Role of PhyR and growth phase-dependent regulation in *Rhodobacter sphaeroides*

zur Erlangung
des akademischen Grades
Doktor der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von

M. Sc. - Biol. Qingfeng Li
aus
V. R. China

angefertigt am
Institut für Mikrobiologie und Molekularbiologie
Fachbereich Biologie und Chemie
Justus-Liebig-Universität Gießen

Giessen，January 2019

Die vorliegende Arbeit wurde angefertigt am Institut für Mikrobiologie und Molekularbiologie des Fachbereiches 08 der Justus-Liebig-Universität Gießen in der Zeit von Oktober 2014 bis Juli 2018 unter der Leitung von Prof. Dr. Gabriele Klug.

1. Gutachterin: **Prof. Dr. Gabriele Klug**

Institut für Mikrobiologie und Molekularbiologie

Justus-Liebig-Universität Gießen

2. Gutachter: **Prof. Dr. Albrecht Bindereif**

Institut für Biochemie

Justus-Liebig-Universität Gießen

Contents

III

Abbreviations

ad	fill up to	µl	microliter
APS	ammonium persulphate	µM	micromolar
bp	base pair	nt	nucleotide
°C	centigrade	OD	optical density
ddH2O	double-distilled water	PAGE	polyacrylamide gel electrophoresis
dATP	deoxyriboadenosine triphosphate	PBS	phosphate-buddered saline
DNA	deoxynucleic acid	PCR	polymerase chain reaction
DTT	1, 4-dithiothreitol	PNK	polynucleotide kinase triphosphate
E. coil	*Escherichia coil*	ROS	reactive oxygen species
EDTA	ethylene diamine tetraacetic acid	rpm	revolution per minute
et al.	*et alii* (and others)	SDS	sodium dodecyl sulfate
EtOH	ethanol	UV	ultraviolet
g	gram		
H	hour		
M	molar (mol/l)		
min	minute(s)		
ml	milliliter		
mM	millimolar		
mRNA	messenger RNA		
µg	microgram		

Publication

The following publication is based on this work

Qingfeng Li[#], Tao Peng[#], Gabriele Klug. (2018) The PhyR homolog RSP_1274 of *Rhodobacter sphaeroides* is involved in defense of membrane stress and has a moderate effect on RpoE (RSP_1092) activity. BMC Microbiology Feb 27; 18(1):18.
[#]equal contributor

1. Introduction

1.1 The general stress response and its regulation

In response to various stimuli, bacteria change their physiology to become more resistant to the severe conditions that they may encounter in their natural habitats, which is called general stress response (GSR). The GSR is a preventive and reversible response to multiple stresses. It is a widely conserved response that allows bacteria to adapt to various stressful conditions. The GSR systems of *Escherichia coli* and *Bacillus subtilis* are studied well, they are not orthologous. However, both of the central regulators are a sigma factor, σ^S and σ^B, respectively. In addition, other central players and complex pathway also have been identified in *Escherichia coli* and *Bacillus subtilis* (Chiang SM*, et al.*, 2010, Hecker M*, et al.*, 2007).

Alphaproteobacteria are a metabolically and habitually diverse class that includes soil-living and aquatic microorganisms, pathogens, and symbionts. Some species belong to this class that also can encode GSR systems. The PhyR-NepR-σ (EcfG) cascade is recognized as a core pathway in general stress response in alphaproteobacteria. Although the regulatory system is conserved in closely related species, the GSR system is not the only system mediating these responses, indicating that there is another system that controls the GSR in bacteria, such as RpoE-RseA in *Escherichia coli* (Ades SE, 2004, Alba BM*, et al.*, 2004). Expression of genes including sigma factors involved in GSRs is not only mediated by transcriptional sigma factors, but also shows growth phase-dependent regulation or together with DNA-binding proteins (Azam TA*, et al.*, 1999).

1.2 Two component regulatory system in bacteria

Over a decade ago, "Two component" was described as a regulatory system found in bacteria. By now, researchers have discovered several hundreds of the systems in eubacteria, archaea, and a few eukaryotic organisms. Two component regulatory systems are essential stimulus response mechanisms to allow organisms to sense and respond to different stresses in severe environmental conditions. Therefore, organisms can cope with and survive in these stressful conditions.

The two component system consists of a membrane-bound sensor histidine kinase (HK) including a conserved kinase core, and a cytoplasmic response regulator protein (RR) including a conserved response domain. HK is composed of a sensory domain and a kinase domain (West AH, *et al.*, 2001). RR is composed of a phosphoryl receiver domain and a variety of signal output domain. The HK domain senses the external signal from the environment, and transfers a phosphoryl group to RR. The phosphorylated receiver domain of RR results in activation of the output domain that elicits the specific response (Fig 1.1). Phosphorylated RR also can be dephosphorylated by hydrolysis. The balance of phosphorylation and dephosphorylation of RR depends on the signals from extracellular.
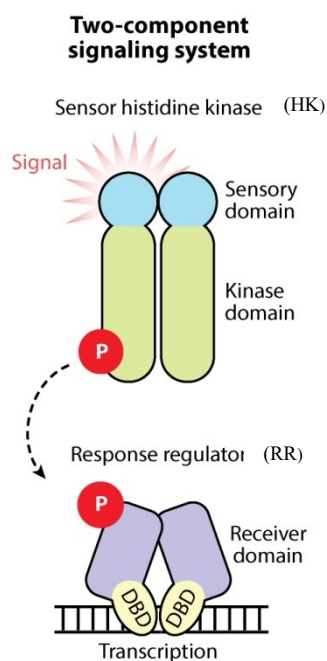
Fig 1.1 A typical two component response system consist of a sensor histidine kinase and a response regulator protein. The sensory domain controls autophosphorylation of kinase domain. Afterwards the phosphoryl residue is transferred to the receiver domain causing activation of the DBD output domain. Adapted from Fiebig *et al.*, 2015.

For the majority of two component regulatory systems, a phosphoryl group is transferred from HK to RR directly, which is common in bacteria. The transduction of phosphorylation pathway involves two phosphor transfer reactions and one phosphoprotein intermediates (Stock AM, *et al.*, 2000):

1. Autophosphorylation: HK-His + ATP↔HK-His~P + ADP

2. Phosphotransfer: HK-His~P + RR-Asp↔HK-His + RR-Asp~P

However, some two component regulatory systems are the multiple phosphotransfer steps known as phosphorelay (Appleby JL, *et al.*, 1996, Perraud AL, *et al.*, 1999).

Four phosphoryl transfer reactions and three phosphoprotein intermediates are involved in phosporelay:

1. Autophosphorylation: HK-HisI + ATP↔HK-HisI~P + ADP

2. Phosphotransfer I: HK-HisI~P + RR-AspI↔HK-HisI + RR-AspI~P

3. Phosphotransfer II: RR-AspI~P + HPt-HisII↔RR-AspI + HPt-HisII~P

4. Phosphotransfer III: HPt-HisII~P + RR-AspII↔HPt-HisII + RR-AspII~P

1.3 Alternative sigma factors

The bacterial sigma factor is a subunit of the RNA polymerase that recognizes and binds to the promoter initiating transcription. The primary sigma factor (σ^{70} in *E. coli*) is responsible for housekeeper genes. Besides primary sigma factor, bacteria also have a different number of alternative sigma factors, which respond to specific stimuli and control a set of genes. Bacterial responses to stresses are principally adjusted at initiation of transcription (Browning DF*, et al.*, 2004), in which alternative sigma factors rapidly reprogram gene expression in response to a variety of stresses via mobilizing RNA polymerase to specific promoters in the cell (Gruber TM*, et al.*, 2003) (Fig 1.2).
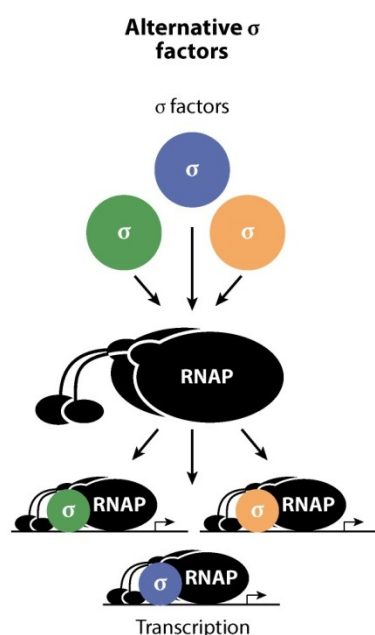
Fig 1.2 Various sigma factors can bind to the core the RNA polymerase in response to different growth conditions. Alternative sigma factors are controlled by complex regulatory mechanisms in the cell. Adapted from Fiebig *et al*., 2015

Regulation of alternative sigma factors varies in different microorganisms. In most cases, the alternative sigma factor is co-transcribed with a cognate regulator, an inner envelope anti-sigma factor, such as RpoE and RseA in *E. coli* (or RpoE and ChrR in *R. sphaeroide*). The anti-sigma factor RseA sequesters the alternative sigma factor RpoE in the cytoplasm, and prevents RpoE from binding to RNA polymerase, thereby inhibiting RpoE dependent transcription (Campbell EA*, et al.*, 2003, DeLasPenas A*, et al.*, 1997, Missiakas D*, et al.*, 1997). However, a few alternative sigma factors are regulated by other sigma factors. For instance, alternative sigma factor RpoHII in *R. sphaeroides* is regulated by another alternative sigma factor RpoE in *R. sphaeroides* through RpoE-dependent promoter in response to singlet oxygen stress (Nuss AM*, et al.*, 2009). Expression of the sigma factor RpoS in *Borrelia burgdorferi* is controlled by another alternative sigma factor RpoN via an RpoN-dependent promoter (Smith AH*, et al.*, 2007). In some bacteria, alternative sigma factors are regulated in multiple ways, like RpoS in *E. coli* also is mediated by small RNA DsrA along with its chaperone Hfq (Mikulecky PJ*, et al.*, 2004, Repoila F*, et al.*, 2001).

1.4 The PhyR-NepR-σ(EcfG) cascade

It emerged that the PhyR-NepR-σ (EcfG) cascade is a core pathway regulating the general stress response in Alphaproteobacteria (Francez-Charlot A*, et al.*, 2015). The PhyR-NepR-σ (EcfG) cascade combines two important regulatory mechanism that the two component regulatory system and alternative sigma factor, encompasses the extracytoplasmic function sigma factor (σ (EcfG)), its anti-sigma factor NepR (for negative regulator of the PhyR response), and the anti-sigma factor antagonist PhyR (phyllosphere regulator). In this cascade, the σ (EcfG) factor is a foundational effector to control target gene expression in resistance to general stress responses. The σ (EcfG)-orthologous sigma factors RpoE2 and SigT exist in *Sinorhizobium meliloti* and *Caulobacter crescentus*, respectively, and are involved in general stress responses (Alvarez-Martinez CE*, et al.*, 2007, Sauviac L*, et al.*, 2007). An anti-sigma factor NepR is encoded downstream of the *ecfG* gene can sequester σ (EcfG), prevent σ (EcfG) from binding RNA polymerase (Sauviac L*, et al.*, 2007). PhyR is central molecule in the signaling pathway in general stress responses, and acts as a anti-sigma factor antagonist. It consists of a C-terminal receiver domain (REC) and an N-terminal σ (EcfG)-like domain (SL) (Francez-Charlot A*, et al.*, 2009, Herrou J*, et al.*,

2010). The REC domain is phosphorylated by a sensor histidine kinase (Herrou J, *et al.*, 2017, Kaczmarczyk A, *et al.*, 2015, Lourenco RF, *et al.*, 2011), destabilizing the interaction interface between the σ (EcfG)-like (SL) domain and C-terminal receiver (REC) domain. Phosphorylated PhyR induces conformational changes.

In the current model, σ (EcfG) factor is sequestered by NepR under non-stress condition; In response to stress, PhyR is phosphorylated, then becomes opened conformation, and interacts with NepR. The σ (EcfG) factor is released and associates with RNA polymerase, controlling gene expressions (Fig 1.3).
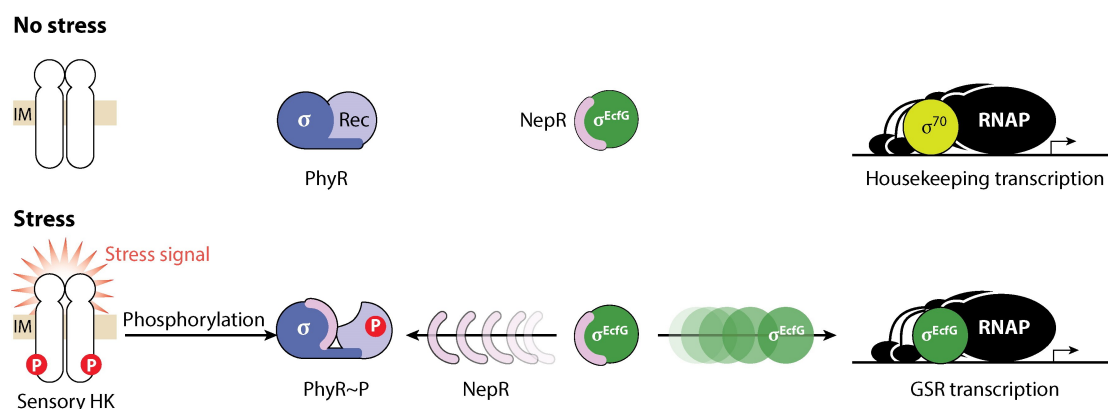
Fig 1.3 Alphaproteobacterial general stress response (GSR) partner switching. Under unstressed conditions, σ (EcfG) (green) is sequestered by the anti-sigma factor NepR (pink). PhyR (purple) is unphosphorylated, in unactivated condition, and has a low affinity for NepR. Under stressed conditions, the receiver domain of PhyR (light purple) can be phosphorylated by a sensor histidine kinase(s) (white). The PhyR conformation is altered, reveals the NepR binding interface and thereby increases the affinity of PhyR for NepR. The PhyR~P-NepR complex releases σ (EcfG) to interact with RNAP (black) and transcription of the GSR regulon is initiated. Taken from Fiebig *et al.*, 2015

1.5 Growth phase dependent regulation in bacteria

Distinct bacterial growth phases can be observed in the lab or in natural environments. During the lag phase, bacteria adapt to growth conditions and start to divide with maximal rate; the exponential phase is a period characterized by cell doubling with a constant rate; in stationary phase bacteria stop to replicate. With entry into stationary phase, the active growth of exponential phase switches into a maintenance growth condition, the abilities of resistance to stresses become stronger (Roop RM, *et al.*, 2003). The accumulation of waste products and depletion of nutrients cause cell death

in prolonged stationary phase. Nutrients are released from dead cells that can support survival cells for a long time in stationary phase with a constant mass of viable cells. Available nutrients in stationary phase result in restarting growth until enter stationary phase again (defined as outgrowth).

In order to adapt to changing environments or harsh growth conditions throughout the growth phases, bacteria need to alter gene expression. Previous studies indicated many genes expression of stress responses presented growth phase dependent regulation. For instance, *rpoS*, which belongs to the sigma 70 family that is involved in GSR, shows growth phase dependent expression in *E. coli*. The earliest expression of *rpoS* is induced in the exponential phase at an OD_{600} of 0.2 (Schellhorn HE*, et al.*, 1992) in *E. coli*. Although maximal expression was usually observed in the stationary phase, revealing that concerted expression of RpoS-regulated genes starts well at the beginning of the stationary phase (Schellhorn HE*, et al.*, 1998). Among these RpoS-regulated genes, *katE*, *otsA*, *osmY*, are induced by hydroperoxide, heat shock or osmotic shock, respectively, showed same expression pattern to *rpoS* (Schellhorn HE*, et al.*, 1998). Under osmotic stress, a nucleoid associated protein, H-NS, also participates in regulation of RpoS activity. H-NS acts as an inhibitor in exponential phase at the post-transcriptional level. It is reported that H-NS relieves repression of *rpoS* and plays a role in stationary-phase induction as well as in hyperosmotic induction of *rpoS* translation (Barth M*, et al.*, 1995). Another sigma factor, RpoE, activates multiple stress responses, such as envelope stress, heat shock and oxidative stress (Alba BM*, et al.*, 2004, Mecsas J*, et al.*, 1993). RpoE activity increases continuously in a growth phase dependent manner from early exponential phase to stationary phase, and has a better preparation for various stresses that may encounter in stationary phase (Costanzo A*, et al.*, 2006). RpoE also leads to lysis of viable but non-culturable cells at the early stationary phase as well as under oxidative stress absence of catalase and superoxide dismutase in *E. coli* (Noor R*, et al.*, 2009).

1.6 Oxidative stress

1.6.1 Source of reactive oxygen species in the cell

Oxygen is considered as the Janus gas. Bacteria get both benefits and damaging side effects in biological processes. Except anaerobic species, organisms require oxygen

for basic energy resource through oxidative phosphorylation generating adenosine-5-triphosphate (ATP). However, bacteria are also attacked by some unwanted byproducts, such as reactive oxygen species (ROS). Some organic macromolecules are destroyed by ROS in the cell.

The oxygen molecule, also called dioxygen (O_2), qualifies as a radical because it has two unpaired electrons in ground state. From a quantum mechanical standpoint, oxygen is a triplet species. Despite oxygen is referred to as a powerful oxidizing agent, the reaction of ground state oxygen with other molecules is "spin forbidden". Because of spin restrict, oxygen prefers to take in one electron at a time, and the continuously addition of one electron results in formation of ROS (Fig 1.4). Acceptance of single electron by an oxygen molecule forms superoxide, $O_2^{\cdot-}$, an superoxide is able to inactivate few enzymes (Zhang Y, *et al.*, 1990). An additional electron is transferred to superoxide generating peroxide (H_2O_2). H_2O_2 does not contain any unpaired electron, so that the activity of oxidation is poor compared to $O_2^{\cdot-}$. Thereby, H_2O_2 is not a radical. However, H_2O_2 crosses the envelope easily and is predicted to oxidize specific protein thiol groups. Even though oxidizing activity of $O_2^{\cdot-}$ and H_2O_2 is limited, they can generate the highly reactive hydroxyl radicals ($OH^{\cdot}$). The Fenton reaction of H_2O_2 with ferrous iron (Fe^{2+}) generates $OH^{\cdot}$ (a) (Sychev AY, *et al.*, 1985). The Haber-Weiss reaction of $O_2^{\cdot-}$ with H_2O_2 catalyzed by Fe or Cu, which also generates $OH^{\cdot}$ (b) (Kehrer JP, 2000).

$$Fe^{2+} + H_2O_2 \rightarrow OH^{\cdot} + OH^{-} + Fe^{3+} \text{ (a)}$$

$$O_2^{\cdot-} + H_2O_2 \rightarrow OH^{\cdot} + OH^{-} + O_2 \text{ (b)}$$
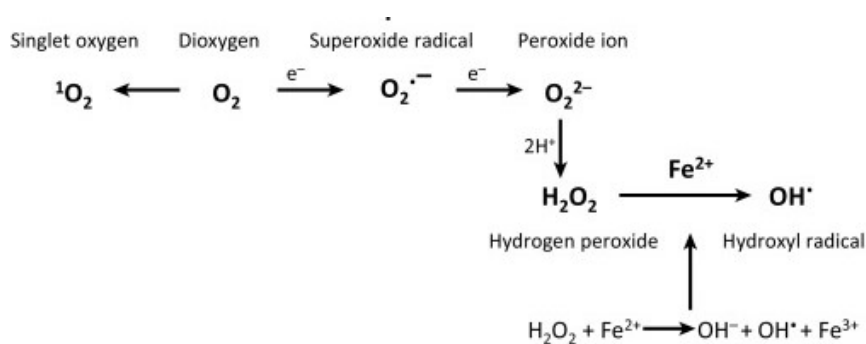
Fig 1.4 Formation of different ROS from oxygen molecule. Taken from Ron Mittler, 2017

The singlet oxygen ($^1O_2$) is different from others ROS that were described in former phrase. Its generation depends on energy rather than electron transfer. It is produced from three different ways. Normally, we consider singlet oxygen stress as photo-oxidative stress, because a major source of singlet oxygen are light dependent processes including two different ways: In photosynthetic organisms, energy transfer to the oxygen molecule (O_2) from excited pigments, such as chlorophyll in plants or bacteriochlorophyll in bacteria (Fig 1.5a). In nonphotosynthetic conditions, energy transfer to the oxygen molecule (O_2) from natural photo-sensitizers such as flavins, rhodopsins, quinones, and porphyrins, or exogenous photo-sensitizers, such as tetrapyrroles and methylene blue or Rose bengal, respectively (Fig 1.5b) (Glaeser J, *et al.*, 2011). However, singlet oxygen also is produced in biological systems in the dark. It is metabolically generated in reduction of O_2 catalyzed by enzymes, such as myeloperoxidase, chloroperoxidase and NADPH-oxidase (Fig 1.5c) (Ryter SW, *et al.*, 1998, Steinbeck MJ, *et al.*, 1992).
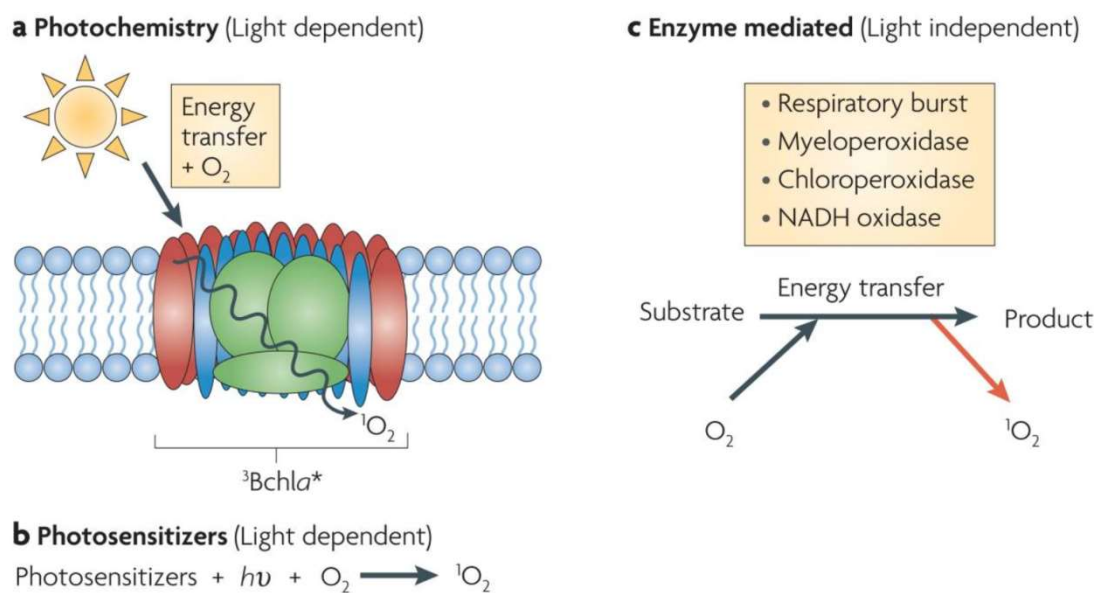
Fig 1.5 Sources of singlet oxygen adapted from *Ziegelhoffer and Donohue*, 2009. a, b: major sources of singlet oxygen dependent light include generation in photosynthetic organism (a), and in nonphotosynthetic organism (b). Light-independent generations of singlet oxygen are catalyzed by different enzymes (c). $h\upsilon$, light energy.

1.6.2 Damage due to oxidative stress

ROS are produced through these reactions (described in 1.3), interact with numerous cellular components, including lipids, proteins, DNA and RNA, which can cause cell

damage. Nonetheless, the damage is highly diverse from different ROS and presented in this chapter.

$O_2^{\cdot-}$, the superoxide radical has one unpaired electron, which itself has limited reactivity. It can oxidize labile (4Fe-4S) to inactive (3Fe-4S) clusters, and release a Fe^{2+} ions (Flint DH, *et al.*, 1993, Kuo CF, *et al.*, 1987). Such reaction at least has two harmful consequences, inactivation of few enzymes including (4Fe-4S) cluster (Gardner PR, *et al.*, 1991, Gardner PR, *et al.*, 1991, Gardner PR, *et al.*, 1991), and DNA damage (Imlay JA, *et al.*, 1988, Keyer K, *et al.*, 1996). DNA damage results from ferrous ions. These ions participate in Fenton reaction (chapter 1.6.1 (a)), and the product hydroxyl radicals (OH$^{\cdot}$) that destroy DNA (Liochev SI, *et al.*, 1994, Srinivasan C, *et al.*, 2000). H_2O_2 damages proteins by oxidizing cysteinyl residues directly (Imlay JA, 2003), or react with $O_2^{\cdot-}$ generating hydroxyl radicals that damage DNA (Kehrer JP, 2000). The extremely reactive hydroxyl radical is considered as principal source in the endogenous oxidative stress (Cadet J, *et al.*, 1999) and reacts with all biological molecules including DNA, RNA, lipids, and proteins (Dizdaroglu M, *et al.*, 2012, Mittler R, 2017). Among these ROS, the most active is singlet oxygen that can react with the majority of macromolecules, for instance membrane lipids, proteins, nucleic acids, carbohydrates and thiols (Ryter SW, *et al.*, 1998), and therefore generate byproduct of organic peroxides and sulfoxides (Glaeser J, *et al.*, 2011).

1.6.3 Response to oxidative stress

To survive oxidative stress, organisms evolved various defense mechanisms that either keep the concentration of ROS in an acceptable level or repair oxidative damages. The former defense mechanisms have two arms including the enzymatic components and non-enzymatic antioxidants. And the latter defense systems include DNA-repair systems and proteolytic and lipolytic enzymes (Cabiscol E, *et al.*, 2000).

The enzymatic antioxidants comprise superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and guaiacol peroxidase (GPX). The removal of superoxide radicals ($O_2^{\cdot-}$) is catalyzed by SOD, Which converts $O_2^{\cdot-}$ to H_2O and O_2 (Mittler R, 2002). CAT, APX and GPX are involved in eliminating H_2O_2, but the products are different,

they are respective H_2O and O_2, H_2O and DHA or H_2O and GSSH (Asada K, 1999, Sharma P*, et al.*, 2004). DHA is reduced by dehydroascorbate reductase to ascorbic acid and GSSH (Eltayeb AE*, et al.*, 2007). And GR uses NADPH as a reductant to reduce GSSG to GSH preventing thiol groups from being oxidized by $OH^{\cdot}$ and $^{1}O_2$.

The non-enzymatic antioxidants consist of ascorbic acid, reduced glutathione (GSH), α-tocopherol, and carotenoids. ascorbic acid acts as a reducing agent to reduce H_2O_2 to H_2O and O_2 by APX. It also reacts with $OH^{\cdot}$ and $^{1}O_2$ to protect cell components from oxidative damages (Shao HB*, et al.*, 2005). GSH exists in almost all cellular compartments, harboring a central cysteine residue. It scavenges $O_2^{\cdot-}$, H_2O_2, $OH^{\cdot}$ and $^{1}O_2$ (Mullineaux PM*, et al.*, 2005). α-tocopherol belongs to the lipophilic antioxidants, which are highly active removal of all ROS, making them obligatory protectors and essential components of biological membranes (Hollander-Czytko H*, et al.*, 2005, Kiffin R*, et al.*, 2006). Carotenoids exhibit its capacity of antioxidant by quenching $^{1}O_2$ (Di Mascio P*, et al.*, 1989) or exciting photo-sensitizer molecules (Muller MG*, et al.*, 2010) to prevent biological molecular damages.

The secondary defense system contains DNA-repair systems, proteolytic and lipolytic enzymes. Endonuclease IV is a DNA repair enzymes, induced by oxidative stress, take effect on duplex DNA cleaning up DNA 3' termini. Prokaryotic cells have some enzymes able to repair some covalent modifications to the primary structure of proteins. One of the most frequent modifications is oxidation of disulfide bonds.

The activity of these defense factors depends on their concentration in the cells. Some of them constantly occur in all organelle of cells (GSH), others are controlled by transcriptional factors such as OxyR and SoxRS in *E.coli*. Previous study revealed that OxyR is induced by H_2O_2 and controls oxidant elimination genes (*katG*, *ahpC* and *ahpF*), the balance between thiol groups and disulphide bonds (*gorA*, *grxA* and *trxC*), and the occurrence of the Fenton reaction via limiting Fe^{2+} availability (*dps* and *fur*) (Christman MF*, et al.*, 1985). SoxRS activated by $O_2^{\cdot-}$ are involved in $O_2^{\cdot-}$ removal (*sodA*), in DNA repair (endonuclease IV (*nfo*)), and in enhancing glutathione-dependent repair reactions (glucose-6-phosphate dehydrogenase (*zwf*)) (Greenberg JT*, et al.*, 1990).

1.7 Membrane stress responses in gram negative bacteria

In gram negative bacteria, cytoplasmic membranes composed of phospholipid bilayer and proteins. The membrane comprises the major boundary outlining the cell cytoplasm, and it controls the transport and diffusion of a huge number of small molecules and secreted proteins between the intracellular and extracellular space. It is the first and major line of defense systems preventing affront from environment. Normally, bacteria live in a complex circumstance, in which they are exposed to toxic agents. The cell envelope is offended, then caused membrane stresses. In order to resist to membrane stresses, bacteria evolved a variety of defense systems.

