The *phrA* gene of *Rhodobacter sphaeroides* encodes a photolyase and is regulated by singlet oxygen and peroxide in a σ^E -dependent manner

Anne-Kathrin Hendrischk, Stephan Braatsch,[†] Jens Glaeser and Gabriele Klug

Correspondence

Gabriele Klug

Gabriele.Klug@mikro.bio.

uni-giessen.de

Institut für Mikrobiologie und Molekularbiologie, University of Giessen, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany

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The genome of the facultatively photosynthetic bacterium *Rhodobacter sphaeroides* encodes three proteins of the photolyase/cryptochrome family. This paper shows that *phrA* (RSP2143) encodes a functional photolyase, which is an enzyme that repairs UV radiation-induced DNA damage in a blue light dependent manner. Expression of *phrA* is upregulated in response to light, with no photoreceptor or the photosynthetic electron transport being involved. The results reveal that singlet oxygen and hydrogen peroxide dependent signals are transmitted by the σ^E factor and the anti- σ^E factor ChrR affecting *phrA* expression, while superoxide anions do not stimulate *phrA* expression. Thus, the σ^E regulon is involved not only in the response to singlet oxygen but also in the hydrogen peroxide response.

INTRODUCTION

All organisms that are exposed to sunlight face the problem of UV light-induced DNA damage. The major UV products are cyclobutane pyrimidine dimers (CPDs), and to a lesser extent, pyrimidine-pyrimidone (6-4) photoproducts (reviewed by Sancar, 2003). Their accumulation blocks DNA replication and transcription and can therefore be lethal to cells. DNA photolyases repair these photoproducts in a blue/near-UV light dependent reaction referred to as photoreactivation (Rupert *et al.*, 1958). Photolyases are closely related to cryptochromes, which belong to the same protein family (Cashmore *et al.*, 1999).

Cryptochromes lack the ability for photoreactivation, but are instead known to function as blue light photoreceptors in diverse organisms by interacting with proteins such as COP1 and clock proteins (Yang *et al.*, 2001; Lin & Shalitin, 2003; Sancar, 2003). Plant cryptochromes mediate e.g. stem elongation and photoperiodic flowering in response to light (reviewed by Lin & Shalitin, 2003). Cryptochromes in animals are light dependent and light independent regulators of the circadian clock (reviewed by Sancar, 2003). Almost all eukaryotic cryptochromes possess C-terminal domains beyond the photolyase-homology region while

prokaryotic cryptochromes lack these C-terminal extensions (reviewed by Partch & Sancar, 2005; Fig. 1). The recently identified cryptochrome *Drosophila*, *Arabidopsis*, *Synechocystis*, Human (cryptochrome DASH) proteins lack the C-terminal extension. This group was found to specifically repair CPDs in single-stranded DNA (Selby & Sancar, 2006) and its members are therefore considered to be photolyases.

Proteins of the photolyase/cryptochrome family contain two noncovalently attached chromophores. The first, reduced FAD, is the catalytic cofactor in photoreactivation. The second cofactor, which serves as photoantenna, is either methenyltetrahydrofolate, 8-hydroxy-7,8-didemethyl-5-deazariboflavin (8-HDF), flavin mononucleotide (FMN) or FAD (reviewed by Sancar, 2003; Ueda *et al.*, 2005; Fujihashi *et al.*, 2007).

Anoxygenic photosynthetic proteobacteria of the genus *Rhodobacter* are metabolically extremely versatile. *R. sphaeroides* uses light for anoxygenic photosynthesis but can also perform aerobic or anaerobic respiration. As a free-living aquatic bacterium, *R. sphaeroides* is exposed to sunlight in its natural habitat (Page *et al.*, 2004). However, solar radiation of high intensity is harmful due to the formation of DNA photoproducts. In the presence of oxygen and bacteriochlorophyll, it generates highly reactive singlet oxygen, which causes photooxidative damage (Borland *et al.*, 1987). To minimize the risk of photooxidative damage, *Rhodobacter* has evolved complex regulatory circuits that control the expression of photosynthesis genes according to environmental conditions. Under high

[†]Present address: Department of Microbiology and Immunology, University of British Columbia Life Sciences Centre, 4557-2350 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada.

Abbreviations: CPD, cyclobutane pyrimidine dimer; DASH, *Drosophila*, *Arabidopsis*, *Synechocystis*, Human; HDF, 8-hydroxy-7,8-didemethyl-5-deazariboflavin.

