer flasks with 200 ml maleate minimal medium (Braatsch et al., 2002). Aerobic growth conditions for oxidative stress experiments were established by gassing cultures with air in flat 50 ml glass bottles (Glaeser & Klug, 2005). For the R. sphaeroides Δlov strain, spectinomycin (10 μg ml⁻¹) was added to the cultures.

**METHODS**

**Bacterial strains and growth conditions.** Bacterial strains and plasmids are listed in Table 1. *R. sphaeroides* wild-type 2.4.1 and 2.4.1Δlov was generated by transferring the plasmid pPHUΔlov into R. sphaeroides 2.4.1. Fragment A (327 bp), containing the upstream region and the 5' part of the gene, was cloned into the suicide vector pPHU281 (Table 1). For the ΔchrR strain, spectinomycin (10 μg ml⁻¹) was added to the cultures.

**Construction of the R. sphaeroides Δlov strain.** *R. sphaeroides* 2.4.1Δlov was generated by transferring the plasmid pPHUΔlov into *R. sphaeroides* 2.4.1. Fragment A (327 bp), containing the upstream region and the 5' part of gene RSP_2228, and fragment B (377 bp), containing the 3' part of the gene and downstream sequences, were amplified by PCR using the oligonucleotides lovforup (5'-GGCTCTG- GAATTCCCTCCAGCC-3'), lovrevup (5'-ACCGAGAGATCCGCGCT- TG-3'),lovforwdown (5'-CATCAGGATCTCGAATTTTC-3') and lovrevdown (5'-CCGTTCCTCGAGATGCTGGC-3'), respectively. Fragment A was cloned into the EcoRI and BamHI sites of the suicide vector pPHU281 with Sp' cassette, flanked by lov upstream and downstream region, and fragment B was cloned into the suicide vector pPHU234 with phrA upstream region.

**Table 1.** Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant features</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JM109</td>
<td>Host strain for plasmid construction</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>S17-1</td>
<td>trα + for diparental conjugation</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td><strong>R. sphaeroides strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.4.1</td>
<td>Wild-type (laboratory strain)</td>
<td>W. R. Sistrom</td>
</tr>
<tr>
<td>2.4.1Δlov</td>
<td>2.4.1 with disruption of lov, Sp'</td>
<td>This study</td>
</tr>
<tr>
<td>TF18</td>
<td>2.4.1 with disruption of rpoEchrR, Tp'</td>
<td>Schilke &amp; Donohue (1995)</td>
</tr>
<tr>
<td>ΔchrR</td>
<td>2.4.1 with disruption of chrR, Tp'</td>
<td>Newman et al. (1999)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pPHU281</td>
<td>Suicide vector for Rhodobacter, Tc'</td>
<td>Hübner et al. (1993)</td>
</tr>
<tr>
<td>pPHUΔlov</td>
<td>pPHU281 with Sp' cassette, flanked by lov upstream and downstream region, Tc'</td>
<td>This study</td>
</tr>
<tr>
<td>pPHUphrA–lacZ</td>
<td>pPHU234 with phrA upstream region, Tc'</td>
<td>Hendrischk et al. (2007)</td>
</tr>
</tbody>
</table>
plasmid pPHU281 (Hübner et al., 1993), fragment B was inserted into the BamHI and PstI sites of the resulting plasmid, and the Sp’ cassette (Prentki & Kirsch, 1984) was cloned into the BamHI site. The resulting plasmid pPHUlov was transferred into Escherichia coli S17-1 and then transferred into R. sphaeroides 2.4.1 by diparental conjugation. Southern blots of spectinomycin-resistant, tetracycline-sensitive clones confirmed that the target gene was knocked out and the cassette was inserted.

**Spectroscopy.** Whole-cell suspensions of R. sphaeroides 2.4.1 and 2.4.1Δlov cultures were grown to identical OD_{660}. The abundance of photosynthetic complexes in cell suspensions was assayed using UV-VIS spectroscopy on a Specord 50 spectrophotometer (Analytik Jena).

**Bacteriochlorophyll measurements.** The relative bacteriochlorophyll content of illuminated cultures is given as the A_{660} after acetone: methanol (7:2) extraction of 1.5 ml cells normalized against the OD_{660}. The first measured relative bacteriochlorophyll value (OD_{660}=0.4) is set to 1.

**β-Galactosidase activity measurements.** For quantification of the γ^D-dependent β-galactosidase activity, overnight cultures were diluted to OD_{660} 0.2 and grown under the appropriate conditions. Samples were taken at OD_{660} 0.2, 0.4 and 0.8. β-Galactosidase activity quantification was performed as described previously (Hübner et al., 1991). For the photoinhibitory stress treatment, 0.05 μM methylene blue was added and the cultures were illuminated with 85 μmol white light m^{-2} s^{-1} during growth.