In gram-negative bacteria, five membrane stress responses have been defined (Fig 1.6). In *E.coli*, the σ^E factor is first identified and plays an essential role in outer membrane protein (OMP) folding. It is activated by misfolded OMPs and protease DegS, or the anti-anti-sigma factor RseB. The σ^E factor transcribes genes that can impact many aspects of the cell envelope such as phospholipid and lipopolysaccharide (LPS) biosynthesis, lipoproteins and signal transduction pathways (Ades SE, 2004, Flores-Kim J*, et al.*, 2014, Lima S*, et al.*, 2013). A typical two-component regulatory system Cpx responds to periplasmic or inner membrane protein misfolding in *E.coli*. Membrane stresses leading to protein misfolding, inactivate the inhibitor CpxP, trigger CpxA-mediated phosphorylation of CpxR, regulated expression of protein foldases and proteases (Raivio TL, 2014). It was found that a complex signal transduction system Rcs was involved in membrane stress in *E. coli*. The Rcs is regulated by a two-component phosphorelay comprising two inner membrane-associated histidine sensor kinase, RscC and RscD, together with a cytoplasmic response regulator, RscB. The phosphorylation of RcsB results in changing in the expression of capsule production, motility, biofilm formation, and other membrane proteins (Majdalani N*, et al.*, 2005). The phage shock protein response (Psp) is activated by disruption of membrane, including the dissipation of the proton motive force (PMF). Signals are transduced through a set of Psp proteins that cause the release of the PspF transcription factor from the inhibitor PspA and the regulation of relative genes expression to mitigate the stress. The Psp system is essential for virulence in *Yersinia enterocolitica* and *Salmonella enterica* (Flores-Kim J*, et al.*, 2014). Bae also controlled by a two-component regulatory system consists of the

sensor kinase BaeS and its cognate partner BaeR in *E.coli*. This pathway is by exposure to membrane damaging agents made by plants, animals, microbes, as well as metals (Cordeiro RP*, et al.*, 2014).
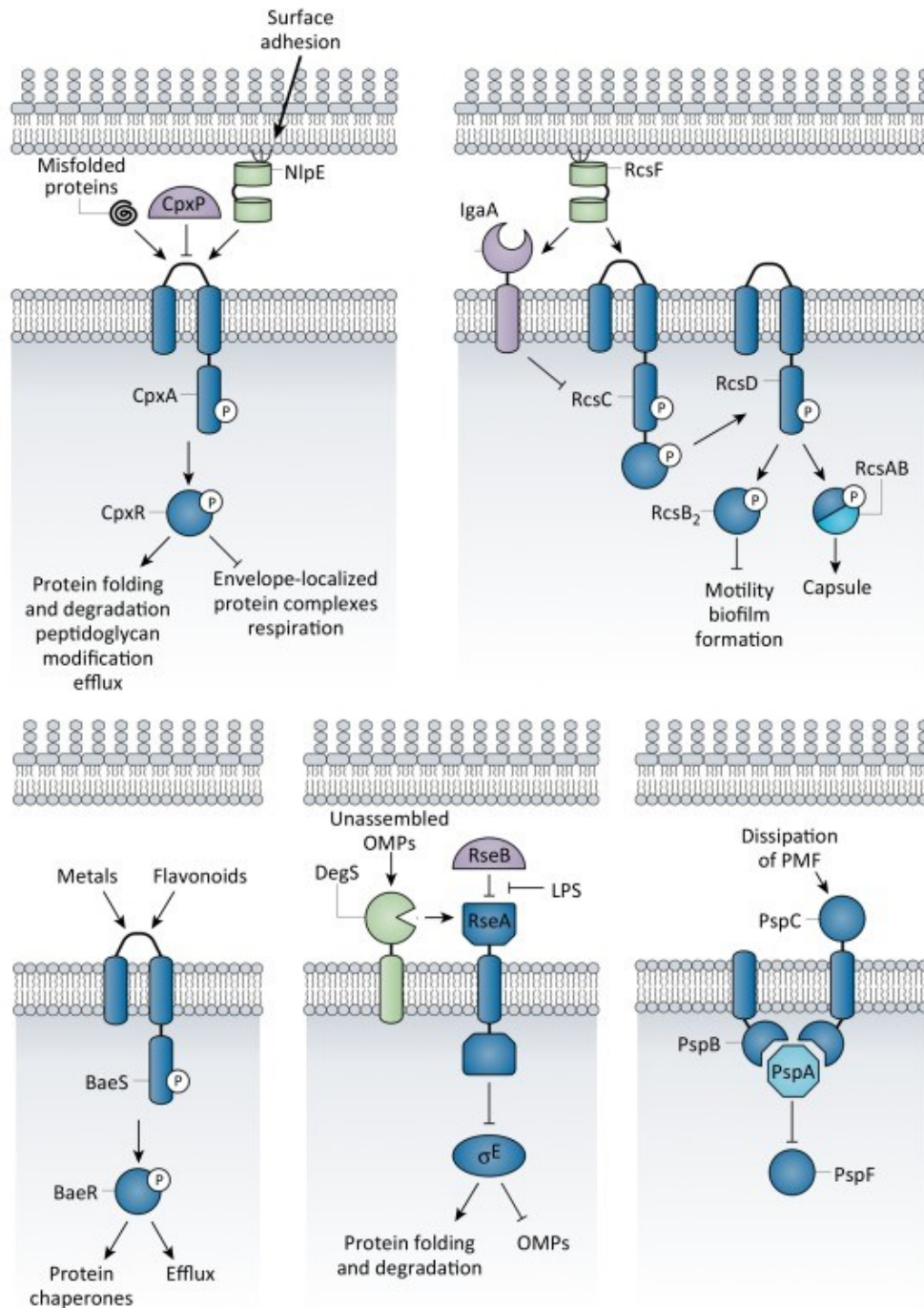
Fig 1.6 Gram-negative membrane stress responses. σ^E, Cpx, Bae and Rcs have been defined in *E. coli*. Psp have been defined in *Y. enterocolitica* and *S. enteric*. Taken from Randi L, *et al*., 2016.

1.8 *Rhodobacter sphaeroides*

1.8.1 Profile

Rhodobacter sphaeroides is a rod-shaped, gram-negative bacterium, belongs to the α-3 proteobacteria. *R. sphaeroides* is spread widely in aquatic habitats in nature. Its genome consists of two circular chromosomes and five endogenous plasmids which have been sequenced. The total genomic size is about 4.6Mbp (Zeilstraryalls JH, *et al.*, 1995), exhibiting a high GC content of ~67%. *R. sphaeroides* is a metabolically diverse organism exhibiting respiratory and photosynthetic lifestyles. The characteristic of growth makes *R. sphaeroides* a model organism for studying various growth features including hydrogen production, fermentation, anoxygenic photosynthesis, regulation of photosynthesis and the structural details of photosynthetic apparatus. For survival in different environments, *R. sphearoides* has a variety of adaption mechanisms and regulatory factors. Particularly it always suffers from $^1O_2$ (generated from photosynthesis) in the presence of oxygen and light. *R. sphaeroides* is a facultative model for studying the mechanism of responses to photo-oxidative stress.

1.8.2 Response to oxidative stress in *Rhodobacter sphaeroides*

General mechanisms of responses to oxidative stress are presented in chapter 1.5. Most of them also appear in *R. sphaeroides*. Previous studies indicated that H_2O_2 is eliminated by catalase (CatE) in *R. sphaeroides*. The expression of *catE* is dependent on OxyR. The OxyR deletion strain also shows more sensitively to H_2O_2 than the wild type 2.4.1 (Zeller T, *et al.*, 2004). The former mentioned major mechanism of defense against to superoxide is the superoxide dismutase (SOD), which catalytically scavenges $O_2^{\cdot-}$. It is reported that *sodB* of *R. sphaeroides* is induced by the superoxide-generating compound paraquat, and also increased in treatment of glutathione-expending reagents such as diamide and the hydroperoxide-generating reagents H_2O_2 and t-BOOH (Li KY, *et al.*, 2003). In *R. sphaeroides*, carotenoids are components of the photosynthetic apparatus and act as a quencher against singlet oxygen, which quench $^1O_2$ directly or prevent formation of $^1O_2$ by quenching triplet bacteriochlorophyll (BChl) (Griffiths M, *et al.*, 1955). It was shown that the concentration of $^1O_2$ is definitely increased in the carotenoids deletion strain (Glaeser

J*, et al.*, 2005). It was also reported that a glutathione peroxidase (RSP_2389) and a Zn-dependent hydrolase, glyoxylase (RSP_0799) were increased under photo-oxidative stress (Glaeser J*, et al.*, 2005). It was indicated that small RNAs are involved in response to oxidative stress (Waters LS*, et al.*, 2009). There are several small RNAs induced by photo-oxidative stress in *R. sphaeroides* (Berghoff BA*, et al.*, 2009). Among these small RNAs, Pos19 (photo-oxidative stress induced sRNA 19) and CcsR1-4 were identified that they negatively and positively affected glutathione concentration in the cell in response to singlet oxygen, respectively (Billenkamp F*, et al.*, 2015, Muller KMH*, et al.*, 2016). The interaction of small RNA and mRNAs often needs an RNA chaperone , the Hfq protein. The Hfq deletion strain have a strong defect of resistant to oxidative stress in *R. spaeroides* (Berghoff BA*, et al.*, 2011).
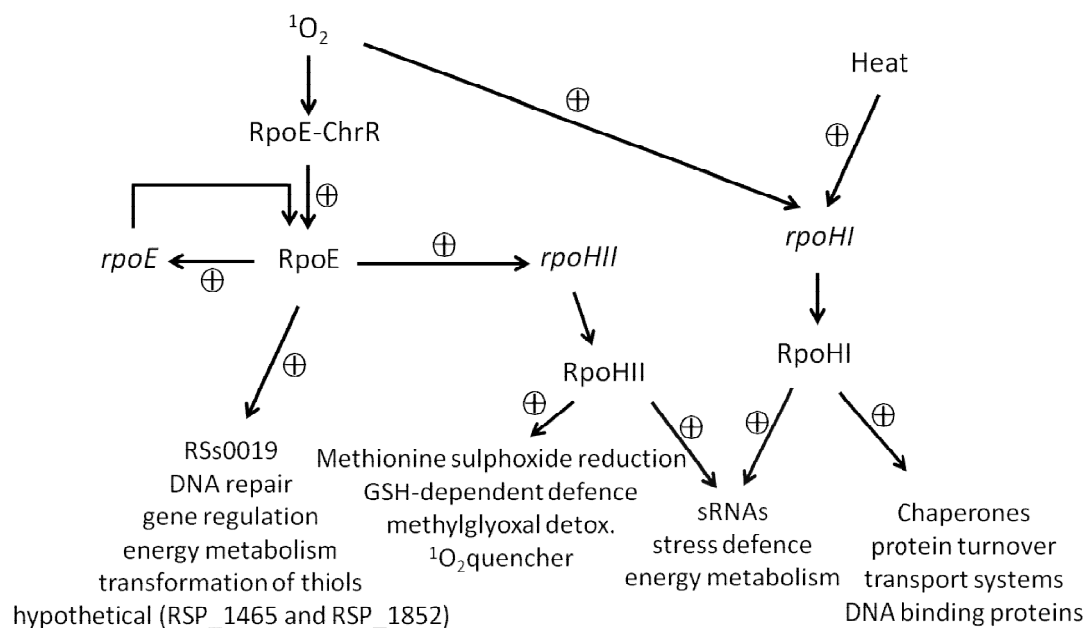
Fig 1.7 Current model of responses to singlet oxygen in *Rhodobacter sphaeroides*. Modified from Nuss *et al.*, 2010.

In *R. sphaeroises*, it emerged that part of response to oxidative stress are triggered by a cascade of alternative sigma factors (Fig 1.7). The alternative sigma factor RpoE that belongs to the group IV sigma factor has a major role in oxidative stress response, which is sequestered by anti-sigma factor ChrR under non-stress condition (Anthony JR*, et al.*, 2004). RpoE is released under $^1O_2$ stress. The activation of RpoE causes the expression of a number of genes. These genes are involved in DNA repair (*phrA*),

14

energy metabolism (cytochrome A, *cycA*; the cyclopropane/cyclopropene fatty acid synthesis RSP_1091 protein), transformation of thiols (RSP_3184 and *dsbG*), hypothetical functions (RSP_1465 and RSP_1852) or are transcribed to small RNA Pos 19 (Anthony JR*, et al.*, 2005, Glaeser J*, et al.*, 2011, Muller KMH*, et al.*, 2016). RpoE also regulates the expressions of its own gene and the gene of another alternative sigma factor, RpoHII (Nuss AM*, et al.*, 2009). A small set of genes related to $^1O_2$ stress are induced by RpoHII, including methionine sulphoxide reduction, GSH-dependent defense, methylglyoxal detox, and $^1O_2$ quenching. Another alternative sigma factor, RpoHI shares 46% identity with RpoHII in *R. sphaeroides*. RpoHI is activated by heat shock stress, then induces several genes expression for chaperones, protein turnover, transport systems and DNA binding proteins (Dufour YS*, et al.*, 2008). The two RpoH sigma factors share an overlapping regulon, including stress defense genes and genes for several sRNAs (Nuss AM*, et al.*, 2010).

1.8.3 PhyR-NepR-σ(EcfG) cascade in *Rhodobacter sphaeroides*

The PhyR-NepR-σ(EcfG) cascade is highly conserved in Alphaproteobacteria. *R. sphaeroides* also harbour a PhyR-NepR-σ(EcfG) cascade, consists of a PhyR homolog (RSP_1274), a NepR homolog (RSP_1273), a sigma factor (RSP_1272) and a histidine kinase(RSP_1271).

1.8.4 Growth phase regulation in *Rhodobacter sphaeroides*

In a previous study (Remes B*, et al.*, 2017), RNA seq and dRNA seq were used for global transcriptional analysis of gene expression levels in different growth phases and following outgrowth in *R. sphaeroides*, which indicated that 7%-11% of genes showed changed expression depending on the length of stationary phase compared to genes expression in the exponential phase. Some genes were induced during outgrowth, which was strongly influenced by the length of the preceding stationary phase. 1.5% of genes were induce in outgrowth after a short stationary phase, and 15.1% of genes were induced in outgrowth after a long stationary phase. A number of these altered genes are known as RpoHI/RpoHII dependent genes. Except RpoH, RpoE and sigma factors of unknown function (RSP_0415 and RSP_3095), genes for putative transcriptional regulators, and genes for sRNAs of unknown function were

induced in outgrowth after stationary phase. Many genes induced in outgrowth have known functions in stress defense (Remes B, *et al.*, 2017).

1.9 Aim of this research

In previous studies, it emerged that the response to oxidative stress is triggered by a cascade of alternative sigma factors RpoE, RpoHI and RpoHII in *R. sphaeroides*. The ECF sigma factor RpoE plays an important role in the oxidative stress response of *R. sphaeroides*, and under non-stress conditions RpoE is sequestered by the anti-sigma factor ChrR. RpoHI is activated by heat shock stress as well as $^1O_2$ stress. A major role of the PhyR-NepR-σ (EcfG) cascade in the general stress response was demonstrated for some bacterial species in Alphaproteobacteria.

The Alphaproteobacterium *R. sphaeroides* harbors a PhyR homolog RSP_1274 in the same genomic context as found in other members of this class. In this study, we focused on the effect of the PhyR homolog RSP_1274 of *R. sphaeroides* which was recognized as involved in general stress response and conserved in Alphaproteobacterium. However, the function of PhyR of *R. sphaeroides* is unknown. Therefore, this study addresses the questions which general stress response the PhyR of *R. sphaeroides* is involved in? whether the PhyR of *R. sphaeroides* plays a role in oxidative stress response?

Moreover, we demonstrate mechanisms of growth dependent gene regulation in *R. sphaeroides* and the link to regulation of the oxidative stress defense. The expression patterns of oxidative stress response related genes throughout the growth phase and outgrowth after 12h, 36 h, 60 h, 132 h of stationary phase were analyzed. In particular, the following questions should be answered: Why do some RpoHI/RpoHII dependent genes show different expression patterns? What are the additional factors involved in growth-dependent regulation?

2 Materials

2.1 Chemicals, reagents and kits

Products	Company
2-propanol	Sigma Aldrich
2-Mercaptoethanol	Roth
Acetic acid	Roth
Acrylamide (30% w/v)/ bis-acryetlamide (0.8% w/v)	Roth
Agarose (LE agarose Biozym)	Roche
Ammonium peroxide sulphate (APS)	Roth
Bovine serum albumin (BSA)	Roth
Bromophenol	Sigma Aldrich
Calcium chloride	Roth
CloneJET PCR cloning kit	Fermentas
CCCP	Sigma Aldrich
Deoxyribonucleoside triphosphates (dNTPs)	Serva
Ethanol	Roth
Ethanolamine	Sigma Aldrich
Ethidium bromide	Roth
Ethylene-diamine tetraacetate (EDTA)	Roth
Formaldehyde (37%)	Roth
Formamid	Sigma Aldrich
GeneRuler 1kb DNA *Ladder* Plus	Fermentas
GeneRuler 100 bp DNA *Ladder*	Fermentas
Glycerol	Roth

Glycine	Roth
Glutathione	Roth
Imidazole	Sigma Aldrich
Lysozyme	Boehringer Ingelheim
Low Range Protein Marker	Biorad
Lumi-Light PLUS Western Blotting Kit	Roche (Grenzach)
Lumi-Light Western Blotting Substrate I and II	Roche
Magnesium chloride	Roth
Magnesium sulphate	Merck
Methanol	Roth
Methylglyoxal	Sigma Aldrich
Methylene blue	Sigma Aldrich
N-2-hydroxyethylpiperazine-N'-2- ethanesulfonic acid	Roth
N, N, N ', N'-tetramethylene diamine	Roth
One-step RT-PCR KIT	Agilent
ortho-nitrophenyl-β-galactoside(ONPG)	
Ponceau-Red	Sigma Aldrich
Phenol/chloroform/isoamyl alcohol mixture (25:24:1)	AppliChem
Potassium chloride	Roth
Phenol (water grade)	Applichem
Phenol/Chloroform	Applichem
Polyvinylpyrrolidon	Sigma Aldrich
Polymyxin-B	Applichem
PEG800	Sigma Aldrich
Prestained Protein Marker, *Broad Range*	NEB

Prime-a-Gene® Labeling System	Promega
Plasmid Isolation KIT	Qiagen
peqGOLD TriFast™ Kit	PEQLAB
QIAEX II Agarose Gel Extraction Kit	Qiagen
Roti-Quant (Bradford-Reagent)	Roth
Sepermidine	Sigma Aldrich
Sodiumthiosulphate × 5H$_2$O	Merck
Spurenelemente (RÄ-medium)	Sigma
Sodium carbonate	Merck
Sodium cloride	Roth
Sodium dodecyl sulphate (SDS)	Roth
t-Butyl hydroperoxide (t-BOOH)	Sigma
Tris-(hydroxymethyl)-aminomethane	Roth
Triton X 100	Roth
Tween 20	Serva
Trypton	Difco
Urea	Roth
Vitamins (RÄ-medium)	Sigma Aldrich
[γ^{32}P] ATP 3000	Hartmann Analytic

2.2 Enzymes used in this study

Enzyme	Manufacturer
DNase I (RNase free)	Fementas or Invitrogen
RNase A	QIAGEN
Restriction endonucleases	New England Biolabs or fermentas
phusion DNA polymerase	QIAGEN

Taq DNA polymerase	QIAGEN
T4 DNA ligase	New England Biolabs
T4 polynucleotide kinase (T4 PNK)	New England Biolabs

2.3 Antibiotic used in this study

Table 2.1 Antibiotic used in this study and final concentration added to bacterial cultures

Antibiotic	*E.coli*	*R. sphaeroides*
Ampicilin	200 µg ml^{-1}	-
Ganamycin	10 µg ml^{-1}	10 µg ml^{-1}
Kanamycin	25 µg ml^{-1}	25 µg ml^{-1}
Tetracycline	20 µg ml^{-1}	1.5 µg ml^{-1}
Spectinomycin	10 µg ml^{-1}	10 µg ml^{-1}

2.4 Buffers used in this study

2.4.1 Commercial reaction buffers

Products	Company
10 × NEB buffer (1, 2, 3, and 4)	New England Biolabs
10 × PCR buffer	QIAGEN
HF-Buffer	QIAGEN
GC-Buffer	QIAGEN
Red Buffer	Fermentas
Green Buffer	Fermentas

2.4.2 Buffers used for gel electrophoresis

TBE (1×)	89 mM Tris/HCl
	89 mM Sodium borate
	2.5 mM EDTA
	pH 8.3
TAE (1×)	40 mM Tris/HCl
	40 mM Acetic acid

	0.4 mM EDTA
	pH 8.0
Separation buffer(4×)	1.5 M Tris/HCl
	0.4% (w/v) SDS
	pH 8.8
Stacking buffer(4×)	0.5 M Tris/HCl
	0.4% (w/v) SDS
	pH 6.8
DNA loading-dye	60% Glycerin
	0.01% Bromphenol blue
	0.01% Xylene cyanol
RNA loading-dye (FU mix)	6 M Urea
	80% Formaide, deionized
	10× TBE
	0.01% Bromphenol blue
	0.01% Xylene cyanol

2.4.3 Buffers used for Northern blot

Church buffer (low stringency)	300 mg BSA
	15 ml Na_2HPO_4 (1 M)
	300 μl EDTA (0.1 M) pH 8
	10.5 ml SDS (20%)
	add ddH_2O up to 30 ml
Washing buffer	200 ml 5× SSC
	0.4 ml SDS (20×)
	add ddH_2O up to 800 ml
Stripping buffer	200 ml 5× SSC
	4 ml SDS (20×)
	add ddH_2O up to 800 ml

2.4.4 Buffers used for β-galactosidase assay

Z-buffer	60 mM Na_2HPO_4

40 mM NaH$_2$PO$_4$

10 mM KCI

1 mM MgSO$_4$

50 mM β-mercaptoethanol

2.4.5 Buffers used in DNA extraction

Solution I	50 mM gluose
	25 mM Tris/HCl pH 8.0
	10 mM EDTA pH 8.0
Solution II	0.4 ml 5M NaOH
	1 ml 10% SDS
	H$_2$O *ad* 10 ml
Solution III	60 ml 5M KCl
	11.5 ml acetic acid
	28.5 ml H$_2$O

2.4.6 Buffers used in RNA extraction

Resuspend buffer	10.27 g Saccharose
	0.33 ml 3M NaOAc
	ad 100 ml ddH$_2$O
Lysis buffer	10 ml 20% SDS
	0.33 ml 3M NaOAc
	H$_2$O *ad* 100 ml

2.4.7 Other buffers used in this study

PBS (1×)	137 mM NaCl
	2.7 mM KCl
	10 mM Na$_2$HPO$_4$
	2.0 mM KH$_2$PO$_4$
	pH 7.4

TE buffer	1 ml 1M Tris HCl pH 8.0
	0.04 g EDTA (disodium salt)
	ddH$_2$O *ad* 100 ml
TBS (1×)	0.15 M NaCl
	0.015 M Tris/HCl
	pH 7.4
SSC (20×)	3.0 M NaCl
	0.3 M Sodium citrate

2.5 Culture medium used in this study

2.5.1 RÄ medium

RÄ liquid medium	
Malic acid	3.0 g
Ammonium sulphate	1.2 g
Magnesium sulphate-7-dihydrate	0.2 g
Calciumchloride dihydrate	0.07 g
Trace elements solution	1.5 ml
ddH$_2$O	1000 ml
Phosphate	20 ml
Vitamins	8 ml
RÄ agar plates	
Bacto Agar (1.6%)	8.0 g
RÄ liquid medium	500 ml
Trace elements solution	
Fe-(II)-citrate	500 mg
MgCl$_2$ × 4 H$_2$O	20 mg
ZnCl$_2$	5 mg
LiCl	5 mg
KI	2.5 mg
KBr	2.5 mg
CuSO$_4$	0.15 mg
Na$_2$MoO$_4$ × 2 H$_2$O	1 mg
CoCl$_2$ × 6 H$_2$O	5 mg

SnCl$_2$ × 2 H$_2$O	0.5 mg
BaCl$_2$	0.5 mg
AlCl$_3$	1 mg
H$_3$BO$_4$	10 mg
EDTA	20 mg
ddH$_2$O	1000 ml

Vitamins solution	
Niacin	3.0 g
Thiamine hydrochloride	1.2 g
Nicotinamide	0.2 g
Biotin	0.07 g
ddH$_2$O	1000 ml

Phosphate solution	
Potassium phosphate dibasic	45 g
Potassium phosphate monobasic	35 g
ddH$_2$O	1000 ml

2.5.2 Standard I medium

Standard I liquid medium	
Standard I Nutrient medium	25 g
Add ddH$_2$O up to	1000 ml

Standard I agar plates	
Bacto Agar (1.6%)	8.0 g
Add ddH$_2$O up to	500 ml

2.5.3 PY medium

PY liquid medium	
Trypton	10 g
Yeast extract	0.5 g
CaCl$_2$ (1M)	2.0 ml
MgCl$_2$ (1M)	2.0 ml
FeSO$_4$ (0.5%)	2.4 ml

Add ddH$_2$O up to	1000 ml	
	pH 7.0	
PY agar plates		
Bacto Agar (1.6%)	8.0 g	
Add PY liquid medium	500 ml	

2.6 Strains and plasmids used in this study

Table 2.2 Strains and plasmids used in this study

Strain or plasmid	Description or relevant features	Source/reference
Strains		
E. coli		
S17-1	*recA pro hsdR RP4-2*-Tc::Mu Km::Tn7 tra$^+$ Km^r Sp^r	(Simon R, *et al.*, 1986)
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi (lac-proAB)*	New England Biolabs
R. sphaeroides		
2.4.1	Wild type	
TF18	*rpoE chrR* mutation in 2.4.1, Tp^r	(Schilke BA, *et al.*, 1995)
ΔrpoH$_{II}$	2.4.1*rpoH$_{II}$*::Sp^r cassette	(Nuss AM, *et al.*, 2009)
ΔrpoH$_I$	2.4.1*rpoHI*::Km^r cassette	(Nuss AM, *et al.*, 2013)
ΔrpoH$_I$ΔrpoH$_{II}$	2.4.1Δ*rpoH$_{II}$rpoH$_I$* ::Km^r cassette	(Nuss AM, *et al.*, 2013)
ΔPhyR	2.4.1RSP_1274::Sp^r cassette	This study
ΔChrR	2.4.1ChrR::Tp^r cassette	(Schilke BA, *et al.*, 1995)
TF18ΔPhyR	TF18 *phyR*::Sp^r cassette	This study
ΔChrR PhyR	ΔChrR *phyR*::Sp^r cassette	This study
Plasmids		
pPHU281	Suicide vector for *R. sphaeroides.* Tc^r	(Hubner P, *et al.*, 1991)
pPHUΔRSP_1274:: Sp	pPHU281 with Sp cassette, flanked by the upstream and downstream regions of RSP_1274, Tc^r	This study
pHP45Ω	Sp cassette, Sp^r	(Prentki P, *et al.*, 1984)
pJET1.2	Ap cassette, Ap^r	Thermo

pBE4352	Km^r, pBE containing RSP_4352 promoter	(Remes B, *et al.*, 2015)
pBE*phyR*	Km^r, pBE4352 containing *phyR* fragment for *cfp* fusion	This study
pBBrI0557p::*lacZ*	pBBrI with promoter of RSP_0557, Gm^r	This study
pBBrI0904lp::*lacZ*	pBBrI with promoter of RSP_0904 (214 nt fragment from start codon), Gm^r	This study
pBBrI0904sp::*lacZ*	pBBrI with promoter of RSP_0904 (100 nt fragment from start codon), Gm^r	This study
pBBrI0960p::*lacZ*	pBBrI with promoter of RSP_0960, Gm^r	This study
pPHU*phrAlacZ*	pPHU234 with promoter of *phrA*, Tc^r	(Hendrischk AK, *et al.*, 2007)