Sco. Phr	--MAAPILFWHRRDLRLSDN	IGIAAARQSAQLIGLCFLD	HH H	H H H	YLOGCLOELQORYQOAGSRL	LLQGDPQHLIQLAQQLQA	(93)	
R.s. PhrA	MMADAPLILWFRDLRLADN	PMIAEAATGRPLIPFLILD	PE---	TEALGAAPKW	RLGLGVEAFARAEGLSRL	VLRGPPALAVLEALVAETGA	(92)	
R.s. 1981	---MNVLIWFRDLRVADH	PAL--ARAADDRLPLAIVD	PAEWADPARSGRGWA	---	FRAECLADLRADLAALGAPL	AVRVGDAQAVLSKLCCKLHRI	(89)	
R.s. 3077	---VTRILLVLGDLSDDL	PALRAADPAADLVMAEVM	EGTYVPHHPQKIA	---	LILAAMRKFAARRLOERGFRV	AYSRLDDPDTGSIGAEILLR	(89)	
Scy. Cry	-----	-----MYCYD	PRQFAQTHQGFATGTGWRSN	---	FLQQSVQNLAESLQKVGKLI	LVTTGCPQEOVIQIAKQINA	(65)	
Sco. Phr	EAIVWNQDIEPYGRDRDQGV	AAALKTAGIRAVQLWDQLH	SPDQ--ILSGSGNPYSVYGP	FWKNWQAO-----	PKP	TPVATPTLVDLSPEQLTAI	(182)	
R.s. PhrA	AGVHWSRLEWPDWRARDEGV	KAGLRQAGIEASHAGHTIF	EPRE--VETGCGGFYRVYTP	FWKAVKDR-----	PVA	ASSPPPARL--LSPAEPVVS	(179)	
R.s. 1981	GRILCLEL--PAFAELDLAL	QAWAGGTGLDF-----	---	IRLK	---	PGA	GAPAAITPLPAVEGPIPPA	(145)
R.s. 3077	RAAETGAREAVATRPQDWRL	IEALEAMPFPVRFLPDDRFL	CPADEFAFWTEGRKQLRMWF	TYREMRRTTGLMEGDEBAG	---	GKWNFTDENRKFAAPDLLRP	(189)	
Scy. Cry	KTIYYHREVTQEELDVERNL	VKQITILGIEAKGYWGSTLC	HEED--LPFSIQDLPLDFTK	---	ERKDIKK-----	KIS	IRPCFFAPS-QULESPNIKLI	(153)
Sco. Phr	APILLSELPTLKQLGFDWDG	GFPVEFGETAIAIRLOEFCF	RA--IADYDPORN-FPAEAG	TSGLS--PAIKFGAIGIRQA	WQASAHAHLSRSDEARNSI	---	(277)	
R.s. PhrA	ERLACWDLGRAMHRGAHVVA	PH-LAVGEAAAERLTFRFLS	GP--IDRYAERD-RPDAPV	TSRLS--ENITYGEISARS	WHAGMRARA-----	EGRAGA	(268)	
R.s. 1981	RAIRLAE-----DRC	PHRPGGRPGQGLLOSFL	RRGALYRDGRNRL-LQAERA	SSRLS--PHLAAGTISREEV	LAQVAARLAERPDADWVRL	---	(232)	
R.s. 3077	RPLRFEPDAEVRVLDLVEA	RFPFRHFGRLRPFWATDRAE	ALRSLDHFIRESLPRFGDEQ	DAMLADDFLSHALLSSSMN	LGLLGPMEV---	CRRATEW	(286)	
Scy. Cry	E-TATAPPEFFPQINFDRHS	VLAFOGGGTAGLARLDQYFW	HGDRKDKYKETRNGMGVADY	SKFES--PWALGCLSPRFI	YQEVKRYEQ---	ERVSNDST	(247)	
Sco. Phr	RVWQOELAWREFFQHALYHF	BSLADGYPYRSLWQOFPENR	---EALFTANTQAOTGYEI	VDAAMRQLTETGWMHNRCEM	IVASFITKDLILDRGEQF	---	(373)	
R.s. PhrA	EKFLQELAWREFFQHALYHT	PEIARRNWRGDWDAFPRGD	---NPDAERWRRTGTEGPF	VDAAMRELFTVTGMHNRRL	IAGSYLTKHLLTDRVQAW	---	(364)	
R.s. 1981	GAFAALASGAPARGAVCP	EGRPGGGSPRGYLDOTEGE	ADALRLRALHAGETGLRF	VDAALRAMAASGWLPARLG	MLASVALHHLGLSEQAAGAA	---	(332)	
R.s. 3077	REGRAPLNAVEGFIRQILGW	REYVRGIWTLSPGDPYIRSNG	LGHSAALPLYWGKPTRMAC	LSAAVAQTRDLAYAHHIQRL	MVTGNFALLAGVPAEVHEW	---	(386)	
Scy. Cry	HWLIFELLWRDFFRFVQKY	GKFLNRGGLLNKNFQOED	---QVRFELWRSGOTGYEL	VDAAMRELNLGFMNSNRGQ	NVASFTCKNLGIDRWGAEW	---	(343)	
Sco. Phr	FMQHLVDGLAANNGGQOWS	ASSGMDPKP-LRIFNE	---	---	---	---	(408)	
R.s. PhrA	FEDCLIDWPASNALGQOWV	AGSGPDASPYFRIENPATQA	EKF--DEESAMRQRFIAEIR	RPGPEALA--FFEAVPRSW	GLRPDRCYPRPVVGLAEGRE	---	(460)	
R.s. 1981	LAAGLTDHSPPIHWAQMRV	ALG-----RPVDERLA	GVL--DEGGAFTRRWLPELAE	VPDRVLQAPWRWEGARG---	--LLGRRYEELVDPSAARA	---	(418)	
R.s. 3077	YLSVYID--ALEWVEAPNT	IGMSQFADHGLLGSKPYVSS	GAYIDRMSDRCGCAYAVKD	RTGPRA--CFENLLYWHFLNR	HRARFERNR--MVQMYRTWD	---	(481)	
Scy. Cry	FESCLIDYVCSNWNQNYT	AGIGNDARD--FRYENIFKQS	QYQ--DEPGTLYRHLWELKN	LPQDKIHQWLLSATEQKQW	GVQLGVDPYPRPCVNFHQVSE	---	(441)	
Sco. Phr	-----	-----	---	---	---	---	(408)	
R.s. PhrA	RALAAYGRNN-----	---	---	---	---	---	(471)	
R.s. 1981	AGRTL--ARRPTARVEVIEG	AAPRRPASDPTQQLCLDL--	---	---	---	---	(454)	
R.s. 3077	RMEETHRARVLTAEAFGLR	LHAGEPV-----	---	---	---	---	(508)	
Scy. Cry	-----ARRKIQMGVIA-	---	---	---	---	---	(453)	

Fig. 1. Sequence alignment of proteins of the photolyase/cryptochrome family. Identical amino acids are boxed in grey. Amino acids which bind the cofactors FAD (F) and 8-HDF (H) are derived from the crystallographic structure of *Synechococcus* sp. 6301 (Tamada *et al.*, 1997; top line) and *Synechocystis* sp. PCC6803 (Brudler *et al.*, 2003; bottom line). The black boxed tryptophans are believed to be involved in intraprotein electron transport (Byrdin *et al.*, 2003). The lengths of the proteins are indicated in parentheses. Sco. Phr, photolyase of *Synechococcus* sp. 6301 (Tamada *et al.*, 1997); R.s. PhrA, photolyase of *R. sphaeroides*; R.s. 1981, RSP1981 gene product of *R. sphaeroides*; R.s. 3077, RSP3077 gene product of *R. sphaeroides*; Scy. Cry, cryptochrome DASH of *Synechocystis* sp. PCC6803 (Brudler *et al.*, 2003). Comparison was carried out using MultAlin software (<http://prodes.toulouse.inra.fr/multalin/multalin.html>).