**Microarray experiments**

**Microaerobic growth.** Overnight cultures of the wild-type 2.4.1 and the Δlov mutant were diluted to OD_{660} 0.4 and grown under microaerobic conditions (approximately 25 μM O_2). The cells were harvested at OD_{660} 0.8 by centrifugation at 10 000 g in a cooled centrifuge.

**Blue light experiments.** Overnight cultures of the wild-type 2.4.1 and the Δlov mutant were diluted to OD_{660} 0.2, adjusted to semiaerobic conditions (approximately 100 μM O_2) by varying the rotation speed of the shaker for one doubling time, and afterwards irradiated with blue light (~λ=450 nm, 20 μmol m^{-2} s^{-1}) through a narrow-band filter with the respective wavelength for 60 min. The cells were then harvested by centrifugation at 10 000 g in a cooled centrifuge. Oxygen tension in R. sphaeroides cultures was monitored by using a Pt/Ag oxygen electrode (Micro Oxygen Sensor 501, UMS). Light quantity was measured at the flask centre (Quantum Sensor, Li-190SA).

**Photooxidative stress experiments.** Overnight cultures of the wild-type 2.4.1 and the Δlov mutant were diluted to OD_{660} 0.2 and gassed to adjust aerobic conditions (approximately 170 μM O_2). Methylene blue (0.2 μM) was added to the cultures to act as a photosensitizer. After one doubling time of growth in the darkness cells were illuminated with 800 W white light m^{-2} for 7 min. The cells were harvested by centrifugation at 10 000 g in a cooled centrifuge. Light quantity was measured at the position of the glass bottle (Pyranometer Sensor, Li-200SA).

**Microarray RNA preparation.** Total RNA was extracted by the hot phenol method, as described earlier (von Gabain et al., 1983; Janzon et al., 1986). Genomic DNA contamination from RNA samples was removed by DNase I treatment (Invitrogen). After DNA digestion, RNA was purified on RNasy MinElut spin columns (Qiagen). All RNA preparations were tested for the lack of genomic DNA contamination by PCR amplification using primers targeting ghoB (RSP0799), as described previously (Glaeser & Klug, 2005).

**Transcriptome profiling.** High-density oligonucleotide R. sphaeroides microarrays (Agilent gene chips corresponding to the whole 4.6 Mb genome; Peuser et al., 2011) were used for transcriptome profiling. The microarray contains probes against 4304 protein coding genes, 79 rRNA and tRNA genes, and 122 intergenic regions; its construction and performance analysis were performed according to the instructions from Agilent (http://www.chem.agilent.com/en-US/ Pages/Homepage.aspx). Three antisense probes with a length of 60 nt were designed for hybridization to each gene.

The ULS Fluorescent Labeling kit for Agilent arrays (Kreatech) was used for RNA labelling and fragmentation. The RNA from three 2.4.1 cultures grown under microaerobic/blue light illumination/photooxidative stress was pooled for hybridization to one array. A total of two arrays were hybridized to RNA from a total of six 2.4.1 cultures. In the same way two arrays were hybridized to RNA from a total of six 2.4.1Δlov cultures. A 2 μg sample of the pooled RNA was incubated with Cy3 dye (2.4.1Δlov) or Cy5 dye (2.4.1) for 15 min at 85 °C in the dark. Labelled RNAs were purified using KREApure columns, and the dye incorporation was measured using a Nanodrop spectrophotometer. Equal quantities of RNAs incorporating Cy3/Cy5 were mixed and incubated with fragmentation buffer for 30 min at 60 °C. Afterwards, the samples were mixed with hybridization buffer, hybridized onto microarray glass slides and incubated overnight (17 h) at 65 °C in a hybridization oven. Genechip hybridizations and scanning were performed according to the specifications of Agilent.

**Microarray data analysis.** Multiarray analysis was performed with the Bioconductor package Limma for R (Smyth, 2005). Background correction and normalization (locally weighted scatterplot smoothing) were performed as described elsewhere (Ritchie et al., 2007; Smyth & Speed, 2003). Genes were considered reliable if the mean intensity (A value) was >9.5 (for microaerobic conditions) and >12 (for blue light illumination/photooxidative stress). To filter out potentially insignificant changes among genes that passed the reliability criterion, a cut-off value was applied, i.e. those genes were retained whose average expression value in the Δlov mutant (ai) compared with the average value of the wild-type culture (aj) was either aj > 1.75 ai or ai ≤ 0.6 aj. Venn diagram analysis was performed with the VENNY tool (Oliveros, 2007).