2.7 Oligonucleotides used in this study

Table 2.3 Oligonucleotides

Name	Sequence	Purpose
1274_for1_kpnI	5´-CGCAGGTTGGTACCTTCGGTG-3´	RSP_1274 deletion
1274_rev2_EcoRI	5´-CGGTGCCCGAATTCGGTTCCG-3´	RSP_1274 deletion
1274_for3_EcoRI	5´-GGAGCCGGAATTCCTCATCACC-3´	RSP_1274 deletion
1274_rev4_XbaI	5´-GAAGATTGGGCGTCTAGAAGGCGC-3´	RSP_1274 deletion
1274check-for	5´-CGAAGTTCGTCCAGGCCTTC-3´	RSP_1274 deletion
1274check-rev	5´-TCTTGCGCAGACGATCCTCG-3´	RSP_1274 deletion
1274check-up	5´-CAGGATGGTGAAGAGCCAGG-3´	RSP_1274 deletion
1274check-down	5´-AACATGTCACAGTTTAATGCGGG-3´	RSP_1274 deletion
1274CF	5´-CATATGATGACCTCCGACACGACG-3´	RSP_1274 cloning
1274CR	5´-GGATCCGGCGCTGAGCGTTTCGGTG-3´	RSP_1274 cloning
1274RTF	5´-GGGACCAACCTGCCGTAT-3´	RT-PCR for RSP_1274
1274RTR	5´-CCAGACGAGATGGAACGC-3´	RT-PCR for RSP_1274
RT1272F	5´-CCTGCGGAACACCTTCTA-3´	RT-PCR for RSP_1272
RT1272R	5´-ATCCTCATAGGCGAAGCC-3´	RT-PCR for RSP_1272
p-0019	5´-GAGATAGCTCATCGGTCAGGTCC-3´	Northern probe
p-5S	5´-CTTGAGACGCAGTACCATTG-3´	Northern probe
RpoZ-A	5´-ATCGCGGAAGAGACCCAGAG-3´	RT-PCR for *rpoZ*
RpoZ-B	5´-GAGCAGCGCCATCTGATCCT-3´	RT-PCR for *rpoZ*
rpoE-A	5´-GTCTGGCAGAAGGCTCAT-3´	RT-PCR for *rpoE*

rpoE-B	5′-GTTCTCCTGCTGCATCTC-3′	RT-PCR for *rpoE*
CatE-A	5′-CTATCCGCTGATCGAGGT-3′	RT-PCR for *catE*
CatE-B	5′-GTCGGCATAGGAGAAGAC-3′	RT-PCR for *catE*
GloA-A	5′-GTCGAACTCACCTACAAC-3′	RT-PCR for g*loA*
GloA-B	5′-CGCACATGTCGTAGATATC-3′	RT-PCR for g*loA*
GloB-A	5′-GAACAATTACGCCTTCTC-3′	RT-PCR for *gloB*
GloB-B	5′-CATCAGCTGGTAGCTCTC-3′	RT-PCR for *gloB*

2.8 Efficiencies of the real-time PCR primers

Table 2.4 Primer efficiencies for real-time RT primers used in this study

Corresponding gene	Efficiency	Reference
RSP_1669 (*rpoZ*)	2.1	This study
rpoE	2.1	This study
RSP_1274	1.9	This study
RSP_1272	2.1	This study
catE	1.9	This study
gloA	2.0	This study
gloB	2.3	This study

2.9 Equipment used in this study

Equipment	Manufacturer
CFX96 Real-Time PCR	Bio-Rad
Cooling centrifuge, Sorvall RC-5C+	Kendro
Cooling centrifuge, Sorvall -5B	Kendro
Cooling centrifuge Z 323K	Hermle
Dialysis tubes (Type 20/32)	Roth
Electroporator (Micro Pulser)	Biorad

Filter paper, Whatman	Hartenstein
Fusion SL4 –Chemiluminesence detector	Vilber Lourmart
Glass wool	Serva
Halogen lamp	Conrad
Incubator Shaker (Model G 25)	New Brunswick Scientific
Microcon *(MWCO 3000)*	Millipore
MicroSpun G-25, G-50 Columns	GE Healthcare
Microarray scanner	Agilent technologies
Nitrocellulose membrane (BA Protran)	Schleicher & Schuell
NanoDrop 1000 spectrophotometer	Peq Lab
PCR cycler S100	Bio-Rad (Li-Cor)
Phosphoimager (*Molecular Imager* FX)	Biorad
Phosphoimager Screens	Biorad/Fuji
pH meter	Schott
Screen Eraser K	Biorad
Semi dry blot- Apparatus(Novablot)	Pharmacia
Sterile filter 0.22 μm	Nalagene
SpeedVac SC 110	ThermoScientific
SuperdexTM75,HiLoadTM16/60 (Gel filtration)	Pharmacia
Scintillation counter (LS 6500)	Beckman Coulter
Tabletop centrifuge Biofuge 13 and fresco , Uni Gen MR	Herolab
Ultrasound machine Sonoplus GM70 (Sonifier)	Bandelin
UV-StratalinkerTM 1800	Stratagene
Vacuum-blotter	Appligene
310 Genetic Analyzer	ABI – Prism

3 Methods

3.1 Microbiological Methods

3.1.1 Liquid cultivation of *Rhodobacter sphaeroides*

R. sphaeroides cells grow on the shaker (140 rpm) at 32°C in RÄ medium (see 2.5.1). The 100 ml-medium has to complemented with 800 µl vitamins (see 2.5.1) and 2 ml phosphate (see 2.5.1). The corresponding antibiotic was added to the media if required according to table 2.1. For inoculation, cells material are taken from glycerin stock or plating culture. The total number of cells in the culture are determined by the photometer.

3.1.1.1 Aerobic cultivation

For aerobic growth, 160 to 180 µM dissolved oxygen have to be in the culture. *R. sphaeroides* cultures are incubated in Erlenmeyer baffled flasks, filled to 30% of their capacity. The cultures are incubated on the shaker (140 rpm) at 32°C. In addition, *R. sphaeroides* cultures are incubated in flat bottles by gassing air to the culture via an aquarium pump, which also leads to an oxygen content of 160 to 180 µM oxygen. The optimal growth temperature of 32°C is here ensured via a water bath.

3.1.1.2 Microaerobic cultivation

For microaerobic growth conditions, Erlenmeyer flasks with a culture volume of 80% were agitated at 140 rpm in 32°C in the dark, resulting in a constant dissolved oxygen concentration of 25 to 30 µM during the mid-exponential growth phase.

3.1.1.3 *Rhodobacter sphaeroides* growth analysis

For comparison of the growth of two or more *R. sphaeroides* strains, pre-cultures of biological triplicates of each strain are prepared under the needed growth conditions, with respective antibiotics added to the medium. The next day the pre-cultures should be in the exponential growth phase (OD_{660} 0.4–0.8) and then diluted to an OD_{660} of 0.1 or 0.2 without the addition of antibiotics. The growth under the desired conditions is monitored by measuring the OD_{660} about each 1.5 h for making the growth curves.

3.1.2 Plate cultivation of *Rhodobacter. sphaeroides*

Agar plates for *R. sphaeroides* cultivation are prepared from molten RÄ agar medium (8 g bacto agar per 500 ml RÄ see 2.5.1) which is supplemented with 10 ml phosphate (see 2.5.1) and 4 ml vitamins (see 2.5.1) per 500 ml. Antibiotics needed for selection are added according to table 2.1 before pouring the lukewarm agar into *Petri* dishes. The plates are then allowed to cool until the agar is solidified. *R. sphaeroides* material cells from a glycerin stock or a liquid culture are streaked on the agar plates with an inoculation loop, respectively. For survival assay, small amounts of cells were spread on the plate, so that the single colony is distinct. Plates are wrapped with cling film and incubated at 32°C in the dark. For incubation the plates are placed upside down, to prevent condensed water from the lid of the Petri dish to drop onto the agar.

3.1.3 Liquid cultivation of *Escherichia coli*

Escherichia coli strains were grown in Standard-I Medium at 37°C in test tubes or Erlenmeyer flasks in the dark, shaking at 180 rpm for aerobic growth condition.

3.1.4 Plate cultivation of *Escherichia coli*

Agar plates for *Escherichia coli* cultivation are prepared from molten Standard-I agar medium (8 g bacto agar per 500 ml Standard-I see 2.5.2) in Petri dishes. Antibiotics needed for selection are added according to table 2.1 before pouring the lukewarm agar into *Petri* dishes. The plates are then allowed to cool until the agar is solidified. *Escherichia coli* material cells from a glycerin stock or a liquid culture are streaked on the agar plates with an inoculation loop, respectively. Plates are wrapped with cling film and incubated at 37°C in the dark. For incubation the plates are placed upside down, to prevent condensed water from the lid of the Petri dish to drop onto the agar.

3.1.5 Glycerol stock

The here described procedure for the preparation of glycerine stocks applies for both, *R. sphaeroides* and *E. coli*. Cells were collected from the overnight liquid culture in the exponential growth phase (OD_{660} 0.4-0.6) by centrifugation at 4°C. The pellet was resuspended by 2 ml relevant medium without antibiotics and mixed with 1 ml 80% glycerol. The suspension was transferred into two cryo-tubes, put in liquid nitrogen immediately, and then stored in -80°C refrigerator.

3.1.6 Optical density measurement of liquid cultures

The spectrophotometer is used for measuring optical density (OD) of fresh bacterial culture, which can determine the total amount of cells in culture. For the measurement 1 ml of the respective liquid culture is transferred into a polystyrene cuvette while 1 ml of fresh medium serves as the reference. The OD of *R. sphaeroides* is measured at 660 nm and the OD of *E. coli* is measured at 600 nm. Samples with an OD higher than 0.8 have to be diluted and measured again.

3.1.7 Preparation of competent *Escherichia coli* cells

Competent *E. coli* cells are used for the transformation of plasmid DNA. The cells are freed of remaining salt from the medium by several washing steps to avoid a fatal short circuit. A fresh overnight culture (30 ml) of the *E. coli* strain (for example, JM109, S17-1 or DH5α) is inoculated into 1 liter of St-I medium and incubated shaking at 37°C in the dark until reached an OD_{600} of 0.6-0.8. The cells are collected in 500 ml centrifuge beakers. The beaker is incubated on ice for 30 min and centrifuged at 6.000 rpm for 8 min (all centrifugation steps at 4°C, in F10-S rotor, Sorvall RC 6 plus/RC 5C plus). The supernatant is discarded, and all cell pellets are resuspended in 350 ml cold Roth H_2O. After 5 min centrifugation, the washing step is repeated once with 200 ml cold Roth H_2O. And then the cell pellets are resuspended in 20 ml cold 10% glycerol solution and is centrifuged for 10 min. To prepare the cells for storage they are resuspended in 3 ml cold 10% glycerol solution and aliquoted into 1.5 ml micro-centrifuge tubes. The aliquots are immediately frozen in liquid nitrogen and stored in -80°C refrigerator.

3.1.8 Electro-transformation of plasmid DNA to *Escherichia coli*

For transformed plasmid DNA to *E. coli* cells, a 2.5 kV electric shock is used for making the uptake of DNA possible into the competent cells (Micro Pulser, BioRad). For the electro-transformation 50 μl of electro-competent cells are thawed on ice. The cells are given into an electro-poration cuvette together with about 200 ng of plasmid DNA. The cuvette is placed in the Micro Pulser, and the voltage of 2.5 kV is set up for 5 ms. 500 μl of St-I medium is added to the cells immediately after the electro pulse, then the mixture is transferred to a virgin 1.5 ml micro-centrifuge tube and incubated on the shaker with 180 rpm at 37°C for 60 min. After the incubation, the

cells are spread on St-I agar plates containing the necessary antibiotics which are incubated at 37°C in the dark overnight.

3.1.9 Plasmid conjugation to *Rhodobacter sphaeroides*

Since electro-transformation of DNA to *R. sphaeroides* is not possible, the di-parental conjugation(horizontal gene transfer) is used to transfer plasmid DNA. First desired plasmid DNA is electrotransferred into *E. coli* S17-1 stains. *R. sphaeroides* acceptor cells pre-cultured until an OD_{660} of about 0.6 and collected at 4000 rpm at room temperature for 5 min, the supernatant was discarded. 500 μl of *E. coli* S17-1 with the desired plasmid overnight culture was taken and together with the former *R. sphaeroides* acceptor cells pellet and spun down at 4000 rpm at room temperature for 5 min, the supernatant was discarded. The cell pellet is resuspended by 100 μl RÄ-medium and the suspension was transferred onto a membrane filter (0.45 μm, 25 mm, Carl-Roth) on a PY agar plate. After at least 4 hours incubation at 32°C, cells on the filter are washed down by 1 ml RÄ-medium, and 100 resuspended cells are spread on RÄ agar plates containing the respective antibiotics. The plates are incubated at 32°C in the dark for three days until single colonies have grown.

3.1.10 Zone of inhibition assay

For the zone of inhibition assay, all *R. sphaeroides* strains of interest are grown in RÄ medium under microaerobic condition to OD_{660} of 0.5. 500 μl of pre-culture is mixed with 5 ml pre-warmed soft agar (RÄ medium containing 0.8% agarose) and poured on solid RÄ agar plates (1.6% agarose in RÄ medium). Disks are placed at the center of plates, 5 μl of 10 mM methylene blue, 750 mM t-BOOH, 700 mM diamide or 700 mM paraquat are added on the filter disks. The plates of methylene blue are incubated under the light (60 W lamp), other plates were incubated in the dark. The diameters of growth inhibition areas were measured after incubation at 32°C for 3 days.

3.1.11 β-galactosidase assay

β-galactosidase assays were performed according to the method of Miller (1972), Which is a fast method to measure the activity of a promoter of interest (transcriptional fusion). For this purpose reporter plasmids carrying the *lacZ* gene, which encodes β-galactosidase, are used for gene fusions. β-galactosidase is an

enzyme which catalyzes the hydrolysis of substrate ortho-nitrophenyl-β-galactoside (ONPG) to galactose and ortho-nitrophenol. The latter has a yellow color which can be measured by photometer at 420 nm, where the amount of yellow color directly correlates with the amount of enzyme and thereby the rate of translation or transcription.

For the measurement of RpoE activity, *R. sphaeroide* strains of interest carrying reporter plasmid (pPHU*phrAlacZ*) were inoculated from a single colony into 40 ml RÄ medium and grown under microaerobic growth condition. Cultures were diluted to an OD_{660} of 0.2 in a flat bottle and allowed to double once under aerobic growth conditions in darkness. Cells were collected before (0h), 1 and 3h under singlet oxygen (high light 880 Wm^{-2} and 50 nM methylene blue) and organic peroxide (360 µM of t-Butyl hydroperoxide (t-BOOH)) stress. The sample is transferred to a 2 ml micro-centrifuge tube and centrifuged with 13.000 rpm at 4°C for 10 min. The supernatant is discarded, and the cell pellet is stored at -20°C

For the demonstration of growth phase-dependent regulation, *R. sphaeroide* strains of interest carrying reporter plasmid were inoculated from a single colony into 40 ml RÄ medium and grown under microaerobic growth condition. The cultures were inoculated into four 500ml Erlenmeyer flasks with 400 ml RÄ medium, and four flasks of culture were diluted to OD_{660} of 0.2 after 24 hours, 48 hours, 72 hours or 144 hours of growth respectively. Cells were collected throughout the growth phase from 0 hour to 144 hours and outgrowth after 24 hours, 48 hours, 72 hours and 144 hours. The sample volume (100-1000µl) depends on the amount of β-galactosidase in the sample which should in total not be as high as the assay reaches its saturation. Therefore a test with different sample volumes should be performed in advance. And the same amount of cells across the growth phase should be collected, so different volumes of sample were collected according to the optical density at 660 nm. The sample is transferred to a 2 ml micro-centrifuge tube and centrifuged with 13.000 rpm at 4°C for 10 min. The supernatant is discarded, and the cell pellet is stored at -20°C

For the assay, each cell pellet is resuspended in 750 µl Z-buffer. In the next step, the cells are lysed by adding 10 µl 10% SDS and 20 µl chloroform and vortexing the samples for 30 sec, and then incubated at 28°C for 5min. The β-galactosidase assay is carried out at 28°C, the reaction can be started by adding 200 µl preheated ONPG (4

mg/ml, 28°C) to each sample and inverting, after an incubation time of 3-5 min(depends on β-galactosidase activity). To stop the reaction, 500 μl sodium carbonate (1M, Na_2CO_3) is added, and the samples are mixed again by inverting. When all reactions are stopped the samples are centrifuged at 13.000 rpm and RT for 3 min to spin down the cell debris. For the measurement, 800 μl of each sample, as well as the blank, are transferred into polystyrene cuvettes, and the absorption is measured at 420 and 550 nm. From these data and the measured OD_{660}, the following calculation can be carried out to receive values for the β-galactosidase activity depicted in Miller Units (MUs).

$$\text{Miller Units} = \frac{[1000 \times (A420 - 1.82 \times A550)]}{(V \times t \times OD660)}$$

V: Sample volume [ml]

t: Reaction time [min]

3.1.12 Ultraviolet assay

For Ultraviolet assay, all *R. sphaeroides* strains of interest are grown in RÄ medium under microaerobic condition to OD_{660} of 0.5 and diluted to a final dilution of 0.5 $\times 10^{-6}$. 50 μl of the dilution was distributed on four plates. Plates were exposed to Ultraviolet (UV) light of 100 J m^{-2} (254 nm) and incubated under the indicated temperature in the light or in the dark. The plates incubated in the light overnight were then transferred to the dark. All the plates are incubated 3 days until the single colony can be distinct. The amount of colonies were counted. Survival rates of UV exposed cells compared with non-exposed cells were calculated.

3.1.13 Survival assays

All *R. sphaeroides* strains of interest are grown in RÄ medium to OD_{660} of 0.5 in microaerobic condition.

For spot survival assays, ethanol (12%), SDS (0.015%) and EDTA (30mM) are added. The growth of viable cells was monitored by spotting 5 μl from consecutive 10-fold dilutions onto agar plates after 30 and 60 minutes of ethanol, 15 and 30 minutes of SDS and EDTA. For counting survival cell numbers, cultures treated in the same way or treated for 60 minutes with polymyxin B (2.5μg/ml), or 60 minutes of CCCP(25

mM) were further diluted 10^{-1} to 10^{-6} (dependent on the strain) and 50 μl are spread on the plate. Colonies were counted after three days incubation at 32 ºC in the dark.

3.1.14 Stress experiment for qPCR

For analyzing stress response of RNA in *R. sphaeroides*, strains of interest were cultured either in aerobic condition or microaerobic condition. After the optical density 660nm of culture reached 0.4 (exponential growth phase), different reagents were added. 250 μM paraquat, 360 μM tBOOH, 1 mM H_2O_2, 500 μM diamide, 500 mM NaCl, or 10 μM $CdCl_2$, 0.005% SDS and 1 mM EDTA, 2.5% ethanol, 1 μg/ml polymyxin B or 10 μM CCCP as final concentration was added in culture in microaerobic growth condition. Photooxidative stress experiment was performed under aerobic conditions by adding 0.2 μM methylene blue to the culture and irradiation with 800 W/m² white light. For the heat shock response, cells were grown at 32°C under microaerobic conditions and shifted to 42°C. All the samples were collected before (0 min) and 7 min after different reagents added or after cultures shifted to 42°C.

3.2 Molecular methods

3.2.1 DNA extraction

3.2.1.1 Chromosomal DNA extraction from *Rhodobacter sphaeroides*

For the extraction, *R. sphaeroides* liquid culture with an OD_{660} of about 0.6 are harvested and centrifuged at 8000 rpm for 10 min at 4°C (RC 5C plus/ RC 6 plus; SS-34rotor). The cells are resuspended in 700 μl TE buffer, which is prepared fresh with 1 mg/ml of lysozyme and are transferred into a virgin 1.5 ml micro-centrifuge tube. The cells are lysed via incubation at RT for 10 min. 30 μl 10% SDS and 16 μl proteinase K (20 mg/ml) are added for the lysis of cellular proteins. The samples are mixed through inverting and are incubated for 1 h at 37°C. After the incubation, 100 μl of 5M NaCl and 80 μl Cetrimonium bromide (CTAB; detergent) are added. The sample is heated at 65°C for 10 min in a heating block. To precipitate the cell debris and proteins, 750 μl of a chloroform/isoamyl alcohol mixture (24:1) are added and mixed by inverting the tube. The mixed sample is centrifuged at 13.000 rpm for 5 min at RT and 650 μl of the upper, aqueous phase is transferred into a virgin 1.5 ml micro-

centrifuge tube. One volume of a phenol/chloroform/isoamyl alcohol mixture (25:24:1) is added, the sample is mixed by inverting, and centrifuged at 13.000 rpm for 5 min at RT. After transferring 550 μl of the upper, aqueous phase into a virgin 1.5 ml micro-centrifuge tube and 0.6 volume of isopropanol is added. The DNA is precipitated by incubation at RT for 5 min and subsequent centrifugation with 3.000 rpm for 3 min at RT. The supernatant is discarded, and the DNA is washed by 200 μl of cold 70% ethanol and repeated centrifugation. The supernatant is pipetted out carefully, the DNA is dried in the SpeedVac for 4 min and dissolved in TE buffer to be stored at 4°C.

3.2.1.2 Plasmid DNA extraction from *Escherichia coli*

For the extraction, *E. coli* containing plasmid liquid culture are harvested in 1.5 ml micro-centrifuge tube. The sample is centrifuged at 8000 rpm at RT for 5 min, then discard supernatant. 200 μl solution I (see 2.4.5) are added and mixed with pellet by vortex. Afterward 200 μl solution II (see 2.4.5) are added and mixed vigorously. Next step 200 μl solution III(see 2.4.5) are added in the former mixture and mixed vigorously. And then the mixture is centrifuged with 13000 rpm at 4°C for 15 min after a 10 min incubation on ice. The supernatant are transferred to a new 1.5 ml micro-centrifuge tube. And 500 μl isopropanol are added in the supernatant and mixed vigorously. And then spin it at 13000 rpm at 4°C for 20 min. Supernatant are discarded, and 300 μl 70% ethanol are added in for washing plasmid DNA pellet with 13000 rpm centrifugation. The supernatant are pipetted out carefully. Plasmid DNA is dried in the SpeedVac for 4 min and dissolved in TE buffer to be stored at 4°C.

3.2.2 Gel extraction

3.2.2.1 Extraction of DNA fragment from agarose gels

For the extraction of interest DNA fragment, a PCR product or a plasmid after restriction are separated by electrophoresis in agarose gel. Then the target DNA fragment is cut out under UV light after EB staining. The DNA fragment was extracted from agarose gels by using QIAEX II Gel Extraction Kit or QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instruction.

3.2.2.2 Extraction of DNA fragment from polyacrylamide gels

To extract DNA from a polyacrylamide gel, the principle of diffusion is used. The part of gel containing the DNA is cut out from the polyacrylamide gel, The gel slice is pounded to pieces and incubated at 37°C in 400 µl diffusion buffer overnight. After the incubation, the supernatant is transferred to a new 1.5 ml Eppendorf tube and the gel pieces are rinsed with 200 µl diffusion buffer. One volume of isopropanol was mixed with the supernatant and incubated at -20°C at least 30 min and subsequently centrifuged at 13,000 rpm for 30 min at 4°C. The pellet is washed once with 80% ethanol. Pellet is dried in the SpeedVac dry for 5 min after the ethanol is discarded. The dried DNA pellet is dissolved in 20 µl Roth H_2O and stored at -20°C.

3.2.3 DNA purification

3.2.3.1 Purification of PCR product

The PCR product is purified by innuPREP DOUBLEpure Kit according to the manufacturer's instruction.

3.2.3.2 N-Butanol purification

To remove various salt contaminations after ligation, a sample of recombinant plasmid DNA was precipitated by n-butanol. First, 30µl Roth H_2O are added into the sample to a final volume 50 µl, then mixed with 500 µl n-butanol. The mixture is shaken by vortex for 30 seconds. The mixture is centrifuged at 13,000 rpm at 4°C for 30 min. The pellet is rinsed once with 80% ethanol and centrifuged at 13,000 rpm for 3 min. Pellet is dried in the Speed Vac for 5 min after the ethanol is discarded. The dried pellet is dissolved in 20 µl Roth H_2O and stored at -20°C.

3.2.4 RNA extraction

To extract total RNA from *R. sphaeroides* cells, two methods can be used. Now the method was used in this study is the so-called hot phenol extraction based on Scherrer and Darnell (1962) and is used to extract high quantities of RNA. It was shown that hot phenol enriches RNAs with a size between tRNAs and large rRNAs (Damm K*, et al.*, 2015). This RNA can be used for Northern blot analysis.

Cells from 20 ml *R. sphaeroides* liquid culture are harvested in centrifuge beakers prefilled with one fourth volume of ice by centrifugation with 10.000 rpm for 10 min at 4°C. The cell pellet is resuspended by 500 µl cold RÄ medium and transferred into a 1.5 ml safe-lock micro-centrifuge tube. The resuspended is centrifuged at 13000 rpm at 4°C for 5 min. The supernatant is discarded. And the pellet is resuspended in 250 µl of cold resuspend buffer. After addition of 250 µl cold lysis buffer which is used for lysing the cells, the sample is mixed by vortex until it is homogenous and incubated in a water bath at 65°C for 90 seconds. In the next step, 500 µl of pre-warmed (65°C) phenol-water is added, the sample is mixed again by vortex and incubated at 65°C for 3 min. The sample is frozen immediately for at least 30 seconds in liquid nitrogen. The tube is taken out from liquid nitrogen and put on the table for 10 seconds avoiding broken the tube during centrifugation. And then the sample is centrifuged at 13.000 rpm for 10 min at RT to separate the phases. The immiscible two phases of water and phenol are distinct, and the denser phenol sits at the bottom of the tube. Approximately of the upper, aqueous phase is transferred into a new 1.5 ml safe-lock micro-centrifuge tube carefully and 500 ml phenol addition, 65°C incubation, freezing and centrifugation is repeated two times. The third repeat of the aqueous phase is transferred into a new 1.5 ml micro-centrifuge tube carefully and mixed with 400 µl chloroform/isoamyl alcohol vigorously to remove the remaining phenol. After centrifugation for 3 min again of the aqueous phase is transferred into a virgin 1.5 ml micro-centrifuge tube and mixed with 500 µl cold 96% ethanol and 1/10 volume of 3M sodium acetate (NaOAc, pH 4.5). The mixture is incubated at -20°C overnight. And the RNA is precipitated by centrifugation with 13.000 rpm, at 4°C for 20 min. The supernatant is discarded, and the RNA rinsed by addition of 200 µl cold 80% ethanol and centrifugation. The ethanol is discarded. The pellet is dried in the Speed Vac for 5 min after the ethanol is discarded. The dried RNA pellet is dissolved in 20 µl Roth H_2O and stored at -20°C.

3.2.5 DNA degradation in RNA sample

The DNA in the RNA sample is degraded by Kit according to the manufacturer's instruction.

3.2.6 Gel electrophoresis

3.2.6.1 Agarose gel electrophoresis

TAE or TBE agarose gels are used to separate and visualize DNA fragments. Different concentration agarose gels are used to separate DNA fragments according to their size. 1% TAE or TBE is for the size from 0.3 to 20 kb of DNA fragment and 1.5-2% TBE is for the size from 0.4 and 2 kb of DNA fragment. The agarose was melted by boiling briefly in TAE/TBE buffer. The deliquescent agarose poured into a taped Gel tray which already has a comb. After freezing, the gel can be used for running the DNA sample in the TAE/TBE buffer respectively. DNA samples are mixed with 10× DNA loading dye before loading into the gel. Electrophoresis was performed at 100 to 150 mA for 30 min. DNA bands are stained by ethidium bromide solution for 8 min. and the stained Gel is observed under UV light.

3.2.6.2 Polyacrylamide gel electrophoresis

Polyacrylamide gel has high resolution and is used to separate and visualize small DNA fragments which have the size less than 300 bp or total RNA. The reaction mix was prepared according to the recipe (Table 3.1). All components are pipetted in a beaker and mixed well. The gel mixture is poured into between a glass and a ceramic plate, which are placed in the gel pouring device. A comb for the needed number of pockets is placed into the gel. After the gel complete polymerization, the comb was removed. The DNA samples are mixed with 10× loading dye and loaded into the gel. The RNA samples are mixed with 6× FUmix and loaded into the gel. Electrophoresis was performed with (1×) TBE for 40 minutes at 150 V. The gel were visualized by staining with ethidium bromide solution.

Table 3.1 The recipe of polyacrylamide gel

Polyacrylamide gel for DNA		Polyacrylamide gel for RNA	
40 % (w/v) Acrylamide	1.5 ml	Urea	8 M
10 x TBE	0.6 ml	40 % (w/v) Acrylamide	1.5 ml
6% APS (w/v)	30 μl	10 x TBE	0.6 ml
TEMED	6 μl	6% APS (w/v)	30 μl

H₂O	3.9 ml	TEMED	6 µl
		H₂O	3.9 ml

3.2.7 Amplification of DNA

All components are pipetted in PCR tube on ice (Table 3.2). The RCR is running in a Thermal cycler (S1000 or T100, BioRad) according to the PCR protocol (Table 3.3)

Table 3.2 Composition of a 10 µl standard PCR

Component	Taq	Phusion
ddH₂O	6.4 µl	6.3 µl
dNTPs [2.5 mM each]	0.4 µl	0.2 µl
Primer forward [10 µM]	0.5 µl	0.2 µl
Primer reverse [10 µM]	0.5 µl	0.2 µl
10x *Taq* buffer	1 µl	-
5x GC-buffer	-	2 µl
Taq polymerase [5 U/µl]	0.2 µl	-
Phusion polymerase [2 U/µl]	-	0.1 µl
DNA Template	1 µl	1 µl

Table 3.3 Standard PCR program

1. Initial Denaturation	95°C (*Taq*) / 98°C (*Phusion*)	5 min
2. Denaturation	95°C (*Taq*) / 98°C (*Phusion*)	30 sec
3. Annealing	Primer-specific temperatue	30 sec
4. Extension	72°C	1 min/kb (*Taq*)/30 sec/kb (*Phusion*)

Back to step 2 for 30 times

5. Final Extension	72°C	10 min

| 6. Hold | 8°C | ∞ |

3.2.8 Restriction

The restriction is performed using the restriction enzymes from New England Biolabs or Fermentas according to the manufacturer's instruction. The restricted products are separated by agarose gel and extracted according to 3.2.6.1.

