Role of sRNAs and a response regulator in response to oxidative stress in *Rhodobacter sphaeroides*

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Contents

Abbreviations

aa	amino acid
ADP	adenosinediphosphate
Ap	ampicillin
APS	ammoniumpersulfate
ATP	adenosinetriphosphateμ
BChl	bacteriochlorophyll
Bp	base pair(s)
BSA	bovine serum albumin
Cpm	counts per minute
℃	centigrade
Da	Dalton
dATP	deoxyriboadenosine triphosphate
dCTP	deoxyribocytosine triphosphate
DMSO	dimethyl sulfoxide
DNA	deoxynucleic acid
DNase	deoxyribonuclase
dNTP	deoxyribonucleotide
DTT	1, 4-dithiothreitol
E. coil	*Escherichia coil*
EDTA	ethylene diamine tetraacetic acid
e.g	*exempli gratia* (for example)
et al.	*et alii* (and others)
etc.	*et cetera* (and other things)
EtOH	ethanol
g	gram
Gm	gentamycin
H	hour (h)
His	histidine
IPTG	isopropyl-β-Dthiogalactopyranoside
kDa	kilodalton
Kb	kilo base pairs
Km	kanamycin
LHI	light-harvesting complex I
LHII	light-harvesting complex II
M	molar (mol/l)
β-ME	β-Mercaptoethanol
min	minute(s)
ml	milliliter
mmol	millimole
mM	millimolar
mRNA	messenger RNA
Ci	microcurie
μg	microgram
μl	microliter
μm	micrometer
μM	micromolar
nm	nano meter
nt	nucleotide
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buddered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluorid
PNK	polynucleotide kinase triphosphate
PS	Photosynthesis
R. sphaeroides	*Rhodobacter sphaeroides*
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
rpm	revolution per minute
SDS	sodium dodecyl sulfate
sec	second(s)
Tc	tetracycline
TCA	trichloroacetic acid
Tris	Trishydroxymethylaminomethane
Tp	trimethoprim
UV	ultraviolet
V	volt

Publications

The following publications are based on this work

1. **Tao Peng**, Bork Berghoff, Jeong-II Oh, Lennart Weber, Johannes Schwarz, Jasmin Schirmer, Jens Glaeser, Gabriele Klug. (2016) Regulation of a polyamine transporter by the conserved 3' UTR-derived sRNA SorX confers resistance to singlet oxygen and organic hydroperoxides in *Rhodobacter sphaeroides*. *RNA biology.* (Epub ahead of print)

2. 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*. 197: 1839-52. [#]equal contributer

1 Introduction

1.1 Regulatory small RNAs in bacteria

Most bacteria are living in changing environmental conditions. In order to adapt to the changing environmental conditions, bacteria have to develop complex regulatory networks to sense and respond to these changes. In recent years, regulatory small RNAs (sRNAs), which range in size from 50 to 300 nucleotides, have obtained attention. The first sRNA MicF was characterized in 1984 (Mizuno *et al*., 1984). MicF has a long sequence that is complementary to the 5' end region of the *ompF* mRNA encoding a major outer membrane protein to repress its translation. Recently, more sRNAs have been identified in bacteria. The sRNAs which have been characterized are divided into two categories, *cis*-encoded and *trans*-encoded. The *cis*-encoded sRNAs are fully complementary to their targets, because they are transcribed from the opposite DNA shared of their target genes (Thomason and Storz, 2010; Brantl, 2007). One example is CopA, a ~90 nucleotide sRNA, which is capable to regulate the copy number of plasmid R1 which contains multi-drug antibiotic resistance genes (Campbell and Mullins, 2007) by base paring to *repA* a replication initiator mRNA (Kolb *et al*., 2000). The *trans*-encoded sRNAs have the ability to bind to the target mRNAs with imperfect bases pairing, since the target genes are located in another chromosomal location (Papenfort and Vogel, 2009). It is known that *trans*-encoded sRNAs are able to regulate multiple target mRNAs by imperfect bases pairing (Lease *et al*., 1998; Majdalani *et al*., 1998).

1.1.1 Gene regulation by sRNAs

Numerous studies have elucidated the functions of sRNAs in the last years. Most sRNAs can modulate gene expression on the post-transcriptional level by short base pairing to target mRNAs (Papenfort and Vogel, 2009). In most cases, the sRNAs which are often induced by stress conditions, negatively regulate the translation and stability of the target mRNAs (Fig 1.1). In some cases, the sRNAs are able to base pair to the targets at the ribosome binding site (RBS) region to mask the RBS and inhibit the 30S ribosomal subunit association, resulting in translational repression. Afterwards, the target mRNAs may be degraded by RNase E, e.g. RyhB of *E. coli*, which is induced by iron limitation, bases pair to the *sodB* mRNA encoding a Fe-containing superoxide dismutases to block the translation, resulting in a rapid degradation of *sodB* by RNAse E dependent cleavage (Prévost *et al*., 2011). In other cases, the sRNAs are able to bind to the coding sequence of the mRNAs to induce

degradation by RNase E. In *E. coli,* MicC binds to the coding sequence of the *ompD* mRNA encoding an outer membrane protein, then guides RNase E to cleave inside the coding sequence of *ompD* (Chen *et al*., 2004).

Some sRNAs are able to positively regulate gene expression (Fig 1.1). In some cases, the RBS binding site of the mRNA is masked by a secondary structure and this secondary structure is released by the sRNA, resulting in translational activation. One example is DsrA which releases the self-inhibitory hairpin of *rpoS* mRNA encoding an alternative sigma factor RpoS and free the RBS, thus activating *rpoS* translation (Sledjeski *et al*., 2001). Some sRNAs can stabilize the target mRNAs by protecting them from degradation by RNase E. In *E. coli*, the *cfa* mRNA encoding a cyclopropane fatty acid synthase is unstable due to the degradation by RNase E. However, the RydC sRNA is able to stabilize it by base pairing at the RNase E-sensitive site (Fröhlich and Vogel, 2009). All these examples confirm that sRNAs can regulate gene expression at the post-transcriptional level.

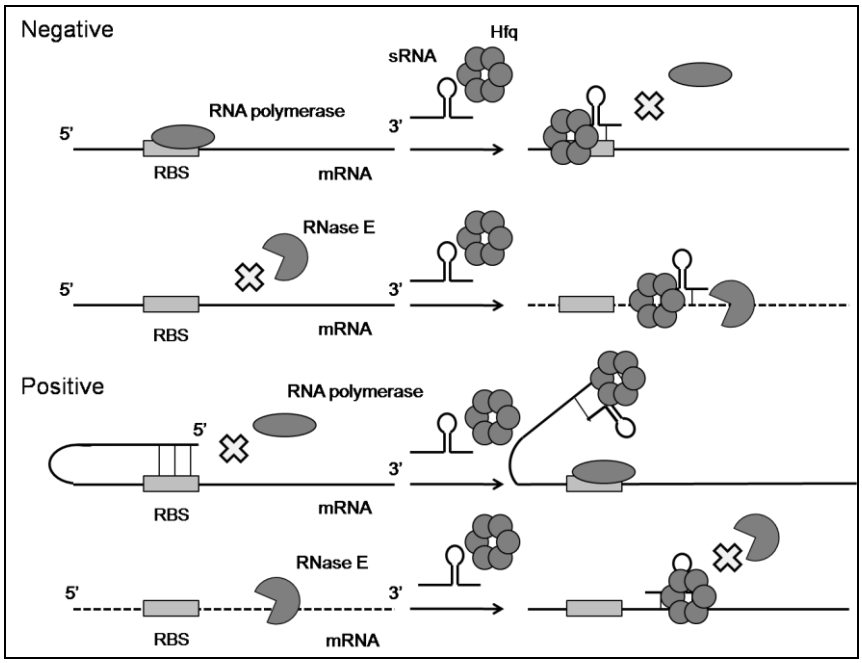

Fig 1.1 Gene regulation by sRNAs. sRNAs are able to negatively or positively regulate gene expression in the cell.

The RNA chaperon Hfq protein

In most cases of *trans*-encoded sRNAs, sRNA-mRNA interactions need the cooperation of the RNA chaperon Hfq (a host factor required for the efficient replication of the RNA bacteriophage QB) in bacteria. The bacterial Hfq protein belongs to the Sm and Sm-like proteins. In eukaryotes, the Sm and Sm-like proteins

have functions in mRNA-splicing and RNA-decapping (Wilusz and Wilusz, 2005). The Hfq protein in *E coli* displays a characteristic Sm-fold and forms a homo-hexamer according to the crystal structure (Schulz and Barabas, 2014). This proves that bacteria contain a hexameric Sm-like protein and indicates that Hfq protein likely has a similar function to Sm proteins in eukaryotes.

In bacteria, Hfq is able to affect the stability and translation of mRNAs. It represses the translation by covering the RBS (Nogueira and Springer, 2000). Hfq also plays a critical role in sRNA-mediated gene regulation. It binds sRNA to moderate the stability and anneal sRNAs to their target mRNAs (Geissmann *et al.*, 2004). Hfq binds to oligo (U) stretches of the sRNA and interacts with the target mRNA to form a nucleoprotein complex, thus promoting the interaction of sRNA and the target mRNA (Møller *et al.*, 2002), e.g. the Hfq protein increases OxyS sRNA interaction with *flhA* which encodes a largellar export pore protein and represses its translation in *E. coli* (Sledjeski *et al.*, 2001).

An endoribonuclease RNase E

RNase E is an endoribonuclease especially for single-stranded RNA and prefers 5'-monophosphorylated ends over 5'-triphosphate ends of primary tanscripts (Mackie, 1998). The N-terminus of RNase E contains an endonuclease activity domain. The C-terminus of RNase E is essential for its membrane localization and function as a scaffold for other proteins to form the degradosome. In *E. coli*, the degradosome is mainly composed of polynucleotide phosphorylase, RNA helicase RhlB and enolase. RNase E is also known to form a complex composed of Hfq, sRNA and mRNA (Callaghan *et al.*, 2005). RNase E is important for mRNA degradation and processing of some structured RNAs (Py *et al.*, 1994). It is also known that mRNA degradation induced by sRNA hybridization is often dependent on RNase E (Miczak *et al.*, 1996). RNase E is able to cleave the mRNA at a distance from the mRNA-sRNA paring site (Prévost *et al.*, 2011). The sRNA MicA become more stable upon deletion of the C-terminus of RNase E indicating that the degradation of this sRNA needs the concerted action of the degradosome.

1.1.2 sRNAs modulate protein activity

Bacterial mRNAs are not the only targets of sRNAs, a number of sRNAs are also known to bind cellular proteins to modulate their activities (Willkomm and Hartmann, 2005). For example, the bacterial 6S RNA is able to inhibit transcription by binding directly to the housekeeping holoenzyme of RNA polymerase in *E. coli*. It occupies

the active site of RNA polymerase to block the binding to promoter DNA. Moreover, 6S RNA is also used as a template for RNA synthesis, resulting in 6S RNA released from RNA polymerase. This regulation by 6S RNA causes a higher survival rate under nutrient-limiting conditions by redirecting resource utilization (Wassarman, 2007).

1.2 Two component regulatory systems in bacteria

Besides the use of sRNAs as regulatory factors in the cell, bacteria also own special two component regulatory systems (TCS) which allow them to sense and to cope rapidly with the changing environment. There is one sensor histidine kinase (HK) and one response regulator (RR) in TCS. A sensory domain and a transmitter domain are in the HK (West and Stock, 2001). The sensory domain is responsible for receiving the external signal form the environment. Two subdomains, a dimerization domain and an ATPase domain are in the transmitter domain. The dimerization domain contains a conserved His residue. The RR contains a receiver domain which can obtain a phosphoryl group from HK and a variety of output domains. If the sensory domain of HK receives the signal from the environment, the HK ATPase domain catalyzes the conserved His phosphorylation. Afterwards, the phosphoryl group is transferred to Asp of the receiver domain to active the RR. Activated RRs often function as transcription factors which can regulate gene expression in response to the signal. The HK also has dephosphorylation activity for RR. The balance of autophosprlaion and dephosphralation activity are controlled by the external signals.

For most TCSs, the phosphoryl group is transferred directly from HK to RR. This phosphor transmission scheme is common in bacteria. However, in some cases, several phosphor transfer steps between HK and RR are involved called phosphorelay (Perraud *et al*., 1999). A special kinase class called hybrid kinase contains both His and Asp in the transmitter domain. The hybrid kinase autophosphralation at His after sensing the signal, then the phosphoryl group is transferred to Asp in its own transmitter domain. Afterwards, the phosphoryl group is transferred from Asp of hybrid kinase to His of a special phosphotransfer protein (Cock and Whitworth, 2007). Finally, the phosphoryl group is transferred from His of phosphotransfer protein to the Asp of RR (Fig 1.2).

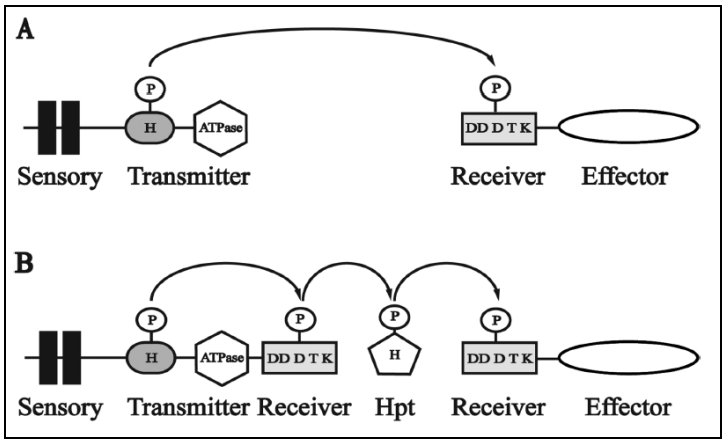

Fig 1.2 Phosphor transmission scheme in TCS from Nguyen *et al*., 2015**.** (A) A phosphoryl group is transferred directly from HK to RR. (B) A phosphoryl group is transferred to the phosphotransfer protein and then transferred to RR.

TCSs are mostly distributed in prokaryotes and rare cases are indentified in eukaryotes. The regulators often cooperate together as a network in the cell. TCS can work with other regulatory factors, such as sRNA, to modulate gene expression (Vogel and Papenfort, 2006). E.g. in enterobacteria the TCS EnvZ/OmpR is able to work with several sRNAs to regulate outer membrane protein expression. Moreover, there are some crosstalks among various TCSs (Alvarez-Martinez *et al*., 2007).

1.3 A two component regulatory system in bacteria: EnvZ/OmpR

EnvZ/OmpR is a well characterized TCS in bacteria. OmpR is a response regulator harboring a DNA binding domain as output domain. In *E. coli*, at low osmolarity, the main outer membrane protein is OmpF. OmpF has a smaller pore and slower flux compared to another outer membrane protein OmpC. At low osmolarity condition, the amount of phosphorylated OmpR protein (OmpR~p) is small, due to the low kinase activity of the HK EnvZ (Mattison and Kenney, 2002). Most of OmpR~p proteins are able to bind to the F1, F2 and F3 OmpR-binding sites in the upstream of *ompF* (Head *et al*., 1998), since these sites possess high affinities to OmpR~p protein. Less of OmpR~p proteins bind to C1, C2 and C3 OmpR-binding sites in the upstream of *ompC* which possess low affinities to OmpR~p, resulting in more *ompF* expression on outer membrane of the cell (Fig 1.3). However, at high osmolarity condition, the number of phosphorylated OmpR protein increases, because the high kinase activity of EnvZ. OmpR~p proteins bind not only to the high affinity binding sites (F1, F2 and F3) in the upstream of the *ompF*, but also to the F4 site which possess a low affinity to OmpR~p protein. As a consequence, a loop forms, resulting in the repression of

ompF gene. Meanwhile, OmpR~p proteins bind to the C1, C2 and C3 sites in the upstream of *ompC* gene, resulting in more *ompC* expression on outer membrane of the cell (Fig 1.3) (Mattison *et al*., 2002). More functions of OmpR have been revealed recently. In *Salmonella*, the *ssrA/B* genes endocing a two-component regulatory system SsrA/B locus on pathogenicity island-2 are under control of the OmpR protein (Feng *et al*., 2003; Lee *et al*., 2000).