oxygen tension, in either the light or dark, the bacterium is virtually unpigmented and performs aerobic respiration. Only when the oxygen tension decreases is the photosynthetic apparatus formed (Zeilstra-Ryalls *et al.*, 1998). When *R. sphaeroides* grows in the absence of oxygen by anaerobic respiration, light has a stimulating effect on the expression of photosynthesis genes (Braatsch *et al.*, 2002). Recent investigations on the protection of *R. sphaeroides* against photooxidative stress showed the involvement of σ^E (RSP1092) and the anti- σ^E factor ChrR (RSP1093) in this response (Anthony *et al.*, 2005; Glaeser & Klug, 2005). σ^E is a member of the extracytoplasmatic function (ECF) family of σ factors. ChrR belongs to a new family of zinc-containing anti- σ factors (Newman *et al.*, 2001) and inhibits the ability of σ^E to form a stable complex with core RNA polymerase by the formation of a σ^E :ChrR complex (Anthony *et al.*, 2004).

The genome of *R. sphaeroides* encodes three proteins, the products of RSP1981, RSP2143 and RSP3077, with similarity to photolyases and cryptochromes. Here we show that RSP2143, putatively named *phrA* (Braatsch *et al.*, 2004), encodes a functional photolyase. RSP3077 also seems to act as a photolyase, while RSP1981 has no function in photoreactivation. Furthermore, analysis of the

expression of *phrA* under different growth conditions revealed its regulation by the σ^E /ChrR system.

METHODS

Bacterial strains and growth conditions. All strains used in this study are listed in Table 1. *Escherichia coli* strains were cultured in Luria-Bertani broth at 37 °C with continuous shaking at 180 r.p.m. *Rhodospirillum rubrum* strains were cultivated at 32 °C in a malate minimal salt medium (Drews, 1983) with continuous shaking at 140 r.p.m.

For semi-aerobic growth of *R. sphaeroides* strains, oxygen concentration in the culture flasks was 104 ± 24 μ M throughout the experiment. Oxygen tension was monitored with a Pt/Ag electrode (microoxygen sensor 501, UMS, Meiningen, Germany) and adjusted by varying the rotation speed of the shaker. Overnight grown cells were adapted to these conditions for one doubling time before illumination and experiments started when cultures had an OD₆₆₀ of 0.2. Blue light in semi-aerobically grown cultures was obtained by passing light from a 500 W halogen lamp or a high-pressure mercury-arc lamp (Schoeffel Instruments Co.) through a combination of heat-absorbing filter KG1 and filter BG12 (Schott). The maximum transmission of this filter is at 400 nm with a half-band width ≤ 80 nm and no detectable transmission at <317 or >515 nm. Photon flux density of each light quality measured at culture level was 10 μ mol photons $m^{-2} s^{-1}$ (3 W m^{-2}) or at least 60 μ mol photons $m^{-2} s^{-1}$ (60 W m^{-2}), respectively.

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics*	Source/reference
<i>E. coli</i>		
JM109	Host strain for plasmid constructions	New England Biolabs
MC1061	Host strain for plasmid constructions, Km ^r Sm ^r	Casadaban <i>et al.</i> (1980)
S17-1	Host strain for plasmid constructions, Sp ^r	Simon <i>et al.</i> (1983)
HB101	Host strain for pRK2013	Ditta <i>et al.</i> (1985)
<i>R. sphaeroides</i>		
2.4.1	Wild-type	van Niel (1944)
TF18	<i>rpoEchrR</i> mutation in 2.4.1, Tp ^r	Schilke & Donohue (1995)
ΔChrR	<i>chrR</i> mutation in 2.4.1, Tp ^r	Newman <i>et al.</i> (1999)
TC67	Tn5 insertion into <i>crtI</i> , Km ^r	Lang <i>et al.</i> (1995)
PUHA1	<i>puhA</i> mutation in 2.4.1, Km ^r	Sockett <i>et al.</i> (1989)
Δ <i>phrA</i>	2.4.1 with disruption of <i>phrA</i> , Km ^r	This study
Δ <i>phrA</i> (pRK <i>phrA</i>)	Transconjugant of Δ <i>phrA</i> with pRK <i>phrA</i> , Km ^r Tc ^r	This study
Δ1981	2.4.1 with disruption of RSP1981, Km ^r	This study
Δ3077	2.4.1 with disruption of RSP3077, Km ^r	This study
2.4.1 (pPHU <i>phrAlacZ</i>)	2.4.1 harbouring pPHU <i>phrAlacZ</i>	This study
TF18 (pPHU <i>phrAlacZ</i>)	TF18 harbouring pPHU <i>phrAlacZ</i>	This study
ΔChrR (pPHU <i>phrAlacZ</i>)	ΔChrR harbouring pPHU <i>phrAlacZ</i>	This study
TC67 (pPHU <i>phrAlacZ</i>)	TC67 harbouring pPHU <i>phrAlacZ</i>	This study
PUHA1(pPHU <i>phrAlacZ</i>)	PUHA1 harbouring pPHU <i>phrAlacZ</i>	This study
Plasmids		
pPHU281	Suicide vector for <i>Rhodobacter</i> , Tc ^r	Hübner <i>et al.</i> (1993)
pUC4K	Vector containing Km cassette	Vieira & Messing (1982)
pRK415	Shuttle vector for expression in <i>Rhodobacter</i> , Tc ^r	Keen <i>et al.</i> (1988)
pRK2013	Mobilizing vector, Km ^r	Ditta <i>et al.</i> (1985)
pPHU234	Vector with promoterless <i>lacZ</i> coding region, Tc ^r	Hübner <i>et al.</i> (1991)
pPHUΔ <i>phrA</i>	pPHU281 with Km cassette, flanked by <i>phrA</i> up- and downstream region	This study
pPHUΔ1981	pPHU281 with Km cassette, flanked by RSP1981 up- and downstream region	This study
pPHUΔ3077	pPHU281 with Km cassette, flanked by RSP3077 up- and downstream region	This study
pRK <i>phrA</i>	pRK415 harbouring <i>phrA</i> coding region, Tc ^r	This study
pRK3077	pRK415 harbouring RSP3077 coding region, Tc ^r	This study
pPHU <i>phrAlacZ</i>	pPHU234 with <i>phrA</i> upstream-region, Tc ^r	This study

*Km^r, kanamycin resistant; Sm^r, streptomycin resistant; Sp^r, spectinomycin resistant; Tc^r, tetracycline resistant; Tp^r, trimethoprim resistant.