3.2.9 Ligation

For ligation of PCR product into pJET cloning vector is performed according to the manufacturer's instruction (CloneJET PCR Cloning Kit handbook, Thermo Fisher Scientific). All the components are pipetted in a tube according to the recipe (Table 3.4) and mixed well. The mixture is incubated at RT for 30 min. After incubation, the mixture is precipitated by n-butanol (see 3.2.3.2).

For standard ligation of the DNA fragment from restriction into a vector, a molar ratio of 3:1 insert to vector is used. The total amount of vector in the reaction is at least 100 ng. The components are pipetted in tube according to table 3.4 and mixed well. The mixture is incubated at 16°C overnight. After incubation, the mixture is precipitated by n-butanol (see 3.2.3.2).

Table 3.4 Reaction components of ligation

pJET cloning vector		Standard ligation	
10 × ligation master mix	2 µl	10 × T4 ligase buffer	2 µl
pJET 1.2 vector (50 ng/µl)	1 µl	Vector	100 ng
PCR product	2 µl	DNA fragment	ratio of 3:1/ DNA:Vector
T4 DNA ligase	1 unit	T4 DNA ligase	1 unit
ddH$_2$O	add to 20 µl	ddH$_2$O	add to 20 µl

3.2.10 Quantitative real-time RT-PCR

For the RT-PCR, total RNA firstly isolated with the Hot phenol method (described in 3.2.4). Before running RT-PCR, the DNA left in the RNA sample have to be degraded (described in 3.2.5). After DNA degradation, the RNA sample is precipitated and

checked quality. Then the RNA samples are diluted to 20 ng/μl. The composition of the RT-PCR reaction using the Brilliant III Ultra - Fast SYBR® Green QRT-PCR master mix kit (Agilent) is shown in table 3.5. Besides an RT-PCR run for the mRNA of interest (target), a run with a primer pair for the house-keeping gene rpoZ (reference) is needed for normalization. The RT-PCR is performed by using a C1000TM Thermal cycler (Bio-Rad Laboratories) and the relative mRNA transcripts were quantified by CFX manage 3.1 (Bio-Rad Laboratories) according to the programme (shown in table 3.6). The calculation of the relative expression ratio (R) of a target gene in comparison to the reference gene (ref) is carried out using the efficiency corrected quantification model by Pfaffl (2001):

$$R = \frac{(E_{target})\Delta Ct_{target}(control-s}{(E_{Ref})\Delta Ct_{Ref}(control-san}$$

E: amplification efficiency

C: amplification cycle

Table 3.5 Composition of a 10 μl RT-PCR reaction

RNA (20 ng/μl)	2 μl
2× RT-PCR master mix	5 μl
Primers mix (10 pmol/μl each)	2 μl
DTT	0.1 μl
RNAse Block	0.5 μl
RNase/DNase-free water	0.4 μl

Table 3.6 Standard RT-PCR programme

Programme	50°C 10 min
	95°C 3 min
	94°C 5 sec
	Annealing temperature 5 sec
	60°C 5 sec
	72°C 5 min

3.2.11 Northern blot with polyacrylamide urea gel

For the Northern blot with polyacrylamide urea gel, total RNA (5 g per lane) is mixed with 10 µl Fu-Mix and incubated at 65°C for 10 min. Electrophoresis of RNA sample is carried out on polyacrylamide gel (described in 3.2.6.2). The running is completed, the RNA is blotted from gel to a nylon membrane (*Roti®-Nylon plus*, pore size 0.45 µm; Roth) by a semi-dry electro-blot. In this step, a stack of blotting paper put in the electroblotter (Albert LabScience). The stack is arranged in the following order from the bottom to top: three blotting papers soaked in 1× TBE, nylon membrane, gel and three blotting papers soaked in 1× TBE. The air bubbles have to be removed among these papers. The electro-blot runs at 250 mA for 2.5h. After the blotting, the gel is checked whether all RNA are transferred to nylon membrane completely, which is performed by UV light after staining the gel. And the RNA is cross-linked to the membrane with UV light twice using the UV (1200 kj UV light) Stratalinker 1800 (Stratgene). For detection of an RNA with a hybridization labeled probe (see 3.2.12).

3.2.12 Radioactive labelling of nucleic acids

To detect an RNA of interest on a membrane from the Northern blot technique described above, A probe is needed for hybridization with that RNA. Here the used probes are end-labeled with $[\gamma^{32}P]$ labeled dATP. The DNA oligo is mixed with T4 PNK plus, PNK buffer A (Thermo Scientific) and 30 µCi $[\gamma^{32}P]$dATP (Hartmann Analytic) (table 3.7). The reaction is incubated at 37°C for 1 h. The reaction is stopped by 40 µl STE buffer. Afterward, the labeled probe is purified by *MicroSpin* G25 illustra-column (GE Healthcare).

Table 3.7: Reaction for oligonucleotide end labeling with polynucleotide kinase (PNK).

Oligonucleotide (10 µM)	2 µl
PNK buffer A	1 µl
T4 PNK (10 U/µl)	1 µl
ddH$_2$O	3 µl
$[\gamma^{32}P]$dATP (10 µCi/µl)	3 µl

4 Results

4.1 The role of PhyR in *Rhodobacter sphaeroides*

4.1.1 Genomic context of the *phyR* gene RSP_1274 in *Rhodobacter sphaeroides*

The PhyR-NepR-σ (EcfG) cascade is conserved and has a central role in general stress responses in all essentially free-living Alphaproteobacteria, and their genes have the same genomic context (Fig 4.1) (Gourion B*, et al.*, 2008, Staron A*, et al.*, 2009). PhyR comprising of an amino terminal output domain and a carboxy-terminal receiver domain, was described first in *Methylobacterium extorquens* AM1 (Gourion B*, et al.*, 2006). It regulates genes expression involved in general stress responses. The amino terminal output domain is a conserved general stress regulator in Alphaproteobacteria and shows high sequence similarity to sigma factors SigT of *Caulobacter crescentus* (Alvarez-Martinez CE*, et al.*, 2007) and RpoE2 of *Sinorhizobium meliloti* (Bastiat B*, et al.*, 2010). The carboxy-terminal receiver domain is also conserved. Phosphorylation of an aspartate in this domain leads to a conformational change which subsequently activates the effector domain (Bourret RB, 2010). PhyR~P titrates anti-sigma factor NepR away from σ (EcfG), therefore releasing the σ-factor which binds to RNA polymerase and initiates transcription of its target genes (Fig 1.3). Thus the phosphorylated PhyR acts as an anti anti-sigma factor.
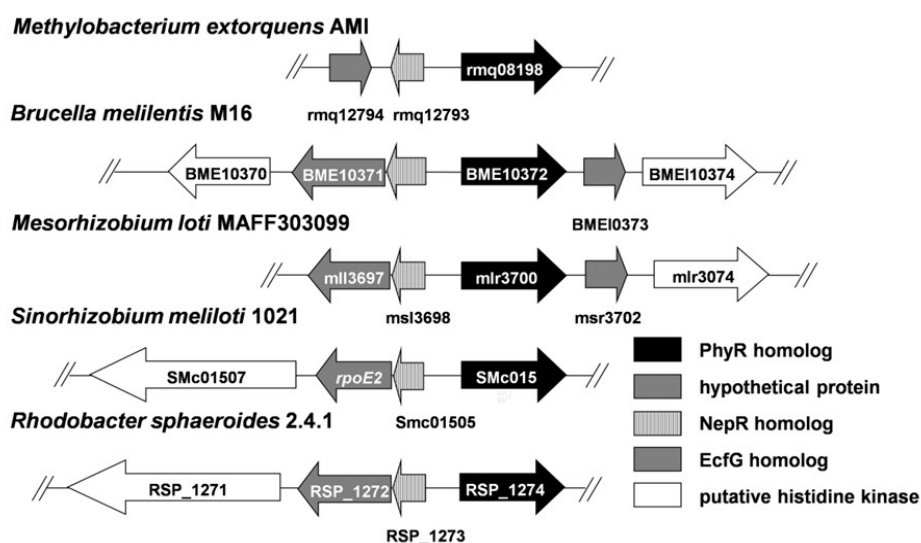
Fig 4.1 Genetic organization of the *phyR* locus in different Alphaproteobacteria (Li QF*, et al.*, 2018).

A single PhyR orthologue RSP_1274 identified by blastp search in *R. sphaeroides* shares 50%, 49%, 49% and 48% identity respectively with the PhyR proteins from *Sinorhizobium meliloti*, *Methylobacterium extorquens* AM1, *Bradyrhizobium japonicum* and *Caulobacter crescentus* (Fig 4.2). An alternative sigma factor (RSP_1272) and a sensor histidine kinase (RSP_1271) are encoded upstream of the *phyR* gene (RSP_1274) on the opposite strand (Fig 4.1) (Francez-Charlot A*, et al.*, 2015). The RNA polymerase sigma factor RSP_1272 shares 45% identity with RpoE2 from *S. meliloti* and 42% identity with SigT from *C. crescentus*. The gene for the 61 aa NepR protein was found between the *ecfG* gene and the *phyR* gene in other alphaproteobacteria. In this position, *R. sphaeroides* encodes a 68 aa protein with 25% identity to NepR. A protein of the Crp-Fnr family is encoded downstream of *phyR*. The gene arrangement for *R. sphaeroides* and selected Alphaproteobacteria is shown in Fig 4.1.

Fig 4.2 Alignment of PhyR in *Rhodobacter sphaeroides* and selected Alphaproteobacteria.

Differential RNA sequencing (dRNAseq) revealed the transcriptional organization for these genes. This method compares RNA samples, which were treated with terminal exonuclease with untreated samples and thus discriminates between RNA 5′ ends with triphosphate (TSS, transcriptional start sites) and RNA with monophosphate at the 5′ end (processing sites) (Sharma CM*, et al.*, 2010). The data strongly suggest that

RSP_1273 and RSP_1272 are cotranscribed from a promoter which is located upstream of RSP_1273, opposite to the coding region of *phyR* (Fig 4.3). Another TSS seems to be present for RSP_1271, however slightly downstream of the ATG start codon of the annotated protein. This ATG overlaps with the TAG terminator codon of RSP_1272 suggesting translational coupling.
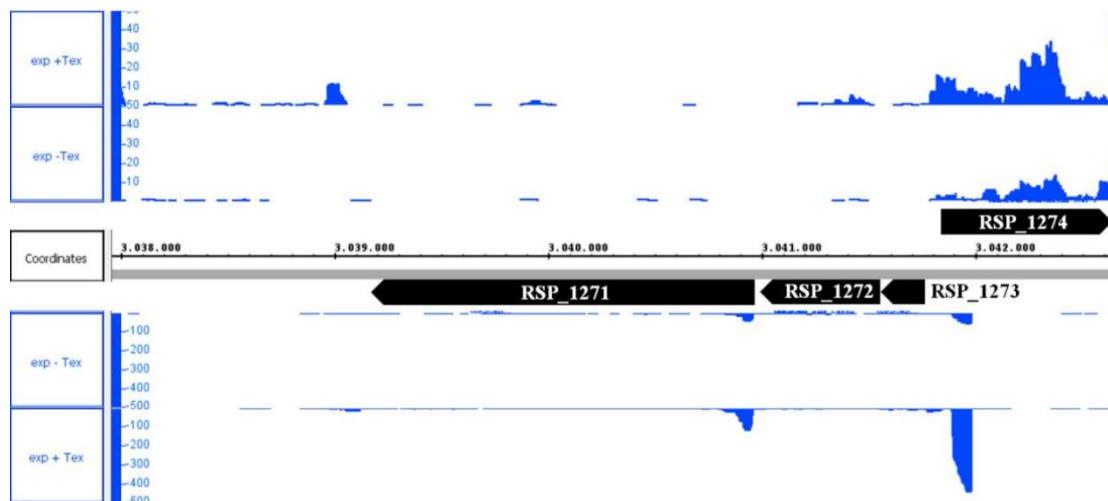
Fig 4.3 Schematic representation and RNA-seq read coverage of the *phyR* operon in *Rhodobacter sphaeroides*. Blue: Read coverage of the *phyR* operon in *R.sphaeroides* visualized by the Integrated Genome Browser. Black: Genes are represented by black boxes. The direction of the arrow indicates the direction of transcription.

4.1.2 RSP_1274 is involved in multiple stress resistance in *Rhodobacter sphaeroides*

The former mentioned PhyR is involved in various stress responses, and induced conditions differ between species. For the purpose of testing which stress responses PhyR is involved in *R. sphaeroides*, a PhyR deletion strain was constructed, which lacks the *phyR* gene and has a spectinomycin cassette inserted instead. In addition, a complemented strain was constructed. The ORF of *phyR* was amplified with the primers 1274CF and 1274CR using chromosomal *R. sphaeroides* DNA as a template. The PCR product was sub-cloned into the pJet cloning vector and ligated into the pBE4352 cloning vector.

Firstly *phyR* (Fig 4.4A) and RSP_1272 (σ (EcfG)) (Fig 4.4B) mRNA levels were tested by real time RT PCR after treatment with different reagents or stressful stimuli. While singlet oxygen (methylene blue), superoxide (paraquat), polymyxin B and

CCCP caused a highly significant increase of the *phyR* mRNA level, diamide and t-BOOH led to a less significant increase of the *phyR* mRNA level, and none of the other stresses result in marked changes in *phyR* mRNA levels. Membrane stress was however the only stress factor that resulted in increased RSP_1272 mRNA levels and this increase is clearly dependent on PhyR.
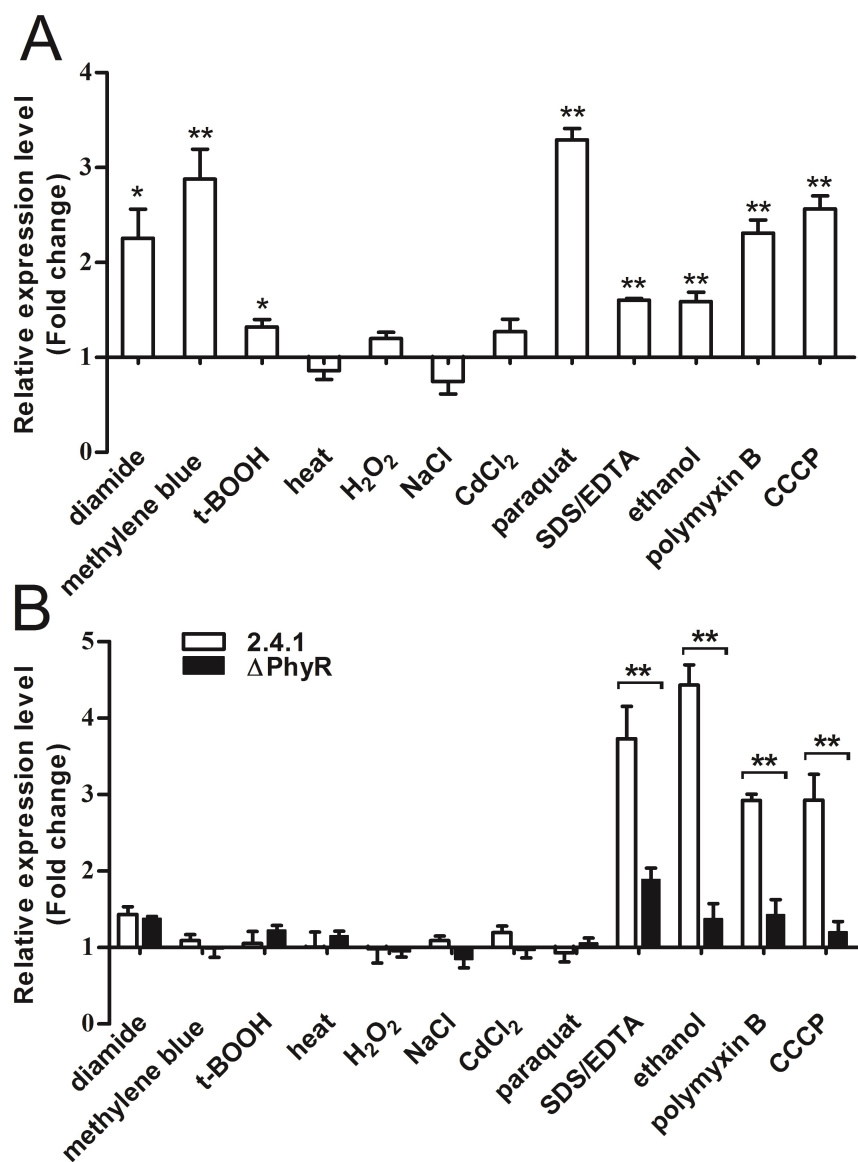
Fig 4.4 Levels of *phyR* (A) and RSP_1272 (B) mRNAs under various stresses as determined by real time RT PCR. The following reagents were added to aerobic cultures at OD_{660} of 0.4 and samples were collected immediately before (0 min) and 7 min after addition: 0.2 μM methylene blue and high light (880 W m-2) , 360 μM t-BOOH, 1 mM H_2O_2, 250 μM paraquat, 500 μM diamide, 500 mM NaCl, or 10 μM CdCl2. For heat shock, microaerobic cultures were shifted to

47

42°C at time 0 min or the following reagents were added: 0.005% SDS and 1mM EDTA, 2.5% ethanol, 1μg/ml polymyxin B or 10μM CCCP, and samples were collected at 0 min and 7 min. The mean of three experiments with standard deviation is shown.

* indicates a significant change (P≤0.05), ** indicates a highly significant change (P≤0.01) according to Students t-test. Published in (Li QF*, et al.*, 2018)

4.1.2.1 The effect of PhyR in oxidative stress response

4.1.2.1.1 Phenotypic analysis of PhyR regulon response to oxidative stress

Rhodobacter sphaeroides is a facultative model for photo-oxidative stress studies. In order to determine whether PhyR is involved in singlet oxygen stress defense, the doubling time was measured. The doubling time of the ΔPhyR strain did not differ from the doubling time of the parental wild type 2.4.1 in aerobic condition and microaerobic condition (Fig 4.5A and C) or during exposure to singlet oxygen in aerobic growth condition (Fig 4.5B). However, the amount of viable cells was different throughout the growth phase between wild type 2.4.1 and ΔPhyR strain in microaerobic growth condition. During the exponential phase, cells of wild type 2.4.1 grew well as of ΔPhyR strain. Entered the stationary phase, the viable cells of ΔPhyR strain were even more compared to the wild type strain (Fig 4.5D).
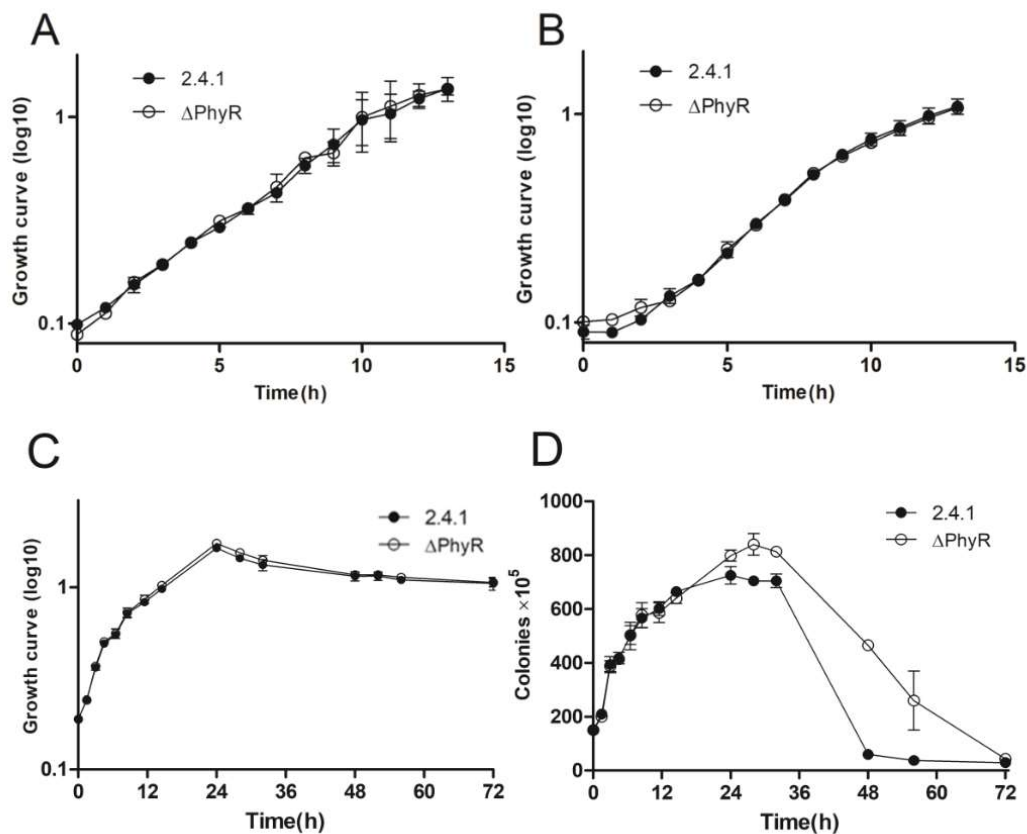
Fig 4.5 Growth curves of *Rhodobacter sphaeroides* wild type strain 2.4.1 and PhyR mutant strain in aerobic condition (A) or during exposure to singlet oxygen (B) and in microaerobic condition (C) were measured by spectrophotometer at 660 nm. Viable cells of wild type strain 2.4.1 and PhyR mutant strain were counted by plating spread assay in microaerobic condition (D). Error bars indicate the standard deviation from three biological replicates.

The singlet oxygen reacts with proteins, lipids and photo-pigments in two ways direct damage or the formation of long-lived reactive organic peroxides. In the zone inhibition assays, t-BOOH is used as a model organic peroxide. In zone inhibition assay, no difference was observed in resistance to singlet oxygen, t-BOOH, superoxide, or diamide between wild type and PhyR mutant (Fig 4.6). The observation in the same experiments showed significantly increased the resistance of ChrR (RSP_1093) mutant strain and decreased resistance in RpoE (RSP_1092) and ChrR (strain TF18) mutant strains against these stress factors (Fig 4.6). Moreover, the same stress resistance was observed between strains lacking ChrR and PhyR together or lacking RpoE/ChrR/PhyR and the parental strains harboring PhyR (Fig 4.6). Thus the effects of higher RpoE activity (strain ΔChrR) or a lack of RpoE (strain TF18) were not altered by an additional lack of PhyR.
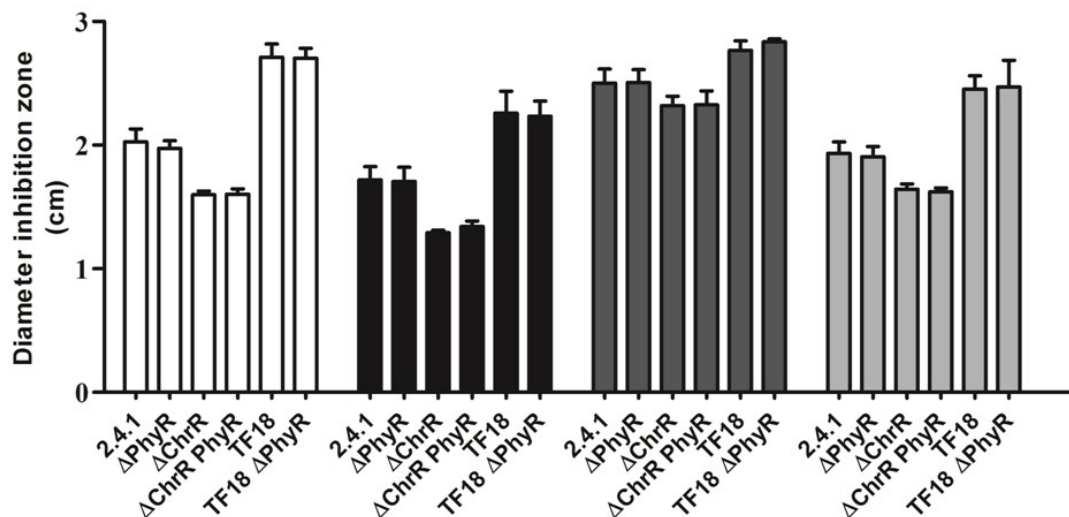
Fig 4.6 Inhibition of growth of the *Rhodobacter sphaeroides* wild type strain 2.4.1 and of mutants lacking the *phyR* or *chrR* gene or *chrR* and *rpoE* (strain TF18) or *phyR* together with *chrR* or *rpoE/chrR*. Following agents were used in zone inhibition assays: t-BOOH (white bars), methylene blue (black bars), diamide (dark grey bars), or paraquat (light grey bars). Error bars indicate the standard deviation of zones of inhibition from three biological replicates. According to Students t-test none of the changes between PhyR deletion strains and the parental strains is significant (P≤ 0.05). Published in (Li QF*, et al.*, 2018)

4.1.2.1.2 Effect of *R. sphaeroides* PhyR on RpoE-dependent gene activation

It is described in chapter 1.8 that RpoE (RSP_1092) plays an important role in response to singlet oxygen stress, and also activates RpoHII in the general stress response. In order to clarify a possible effect of PhyR on the activity of this sigma factor, the β-galactosidase activity was determined for checking the activity of RpoE in *R. sphaeroides* wild type 2.4.1 and PhyR mutant. The *phrA* gene of photolyase is directly controlled by RpoE. The β-galactosidase activity in strain TF18, which lacks RpoE and ChrR, is very low in absence or presence of singlet oxygen (generated by addition of methylene blue and illumination) (Hendrischk AK*, et al.*, 2007). The β-galactosidase activity of the strain lacking PhyR increased after 1h and 3h treatment with singlet oxygen stress, which was however less than the observation in the wild type 2.4.1 (Fig 4.7A). As a result, the β-galactosidase activity of wild type 2.4.1 showed significantly higher after 1h and 3 h of stress (Fig 4.7A). The β-galactosidase activity was also measured after treatment with t-BOOH (Fig 4.7B). The PhyR mutant showed an increase in β-galactosidase activity, which was significantly lower

compared to the wild type 2.4.1. When the mutant was complemented with PhyR, the β-galactosidase activity showed a similar or higher level than that of the wild type 2.4.1, proving that PhyR indeed altered RpoE activity.
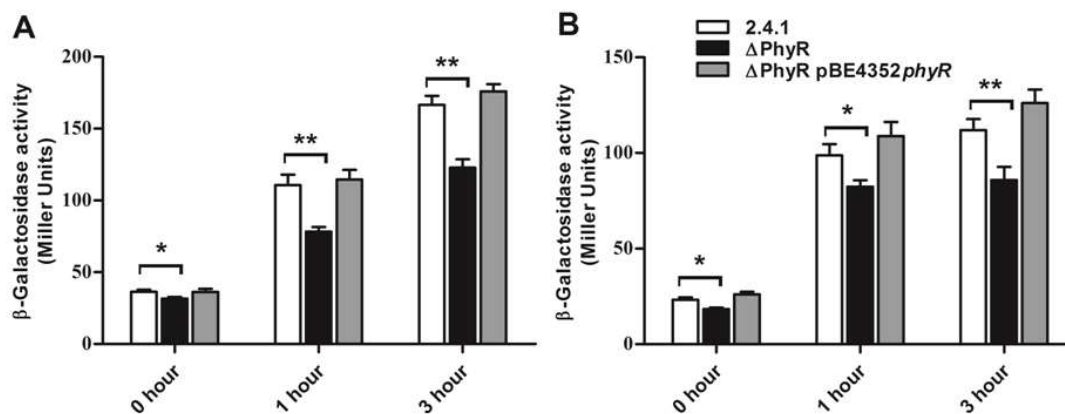
Fig 4.7 β-galactosidase actvity of *R. sphaeroides* strains harboring the reporter plasmid pPHU*phrAlacZ*. Cells were grown in aerobic conditions in the dark and were exposed to high light intensity (880 W m^{-2}) and 50 nM methylene blue (A) or to 360 μM of t-BOOH (B) for the indicated time periods. The mean of three experiments and standard deviations are shown.* indicates a significant change (P≤0.05), ** indicates a highly significant change (P≤0.01) according to Students t-test. Published in (Li QF, *et al.*, 2018)