**RNA quantification.** Real-time RT-PCR was performed according to the specification of the OneStep RT-PCR kit (Qiagen) with a final RNA concentration of 2 ng μl^{-1} (or 0.02 ng μl^{-1} for puc2AB). SYBR green I (Sigma Aldrich) was added at a final dilution of 1 : 50 000 to the RT-PCR Master Mix. Quantification of amplified products was performed using a CFX96 Real-Time PCR Detection System on a Bio-Rad S1000 thermal cyclor. For data analysis the CFX Manager Software (Bio-Rad) was used. Relative expression levels were quantified and normalized against mRNA levels of the rpoZ gene, as described elsewhere (Pappas et al., 2004). The fold change of target genes was calculated relative to that of rpoZ and control cultures grown in the dark (Pfaffl, 2001). Sequences and efficiencies of primers can be found in Table 2.

**RESULTS**

RsLOV affects the formation of photosynthetic complexes in R. sphaeroides

Two photoreceptors have been identified in the past in R. sphaeroides that influence the formation of pigment protein complexes and consequently the absorbance spectra: the BLUF domain of AppA and the cryptochrome-like CryB protein (Braatsch et al., 2002; Hendrichs et al., 2009a). We aimed to elucidate the biological function of the RsLOV protein, which shows the typical photocycle of LOV-domain
proteins, but in contrast to other short LOV-domain proteins has an extended C-terminal helix and shows very slow dark state recovery (Hendrichsk et al., 2009b). Therefore a strain was constructed that had the chromosomal copy of RSP_2228 replaced by an Sp’ cassette. The wild-type and the mutant were grown under different conditions with regard to oxygen tension and light, and whole-cell spectra were monitored. Neither strain showed significant differences in growth rates (growth curves for microaerobic conditions are shown in Supplementary Fig. S1). When cells were grown under microaerobic conditions in the dark to OD<sub>660</sub> 0.8 the mutant strain 2.4.1Δlov showed higher absorbance peaks than the wild-type 2.4.1 (Fig. 1a). At higher or lower optical densities only minor differences in absorbances were observed. The higher absorbance peaks at an OD<sub>660</sub> of 0.8 correlated with increased bacteriochlorophyll levels (Fig. 1b). When cultures were grown in the absence of oxygen under illumination (phototrophically), a slightly higher absorbance was observed for 2.4.1Δlov compared with the wild type (data not shown).

### Effect of RsLOV on the transcriptome of *R. sphaeroides* 2.4.1

The effect of RsLOV on the formation of pigment–protein complexes suggested that this photoreceptor affects the expression of photosynthesis genes. Real-time RT-PCR experiments indeed verified a stronger expression of the *puc* genes for proteins of the light-harvesting II complex in the mutant compared with the wild-type (Fig. 2). In order to get a more general view of the effect of the RsLOV protein on gene expression, we used a DNA microarray covering 4304 protein coding genes and 122 genes for sRNAs.

In a first analysis we cultivated the wild-type 2.4.1 and the Δlov mutant under low oxygen tension, when photosynthesis gene expression is high. Under these conditions 5% (227) of all genes showed different expression levels in the two strains (Supplementary Table S1). Most of these genes (210) showed higher expression (+twofold or greater) in the Δlov mutant compared with the wild-type 2.4.1, while only a few genes (17) showed higher expression in the wild-type (−twofold or greater). As expected from the observed difference in colour of the two strains, several photosynthesis genes showed differential expression in the mutant strain (Table 3). This was true for *pucB*, *pucA*, *puc2B*, *puc2A* and *pucC*, encoding pigment-binding proteins, as well as *pufQ* and *pufL*. However, *pufB*, *pufA* and *pufM* showed no or minor differences in expression. The same was true for the *puhA* gene, which encodes the non-pigment-binding subunit H of the reaction centre. Similarly, some genes for bacteriochlorophyll or carotenoid synthesis were expressed at higher levels in the mutant, while others showed no significant expression differences (Table 3). Different expression levels in the two strains were also observed for the alternative sigma factors *rpoE* and *rpoHI*, both known to be involved in the response to singlet oxygen (Anthony et al., 2005; Glaeser & Klug, 2005; Glaeser et al., 2007; Nuss et al., 2009). Table 3 summarizes results that show that most of the RpoE-dependent genes were also more strongly expressed in the mutant strain. The expression of the *lov* gene (RSP_2228) itself was not affected by photooxidative stress, and the Δlov mutant itself did not prove to be more sensitive to the photosensitizer methylene blue in inhibition zone assays (data not shown).

To test the results from the microarrays, real-time RT-PCR for quantification of selected mRNAs was performed (Fig. 2). The results mostly verified the microarray data.
with regard to the observed differences in expression levels. In general, the relative change as determined by real-time RT-PCR was larger than that calculated from the microarray data, although for most genes the difference between the two datasets was not significant.

