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regO: a novel locus in the regulation of tetrapyrrole biosynthesis in *Rhodospirillum rubrum*

Manar Mansour* and Khaled Abou-Aisha

Abstract

Purpose A new locus, *regO*, involved in the regulation of photosynthesis gene expression in response to oxygen and light, has been studied in *Rhodosprillum rubrum* ATCC1117 (*Rsp. rubrum*) for identification of its function.

Methods Inactivation of *regO* by interposon mutagenesis resulted in the inability of cells to grow photosynthetically, (i.e. become PS^{-}). Protein domain analysis of RegO using the BLAST engine was also performed.

Results The mutant strain was able to grow only anaerobically in the dark in the presence of DMSO as an external electron acceptor. Under these conditions, the mutant strain produced substantially lower amounts of photosynthetic membranes, indicating that *regO* is involved in the regulation of photosynthetic gene expression in response to anaerobiosis. The *Rsp. rubrum* $REGO^{-}$disrupted mutant recovered the synthesis of photosynthetic membranes and retained regulation by light and/or oxygen tension when wild-type *regO* was provided *in-trans*.

Protein domain analysis of RegO revealed that it encodes a multi-domain sensor histidine kinase (HK). The signal-input domains, or PAS domains, bear strong similarities to putative heme-bound sensors involved in sensing light, redox potential, and/or oxygen. The output HK domain exhibits strong homology to sensor domains from bacterial two-component systems involved in signal transduction in response to the same environmental signals.

Conclusion *regO* is coding for a sensor histidine kinase that belongs to bacterial two-component systems responsible for signal transduction in response to light and oxygen, particularly in the absence of oxygen. It is believed to be involved in the regulation of tetrapyrrole biosynthesis, which was shown as a lack of photosynthetic membranes in the mutant strain $REGO^{-}$. Unlike other sensor kinase homologues from related anoxygenic phototrophic bacterial species, although functionally similar to RegB and PrrB, RegO is predicted to lack transmembrane domains and is thus expected to be a cytosolic member of a two-component signal transduction system. RegO also differs from its functional homologues, Reg B/PrrB sensor protein kinases, of the two component systems in that it lacks the second component of this two-component signal transduction system found in the neighboring genes. That encouraged us to give it the name RegO, indicating the lack of a cognate response regulator similar to Reg A/PrrA on other closely related anoxygenic *Rhodobacter* species.

Keywords Sensor histidine kinase, *Rhodosprillum rubrum*, Tetrapyrrole biosynthesis, Two-component systems

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Introduction

In order to survive in a continuously varying environment, living organisms must be able to adjust their metabolic functions to their surrounding conditions, with factors such as the availability of nutrients, oxygen, pH

value, temperature, and light having direct effects on the development and differentiation of organisms (Armitage 1997). In unicellular organisms, their entire surface is in direct contact with their environments and must therefore quickly adapt to the prevailing conditions in order to avoid cell damage or death (Lee and Wang 2019). In those cells, environmental conditions are sensed by a variable number of sensor proteins (receptors) (Stein et al. 2020).

The activation of these proteins leads to the transduction of the respective signals to the so-called effector proteins, which in turn can elicit, at variable levels, the required adaptive effect. The most frequent form of modification is by phosphorylation. Whereas eukaryotes use, in the main, the amino acids serine, threonine, and tyrosine in phosphorylation, bacterial kinases preferentially utilise histidine and aspartate for phosphorylation (Singh et al. 2003). Although serine-, threonine-, and tyrosine kinases are functionally very similar to histidine kinases, decisive differences do exist. Prokaryotic kinases play a crucial role in signal transduction, and in many cases, environmental signals are perceived by the so-called two-component systems (Francis et al. 2018; Francis and Porter 2019).

As their name implies, two-component systems consist of two individual components: a sensor kinase and a cognate response regulator, which become phosphorylated upon sensor activation (Desai and Kenney 2017). The phosphorylation of the response regulator enables the specific regulation of the target gene(s). In organisms such as *Escherichia coli* (*E. coli*), Bacillus subtilis, and Synechocystis sp., 30 to 40 different two-component systems have been identified, where each individual system responds to a different signal and activates a specific gene (Hoch and Varughese 2001).

The activation of the sensor kinases described above by binding a signalling molecule, either from the external environment or conveyed from another protein, leads to their autophosphorylation (Stock et al. 2000; Buelow and Raivio 2010). The histidine-protein kinases of prokaryotes are characterised by the presence of a conserved amino acid sequence of approximately 200 amino acids that contains an ATP-binding domain. This sequence is flanked by other domains that show low homology when several histidine kinases are compared (Casino et al. 2009). These regions possess regulatory functions and are thus specific for the signal that a given kinase perceives (Mitrophanov and Groisman 2008; Skerker et al. 2008). Many histidine-protein kinases possess extensive N-terminal domains with long stretches of hydrophobic amino acids. These regions serve to anchor the protein in the cytoplasmic membrane (Mascher et al. 2006). Some of these transmembrane kinases possess external sensor domains, which enable the protein to accept signals at the cell surface leading to the phosphorylation of the protein(Isaacson et al. 2006). In all cases studied so far, phosphorylation could be identified as a bimolecular reaction: autophosphorylation leads to the formation of a homodimer of the kinase. The phosphorylation of the histidine kinase monomer is catalyzed by a second monomer of the protein (Creager-Allen et al. 2013). Histidine-protein kinases can vary greatly in their structure. Thus, whereas some proteins, as mentioned above, have a membrane anchoring domain, others, such as NtrB, are cytoplasmic proteins (Martínez-Argudo et al. 2002). In these cases, the transfer of the phosphate group to an internal aspartate residue in the response regulator domain is possible. Response regulators are classified into four different families: the CheY-, NtrB-, FixJ-, and OmpR-families. The CheY family contains the response regulators, which consist of a single domain. All other response regulators are built of multiple domains. Most response regulators are transcription regulators. They carry in their C-termini DNA binding domains, which enable them to interact with DNA. CheY, however, does not carry such a DNA-binding region. Instead, the protein receives a phosphate group from the corresponding sensor kinase CheA in a chemotaxis system. The phosphorylated CheY interacts with the flagellar motor protein of *E. coli*, thereby affecting its swimming motility (Galperin 2006; West and Stock 2001).