In addition, another gene encodes the small RNA Pos19 (Rss0019), which is a part of RpoE regulon (Berghoff BA, *et al.*, 2009, Muller KMH, *et al.*, 2016). The gene was used for analyzing the effect of PhyR on RpoE activity by northern blot. The observation of northern blot showed that Pos19 was induced stronger in the wild type 2.4.1 compared to that in the *phyR* mutant upon treatment with organic peroxide (Fig 4.8).
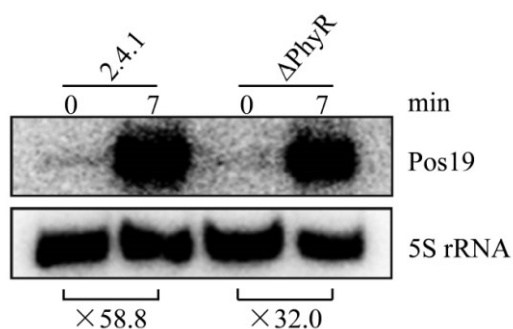
Fig 4.8 Northern blot analysis of Pos19. Cells were treated with t-BOOH and harvested at time point 0 and 7 min. Pos19 bands were normalized to the 5S rRNA and the calculated fold change is indicated. Published in (Li QF, *et al.*, 2018)

4.1.2.1.3 Effect of *R. sphaeroides* PhyR on stress-dependent mRNA levels

Real time RT-PCR analysis revealed that the *rpoE* mRNA levels of wild type strain and complemented mutant increase significantly stronger compared to the strain lacking PhyR after treatment with t-BOOH (Fig 4.9A). The effect of PhyR was also tested on some other genes with a role in the oxidative stress response in *R. sphaeroides* (Berghoff BA*, et al.*, 2013, Zeller T*, et al.*, 2004). The *catA* (RSP_2779) mRNA level of the strain lacking PhyR increased significantly more than in the wild type 2.4.1 after 7 min of hydrogen peroxide treatment (Fig 4.9B). The *catA* gene encodes catalase. The genes *gloA* (RSP_0392) and *gloB* (RSP_2294) encode putative glyoxalases. The mRNAs level of *gloA* and *gloB* were induced higher in response to singlet oxygen in the wild type and the complemented PhyR mutant compared to the PhyR mutant (Fig 4.9B). The expressions of *gloA* and *gloB* genes are dependent on RpoHII-dependent promoters (Nuss AM*, et al.*, 2009), while *catA* is not dependent on a promoter sequence for an alternative sigma factor. In previous studies, the *rpoHII* gene is regulated by RpoE (Nuss AM*, et al.*, 2009). Thus the changed expression levels of *gloA* and *gloB* mRNAs are most likely a consequence of the PhyR effect on RpoE activity.
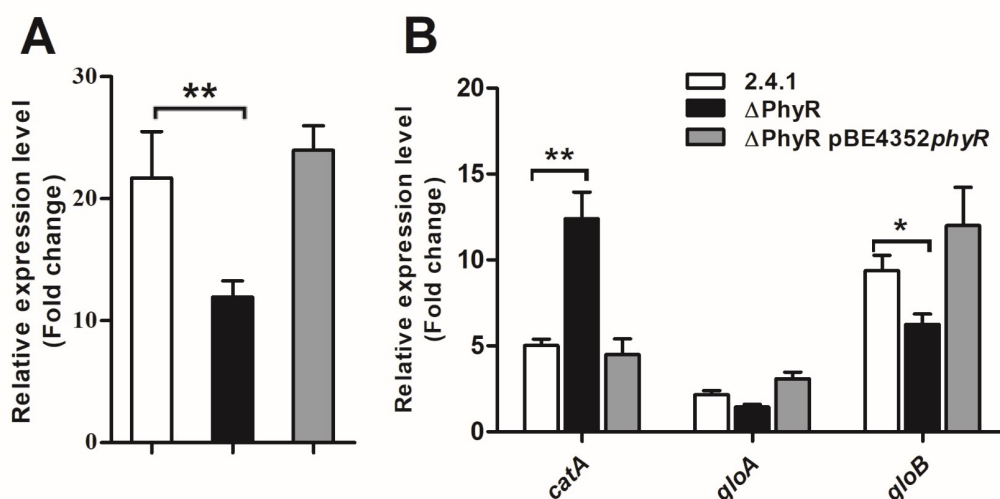
Fig 4.9 (A) Levels of *rpoE* mRNA as determined by real time RT PCR in wild type 2.4.1, *phyR* mutant and complemented strain. The fold change of 7 min versus 0 min under t-BOOH after normalization to *rpoZ* mRNA level is shown. (B) Levels of relative expression are shown for *catA* in response to hydrogen peroxide and *gloA*, *gloB* in response to $^1O_2$ exposure in wild type 2.4.1, *phyR* mutant and complemented strain. Exposure to hydrogen peroxide or $^1O_2$ was performed for 7 minutes. Values for relative expression levels represent the increase in gene expression

compared to that of the control at time point 0 min and were normalized to mRNA levels determined for *rpoZ*. The mean of three experiments with standard deviation is shown. Published in (Li QF, *et al.*, 2018)

Moreover, we checked the *rpoE* mRNA levels in response to other stress conditions in the wild type 2.4.1 and the PhyR mutant strain (Fig 4.10). The observation of *rpoE* mRNA level showed a significant difference between the two strains for singlet oxygen stress (methylene blue in the light). Diamide, heat shock, SDS/EDTA or ethanol had only minor effects on *rpoE* mRNA levels, while superoxide (paraquat treatment) and hydrogen peroxide resulted in increased *rpoE* mRNA levels, which were slightly lower in the mutant, but these differences were statistically not significant (Fig 4.10).
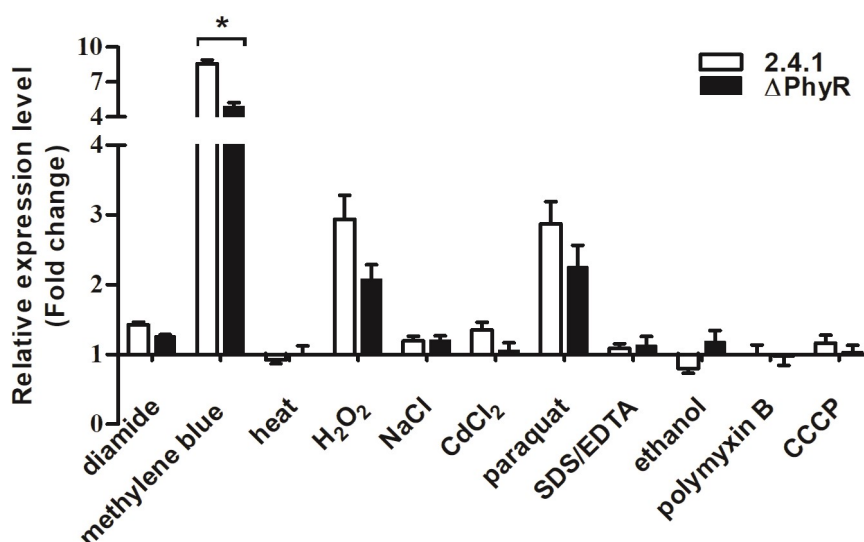
Fig 4.10 Relative *rpoE* mRNA levels under different stress conditions (described in Fig 3.4) as determined by real time RT PCR in the wild type and the mutant lacking PhyR. The mean of three experiments with standard deviation is shown. Published in (Li QF, *et al.*, 2018)

4.1.2.2 The effect of PhyR on membrane stress response

To confirm whether PhyR has a role in resistance to membrane stress, spot plating assays were applied for checking cells survival after SDS and EDTA treatment or ethanol treatment in PhyR mutant and wild type 2.4.1 strains. No growth difference was observed in none treatment condition. However, after 15 min of treatment with 0.015% SDS and 30mM EDTA the wild type 2.4.1 showed much better survival than the PhyR mutant (Fig 4.11A), which indicates a role of the PhyR homolog in defense

of membrane stress. After 30 min or 60 min of treatment with ethanol, the survival of wild type was slightly better than that of the ΔPhyR mutant (Fig 4.11B). When a plasmid-encoded *phyR* gene was introduced into the PhyR mutant (strain ΔPhyR (pBE::*phyR* eCFP)), survival was identical as for the wild type 2.4.1 for both stresses (Fig 4.11A and B).
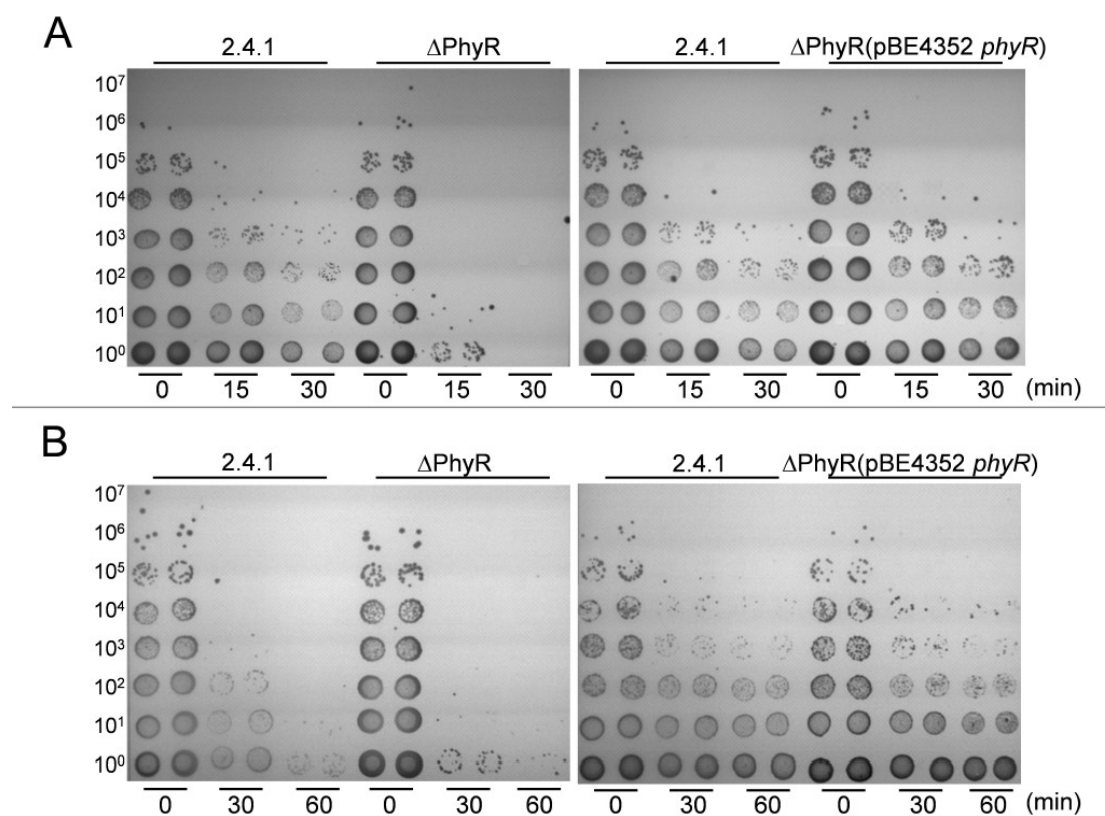
Fig 4.11 Spot survival assays for *R. sphaeroides* and *phyR* mutant after membrane stress. All strains were cultured to an OD$_{660}$ of 0.5 in microaerobic condition. 5 μl from consecutive 10-fold dilutions were spotted onto agar plates before and after 15 and 30 minutes of SDS (0.015%) and EDTA (30mM) treatment(A) or after 30 and 60 minutes of ethanol (12%) treatment. Published in (Li QF, *et al.*, 2018)

In addition to spot plating assays, a significant effect of PhyR on the survival was also confirmed by experiments of spread plating assays in the presence of SDS/EDTA or ethanol (Fig 4.12). Additional reagents polymyxin B and CCCP (carbonyl cyanide m-chlorophenyl hydrazone) were used for checking the effect of PhyR on the defense of membrane stress. Polymyxin B is primarily used for resistant gram-negative bacteria infections through altering membrane structure and making it more permeable. The

survival rate of *phyR* mutation was significantly lower than in the wild type 2.4.1 upon treatment with polymyxin B and CCCP (Fig 4.12).
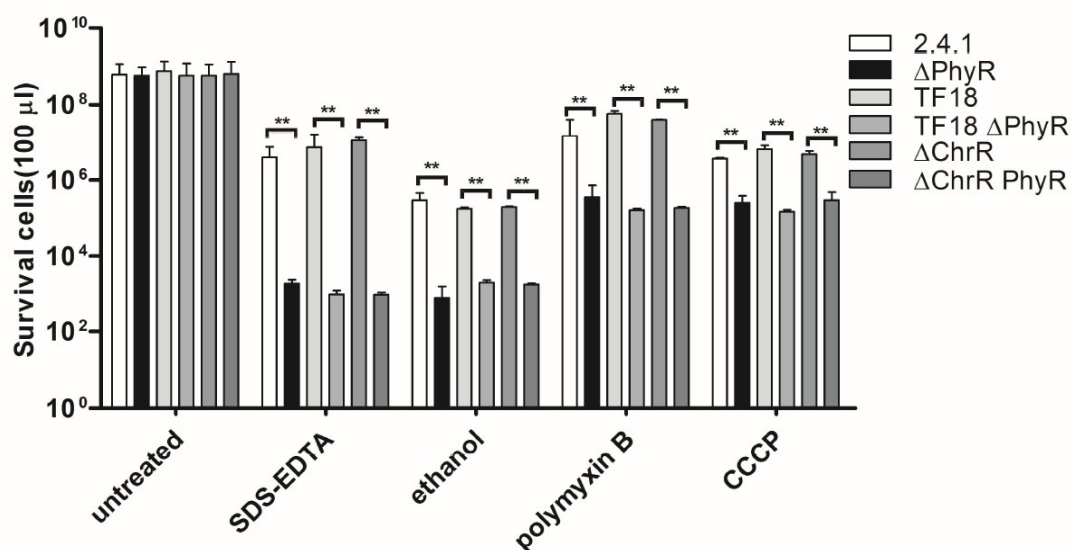
Fig 4.12 Survival rates of *R. sphaeroides* strains as determined by spread plating assays and colony counting before and after 15 min of addition of SDS (0,015%) and EDTA (30mM), 30 min of ethanol (12%), 60 min of polymyxin B (2.5 mg/ml), or 60 min of CCCP (25 µM). Error bars indicate the standard deviation from three biological replicates. ** indicates a highly significant change (P≤0.01) according to Students t-test. Published in (Li QF, *et al.*, 2018)

To test whether the RpoE/ChrR system mediates the effect of PhyR on membrane stress response, two mutants lacking ChrR or ChrR/RpoE together with PhyR were constructed. The similar resistance to membrane stress was observed for strains ΔChrR and wild type, or TF18 and wild type (Fig 4.12). The results clearly demonstrate that the effect on membrane stress is solely due to PhyR.

4.1.2.3 The effect of PhyR on UV stress response

The effect of *R.spaeroides* PhyR on UV stress was also checked. *R. sphaeroides* cells were incubated in the dark or light after exposed to UV light. When the cells were incubated in the dark, the PhyR mutant strain showed significantly lower survival rate compared to the wild type. When *phyR* gene was introduced to the mutant, the survival rate was identical as for the wild type. When the cells were incubated in the light, no difference in survival rates was observed for the two strains (Fig 4.13). These results revealed that PhyR does not affect the photolyase activity of *R. sphaeroides*, but rather processes for UV repair that is light-independent.
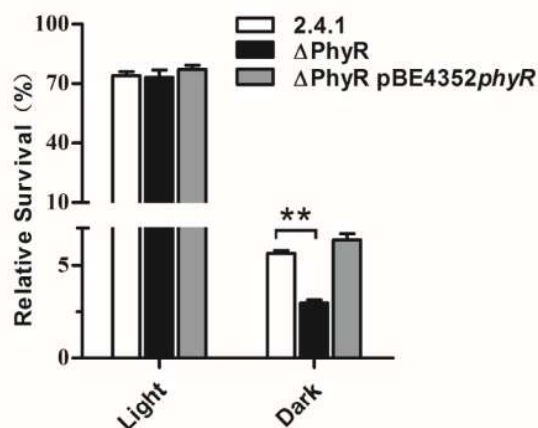
Fig 4.13 Survival of *R. sphaeroides* after UV light exposure. Cells of the indicated strains were exposed to UV light of 100 J m^{-2} (254 nm), spread on agar plates and kept in the light (60 W lamp) or in the dark. The survival rates are given as the mean of three experiments with standard deviation. ** indicates a highly significant change (P≤0.01) according to Students t-test. Published in (Li QF, *et al.*, 2018)

4.1.2.4 The effect of PhyR on heat shock response

In order to test the effect of PhyR on heat stress in *R.sphaeroides* as well as PhyR in *Methylobacterium. extorquens* (Francez-Charlot A, *et al.*, 2009), the growth was tested at elevated temperature in wild type 2.4.1 and PhyR mutant. Plates were incubated at 42°C for 24 h after streaking and further incubation was at 32°C for 72 h. Plates were incubated permanently at 32°C as a control. A clear growth defect was observed in strains lacking RpoHI or RpoHI and RpoHII after heat stress. In previous studies, it was verified that both sigma factors are involved in the heat shock response of *R. sphaeroides* (Dufour YS, *et al.*, 2012, Nuss AM, *et al.*, 2009). There was no significant effect of elevated temperature on growth of the strain lacking PhyR (Fig 4.14).
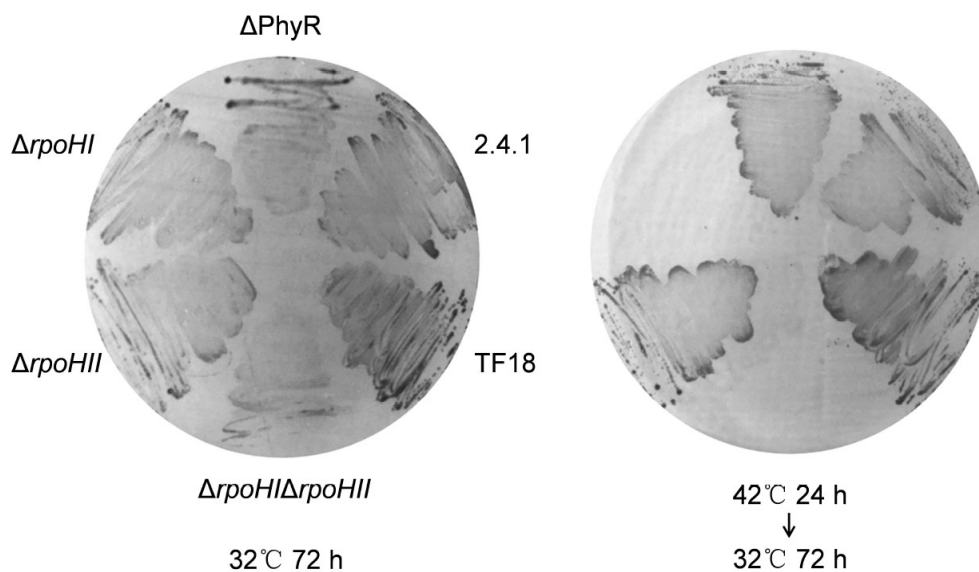
Fig 4.14 Growth of the *Rhodobacter sphaeroides* wild type 2.4.1 and various mutant strains after heat shock. Cultures were grown at 32°C to exponential phase in microaerobic conditions and diluted to OD_{660} of 0.1. For each strain 5 µl of diluted culture were spread on agar plates and incubated under the indicated temperature in the dark. The agar plates incubated at 42°C were shifted to 32°C after 24 h. Published in (Li QF, *et al.*, 2018)

4.1.3 Effect of *R. sphaeroides* PhyR on cross-resistances

In some Alphaproteobacteria, PhyR affects cross-resistance, when a second stress factor follows a first stressor (Gourion B, *et al.*, 2008, Gourion B, *et al.*, 2009). To test for a similar function of PhyR in *R. sphaeroides*, the non-lethal concentration of different reagents were firstly settled: 500mM NaCl, 1.2mM t-BOOH, 25mM methylglyoxal, 2mM paraquat and 10mM H_2O_2, and lethal concentration of different reagents were settled: 75mM methylglyoxal, 3.6mM t-BOOH and 0.5mM methylene blue. And then different combinations of stressful reagents were applied.

The wild type showed transiently increased survival of t-BOOH stress after pretreatment with 500mM of NaCl (Fig 4.15A). After this initial phase, the survival rate dropped faster than in cultures, which were treated with t-BOOH without NaCl pretreatment. While the PhyR mutant showed identical survival as the wild type in 500mM NaCl or 3.6 mM t-BOOH, the survival rate dropped more quickly when t-BOOH was added in the presence of 500mM NaCl (Fig 4.15A). A similar effect was observed when methylglyoxal was applied upon pre-exposure to 1.2mM t-BOOH (Fig

57

4.15B). No differences were observed between mutant and wild type when applying paraquat/methylglyoxal, hydrogen peroxide/methylglyoxal, paraquat/t-BOOH, hydrogen peroxide/t-BOOH, NaCl/methylglyoxal or NaCl/ methylglyoxal (Fig 4.16).
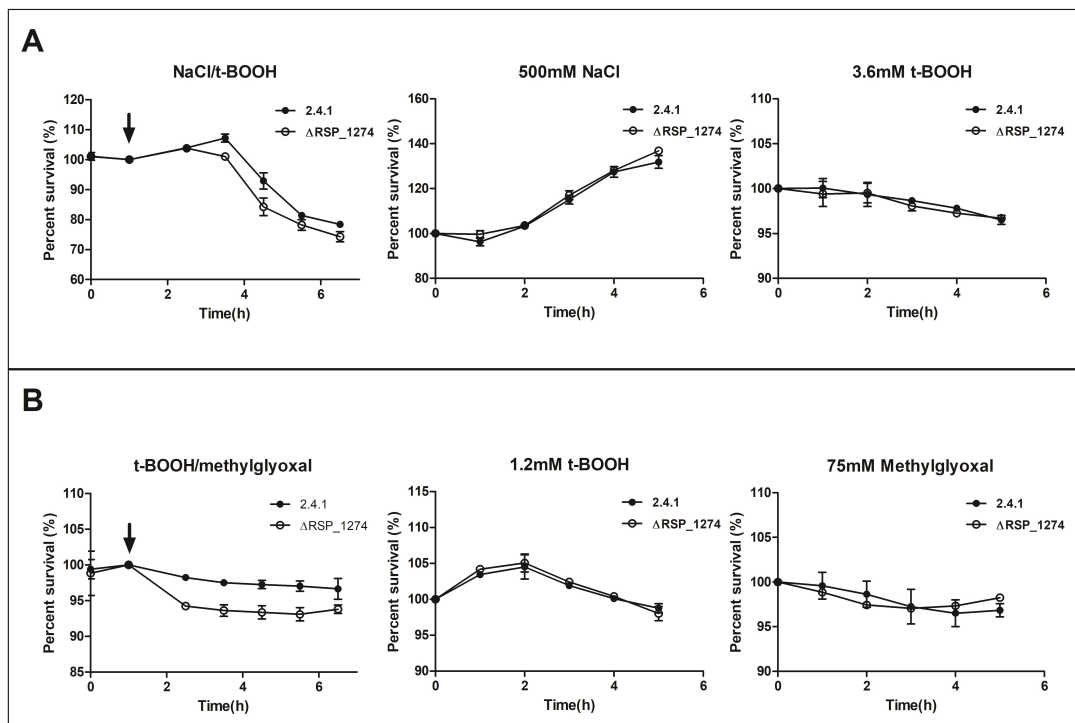
Fig 4.15 Cross-resistance in *Rhodobacter sphaeroides*. (A) NaCl (500 mM) was added to the culture when an OD_{660} reached 0.5. After treatment for 1 h, the t-BOOH (3.6 mM) was then added to the culture. (B) Cells were cultured to an OD_{660} of 0.5 and then t-BOOH (1.2 mM) was added to the culture, the second stressor methylglyoxal (75 mM) was added after 1h of t-BOOH treatment. Cultures treated with only one stressor served as control. The mean of three experiments with standard deviation is shown.
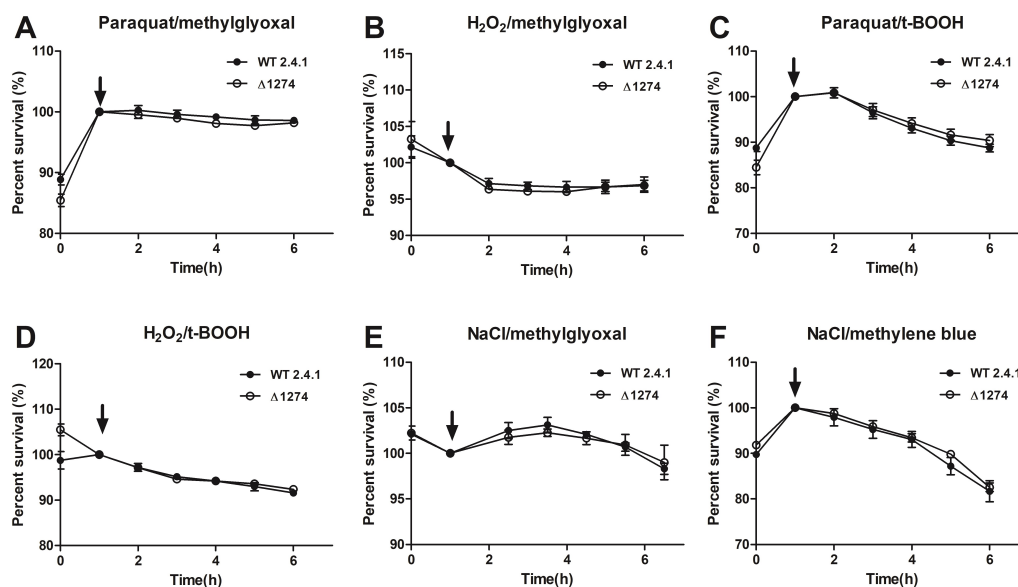
Fig 4.16 Cross-resistance in *Rhodobacter sphaeroides*. Cells were cultured to an OD_{660} of 0.5 and then the first reagent was added to the culture, the second stressor was added after 1h of first stress treatment. (A) The first reagent is 2mM paraquat, second reagent is 75mM methylglyoxal. (B) The first reagent is 10mM H_2O_2, second reagent is 75mM methylglyoxal. (C) The first reagent is 2mM paraquat, second reagent is 3.6mM t-BOOH. (D) The first reagent is 10mM H_2O_2, second reagent is 3.6mM t-BOOH. (E) The first reagent is 500mM NaCl, second reagent is 75mM methylglyoxal. (F) The first reagent is 500mM NaCl, second reagent is 0.5mM methylene blue. The mean of three experiments with standard deviation is shown.

4.2 Growth phase-dependent regulation in *R. sphaeroides*

For analysis of Growth phase-dependent regulation in *R. sphaeroides*, we selected few promoters with growth phase-dependent expression pattern. The expressions of selected promoters were monitored throughout the growth phase by the *lacZ* reporter system, in which promoters from selected genes were transcriptionally fused with the *lacZ* gene in the reporter plasmid pBBRI MCS5.

4.2.1 Gene expression patterns throughout the growth phase in *R. sphaeroides*

4.2.1.1 Expression pattern of RSP_0557 in *R. sphaeroides* wild type

The gene RSP_0557, has an RpoH-dependent promoter, encodes a putative small protein, and is induced by singlet oxygen stress (Berghoff BA*, et al.*, 2013). To test

the activity of the RSP_0557 promoter across growth phase, the reporter plasmid (pBBRI 0557p::*lacZ*) was transferred into wild type 2.4.1, the RpoHII deletion strain, the RpoHI/RpoHII double deletion strain and TF18 (RpoE-ChrR deletion strain). Cells from reporter strains were harvested for β-galactosidase (β-Gal) activity assay from dozens of time points across growth phases (0h-144h of growth) and outgrowth after 12 hours, 36 hours, 60 hours and 132 hours of entry stationary phase in microaerobic growth condition.