**Blue light- and photooxidative stress-specific RsLOV-mediated effects on gene expression**

To further analyse the effect of the lov deletion after blue light illumination and under photooxidative stress was analysed. Established blue light illumination conditions were used to determine a possible light-dependent physiological reaction mediated by the photoreceptor RsLOV (Braatsch et al., 2002). While the protein showed a characteristic spectroscopic reaction to blue light that indicates the formation of a flavin–cysteinyldioxiduct, no direct *in vivo* effect of blue light could be observed (Hendrischk et al., 2009b). Due to the results of the microaerobic transcriptome analysis, which indicated a connection to the RpoE-dependent photooxidative stress response, gene expression after treatment with the photosensitizer methylene blue and high-light conditions (Glaeser & Klug, 2005) was also analysed.

Under blue light illumination conditions, 7% (305) of all genes showed different expression levels between 2.4.1Δlov and the wild-type 2.4.1. Most of these genes (225) showed higher expression (+ two-fold or greater) in the Δlov mutant compared with the wild-type 2.4.1, while a smaller number (80) of transcripts was expressed at a higher level in the wild-type. The highest number of differentially expressed genes was detected under photooxidative stress conditions. Here, 10% (446) of the transcripts showed different expression levels between the two strains. Again, more genes showed higher expression in the Δlov mutant (347) than in the wild-type 2.4.1 (99). Venn diagram analysis of the three microarray sets (Fig. 3) showed a strong overlap between the genes affected under blue light and photooxidative stress conditions, but only a small overlap with the genes affected under microaerobic growth conditions. One group of genes that showed higher expression in the Δlov mutant under all tested conditions included the operons encoding the pigment-binding proteins of the light-harvesting complex II (puc2BA and pucBAC). Other genes of the photosynthetic gene cluster were again only slightly changed in their expression levels or were below our selection criteria (Table 3). While genes of the photooxidative stress response in *R. sphaeroides* showed a stronger expression in the Δlov mutant under microaerobic conditions, this effect was not visible for...
blue light illumination and photooxidative stress (Table 3). The biggest group of genes that was affected under all tested conditions and also showed the strongest differences were linked to the carbon metabolism of *R. sphaeroides* (Table 4). These differentially expressed genes encode sugar transporters (e.g. *yurM*, RSP_3948, RSP_3501), transcriptional regulators of carbon metabolism (e.g. the DeoR-family proteins RSP_4038 and RSP_0457 or the GntR-family proteins RSP_1441 and RSP_1925), vitamin B12 synthesis (*cobN*, *cobB*, *cobV*, *cobL* and *corA*) and metabolic proteins [e.g. the subunits of the pyruvate dehydrogenase (*pdhAa*, *pdhAb* and *pdhB*), an aldehyde dehydrogenase (RSP_3333), an enoyl-CoA hydratase (RSP_3535) and the phosphoribulokinase (*prkA* and *prkB*). With the exception of the genes encoding pyruvate dehydrogenase, all these genes showed higher expression in the Δlov mutant. The last functional group of genes that showed differential expression in the Δlov mutant comprised several chemotaxis operons of *R. sphaeroides* (Table 5). Interestingly, some genes in this group were expressed more strongly in the wild-type (*fliC*, *flgB*, *flgM*, *flgE*, *cheW2* and *cheW3*), while others showed weaker expression (*flgI*, *flgR*, *fliH* and *fliB*).

To verify the results from the microarrays we again performed real-time RT-PCR for quantification of selected mRNAs (Fig. 4). The obtained expression levels confirmed the data from the microarray analysis.

### Effect of RsLOV on RpoE-dependent gene expression

Our microarray data revealed an effect of RsLOV on the expression of the *rpoE* gene and on genes which are under control of the alternative sigma factor RpoE (Table 3).
further analyse this effect, we followed the activity of the RpoE-dependent promoter phrA (for photolyase) by a reporter gene assay in different strains under different growth conditions (Fig. 5). The phrA–lacZ reporter plasmid was expressed in the wild-type 2.4.1, in strain 2.4.1Δlov, in strain TF18, which lacks the rpoE and chrR genes, and in strain ΔchrR, which lacks the anti-sigma factor ChrR. In agreement with the higher expression levels of rpoE as Fig. 3.

Venn diagram analysis of the data obtained in the three microarray experimental setups that were more strongly (+twofold or greater) (a) or more weakly expressed (−twofold or greater) (b) in the Δlov mutant and the wild-type. A list of the genes in the respective groups can be found in Supplementary Table S2.