All fully sequenced bacterial and archaeal genomes have been found to contain, in their HK sensor proteins, specific signalling modules, called PAS domains (Taylor and Zhulin 1999). PAS domains containing signal-transducing proteins are always located intracellularly (Szurmant et al. 2007). These domains track changes in light, redox potential, oxygen, small ligands, and the cell's overall energy level. PAS domains may also sense external environmental factors that cross the cell membrane and/or affect cell metabolism (Möglich et al. 2009). The cytoplasmic location of PAS domains suggests that they sense changes in the intracellular environment, but PAS domains can directly sense the environment outside the cell for stimuli that enter the cell, such as light (Taylor and Zhulin 1999). The advantage of detecting oxygen, light, redox potential, and energy levels for cell survival has long been recognised (Yang and Tang 2000). An intracellular location of single and multiple PAS domains was predicted in all analysed sensor proteins (Möglich et al. 2009).

In some signalling pathways, the signal from the receptor is itself transduced into a different form of energy by a second protein. FixL is an oxygen receptor, in which oxygen binds directly to a heme that is coordinated to a histidine residue within a PAS domain (Key and Moffat 2005). Other PAS proteins, such as Aer, are transducers

that detect oxygen indirectly by sensing redox changes as the electron transport system responds to changes in oxygen concentration (Taylor 2007). Adaptation of the PAS domain structure to sense various stimuli such as oxygen, ligands, light, and redox potential is found in prokaryotes, and the presence of divergent PAS domains in a single protein may be functionally discriminated to sense different stimuli (Taylor and Zhulin 1999; Mann and Shapiro 2018).

Rhodosprillum rubrum is a facultative anoxygenic photosynthetic bacterium that exhibits a versatile metabolism that allows it to adapt to rapidly changing growth conditions in its natural environment and, therefore, has been utilized as a model organism for cellular redox studies (Ghosh et al. 1994; Grammel et al. 2003).

In the presence of oxygen, *Rsp. rubrum* performs aerobic respiration. Under anaerobic conditions, *R. sp. rubrum* can, in the presence of light, grow photosynthetically. Reduction of oxygen partial pressure induces the synthesis of photosynthetic complexes. In the closely related species Rhodobacter sphaeroides and Rb. capsulatus, the expression of photosynthetic genes and genes required for carbon dioxide fixation are largely controlled by the well-conserved global two-component systems referred to as RegA/RegB in Rb. capsulatus or its homologue PrrA/PrrB in Rb. sphaeroides (Grammel et al. 2003). The physical gene organisation of the two regulatory circuitries is maintained in both species and in other members of purple bacteria (Eraso et al. 2008). The regulation of photosynthesis gene expression in species other than Rhodobacter has also been examined (Masuda et al. 1999).

Tetrapyrroles are a "color palette" of physiologically packed metal ions that play critical roles in both anabolic and catabolic metabolisms, and the kinds of tetrapyrroles synthesised or acquired by cells define their metabolic capabilities (Sebastien et al. 2010). The presence of two tetrapyrrole species, heme and bacteriochlorophyll (BChl), made anoxygenic photosynthesis—their most distinctive feature—possible.

Transcription of the tetrapyrrole biosynthesis genes—just like photosynthesis genes—is also responsive to oxygen, since the need for heme and BChl is dictated to a significant degree by what form of energy metabolism is used by the cell (Sebastien et al. 2010).

In the presence of high oxygen tensions, heme biosynthesis is necessary in order to form respiratory cytochromes, the cells have no need for, nor do they produce, BChl. But when oxygen tensions fall, BChl levels are estimated to increase more than 100-fold (Kořený et al. 2021), while at the same time heme production also increases, as both are required for photosynthesis (Flory and Donohue 1997).

In this study, we investigated, in *Rsp. rubrum*, a gene called regO, as suggested by the author, encoding a putative sensor HK using interposon mutagenesis and complementation analysis. We show that the encoded protein is functionally analogous to the previously identified sensor HKs from other anoxygenic phototrophic bacteria, e.g., RegB/PrrB in Rb. capsulatus and Rb. sphaeroides, respectively

Material and methods

Bacterial strains and growth conditions

The characteristics of the bacterial strains and plasmids used in this study are listed in Table 1.

Wild-type *Rsp. rubrum* S1 (ATCC 11170) cells were grown at 30°C, in Sistrom's basal medium (Table 2) (M-medium). Cultures were grown aerobically in the dark (chemotrophically) or anaerobically in the light (phototrophically). *Rsp. rubrum* was grown aerobically in the dark by inoculating 100 ml of Sistrom's medium in 250 ml baffled Erlenmeyer flasks with vigorous shaking at 200 rpm.

To grow *R. rubrum* anaerobically (phototrophically), 100-ml screw-cap Pyrex bottles were filled with Sistrom medium, supplemented with succinate as a carbon source, and incubated in the dark for 24 h to deplete oxygen from the medium. Cultures were then incubated in light using a 160-W lamp at an intensity of 10 W/m^2.