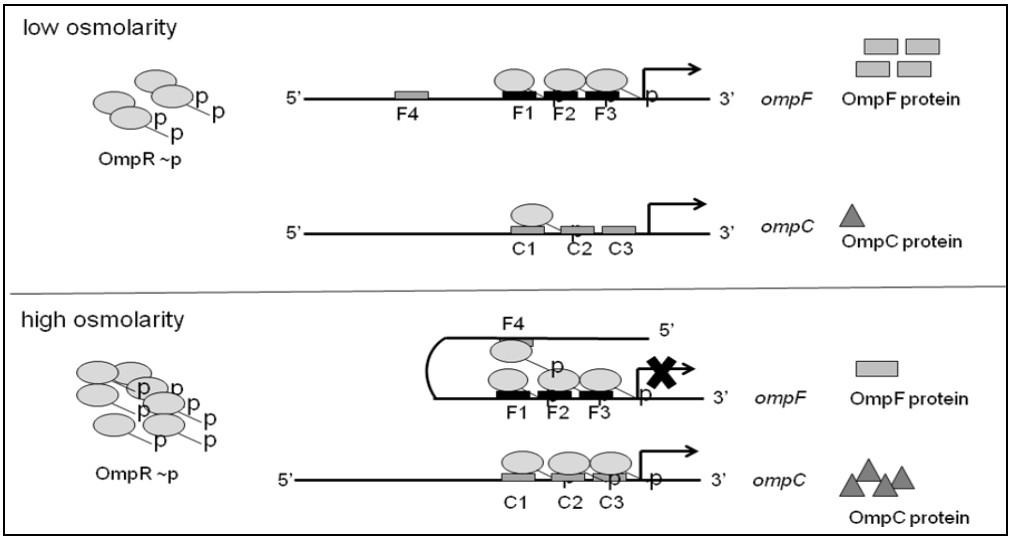

Fig 1.3 The expression of *ompF* and *ompC* is regulated by OmpR dependent on osmolarity level in the environment.

1.4 Reactive oxygen species in the cell

Bacteria obtain benefits from oxygen. The oxygen is crucial to many metabolic pathways in the cell. Molecular oxygen is essential for cellular respiration in all aerobic organisms. However, bacteria have a limited tolerance for oxygen, due to many reactive oxygen species (ROS) which are produced in the cell.

Molecular oxygen is a stable diradical with two unpaired, spun-aligned electrons in the outer p (molecular) orbital, thus making oxygen a molecule that is able to accept electrons. The type I mechanism in which one electron is transferred to molecule oxygen is able to produce superoxide (O_2^-) (Imlay, 2003). Furthermore, in biological systems hydrogen peroxide (H_2O_2) is generated by an electron transferred to superoxide. If H_2O_2 reacts with ferrous iron (Fe^{2+}), then a hydroxyl (OH•) is generated (Halliwell and Gutteridge, 2010) (Fig 1.5).

The type II mechanism in which energy is transferred from a excited molecule used as a photosensitizer to molecular oxygen is able to produce singlet oxygen ($^1O_2$) (Briviba *et al*., 1997) (Fig 1.5). If the excitation energy is transferred to molecular oxygen, the

outer most electrons of molecular oxygen are rearranged, resulting in an outer p orbital structure in which one orbital contains paired electrons, while the other orbital contains no electrons. The spun restriction which normally prevents the reaction with most organic molecules in the cell is removed, therefore, singlet oxygen is the most reactive oxygen specie.

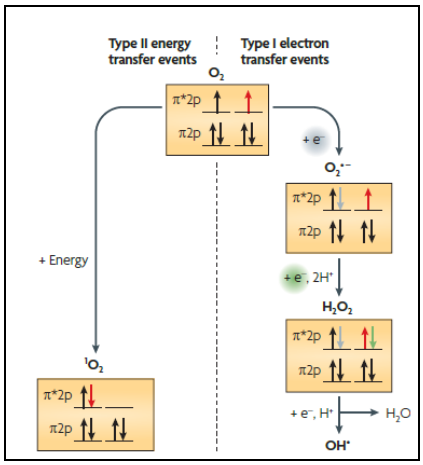

Figure 1.5 Reactive oxygen species are generated by type I and type II mechanisms taken from Ziegelhoffer and Donohue, 2009.

ROS are more reactive than molecular oxygen. They can damage the DNA, proteins and membranes, thus ROS are toxic to the cell. Superoxide can oxidize Fe-S cluster, then iron is released into the cytosol and reacts with hydrogen peroxide, generating the hydroxyl radical which reacts with DNA (Keyer and Imlay, 1996). Such species are inevitable by-products of aerobic metabolism. In *E. coli*, superoxide and hydrogen peroxides are generated by NADH dehydrogenase II, succinate dehydrogenase, sulfite reductase and fumarate reductase in the electron transport chains if exposed to oxygen (Imlay, 1995; Messner and Imlay, 1999). A rapid response of the cell to ROS is crucial for its survival. Some transcription factors are involved in response to oxidative stress in bacteria. OxyR is activated by hydrogen peroxides. It is able to regulate the genes (*katG*, *ahpC* and *ahpF*) related to oxidant elimination and some genes (*dps* and *fur*) which are involved in reduction of Fe^{2+} availability, which reduces hydroxyl radical generation (Christman *et al*., 1985). Superoxide can increase SoxR activity, another factor SoxS is activated by SoxR. SoxRS can regulate gene expression encoding manganese superoxide dismutase and superoxide reductase (Greenberg *et al*., 1990). Many reports have elucidated that peroxidases and catalases scavenge hydrogen peroxides in the cell. Superoxide reductase and superoxide dismutase help the cell to defense superoxide (Seaver and Imlay, 2004; Pomposiello *et al*., 2001).

1.5 Source of singlet oxygen in the cell

Among all the ROS, singlet oxygen is the most reactive, because the spun restriction is removed. It can react with most organic molecules in the cell, such as membrane lipids, sterols, proteins, amino acids, nucleic acids (DNA and RNA), carbohydrates, and thiols (Ryter and Tyrrell, 1998).

In the cell, singlet oxygen is mainly produced dependent on light. In photosynthetic bacteria, bacteriochlorophyll is usually used as photosensitizer to generate singlet oxygen in the presence of light and oxygen. In a photosynthetic bacterium like *R. sphaeroides*, when oxygen and light are present simultaneously, the energy is transferred to oxygen by bacteriochlorophyll to generate the singlet oxygen which is a kind of photooxiditive stress (Fig 1.6). In nonphotosynthetic bacteria, several endogenous photosensitizes, such as porphyrins, rhodopsins or flavins, are present (Ryter and Tyrrell, 1998). They are able to produce singlet oxygen whenever light and oxygen are present (Redmond and Gamlin, 1999) (Fig 1.6).

However, singlet oxygen is also produced even without light. In the cell, many pathways can generate singlet oxygen (Fig 1.6). If hydrogen peroxides are produced in the cell, reduction of hydrogen peroxides is able to generate hypochlorous acids. Consequently, singlet oxygen is produced by the spontaneous reaction of hydrogen peroxides with hypochlorous acids (Davies, 2004).

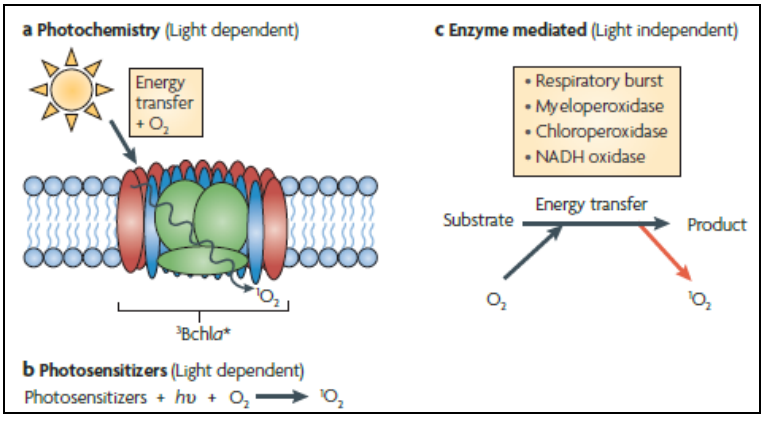

Figure 1.6 Source of singlet oxygen in the cell taken from Ziegelhoffer and Donohue, 2009. a, b: Singlet oxygen is produced dependent of light. c: Singlet oxygen is produced independent of light.

1.6 Physiology of *R. sphaeroides*

R. sphaeroides is a gram negative bacterium and belongs to the α-3 group of proteobacteria. It is a purple non-sulfur bacterium and distributed in mud, and other

aquatic environmental zones in nature. The genome was sequenced in 1989. It contains two circlular chromosomes and five plasmids. The total genome size is 4.6 Mb and G+C content is 67.3 mol (Suwanto and Kaplan, 1989). Interestingly, *R. sphaeroides* has the ability to perform diverse metabolic pathways. It is capable to perform anoxygenic photosynthesis in the presence of light and grow by aerobic respiration under aerobic condition. *R. sphaeroides* has been the model organism for studying anoxygenic photosynthesis, particularly regarding the process and control of photosynthesis. Since singlet oxygen is produced in *R. sphaeroides*, it is also used as a model for studying the photooxiditive stress response.

1.7 Regulation of photosynthesis genes in *R sphaeroides*

Singlet oxygen is generated by photosynthetic complexes if light and oxygen are simultaneously present in *R. sphaeroides*. It is known that singlet oxygen is harmful to the cell. In order to avoid the generation of singlet oxygen, the synthesis of photosynthetic complexes are regulated by oxygen tension and light in *R. sphaeroides*.

At high oxygen tension, transcription of photosynthesis related genes is repressed by PpsR, which directly binds to the promoter region of photosynthesis genes (Gomelsky and Kaplan, 1995). When oxygen tension is decreased, the AppA protein senses the oxygen signal by a heme binding redox sensing domain. then binds to PpsR. As a consequence, the PpsR protein is released from the DNA, resulting in photosynthesis gene transcription (Fig 1.7). Binding of AppA to PpsR also depends on light. AppA can sense the blue light by a flavin-adenine dinucleotide binding domain, which prevents the interaction of AppA with PpsR (Braatsch et al., 2002). This regulation ensures that there is no harmful singlet oxygen produced by photosynthetic complexes when light and oxygen are simultaneously present. Moreover, if oxygen tension is low, a HK PrrB (belongs to a TCS) undergoes autophosphorylation and transfers the phosphoryl group to the response regulator PrrA (Oh and Kaplan, 1995), then PrrA~p protein activates photosynthesis gene transcription. Another regulator FnrL also can activate transcription of photosynthesis related genes. FnrL is an anaerobic regulator and its activity is induced by low oxygen tension (Zeilstra-Ryalls *et al.*, 1995). Moreover, a sRNA PcrZ, which transcribed is dependent on PrrA and FnrL, is capable to repress photosynthesis gene expression. The *appA* gene is also repressed by PcrZ (Mank *et al*., 2012), resulting in a stronger repression of photosynthesis gene by PpsR. CryB belongs to a cryptochrome family can regulate photosynthesis gene expression

by directly binding to AppA dependent on light (Metz *et al*., 2012). A *ppaA* gene encoding a regulator can activate some photosynthesis genes at high oxygen tension, while the *ppaA* gene is repressed by PpsR (Fig 1.7) (Eraso *et al*., 2008; Gomelsky *et al*., 2008). This regulation also avoids singlet oxygen produced in the cell.

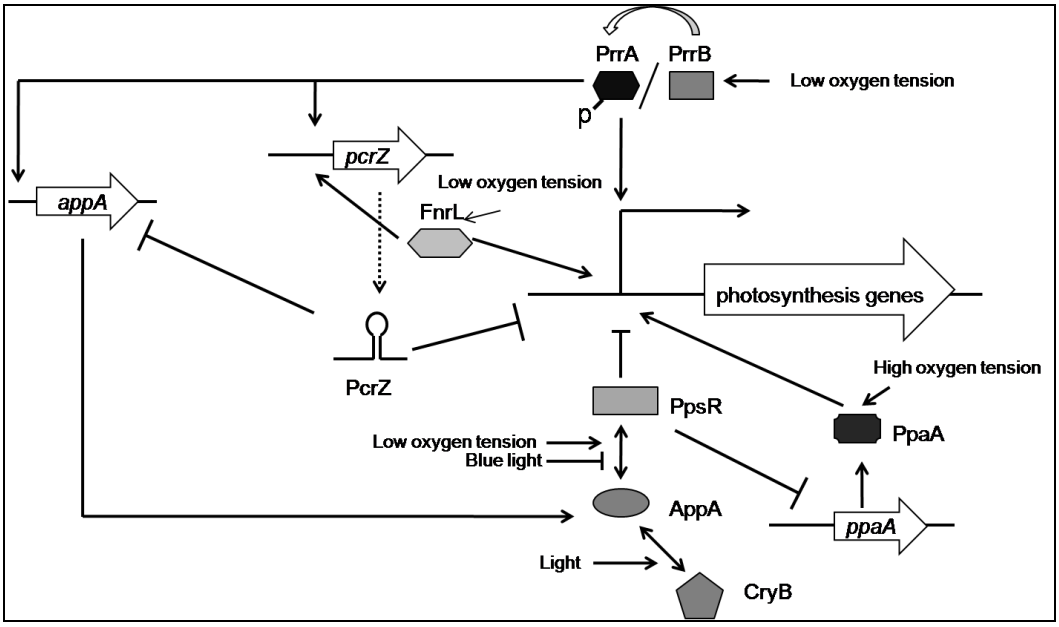

Figure 1.7 Regulation of photosynthesis genes by light and oxygen tension in *R sphaeroides.*

1.8 Singlet oxygen response in *R. sphaeroides*

1.8.1 Carotenoids: quenchers of singlet oxygen

In *R. sphaeroides*, the protective role of carotenoids as quenchers against singlet oxygen was recognized 60 years ago (Griffiths *et al*., 1955). A carotenoid-deficient mutant of *R. sphaeroides* is rapidly killed when light and oxygen are present simultaneously. It was reported that high levels of singlet oxygen are produced in a mutant lacking carotenoids (Glaeser and Glug, 2005). These studies indicate carotenoids play an important role in response to singlet oxygen in *R. sphaeroides*

Carotenoids are able to defend against singlet oxygen by quenching excited chlorophylls and bacteriochlorophylls or by directly quenching singlet oxygen (Cogdell *et al*., 2000). Besides carotenoids, more singlet oxygen scavengers are present in the cell, such as glutathione (GSH) (Devasagayam *et al*., 1991). It reacts with singlet oxygen to prevent the damage to the cell. GSH is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side-chain and the amine group of cysteine. If GSH is at reduced state, the thiol group of cysteine is

able to donate a reducing equivalent to other unstable molecules, such as ROS. As a consequence, GSH is oxidized to GSSG. Glutathione reductase is responsible for reduction of GSSG to GSH using NADPH as an electron donor (Ryter and Tyrrell, 1998; Davies *et al*., 2004).

1.8.2 The role of the sigma factor RpoE in the singlet oxygen response

In addition to carotenoids used as quenchers of singlet oxygen, previous studies demonstrated that a ECF sigma factor RpoE (RSP_1092) is involved in singlet oxygen response in *R. sphaeroides* (reviewed in Glaeser *et al*., 2011). It was reported that an increase in RpoE activity under singlet oxygen stress in *R. sphaeroides* (Anthony *et al*., 2005). RpoE belongs to the group Ⅳ sigma factor (Campbell *et al*., 2007). In the cell, RpoE is at inactive state by binding to an anti-sigma factor ChrR (RSP_1093) to form a 1:1 heterodimeric complex under non-stress condition (Dufour *et al*., 2008). The *chrR* gene is located in the same operon and transcribed with *rpoE*. The ChrR protein contains a zinc containing anti-sigma domain and a cupin like domain (Anthony *et al*., 2005). If the cell is exposed to singlet oxygen, ChrR is degraded by the proteases DegS and RseP (Nuss *et al*., 2013) to release RpoE, afterwards, RpoE binds to the RNA polymerase and actives the expression of target genes and a sRNA, RSs0019 (Berghoff *et al*., 2009). However, There are only a few genes directly regulated by RpoE including its own *rpoE* gene (Fig 1.8). RpoE-ChrR homologs are distributed in many α-, β-and γ-proteobacteria. In the α-proteobacterium *Caulobacter crescentus*, the activity of RpoE is not only induced by singlet oxygen, but also by organic hydroperoxides (tert-butyl-hydroperoxide, tBOOH) (Nam *et al*., 2013). It is also true for *R. sphaeroides* that organic hydroperoxide can induce RpoE activity. In *R. sphaeroides*, deletion of *chrR* causes enhanced resistance to singlet oxygen and deletion of the *rpoE-chrR* operon causes a higher sensitivity to singlet oxygen (Nuss *et al*., 2010). These data demonstrate that RpoE plays an important role in the singlet oxygen response in *R. sphaeroides*.