Table 4. Selected carbon transporters and metabolic genes affected in the 2.4.1Δlov mutant

<table>
<thead>
<tr>
<th>Carbon transport and metabolism gene RSP number</th>
<th>Gene</th>
<th>Δlov/2.4.1 OD660=0.8*</th>
<th>Δlov/2.4.1 blue light*</th>
<th>Δlov/2.4.1 1O2 stress†</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSP_3943</td>
<td>yurM</td>
<td>+2.0</td>
<td>+2.0</td>
<td>+2.7</td>
<td>ABC sugar transporter, inner-membrane subunit</td>
</tr>
<tr>
<td>RSP_3948</td>
<td>-</td>
<td>+2.0</td>
<td>+2.0</td>
<td>+2.6</td>
<td>ABC sugar transporter, ATPase subunit</td>
</tr>
<tr>
<td>RSP_3501</td>
<td>-</td>
<td>+1.8</td>
<td>+2.9</td>
<td>+2.7</td>
<td>ABC sugar transporter, inner-membrane subunit</td>
</tr>
<tr>
<td>RSP_4038</td>
<td>glpR</td>
<td>+1.6</td>
<td>+28.0</td>
<td>+9.0</td>
<td>Transcriptional regulator, DeoR family</td>
</tr>
<tr>
<td>RSP_0457</td>
<td>glpR</td>
<td>+1.3</td>
<td>+2.7</td>
<td>+2.2</td>
<td>Probable glycerol 3-phosphatase repressor</td>
</tr>
<tr>
<td>RSP_1441</td>
<td>-</td>
<td>+1.5</td>
<td>+3.3</td>
<td>+4.0</td>
<td>Regulatory protein, GntR family</td>
</tr>
<tr>
<td>RSP_1925</td>
<td>-</td>
<td>+1.5</td>
<td>+20.6</td>
<td>+7.2</td>
<td>Regulatory protein, GntR family</td>
</tr>
<tr>
<td>RSP_4047</td>
<td>pdhAa</td>
<td>-2.1</td>
<td>-2.0</td>
<td>-2.5</td>
<td>Pyruvate dehydrogenase E1 component</td>
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<td>RSP_4049</td>
<td>pdhAb</td>
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<td>-2.0</td>
<td>-2.4</td>
<td>Dihydrolipoamide acetyltransferase</td>
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<tr>
<td>RSP_4050</td>
<td>pdhB</td>
<td>-2.0</td>
<td>-2.1</td>
<td>-2.5</td>
<td>E2 component of pyruvate dehydrogenase complex</td>
</tr>
<tr>
<td>RSP_1284</td>
<td>prkA</td>
<td>+3.2</td>
<td>+4.7</td>
<td>+3.2</td>
<td>Phosphoribulosinase</td>
</tr>
<tr>
<td>RSP_3267</td>
<td>prkB</td>
<td>+2.7</td>
<td>+4.8</td>
<td>+3.9</td>
<td>Phosphoribulosinase</td>
</tr>
<tr>
<td>RSP_3533</td>
<td>-</td>
<td>+2.2</td>
<td>+3.1</td>
<td>+4.3</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>RSP_3535</td>
<td>-</td>
<td>+2.8</td>
<td>+7.0</td>
<td>+6.1</td>
<td>3-Hydroxyacyl-CoA dehydrogenase/short-chain enoyl-CoA hydratase</td>
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<tr>
<td>RSP_2827</td>
<td>cobN</td>
<td>+1.4</td>
<td>+2.2</td>
<td>+1.8</td>
<td>Cobaltocelatase subunit CobN</td>
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<tr>
<td>RSP_3185</td>
<td>cobB</td>
<td>+1.6</td>
<td>+3.5</td>
<td>+2.9</td>
<td>Cobyrinic acid a,c-diamide synthase</td>
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<tr>
<td>RSP_2608</td>
<td>corA</td>
<td>+1.6</td>
<td>+2.3</td>
<td>+2.3</td>
<td>Mg²⁺/Co²⁺ transporter</td>
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<tr>
<td>RSP_2427</td>
<td>cobV</td>
<td>+1.6</td>
<td>+2.8</td>
<td>+2.4</td>
<td>Cobalamin-5’-phosphate synthase</td>
</tr>
<tr>
<td>RSP_2823</td>
<td>cobL</td>
<td>+1.6</td>
<td>+7.0</td>
<td>+2.6</td>
<td>Putative precorrin-6γ C5,15-methyltransferase</td>
</tr>
</tbody>
</table>

*Significant changes are in bold type. Selected genes that missed the cut-offs are included in the table to fully represent the functional groups discussed.
†¹O₂, singlet oxygen.
In general, the deletion of the LOV protein leads mostly to increased gene expression in the cell, with 5–10% of all transcripts being more strongly expressed, while only 0.5–1.9% of all transcripts were more weakly expressed compared with the wild-type. These results could be verified by real-time RT-PCR (Figs 2 and 4), supporting a repressing role of LOV in gene regulation. Microarray analysis of a deletion of the strong repressor of photosynthesis genes PpsR (Moskvin et al., 2005) led to a slightly more accented ratio of affected genes (18% more strongly expressed to 2% more weakly expressed genes). PpsR acts directly as a repressor by binding to DNA, while LOV-dependent effects on gene expression are expected to be indirect due to the lack of a DNA-binding domain.