Transformed *E. coli* strains, containing different plasmid vectors (see Table 1), were grown in Lysogeny Broth (LB) medium supplemented, when required, with 100 µg/ml ampicillin, 34 µg/ml chloramphenicol, 40 µg/ml streptomycin plus spectinomycin, 50 µg/ml kanamycin, or 10 µg/ml tetracycline, for selection. Cells were incubated at 37 °C.

Interposon mutagenesis of regO locus

Genomic DNA of *Rsp. rubrum* was isolated by the rapid isolation protocol of genomic DNA of gram-negative bacteria as described by (Neumann et al. 1992). The protocol starts with the lysis of bacterial cells by Lysozyme 1 mg/mL (Roth, Germany) 37 °C for 30 min. Following the addition of 10% SDS and protinase K 1 mg/ml (Roth, Germany) pre-incubated at 37 °C, the tube was gently inverted to obtain a homogenous solution. NaCl (5 M) in a ratio of 1/3 volume was then added. After transferring the clear supernatant into a new tube, an equal volume of chloroform was also added. The tube was gently inverted, and the mixture was incubated at room temperature for 30–60 min with continuous gentle mixing throughout the incubation period. The aqueous phase was then transferred after centrifugation to a new tube. The DNA were precipitated by addition of 1 volume isopropanol,

Table 1 Bacterial strains and plasmids

Bacterial strain	Genotype or phenotype	Reference/source
E. coli		
XL1-Blue	*recA1*, *endA1 gyrA96* thi-1 *hsdr17 sup E44 relA1* lac^q ZΔ M15 Tn 10 (Tet^r)]	Stratagen
Rsp. rubrum	Wild type	ATCC11170
REGO$^-$	mutants *R. rubrum regO* Ω, sm^r, sp^r	This work
REGO pRK415 *regO*	*regO* Ω, sm^r, sp^r , pRK415 Tet^r*regO* PCR fragment	This work
Plasmids		
pBluescript KSII	Cloning vector (Ap^r); with T3 and T7 promotors	Stratagen
pHP45Ω	Source of the Ω Sm^r Sp^r cassete	(Prentki and and Henry M. Krisch 1984)
pSUP202	Suicide vector for *R. rubrum*, ap^r cm^r tc^r; mob$^+$	(Simon et al. 1983)
pRK2013	kan^r; tra$^+$	(Lois et al. 1993)
pRK415	tet^r	/ (Lois et al. 1993)
pBS-515	1545 bp PCR fragment from *R. rubrum* was cloned in pBSKS(+), ap^r	This work
pBS-515Ω	3545 bp, PCR fragment of *R. rubrum* with Ω, sm^r sp^r at *Nde*I site of *regO* was cloned in pBSKS(+), ap^r	This work
pPSUP-515Ω	3500 bp, PCR fragment of *R.* ` with Ω, sm^r sp^r at *Nde*I site of *regO*was cloned in pSUP202, ap^r,cm^r	This work

Table 2 Composition of Sistrom's basal medium

Ingredient	Concentration (mM)
K_2HPO_4	199.7 mM
or KH_2PO_4	199.8 mM
$(NH_4)_2SO_4$	37.8 mM
or NH_4Cl	36.55 mM
Succinic acid	338.7 mM
L-glutamic acid	5.34 mM
L-aspartic acid	3 mM
NaCl	86.2 mM
Nitrilotriacetic acid	10.4 mM
$MgSO_4 . 7H_2O$	12.17 mM
$CaCl_2 . 2H_2O$	2.27 mM
$FeSO_4 . 7H_2O$	0.072 mM
$(NH_4)_6Mo_70_{24}$ (1% solution)	0.2 ml
Trace Elements Solution	1 ml
Vitamins Solution	1 ml

washed twice with 70% EtOH, vacuum dried and dissolved in 1× TE.

The regO gene was amplified by PCR using, a forward primer: 5′-**AAG CTT** GGC CAT GGG CGG CGA GCC CGT CGG TCT G-3′, and a reverse primer: 5′-**GGA TCC** TTC ACC CGC CCC CGT CGA TAA-3′ with HindIII and BamHI restriction sites, (bold underlined), respectively (Invitrogen, USA). PCR products were then purified from agarose gel by QIAquick gel extraction kit (Qiagen, Germany). The PCR product was double digested by HindIII and BamHI using a compatible buffer according to the manufacturer's recommendations (NEB, Germany). The fragment was then cloned into a pBSKs II plasmid vector that was double digested using the same restriction enzymes and overnight ligated by T4 DNA ligase (NEB, Germany) at 16 °C, *E. coli* XL1 was then transformed with the new construct, now called pBS-515. Transformed cells were selected on LB supplemented with 100 µg/ml ampicillin.

A spectinomycin-streptomycin resistant omega Ω cassette (Gene 29, 303 313) (Prentki and Krisch 1984) was cloned at the NdeI unique site of regO located 930 bp downstream from the start codon. The NdeI digested regO in pBSKsII was blunted by DNA polymerase I Klenow fragment (NEB, Germany) at 21 °C. Cloning of the Ω interposon into the regO at the NdeI blunted site was performed using overnight blunt end ligation by T4 DNA ligase at 16 °C, followed by transformation of the new construct (pBSKsII + regO Ω) into *E. coli*. XL1. Transformed cells were selected on LB plates supplemented by ampicillin; streptomycin, and spectinomycin.

regO Ω was cut out of the pBSKsII plasmid by PvuII, purified using the QIAquick gel extraction kit, and then sub-cloned into the suicide plasmid vector pSUP202 previously linearized by *Eco*RV. All constructs were verified by Sanger sequencing.