1.8.3 The role of the singma factors RpoHI/II in the singlet oxygen response

The *rpoHII* gene encoding a sigma factor is induced by singlet oxygen. The *rpoHII* gene is transcribed from a promoter dependent on RpoE and the RpoHII protein is capable to activate more genes in response to singlet oxygen (Nuss *et al*., 2009), such as genes related to oxidative-stress defense systems, including the degradation of methylglyoxal, detoxification of peroxides, singlet oxygen scavenging, and redox and

iron homeostasis (Fig 1.8). RpoHII belongs to the group Ⅳ sigma factor and is widely distributed in the α-proteobacteria (Nuss *et al*., 2009). Another sigma factor, RpoHI, is also activated by singlet oxygen by a unknown mechanism and regulates gene transcription (Nuss *et al*., 2010). RpoHI and RpoHII are both involved in the singlet oxygen and the heat stress response in *R. sphaeroides*. The RpoHII regulon has an overlap with the RpoHI regulon. Several sRNAs are induced both by RpoHI and RpoHII (Fig 1.8) (Dufour *et al.*, 2012; Nuss *et al*., 2010). A previous study showed that RpoHII has the major function in response to singlet oxygen, however, RpoHI is more important in response to heat stress (Nuss *et al.*, 2010).

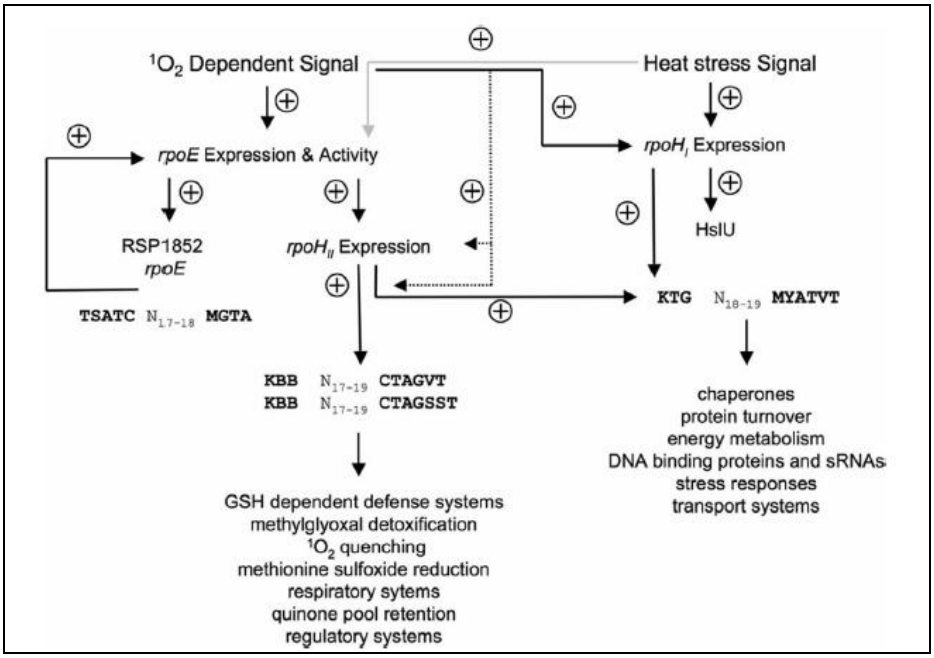

Figure 1.8 Several sigma factors are involved in response to singlet oxygen in *R. sphaeroides* from: Nuss *et al*., 2010.

1.8.4 An OmpR homologous protein induced by singlet oxygen

In *R. sphaeroides*, the RSP_0847 gene encodes an OmpR homologous response regulator which contains a receiver domain and a DNA binding domain. *ompR* is transcribed from a promoter dependent on RpoHI/HII (Berghoff *et al*., 2009). It was reported that OmpR protein is induced by singlet oxygen based on proteome analysis (Glaeser *et al*., 2007) (Fig 1.9). This indicates OmpR protein is likely involved in the singlet oxygen response in *R. sphaeroides*.

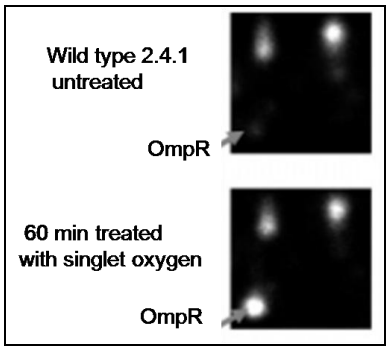

Figure 1 9 An OmpR protein is induced by single oxygen in *R. sphaeroides* based on proteome analysis (Glaeser *et al*., 2007)**.** Protein extracts were prepared from cells growing exponentially in the presence of methylene blue and high light (0.2 µM, 800 Wm^{-2}) for 60 min. For radioactive labeling, 10 µCi L-[^{35}S]-methionine was added to 7 mL samples of the cultures and incubated for 10 min. Protein spots indicated by arrows illustrate the response to singlet oxygen..

1.8.5 sRNAs are involved in the singlet oxygen response in *R. sphaeroides*

There are several sRNAs induced by singlet oxygen in *R. sphaeroides* (Berghoff *et al*., 2009). The interactions of most *trans*-encoded sRNAs with mRNAs need the cooperation of a RNA chaperone, the Hfq protein. At least 25 sRNAs and mRNAs encoding cell division proteins and ribosomal proteins were indentified to bind to Hfq by co-immunoprecipitation (Berghoff *et al*., 2011a). Deletion of *hfq* causes a higher sensitivity to singlet oxygen in *R. sphaeroides.* This indicates that Hfq is likely involved in the singlet oxygen response in *R. sphaeroides.* Further analysis showed that deletion of *hfq* affects amino acid transport and metabolic functions, and also causes a reduced RpoE activity and a disordered induction of RpoHII-dependent genes (Berghoff *et al*., 2011a). All these data suggest that the Hfq protein is able to regulate several fundamental cellular processes in *R. sphaeroides* (Fig. 1.10).

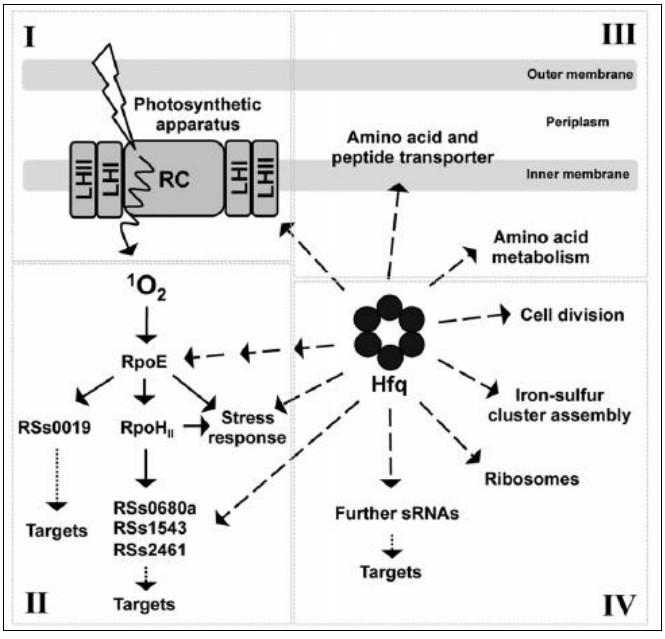

Figure 1.10 Several fundament cellular processes are controlled by Hfq in *R. sphaeroides* take from Berghoff *et al*., 2011a.

SorX, an sRNA derived from the 3' UTR of the *ompR* gene

SorX was originally identified as RSs2461 in *R. sphaeroides*. It is located in the 3' UTR of the gene RSP_0847 encoding a homologue of OmpR (Berghoff *et al*., 2009; Nuss *et al*., 2010). *sorX* is transcribed with the *ompR* gene from an RpoHI/HII promoter (Fig 1.11). The 116 nt length RSs2461 is further processed into a shorter 75 nt fragment RSs2461 (Berghoff *et al*., 2009). The shorter 75 nt fragment RSs2461 was renamed as SorX (singlet oxygen resistance small RNA X) and the 116 nt length fragment RSs2461 was renamed as pre-SorX (Peng *et al*., 2016).

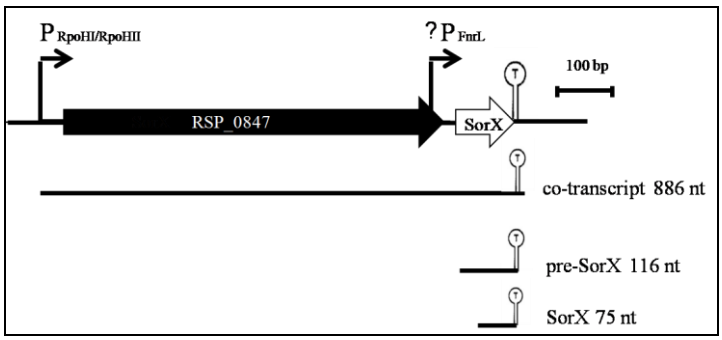

Figure 1.11 Genetic location of *sorX*. *sorX* is transcribed together with the upstream gene RSP_0847 from an RpoHI/HII promoter. A promoter dependent on FnrL before pre-SorX was predicted based on Chip-seq experiments. The 116 nt long pre-SorX is further processed into a 75 nt SorX fragment. The lollipop indicates a Rho-independent terminator.

A previous study showed an accumulation of the 75 nt SorX fragment after 10 min treatment with singlet oxygen. However, it was not accumulated in a Hfq deletion stain. Furthermore, the half-life of SorX was decreased from ~17 min in wild type to ~4 min in the *hfq* mutant indicating that SorX was stabilized by the Hfq protein. SorX binding to Hfq was confirmed by co-immunoprecipitation (Berghoff *et al*., 2011a). The 75 nt SorX fragment is considerably conserved among *Rhodobacterales* species (Berghoff *et al*., 2009). Recently, a promoter dependent on FnrL in the upstream of the *sorX* gene was predicted based on Chip-seq data (Fig 1.10) (Imam *et al*., 2014).

CcsR1-4, sRNAs derived from the 3' UTR of the RSP_6037 gene

CcsR1-4 are four homologous sRNAs originally named as RSs0680a-d and induced by singlet oxygen in *R. sphaeroides* (Berghoff *et al*., 2009). CcsR1-4 are transcribed with the upstream gene RSP_6037 which encodes a protein harboring a conserved unknown function domain DUF1127 from a RpoHI/HII dependent promoter (Fig 1.12) (Berghoff *et al*., 2009).

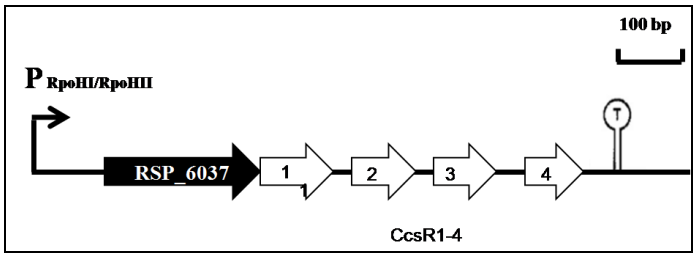

Fig 1.12 Genetic location of CcsR1-4. The four sRNAs CcsR1–4 are transcribed together with an upstream gene RSP_6037. Transcription is initiated at an RpoHI/RpoHII dependent promoter and terminated at a rho-independent stem-loop structure.

CcsR1-4 are induced by oxidative stresses, caused by organic hdyroperoxides, superoxide and hdyroperoxides (Fig 1.13). Each of CcsR carries a conserved CCUCCUCCC-motif. Therefore, RSs0680a-d were renamed as CcsR1-4 (conserved CCUCCUCCC-motif stress-induced RNAs).

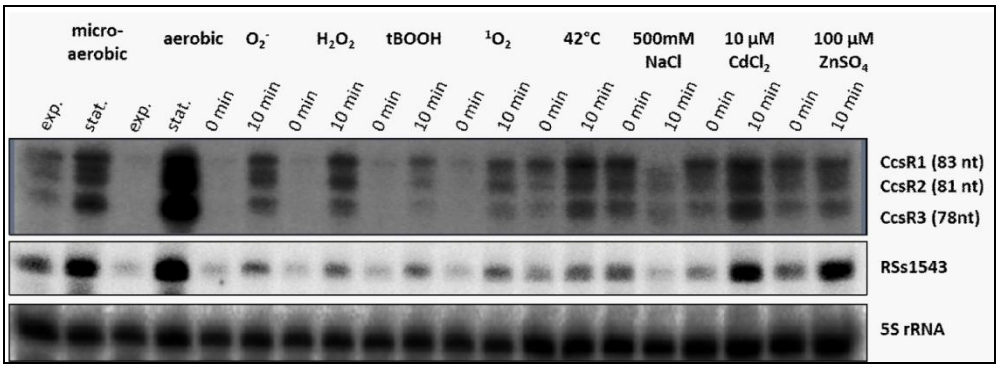

Figure 1.13 Expression level of CcsR under various stress conditions are shown by Northern blot from Billenkamp *et al.*, 2015. Samples were taken at before (0 min) and 10 min after adding the agents or treatment with heat (42 ℃). 5S rRNA was used as loading control. SorY (RSs1543) was also detected by Northern blot.

Other sRNAs induced by singlet oxygen

Several sRNAs, such as SorY, RSs0680 and RSs0019 are also induced by singlet oxygen. RSs0019 is transcribed from a promoter dependent on RpoE (Berghoff *et al.*, 2009). The *sorY* gene is transcribed from a RpoHI/HII promoter and is induced by singlet oxygen and other stress conditions (Adnan *et al.*, 2015). A *takP* mRNA encoding a TRAP-T transporter was verified as a target of SorY. It was shown that SorY reduces the metabolic flux into the tricarboxylic acid cycle by reducing malate import through TakP, thus it contributes to a metabolic switch, which reduces the production of the pro-oxidant NADH in favor of the production of the anti-oxidant NADPH (Adnan *et al.*, 2015).

1.9 Aims of this work

In the anoxygenic photosynthetic bacterium *R. sphaeroides*, singlet oxygen is produced in the presence of oxygen and light. Singlet oxygen is a challenge to the cell, due to its ability to damage many components in the cell.

Several sRNAs are induced by singlet oxygen (Berghoff *et al*., 2009). In this study, we focused on the sRNAs, CcsR1-4 and SorX. SorX and CcsR1-4 are transcribed from the RpoHI/RpoHII-dependent promoters and are derived from the 3' UTR of upstream genes RSP_0847 and RSP_6037. SorX and CcsR1-4 can bind to the RNA chaperon Hfq (Berghoff *et al*., 2009). It is known that many sRNAs are involved in adaptation to stress conditions. However, the functions of SorX and CcsR1-4 in oxidative stress response in *R. sphaeroides* were still unknown. Therefore, this study addresses the question which gene is the direct target of SorX? What are the roles of SorX and CcsR1-4 in response to singlet oxygen in *R. sphaeroides*?

Moreover, an OmpR homologous response regulator (RSP_0847) is induced by singlet oxygen (Glaeser *et al*., 2007). This indicates that OmpR protein likely plays an important role in the singlet oxygen response in *R. sphaeroides*. Therefore, the role of the OmpR protein in response to oxidative stress is also investigated in this study.