The reporter assay confirmed expression of RSP_0557 is not constant throughout the growth phase of culture and changed with respect to the growth phase in the wild type strain (Fig 3.17). The expression level was low in lag phase, as can be seen by β-Gal activity. As the culture entered and progressed through the exponential phase, the β-Gal activity began to increase fluctuatingly. In the late exponential and transition phase (10 h of growth), the β-Gal activity reached the peak value, which dropped quickly when entering stationary phase (12h of growth) (Fig 4.17). After 12h of entry in stationary phase (early stationary phase), the β-Gal activity recovered to a high level from the lowest level at 24h of growth. From 30h to 80h of the stationary phase, β-Gal activity kept in a constantly high level. β-Gal activity dropped again after 80h of stationary phase (Fig 4.17). When cultures were diluted to OD_{660} of 0.2 at 24h, 48h, 96h and 144h of growth (12h, 36h, 84h and 132h of stationary phase), a transitory increase of the β-Gal activity was observed. Upon further incubation, the activity declined steadily (Fig 4.17).
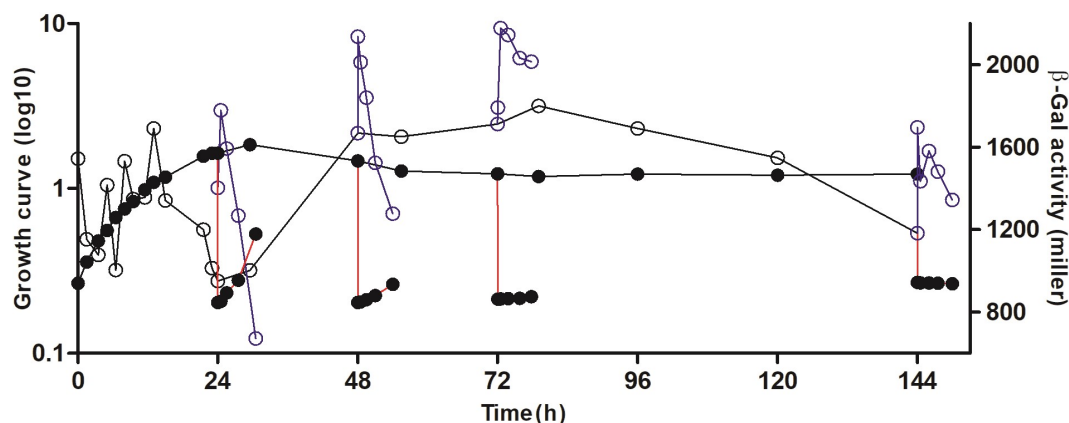
Fig 4.17 Activity of the RSP_0557 promoter is affected by growth phase. *R. sphaeroides* strain wild type 2.4.1 (with 0557p::*lacZ* reporter plasmid) was grown in RÄ medium 32°C in microaerobic condition. Optical density at 660nm (OD$_{660}$) was measured over time, and β-galactosidase activity was measured throughout the growth of the culture and outgrowth after stationary phase at four time points. Black spots and line represent growth curve throughout the growth phase; Black spots and red line represent growth curve of outgrowth; Black open circles and line represent β-Gal activity throughout the growth phase; Blue open circles and line represent β-Gal activity of outgrowth.

4.2.1.2 Effect of RpoE on regulation of RSP_0557

For further demonstration of expression of RSP_0557, β-Gal activity also was measured in TF18 (RpoE-ChrR deletion strain). As we know, RpoE has the function affecting *rpoHII* expression, and the promoter RSP_0557 is controlled by RpoHII. The alternative sigma factor RpoE may affect the expression of RSP_0557. Nevertheless, a similar pattern of growth phase-dependent activity of the RSP_0557 promoter was observed in TF18 strain compared to in wild type throughout the growth phase (Fig 4.18A). The differences occurred in the β-Gal activity after dilution at 24h, 48h, 96h and 144h of growth between wild type 2.4.1 and TF18. A transient increase of β-Gal activity after dilution was observed in wild type 2.4.1. Conversely, The β-Gal activity decreased directly after dilution in TF18. Upon further incubation, the β-Gal activity increased and recovered to a high level (Fig 4.18A).

4.2.1.3 Effect of RpoH on regulation of RSP_0557

In the RpoHII deletion strain, the expression pattern of RSP_0557 had a great difference from that in wild type and TF18 strain (Fig 4.18B). Overall, the β-Gal

activity in the RpoHII mutant strain was definitely lower than in wild type or in TF18 strains. However, the β-Gal activity is not constant across the growth phase. From lag phase to early stationary phase β-Gal activity increased smoothly, and maintained a high level with ongoing stationary phase in absence of RpoHII. The β-Gal activity dropped after 100h of growth (Fig 4.18B). During the outgrowth after 24h and 48h of growth (12h and 36h of stationary phase), the changes of β-Gal activity were the same as the changes in the wild type. During the outgrowth after 72h and 144h of growth (60h and 132h of stationary phase), the β-Gal activity kept in an invariable high value as well as the value in preceding stationary phase differed from in wild type and TF18 (Fig 4.18B).

The observation of expression pattern of RSP_0557 in the RpoHI/RpoHII double deletion strain was totally different from that in the wild type, RpoHII deletion strain, or RpoE-ChrR deletion strain (Fig 4.18C). The global β-Gal activity in the RpoHI/RpoHII double deletion strain was lower than in the RpoHII deletion strain. The β-Gal activity increased in late exponential and transition phase, which dropped again when the culture entered stationary phase. During the outgrowth after 24h, 48h, 72h and 144h of growth, the β-Gal activity kept in a constant value as well as the value before dilution (Fig 4.18C).
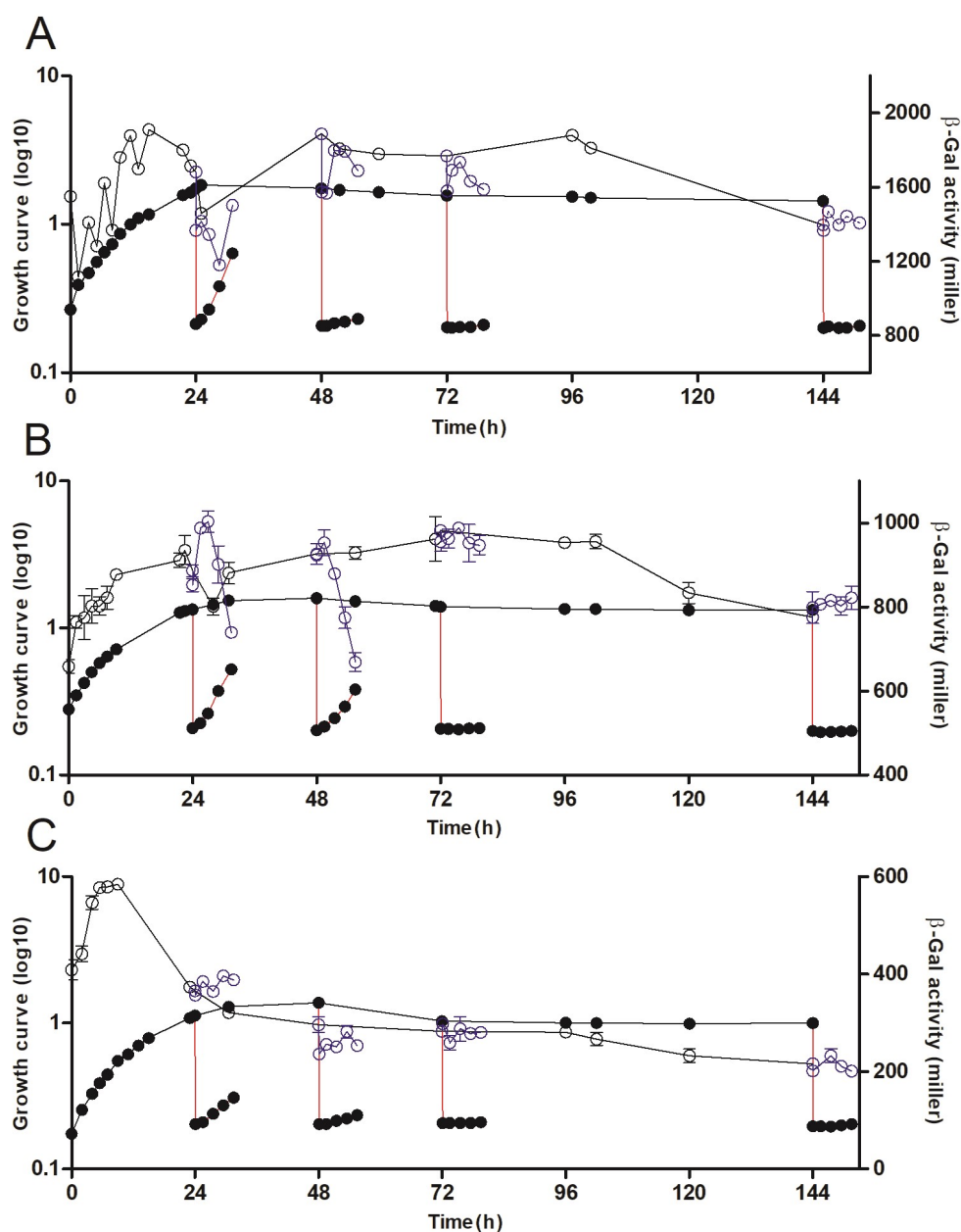
Fig 4.18 Activity of the RSP_0557 promoter is affected by growth phase. *R. sphaeroides* strain TF18 (A), RpoHI deletion (B) and RpoHI/RpoHII double deletion (C) strains (with 0557p::*lacZ* reporter plasmid) were grown in RÄ medium 32°C in microaerobic condition. Optical density at 660nm (OD$_{660}$) was measured over time, and β-galactosidase activity was measured throughout the growth of the culture and outgrowth after stationary phase at four time points. Black spots and line represent growth curve throughout the growth phase; Black spots and red line represent growth curve of outgrowth; Black open circles and line represent β-Gal activity throughout the growth phase; Blue open circles and line represent β-Gal activity of outgrowth.

In the course of these experiments, however, even though the β-Gal activities were significantly different in overall level among the four strains, it was still regulated with respect to the growth phase of the culture (Fig 4.17 and Fig 4.18). The same changed expression of RSP_0557 was observed in four different strains from the lag phase to late exponential phase. After entry into the stationary phase and following dilutions, the expression of RSP_0557 presented different patterns during different strains. These results also suggested that the responsibility for expression of RSP_0557 after entry into the stationary phase was part of sigma factor RpoHI.

4.2.2 Expression pattern of RSP_0904 throughout the growth phase

Gene RSP_0904 (*sitA*), is involved in iron transport, showed increased expression in the stationary phase (Remes B, *et al.*, 2017). The gene has no consensus promoter. In order to understand more about the expression of RSP_0904 throughout the growth phase, two reporter plasmids were constructed: a ~214 nt upstream fragment of RSP_0904 or a shortening of the upstream fragment to only 100 nt was fused to the *lacZ* gene. Wild type 2.4.1, RpoHII deletion strain, RpoHI/RpoHII double deletion strain and TF18 (RpoE-ChrR deletion strain) harbouring the reporter plasmids were cultured in microaerobic condition. Cells were harvested for β-galactosidase activity assay.

4.2.2.1 Expression pattern of RSP_0904 in *R. sphaeroides* wild type

The measurement of β-Gal activity in wild type strain confirmed increased expression of RSP_0904 in stationary phase compared with in exponential phase (Fig 4.19). Moreover, many differences occurred in the activity of RSP_0904 promoter with different length of the upstream fragment. Overall, the activity of the promoter with ~130 nt upstream fragment (from TSS) was significantly higher than the promoter with ~30 nt upstream fragment (from TSS) (Fig 4.19). The lowest activity of the promoter with ~30 nt upstream fragment (from TSS) was particular to cells at a low cell density in fresh medium. However, the β-Gal activity increased along with the density of culture and kept in the highest level after the late stationary phase (72h of growth) for a long time of incubation (Fig 4.19B). The activity of the promoter with ~130 nt upstream fragment (from TSS) was different from the promoter with ~30 nt upstream fragment (from TSS). The lowest β-Gal activity was in the mid-exponential

phase, after that increased steadily until early exponential phase (30h of growth). After the early stationary phase, the β-Gal activity then kept at a constant level (Fig 4.19A). Nevertheless, the changes of β-Gal activity during outgrowth were same between RSP_0904 promoter with different length of upstream fragments. After dilution at early stationary phase (24h of growth), the activity of RSP_0904 promoter dropped quickly from a high level. Different from the dilution at early stationary phase, the β-Gal activity were transiently increased and then dropped down slowly after dilution at middle stationary phase (48h of growth) and later stationary phase (72h of growth) (Fig 4.19).
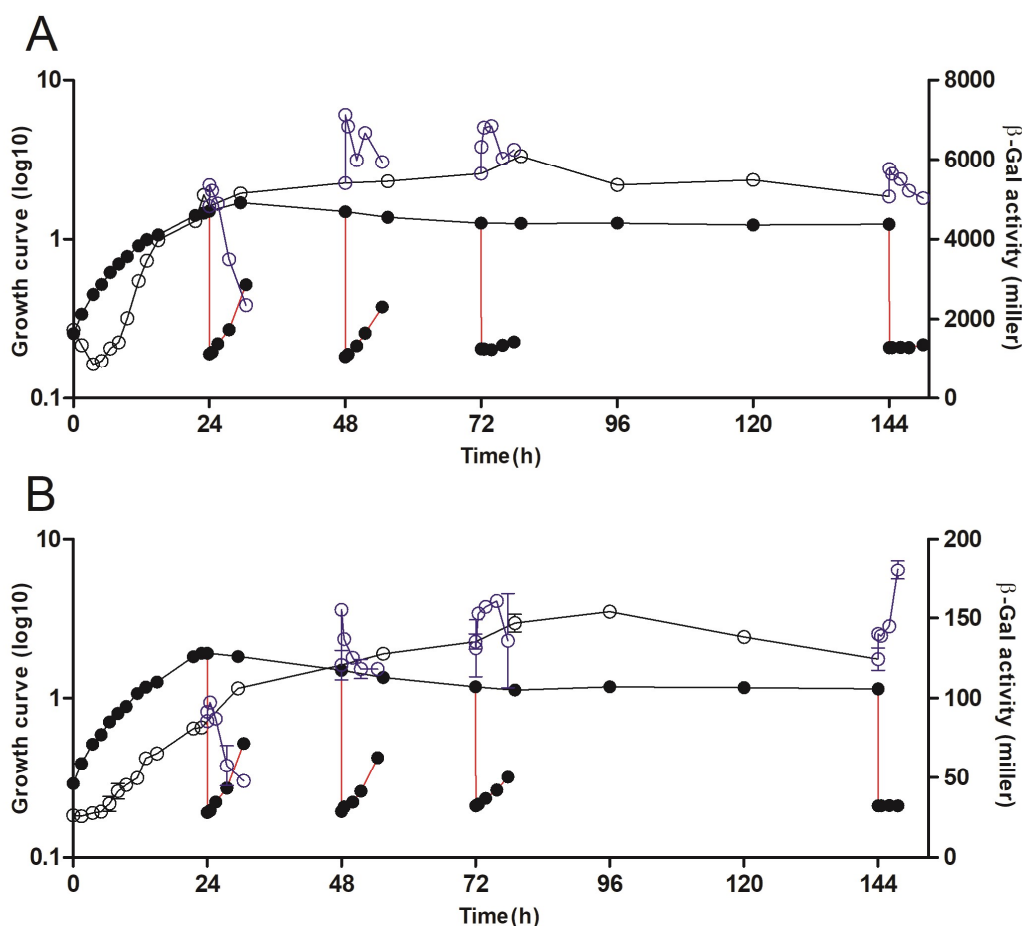
Fig 4.19 The promoter of the RSP_0904 activity is affected by growth phase in *R. sphaeroides* wild type strain. (A) the activity of RSP_0904 promoter with ~130 nt upstream fragment (from TSS), (B) the activity of RSP_0904 promoter with ~30 nt upstream fragment (from TSS). Black spots and line represent growth curve throughout the growth phase; Black spots and red line

represent growth curve of outgrowth; Black open circles and line represent β-Gal activity throughout the growth phase; Blue open circles and line represent β-Gal activity of outgrowth.

4.2.2.2 Effects of RpoE and RpoH on regulation of RSP_0904

In order to investigate the expression of RSP_0904 throughout the growth phase, the activity of RSP_0904 promoter also was measured in TF18, RpoHI mutant and RpoHI/RpoHII mutant strains. As a result, the promoter with ~30 nt upstream fragment (from TSS) had no activity in the three mutants. Although the promoter with ~130 nt upstream fragment (from TSS) still exhibited high activity, its activity was significantly decreased in TF18, RpoHI mutant and RpoHI/RpoHII mutant strains compared to that in the wild type strain throughout the growth phase. However, even though the activity of the promoter was remarkably decreased, it was still regulated with respect to the growth phase of the culture (Fig 4.20). The β-Gal activity in TF18, RpoHI mutant and RpoHI/RpoHII mutant strains was again the lowest in mid-exponential phase (Fig 4.20) and then increased continuously until entering stationary phase. The β-Gal activity reached the highest at the early stationary phase (24h of growth) and kept in this level in a prolonged stationary phase (Fig 4.20). When the cultures were diluted to an OD_{660} of 0.2 after 24h or 48h of growth (12h or 36h of stationary phase), a small transient increase of the β-Gal activity was observed in TF18, RpoHI mutant and RpoHI/RpoHII mutant strains (Fig 4.20). Upon further incubation, the β-Gal activity dropped steadily. When the cultures were diluted after 72h or 144h (60h or 132h of stationary phase), β-Gal activity showed a similar rate to that before dilution in TF18 and RpoHI mutant strains (Fig 4.20A B) and a strong increase in RpoHI/RpoHII mutant strains (Fig 4.20C). Upon further incubation, the β-Gal activity kept at a constant rate.
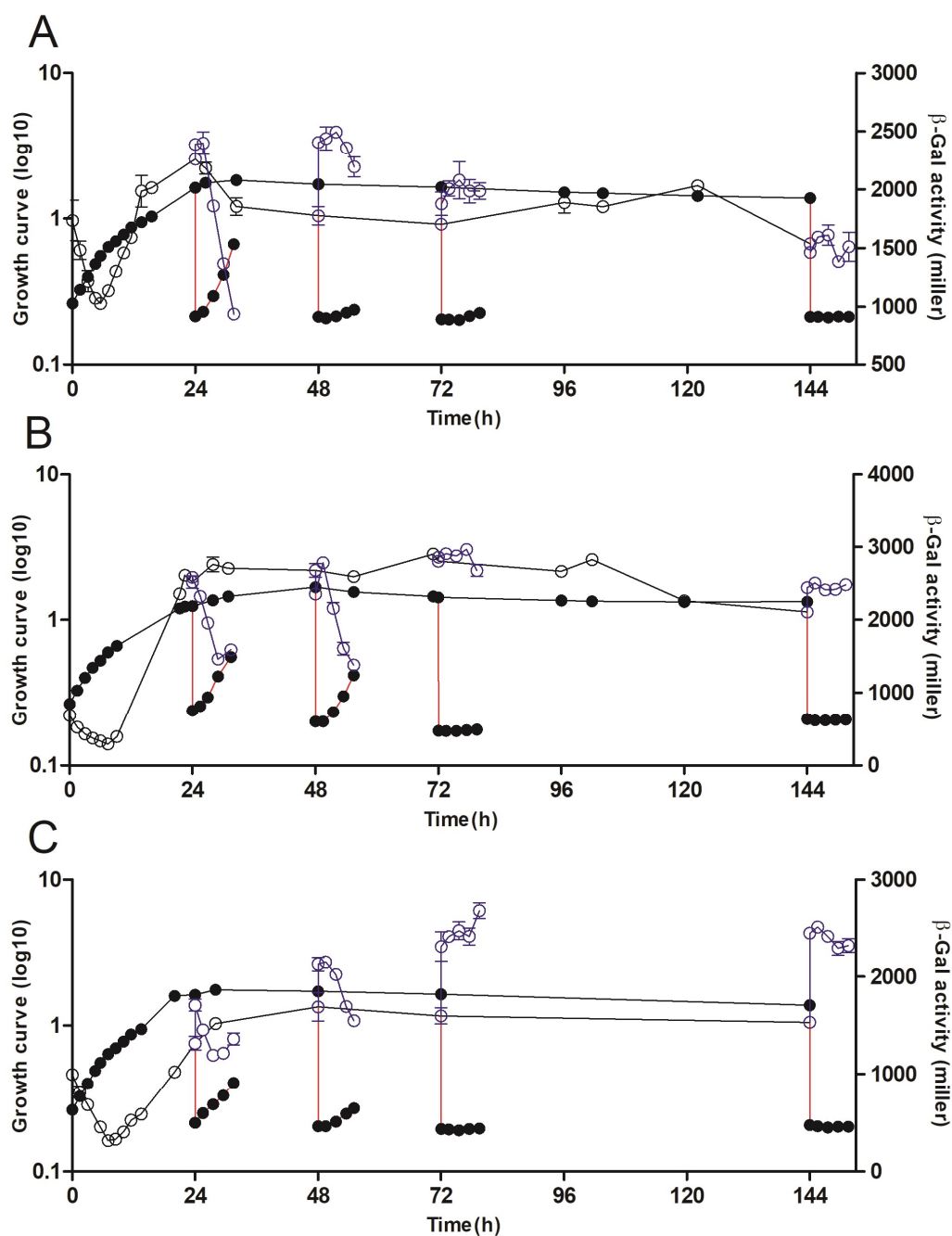
Fig 4.20 The promoter of the RSP_0904 activity is affected by growth phase. *R. sphaeroides* strains: TF18 (A), RpoHI deletion (B) and RpoHI/RpoHII deletion (C) strains (with 0904p::*lacZ* reporter plasmid) were grown in RÄ medium 32°C in microaerobic condition. Optical density at 660nm (OD$_{660}$) was measured over time, and β-Gal activity was measured throughout the growth of the culture and outgrowth after stationary phase at four time points. Black spots and line represent growth curve throughout the growth phase; Black spots and red line represent growth curve of outgrowth; Black open circles and line represent β-Gal activity throughout the growth phase; Blue open circles and line represent β-Gal activity of outgrowth.

In the course of these experiments, the promoter with ~30 nt upstream fragment (from TSS) showed an increased activity along with cells density increased in wild type strain, but no activity in TF18, RpoHI mutant and RpoHI/RpoHII mutant strains. However, the promoter with ~130 nt upstream fragment (from TSS) showed lower activity in TF18, RpoHI mutant and RpoHI/RpoHII mutant strains compared to that in wild type strain across the growth phase and during outgrowth. It had a similar expression pattern in wild type, TF18, RpoHI mutant and RpoHI/RpoHII mutant strains throughout the growth phase and there were slight differences during outgrowth among the four strains.

4.2.3 Expression pattern of RSP_0960 throughout the growth phase

The gene RSP_0960 (crotonyl-CoA carboxylase/reductase), is involved in carbon fixation, has no consensus promoter. To test the activity of the RSP_0960 promoter across the growth phase, the reporter plasmid (pBBRI 0960p::*lacZ*) was transferred into the wild type 2.4.1. A 250 nt upstream fragment of RSP_0960 gene was inserted into the pBBRIMCS5 reporter plasmid. Cells from reporter strains were harvested for β-Gal activity assay from dozens of time points across the growth phase (0h-144h of growth) and outgrowth after 12 h, 36 h, 60 h and 122 h of entry into the stationary phase (24h, 48h, 72h and 144h of growth) in microaerobic growth condition or aerobic condition.

No matter cells grew in microaerobic condition or in aerobic condition, β-Gal activity was not in a constant level throughout the growth phase or during outgrowth. The β-Gal activity varied with respect to the growth phase. High β-Gal activity was observed at the beginning of growth. As the culture progressed through the exponential phase, β-Gal activity began to decrease (Fig 4.21). The β-Gal activity decreased continuously until entry into the stationary phase in microaerobic condition, the lowest occurred at the 12h of entry into stationary phase. Along with prolonging stationary phase, β-Gal activity recovered to a high level (Fig 4.21A). After the culture was diluted to an OD_{660} of 0.2 after 24h of growth (12h of stationary phase), a steady increase of β-Gal activity was observed (Fig 4.21A). When the cultures were diluted to an OD_{660} of 0.2 after 48h, 72h or 144h of growth (36h, 60h or 132h of stationary phase), a similar β-Gal activity was observed. Upon further incubation, the

β-Gal activity decreased steadily (Fig 4.21A). The change of β-Gal activity in aerobic condition differed from that in microaerobic condition. The β-Gal activity decreased continuously until entry into the transition phase in aerobic condition, the lowest activity occurred in the early stationary phase. After entry into the stationary phase, the β-Gal activity began to increase. The β-Gal activity recovered to the highest level at 24h of growth and kept until 48h of growth. After that the β-Gal activity decreased. The lowest activity occurred at 72h of growth and remained at the lowest level after prolonged stationary phase (Fig 4.21B). After the culture was diluted to an OD_{660} of 0.2 after 24h of growth (12h of stationary phase), a persistent decrease of β-Gal activity was observed (Fig 4.21B). When the culture was diluted to an OD_{660} of 0.2 after 48h of growth (36h of stationary phase), a transient increase of β-Gal activity was observed. The culture was diluted to an OD_{660} of 0.2 after 72h or 144h of growth (60h or 132h of stationary phase), the β-Gal activity showed a strong increase by a factor of about 3. Upon further incubation, the β-Gal activity decreased steadily (Fig 4.21B).

Overall, these results indicated that the expression of RSP_0960 was not constant throughout the growth of culture and changed with respect to the growth phase in wild type strain in microaerobic condition or in aerobic condition. Moreover, the expression patterns of RSP_0960 varied in different growth conditions.
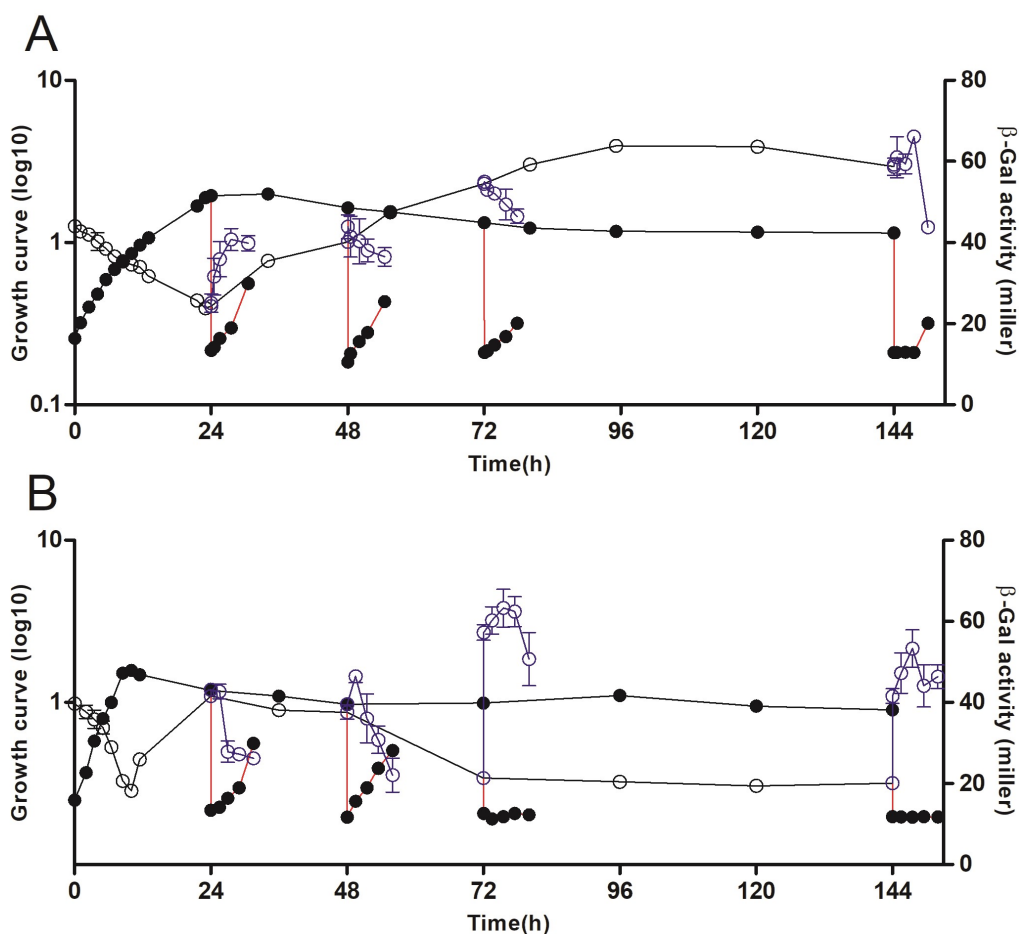
Fig 3.21 Activity of the RSP_0960 promoter is affected by growth phase. *R. sphaeroides* strain wild type 2.4.1 strain (with 0960p::*lacZ* reporter plasmid) was grown in RÄ medium 32°C in microaerobic condition (A) or aerobic condition (B). Optical density at 660nm (OD_{660}) was measured over time, and β-Gal activity was measured throughout the growth of the culture and outgrowth after stationary phase at four time points. Black spots and line represent growth curve throughout the growth phase; Black spots and red line represent growth curve of outgrowth; Black open circles and line represent β-Gal activity throughout the growth phase; Blue open circles and line represent β-Gal activity of outgrowth.

4.3 The effect of nucleoid -associated proteins on growth phase dependent regulation

One feature of NAPs (nucleoid-associated proteins) that makes them excellent regulators of gene expression at the global level is interaction with DNA. In order to demonstrate the effect of NAPs on genes expression with a role in stress response in

R.sphaeroides, we checked gyrase activity throughout the growth phase in *R.sphaeroides* wild type and three mutant strains (H-NS, Lrp and IhfB).