Mobilization of the new construct by tri-parental conjugation into Rsp. Rubrum

The recipient strain, *Rsp. rubrum*, was grown on liquid culture to log phase, and concentrated to obtain a density of (5.0×10^8) cells/ml. The donor *E. coli* XL1 and the

helper strains were grown separately, overnight, in liquid LB culture containing the appropriate antibiotics. OD660 of recipient, donor, and helper cultures were measured, mixed in an Eppendorf tube (ratio 1:1 in *E. coli* mating and 1:100 in *E. coli -Rsp. rubrum* mating). The mating mixtures were carefully suspended in 15–20 µl 0.9% sterile NaCl and spread onto a nitrocellulose filter paper on LB agar plate, incubated at 37 °C for 6 h. Mating mixtures were resuspended and diluted in a 0.9% NaCl solution, and plated onto M plates supplemented with appropriate antibiotics. Selected mutants, *Rsp. rubrum*, were selected following incubation anaerobically at 30 °C for 3–5 days.

Complementation analysis of regO gene

Complementation analysis of *Rsp. rubrum* regO mutants was carried out by cloning the wild-type regO gene into the broad-host-range vector pRK415 and introducing it into the regO disrupted mutant strain REGO^{-}.

Alignments and gene annotation

Database searches were performed using National Center for Biotechnology Information (NCBI) BLASTP (http://www.ncbi.nlm.nih.gov/BLAST.cgi), with *regO* from *Rsp. rubrum* ATCC11170 as a query from https://img.jgi.doe.gov/cgi-bin/m/main.cgi?section=GeneDetail&page=geneDetail&gene_oid=637827484. A protein sequence alignments were generated using Vector NTI–Advance-Version 11 software. Domain structure analysis was performed by SMART (http://smart.embl-heidelberg.de/smart/show_motifs.pl/). Protein interaction network was performed using STRING http://smart.embl-heidelberg.de/smart/show_motifs.pl .11 (https://version11.string-db.org/cgi/network.pl?taskId=kRRkIT0mYzV2) scan the attached QR code for more details.

Results

Gene annotation and alignment of regO

Sequence analysis of published genomes from several phototrophic bacteria (http://img.jgi.doe.gov/cgi-bin/

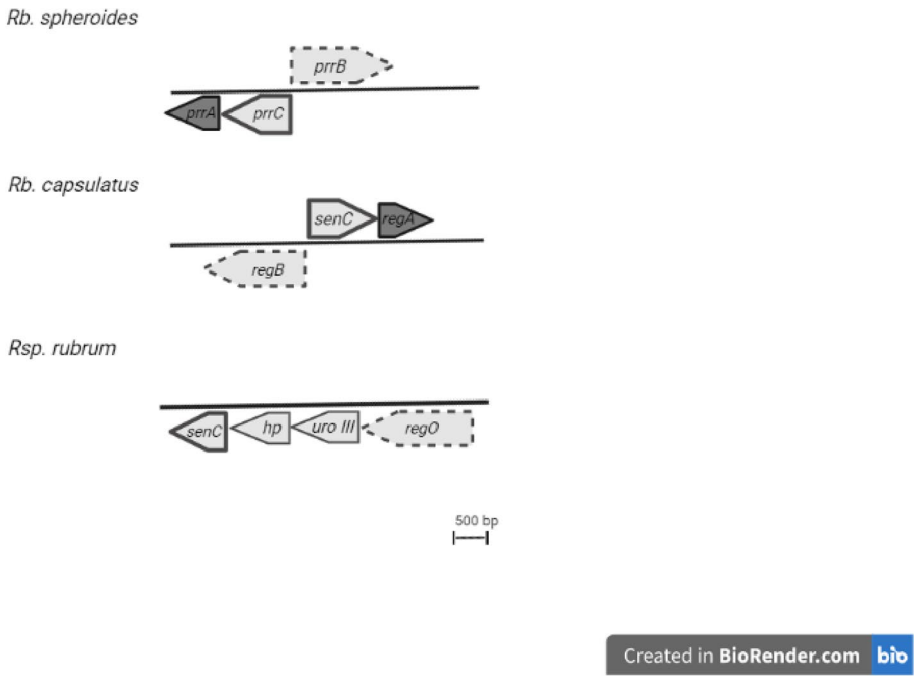

Fig. 1 Physical and genetic maps of the photosynthetic regulatory gene cluster in different *Rhodobacter* species compared to *Rhodosprillum*. ORFs and their directions of transcription are represented by open arrows. *Rba., Rhodobacter* capsulatus; *Rba., Rhodobacter sphaeroides*. (drawn by Biorender). Gene organization in the *regO* gene locus of *Rhodosprillum rubrum* (*Rsp. rubrum*); hp, hypothetical protein; uroIII, predicted uroporphyrinogenIII methyltransferase