2. Materials

2.1 Chemicals used in this study

Acetic acid	Roth
Acrylamide (30% w/v)/ bisacryet lamide (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
Coomassie Brilliant Blue G-250	Difco
Deoxyribonucleoside triphosphates (dNTPs)	Serva
Ethanol	Roth
Ethanolamine	Sigma Aldrich
Ethidium bromide	Roth
Ethylene-diamine tetraacetate (EDTA)	Roth
Formaldehyde (37%)	Roth
Formamid	Sigma Aldrich
Glycerol	Roth
Glycine	Roth
Glutathione	Roth
Imidazole	Sigma Aldrich
Isopropyl-β-D-thiogalactoside (IPTG)	Roth
Lumi-Light Western Blotting Substrate I and II	Roche
Magnesium chloride	Roth
Magnesium sulphate	Merck
Methanol	Roth
Methylene blue	Sigma Aldrich
Nickel NTA	Qiagen
N-2-hydroxyethylpiperazine-N'-2- ethanesulfonic acid	Roth
N, N, N ', N'-tetramethylene diamine	Roth
Ponceau-Red	Sigma Aldrich
Phenyl-methyl-sulfonylfluoride (PMSF)	Sigma Aldrich
Potassium dihytrogen phosphate	Roth
Potassium chloride	Roth

Phenol (water grade)	Applichem
Phenol/Chloroform	Applichem
Polyvinylpyrolidon	Sigma Aldrich
PEG800	Sigma Aldrich
Roti-Quant (Bradford-Reagent)	Roth
Sepermidine	Sigma Aldrich
Sodiumthiosulphate $\times 5H_2O$	Merck
Spurenelenmente (RÄ-medium)	Sigma
Sodium carbonate	Merck
Sodium dodecyl sulphate (SDS)	Roth
Tris-(hydroxymethyl)-aminomethane	Roth
Triton X 100	Roth
Tween 20	Serva
Trypton	Difco
Urea	Roth
Vitamins (RÄ-medium)	Sigma Aldrich

2.2 Markers used in this study

Marker	**Manufacture**
Prestained Protein Marker, *Broad Range*	NEB
Low Range Protein Marker	Biorad
GeneRuler 1kb DNA *Ladder* Plus	Fermentas
GeneRuler 100 bp DNA *Ladder*	Fermentas

2.3 Radioactive nucleotides used in this study

Nucleotides Conc. used (Ci/mmol)	**Manufacturer**
[γ^{32}P] ATP 3000	Hartmann Analytic
[α^{32}P] dCTP 3000	Hartmann Analytic

2.4 Enzymes used in this study

Enzyme	**Manufacturer**
DNase I (RNase free)	Fementas or Invitrogen
Taq DNA polymerase	QIAGEN
T4 DNA ligase	New England Biolabs
T4 polynucleotide kinase (T4 PNK)	New England Biolabs
Restriction endonucleases	New England Biolabs or fermentas

phusion DNA polymerase	QIAGEN
RNase A	QIAGEN

2.5 Molecular biological kits used in this study

Kit	**Manufacturer**
Prime-a-Gene® Labeling System	Promega
Plasmid Isolation KIT	Qiagen
One-step RT-PCR KIT	Agilent
QIAEX II Agarose Gel Extraction Kit	Qiagen
Lumi-Light PLUS Western Blotting Kit	Roche (Grenzach)
ULS™ Labeling Kit for Agilent arrays (Cy5)	Kreatech (Amsterdam)
ULS™ Labeling Kit for Agilent arrays (Cy3)	Kreatech (Amsterdam)
CloneJET PCR cloning kit	Fermentas
peqGOLD TriFast™ Kit	PEQLAB

2.6 Antibody used in this study

Antibody	**Manufacturer**
Anti-OmpR (Rabbit, Antiserum)	Davids Biotechnology

2.7 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***
Tetracycline	20 μg ml^{-1}	1.5 μg ml^{-1}
Kanamycin	25 μg ml^{-1}	25 μg ml^{-1}
Ampicilin	200 μg ml^{-1}	-
Spectinomycin	10 μg ml^{-1}	10 μg ml^{-1}

2.8 Buffers used in this study

2.8.1 Commercial reaction buffers

10 × NEB buffer (1, 2, 3, and 4)	New England Biolabs
10 × PCR buffer	QIAGEN
5 × Q-solution	QIAGEN
HF-Buffer	QIAGEN
Red, green,	Fermentas

2.8.2 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
Laemmli buffer(1 ×)	25 mM Tris/HCl
	192 mM Glycin
	0.1% (w/v) SDS
	pH 8.2
SDS-loading gel buffer	200 mM Tris/HCl (pH 6.8)
	400 mM DTT
	8% SDS
	0.4% bromophenol blue
	40% glycerol

2.8.3 Buffers used for western blot

Transfer buffer	25 mM Tris/HCl
	192 mM Glycin
	20% Methanol
TBS (10 ×)	0.5 M Tris/HCl
	2 M NaCl
	pH 7.4
Blocking solution	5% (w/v) milk powder

2.8.4 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
Fu-mix buffer	50 µl 10 × MOPS
	250 µl formamide
	89 µl formaldehyde
	11 DEPC H_2O

2.8.5 Other buffers used in this study

PBS (1 ×)	137 mM NaCl
	2.7 mM KCl
	10 mM Na_2HPO_4
	2.0 mM KH_2PO_4
	pH 7.4
TBS (1 ×)	0.15 M NaCl
	0.015 M Tris/HCl
	pH 7.4
Z-Buffer forβ-galactosidase assay	0.06 M Na_2HPO_4 × H_2O
	0.04 M NaH_2PO_4 × H_2O
	0.01 M KCl
	0.001 M $MgSO_4$ × 7 H_2O
	Add β-Mercaptoethanol fresh
	pH 7.4
SSC (20 ×)	3.0 M NaCl
	0.3 M Sodium citrate
Diffusion buffer	0.5 M Ammonium acetate
	10 Mm Magnesium acetate

	1 Mm EDTA pH 8.0
	0.1% SDS
GSH buffer	143 mM $NaH_2PO_4 \times H_2O$
	6 mM EDTA
	adjust with NaOH to pH 7.5
Coomassie staining solution	
Water	50% (w/v)
Methanol	40% (w/v)
Acetic acid	10% (w/v)
Coomassie Brilliant Blue (G-250)	0.2% (w/v)
Coomassie destaining solution	
Methanol	20% (w/v)
Acetic acid	10% (w/v)
Water	70% (w/v)

2.9 Culturing media used in this study

2.9.1. Standard I medium

Standard I liquid medium	
Standard I Nutrient medium	25 g
Add ddH_2O up to	1000 ml
Strandard I agar plates	
Bacto Agar (1.6%)	8.0 g
Add ddH_2O up to	500 ml

2.9.2 RÄ 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_2O up to	1000 ml
	pH 7.0

PY agar plates

Bacto Agar (1.6%)	8.0 g
Add PY liquid medium	500 ml

2.10 Strains and plasmids used in this study

Table 2.2 Strains and plasmids

Strain	Description	Source/ Reference
Rhodobacter sphaeroides		
2.4.1	Wild type	van Niel, 1944
2.4.1ΔSorX	SorX deletion strain	This study
2.4.1Δ*potA*	*potA* deletion strain, Km^r	This study
2.4.1Δ*hfq*	Hfq deletion strain, Sp^r	Glaeser *et al*., 2007
2.4.1Δ*flhR*	*flhR* deletion strain, Km^r	Billenkamp *et al*., 2015
2.4.1$rne^{E.c.(ts)}$	RNase E variant with *rne-3071* (ts) mutation of *E. coli* N3431 instead of its native RNase E	By Lennart Weber
JZ1678	*fnrL* deletion strain, Kmr	Zeilstra-Ryalls and Kaplan,1995
PrrA2	*prrA* deletion strain, Spr	Eraso and Kaplan, 1994
E. coli		
S17-1	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn7 tra^+ Km^r, Sp^r	Simon *et al*., 1983
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi (lac–proAB)*	New England Biolabs
Plasmid	**Description**	**Source/ Reference**
pRK415	Tc^r, broad-host-range cloning vector	Keen *et al*., 1988
pRK4352	Tc^r, pRK415 containing 16S rRNA promoter	Mank *et al*., 2012
pBBR4352	Km^r, pBBR1MCS-2 containing 16S rRNA promoter	Mank *et al*., 2012
pLO1	Km^r, *sacB* RP4-*oriT* ColE1-*oriV*	Lenz *et al*., 1994
pLO1ΔSorX	pLO1::0.61-kb SphI-SalI fragment containing ΔSorX	This study
$pBBRSorX^{144}$	Km^r, pBBR4352 containing the 116-bp *sorX* gene together with its 28-bp upstream region	This study

pBBRSorX^{76}	Km^r, pBBR4352 containing the 76-bp 3' part of the *sorX* gene	This study
pBBRSorX^{144}-M3	Km^r, pBBR4352 containing the 116-bp *sorX* gene with a triple mutation (CTC → GAG) together with its 28-bp upstream region	This study
pRKSorX^{144}	Tc^r, pRK4352 containing the 116-bp *sorX* gene together with its 28-bp upstream region	This study
pRKOmpRfalg	Tc^r, pRK4352 containing the *ompR* gene fusion with flag Tag	This study
pRCcsR1	Tc^r, pRK4352 containing *ccsR1* gene	Billenkamp *et al*., 2015
pRCcsR2	Tc^r, pRK4352 containing *ccsR2* gene	Billenkamp *et al*., 2015
pRCcsR1+2	Tc^r, pRK4352 containing *ccsR1*and *ccsR2* gene	Billenkamp *et al*., 2015
pRCcsR1-3	Tc^r, pRK4352 containing *ccsR1, ccsR2* and *ccsR3* gene	Billenkamp *et al*., 2015
pRCcsR1-4	Tc^r, pRK4352 containing *ccsR1, ccsR2, ccsR3* and *ccsR4* gene	Billenkamp *et al*., 2015
pPHU281	Tc^r, suicide vector for *R. sphaeroides*	Hübner *et al*., 1991
pUC4K	Km^r, source for Km cassette	Vieira *et al*., 1982
pPHU4352	Tc^r, pPHU235 containing 16S rRNA promoter	Mank *et al*., 2012
pPHU*potA*	Tc^r, pPHU4352 containing a *potA-lacZ* fusion	This study
pPHU*potD*	Tc^r, pPHU4352 containing a *potD-lacZ* fusion	This study
pPHU*potA*-M3	Tc^r, pPHU4352 containing a *potA-lacZ* fusion with a triple mutation (GAG → CTC) in *potA*	This study
pBE4352::eCFP: eCFP	Km^r, Fluorescence-based (eCFP) in vivo reporter plasmid	Remes *et al*., 2015
pBE::P_{RSP_6037}: eCFP	Km^r, pBE4352::eCFP:eCFP containing promoter(200 bp) of RSP_6037 fused to e*cfp*	This study
pBE::SP_{RSP_6037}: eCFP	Km^r, pBE4352::eCFP:eCFP containing promoter(100 bp) of RSP_6037 fused to e*cfp*	This study
pBE::P_{SorY}: eCFP	Km^r, pBE4352::eCFP:eCFP containing promoter of SorY fused to e*cfp*	This study
pBE::P_{OmpR}: eCFP	Km^r, pBE4352::eCFP:eCFP containing promoter of *ompR* fused to e*cfp*	By Benjamin D. Eisenhardt
pBE::P_{RpoHI}: eCFP	Km^r, pBE4352::eCFP:eCFP containing promoter of *rpoHI* fused to e*cfp*	By Benjamin D. Eisenhardt
pJET1.2/blunt	Ap^r, cloning vector	Fermentas
pDrive	Km^r; Amp^r, cloning vector	Qiagen

2.11 Oligonucleotides used in this study

Table 2.3 Oligonucleotides

Oligonucleotide	Sequence (5' to 3')	Source/ Reference
Deletion-f	GGCTTGTTGCGTGAGATCTTAGAGACACGGACCCTC	This study
Outrev-SalI	AAAGTCGACCAGCGCCTCGCGCAGGC	This study
Deletion-r	GAGGGTCCGTGTCTCTAAGATCTCACGCAACAAGCC	This study
Outfor-SphI	AAAGCATGCGCACGAACAATCAGAGG	This study
1882KO-UP-F-XbaI	TCTAGACGCTCTGGCCCTTGCGGC	This study
1882KO-UP-R-PstI	CTGCAGGTGACGGAAGCGAACCGGC	This study
1882KO-DO-F-PstI	CTGCAGCTCCGTAGTAACGGACCTGC	This study
1882KO-DO-R-HindIII	AAGCTTCATCAGCGCCATCTCGAGCG	This study
1882OUT-F	GGATCTGATCGTGCCCCAG	This study
1882OUT-R	CCTCCCACCAGATCAGGTC	This study
SorXMU-F	CACGGACCGAGCCTGTTGGATCTGGCCG	This study
SorXMU-R	CAACAGGCTCGGTCCGTGTCTCTAACCC	This study
1882MU-F	AAGATGGGCTCAATGGGGTGGTGGCTTC	This study
1882MU-R	CCCCATTGAGCCCATCTTGACCGGCAGG	This study
1882RE-F-BamHI	GGATCCCCTTCCGCTTTAAACAATGAC	This study
1882RE-R-HindIII	AAGCTTGTCGTCCTGGGTAGAAGCC	This study
1883RE-F-BamHI	GGATCCCTCCGTAGTAACGGACCTG	This study
1883RE-F-HindIII	AAGCTTCACGGCCGACAAGGCCG	This study
6037P-F-SacII	CCGCGGCCGAAGCGGGCGAAGCG	This study
6037P-R-BamHI	CGCGGATCCGAGACCGTAGGCGGCTTC	This study
SorYP-F-XbaI	TCTAGAGCCTTTTCGGGCTAAGC	This study
SorYP-R-BamHI	GGATCCGGCCCAAGTCCAACAGG	This study
SorX-up0-BamHI	GGATCCGATCAAATTAAGGATCACACTCCTATC	This study
SorX-over-R-XbaI	TCTAGAGAAGGCTGGCACGAAGC	This study
Term-SorX-EcoRI	GAATTCGAAGGCTGGCACGAAG	This study
SorXSO-F-BamHI	GGATCCGTTCCCTCCCGTGTCG	This study
Start-RSs2461-BamHI_forw	GGATCCTATAAAGACAGTAGAGACGGCGG	This study
Term-RSs2461-EcoRI rev	GAATTCGAAGGCTGGCACGAAGC	This study
1883RT-F	CACAAGCCCTTCACCGCC	This study
1883RT-R	CGCCGGATCGATCTCCTC	This study
1882RT-F	CGACCCGCAGCTCGTGC	This study
1882RT-R	CGGCGATCCGGTCCGAC	This study
rpoZ-A	ATCGCGGAAGAGACCCAGAG	Zeller and Klug, 2004

rpoZ-B	GAGCAGCGCCATCTGATCCT	Zeller and Klug, 2004
R.s.rneUP_f	AGTACTCGGATCTCTATTACCGGCTCG	This study
R.s.rneUP_r	CATATGATTCCATGGCACCCG	This study
R.s.rneDOWN_f	GGTACCGCTGATCTCCAGAGATTGACAC	This study
R.s.rneDOWN_r	GAATTCTGCATTGGACAGGAAGAAGG	This study
E.c.rne_fw	CATATGAAAAGAATGTTAATCAACGCAAC	This study
E.c.rne_rev	TCTAGAAACTGGGTATGTTTTGTCTGCC	This study
p-SorX-R	CCGGGAAGCGCGAGAGAAG	This study
p-1543	ATGAAGCGGACGAGAGAACCCTC	Nuss *et al.*, 2010
p-5S	CTTGAGACGCAGTACCATTG	Berghoff *et al.*, 2009
p-0680a	CGTCGCCGCTGCTGCTACAGGTC	Berghoff *et al.*, 2009
RSP_2579-RT-F	CTGAAGGACAGCGTGATG	This study
RSP_2579-RT-R	GCGCAGATAGGTCCAGA	This study
RSP_2410-RT-F	GATCGCCAAGGATCT	Nuss *et al.*, 2009
RSP_2410-RT-R	CTGGTCGCTGTCTTCA	Nuss *et al.*, 2009
RSP_1409-RT-F	CAGCTTCACCACGCTCCT	This study
RSP_1409-RT-R	GGTCATGCCTTCCGTCAG	This study
RSP_1020-RT-F	CTCGGGCACCTCGCTGG	This study
RSP_1020-RT-R	GAAGCCGTCCACCTCGAC	This study
RSP_3622-RT-F	GGGAACCCCTGTCCTTC	This study
RSP_3622-RT-R	CTGCCGACCGAACCCAC	This study
RSP_3106-RT-F	CCCTGCTCATTACGGCG	This study
RSP_3106-RT-R	GCACAGGATCGAGGAGG	This study
RSP_4209-RT-F	CGCTGATCGCGAACAGTG	This study
RSP_4209-RT-R	GAAGTTCAGCCAGCGCG	This study
RSP_2579-RT-F	CATCCGCTCGTTCAATGC	Billenkamp *et al.*, 2015
RSP_2579-RT-R	CAGACATGGCACTCCGAC	Billenkamp *et al.*, 2015
RSP_2877-RT-F	TACGAGCAGGCCAAGGAT	Billenkamp *et al.*, 2015
RSP_2877-RT-R	GGCGGTTGTTCACCAGTT	Billenkamp *et al.*, 2015
RSP_4050-RT-F	GCACCGCACGAATATCAG	Billenkamp *et al.*, 2015
RSP_4050-RT-R	CGAGAGCGTCATCGACAT	Billenkamp *et al.*, 2015
RSP_6132-RT-F	GGAAGACCCCGCGTTAC	Billenkamp *et al.*, 2015
RSP_6132-RT-R	CTTGCGCTTGGCGCAGG	Billenkamp *et al.*, 2015

RSP_2580-RT-F	GATCCGCGCTCTCCTGC	Billenkamp *et al.*, 2015
RSP_2580-RT-R	CAGGGGCGAATTCGCCG	Billenkamp *et al.*, 2015
RSP_0847-RT-F	GGACAGCGACACCATTCT	This study
RSP_0847-RT-R	CGTCCACCAGCATCTTCT	This study

2.12 Efficiencies of the real-time primers

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

Corresponding gene	Efficiency	Reference
RSP_1669 (*rpoZ*)	2.1	Zeller and Klug, 2004
RSP_1882	2.2	This study
RSP_1883	2.0	This study
RSP_6037	2.2	This study
RSP_2410 (*rpoHI*)	1.9	This study
RSP_1409	2.0	This study
RSP_1020	2.0	This study
RSP_3622	2.3	This study
RSP_3106	2.0	This study
RSP_4209	1.9	This study
RSP_2579 (*cycB*)	2.1	Billenkamp *et al*., 2015
RSP_2877 (*cosL*)	2.0	Billenkamp *et al*., 2015
RSP_4050 (*pdhB*)	2.1	Billenkamp *et al*., 2015
RSP_6132 (pqqA)	1.9	Billenkamp *et al*., 2015
RSP_2580 (*xoxJ*)	2.1	Billenkamp *et al*., 2015
RSP_0847 (*ompR*)	2.0	This study

2.13 Equipments used in this study

Equipment	Manufacturer
CFX96 Real-Time PCR	Bio-Rad
Centricon 10 membrane	Millipore
Cooling centrifuge, Sorvall RC-5C+	Kendro
Cooling centrifuge, Sorvall -5B	Kendro
Cooling centrifuge Z 323K	Hermle
Dialyse tubes (Type 20/32)	Roth

Electroporator (Micro Pulser)	Biorad
Filter paper, Whatman	Hartenstein
Fusion SL4 –Chemiluminasence 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 *E. coli* liquid culture

E. coli strain was grown in a flask with STI-medium on a shaker at a speed of 180 rpm at 37 ℃ containing the corresponding antibiotic if required. The total number of *E. coli* cells in the liquid culture was calculated photometrically by the optical density (OD) at 600 nm.