For aerobic growth, overnight cultures with an OD₆₆₀ of 0.2 were transferred to a flask and gassed with air, resulting in an oxygen concentration of approximately 180 μM. To test for the effect of illumination, cultures grown under dark aerobic conditions to an OD₆₆₀ of 0.4 were illuminated with a 500 W halogen lamp with a light intensity of 800 W m⁻² (750 μmol photons m⁻² s⁻¹; high intensity white light), or 800 W m⁻² (380 μmol photons m⁻² s⁻¹; high intensity red light) or 30 W m⁻² (18 μmol photons m⁻² s⁻¹; low intensity white light). A filter transmitting wavelength greater 600 nm was used for red light illuminations. Light energy in W m⁻² was measured with a LI-250 light metre equipped with a LI-200SA pyranometer sensor (spectral response of 400–1100 nm, LI-Cor) and photon flux in μmol photons m⁻² s⁻¹ was measured with an LI-189 light metre equipped with a LI-190SA quantum sensor (spectral response of 400–700 nm, LI-Cor).

When required, antibiotics were used at the following concentrations: kanamycin 25 μg ml⁻¹; tetracycline 20 μg ml⁻¹ (*E. coli*) or 2 μg ml⁻¹ (*R. sphaeroides*); trimethoprim 50 μg ml⁻¹ (*R. sphaeroides*). In the

presence of light, no tetracycline was added. For the generation of singlet oxygen, methylene blue (Sigma-Aldrich) was added to a final concentration of 0.2 μM. To test the effect of hydrogen peroxide (H₂O₂) and superoxide anions on *phrA* expression, H₂O₂ and paraquat were added to a final concentration of 1 mM to *R. sphaeroides* cultures with an OD₆₆₀ of 0.5.

Photoreactivation activity. Overnight cultures of *Rhodobacter* strains grown to an OD₆₆₀ of 0.8 were diluted 1 : 100 in malate minimal salt medium. After exposure to various doses of UV light at 254 nm (UVcross-linker 1800, Stratagene), the cells were plated on agar plates in a dark room with dim red light, and afterwards exposed to the light of a 58 W fluorescent lamp (TLD 58W/25, Philips). For photoreactivation, the plates were covered with the plastic cover of the Petri dish. Photon flux density measured at culture level was 35–40 μmol photons m⁻² s⁻¹ (LI-190SA quantum Sensor, LI-Cor; spectral response 400–700 nm). Control plates were wrapped in aluminium foil for growth in the dark. After 3 days, the colonies were counted and survivals relative to UV-unirradiated controls were calculated.

Genetic techniques. DNA cloning was performed according to standard protocols (Sambrook & Russell, 2001). All plasmids used are listed in Table 1. Oligonucleotides generating suitable recognition sites for cloning or used as primers for RT-PCR analyses were synthesized by Roth. DNA sequencing was performed with an ABI-Prism 310 genetic analyser (Applied Biosystems).

Construction of *R. sphaeroides* Δ *phrA*, Δ 1981 and Δ 3077. *R. sphaeroides* Δ *phrA*, Δ 1981 and Δ 3077 were generated by transferring plasmid pPHU Δ *phrA*, pPHU Δ 1981 or pPHU Δ 3077 into *R. sphaeroides*. Fragments (500 bp) of the 5' and 3' ends of the genes were amplified by PCR using the oligonucleotides *phrA*HindIII_{up} (5'-GGGAAGCTTCAGGCCGATCTCCCC-3'), *phrA*EcoRI_{up} (5'-GGAATTCGAAGATCGTATGGCCC-3'), *phrA*EcoRI_{down} (5'-GAA-TTCGTGACCGGCACCATG-3') and *phrA*XbaI_{down} (5'-GC-TCTAGACCGTCATCTCGTGTC-3') for *phrA*; 1981HindIII_{up} (5'-GGGAAGCTTCGCGACCTGCCTCG-3'), 1981ScaI_{up} (5'-CCG-GCCAGTACTGCAGG-3'), 1981ScaI_{down} (5'-GTGGATGCAGT-ACTGCGCG-3') and 1981XbaI_{down} (5'-CGTCTAGAAACATGA-CCAATCCGCC-3') for RSP1981 and 3077HindIII_{up} (5'-CCCA-AGCTTGGGCTCGACTGGAGCCGA-3'), 3077ScaI_{up} (5'-AAAA-GTACTTTTCGATCAGCCGCCAGTC-3'), 3077ScaI_{down} (5'-AAAAGTACTTTTCGCGCTGCTGGCGGG-3') and 3077XbaI_{down} (5'-GCTCTAGAGCTTCCGCCTCGGTCAGA-3') for RSP3077. The *Km^r* gene (Vieira & Messing, 1982), flanked by the corresponding fragments, was inserted in the suicide plasmid pPHU281 (Hübner *et al.*, 1993). The plasmids were transferred into *E. coli* S17-1 and each was then transferred into *R. sphaeroides* 2.4.1 by biparental conjugation. Southern blotting was performed of kanamycin-resistant, tetracycline-sensitive clones, to confirm that the target genes had been replaced by the inactive gene through double crossover.