pub/main.cgi) revealed that genes encoding two-component system proteins are frequently organised in a regulatory locus on the chromosome. In addition to the sensor kinase (e.g., regB in Rb. capsulatus or its homologue prrB in Rb sphaeroides) and its cognate response regulator (regA or prrA), this regulatory locus also carries a third gene, senC (or prrC) with strong homology to scoI (Smith et al. 1996), (Happ et al. 2005) encoding inner mitochondrial membrane protein (Fig. 1). Despite their conservation in all bacterial species studied so far, a search of the published genome of *Rsp. rubrum* for homologues of both regulatory (reg/prr) genes in the Integrated Microbial Genome database (http://img.jgi.doe.gov/cgi.bin/pub/main.cgi) returned no results. Apparently, *Rsp. rubrum* ATCC11170 has no sequences coding for PrrA/RegA or PrrB/RegB proteins (Zeilstra-Ryalls 2009). However, the third gene of the abovementioned regulatory locus, senC (prrC), seems to be conserved in *Rsp. rubrum* and possesses strong homology to its Rhodobacter homologues. Since the physical organisation of the kinase and regulator genes is often maintained in two-component systems (Nijhoff et al. 1984; Stock et al. 1989), we reasoned that, at least, a regB-homologue might be adjacent to senC. Indeed, sequence analysis of the upstream region of *Rsp. rubrum* senC-homologue indicated the presence of an open reading frame (orf515, Gene ID: 637827484), which is predicted to encode a PAS/PAC sensor kinase (Fig. 2). Whereas regB from other purple bacterial species formed a divergent transcriptional unit relative to regA and senC (Masuda et al. 1999), orf515 is flanked by two hypothetical proteins and transcribes in the same orientation as senC (Fig. 1). One striking observation is the presence, in the same locus, of four other open reading frames that have been annotated as being involved in the cobalamin biosynthetic pathway. Those ORFs included a uroporphoryinogen III methyltransferase, known to be involved in the early stages of the general tetrapyrrole biosynthetic pathway. Local alignment of either the nucleotide- or the protein sequences of orf515 (hereafter designated regO) against regB, and prrB showed that regO shares no significant sequence similarity (13% of similarity with Rb sphaeroides and 11% of similarity with Rb capsuatus) with either genes.

A BLAST similarity search for RegO homologues in the NCBI database returned more than one hundred proteins that share significant similarity with the query (regO); none of them belonged to either Rb. capsulatus or Rb. sphaeroides. The alignment of RegO with the top ten protein homologies obtained from the BLAST search is shown in Table 3.

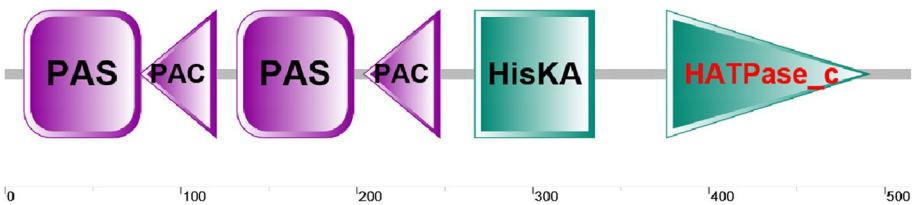

Fig. 2 Domain structure of RegO generated by SMART (http://smart.ambl-heidelberg.de/). Multi-domain hybrid structure of RegO in *Rsp. rubrum*. RegO possesses two PAS domains, a histidine kinase (HisKA) domain and HATPase domain

Table 3 Amino acid lengths, percent identities, and similarities of the proteins with highest homology to RegO and their respective *E* values, as obtained from the BLAST search

Source	Length	Identity %	Similarity %	*E* value
Geobacter sp. (strain FRC-32)	737	38	57	4.0×10^{-62}
Methanosarcina acetivorans C2A	1456	31	51	2.0×10^{-59}
Magnetospirillum magnetotacticum MS-1	380	39	54	6.0×10^{-58}
Magnetospirillum magneticum AMB-1	626	36	54	6.0×10^{-58}
Magnetospirillum gryphiswaldense MSR-1	730	33	49	1.0×10^{-57}
Candidatus Kuenenia stuttgartiensis	494	33	49	4.0×10^{-57}
Magnetospirillum magneticum AMB-1	368	40	53	3.0×10^{-56}
Nitrosococcus oceani ATCC 19707	368	36	54	6.0×10^{-56}
Magnetospirillum magnetotacticum MS-1	625	37	55	1.0×10^{-55}
Candidatus Methanosphaerula palustris	640	45	60	3.0×10^{-55}

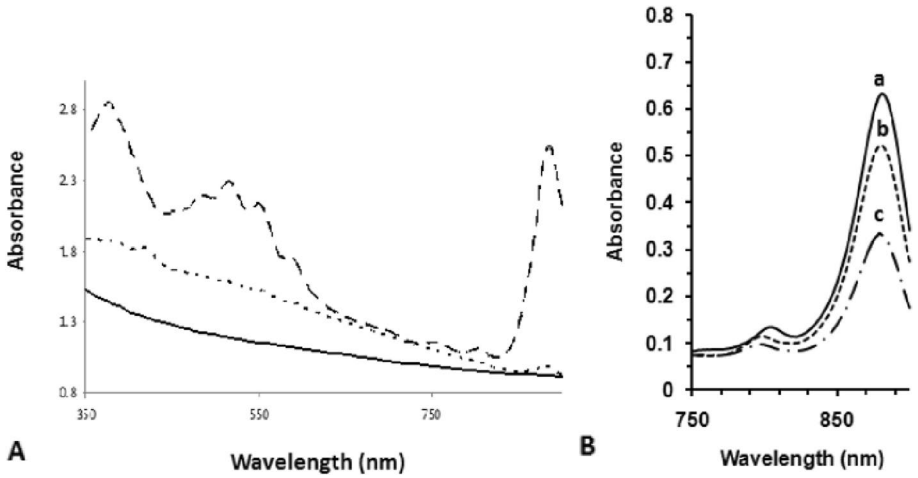

Fig. 3 Absorption spectra of whole cultures of *Rsp. Rubrum*. **A** Absorption spectra of whole cultures of the wild-type strain of *Rsp. rubrum* grown phototrophically anaerobically (dashed line), chemophototrophically, grown aerobically (no photosynthetic membranes) (dotted line) and the mutant strain *Rsp. rubrum* REGO$^-$ (solid line) grown anaerobically in the dark at 30 °C in presence of DMSO. **B** (a) Absorption spectrum of whole culture of wild-type strain of *Rsp. rubrum* grown phototrophically. (b) Absorption spectrum of *Rsp. rubrum* of the complementation strpRK-REGO grown phototrophically is also shown. (c) Wild-type *Rsp. rubrum* grown chemophototrophically in presence of DMSO as an external electron acceptor