One of the early observations on the pigment composition of the Δlov strain was a stronger pigmentation and higher bacteriochlorophyll content of the cells while growing in late exponential phase (OD660 0.8) that was lost when the cultures reached stationary phase (Fig. 1). This effect was paralleled by changes in the expression of photosynthesis genes in the microarray analysis. Interestingly, only the operons encoding the protein components of the light-harvesting complex II seem to be more strongly expressed in the Δlov mutant, while expression of protein components of the light-harvesting complex I and the photosynthetic reaction centre are mostly unaffected (Table 3). Microarray analyses revealed that other photosynthesis gene expression regulators such as the activator PrrA (Eraso et al., 2008) and the repressor PpsR (Moskvin et al., 2005) always affected all groups of photosynthesis genes. The same was true for hydrogen peroxide stress (Zeller et al., 2005) or iron limitation (Peuser et al., 2011). A specific increase of light-harvesting complex II formation under microaerobic conditions however can be generated

### DISCUSSION

Light perception in *R. sphaeroides* is one of the main factors responsible for the regulation of gene expression. Intensive research has outlined the effect of AppA on the repressor PpsR in photosynthesis gene expression (Gomelsky & Kaplan, 1997; Braatsch et al., 2002; Masuda & Bauer, 2002), and with the cryptochrome CryB a second blue light receptor that plays a role in photosynthesis gene expression has been identified (Hendrischk et al., 2009a). Our transcriptome approach to the physiological role of a third blue light photoreceptor, the *R. sphaeroides* LOV protein (Hendrischk et al., 2009b), also indicates an influence on the regulation of photosynthesis genes.

### Table 5. Selected chemotaxis genes affected in the 2.4.1Δlov mutant

<table>
<thead>
<tr>
<th>Chemotaxis gene RSP number</th>
<th>Gene</th>
<th>Ratio</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Δlov/2.4.1 OD660=0.8*</td>
<td>Δlov/2.4.1 blue light*</td>
</tr>
<tr>
<td>RSP_0069</td>
<td>fliC</td>
<td>−1.2</td>
<td>−3.5</td>
</tr>
<tr>
<td>RSP_0083</td>
<td>fliB</td>
<td>+1.4</td>
<td>−2.0</td>
</tr>
<tr>
<td>RSP_0037</td>
<td>fliM</td>
<td>+1.0</td>
<td>−2.1</td>
</tr>
<tr>
<td>RSP_0080</td>
<td>fliE</td>
<td>+1.4</td>
<td>−2.0</td>
</tr>
<tr>
<td>RSP_1307</td>
<td>fliI</td>
<td>+1.4</td>
<td>+2.2</td>
</tr>
<tr>
<td>RSP_1321</td>
<td>fliR</td>
<td>+1.5</td>
<td>+3.6</td>
</tr>
<tr>
<td>RSP_1311</td>
<td>fliH</td>
<td>+1.3</td>
<td>+1.8</td>
</tr>
<tr>
<td>RSP_1322</td>
<td>fliB</td>
<td>+1.7</td>
<td>+3.2</td>
</tr>
<tr>
<td>RSP_1585</td>
<td>cheW2</td>
<td>+1.2</td>
<td>−2.0</td>
</tr>
<tr>
<td>RSP_1586</td>
<td>cheW3</td>
<td>+1.3</td>
<td>−2.0</td>
</tr>
</tbody>
</table>

*Significant changes are in bold type. Selected genes that missed the cut-offs are included in the table to fully represent the functional groups discussed.
†1O2 singlet oxygen.

Determined by microarrays and real-time RT-PCR for the mutant 2.4.1Δlov when compared with the wild-type, we found slightly increased β-galactosidase activities of the *phrA-lacZ* reporter in the mutant under microaerobic growth conditions. Since RpoE has constitutive high activity when the anti-sigma factor is missing, β-galactosidase activities were very high in strain ΔchrR under all tested conditions. Under aerobic conditions in the presence of methylene blue and light (high levels of singlet oxygen), the activity of the *phrA* promoter in the wild-type was clearly higher than that under microaerobic growth conditions at optical densities of 0.4 or 0.8. Under these conditions and at these cell densities, *phrA* expression was lower in the mutant than in the wild-type, which is in agreement with the lower levels of *rpoE* transcripts. The lack of RsLOV had, however, a much weaker effect than the lack of RpoE and ChrR, supporting the view that RsLOV is involved in singlet oxygen-dependent regulation but is not a main regulator of this response.
by blue and red light (Hunter et al., 2005). These data suggest that RsLOV does not affect photosynthesis gene expression through interaction with one of the other known regulatory factors.