Complementation of *R. sphaeroides* Δ *phrA* and Δ 3077. For complementation of *R. sphaeroides* Δ *phrA* or Δ 3077, the genes *phrA* or RSP3077 with around 100 bp of the flanking sequences on each end were generated with primers *phrA*HindIII_{up} and *phrA*XbaI_{down} or 3077HindIII_{up} and 3077XbaI_{down}, respectively. The fragments were ligated into plasmid pRK415, and the resulting plasmids pRK*phrA* and pRK3077 were transferred into the recipient strain by triparental mating as described earlier (Klug & Drews, 1984).

Construction of the *phrA* : *lacZ* reporter plasmid. The putative promoter region of *phrA* (−108 to +130 bp) was amplified with the oligonucleotides *phrA*AlaCZ_{up} (5'-GCGAATTCAGGCCGATCTCCC-CC-3') and *phrA*AlaCZ_{down} (5'-CGGTCTCGGGATCCAGAATGAAC-3') and ligated in the vector pPHU234, carrying the promoterless *lacZ* gene. The construct was conjugated into *R. sphaeroides* strains, as well as the plasmid pPHU234, as a control.

β -Galactosidase assay. This was carried out as described previously (Hübner *et al.*, 1991).

RNA extraction and real-time RT-PCR. Total RNA of *R. sphaeroides* cells grown under different conditions was isolated as described previously (Nieuwlandt *et al.*, 1995) and quantified by spectrophotometric analysis (absorbance at 260 nm). For quantitative real-time RT-PCR, 40 ng total RNA was used and the One-Step RT-PCR kit (Qiagen) and Reverse-iT One-Step RT-PCR Kit (ABgene), following the manufacturer's instructions. The amplification of PCR products [oligonucleotides *phrA*-A (5'-GTTTCGAGGACTGCCTGAT-3') and *phrA*-B (5'-AGGAACCTCTGGCGATAG-3') for *phrA*, *lacZ*-A (5'-CATTGAGGCTGCGCAACT-3') and *lacZ*-B (5'-TTAA-TCGCCTTGCAGCAC-3') for *lacZ* and *rpoZ*-A (5'-ATCGCGGAAG-CCAGAG-3') and *rpoZ*-B (5'-GAGCAGCGCCGATCCT-3') for *rpoZ*] was monitored with SYBR Green using a Rotor-Gene 3000 real-time PCR cyclor (LTF). Relative expression of *phrA* and *rpoZ*

(a housekeeping σ -factor) mRNA were calculated by the method of Pfaffl (2001).

RESULTS AND DISCUSSION

R. sphaeroides harbours three members of the photolyase/cryptochrome family

The *R. sphaeroides* 2.4.1 genome sequence (<http://mmg.uth.tmc.edu/sphaeroides/>) contains three ORFs (*phrA*, RSP1981 and RSP3077) encoding proteins with similarity to photolyase/cryptochrome proteins. According to DNA microarray studies, all three genes are transcribed (our unpublished microarray chip data). We recently identified and characterized a CPD-photolyase from the related organism *Rhodobacter capsulatus* (Braatsch & Klug, 2004). In contrast to *R. sphaeroides* this protein appears to be the only member of the photolyase/cryptochrome family in this organism. *PhrA*, RSP1981 and RSP3077 exhibit 61 %, 31 % and 24 % sequence identity, respectively, to the protein encoded by ORF90. Primary sequence comparison with the CPD-photolyase derived from *Synechococcus* sp. 6301 (Tamada *et al.*, 1997) revealed several conserved residues shown to be involved in cofactor binding (Tamada *et al.*, 1997; Fig. 1). Photolyases provide three conserved tryptophan residues, which were supposed to be involved in electron transport to reduce the FADH₂ (Byrdin *et al.*, 2003; Fig. 1). These residues are present in *PhrA*, but are notably absent in RSP1981 and RSP3077. However, since blocking photoreduction through this tryptophan chain by point mutations has little or no physiological significance for the *in vivo* activity of the *E. coli* photolyase (Kavakli & Sancar, 2004), the absence of the tryptophan triad does not necessarily correlate with a loss of photolyase function in RSP1981 and RSP3077.

phrA and RSP3077, but not RSP1981, are responsible for photoreactivation in *R. sphaeroides*

A quantitative analysis of UV light challenge revealed that *R. sphaeroides* 2.4.1 indeed shows photoreactivation (Fig. 2). While only 8 % of *R. sphaeroides* wild-type cells survived 100 J m^{−2} UV light exposure without photoreactivation, subsequent illumination increased the survival rate by a factor of 6.6 to 53 %. To test for a photolyase function, the gene *phrA*, RSP1981 or RSP3077 was inactivated by insertion of a kanamycin resistance cassette, and the resulting mutants were tested for photoreactivation. All mutants showed a comparable survival after UV light as the wild-type when light was not available (Fig. 2). When *R. sphaeroides* Δ *phrA* was illuminated after exposure to UV light, we observed a significantly decreased survival rate (3 %) in comparison to the wild-type (53 %, Fig. 2). Complementation of *R. sphaeroides* Δ *phrA* by plasmid-borne *phrA* restored photoreactivation properties and increased the survival rate of the deletion mutant 14-fold.

Fig. 2. Survival of *R. sphaeroides* after UV light exposure. Cells of the indicated strains were exposed to UV light of 100 J m^{-2} (254 nm), spread on agar plates and kept in the light (58 W lamp) or in the dark. The survival rates are given as the mean of three experiments with maximal deviation.

The complementation of the photolyase mutant by the *phrA* gene is a clear proof for its function as photolyase.

Mutation of RSP1981 leads to no significant difference in survival compared to the wild-type, except a slightly increased survival (66%) after UV light exposure and subsequent illumination. This gene is obviously not involved in photorepair.