Table 4 Predicted domains, repeats, motifs and features, generated using SMART

Name	Begin	End	*E* value
PAS	11	77	1.88e−06
PAC	77	120	1.80e−03
PAS	132	198	1.12e−04
PAC	204	247	1.04e+01
HisKA	267	335	5.28e−04
HATPase_c	376	491	1.02e−28

Phenotype of regO-disrupted mutant Rsp. rubrum REGO$^-$

A RegO interrupted mutant was constructed as described before. The mutant strain was unable to grow photosynthetically, i.e., became PS$^-$ at any light intensity. The mutant strain showed reduced synthesis of photosynthetic pigments compared to those of the wild type, when grown anaerobically in the dark in the presence of dimethyl sulfoxide (DMSO) as an external electron acceptor (Fig. 3A). Compared to the wild type, levels of A_{880} were dramatically reduced. In *Rsp. rubrum*, A_{880} value is routinely used as a simple reliable screen for variations in levels of spectral complex-synthesis of the light harvesting system (LH1) under all growth conditions (Ghosh et al. 1994).

Complementation analysis of regO

DNA fragments containing wild-type *regO* were cloned into broad-host-range vector pRK415 and introduced into the *Rsp. rubrum regO* disrupted mutant strain REGO$^-$. Transconjuants *Rsp. rubrum* containing the plasmid pRK-REGO, complemented the chromosomal disruption of *regO* see (Fig. 3B).

Domain structure analysis and hydropathicity of RegO

Analysis of the domain structure of RegO using the BLAST engine revealed that the deduced amino acid sequence possesses multiple domains: two PAS domains at the C-terminus, a histidine kinase (HisKA) domain, and a HATPase domain at the N-terminus (Fig. 2) (Table 4).

PAS domains are known as signalling modules that monitor changes in light, redox potential, oxygen, small ligands, and the overall energy level of a cell (see "Introduction" section). The histidine kinase domain includes a histidine-containing block, which is known to be the site of autophosphrylation in several two-component regulatory systems. Unlike many other sensor modules, PAS domains are located in the cytosol (Taylor and Zhulin 1999). A Kyte and Dolittle hydropathy plot (Fig. 4) predicts the lack of membrane-spanning domains over the entire length of RegO. The same prediction was reached when other hydropathy parameters were used (e.g., using TMIM).

Protein interaction network analysis

A protein-interaction network analysis was generated using the STRING 11 freeware (http://string-db.org/) engine employing a medium confidence scale. The retrieved

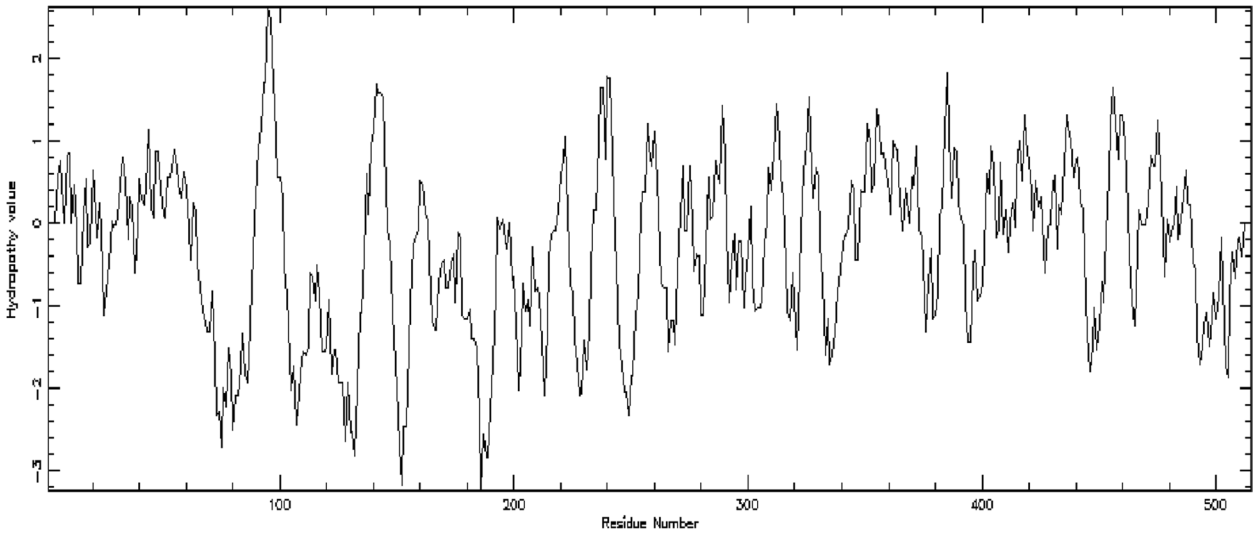

Fig. 4 Hydropathy plot of RegO, using Kyte and Dolittle hydropathy parameters. Showing that RegO is a cytoplasmic not a membrane bound sensor his Kinase