Our microarray analysis points to a role of RsLOV in the RpoE-dependent response to photooxidative stress. Under microaerobic conditions almost all described genes of the RpoE regulon (Anthony et al., 2005; Glaeser & Klug, 2005; Glaeser et al., 2007; Nuss et al., 2009) are more strongly expressed in the mutant (Table 3). Additionally β-galactosidase measurements also verify a connection between the RsLOV deletion and the photooxidative stress response (Fig. 5). Expression of rpoE, rpoHI, rpoHII and some other RpoE-dependent genes was similar in both strains when grown under blue light or under photooxidative stress. RSP_4257, which is more strongly expressed in the mutant, shows high homology to the STAS transporter family, which plays an important role in the cellular response to osmotic stress (Aravind & Koonin, 2000). A STAS domain is also found in the YtvA protein of Bacillus subtilis. This LOV domain protein acts as an activator of the sigma B-dependent general stress response in Bacillus subtilis and comprises an N-terminal LOV and a C-terminal STAS domain (Avila-Pérez et al., 2006) that have been shown to interact during blue light illumination (Avila-Pérez et al., 2009). The transcriptional regulator RSP_3188 shows strong homology to the E. coli RseC protein, which plays a major role in RpoE activation in this organism (Missiakas et al., 1997) and is connected to thiamin (vitamin B1) synthesis in Salmonella (Beck & Downs, 1998). While a link of RsLOV to the RpoE response in R. sphaeroides is supported by our data, a clear growth difference under photooxidative stress was not visible in inhibition zone assays, and the expression of the LOV gene (RSP_2228) was also unaffected (results not shown), indicating that the influence of the LOV protein

![Fig. 4. Validation of microarray data by real-time RT-PCR. Ratios of the expression levels between R. sphaeroides Δlov and 2.4.1 grown under blue light illumination (a) and photooxidative stress (b) determined by real-time RT-PCR (white bars) in comparison with ratios calculated from the microarrays (black bars). Values are normalized to rpoZ. The means of at least three independent experiments were calculated and error bars indicate SD.](image1)

**Fig. 4.** Validation of microarray data by real-time RT-PCR. Ratios of the expression levels between R. sphaeroides Δlov and 2.4.1 grown under blue light illumination (a) and photooxidative stress (b) determined by real-time RT-PCR (white bars) in comparison with ratios calculated from the microarrays (black bars). Values are normalized to rpoZ. The means of at least three independent experiments were calculated and error bars indicate SD.

![Fig. 5. β-Galactosidase assays of an RpoE-dependent reporter construct under different growth and stress conditions in the strains R. sphaeroides 2.4.1, Δlov, TF18 and ΔchrR. Cultures were grown under aerobic (a) and aerobic conditions with added methylene blue and white light illumination (b). Samples were taken at OD660 0.2 (white bars), 0.4 (black bars) and 0.8 (hatched bars). Average values of two different experiments that showed the highest variation are presented.](image2)

**Fig. 5.** β-Galactosidase assays of an RpoE-dependent reporter construct under different growth and stress conditions in the strains R. sphaeroides 2.4.1, Δlov, TF18 and ΔchrR. Cultures were grown under aerobic (a) and aerobic conditions with added methylene blue and white light illumination (b). Samples were taken at OD660 0.2 (white bars), 0.4 (black bars) and 0.8 (hatched bars). Average values of two different experiments that showed the highest variation are presented.
on RpoE-dependent gene expression is indirect. There is a strong overlap in the Venn analysis (Fig. 3) between blue light- and photooxidative stress-affected genes. This may indicate that the effects of the photooxidative stress experiments are indeed due to the blue light portion (approximately 20 μmol m⁻² s⁻¹) of the white light treatment during the stress experiment. On the other hand, low levels of photooxidative stress may also be generated in the cultures during blue light illumination. For some genes (e.g. RSP_4038 and RSP_1925) the effects of blue light and photooxidative stress differed significantly. This suggests specific effects of light or oxidative stress at least for those genes. It has been recently suggested that bacterial LOV domain proteins may also function as redox sensors (Purcell et al., 2010). That study demonstrated that FMN bound to LovK has a midpoint potential which is congruent with the redox potential of the Gram-negative cytoplasm. Chemical reduction of the FMN cofactor attenuated the light-dependent ATPase activity of LovK and demonstrated that LovK is regulated by the redox state of the cellular environment. The authors present a model in which the redox state of the cytoplasm can regulate LovK photoactivity. The hypothesis that LOV domain proteins may function as redox sensors is supported by our finding that the expression of more genes is affected in the mutant by singlet oxygen than by blue light.