R. sphaeroides lacking RSP3077 showed photoreactivation; survival increased about 3.5-fold after illumination. Interestingly, the RSP3077 mutant showed 2.5-fold reduced survival (21%) in comparison to the wild-type (53%). When this strain was complemented, illumination increased the survival to 67%, similar to that of the complemented *phrA* mutant. Selby & Sancar (2006) reported recently that members of the previously found cryptochrome DASH family (Brudler *et al.*, 2003; Daiyasu *et al.*, 2004) repair single stranded DNA. Although RSP3077 shows only minor (up to 9%) sequence identity to members of this family, we can not exclude a function of RSP3077 in this regard and a role as putative second photolyase.

Expression of *phrA* is regulated by σ^E and the anti- σ^E factor ChrR

To date, there has been no evidence of a specific regulatory mechanism for the photolyase gene in any organism studied in this regard. Previous analysis of the promoter region of *phrA* revealed the presence of a putative σ^E DNA-binding site, which is identical to the *rpoE* consensus sequence (Braatsch *et al.*, 2004). Additional transcriptome analysis and *in vitro* transcription studies support the view that *phrA* is under control of σ^E (Anthony *et al.*, 2005).

In order to confirm these data and to test for the involvement of the anti- σ^E factor ChrR, we compared the expression level of *phrA* in the wild-type strain *R. sphaeroides* 2.4.1 and in the derived mutant strains TF18 and ΔChrR grown at high oxygen tension in the dark. Mutant strain TF18 has the *rpoE* and the *chrR* genes deleted (Schilke & Donohue, 1995), while mutant strain ΔChrR has only *chrR* inactivated (Newman *et al.*, 1999). When a *phrA* : *lacZ* reporter plasmid (pPHU*phrAlacZ*) was introduced into the wild-type strain, we observed a low β -galactosidase activity of 6 Miller units (Fig. 3a). In strain TF18, the activity was only 1 unit (Fig. 3a), which was the same as background (not shown). In contrast, we obtained about 200 Miller units in strain ΔChrR harbouring the reporter plasmid (Fig. 3a). These data suggest that σ^E is required for transcription of *phrA* and that ChrR strongly counteracts σ^E -mediated transcription, as was shown previously (Anthony *et al.*, 2004).

The fact that the photolyase of the related *R. capsulatus*, encoded by ORF90, has 61% sequence identity to PhrA suggests the presence of a similar regulatory mechanism of this photolyase gene. Interestingly, analysis of the genome of *R. capsulatus* showed neither an ORF with high similarity to σ^E or to ChrR of *R. sphaeroides*, nor an σ^E -like binding region in the promoter of ORF90.

Upregulation of *phrA* expression depends on light intensity and can be enhanced by the photosensitizer methylene blue

Although photolyase activity is only required upon the formation of pyrimidine dimers, *phrA* seems to be expressed even in the absence of light (Fig. 3a and our unpublished microarray chip data). However, we have reported before that blue light stimulates *phrA* expression (Braatsch *et al.*, 2004). In the fungus *Trichoderma harzianum* (Berrocal-Tito *et al.*, 1999) and in the goldfish *Carassius auratus* (Mitani *et al.*, 1996; Mitani & Shima, 1995; Yasuhira *et al.*, 1991) blue light as well as H_2O_2 induces the transcription of the photolyase gene. Kihara *et al.* (2004) showed that near-UV light (300–400 nm) and sunlight enhance the expression of the photolyase in the fungus *Bipolaris oryzae*. Therefore, it is possible that *phrA* gene expression is upregulated in response to general stressors. To test this hypothesis, we used the *phrA* : *lacZ* reporter plasmid and performed real-time RT-PCR to monitor σ^E /ChrR dependent *phrA* expression in cultures grown under different light conditions. We used methylene blue as a photosensitizer to generate singlet oxygen in irradiated cultures and tested whether *phrA* may also respond to other reactive oxygen species, i.e. H_2O_2 or superoxide anions, in a σ^E /ChrR dependent manner.

R. sphaeroides 2.4.1 and mutant strains TF18 and ΔChrR carrying the reporter plasmid pPHU*phrAlacZ* were grown in the dark under high oxygen concentration and then shifted to high intensity white light (800 W m^{-2} or $750 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) in the presence or absence of

Fig. 3. Expression of *phrA* in response to high intensity light irradiation and methylene blue treatment. Cells grown aerobically in the dark to an OD₆₆₀ of 0.4 were exposed to high intensity light irradiation and treated simultaneously with 0.2 μ M methylene blue where indicated. The data represent the mean of three independent experiments. Error bars indicate maximal deviation. (a) β -Galactosidase activity of strains harbouring a *phrA* : *lacZ* reporter plasmid. The cells were exposed to high intensity white light irradiation for 120 min. *This wild-type strain was illuminated with high intensity red light. †Strain TC67 was exposed to low intensity white light irradiation. (b) The same assay as (a) but showing the fold changes of β -galactosidase activity. (c) *phrA* expression levels normalized to dark-grown cells as determined by real-time RT-PCR. Cultures were exposed to high intensity white light irradiation for 5 or 15 min, as indicated.

methylene blue. In all strains tested the addition of 0.2 μ M methylene blue in the dark did not affect the β -galactosidase activity (data not shown). In the wild-type, the β -galactosidase activity increased from 5.5 to 19 Miller units after 120 min of white light illumination (Fig. 3a, b). An identical light shift experiment in the presence of methylene blue resulted in a slightly increased β -galactosidase activity (25 Miller units, Fig. 3a, b). No such light dependent increase in activity was observed in strains TF18 or Δ ChrR (Fig. 3a, b). Here, methylene blue did not affect the β -galactosidase activity of the illuminated mutant strains (Fig. 3a, b).