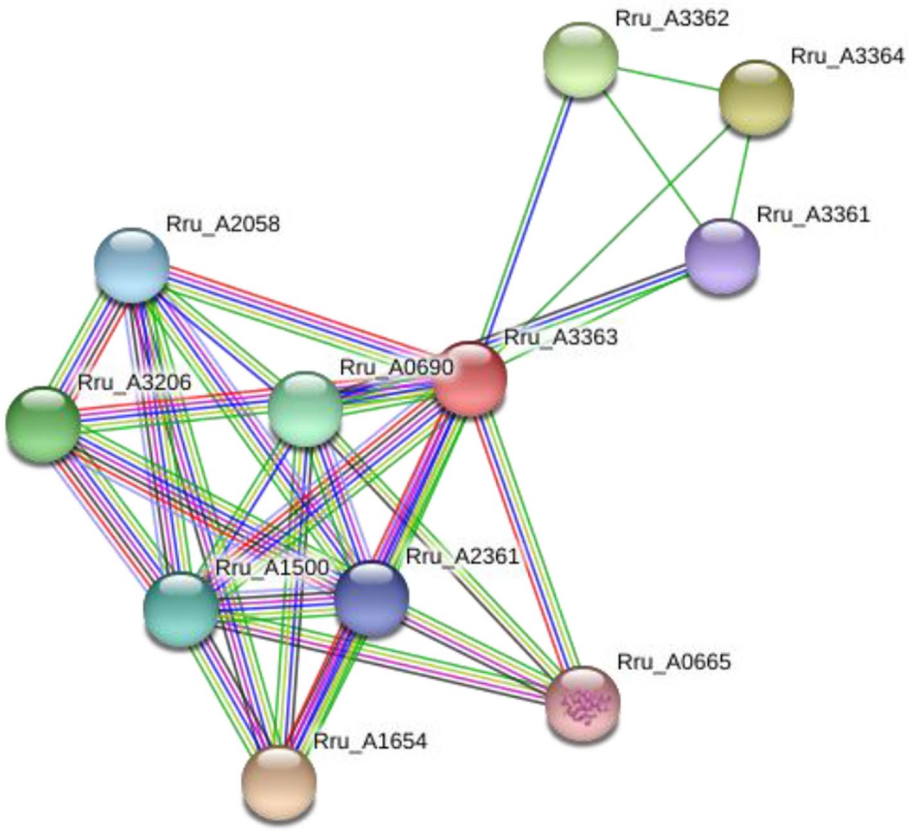

Fig. 5 Protein-interaction network display, showing possible cognate functional partner proteins of RegO. Generated by STRING V.11 https://version11.string-db.org/cgi/network.pl?taskId=n9ljuDp6PKvU scan the attached QR code (designated Rru_A3363 in the figure). Description and designations of the predicted functional proteins are listed in Table 5

Table 5 Predicted functional partners of RegO as produced by STRING (version 11). (Scanning the attached QR code directs to the online analysis page)

Rru_A1654	Response regulator receiver domain protein (CheY) (139 aa)
Rru_A3364	Putative uncharacterized protein (409 aa)
Rru_A0665	Response regulator receiver domain protein (CheY) (148 aa)
Rru_A3362	Uroporphyrin-III C-methyltransferase-like (291 aa)
Rru_A3361	Putative uncharacterized protein (255 aa)
Rru_A3206	Response regulator receiver domain protein (CheY) (126 aa)
lepA	Small GTP-binding protein domain (621 aa)
Rru_A3360	Electron transport protein SCO1/SenC (210 aa)
Rru_A3359	Cobyrinate a,c-diamide synthase/hydrogenobyrinic acid a,c-diamide synthase (Glutamine-hydroly (460 aa)
Rru_A3366	Hydrogenobyrinic acid a,c-diamide cobaltochelatase (1240 aa)

results are shown in (Fig. 5) and the predicted functional partners are listed in Table 5. Screening of the *Rsp. rubrum* proteome for potential interaction partners predicts that three CheY chemotaxis response regulators are strong candidates for direct interaction with RegO. Other interesting predictions include potential interactions with the cobalamin biosynthetic genes and the *scoI* homologue *senC*, all existing in the vicinity of *regO*.

Discussion

In this study, a novel regulatory gene, regO, is described and shown to be clearly involved in the positive regulation of photosynthesis gene expression in response to removal of oxygen from cultures of the wild-type strain *Rsp. rubrum* S1. Disruption of regO by interposon mutagenesis produced the mutant strain *Rsp. rubrum* REGO$^-$, which is unable to grow phototrophically at low light intensities and could only be grown either anaerobically in the dark in the presence of DMSO (an external electron acceptor) or aerobically in the dark, albeit very slowly. Under the microscope, the mutant strain possessed elongated dark cells, reminiscent of fts mutations in bacterial cell division machinery proteins (fts = filamentous temperature sensitive). Sequence comparisons indicated that regO is another member of the sensor kinase family of prokaryotic sensory transduction factors known to regulate a number of diverse cellular processes. In this respect, regO seems to be a functional homologue of regB in Rb. capsulatus and prrB in Rb. sphaeroides, which were identified earlier (Mosley et al. 1995; Eraso and Kaplan 1994). Interestingly, each of the three members of the histidine kinase family is physically adjacent to another gene, designated senC in both Rsp. rubrum and Rb. capsulatus and prrC in Rb. sphaeroides. The senC gene bears strong similarity to a family of genes containing a putative membrane-spanning domain, among which are the sco1 and sco2 genes from S. cerevisiae. In the latter organism, these genes are believed to play a role in cytochrome c oxidase subunit assembly in the mitochondria. However, in anoxygenic phototrophic bacteria, senC is located upstream of the two-component RegB/A activation system but downstream of the cbb3 cytochrome c oxidase. It was suggested that SenC functions as a transducer of the oxygen and therefore the redox signal from Cbb3 to RegB (Eraso and Kaplan 1994). Despite the predicted functional similarity between RegB, PrrB, and RegO, the latter shows poor sequence homology with the former two proteins. Indeed, homology searches using either the nucleotide or amino acid sequences of RegB/PrrB, showed the absence of homologous genes and/or proteins in *Rsp. rubrum*. In addition to the sensor kinase, the second component of this two-component signal transduction system is the response regulator. For the RegB/PrrB sensor proteins, the cognate response regulators RegA/PrrA were identified in Rb. capsulatus and Rb. sphaeroides, respectively. Both components form two divergent transcriptional units in most phototrophic bacterial species investigated (see Fig. 1). In *Rsp. rubrum*, none of the regO neighboring genes is predicted to encode a candidate response regulator. The general features of the system, however, seem to be maintained in *Rsp. rubrum*: the transduction of an environmental signal, such as anaerobiosis, involves a phosphorylation cascade. This signal is initiated when the sensor kinase undergoes autophosphorylation at a conserved histidine residue, forming a high-energy state phosphate that can subsequently be transferred to a conserved aspartate residue on the cognate response regulator. The highly conserved histidine residue (H277) in RegO suggests that this protein maintains the phosphorylation feature of other sensor kinases. Although not investigated in this study, many sensor kinases also exhibit phosphatase activity with the capability of removing phosphate. This activity has been shown to be affected by the extent of the gene disruption during the construction of mutant strains. Thus, Mosley et al. (Mosley et al. 1995) found that a RegB mutant with a simple point mutation has a significantly lower level of aerobic expression than does a putative knockout mutation in RegB. They explained that the first mutant has possibly lost the kinase activity but retained the phosphatase activity, whereas the second mutant has lost both the kinase and phosphatase activities. Similarly, dephosphorylation has been shown to play a role in the oxygen regulation of expression regulated by FixL in Rhizobium meliloti (Lois et al. 1993). Although a RegA response-regulator-homologue was not identified