Firstly, we tested the supercoiling activity of gyrase *in vitro* throughout the growth phase in the wild type in aerobic growth condition or microaerobic growth condition. The results showed high supercoiling activity of gyrase before 36h of the stationary phase (48h of growth) in aerobic growth condition. After 48h of the stationary phase (60h of growth), the supercoiling activity of gyrase became significantly low. The similar results also were observed in microaerobic growth condition (Fig 4.22).
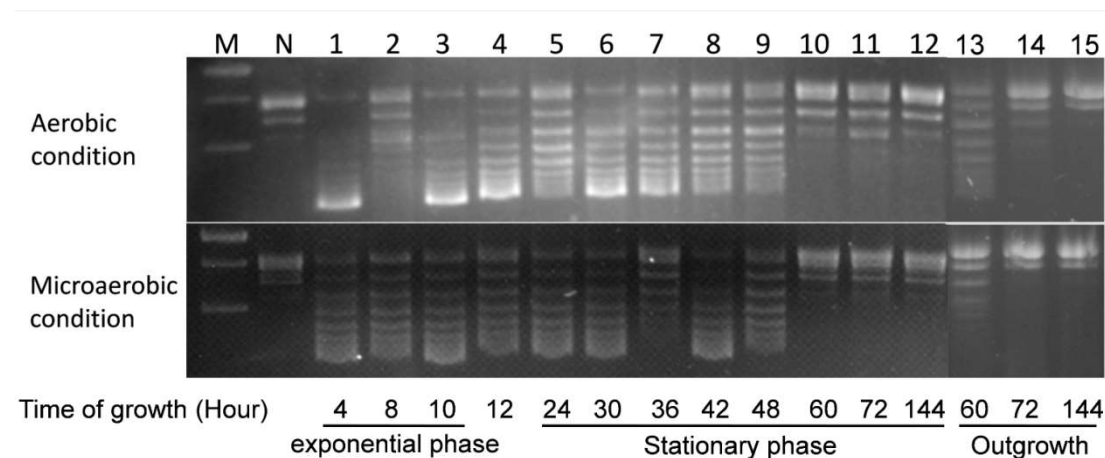
Fig 3.22 Gyrase supercoiling activity in *R.sphaeroides* throughout the growth phase in aerobic or microaerobic condition. Two micrograms of total protein (cell extract) as determined by Bradford assay were incubated at room temperature in buffer with 0.2 mg of relaxed plasmid pBluescript DNA, for 2 h at 37°C. M, DNA marker. N, negative control without cell extract. 1-3, cell extract from exponential phase. 4, cell extract from transition phase. 5-12, cell extract from stationary phase. 13-15, cell extract from outgrowth phase.

Additional experiments were used for checking the effect of NAPs on gyrase supercoiling activity. We tested gyrase supercoiling activity in strains lacking H-NS (histone-like nucleoid-structuring protein), Lrp (leucine-responsive regulatory protein) or IhfB (Integration host factor subunit beta) in microaerobic condition. The result showed the supercoiling activity of gyrase was significantly low after 48h of stationary phase (60h of growth) in Lrp mutant strain as well as in wild type. However, the supercoiling activity of gyrase recovered during outgrowth at 48h of stationary phase (60h of growth) (Fig 4.22). The strain lacking H-NS showed higher gyrase activity after 48h of stationary phase (60h of growth) than wild type. In the previous study, it emerged that H-NS can constrain supercoils in DNA (Hinton JCD, *et al.*,

1992). The supercoiling activity of gyrase was higher in IhfB mutant after 48h of stationary phase than that in wild type. During outgrowth, the same result was observed in the three mutants.
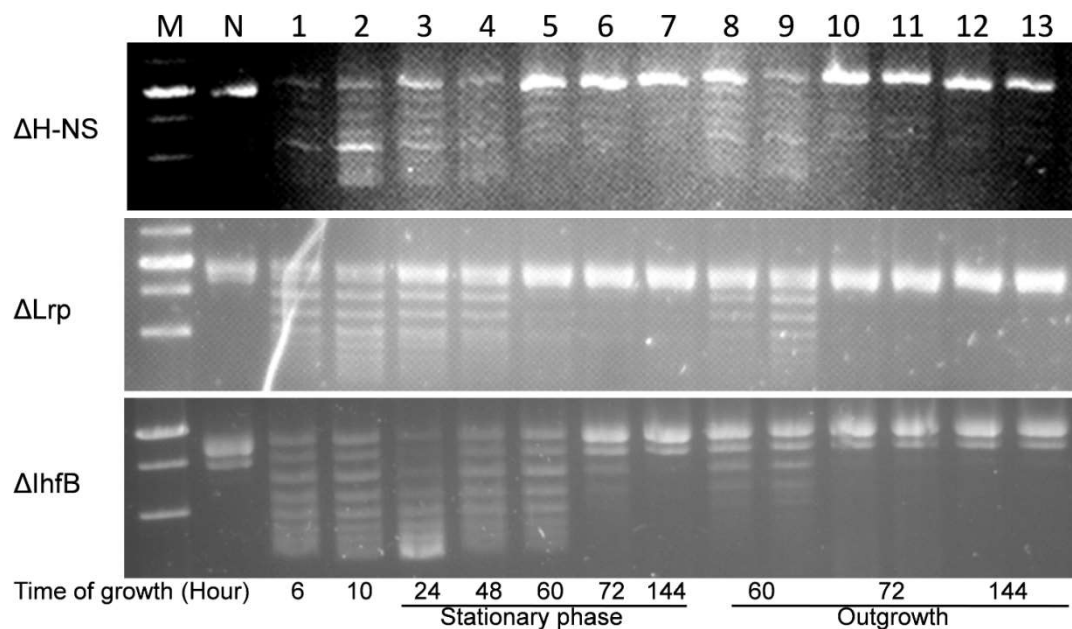
Fig 3.23 Gyrase supercoiling activity in mutants throughout the growth phase and outgrowth in microaerobic condition. Two micrograms of total protein (cell extract) as determined by Bradford assay were incubated at room temperature in buffer with 0.2 mg of relaxed plasmid pBluescript DNA, for 2 h at 37°C. M, DNA marker. N, negative control without cell extract. 1, cell extract from exponential phase. 2, cell extract from transition phase. 3-7, cell extract from stationary phase. 8-13, cell extract from outgrowth phase.

5 Discussion

5.1 The role of PhyR-NepR-σ(EcfG) in *Rhodobacter sphaeroides*

In the present study, we investigated whether PhyR of *R. sphaeroides* plays a role in the general stress response. *R. sphaeroides* has several sigma factors. The sigma factors RpoE, RpoHI and RpoHII have been well studied, and are involved in oxidative stress response (Anthony JR, *et al.*, 2004, Nuss AM, *et al.*, 2009). RpoHI also is a major player in the heat stress response (Nuss AM, *et al.*, 2010). Another sigma factor, EcfG (RSP_1272) attracted our interest because its gene is linked to another gene (RSP_1274, now renamed *phyR*), whose orthologue in *Sinorhizobium meliloti*, *Methylobacterium extorquens* AM1, *Bradyrhizobium japonicum* and *Caulobacter crescentus* encodes a regulator, PhyR, involved in the general stress response (Francez-Charlot A, *et al.*, 2015). The *ecfG* or *phyR* mutants increased sensitivity towards different types of stresses. However the spectrum of inducing conditions and the degree to which mutants are sensitive to a particular stress differ between species (Table 5.1). Therefore it is interesting to learn the role of PhyR-NepR-σ (EcfG) in *R. sphaeroides*.

Table 5.1. Phenotypes of *ecfG* or *phyR* mutants in selected Alphaproteobacteria

Species	Representative phenotypes of *ecfG* or *phyR* mutants	References
Bradyrhizobium japonicum USDA110	*ecfG* or *phyR* mutant: delayed nodulation and nitrogen fixation, induction of partly aberrant root nodules, increased sensitivity to heat shock and desiccation	(Gourion B, *et al.*, 2009)
Caulobacter crescentus CB15 or NA1000	*sigT* (*ecfG*) mutant: increased sensitivity to osmotic and oxidative stress	(Alvarez-Martinez CE, *et al.*, 2007, Foreman R, *et al.*, 2012, Lourenco RF, *et al.*, 2011)
Methylobacterium extorquens AM1	*phyR* mutant: impaired phyllosphere colonization, increased sensitivity to heat shock, desiccation, UV, ethanol,	(Gourion B, *et al.*, 2006, Gourion B, *et al.*, 2008)

	osmotic and oxidative stress	
Sinorhizobium meliloti 1021	*rpoE2* (*ecfG*) mutant: increased sensitivity to heat shock, osmotic, oxidative stress and desiccation	(Flechard M*, et al.*, 2009, Flechard M*, et al.*, 2010, Humann JL*, et al.*, 2009)

5.1.1 PhyR has a role in response to UV stress

Significant difference in susceptibility to UV irradiation is observed between wild type and *phyR* mutant strain, when the light-dependent repair mechanisms were excluded in this study (Fig 4.13). This suggests that the wild type is more resistance than *phyR* mutant to UV stress, but this resistance was only observable in the absence of light. This shows the importance of PhyR for protection against UV light, but the mechanism is unknown. In *Methylobacterium extorquens* AM1, *uvrA* is part of the PhyR regulon, and encodes a subunit of the UvrABC complex that is responsible for DNA repair, which suggests that PhyR is involved in UV resistance (Truglio JJ*, et al.*, 2006). The expression level of *uvrA* was found to be controlled by the SOS response (Janion C, 2001). In *R. sphaeroides*, a SOS box also is present upstream of the *uvrA* promoter (de Henestrosa ARF*, et al.*, 1998). Therefore, PhyR may be involved in UV stress response via regulating *uvrA* gene expression in *R. sphaeroides*.

5.1.2 PhyR has a role in response to membrane stress

Significant differences of phenotype are observed between wild type and PhyR mutant in resistance to membrane stress. The PhyR mutant is more sensitive to membrane stress (Fig 4.11 and 4.12). This suggests that PhyR has a role in response to membrane stress. However the mechanism is unknown. The mRNA of RSP_1272 encoding the σ(EcfG) protein of the PhyR-NepR-σ (EcfG) locus was clearly induced by membrane stress. In contrast, the RSP_1272 mRNA levels show only a slight increase in absence of PhyR under membrane stress (Fig 4.4B). Therefore, the effect of PhyR on RSP_1272 mRNA level agrees with signal transfer within the PhyR-NepR-σ (EcfG) cascade, ie the anti-anti sigma factor activates the EcfG upon stress. These results indicate that the PhyR-NepR-σ (EcfG) cascade in *R. sphaeroides* plays a major role in the defense of membrane stress.

5.1.3 PhyR has a moderate effect on RpoE (RSP_1092) activity but no major role in oxidative stress response

The main goal of this study was to investigate whether PhyR was involved in the oxidative stress response. Our data provided clear evidence for an effect of PhyR on *rpoE* mRNA level and RpoE-dependent gene activation (Fig 4.7, 4.8, 4.9 and 4.10). However, the same resistance to oxidative stress was observed in wild type and PhyR mutant (Fig 4.6). Why is the influence of PhyR on RpoE activity not manifested as altered stress resistance in the mutant? The change of *rpoE* mRNA level is of course much more significant in mutants lacking ChrR or RpoE and ChrR together (strain TF18) than in the PhyR mutant under the tested conditions. In the phenotypic assay, ΔChrR or TF18 mutants have obviously increased or decreased resistance to oxidative stress compared to wild type strain (Fig 4.6). However, strains ΔChrR or TF18 lacking PhyR showed the same resistance as parental strains implying that PhyR cannot even partially compensate the loss of these main regulators. In previous studies, it was revealed that a complex regulatory network including protein and sRNA is involved in stress responses. The moderate changes of RpoE activity caused by the lack of PhyR may not be sufficient to cause a clear phenotype since compensation by other players in the regulatory network may take place. This suggests that RpoE plays a major role in response to oxidative stress rather than PhyR.

5.1.4 PhyR has a role in response to cross-resistance

Although PhyR was not found to be involved in oxidative stress response by the zone inhibition assay, the PhyR mutant is more sensitive to organic peroxide stress (generated by t-BOOH) than the wild type strain after NaCl treatment (Fig 4.15A). This finding that PhyR is involved in the oxidative stress response was substantiated by the finding that hypertonicity induces protection against oxidative stress and that this protection is at least in part dependent on PhyR. A similar effect was observed when methylglyoxal was applied upon pre-exposure to organic peroxide stress (generated by t-BOOH) (Fig 4.15B). This suggests that the stress response from PhyR is induced insufficiently by organic peroxide stress, but took place after an additional stress factor was added.

According to these results, the PhyR-NepR-σ (EcfG) cascade plays a major role in response to membrane and UV stress rather than to oxidative stress in *R. sphaeroides* compared to the other Alphaproteobacteria listed in Table 5.1. This suggests that PhyR has a more specialized role in *R. sphaeroides*. So far, the role of PhyR in stress responses was not analyzed for any member of the Rhodobacterales. Therefore it is possible that a more specialized function of PhyR is not limited to *R. sphaeroides* but may apply to a certain sub-branch of the Alphaproteobacteria.

5.2 Growth phase-dependent regulation in *Rhodobacter sphaeroides*

Our knowledge of growth phase-dependent regulation in *R. sphaeroides* is limited. A previous study showed that alternative sigma factors play an important role in the regulation. It was revealed that the alternative sigma factors RpoHI and RpoHII play a major role during outgrowth after prolonged stationary phase, and they are involved in the response to oxidative stress and heat shock (Remes B*, et al.*, 2017). The gene for RpoE showed decreased expression in outgrowth after a short stationary phase (28h of stationary phase) and increased expression in outgrowth after a long stationary phase (72h of stationary phase) (Remes B*, et al.*, 2017). The sigma factor RpoE has a main function in activating a small subset of genes including *rpoHII* in response to singlet oxygen stress (Glaeser J*, et al.*, 2007, Nuss AM*, et al.*, 2009). In *E. coli*, the RpoE activity also varied with respect to the growth phase (Costanzo A*, et al.*, 2006). The PhyR-NepR-σ(EcfG) cascade was recognized as a core pathway regulating the general stress response in Alphaproteobacteria (Francez-Charlot A*, et al.*, 2015). A role of the PhyR-NepR-σ(EcfG) cascade in growth phase adaptation has not been reported to date.

5.2.1 Growth phase-dependent regulation on PhyR

The growth conditions differ in different growth phases. Bacteria need to change their physiology to survive or adapt to different growth conditions. Especially in the stationary phase, in which bacteria suffer from environmental stresses including oxidative stress, extreme pH, and nutrient deprivation (Roop RM*, et al.*, 2003). PhyR is the main regulator of the general stress response system that protects bacteria against a wide variety of stress condition. We suggested that PhyR could make cells

grow better in stationary phase. Conversely, however, there are even more viable cells in a PhyR mutant culture than in the wild type 2.4.1 under microaerobic growth conditions. No difference was observed in the exponential phase (Fig 5.4D). This suggests that PhyR had no function in the exponential phase and a negative effect on cell viability in stationary phase in *R. sphaeroides*. The mechanism is still unknown.

5.2.2 Growth phase-dependent regulation on RSP_0557

The gene RSP_0557 has an RpoH-dependent promoter, encodes a putative protein, and is induced by singlet oxygen stress and heat shock (Dufour YS*, et al.*, 2012). The gene RSP_0557 is homologous to RSP_6037 that is cotranscribed with the four sRNA CcsR1-4 (Berghoff BA*, et al.*, 2009, Billenkamp F*, et al.*, 2015). The proteins of RSP_0557 and RSP_6037 contain a domain of unknown function (DUF1127), according to the Pham database (Finn RD*, et al.*, 2016), that is found in several hypothetical bacterial proteins. In some cases, DUF1127 represents the C-terminal region, whereas in others it represents the whole protein sequence. The protein RSP_0557 has a regulatory function by binding DNA or RNA, and may regulate sulfur genes and maintain a balanced GSH level (Muller KMH*, et al.*, 2016). It is known that RSP_0557 is negatively regulated by the sRNA Pos19 depending on Hfq, and that Pos19 is stress induced in a RpoE-dependent manner (Muller KMH*, et al.*, 2016). In order to test the growth phase regulation on RSP_0557, the activity of the RSP_0557 promoter was measured throughout the growth phase by the LacZ reporter system.

5.2.1.1 Expression pattern of RSP_0557 in wild type strain

The β-Gal assays showed that the activity of the RSP_0557 promoter was not constant throughout the growth phase. Instead, it varied with respect to the growth phase in the wild type strain under microaerobic growth conditions. The activity of the RSP_0557 promoter increased from the exponential phase to the transition phase. As cells entered and progressed into stationary phase, large changes in cellular metabolism occur that render the cells more stress resistant to diverse environmental stresses including oxidative stress, acidic pH, and nutrient deprivation (Roop RM*, et al.*, 2003). The gene RSP_0557 is induced by singlet oxygen stress and heat shock (Dufour YS*, et al.*, 2012). The reporter assay confirms that the activity of the

RSP_0557 promoter markedly increased upon entry into stationary phase and remained at a high level after 48h of stationary phase (Fig 4.17). The results also showed a transitory increased activity in the RSP_0557 promoter at the beginning of outgrowth after 24h, 48h, 96h and 144h of growth under microaerobic conditions. The activity dropped again along with extended cultivation (Fig 4.17). In a previous study, it was shown that the oxygen level in the cultures had a sudden rise and then dropped again after dilution from low-aeration stationary phase cultures (Remes B*, et al.*, 2017). The high correlation between the RSP_0557 promoter activity and oxygen level in the culture suggests that regulation of RSP_0557 after outgrowth could be affected by oxygen levels.

5.2.1.2 Role of RpoE in the regulation of RSP_0557

Does RpoE have any role in the growth phase-dependent regulation of RSP_0557? It emerged that RSP_0557 expression is negatively regulated by the sRNA Pos19 depending on Hfq, but the regulation of RSP_0557 on the transcriptional level was excluded. The Pos19 is stress induced in a RpoE-dependent manner upon singlet oxygen and peroxide stresses (Muller KMH*, et al.*, 2016). In this study, the promoter of RSP_0557 was transcriptionally fused to *lacZ*, thus the results should reflect the regulation of RSP_0557 at the transcriptional level. We observed that the pattern of the RSP_0557 promoter activity in TF18 (the strain lacks RpoE-ChrR) is similar to that in the wild type 2.4.1 throughout the growth phase and during outgrowth (Fig 4.18A). Our results support the evidence that the effect of Pos19 on RSP_0557 is not at transcriptional level. On the other hand, the expression of RSP_0557 is partly controlled by RpoHII which is activated by RpoE in response to singlet oxygen (Muller KMH*, et al.*, 2016, Nuss AM*, et al.*, 2009). According to this model, the expression pattern of RSP_0557 should be altered in TF18 strain. Nevertheless, this was not the case. If RpoE does not contribute to the growth phase-dependent regulation of RSP_0557 in this pathway, then we predict that the strength of oxidative stress is too low to make RpoE elevate *rpoHII* expression. The expression of *rpoHII* is induced by oxidative stress directly (Glaeser J*, et al.*, 2007, Nuss AM*, et al.*, 2009). As such, RpoE does not have a role in growth phase-dependent regulation of RSP_0557 under microaerobic growth conditions.

5.2.1.3 Role of RpoHI and RpoHII in the regulation of RSP_0557

Does RpoHII have any role in the growth phase-dependent regulation of RSP_0557? RpoHII is clearly required to set the overall activity of the RSP_0557 promoter in the cell under microaerobic conditions. However, a slight change in the expression pattern was observed in the RpoHII deletion strain across the growth curve and during outgrowth compared to the wild type under microaerobic conditions. The observation that the increase in the activity of the RSP_0557 promoter between early exponential phase and entry into stationary phase was higher in wild type than in the cells lacking RpoHII (2-fold increase in wild type, 1.2-fold increase in ΔRpoHII cells) could reflect a contribution by RpoHII to the growth phase-dependent regulation of RSP_0557 (Fig 4.17 and Fig 4.18B). During the outgrowth after 60h of stationary phase, the activity of the RSP_0557 promoter was kept at a constant level. This suggests that the effect of RpoHII on the regulation of RSP_0557 during the outgrowth depends on the duration of stationary phase. Despite that, the promoter of RSP_0557 did not lose activity totally, which reveals that RpoHII is not absolutely required for the expression level of RSP_0557 under microaerobic conditions.

Does RpoHI have any role in the growth phase-dependent regulation of RSP_0557? We observed that the overall activity of the RSP_0557 promoter in the RpoHI/RpoHII double deletion strain was significantly lower compared to the RpoHII deletion strain throughout the growth phase, which could reflect a contribution of RpoHI to the promoter activity of RSP_0557 under microaerobic conditions. The expression pattern of RSP_0557 in the ΔRpoHI/RpoHII strain was greatly different from that in the ΔRpoHII strain (Fig 4.18C). The observation that the activity of the RSP_0557 promoter was maintained at a low and constant level in the ΔRpoHI/RpoHII strain in the stationary phase (Fig 4.18C) initially led us to believe that RpoHI plays a crucial role in the stationary phase for regulation of RSP_0557. The activity of the RSP_0557 promoter was constant during outgrowth and constant with the activity before dilution in the ΔRpoHI/RpoHII strain. It is therefore conceivable that RpoHI is also important for the regulation of RSP_0557 during outgrowth. In addition, we cannot exclude that both RpoHI and RpoHII affect the expression of RSP_0557, because the promoter of RSP_0557 might be recognized by either sigma factors RpoHI or RpoHII. As a consequence, the activity of the RSP_0557 promoter in the ΔRpoHI/RpoHII strain

was significantly different from that in the wild type 2.4.1 in the late stationary phase and following outgrowth phase.

5.2.3 Growth phase-dependent regulation on RSP_0904

Some enzymes and regulatory proteins require iron for their biological function. Therefore, it is important to uptake sufficient amounts of iron from the environment. However, excess amounts of iron are harmful for the living organism. Ferrous iron increases oxidative toxicity by the production of hydroxyl radicals in the Fenton reaction. Thus cells require strict regulation of iron metabolism in the presence of oxygen. The gene RSP_0904 (*sitA*) is involved in iron and manganese transport and negatively regulated by the Fur/Mur protein in a manganese-dependent manner (Peuser V*, et al.*, 2011). The expression of RSP_0904 also showed an increase in the stationary phase compared to the middle exponential phase (Remes B*, et al.*, 2017). In order to better understand the expression of RSP_0904 throughout the growth phase, the activity of the RSP_0904 promoter was monitored across the growth curve.

5.2.2.1 Expression pattern of RSP_0904 in wild type strain

The activity of variants of the RSP_0904 promoter with different length of upstream fragments suggests that there is an important regulatory element upstream of the promoter (Fig 4.19). The activity of the RSP_0904 promoter with the longest upstream region (~130 nt upstream fragment (from TSS)) was not constant throughout the growth phase, instead varied with respect to the growth phase in the wild type strain under microaerobic growth conditions (Fig 4.19A). The curve of promoter activity matched with that of oxygen saturation (The curve was published in (Remes B*, et al.*, 2017)) indicating that the expression of RSP_0904 was related to oxygen saturation. After entry into stationary phase, the activity of the RSP_0904 promoter was increased in response to high oxygen saturation. The lowest oxygen saturation occurs in the late exponential phase, while the activity of the RSP_0904 promoter is the lowest in the late exponential phase (Fig 4.19A). The activity of the RSP_0904 promoter have a transient increase after dilution at 24h, 48h, 72h and 144h of growth, upon further incubation, the activity declined steadily (Fig 4.19A). The same trendline was also observed in oxygen saturation after outgrowth. These experiments suggested that expression of RSP_0904 may be related to oxygen

saturation. Previous studies also revealed that iron starvation caused a strongly increased ROS accumulation in the wild type cells (Peuser V, *et al.*, 2011, Remes B, *et al.*, 2014).

5.2.2.2 Role of alternative sigma factors in the regulation of RSP_0904

Do sigma factors have any role in the growth phase-dependent regulation of RSP_0904? The overall activity of the RSP_0904 promoter in TF18, RpoHII deletion or RpoHI/RpoHII double deletion cells was significantly lower compared to the wild type cells across the growth phase reflecting a contribution of these sigma factors to the activity of the RSP_0904 promoter under microaerobic conditions (Fig 4.20). The change in the activity of the RSP_0904 promoter is the same in TF18, RpoHII deletion or RpoHI/RpoHII double deletion strains compared to the wild type. Nevertheless, the question remains: how do these sigma factors affect the expression of RSP_0904? For this, we predicted that RpoE affected the activity of the RSP_0904 promoter indirectly via RpoHII, thus the overall activity of the RSP_0904 promoter is the same in TF18 and RpoHII mutant strain throughout the growth phase (Fig 4.20A and B). Comparison of the promoter activity in the RpoHII mutant to the RpoHI/RpoHII mutant suggests that the RpoHI deletion has a slight effect on the activity of the RSP_0904 promoter. However, while the expression was weaker in the mutants compare the expression pattern of RSP_0904 was nonetheless constant among the four strains throughout the growth phase and during outgrowth. This indicates that RpoE, RpoHI or RpoHII might not have a specific role in growth phase-dependent regulation of RSP_0904.

5.2.4 Growth phase-dependent regulation on RSP_0960

The gene RSP_0960 (*ccr* (crotonyl-CoA carboxylase/reductase)), is involved in carbon fixation. Increased expression of RSP_0960 was observed in the stationary phase and during outgrowth by microarray and RNAseq. In order to better understand the growth phase-dependent regulation of RSP_0960, the activity of the RSP_0960 promoter was monitored across the growth curve.

The results showed that the expression of RSP_0960 is not constant throughout the growth phase and during outgrowth under microaerobic growth conditions or aerobic growth conditions (Fig 4.21). Rather, the expression pattern differs between

microaerobic growth conditions or aerobic growth conditions. This indicates that oxygen saturation can affect the expression of RSP_0960. Nevertheless, the activity of the RSP_0960 promoter varied with respect to the growth phase under aerobic growth conditions (Fig 4.21B). This suggests that oxygen saturation is not the sole factor influencing the expression of RSP_0960, because the oxygen saturation is constant throughout the growth phase.

5.3 Gyrase activity throughout the growth phase and outgrowth

Many investigations in the past have demonstrated that several elements including NAPs (nucleoid-associated proteins), stress factors, transcriptional regulatory factor and growth phase can affect DNA supercoiling. In order to test whether gyrase can affect gene expression in different growth phase in *R. sphaeroides*, we checked the supercoiling activity of gyrase throughout the growth phases and during outgrowth *in vitro*. The similar pattern of gyrase activity in the wild type was observed under aerobic growth conditions and microaerobic growth conditions, which suggests that oxygen saturation does not alter gyrase activity. After 60h of growth the cells almost lost supercoiling activity, but recovered it as soon as nutrients became available at 60h of growth. This agrees with a previous study (Reyes-Dominguez Y*, et al.*, 2003). In this study, the stationary phase cells of *E.coli* showed the ability to recover plasmid supercoiling when nutrients became available. RpoS plays a role in the regulation of plasmid topology during the stationary phase, and the DNA relaxation that occurs in the stationary phase cells has an effect on the transcription of RpoS-dependent genes (Reyes-Dominguez Y*, et al.*, 2003). In *R. sphaeroides*, there is no a RpoS homologous, but RpoHI and RpoHII contribute to survival in outgrowth after a long stationary phase and thus imply an RpoS-like function (Remes B*, et al.*, 2017). RSP_0557 is an RpoHI/II-dependent gene. Some differences in the RSP_0557 promoter activity were observed throughout the growth phase and during outgrowth between the wild type and the RpoH deletion strain (Fig 4.17 and 4.18 BC). There is a possibility that RpoHI/II regulate the expression of RSP_0557 throughout growth phase by affecting DNA supercoiling. When fresh nutrients were added at 72h or 144h of growth, the recovery of supercoiling activity did not take place. For this we proposed that a 30 min incubation after addition of fresh nutrients was too short to observe a recovery in supercoiling activity.

Furthermore, the supercoiling activity of gyrase was checked in H-NS, Lrp and IhfB mutant strains under microaerobic growth conditions. The supercoiling activity was significantly lower after 72h of growth and following outgrowth in the wild type compared to the H-NS mutant. The ΔH-NS cells still had supercoiling activity after 72h of growth and following outgrowth, which suggests that H-NS negatively affects supercoiling activity in the stationary phase. It was found that H-NS can constrain supercoils in DNA (Higgins CF, *et al.*, 1988). The change in supercoiling activity in the ΔLrp strain was comparable to the wild type strain, revealing that Lrp had no effect on the supercoiling activity of gyrase. The ΔIhfB mutant also still had supercoiling activity after 72h of growth, but the activity was not recovered during the following outgrowth. This suggests that IhfB can influence the supercoiling activity of gyrase after 60h of the stationary phase, but not during outgrowth. Further, in order to demonstrate an effect of these NAPs on gene expression, the activity of selected gene promoters in these strains should be measured throughout the growth phase.

6 Summary

The data obtained in this present work demonstrated that PhyR has no major role in the general stress response of the alphaproteobacterium *R. sphaeroides*, in contrast to other bacteria. We could attribute a role for PhyR in response to membrane stress and UV stress in the dark rather than in oxidative stress in *R. sphaeroides*, supporting a more specialized function in this bacterium. PhyR has no major contribution to the complex regulatory network (Billenkamp F*, et al.*, 2015, Cabiscol E*, et al.*, 2000, Glaeser J*, et al.*, 2011, Hess WR*, et al.*, 2014) of protein and sRNA regulators that control the oxidative stress response in *R. sphaeroides*.