To confirm the reporter gene studies, we used real-time RT-PCR for determination of the relative increase in *phrA* levels due to illumination in the presence or absence of methylene blue (Fig. 3c). For the wild-type, we observed a relative fourfold upregulation in *phrA* expression as early as 5 min of irradiation. A prolonged illumination of 15 min resulted in a fivefold increase in *phrA* transcript level compared to the dark aerobic control. No such significant upregulation was observed for the mutant strains TF18 and ChrR, which is in line with our reporter gene studies (Fig. 3a, b). Interestingly, illumination of wild-type cultures in the presence of methylene blue caused a further 1.8-fold upregulation of *phrA* expression at the given time points while no effect of the photosensitizer was detectable for the mutant strains (Fig. 3c). The higher *phrA* transcript level observed in the wild-type cultures treated with the photosensitizer suggests that singlet oxygen is a trigger for this response. It is conceivable that the β -galactosidase activity is impaired by the presence of singlet oxygen. However, our results strongly suggest that (i) white light illumination triggers a *phrA* upregulation in aerobically grown *R. sphaeroides* cells and that (ii) the σ^E /ChrR pathway is responsible for signal transduction, resulting in a fast transcriptional response.

When we illuminated semi-aerobically grown wild-type cells with blue light of 10 μ mol photons $\text{m}^{-2} \text{s}^{-1}$ (3 W m^{-2}), we did not see any significant change in *phrA* expression as determined by the *phrA* : *lacZ* reporter plasmid or by real-time RT-PCR (not shown). This is not in agreement with previously published data (Braatsch *et al.*, 2004), where a 3.9-fold increase in *phrA* expression was described after 5 min blue light illumination with 10 μ mol photons $\text{m}^{-2} \text{s}^{-1}$ of the wild-type. In the latter case light intensities were monitored with a lux metre (DX-100), so it is conceivable that the light intensities used for the previous experiments were indeed higher than the 10 μ mol photons $\text{m}^{-2} \text{s}^{-1}$ used in the experiments reported here and monitored by a quantum sensor (LI-190SA). Indeed, when we increased the intensity of blue light to 60 μ mol photons $\text{m}^{-2} \text{s}^{-1}$, the β -galactosidase activity in wild-type cells increased twofold (not shown).

When wild-type cells were grown under high oxygen tension and illuminated with red light of high intensity (800 W m^{-2} or 380 μ mol photons $\text{m}^{-2} \text{s}^{-1}$) we

determined a 2.8-fold increase in *phrA* expression by the reporter assay and a 4.8-fold increase in the presence of methylene blue (Fig. 3a, b). Controls in the dark with or without methylene blue did not show any significant changes in β -galactosidase activity. These data reveal that *phrA* does not specifically respond to blue light and that no specific photoreceptor appears to be involved.

***phrA* expression of *R. sphaeroides* 2.4.1 responds to singlet oxygen**

The amplifying effect of methylene blue on *phrA* expression of illuminated cells (Fig. 3a–c) suggests that singlet oxygen is a trigger for this response. To further support this assumption, we monitored *phrA* expression in strain TC67, which lacks carotenoids. Carotenoids are essential constituents of the photosynthetic apparatus and are assumed to prevent the formation of singlet oxygen by quenching of triplet bacteriochlorophyll *a* *in vivo* (Borland *et al.*, 1989). It was shown previously that, in the presence of oxygen and high light, *R. sphaeroides* forms small amounts of singlet oxygen *in vivo*, which do not adversely affect cell growth (Glaeser & Klug, 2005). Higher amounts of singlet oxygen were generated by bacteriochlorophyll *a* in the carotenoid-deficient strain TC67, causing a decrease in growth and survival rates (Glaeser & Klug, 2005). When strain TC67(pPHU*phrAlacZ*) was illuminated with white light of low intensity (30 W m⁻²), the β -galactosidase activity increased about fivefold within 2 h (Fig. 3a, b). The induction of a comparable response in the wild-type required a 26-fold higher light intensity (800 W m⁻²; Fig. 3a, b). The more light sensitive *phrA* upregulation in strain TC67 supports our view that singlet oxygen can trigger this response.

We also monitored *phrA* expression by using the reporter plasmid in strain PUHA1, which lacks the H-subunit of the reaction centre (RC). In the RC all the cofactors are bound to the L and M subunits. A RC complex in which the H peptide has been stripped away, leaving an LM remnant, retains full primary photoactivity. However, in these LM complexes, the Q_A to Q_B reaction is impaired and the complexes are significantly less stable than LMH (Debus *et al.*, 1985). *In vivo*, reaction centres lacking the H subunit are virtually absent as determined by spectroscopy (Tehrani *et al.*, 2003) and as a consequence, strain PUHA1 can not grow phototrophically. Illumination of PUHA1 by high white light intensity resulted in about sixfold increase of *phrA* expression, high light and methylene blue in about sevenfold increase (Fig. 3a, b). This is significantly higher than in the wild-type (3.5- and 4.2-fold, respectively), suggesting that the lack of RC may amplify the signal acting on *phrA*. Most likely, energy transfer from excited bacteriochlorophyll to oxygen is enhanced when no photosynthetic electron transport can be initiated in the RC. Furthermore, we conclude that photosynthetic electron transport is not involved in light dependent regulation of *phrA* expression, as demonstrated

for the upregulation of photosynthesis genes (Happ *et al.*, 2005).

***phrA* expression of *R. sphaeroides* 2.4.1 responds to hydrogen peroxide but not to superoxide anions**

In order to test whether *phrA* expression may also respond to other reactive oxygen species, we quantified its expression in the presence of H₂O₂ and the superoxide-generating agent paraquat. By using the *phrA* : *lacZ* reporter plasmid we could not observe any significant expression changes in response to H₂O₂ (Fig. 5) or paraquat (not shown). However, we had observed before that the *lacZ* gene is not suitable to monitor the response to H₂O₂ and superoxide anions (see below), presumably due to loss of activity of the β -galactosidase under oxidative stress.