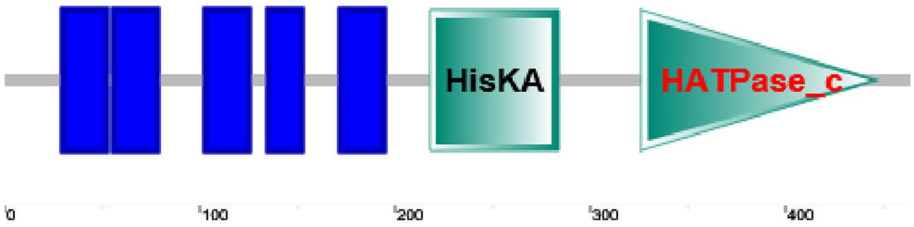

Fig. 6 Domain structure of RegB in *Rhodobacter capsulatus*, generated by SMART (http://smart.embl-heidelberg.de/smart/show_motifs.pl). RegB has a histidin kinase (HisKA) domain and HATPase_c domain. No PAS domains

in this study, a network analysis using the programme STRING 8.2 predicted two potential CheY-like proteins as possible candidates (Fig. 5). CheY is known as the response regulator of the chemotaxis sensory protein CheA (Thakor et al. 2011; Hirschman et al. 2001). The predicted cytosolic nature of RegO (see "Results" section) suggests a possible crosstalk between RegO and other response regulators that are likely to obtain the phosphate from RegO. Screening of the genome of *Rsp. rubrum* for potential response regulators revealed the presence of more than 200 potential proteins possessing DNA binding domains, characteristic for this type of interaction (data not shown). As described in Results, amino acid sequence analysis of RegO using BLAST, SMART, or Prosite engines predicted two Per-Arnt-Sim (PAS) domains at the N-terminus, a middle histidine kinase domain, and a histidine ATP kinase at the C-terminus (Fig. 2) (Table 4). Each of the predicted PAS domains has a photoactivated adenylyl cyclase (PAC) at its C-terminus. The latter motifs are suggested to contribute to the PAS fold (Taylor and Zhulin 1999). One major difference between RegO and other sensor kinases that respond to changes in oxygen levels, e.g., the RegB (PrrB) system in most purple bacterial species investigated (Masuda et al. 1999; Sebastien et al. 2010), and FixL in Rhizobium meliloti and ArcB in *E. coli*, is that these kinases lack the N-terminus PAS/PAC domain (Fig. 6). Instead, these proteins exhibit membrane-spanning domains. However, mutational studies show that apparently the membrane-spanning and periplasmic regions of these kinases do not function as receptors but act as anchors to the membrane, which are required for optimal in vivo activity (Lois et al. 1993). On the other hand, FixL was found to interact directly with oxygen via a cytoplasmic heme moiety (Gilles-Gonzalez et al. 1991). However, FixL proteins from Bradyrhizobium japonicum (Anthamatten and Hennecke 1991; Lois et al. 1993) do not appear to have any transmembrane region and apparently are soluble cytoplasmic proteins. Both oxygen sensing and kinase activities appear to be similar in membrane-bound and soluble FixL proteins (Gilles-Gonzalez et al. 1991; Gong et al. 1998). ArcB, was shown to detect changes in aerobic or anaerobic culture conditions by monitoring cell redox poise, through direct interaction with the cytoplasmically localized respiratory chain (Jung et al. 2008). A Kyte-Doolittle hydropathy plot of the deduced RegO polypeptide sequence revealed the lack of any putative transmembrane domains, and therefore RegO appears to be a cytosolic protein (Fig. 4).

The presence of several tetrapyrrole-encoding genes near regO encourages us to hypothesize that a heme group is a possible "sensing" ligand of the predicted PAS domains. Further, both PAS-domain duplication and the predicted interaction with multiple response regulators suggest that the described cytosolic sensor RegO might respond to multiple signals.

Conflict of interest
The authors declare no conflict of interest

Authors' contributions
Conceptualization: K. A. Methodology: K.A. Investigation: M. M. Writing original draft: M.M. Writing review and editing: K. A. Supervision: K.A. All authors read and approved the final manuscript.

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Declarations

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Not applicable

Consent for publication
Not applicable

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