3.1.2 *E. coli* plating culture

E. coli strain was grown on the STI-agar containing the corresponding antibiotic if required at 37 ℃.

3.1.3 *R. sphaeroides* liquid culture

R. sphaeroides strain was grown at 32 ℃ with RÄ medium on a shaker at a speed of 140 rpm in dark. Corresponding antibiotic was added to the media if required. The total number of *R. sphaeroides* cells in the liquid culture was calculated photometrically by the optical density at 660 nm.

3.1.3.1 Aerobic growth condition

For aerobic growth conditions, *R. sphaeroides* strain was cultured by gassing air to the culture in flat glass bottles or by continuous shaking of Erlenmeyer flasks at 140 rpm with a total culture volume of 20% (160 to 180 µM of dissolved oxygen in the culture).

3.1.3.2 Microaerobic growth condition

For microaerobic growth condition, *R. sphaeroides* strain was cultured in Erlenmeyer flask at 140 rpm with a total volume of 80% (25 µM dissolved oxygen in the culture).

3.1.3.3 Phototropic growth condition

For phototropic conditions, *R. sphaeroides* strain was cultured in the flat bottle which was filled by 100% volume with culture and incubated at 32 ℃ in the presence of 60 Wm^{-2} white light.

3.1.3.4 Oxygen shift assay

The strain was cultured overnight and diluted to optical density at 660 nm of 0.1, then the strain was cultured at high oxygen tension (400 ml culture in 1 L flat glass bottle) until the optical density reached at 0.4. The strain was shifted to low oxygen tension

(400 ml culture in 500 ml flat glass) and grown in the low oxygen tension.

3.1.3.5 Stress experiment

For analysis stress response in *R. sphaeroides*, stains were cultured either on aerobic growth condition or microaerobic growth condition. At an OD_{660} of 0.4 (exponential growth phase), 250 µM paraquat which can generate superoxide, 100 µM tBOOH (*tert*-butyl hydroperoxide) used as organic hydroperoxides or 1 mM H_2O_2 as final concentration was added. Photooxidative stress experiment was performed under aerobic conditions by adding 0.2 µM methylene blue to the culture and irradiation with 800 W/m ²white light for 7 and 15 min. 10 µM $CdCl_2$ or 1.5 mM diamide which is a tioloxidant as final concentration were added to the cultures grown under microaerobic condition until the OD_{660} reached at 0.4. For analysis of the heat shock response, cells were grown at 32 ℃ under microaerobic conditions and shifted to 42 ℃ incubated for 7 and 15 min. Cells were collected on ice by centrifugation at 10,000 *g* and 4 ℃.

3.1.4 *R. sphaeroides* plate culture

R. sphaeroides strain was grown on the RÄ agar containing the corresponding antibiotic if required at 32 ℃ in the dark.

3.1.5 Preparation of glycerol stocks for the -80 ℃ strain collection

Cells were collected from liquid overnight culture of *E. coli* or *R. sphaeroides* in late exponential growth phase by centrifugation at 4 ℃. The pellet was resuspended by 2 ml relevant medium without antibiotics and 1 ml 80% glycerol, the suspension was transferred into two cryo-tubes, freezed in liquid nitrogen and stored at -80 ℃.

3.1.6 Transfer plasmid into host cell by diparental conjugation

First *Rhodobacter* acceptor cells were collected at 4000 rpm at room temperature, the supernatant was discarded. 500 µl of *E. coli* S17-1 over night culture was taken and mixed with the pellet and spun down at 4000 rpm at room temperature The cell pellet was resuspended by 100 µl RÄ-medium and the suspension was transferred onto a membrane filter (0.45 µm, 25 mm, Carl-Roth) and the cell mixture was incubated on a PY agar plate at 32 ℃ for at least 4 hours.

3.1.7 Zone of inhibition assay

To verify the sensitivity of *R sphaeroides* strains to various stress conditions, zone of inhibition assays were used. Over-night cultures were diluted to an OD_{660} of 0.2 and

cultured under micoaerobic conditions at 32 ℃. After the OD_{660} reached at 0.4, 500 µl of the culture were mixed with 5 ml of soft agar (minimal salt medium containing 0.8% agarose) and poured on top of an agar plate (1.6% agarose in minimal salt medium) without antibiotic. Filters soaked with 5 µl of 1 M tBOOH or 10 µl methylene blue were placed on top of the plates. The plates for methylene blue were incubated under light (60 lamp), other plates were incubated at 32 ℃ in dark for 3 days. The diameter of the inhibition zones were measured.

3.1.8 Intracellular GSH measurement

For glutathione (GSH) assays, cultures were grown under microaerobic conditions to an OD_{660} of 0.4. Cells from 2 ml culture were harvested on ice and sedimentated by centrifugation at 10,000 × *g*. Cells were resuspended in 3.75% 5-sulfosalicylic acid for lysis by vigorous shaking. Cell debris and proteins were sedimentated by centrifugation at 10,000 × *g* and the supernatant was used to measure GSH. Ellman's reagent 5,5'-dithiobis-(2-nitrobenzoic acid) was used for colorimetric determination of GSH level and prepared as a 6 mM solution in reaction buffer (143 mM NaH_2PO_4, 6 mM EDTA, pH 7.5). Reaction mixtures with a volume of 1 ml were set up as follows: 845 µl reaction buffer containing NADPH (0.3 mg ml^{-1}) were mixed with 50 µl supernatant and 100 µl DTNB (6 mM). Samples were kept at room temperature for 10 min to allow GSH to be completely oxidized by DTNB. Afterwards reactions were started by adding 5 µl of glutathione reductase (100 units ml^{-1}) from baker's yeast (Sigma-Aldrich) and color was monitored at 412 nm over time. Total GSH concentrations were calculated from initial slopes in comparison to a calibration curve prepared from a standard GSH solution in reaction buffer. Total GSH concentrations were normalized to OD_{660} values.

3.1.9 β-galactosidase assays in *R. sphaeroides*

To perform β-galactosidase assay, *R. sphaeroides* strains were cultured at 32 ℃ when the OD_{660} reached at 0.4, 500 µl of each sample were taken. Samples were centrifuged at 10,000 rpm (4 ℃) for 10 min and the supernatant was discarded. The cell pellet was dissolved in 750 ml of Z-buffer (with fresh β-mercaptoethanol added), 20 µl of chloroform and 10 µl of 0.1% SDS. The mixture was vortexed for 30 sec. The samples were pre-heated for 5 min at 28 ℃ along with the ONPG. The color reaction was started by adding 200 µl of ONPG solution (4 mg/ml). After exactly 5 min the reaction was stopped by adding 500 µl of Na_2CO_3 (1 M) and the samples were centrifuged at 13,000 rpm for 3 min. Finally, the absorbance of the supernatant was

measured photometrically at 420 nm and 550 nm. The enzyme activity was calculated using the following formula which gives the β-galactosidase activity in Miller units.

Formula for Miller units = $(1000 \times (Abs_{420} - 1.82 \times Abs_{550}) / Abs_{660} \times V \times T$
Abs_{420} = absorbance of the yellow color
Abs_{550} = absorbance from cell debris
T = employed reaction time in min (5 min)
V = employed culture volume (1 ml)
Abs_{660} = reflects cell density

3.1.10 Reactive oxygen species measurement

The level of ROS was measured as mentioned previously (Remes *et al*., 2014) using an oxidation-sensitive fluorescent probe, 2,7-dihydrodichlorofluorescein diacetate (DCFH-DA; Molecular Probes). 95 µl of culture were incubated with 5 µl of the 100 µM fluorescent probe for 1 hour at 32°C in dark. The fluorescence intensities (excitation 492 nm, emission 525 nm) were measured in an Infiniti M200 microplate reader (Tecan).

3.2 Molecular methods

3.2.1 DNA preparation

3.2.1.1 Chromosomal DNA isolation

Cells (20 ml overnight liquid culture) were collected by centrifugation at 8,000 rpm for 10 min at 4 ℃. The pellet was washed with 1 ml PBS once and resuspended by 0.5 ml SET buffer containing 1 mg/ml lysozyme, then incubated at 37 ℃ for 30 min. 50 µl 10% SDS and 25 µl 20 mg/ml proteinase K were added and the mixture was incubated at 55 ℃ for 2 h, inverted several times during the incubation. Afterwards, 1/3 volume 6 M NaCl was added and inverted several times. 1 volume chloroform was added and the mixture was incubated at room temperature for 30-60 min with end-over-end rotation. After centrifugation, the chromosomal DNA from the supernatant was precipitated by 1 volume isopropanol and DNA was washed with 75% ethanol. Then the dry DNA was dissolved in 1 ml ddH_2O. The DNase was deactivated at 65 ℃ for 10 min, the chromosomal DNA was stored at 4 ℃.

3.2.2.2 Plasmid minipreparation by alkaline lysis

Plasmid DNA from cell was isolated by minipreparation according to the alkaline lysis principle in a small culture volume. 1.5 ml of overnight culture was centrifuged at 8000 rpm for 5 min at room temperature. The supernatant was discarded and the pellet was resuspended in 200 µl solution I (ice cold) containing 4 µl/ml RNase A and

incubated for 5 min at room temperature. Then solution II was added and inverted several times. To precipitate the chromosomal DNA of the cells, 200 µl solution III (ice cold) was added and incubated on ice for 10 min. Chromosomal DNA was visible as a white, filamentous precipitate. Afterwards the mixture was centrifuged at 13,000 rpm at 4 ℃ for 10 min. Cell debris and the chromosomal DNA were in the pellet after centrifugation while the plasmid DNA remained in the supernatant. The supernatant was transferred into a new Eppendorf tube and the same volume of chilled isopropanol was added to precipitate the plasmid DNA by centrifugation at 13,000 rpm at 4 ℃ for 20 min. The pellet was washed with 200 µl of 80% ethanol once and centrifuged at 13,000 rpm for 10 min. The pellet was dried in a Speed Vac to remove the left over ethanol and then the plasmid was dissolved in 50 µl ddH_2O.

3.2.2 Gel extraction

3.2.2.1 Extraction of DNA fragment from agarose gels

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

The piece containing the DNA form the polyacrylamide gel was cut, the gel piece was dissolved in 400 µl diffusion buffer and incubated at 37 ℃ overnight. The supernatant was transferred to a new Eppendorf tube and the gel piece was rinsed with 200 µl diffusion buffer. The supernatant was taken to the same tube. A equal volume of isopropanol was mixed with the supernatant and incubated at -20 ℃ at least 30 min, then centrifuged at 13,000 rpm for 30 min at 4 ℃. The pellet was washed with 80% ethanol once and dried in the Speed Vac. DNA was eluted in 20 ddH_2O.

3.2.3 n-Butanol precipitation

To remove various salt contaminations in the sample, sample of nucleic acids was precipitated by n-Butanol. The sample was mixed with ddH_2O to a final volume 50 µl, then mixed with 500 µl n-Butanol. The mixture was shaken vigorously and then centrifuged at 13,000 rpm (4 ℃) for 30 min. The pellet was washed with 80% ethanol and centrifuged at 13,000 rpm for 10 min. The pellet was dried in Speed Vac and dissolved in 20 µl ddH_2O.

3.2.4.Gel electrophoresis of DNA

3.2.4.1 Agarose gel electrophoresis

1% TAE or TBE agarose gels were used to separate and visualize DNA fragments which have the size from 0.3 to 20 kb. The agarose was melted by boiling briefly in TAE/TBE buffer and poured into a taped Gel tray which already has a comb. After polymerization, the gel was transferred into the chamber which has the respective TAE/TBE buffer (1 ×) for running the gel. DNA samples were mixed with 6 × DNA loading dye (15% of the total volume) before loading into the gel. Electrophoresis was performed at 70 to 100 mA for 40 min. DNA bands were visualized by staining with ethidium bromide solution for 8 min.

3.2.4.2 Polyacrylamide gel electrophoresis

Polyacrylamide gels were used to separate and visualize DNA fragments which have the size less than 300 bp. The reaction mix was prepared according to the recipe. Afterwards the gel was poured into the mini-gel system, After its complete polymerization, the comb was removed and DNA samples were mixed with 6 × loading dye and loaded into the gel. Electrophoresis was performed with (1 ×) TBE for 40 minutes at 120 V. DNA bands were visualized by staining with ethidium bromide solution.

Polyacrylamide gel

40 % (w/v) Acrylamide	1.5 ml
10 x TBE	0.6 ml
6% APS (w/v)	30 µl
H_2O	3.9 ml
TEMED	6 µl

Add ddH_2O up to 6 ml

3.2.5 Standard PCR

***Taq* DNA polymerase**

Reaction components	100 ng template DNA 1 µl 10 × PCR buffer 0.5 µl dNTP mix (4 mM each) 1 µl primers mix (50 pmol/µl each) 1 unit *Taq* DNA polymerase add H_2O to 10 µl

Program

94 ℃ 3 min
94 ℃ 30 sec
Annealing temperature 30 sec } 30 Cycles
72 ℃ 1 min/kb
72 ℃ 10 min

***Phusion* DNA polymerase,**

Reaction components

100 ng template DNA
5.0 µl 10 × PCR buffer
3.0 µl dNTP mix (4 mM each)
6 µl primers mix (50 pmol/µl each)
1 unit *phusion* DNA polymerase
add H_2O to 50 µl

Programme

98 ℃ 3 min
98 ℃ 20 sec
Annealing temperature 40 sec } 30 Cycles
72 ℃ 1 min/kb
72 ℃ 10 min

PCR products were purified by the kit (innuPREP DOUBLEpure Kit) according to the manufacture's instruction

3.2.6 Restriction

The restriction was performed using the restriction enzymes form New England Biolabs or Fermentas according to the manufacture's instruction. The restricted products were precipitated by agorose gel purification (innuPREP DOUBLEpure Kit) kit after the restriction.

3.2.7 Ligation

3.2.7.1 Standard ligation

Reaction components

30 fmoles vector
90 fmoles insert
1 unit T4 DNA ligase
2 µl 10 × T4 ligase buffer
add H_2O to 20 µl

The reaction mixture was incubated at room temperature for 2-3 hours or 16 ℃ overnight. The ligase was inactivated at 65 ℃ for 10 min. 30 µl H_2O and 500 µl n-Butanol was added to the mixture, vigorously mixed and centrifuged at maximum speed for 30 min in a microfuge. The pellet was washed once with 100 µl 80% ethanol and the pellet was dried in a speed Vac to remove the left over ethanol. The pellet was dissolved in 20 µl ddH_2O.