Real-time RT-PCR analysis of *phrA* expression revealed a transient upregulation due to H₂O₂ exposure (Fig. 4). As soon as 7 min after H₂O₂ exposure, we observed a 5.6-fold increase in *phrA* transcript level (Fig. 4). In strains TF18 and Δ ChrR, which lack σ^E and ChrR or ChrR, respectively, the *phrA* activity dropped about twofold upon 7 min exposure to H₂O₂ (Fig. 4). However, after 30 min of H₂O₂ treatment a relative ~1.5-fold increase in *phrA* transcript levels was observed in all three strains (Fig. 4). Based on gene expression dynamics we conclude that the σ^E /ChrR pathway is responsible for the fast upregulation of *phrA*. For unknown reasons, expression of this gene was found to be slightly downregulated in mutant strains TF18 and Δ ChrR after 7 min (Fig. 4). Since a prolonged exposure to

Fig. 4. H₂O₂ and paraquat dependent *phrA* expression in *R. sphaeroides* strains. Cells grown semi-aerobically in the dark to exponential phase were exposed to 1 mM H₂O₂ or 1 mM paraquat for 7 and 30 min. *phrA* transcript levels were determined by real-time RT-PCR. Values represent the relative fold change of *phrA* expression in treated compared to untreated cells. The mean and maximal deviations of three independent experiments are indicated.

H₂O₂ resulted in a similar ~1.5-fold increase in *phrA* transcript levels, we suggest that other factors besides σ^E and ChrR are involved in a retarded upregulation of *phrA* expression. A fast and transient response is expected, since the *R. sphaeroides* catalases detoxify H₂O₂ very quickly (Zeller & Klug, 2004).

In contrast to H₂O₂, paraquat had no significant effect on *phrA* expression in the wild-type (Fig. 4). Thus, superoxide anions generated by paraquat do not lead to σ^E dependent stimulation of *phrA*.

Anthony *et al.* (2005) reported that σ^E together with its anti- σ factor ChrR transmit singlet oxygen dependent signals but are not involved in the response to H₂O₂, diamide and superoxide anions. An increase of *rpoE* expression in the presence of H₂O₂ was not observed according to *rpoE* : *lacZ* reporter gene studies (Anthony *et al.*, 2005). In contrast to this previous study, we found a significant upregulation of *phrA* (Fig. 4) and a similar upregulation in *rpoE* expression (not shown) due to H₂O₂ stress using real-time RT-PCR and DNA microarray analysis (Zeller *et al.*, 2005).

In the non-phototrophic α -proteobacterium *Caulobacter crescentus* cadmium sulphate (6 μ M) caused a sixfold upregulation of the *phrA* homologue CC1428 (Hu *et al.*, 2005). Cd²⁺ binds to glutathione in Gram-negative bacteria, which subsequently results in the formation of a considerable amount of H₂O₂ (reviewed by Nies, 1999). Interestingly, the upstream regions of CC1428 and *rpoE/chrR* (CCR0645/CCR0644) encode a putative *R. sphaeroides* σ^E -dependent promoter sequence. Therefore it is likely that in *C. crescentus* (i) H₂O₂ formation due to Cd²⁺ stress results in a σ^E /ChrR dependent upregulation of the *phrA* homologue CCR1428 and (ii) *rpoE* is transcribed by its own product as suggested for *R. sphaeroides* (Newman *et al.*, 1999).

β -Galactosidase-activity in *R. sphaeroides* is affected by H₂O₂

We observed frequently that *lacZ* reporter assays in *R. sphaeroides* are not suitable to monitor responses to H₂O₂ and superoxide anions, indicating sensitivity of the β -galactosidase enzyme. The data presented in this paper suggest that this might also be the case for singlet oxygen.

We analysed by real-time RT-PCR whether the expression of *lacZ* in the *phrA* : *lacZ* reporter plasmid is enhanced in the wild-type after 7 min incubation with 1 mM H₂O₂. We measured a 2.6-fold increase in *lacZ*-mRNA level (Fig. 5), and a 5.6-fold increase of the mRNA transcribed from the chromosomal copy of *phrA* (5.6-fold, Figs 4 and 5). When the wild-type harbouring the reporter plasmid was cultured under high intensity white light conditions for 15 min, we observed a 7.4-fold increase of *lacZ* mRNA level (Fig. 5), which is similar to the increase of *phrA* mRNA level (Figs 3c and 5).

Based on the result that the *lacZ* gene expression but not the β -galactosidase enzymic activity increased, we suggest

that this activity is somehow affected by reactive oxygen species. Topatchieva *et al.* (2003) found that intrinsic tellurite toxicity prevented the use of the *lacZ* fusion as a reporter for regulatory studies in *Proteus mirabilis*. Since tellurite oxidizes thiol groups, it contributes to oxidative stress and might affect the β -galactosidase enzyme in a similar way as ROS.

Conclusion

Our data reveal that the *phrA* gene of *R. sphaeroides* encodes a functional photolyase. A response of increased *phrA* expression caused by light is indeed triggered by singlet oxygen, which is generated by energy transfer from bacteriochlorophyll to molecular oxygen, and the response requires the σ^E /ChrR system. Involvement of photoreceptors or photosynthetic electron transport can be excluded. In contrast to previous studies, we provide evidence that the σ^E /ChrR system is also stimulated by H₂O₂. Future investigations will focus on determining by which mechanisms singlet oxygen or H₂O₂ affect σ^E :ChrR complex dissociation.

Fig. 5. Comparison of *phrA* and *lacZ* expression in *R. sphaeroides* 2.4.1 in response to H₂O₂ and high intensity white light irradiation and methylene blue treatment. β -Galactosidase activity was measured from the *phrA* : *lacZ* reporter plasmid. *phrA* mRNA levels were determined from the gene expressed from chromosomal DNA and *lacZ* mRNA levels from the *phrA* : *lacZ* reporter plasmid in *R. sphaeroides* wild-type. Wild-type cells were grown semiaerobically to exponential phase and exposed to 1 mM H₂O₂ for 7 min for determining expression levels by real-time RT-PCR or 45 min for β -galactosidase activity tests. For the generation of singlet oxygen, cells were grown aerobically and treated with high intensity white light and 0.2 μ M methylene blue for 15 min to determine mRNA levels by real-time RT-PCR or 120 min for β -galactosidase assays. *phrA* and *lacZ* expression was normalized to untreated cells. The data represented are the mean of three independent experiments; the β -galactosidase activity test for H₂O₂ dependent expression was done twice. Error bars indicate maximal deviation.

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