PhyR was found to have a function in cell viability in the stationary phase. In addition, the activity of three gene promoters (RSP_0557, *sitA* (RSP_0904) and *ccR* (RSP_0960)) was determined throughout the growth phase and outgrowth. These genes were selected on the basis of differential regulation as determined by microarray and RNAseq approaches. Transcriptional fusions were constructed between these promoters (genes RSP_0557, *sitA* (RSP_0904) and *ccR* (RSP_0960)) and the *lacZ* reporter system. The three promoters showed growth phase-dependent regulation. The sigma factors RpoHI and RpoHII are clearly important for the overall activity of the RSP_0557 promoter and the *sitA* promoter under microaerobic growth conditions. However, while RpoHI and RpoHII have a function on the activity of the RSP_0557 promoter in the stationary phase and following outgrowth, they have no function on the growth phase-dependent regulation of *sitA* under microaerobic growth conditions. Oxygen saturation also has an important role on the regulation of RSP_0557, *sitA* (RSP_0904) and *ccR* (RSP_0960) throughout the growth phase. The supercoiling activity of gyrase was also investigated throughout the growth phase. In the wild type strain, after 60h of growth, the cells almost completely lost supercoiling activity, but this was recovered as soon as nutrients became available. Furthermore, it was demonstrated that H-NS and IhfB had an influence on the supercoiling activity of gyrase in the late stationary phase and following outgrowth.

7 References

Ades SE (2004) Control of the alternative sigma factor sigma(E) in *Escherichia coli*. *Current Opinion in Microbiology* vol. 7: 157-162

Alba BM, Gross CA (2004) Regulation of the *Escherichia coli* sigma(E)-dependent envelope stress response. *Molecular Microbiology* vol. 52: 613-619

Alvarez-Martinez CE, Lourenco RF, Baldini RL, Laub MT, Gomes SL (2007) The ECF sigma factor sigma(T) is involved in osmotic and oxidative stress responses in *Caulobacter crescentus*. *Molecular Microbiology* vol. 66: 1240-1255

Anthony JR, Newman JD, Donohue TJ (2004) Interactions between the *Rhodobacter sphaeroides* ECF sigma factor, sigma(E), and its anti-sigma factor, ChrR. *J Mol Biol* vol. 341: 345-360

Anthony JR, Warczak KL, Donohue TJ (2005) A transcriptional response to singlet oxygen, a toxic byproduct of photosynthesis. *Proc Natl Acad Sci USA* vol. 102: 6502-6507

Appleby JL, Parkinson JS, Bourret RB (1996) Signal transduction via the multi-step phosphorelay: not necessarily a road less traveled. *Cell* vol. 86: 845-848

Asada K (1999) THE WATER-WATER CYCLE IN CHLOROPLASTS: Scavenging of Active Oxygens and Dissipation of Excess Photons. *Annu Rev Plant Physiol Plant Mol Biol* vol. 50: 601-639

Azam TA, Iwata A, Nishimura A, Ueda S, Ishihama A (1999) Growth phase-dependent variation in protein composition of the *Escherichia coli* nucleoid. *J Bacteriol* vol. 181: 6361-6370

Barth M, Marschall C, Muffler A, Fischer D, Hengge-Aronis R (1995) Role for the histone-like protein H-NS in growth phase-dependent and osmotic regulation of sigma S and many sigma S-dependent genes in *Escherichia coli*. *J Bacteriol* vol. 177: 3455-3464

Bastiat B, Sauviac L, Bruand C (2010) Dual control of *Sinorhizobium meliloti* RpoE2 sigma factor activity by two PhyR-type two-component response regulators. *J Bacteriol* vol. 192: 2255-2265

Berghoff BA, Glaeser J, Sharma CM, Vogel J, Klug G (2009) Photooxidative stress-induced and abundant small RNAs in *Rhodobacter sphaeroides*. *Mol Microbiol* vol. 74: 1497-1512

Berghoff BA, Glaeser J, Sharma CM, Zobawa M, Lottspeich F, Vogel J, Klug G (2011) Contribution of Hfq to photooxidative stress resistance and global regulation in *Rhodobacter sphaeroides*. *Mol Microbiol* vol. 80: 1479-1495

Berghoff BA, Konzer A, Mank NN, Looso M, Rische T, Forstner KU, Kruger M, Klug G (2013) Integrative "Omics"-Approach Discovers Dynamic and Regulatory Features of Bacterial Stress Responses. *Plos Genet* vol. 9: e1003576

Billenkamp F, Peng T, Berghoff BA, Klug G (2015) A cluster of four homologous small RNAs modulates C1 metabolism and the pyruvate dehydrogenase complex in *Rhodobacter sphaeroides* under various stress conditions. *J Bacteriol* vol. 197: 1839-1852

Bourret RB (2010) Receiver domain structure and function in response regulator proteins. *CURR OPIN MICROBIOL* vol. 13: 142-149

Browning DF, Busby SJ (2004) The regulation of bacterial transcription initiation. *Nat Rev Microbiol* vol. 2: 57-65

Cabiscol E, Tamarit J, Ros J (2000) Oxidative stress in bacteria and protein damage by reactive oxygen species. *Int Microbiol* vol. 3: 3-8

Cadet J, Delatour T, Douki T, Gasparutto D, Pouget JP, Ravanat JL, Sauvaigo S (1999) Hydroxyl radicals and DNA base damage. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis* vol. 424: 9-21

Campbell EA, Tupy JL, Gruber TM, Wang S, Sharp MM, Gross CA, Darst SA (2003) Crystal structure of *Escherichia coli* sigmaE with the cytoplasmic domain of its anti-sigma RseA. *Mol Cell* vol. 11: 1067-1078

Chiang SM, Schellhorn HE (2010) Evolution of the RpoS Regulon: Origin of RpoS and the conservation of RpoS-Dependent Regulation in Bacteria. *Journal of Molecular Evolution* vol. 70: 557-571

Christman MF, Morgan RW, Jacobson FS, Ames BN (1985) Positive Control of a Regulon for Defenses against Oxidative Stress and Some Heat-Shock Proteins in *Salmonella-Typhimurium*. *Cell* vol. 41: 753-762

Cordeiro RP, Krause DO, Doria JH, Holley RA (2014) Role of the BaeSR two-component regulatory system in resistance of *Escherichia coli* O157:H7 to allyl isothiocyanate. *Food Microbiol* vol. 42: 136-141

Costanzo A, Ades SE (2006) Growth phase-dependent regulation of the extracytoplasmic stress factor, sigmaE, by guanosine 3',5'-bispyrophosphate (ppGpp). *J Bacteriol* vol. 188: 4627-4634

Damm K, Bach S, Muller KMH, Klug G, Burenina OY, Kubareva EA, Grunweller A, Hartmann RK (2015) Impact of RNA Isolation Protocols on RNA Detection by Northern Blotting. *Methods Mol Biol* vol. 1296: 29-38

de Henestrosa ARF, Rivera E, Tapias A, Barbe J (1998) Identification of the *Rhodobacter sphaeroides* SOS box. *Mol Microbiol* vol. 28: 991-1003

DeLasPenas A, Connolly L, Gross CA (1997) The sigma(E)-mediated response to extracytoplasmic stress in *Escherichia coli* is transduced by RseA and RseB, two negative regulators of sigma(E). *Molecular Microbiology* vol. 24: 373-385

Di Mascio P, Kaiser S, Sies H (1989) Lycopene as the Most Efficient Biological Carotenoid Singlet Oxygen Quencher. *Archives of Biochemistry and Biophysics* vol. 274: 532-538

Dizdaroglu M, Jaruga P (2012) Mechanisms of free radical-induced damage to DNA. *Free Radical Research* vol. 46: 382-419

Dufour YS, Landick R, Donohue TJ (2008) Organization and Evolution of the Biological Response to Singlet Oxygen Stress. *J Mol Biol* vol. 383: 713-730

Dufour YS, Imam S, Koo BM, Green HA, Donohue TJ (2012) Convergence of the Transcriptional Responses to Heat Shock and Singlet Oxygen Stresses. *Plos Genet* vol. 8: e1002929

Eltayeb AE, Kawano N, Badawi GH, Kaminaka H, Sanekata T, Shibahara T, Inanaga S, Tanaka K (2007) Overexpression of monodehydroascorbate reductase in transgenic tobacco confers

enhanced tolerance to ozone, salt and polyethylene glycol stresses. *Planta* vol. 225: 1255-1264

Finn RD, Coggill P, Eberhardt RY, Eddy SR, Mistry J, Mitchell AL, Potter SC, Punta M, Qureshi M, Sangrador-Vegas A, et al (2016) The Pfam protein families database: towards a more sustainable future. *Nucleic Acids Res* vol. 44: D279-D285

Flechard M, Fontenelle C, Trautwetter A, Ermel G, Blanco C (2009) *Sinorhizobium meliloti* rpoE2 is necessary for H(2)O(2) stress resistance during the stationary growth phase. *Fems Microbiol Lett* vol. 290: 25-31

Flechard M, Fontenelle C, Blanco C, Goude R, Ermel G, Trautwetter A (2010) RpoE2 of *Sinorhizobium meliloti* is necessary for trehalose synthesis and growth in hyperosmotic media. *Microbiology* vol. 156: 1708-1718

Flint DH, Tuminello JF, Emptage MH (1993) The Inactivation of Fe-S Cluster Containing Hydro-Lyases by Superoxide. *J Biol Chem* vol. 268: 22369-22376

Flores-Kim J, Darwin AJ (2014) Regulation of bacterial virulence gene expression by cell envelope stress responses. *Virulence* vol. 5: 835-851

Foreman R, Fiebig A, Crosson S (2012) The LovK-LovR two-component system is a regulator of the general stress pathway in *Caulobacter crescentus*. *J Bacteriol* vol. 194: 3038-3049

Francez-Charlot A, Frunzke J, Reichen C, Ebneter JZ, Gourion B, Vorholt JA (2009) Sigma factor mimicry involved in regulation of general stress response. *Proc Natl Acad Sci USA* vol. 106: 3467-3472

Francez-Charlot A, Kaczmarczyk A, Fischer HM, Vorholt JA (2015) The general stress response in Alphaproteobacteria. *Trends in Microbiology* vol. 23: 164-171

Gardner PR, Fridovich I (1991) Superoxide Sensitivity of the Escherichia-Coli 6-Phosphogluconate Dehydratase. *J Biol Chem* vol. 266: 1478-1483

Gardner PR, Fridovich I (1991) Superoxide Sensitivity of the *Escherichia-Coli* Aconitase. *J Biol Chem* vol. 266: 19328-19333

Gardner PR, Fridovich I (1991) Quinolinate Synthetase - the Oxygen-Sensitive Site of De-Novo Nad(P)+ Biosynthesis. *Archives of Biochemistry and Biophysics* vol. 284: 106-111

Glaeser J, Klug G (2005) Photo-oxidative stress in *Rhodobacter sphaeroides*: protective role of carotenoids and expression of selected genes. *Microbiology* vol. 151: 1927-1938

Glaeser J, Zobawa M, Lottspeich F, Klug G (2007) Protein synthesis patterns reveal a complex regulatory response to singlet oxygen in *Rhodobacter*. *J Proteome Res* vol. 6: 2460-2471

Glaeser J, Nuss AM, Berghoff BA, Klug G (2011) Singlet Oxygen Stress in Microorganisms. *Advances in Microbial Physiology, Vol 58* vol. 58: 141-173

Gourion B, Rossignol M, Vorholt JA (2006) A proteomic study of *Methylobacterium extorquens* reveals a response regulator essential for epiphytic growth. *Proc Natl Acad Sci USA* vol. 103: 13186-13191

Gourion B, Francez-Charlot A, Vorholt JA (2008) PhyR is involved in the general stress response of *Methylobacterium extorquens* AM1. *J Bacteriol* vol. 190: 1027-1035

Gourion B, Sulser S, Frunzke J, Francez-Charlot A, Stiefel P, Pessi G, Vorholt JA, Fischer HM (2009) The PhyR-sigma(EcfG) signalling cascade is involved in stress response and symbiotic efficiency in *Bradyrhizobium japonicum*. *Mol Microbiol* vol. 73: 291-305

Greenberg JT, Monach P, Chou JH, Josephy PD, Demple B (1990) Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. *Proc Natl Acad Sci U S A* vol. 87: 6181-6185

Griffiths M, Sistrom WR, Cohenbazire G, Stanier RY (1955) Function of Carotenoids in Photosynthesis. *Nature* vol. 176: 1211-1214

Gruber TM, Gross CA (2003) Multiple sigma subunits and the partitioning of bacterial transcription space. *Annual Review of Microbiology* vol. 57: 441-466

Hecker M, Pane-Farre J, Volker U (2007) SigB-dependent general stress response in *Bacillus subtilis* and related gram-positive bacteria. *Annual Review of Microbiology* vol. 61: 215-236

Hendrischk AK, Braatsch S, Glaeser J, Klug G (2007) The *phrA* gene of *Rhodobacter sphaeroides* encodes a photolyase and is regulated by singlet oxygen and peroxide in a sigma(E)-dependent manner. *Microbiology* vol. 153: 1842-1851

Herrou J, Foreman R, Fiebig A, Crosson S (2010) A structural model of anti-anti-sigma inhibition by a two-component receiver domain: the PhyR stress response regulator. *Molecular Microbiology* vol. 78: 290-304

Herrou J, Crosson S, Fiebig A (2017) Structure and function of HWE/HisKA2-family sensor histidine kinases. *Current Opinion in Microbiology* vol. 36: 47-54

Hess WR, Berghoff BA, Wilde A, Steglich C, Klug G (2014) Riboregulators and the role of Hfq in photosynthetic bacteria. *Rna Biol* vol. 11: 413-426

Higgins CF, Dorman CJ, Stirling DA, Waddell L, Booth IR, May G, Bremer E (1988) A Physiological-Role for DNA Supercoiling in the Osmotic Regulation of Gene-Expression in *S-Typhimurium* and *Escherichia-Coli*. *Cell* vol. 52: 569-584

Hinton JCD, Santos DS, Seirafi A, Hulton CSJ, Pavitt GD, Higgins CF (1992) Expression and Mutational Analysis of the Nucleoid-Associated Protein H-Ns of *Salmonella-Typhimurium*. *Mol Microbiol* vol. 6: 2327-2337

Hollander-Czytko H, Grabowski J, Sandorf I, Weckermann K, Weiler EW (2005) Tocopherol content and activities of tyrosine aminotransferase and cystine lyase in Arabidopsis under stress conditions. *J Plant Physiol* vol. 162: 767-770

Hubner P, Willison JC, Vignais PM, Bickle TA (1991) Expression of regulatory nif genes in *Rhodobacter capsulatus*. *J Bacteriol* vol. 173: 2993-2999

Humann JL, Ziemkiewicz HT, Yurgel SN, Kahn ML (2009) Regulatory and DNA Repair Genes Contribute to the Desiccation Resistance of *Sinorhizobium meliloti* Rm1021. *Appl Environ Microb* vol. 75: 446-453

Imlay JA, Linn S (1988) DNA Damage and Oxygen Radical Toxicity. *Science* vol. 240: 1302-1309

Imlay JA (2003) Pathways of oxidative damage. *Annual Review of Microbiology* vol. 57: 395-418

Janion C (2001) Some aspects of the SOS response system - A critical survey. *Acta Biochim Pol* vol. 48: 599-610

Kaczmarczyk A, Hochstrasser R, Vorholt JA, Francez-Charlot A (2015) Two-tiered histidine kinase pathway involved in heat shock and salt sensing in the general stress response of *Sphingomonas melonis* Fr1. *J Bacteriol* vol. 197: 1466-1477

Kehrer JP (2000) The Haber-Weiss reaction and mechanisms of toxicity. *Toxicology* vol. 149: 43-50

Keyer K, Imlay JA (1996) Superoxide accelerates DNA damage by elevating free-iron levels. *Proc Natl Acad Sci U S A* vol. 93: 13635-13640

Kiffin R, Bandyopadhyay U, Cuervo AM (2006) Oxidative stress and autophagy. *Antioxid Redox Sign* vol. 8: 152-162

Kuo CF, Mashino T, Fridovich I (1987) Alpha,Beta-Dihydroxyisovalerate Dehydratase - a Superoxide-Sensitive Enzyme. *J Biol Chem* vol. 262: 4724-4727

Li KY, Pasternak C, Klug G (2003) Expression of the *trxA* gene for thioredoxin 1 in *Rhodobacter sphaeroides* during oxidative stress. *Archives of Microbiology* vol. 180: 484-489

Li QF, Peng T, Klug G (2018) The PhyR homolog RSP_1274 of *Rhodobacter sphaeroides* is involved in defense of membrane stress and has a moderate effect on RpoE (RSP_1092) activity. *Bmc Microbiology* vol. 18: 18

Lima S, Guo MS, Chaba R, Gross CA, Sauer RT (2013) Dual Molecular Signals Mediate the Bacterial Response to Outer-Membrane Stress. *Science* vol. 340: 837-841

Liochev SI, Fridovich I (1994) The Role of O_2-Center-Dot- in the Production of Ho-Center-Dot - *inVitro* and *in Vivo*. *Free Radical Biology and Medicine* vol. 16: 29-33

Lourenco RF, Kohler C, Gomes SL (2011) A two-component system, an anti-sigma factor and two paralogous ECF sigma factors are involved in the control of general stress response in *Caulobacter crescentus*. *Mol Microbiol* vol. 80: 1598-1612

Lourenco RF, Kohler C, Gomes SL (2011) A two-component system, an anti-sigma factor and two paralogous ECF sigma factors are involved in the control of general stress response in *Caulobacter crescentus*. *Molecular Microbiology* vol. 80: 1598-1612

Majdalani N, Gottesman S (2005) The Rcs phosphorelay: a complex signal transduction system. *Annual Review of Microbiology* vol. 59: 379-405

Mecsas J, Rouviere PE, Erickson JW, Donohue TJ, Gross CA (1993) The Activity of Sigma(E), an *Escherichia-Coli* Heat-Inducible Sigma-Factor, Is Modulated by Expression of Outer-Membrane Proteins. *Genes & Development* vol. 7: 2618-2628

Mikulecky PJ, Kaw MK, Brescia CC, Takach JC, Sledjeski DD, Feig AL (2004) *Escherichia coli* Hfq has distinct interaction surfaces for DsrA, *rpoS* and poly(A) RNAs. *Nature Structural & Molecular Biology* vol. 11: 1206-1214

Missiakas D, Mayer MP, Lemaire M, Georgopoulos C, Raina S (1997) Modulation of the *Escherichia coli* sigmaE (RpoE) heat-shock transcription-factor activity by the RseA, RseB and RseC proteins. *Molecular Microbiology* vol. 24: 355-371

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci* vol. 7: 405-410

Mittler R (2017) ROS Are Good. *Trends Plant Sci* vol. 22: 11-19

Muller KMH, Berghoff BA, Eisenhardt BD, Remes B, Klug G (2016) Characteristics of Pos19-A Small Coding RNA in the Oxidative Stress Response of *Rhodobacter sphaeroides*. *Plos One* vol. 11: e0163425

Muller MG, Lambrev P, Reus M, Wientjes E, Croce R, Holzwarth AR (2010) Singlet Energy Dissipation in the Photosystem II Light-Harvesting Complex Does Not Involve Energy Transfer to Carotenoids. *Chemphyschem* vol. 11: 1289-1296

Mullineaux PM, Rausch T (2005) Glutathione, photosynthesis and the redox regulation of stress-responsive gene expression. *Photosynth Res* vol. 86: 459-474

Noor R, Murata M, Yamada M (2009) Oxidative Stress as a Trigger for Growth Phase-Specific sigma(E)-Dependent Cell Lysis in *Escherichia coli*. *Journal of Molecular Microbiology and Biotechnology* vol. 17: 177-187

Nuss AM, Glaeser J, Klug G (2009) RpoH(II) activates oxidative-stress defense systems and is controlled by RpoE in the singlet oxygen-dependent response in *Rhodobacter sphaeroides*. *J Bacteriol* vol. 191: 220-230

Nuss AM, Glaeser J, Berghoff BA, Klug G (2010) Overlapping Alternative Sigma Factor Regulons in the Response to Singlet Oxygen in *Rhodobacter sphaeroides*. *J Bacteriol* vol. 192: 2613-2623

Nuss AM, Adnan F, Weber L, Berghoff BA, Glaeser J, Klug G (2013) DegS and RseP Homologous Proteases Are Involved in Singlet Oxygen Dependent Activation of RpoE in *Rhodobacter sphaeroides*. *Plos One* vol. 8: e79520

Perraud AL, Weiss V, Gross R (1999) Signalling pathways in two-component phosphorelay systems. *Trends in Microbiology* vol. 7: 115-120

Peuser V, Metz S, Klug G (2011) Response of the photosynthetic bacterium *Rhodobacter sphaeroides* to iron limitation and the role of a Fur orthologue in this response. *Env Microbiol Rep* vol. 3: 397-404

Prentki P, Krisch HM (1984) *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* vol. 29: 303-313

Raivio TL (2014) Everything old is new again: An update on current research on the Cpx envelope stress response. *Biochimica Et Biophysica Acta-Molecular Cell Research* vol. 1843: 1529-1541

Remes B, Berghoff BA, Forstner KU, Klug G (2014) Role of oxygen and the OxyR protein in the response to iron limitation in *Rhodobacter sphaeroides*. *Bmc Genomics* vol. 15: 794

Remes B, Eisenhardt BD, Srinivasan V, Klug G (2015) IscR of *Rhodobacter sphaeroides* functions as repressor of genes for iron-sulfur metabolism and represents a new type of iron-sulfur-binding protein. *Microbiologyopen* vol. 4: 790-802

Remes B, Rische-Grahl T, Muller KMH, Forstner KU, Yu SH, Weber L, Jager A, Peuser V, Klug G (2017) An RpoHI-Dependent Response Promotes Outgrowth after Extended Stationary Phase in the Alphaproteobacterium *Rhodobacter sphaeroides*. *J Bacteriol* vol. 199: e00249-17

Repoila F, Gottesman S (2001) Signal transduction cascade for regulation of RpoS: temperature regulation of DsrA. *J Bacteriol* vol. 183: 4012-4023

Reyes-Dominguez Y, Contreras-Ferrat G, Ramirez-Santos J, Membrillo-Hernandez J, Gomez-Eichelmann MC (2003) Plasmid DNA supercoiling and gyrase activity in *Escherichia coli* wild-type and *rpoS* stationary-phase cells. *J Bacteriol* vol. 185: 1097-1100

Roop RM, Gee JM, Robertson GT, Richardson JM, Ng WL, Winkler ME (2003) *Brucella* stationary-phase gene expression and virulence. *Annu Rev Microbiol* vol. 57: 57-76

Roop RM, Gee JM, Robertson GT, Richardson JM, Ng WL, Winkler ME (2003) *Brucella* stationary-phase gene expression and virulence. *Annual Review of Microbiology* vol. 57: 57-76

Ryter SW, Tyrrell RM (1998) Singlet molecular oxygen (O-1(2)): A possible effector of eukaryotic gene expression. *Free Radical Biology and Medicine* vol. 24: 1520-1534

Sauviac L, Philippe H, Phok K, Bruand C (2007) An extracytoplasmic function sigma factor acts as a general stress response regulator in *Sinorhizobium meliloti*. *J Bacteriol* vol. 189: 4204-4216

Schellhorn HE, Stones VL (1992) Regulation of *katF* and *katE* in *Escherichia coli* K-12 by weak acids. *J Bacteriol* vol. 174: 4769-4776

Schellhorn HE, Audia JP, Wei LI, Chang L (1998) Identification of conserved, RpoS-dependent stationary-phase genes of *Escherichia coli*. *J Bacteriol* vol. 180: 6283-6291

Schilke BA, Donohue TJ (1995) ChrR positively regulates transcription of the *Rhodobacter sphaeroides* cytochrome c2 gene. *J Bacteriol* vol. 177: 1929-1937

Shao HB, Liang ZS, Shao MA, Sun Q (2005) Dynamic changes of anti-oxidative enzymes of 10 wheat genotypes at soil water deficits. *Colloids and Surfaces B-Biointerfaces* vol. 42: 187-195

Sharma CM, Hoffmann S, Darfeuille F, Reignier J, Findeiss S, Sittka A, Chabas S, Reiche K, Hackermuller J, Reinhardt R, et al (2010) The primary transcriptome of the major human pathogen *Helicobacter pylori*. *Nature* vol. 464: 250-255

Sharma P, Dubey RS (2004) Ascorbate peroxidase from rice seedlings: properties of enzyme isoforms, effects of stresses and protective roles of osmolytes. *Plant Science* vol. 167: 541-550

Simon R, Oconnell M, Labes M, Puhler A (1986) Plasmid Vectors for the Genetic-Analysis and Manipulation of *Rhizobia* and Other Gram-Negative Bacteria. *Method Enzymol* vol. 118: 640-659

Smith AH, Blevins JS, Bachlani GN, Yang XF, Norgard MV (2007) Evidence that RpoS (sigmaS) in *Borrelia burgdorferi* is controlled directly by RpoN (sigma54/sigmaN). *J Bacteriol* vol. 189: 2139-2144

Srinivasan C, Liba A, Imlay JA, Valentine JS, Gralla EB (2000) Yeast lacking superoxide dismutase(s) show elevated levels of "free iron" as measured by whole cell electron paramagnetic resonance. *J Biol Chem* vol. 275: 29187-29192

Staron A, Sofia HJ, Dietrich S, Ulrich LE, Liesegang H, Mascher T (2009) The third pillar of bacterial signal transduction: classification of the extracytoplasmic function (ECF) sigma factor protein family. *Mol Microbiol* vol. 74: 557-581

Steinbeck MJ, Khan AU, Karnovsky MJ (1992) Intracellular Singlet Oxygen Generation by Phagocytosing Neutrophils in Response to Particles Coated with a Chemical Trap. *J Biol Chem* vol. 267: 13425-13433

Stock AM, Robinson VL, Goudreau PN (2000) Two-component signal transduction. *Annual Review of Biochemistry* vol. 69: 183-215

Sychev AY, Duka GG (1985) Catalysis of the Tartaric Acid Oxidation by Oxygen in the Presence of Iron(Iii) Ions. *Zhurnal Fizicheskoi Khimii* vol. 59: 1933-1936

Truglio JJ, Croteau DL, Van Houten B, Kisker C (2006) Prokaryotic nucleotide excision repair: The UvrABC system. *Chem Rev* vol. 106: 233-252

Waters LS, Storz G (2009) Regulatory RNAs in bacteria. *Cell* vol. 136: 615-628

West AH, Stock AM (2001) Histidine kinases and response regulator proteins in two-component signaling systems. *Trends in Biochemical Sciences* vol. 26: 369-376

Zeilstraryalls JH, Kaplan S (1995) Aerobic and Anaerobic Regulation in *Rhodobacter Sphaeroides* 2.4.1 -the Role of the Fnrl Gene. *J Bacteriol* vol. 177: 6422-6431

Zeller T, Klug G (2004) Detoxification of hydrogen peroxide and expression of catalase genes in *Rhodobacter*. *Microbiology* vol. 150: 3451-3462

Zhang Y, Marcillat O, Giulivi C, Ernster L, Davies KJA (1990) The Oxidative Inactivation of Mitochondrial Electron-Transport Chain Components and Atpase. *J Biol Chem* vol. 265: 16330-16336

Acknowledgements

I would like to take this opportunity to thank all the people, without whom this dissertation would not have existed.

First and foremost, I would like to express my sincere gratitude to my supervisor, Prof. Dr. Gabriele Klug for her immense knowledge, constructive ideas, discussion, and support. It was a great honor for me to be her student. I also thank Prof. Dr. Albrecht Bindereif for being my co-supervisor and the referee of my dissertation. I thank Apl. Prof. Dr. Elena-Evguenieva-Hackenberg and Prof. Dr. Reinhard Dammann for being the referees on my defense.

In addition, I really appreciate that my wonderful colleagues, they gave me suggestions on my experiments and help me a lot on my life. In particular, I would like to express gratitude to Dr. Peng for teaching me a lot of methods of molecular biology and to Dr Remes, Dr Kathrin and Dr Zhao for their help on my study. I also thank Dr. Berghof and Apl. Prof. Dr. Elena Evguenieva-Hackenberg for their instructive suggestions. I would like to thank Angelika, Carmon, Andreas and Kerstin for their technical assistances.

I would also like to thank Chinese scholarship council for the financial support. I greatly appreciate Dr, Fei Song, Xin Nie, Yu Sun, Shaoshuai Liu, Fang Xu and all my Chinese friends for their kind help and support during my study and life in Giessen.

Finally, I express my appreciation to my parents for their unconditional love and support throughout my life.

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig verfasst habe und dabei keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Zitate sind als solche gekennzeichnet.

Giessen, den 15. Januar 2019

Qingfeng Li

Erklärung