3.2.7.2 Ligation using the pJET 1.2 vector

Reaction components	2 µl 10 × ligation master mix 1 µl pJET 1.2 vector (50 ng/µl) 90 fmoles insert 1 unit T4 DNA ligase add ddH_2O to 20 µl

The ligation was performed according to the manufacturer's instruction (CloneJET PCR Cloning Kit handbook, Thermo Fisher Scientific). The reaction components were briefly mixed as described above and then incubated for 30 min at room temperature. n-Butanol precipitation was performed as descried before transformation.

3.2.7.3 Ligation using the pDrive vector

Reaction components	5 µl 2 × ligation master mix 1 µl pDrive vector (50 ng/µl) 90 fmoles insert 1 unit T4 DNA ligase add ddH_2O to 10 µl

The ligation was performed according to the manufacturer's instruction (QIAGEN PCR Cloning Handbook, QIAGEN). The reaction components were briefly mixed as described above and then incubated for 1 hour at room temperature. n-Butanol precipitation was performed as descried before transformation.

3.2.8 Quantitative real-time RT-PCR

One-Step Brilliant III QRT-PCR Master Mix Kit (Agilent)

Reaction components	2.0 µl RNA (20 ng/µl) 5.0 µl 2 × RT-PCR master mix 2 µl primers mix (10 pmol/µl each) 0.1 µl DTT 0.5 µl RNAse Block 0.4 µl RNase/DNase-free water Add water to 10 µl
Programme	50 ℃ 10 min 95 ℃ 3 min 94 ℃ 5 sec Annealing temperature 5 sec } midexponential cycles 60 ℃ 5 sec 72 ℃ 5 min

The Real Time RT-PCR was 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). The One-Step Brilliant III QRT-PCR Master Mix Kit (Agilent) was used for reverse transcription followed as described in the manufacturer's manual. A final concentration of 4 ng total RNA μl^{-1} without DNA left was used. The relative expression ratio (R) of a target gene was calculated based on real-time PCR efficiency (E) and the cross point (CP) defined as the point at which the fluorescence rises appreciably above the background fluorescence for each transcript according to the method of Pfaffl (Pfaffl, 2001). Relative expression of mRNA was calculated relative to the control and normalized to standard gene *rpoZ* (Pappas *et al.*, 2004).

3.2.9 Preparation of *E. coli* competent cells for electroporation

E. coli overnight culture was diluted into 1 l STI-medium with 1:100 dilution and cultured at 37 ℃ with vigorous shaking until the optical density at 600 nm reached 0.5~1.0. After the cells were kept on ice for 15~30 min, cells were collected by centrifugation in a cold rotor at 4,000 × g for 15 min. The pellet was resuspended by 1 l cold sterile water, spun down; resuspended in 0.5 l cold sterile water, spun down; resuspended in 50 ml 10% ice-cold sterile glycerol, spun down; and resuspended in 6 ml 10% ice-cold sterile glycerol. The suspension was freezed in aliquots in liquid nitrogen and stored at -80 ℃.

3.2.10 Transformation by electroporation

Ligated DNA or plasmid was mixed with 120 µl *E. coli* competent cells on ice in the Gene Pulser cuvette (Bio-rad). The Gene Pulser cuvette (Bio-rad) should be autoclaved by UV-Stratalinker 1800 (Stratagene) using "Auto Crosslink" two times and chilled on ice before use. A pulse of electricity (2.4 KV, 5 milliseconds) was applied to the mixture. Afterwards the DNA/cell mixture was transferred to 1 ml STI-medium without any antibiotic and incubated at 37 ℃ with vigorous shaking for 1 h. 25 µl or 100 µl mixture was placed on STI-agar plate containing corresponding antibiotic and the agar-plates were incubated at 37 ℃ overnight.

3.3 Extraction, purification and analysis of mRNA from *R. sphaeroides*

3.3.1 RNA isolation

Hot phenol method was used for isolating RNA. This RNA was further used for Northern blot. Cells were collected by centrifugation at 10000 rpm at 4 ℃. Cell pellet

was resuspended by 250 µl RNA extraction solution I and 250 µl solution II. After vigorous mixed, the suspension was incubated at 65 ℃ for 1.5 min, 500 µl phenol-water was added, vortexed, incubated at 65 ℃ for 3 min, freezed in liquid nitrogen for at least 30 sec, centrifuged 10 min at 13000 rpm. The upper phase was taken to a new eppendof tube. Phenol extraction was repeated three times. 40 µl 3 M NaOAc (pH 4.5) and 1 ml ethanol were added to the upper phase and the mixture was kept at -20 ℃ at least 30 min. After centrifugation (4 ℃, 13000 rpm, 30 min), the pellet was washed with 1 ml 75% ethanol, dried in Speed Vac for 5 min and dissolved in 180 µl DEPC-H_2O. 20 µl 10 × DNase I buffer and DNase I (15 unit per 1 µg RNA) were added to the mixture. The mixture was incubated at room temperature for 20 min. 200 µl phenol/chloroform/isoamylalcohol was added and centrifuged (13000 rpm, 5 min). The upper phase was treated again with phenol/chloroform/isoamylalcohol. 40 µl 3 M NaOAc (pH 7.0) and 1 ml ethanol were added to the upper phase. The mixture was incubated at -20 ℃ for more than 30 min. After centrifugation (4 ℃, 13000 rpm, 30 min), the pellet was washed with 1 ml 75% ethanol, dried in Speed Vac for 5 min and dissolved in 50 µl DEPC-H_2O. For real time RT-PCR, The total RNA isolation was performed with peqGOLD TriFastTM Kit (PEQLAB) as described in the manufacturer's manual.

3.3.2 Northern blot

Northern blot was performed as before (Berghoff *et al.*, 2009). In short, total RNA was dissolved (5 g per lane) in 10 µl Fu-Mix (formamide-urea buffer), incubated at 65 ℃ for 10 min. Electrophoresis was carried out on 8 M urea polyacrylamide gel for 3 h at 300 V in 1 × TBE buffer. After running was completed, the gel was washed with 1x TBE buffer and placed on top of a nylon membrane. Blotting was performed at 250 V for 120 min and the RNA was fixed on the membrane by UV cross linking (UV-Stratalinker 1800; Stratagene). For detection of SorX, the random probes were labeled by [α^{32}P] dCTP using the Prime-a-Gene® Labeling System (Promega) as described in the manufacturer's manual. For detection CcsR1 and SorY, the probes were labeled as published previously (Berghoff *et al*., 2009; Billenkamp *et al*., 2015). Membrane hybridization was incubated at 42 ℃ for overnight with a low stringency Church buffer. 5 × SCC buffer containing 0.01% SDS was used to wash the membrane. Afterwards, the membrane was exposed on phosphoimaging screens (Bio-Rad) and the image was analyzed by the 1D-Quantity One software (Bio-Rad).

3.4 Protein techniques

3.4.1 Bradford protein concentration assay

Reaction components	5 µl protein
	900 µl deionized water
	250 µl Roth-Quant (Carl-Roth)

The mixture was inverted several times, incubated at room temperature for 10 min and absorbance of the sample at 595 nm was recorded. BSA was used as standard.

3.4.2 SDS-polyacrylamide gel electrophoresis

The protein sample was mixed with 4 × SDS gel-loading buffer in a ratio of 4:1 (v/v) and denatured at 95 ℃ for 5 min. The sample was loaded in an SDS-polyacrylamide gel of appropriate percentage and run the gel at 150~200 volts for 3~5 hours.

Separation gel (12%)	
40 % (w/v) Acrylamide	7.5 ml
4x SDS-separation buffer	6.25 ml
6% APS (w/v)	200 µl
TEMED	20 µl
Add ddH_2O up to 25 ml	
Stacking gel (4%)	
40 % (w/v) Acrylamide	1.6 ml
4 x SDS-stacking gel buffer	4.0 ml
6 % APS (w/v)	120 µl
TEMED	12 µl
Add ddH_2O up to 16 ml	

3.4.3 Western blot

The protein samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to Protran nitrocellulose membrane (Carl-Roth). The electrophoretic transfer was performed using blotter (Amersham Pharmacia) at 1.5 mA/cm^2 for 1 h. Whatman paper was used with transfer buffer as a reservoir. The membrane was incubated for 5 minutes in Ponceau solution to fix the proteins on the membrane until all the bands were completely visible which is used as loading control. The stained membrane was quickly distained with water and few drops of 0.1 M NaOH before using it further. The membrane was incubated in blocking agent (milk powder) at 4 ℃ overnight. The membrane was incubated with specific antibody in TBS on a shaker at

room temperature for 2 hours, and then washed by shaking in TBS three times, 5 min each time. Then the blot was incubated with the second antibody conjugated with peroxidase in TBS on a shaker at room temperature for 1 hours, and washed by shaking in TBS three times, 5 min each time. For detection of peroxidase, lumi-light western blotting substrate 1 and 2 (v:v=1:1) were applied on the blot, and the chemiluminescence was captured on an X-ray film. The signals were quantified by software QUANTITY ONE (Bio-Rad).

3.4.4 Coomassie blue staining of SDS-polyacrylamide gels

The gel was stained with staining solution for 20 min on a shaker, rinsed with deionized water and the gel was distained by distaining solution until the background was nearly clear.

4 Results

4.1 CcsR1-4 increase resistance to oxidative stress by affecting the cellular glutathione level and C1 metabolism

4.1.1 CcsR1-4 enhance resistance to oxidative stress

This project was performed and published together with Fabian Billenkamp (Billenkamp *et al.*, 2015). The figures shown in the result part are from the work of Tao Peng.

CcsR1-4 were originally identified as sRNAs RSs0680a-d induced by singlet oxygen in *R. sphaeroides* (Berghoff *et al.*, 2009). RSs0680a-d contain four homologues sRNAs, each sRNA carries a conserved CCUCCUCCC-motif. RSs0680a-d were renamed as CcsR1-4 (conserved CCUCCUCCC-motif stress-induced RNAs). RSs0680a-d are tanscribed with the upstream gene RSP_6037 from a RpoHI/HII dependent promoter (Berghoff *et al.*, 2009; Billenkamp *et al.*, 2015). Since CcsR1-4 are induced by various stress conditions (Billenkamp *et al.*, 2015), specifically the oxidative stress, we assumed CcsR1-4 are involved in the oxidative stress response in *R. sphaeroides*.

In order to uncover the function of CcsR1-4 in *R. sphaeroides*, it was attempted to delete CcsR1-4 from chromosome. However, there was no CcsR1-4 deletion strain obtained indicating CcsR1-4 are essential to the cell (Billenkamp *et al.*, 2015). Afterwards, the CcsR1-4 overexpression strain (2.4.1pRCcsR1-4) was constructed and it showed enhanced resistance to superoxide stress and organic hydroperoxides (Fig 4.2). Zone inhibition assay was used to verify the sensitivity of *R. sphaeroides* strain to oxidative stress (Fig 4.1).

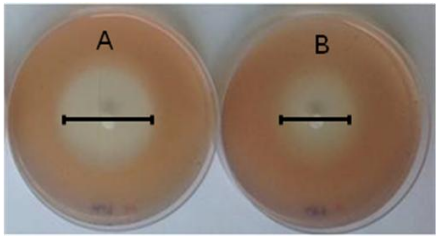

Fig 4.1 The diameter of the zone inhibition correlates to the sensitivity of bacteria to oxidative stress.

To test whether a single or a minimal number of copies of CcsR1-4 are sufficient for enhancing resistance to oxidative stress, CcsR1 (pRCcsR1) or CcsR2 (pRCcsR2) alone, CcsR1 and CcsR2 together (pRCcsR1+2) or CcsR1, CcsR2 and CcsR3 together (pRCcsR1-3) overexpression strains were constructed (Billenkamp *et al.*, 2015). The results showed that overexpression of either CcsR1 or CcsR2 alone, or overexpression

of several copies of CcsR1-4 together increased resistance to superoxide stress and organic hydroperoxides. Maximal resistance was only observed if all the CcsR1-4 copies (pRCcsR1-4) were overexpressed together (Fig 4.2). This indicates that a single copy of CcsR1-4 is sufficient for the function, while several copies of CcsR1-4 enhance the function.

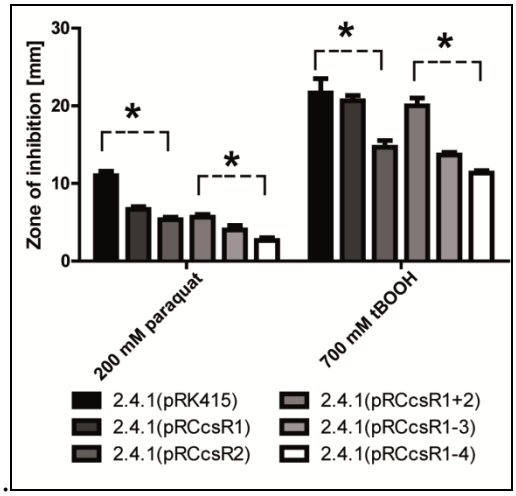

Figure 4.2 Effects of CcsR1-4 overexpression on resistance to superoxide stress and organic hydroperoxides shown by zone inhibition assays from Billenkamp *et al.*, 2015. Filter disks were soaked with either 200 mM paraquat or with 700 mM tBOOH for the zone inhibition assays. CcsR1 (pRCcsR1) or CcsR2 (pRCcsR2) alone, CcsR1 and CcsR2 together (pRCcsR1+2) , CcsR1, CcsR2 and CcsR3 together (pRCcsR1-3) or all the copies of CcsR1-4 (pRCcsR1-4) were overexpressed in *R. sphaeroides* wild type 2.4.1. The error bars indicate the standard deviation from the mean of two biological repeats with three technical repeats. Asterisks indicate a statistically significant change on inhibition zone ($p \leq 0.05$).

4.1.2 Effect of CcsR1-4 on the cellular glutathione level and C1 metabolism

In order to search for the direct targets of CcsR1-4, global transcriptome analysis by microarray was performed with the wild type containing an empty vector pRK415 (2.4.1pR415) in comparison to the CcsR1-4 overexpression strain (2.4.1pRCcsR1-4) (Billenkamp *et al*., 2015).

According to the microarray data, the mRNA levels of genes related to C1 metabolism like *pqqA* (RSP_6132, putative coenzyme PQQ synthesis protein), *xoxJ* (RSP_2580, putative methanol oxidation protein), *xoxF* (RSP_2578, putative PQQ dehydrogenase protein), *cycB* (RSP_2579, cytochrome c533i), and *coxS* and *coxL* (RSP_2878 and RSP_2877, two subunits of a putative carbon monoxide dehydrogenase) were down-regulated by CcsR1-4 overexpression. Moreover, *pdhD* (RSP_2968, dihydrolipoamide dehydrogenase), *pdhAb* (RSP_4049, dehydrolipoamide

acetyltransferase), and *pdhB* (RSP_4050, deydrolipoamide acetyltransferase E2 component) encoding subunits of the pyruvate dehydrogenase complex showed lower expression levels in the CcsR1-4 overexpression strain. Interestingly, the mRNA of *flhR* encoding a putative transcriptional activator also showed lower expression level according to the microarray data. *coxL* (RSP_2877), *pdhB* (RSP_4050), *pqqA* (RSP_6132), and *flhR* (RSP_2591) were predicted as putative target genes of CcsR1-4 by the software tool IntaRNA, however, only the mRNA of *flhR* was verified as a direct target of CcsR1-4 by a *lacZ* based *in vivo* reporter system and gel shift assay (Billenkamp *et al*., 2015). The next experiments were focus on the target gene: *flhR* mRNA.

To reveal the function of FlhR in the oxidative stress response in *R. sphaeroides*, a *flhR* deletion strain was constructed (Billenkamp *et al*., 2015). Since *flhR* encodes a putative transcriptional activator of glutathione (GSH)-dependent methanol/formaldehyde metabolism, the intracellular glutathione (GSH) level was measured in the CcsR1-4 overexpression strain and the *flhR* deletion strain compared to the corresponding control. The results demonstrated that the GSH level was increased by CcsR1-4 overexpression in wild type (Fig 4.3A). However, there was no difference in the GSH level in the strain lacking FlhR compared to wild type. CcsR1-4 overexpression in the *flhR* deletion strain failed to increase the GSH level (Fig 4.3A). This indicates that CcsR1-4 affect the GSH level by targeting the transcriptional activator FlhR. CcsR1-4 overexpression enhanced resistance to oxidative stress (Fig 4.1), however, deletion of *flhR* had no effect on resistance to oxidative stress shown by zone inhibition assays (Fig 4.3B). CcsR1-4 overexpression in the *flhR* deletion strain failed to induce resistance to organic hydroperoxides (Fig 4.3B). This supports the view that CcsR1-4 increases resistance to oxidative stress by targeting *flhR*.