The biggest physiological group of differentially expressed genes comprised genes for the carbon metabolism of R. sphaeroides (Table 4). Most genes in this group are more strongly expressed in the mutant strain. The only exception is the operon encoding the pyruvate dehydrogenase (RSP_4047–RSP_4050), which shows around twofold lower expression in the mutant. This thiamin-dependent enzyme complex catalyses the transformation of pyruvate into acetyl-CoA, and thus is needed for the transition from glycolysis to the citrate cycle. Lower expression in the mutant would indicate an enhancing role of LOV in central carbon metabolism. Nevertheless, all other genes of carbon metabolism, such as several sugar transporters (yurM, RSP_3948 and RSP_3501), an aldehyde dehydrogenase (RSP_3533), an enoyl-CoA hydratase (RSP_3535) and the phosphoribulokinase (prkA and prkB), are expressed more in the mutant strain, suggesting a repressing effect mediated by LOV on carbohydrate uptake and processing. This is especially plausible if one takes into account the most strongly affected genes in the Δlov mutant—the transcription factors. These genes all show a greatly increased expression in the mutant. The two GntR transcription regulators RSP_1441 and RSP_1925 (Haydon & Guest, 1991) both possess a DNA-binding GntR domain linked to a sugar-binding domain and show strong homology to sugar transport regulators. RSP_1441 contains a NagC kinase domain (Plumbbridge, 1995, 2001), while RSP_1925 has a high sequence similarity to the galactonate repressor NatR (van Aalten et al., 2000). RSP_4038 and RSP_0457 are both DeoR transcriptional regulators that also feature a GntR DNA-binding domain, and in addition a GlpR domain that has been described as playing an important role in carbon metabolism in E. coli (Boyd et al., 2000, Dandanell et al., 1991). Several of the proteins encoded by the RsLOV-affected carbon metabolism genes can affect the redox state of the cell through generation of reducing power. This response would therefore be in agreement with a role for RsLOV as a redox mediator.

It is also notable that most of the genes necessary for cobalamin (Vitamin B12) synthesis (cobN, cobB, cobV, cobL and corA) are more strongly expressed in the mutant. Cobalamin is an important cofactor for haem and bacteriochlorophyll synthesis (Pollich & Klug, 1995; Pollich et al., 1996), and the increase in expression may contribute to the increase in absorbance spectra and the relative bacteriochlorophyll content (Fig. 1).

The last functional group affected by the deletion of the LOV photoreceptor belongs to the group of the chemotaxis genes, where some genes are more strongly expressed (flIC, flgB, flgM, flgE, cheW2 and cheW3) while others show weaker expression (flgI, flgIR, flIL and flIB) in the mutant. Almost all of these genes can be found in the same or nearby operons on the chromosome and are under control of the alternative sigma factors of the RpoN group (Peña-Sánchez et al., 2009). The involvement of a photoreceptor in the regulation of these genes suggests an effect on phototaxis. This hypothesis could not be tested, since strain 2.4.1 has lost phototactic responses.

In summary, our results demonstrate that although lacking any output domain, RsLOV affects a large set of genes with different physiological functions in R. sphaeroides. It constitutes the third flavin-binding photoreceptor in R. sphaeroides, besides AppA and CryB, that affects photosynthesis gene expression. Certainly, AppA is a main regulator of photosynthesis genes, since its deletion results in an almost complete lack of pigment–protein complexes (Gomelsky & Kaplan, 1995). The effects of RsLOV and CryB on photosynthesis gene expression and pigment–protein complex formation are much smaller, implicating a function in fine-tuning, possibly in response to certain growth conditions. RsLOV may counteract the activating effect of AppA when cells enter stationary phase and energy-generating metabolism is slowed down. How RsLOV can affect gene expression needs to be elucidated in future experiments. Since it lacks any DNA-binding domain, it is likely to act through protein interactions, and different interaction partners may affect different sets of genes. In general, the picture emerges that light-dependent regulation of bacterial photosynthesis genes involves a complex regulatory network including multiple photoreceptors. This may reflect the need for a balance between the use of light as an energy source and the need to avoid the production of reactive oxygen species or damage by UV light.

**ACKNOWLEDGEMENTS**

We thank Jürgen Tomasch for instructions and help on using Agilent gene chips, Gregor Langen for technical support on the Agilent
scanning, and Birgit Samans for helpful discussions and advice on microarray data analysis. This work was supported by the Deutsche Forschungsgemeinschaft (Ki563/22-1).

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Light-regulated gene expression in R. sphaeroides


Edited by: D. J. Kelly