Several mRNAs of genes related to C1 metabolism showed lower expression levels in CcsR1-4 overexpressing strain compared to wild type according to microarray data. To confirm this, real time RT-PCR was performed. Lower expression levels of these genes were confirmed in the CcsR1-4 overexpression strain (Fig 4.3C). Moreover, the *pdhB* gene encoding a component of the pyruvate dehydrogenase complex also showed lower expression level (Fig 4.3C). Lower expression levels of these genes in the *flhR* deletion strain (Fig 4.3C) were also observed. Consequently, CcsR1-4 overexpression in the *flhR* deletion strain had no effect on *cycB, xoxJ, pdhB,* and *coxL* mRNA levels indicating that their expression is dependent of FlhR (Fig 4.3D).

However, the mRNA levels of *pqqA* and *adhI* were still regulated by CcsR1-4 overexpression in the *flhR* deletion strain indicating their regulation is independent of FlhR.

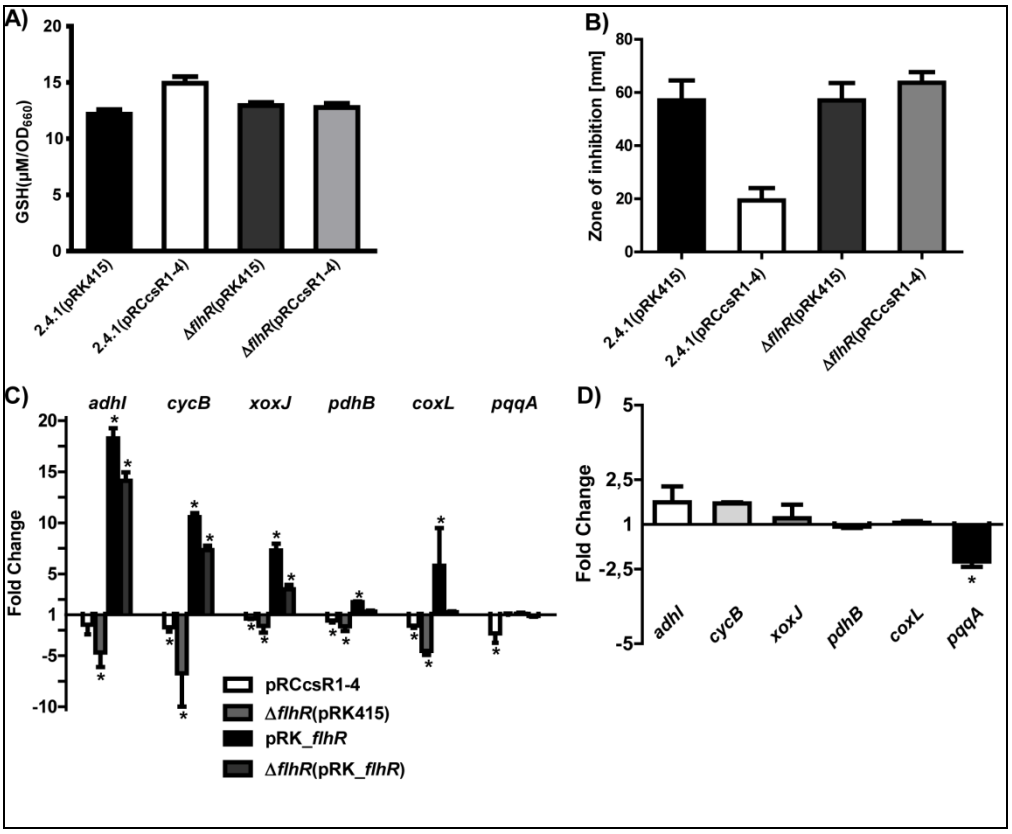

Figure 4.3 A) Intracellular GSH was measured in the CcsR1-4 overexpression strain (pRCcsR1-4) and the *flhR* deletion strain, as well as in the respective control strains harboring empty vector pRK415. B) Effect of CcsR1-4 overexpression in the *flhR* deletion strain on resistance towards oxidative stress determined by zone inhibition assay. C) Expression levels of selected genes from C1 metabolism and *pdhB* was validated by real time RT-PCR. Relative expression level was given as fold change D) The mRNA levels of selected genes in the *flhR* deletion strain affected by CcsR 1-4 overexpression was determined by real time RT- PCR. Relative expression level was given as fold change. The error bars indicate the standard deviation of the mean of two biological repeats consisting of three technical repeats each. Asterisks indicate a significant change in gene expression ($p \leq 0.05$) (Billenkamp *et al*., 2015).

Based on these data, we conclude that CcsR1-4 are able to regulate cellular glutathione level and C1 metabolism by targeting *flhR* which encodes an activator of glutathione (GSH)-dependent methanol/formaldehyde metabolism. GSH is one of major scavengers of ROS in the cell. The GSH level is increased by CcsR1-4 overexpression resulting in enhanced resistance to oxidative stress.

4.2 SorX enhances resistance to singlet oxygen and organic hydroperoxides by regulating a polyamine transporter *potA*

4.2.1 SorX is generated by RNase E cleavage

SorX was originally identified as RSs2461 induced by singlet oxygen in *R. sphaeroides* (Berghoff *et al*., 2009). The *sorX* gene is transcribed together with the upstream gene RSP_0847 from an RpoHI/HII dependent promoter. The 116 nt pre-SorX transcript is processed into a more abundant 75 nt fragment SorX (Berghoff *et al*., 2009), however, which RNase is responsible for this process was still unknown. By using a temperature-sensitive RNase E variant from *E. coli* in *R. sphaeroides* (constructed by Lennart Weber), it was proven that SorX is generated by RNase E cleavage: SorX was not detectable at the non-permissive temperature as shown by Northern blot (Fig 4.4).

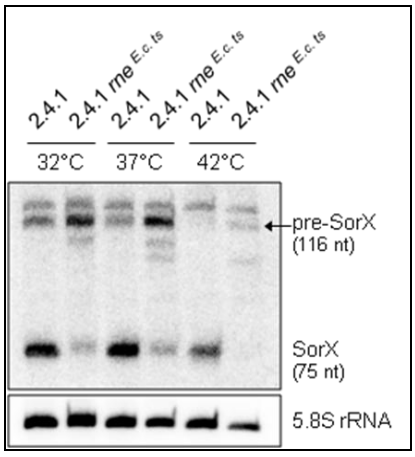

Figure 4.4 Expression levels of SorX and pre-SorX in *R. sphaeroides* wild type 2.4.1 and a strain that expresses a temperature-sensitive RNase E variant (*rne*-3071$^{(ts)}$ mutation) from *E. coli* (2.4.1*rne*$^{E.c.\ ts}$) shown by Northern blot (Data from Lennart Weber) The strains were analyzed at different temperatures (32 ℃ to 42 ℃). The upper band is an unspecific signal. 5.8S rRNA signals are displayed as a loading control.

Imam *et al.*, predicted a FnrL-dependent promoter in the upstream region of pre-SorX based on Chip-seq data (Imam *et.al*., 2014). In order to prove the prediction, the wild type, a *fnrL* deletion strain and a *prrA* (encoding a response regulator) deletion strain (used as control) were cultured at high oxygen tension and shifted to low oxygen tension to induce FnrL activity. FnrL is an anaerobic regulator. Reduction of oxygen tension will increase the activity of FnrL (Zeilstra-Ryalls *et al.*, 1995). Samples were taken before (0 h) and 0.5, 1, 1.5, 2 or 4 h after the shift. SorX was analyzed by Northern blot. The Fig 4.5 shows that SorX is induced by low oxygen tension in the wild type, and this induction is also observed in the *prrA* deletion strain, but not in the *fnrL* deletion strain. This indicates that FnrL has a clear effect on the induction of SorX.

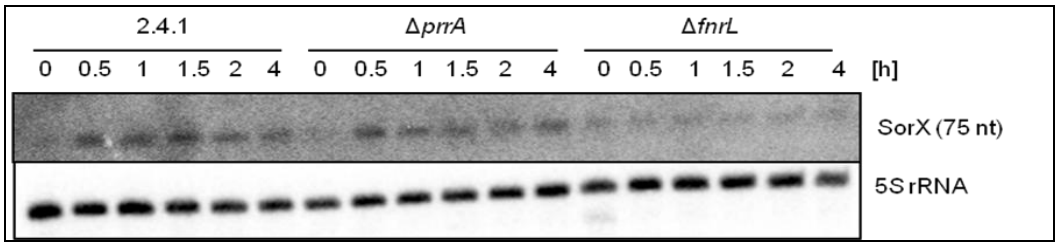

Figure 4.5 Effect of oxygen tension on the expression level of SorX shown by Northern blot. Samples were taken before (0 h) and 0.5, 1, 1.5, 2, 4 h after the cultures were shifted from high oxygen level to low oxygen level. 5S rRNA was used as a loading control.

4.2.2 SorX is induced by various stress conditions

It is known that the genes transcribed form an RpoHI/HII promoter are induced by several stress conditions like heat stress and the oxidative stresses caused by hydrogen peroxide, superoxide, organic hydroperoxides and cadmium chloride (Billenkamp *et al*., 2015). Since *sorX* is transcribed with RSP_0847 from an RpoHI/HII dependent promoter, the expression level of SorX was analyzed by Northern blot under various stress conditions.

The results showed that SorX was induced moderately by singlet oxygen, organic hydroperoxides, diamide, or heat stress (42 ℃). However, SorX was not induced by SDS/EDTA and only slightly induced by superoxide, hydrogen peroxide, or $CdCl_2$ according to the Northern blot (Fig 4.6). SorX is induced by several stress conditions, especially by oxidative stress condition. This indicates that SorX likely plays an important role in the oxidative stress response in *R. sphaeroides*. To uncover the function of SorX in *R. sphaeroides*, a *sorX* deletion strain and constitutive overexpression strains were constructed.

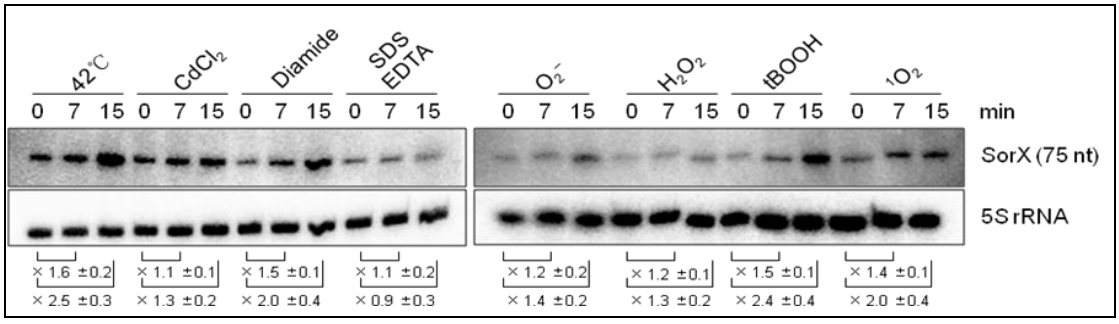

Figure 4.6 Expression levels of SorX induced by various stress conditions shown by Northern blot. *R. sphaeroides* wild type 2.4.1 was grown under aerobic conditions to apply singlet oxygen ($^1O_2$), superoxide (O_2^-), organic hydroperoxide (tBOOH), and hydrogen peroxide (H_2O_2) stress. Growth under microaerobic condition was conducted to induce heat stress (42 ℃) or to treat the cells with $CdCl_2$, SDS/EDTA, and diamide. Total RNA isolated at the indicated time points was analyzed on Northern blots. Results for the 75-nt SorX 3' fragment are shown. Band

intensities for SorX were normalized to the 5S rRNA. Fold changes and standard deviations were calculated from biological triplicates.

Construction of a *sorX* deletion strain

For construction of a *sorX* deletion mutant, overlap extension polymerase chain reaction was performed using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) (Fig 4.7). Using chromosomal DNA of *R. sphaeroides* 2.4.1 as template, two primary PCR reactions were performed with primers Deletion-f and Outrev-SalI and with primers Deletion-r and Outfor-SphI to generate two 36-bp overlapping DNA fragments (345 and 350 bp, respectively), both arounding the same 83-bp deletion within the *sorX* gene in the region of overlap. The 36-mer primers Deletion-f and Deletion-r are complementary to each other. The primary PCR products were used as templates for the secondary PCR. The overlap in sequence allowed the DNA fragments to recombine during the secondary PCR reaction, producing a complete duplex fragment that served as the template for the secondary PCR with primers Outfor-SphI and Outrev-SalI. The 659-bp secondary PCR product containing the deletion within *sorX* was purified with QIAEX II Gel Extraction Kit (Qiagen), restricted with enzymes SphI and SalI, and cloned into the pDrive vector, resulting in pDriveΔSorX. The 659-bp insert in pDriveΔSorX was sequenced to confirm the deletion within *sorX*. Finally the 659-bp SphI/SalI fragment was cloned into the suicide vector pLO1 digested with the same restriction enzymes, yielding the plasmid pLO1_ΔSorX (Fig 4.6). The resulting plasmid pLO1_ΔSorX was introduced into *R. sphaeroides* 2.4.1 by conjugation to generate a ΔSorX mutant. Heterogenotes of *R. sphaeroides*, generated by a single recombination event, were selected for their kanamycin resistance on RÄ agar plates. Isogenic homogenotes were obtained from the heterogenotes after a second recombination by selecting for sucrose resistance on RÄ agar plates containing 10% (w/v) sucrose. The allelic exchange was verified by PCR.

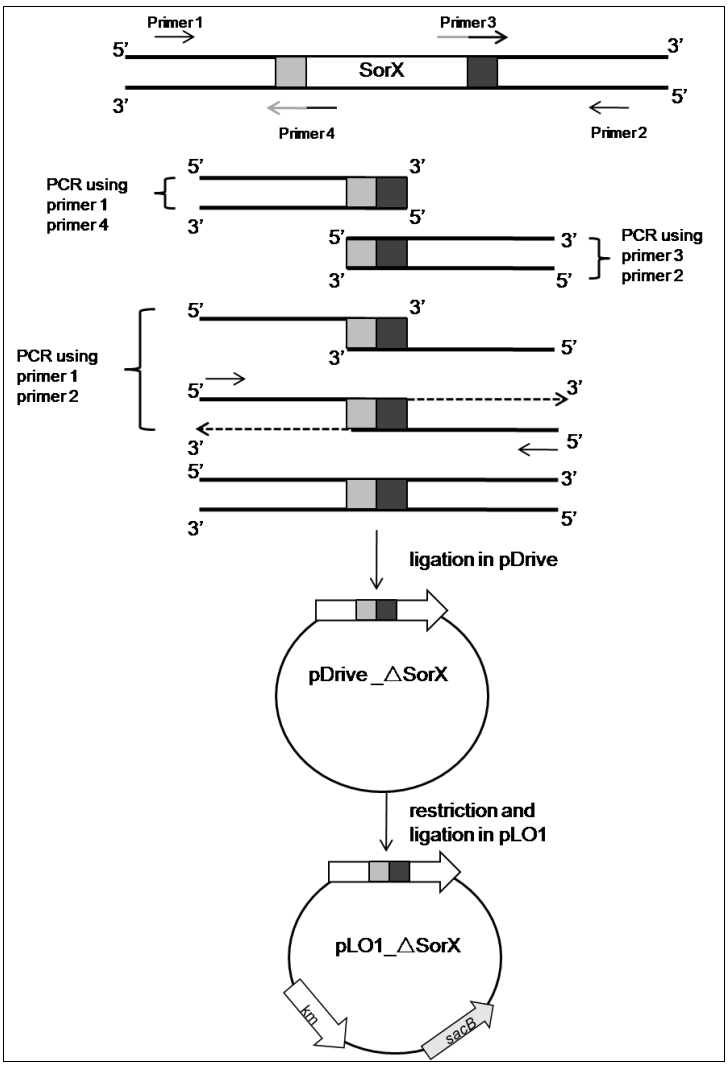

Figure 4.7 Construction of suicide vector pLO1_ΔSorX for the SorX deletion strain. Overlap extension polymerase chain reaction was used to produce the fragment containing the deletion within *sorX*.

Construction of pre-SorX overexpression strain

In order to constitutively overexpress pre-SorX in the cell, a 176 bp fragment containing the 116 bp *sorX* gene and its 28 bp upstream region was amplified by PCR using the primers SorX-up0-BamHI and SorX-over-R-XbaI. The fragment was subcloned to pJET1.2. Afterwards, the fragment was restricted from pJET1.2 and ligated with pBBR4352 containing a 16S promoter (Mank *et al.*, 2014) after restriction by the corresponding enzymes, resulting in plasmid pBBRSorX^{144} (Fig 4.8). The same procedure was used to produce pRKSorX^{144} by primers SorX-up0-BamHI and Term-SorX-EcoRI (made by Jasmin Schirmer). Afterwards, the plasmid pBBRSorX^{144} or pRKSorX^{144} was transferred to *R. sphaeroides* 2.4.1 by conjugation.

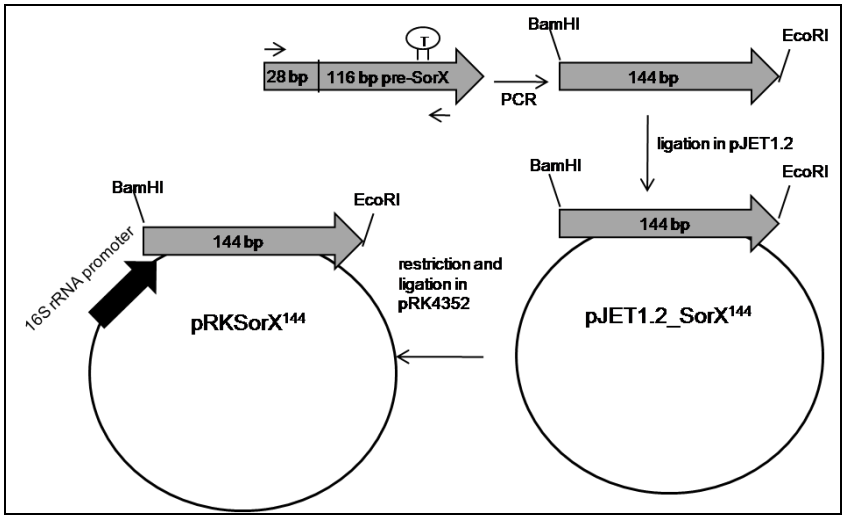

Figure 4.8 Construction of pre-SorX constitutive overexpression plasmid pBBRSorX^{144}.

Construction of SorX overexpression strain

A 108 bp fragment containing 76 bp *sorX* gene was amplified by PCR using the primers SorXSO-F-BamHI and SorX-over-R-XbaI. The fragment was introduced to pJET1.2. After restriction from pJET1.2, the fragment was subsequently ligated into pBBR4352 after restriction by the corresponding enzymes. This yielded the plasmid pBBRSorX^{76} (Fig 4.9). The plasmid was transferred to *R. sphaeroides* 2.4.1 by conjugation.

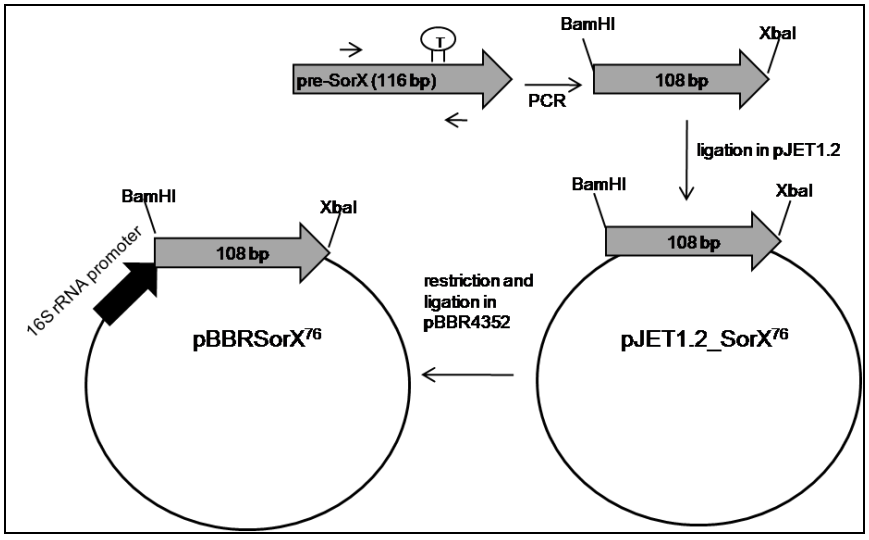

Figure 4.9 Construction of SorX constitutive overexpression plasmid pBBRSorX^{76}.

4.2.3 Validation of SorX mutant and overexpression strains

In order to validate the SorX deletion and constitutive overexpression strains, Northern blot was performed to monitor the expression levels of SorX in the cell.

SorX and pre-SorX were not detectable in the SorX deletion strain. Both pre-SorX and SorX were accumulated in pre-SorX overexpression strain (2.4.1pBBRSorX^{144}) (Fig 4.10A).

The SorX overexpression plasmid (pBBRSorX^{76}) was transferred to a RNA chaperon Hfq deletion strain for further experiments (see below). In the *hfq* mutant, the half-life of SorX was decreased compared to wild type and induction of SorX by singlet oxygen was not observed (Berghoff *et al.*, 2009). If the SorX overexpression plasmid (pBBRSorX^{76}) was transferred to the *hfq* deletion strain, only SorX was accumulated as shown by Northern blot (Fig 4.10B). This proves that overexpression of SorX was successful in the cell.

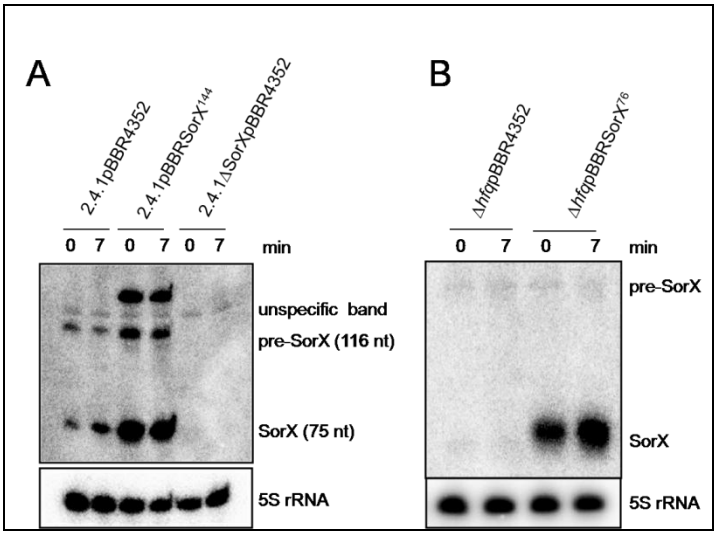

Figure 4.10 Validation of SorX deletion and overexpression strains by Northern blot. (A) Expression levels of pre-SorX and SorX in *R. sphaeroides* wild type 2.4.1 harboring an empty vector (2.4.1pBBR4352) and a pre-SorX overexpression plasmid (2.4.1pBBRSorX^{144}). (B) Expression levels of pre-SorX and SorX in a *hfq* deletion strain harboring an empty vector (pBBR4352) or SorX overexpression plasmid (pBBRSorX^{76}). Total RNA was isolated from each strain before and 7 min after adding 100 µM tBOOH. 5S rRNA was used as a loading control.

4.2.4 Characterization of the SorX deletion strain

4.2.4.1 Effect of SorX on growth

The pre-SorX overexpression plasmid (pRKSorX^{144}) was transferred to SorX mutant to obtain the complemented strain (ΔSorXpRKSorX^{144}). Growth of the SorX deletion strain, the pre-SorX overexpression strain, the complemented strain were compared to that of the wild type harboring an empty vector plasmid pRK4352. A slower growth by deletion of SorX was observed. The doubling time of the SorX deletion strain was

increased compared to that of the wild type under aerobic condition. Moreover, pre-SorX overexpression (pRKSorX^{144}) also moderately increased the doubling time in the wild type as shown in Fig 4.11.

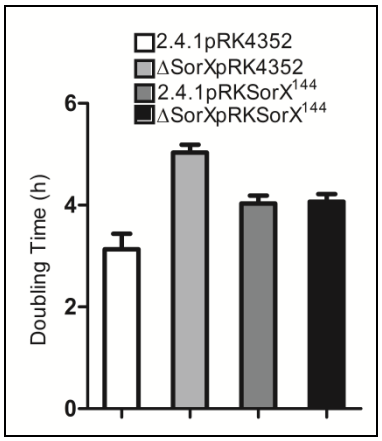

Figure 4.11 Effect of SorX on doubling time. The strains were grown under aerobic condition. Doubling time was calculated based on the chage of OD_{660} measured in the middle exponential phase. The error bars indicate the standard deviation of the mean of three biological replicates.

4.2.4.2 Effect of SorX on the expression of *ompR*

SorX is generated from the 3' UTR of the *ompR* transcript, therefore, the question is whether SorX can regulate the OmpR protein level in the cell. Western blot using an OmpR specific antibody showed that deletion of *sorX* had no effect on the expression of *ompR* (Fig 4.12).

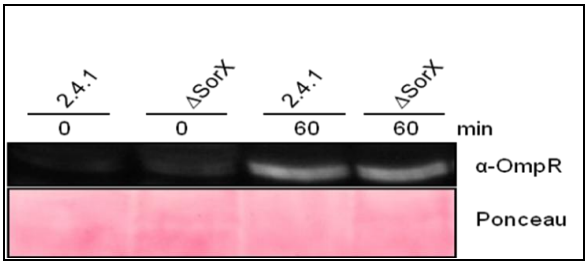

Figure 4.12 Effect of SorX on expression of *ompR* shown by western blot. The samples were taken before (0 min) and 60 min after treatment with singlet oxygen. Ponceau staining was used as a loading control. 50 µg of total protein was applied in each lane.

4.2.4.3 Effect of SorX on resistance to oxidative stress

SorX is induced by oxidative stress supporting that SorX is likely involved in oxidative stress response in *R. sphaeroides*. To test the effect of deletion of *sorX* on resistance to oxidative stress, zone inhibition assays were performed. The results showed that deletion of SorX caused a higher sensitivity against organic hydroperoxides and singlet oxygen. Moreover, pre-SorX overexpression enhanced resistance towards singlet oxygen and organic hydroperoxides (Fig 4.13A).

In order to test whether pre-SorX or SorX is functional in the cell, the same zone

inhibition assays were performed using the SorX mutant harboring the pre-SorX (pBBRSorX^{144}) or SorX (pBBRSorX^{76}) overexpression plasmid. Fig 4.13B shows that SorX overexpression recovers resistance of the mutant to singlet oxygen and organic hydroperoxides.

Based on these data, it was assumed that SorX is involved in response to singlet oxygen and organic hydroperoxides in *R. sphaeroides* and the small pre-SorX processing product SorX is sufficient for this function. Therefore, the 75 nt fragment product was renamed as SorX (for singlet oxygen resistance RNA X) , the 116 nt fragment was renamed as pre-SorX.

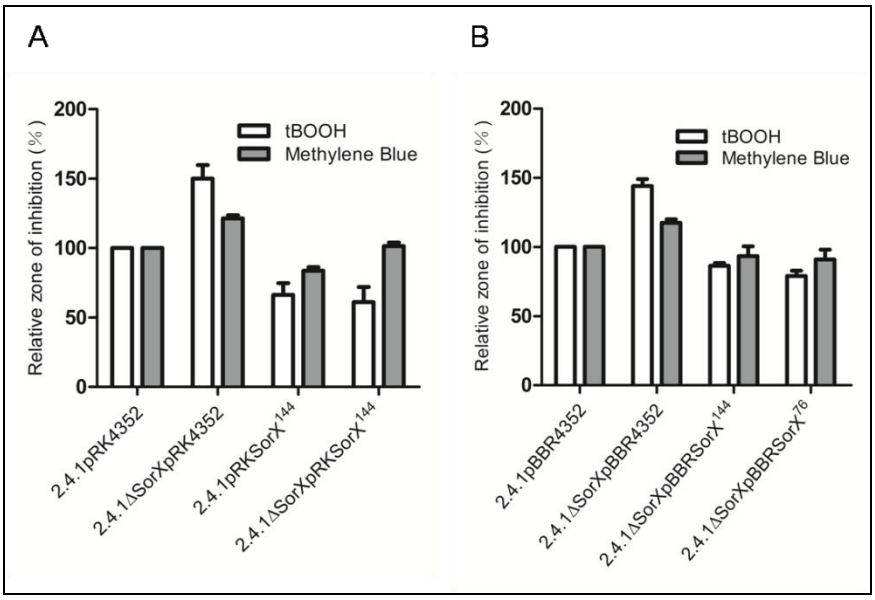

Figure 4.13 Effect of SorX on resistance to singlet oxygen and organic hydroperoxides monitored by zone inhibition assays. Zones of inhibition for each strain were calculated relative to their respective control strain (100%). 1 M tBOOH or 50 mM methylene blue in presence of light was used to generate organic hydroperoxides or singlet oxygen stress, respectively. (A) The SorX deletion strain (2.4.1ΔSorXpRK4352), the pre-SorX overexpression strain (2.4.1pRKSorX^{144}) and the complemented SorX deletion strain (2.4.1ΔSorXpRKSorX^{144}) were compared to wild type 2.4.1 carrying an empty vector control (2.4.1pRK4352). (B) The SorX deletion was complemented either with the pre-SorX overexpression plasmid (pBBRSorX^{144}) or the SorX overexpression plasmid (pBBRSorX^{76}). The corresponding strains were compared to wild type 2.4.1 (2.4.1pBBR4352) and the SorX deletion strain (2.4.1ΔSorXpBBR4352), each carrying an empty vector control. The error bars indicate the standard deviation from the mean of biological triplicates.

4.2.5 Effect of SorX on global gene expression

4.2.5.1 Global transcriptome analysis

In order to search for direct target genes of SorX, global transcriptome analysis by microarray was performed with the wild type harboring an empty vector (2.4.1pRK415) compared to the pre-SorX overexpression strain (2.4.1pRKSorX^{144}). Microarray analysis was performed as described previously (Berghoff *et.al*., 2013). Data shown in this study represent the results from two individual microarrays (biological replicates) and one technical replicate, each contains a pool of three independent experiments for each sample. The genes regulated by SorX are listed in Table 4.1.

Table 4.1 Genes with a log_2 fold change of > 0.6 or < -0.6 were shown according to microarray data

RSP no.	Gene	Description	log_2 ratio	Regulated by*	
				CcsR	SorY
RSP_0069	*fliC*	Flagellar filament protein	-0.7		
RSP_0097	*takP*	TRAP-T family sorbitol/mannitol transporter,			
		periplasmic binding protein, SmoM	-1.1		yes
RSP_0099		TRAP-T family sorbitol/mannitol transporter,			
		DctQ (4TMs) subunit	-0.6		
RSP_0161		Spermidine/putrescine-binding periplasmic protein	-0.7		
RSP_0381		hypothetical protein	-0.9		
RSP_0576		Na+/solute symporter	-0.6		
RSP_0577		hypothetical protein	-0.8		
RSP_0842		putative porin	-0.6		
RSP_0910	*dctP*	TRAP-T family transporter,			
		C4-dicarboxylate-binding protein DctP	-0.9		
RSP_0911	*dctQ*	TRAP-T family C4-dicarboxylate transporter,			
		DctQ (4TMs) subunit	-0.9		
RSP_0912	*dctM*	TRAP-T family C4-dicarboxylate transporter,			
		DctM (12TMs) subunit	-0.8		
RSP_1140	*ilvE*	branched-chain amino acid aminotransferase	-0.8		
RSP_1413		TRAP-T family transporter, periplasmic binding component	-0.6		
RSP_1613		TRAP-T family transporter, DctP subunit	-0.9		
RSP_1877	*coxI*	Cytochrome c oxidase, aa3 type, subunit I	-0.7		
RSP_1882	*potA*	ABC polyamine/opine transporter, ATPase subunit	-0.6		
RSP_2310	*groES*	Chaperonin Cpn10 (GroES)	-0.8		
RSP_2311	*groEL*	chaperonin GroEL	-0.7		
RSP_2578	*xoxF*	putative pqq dehydrogenase protein	-1.0	yes	yes
RSP_2579	*cycB*	cytochrome c553i	-0.6	yes	yes
RSP_2585		hypothetical protein	-0.9		
RSP_2877	*coxL*	Putative carbon monoxide dehydrogenase large chain	-0.9	yes	

RSP_2878	*coxS*	Putative carbon-monoxide dehydrogenase small chain	-1.2	yes
RSP_2879		hypothetical protein	-0.9	yes
RSP_2968		dihydrolipoamide dehydrogenase	-0.9	
RSP_4047	*pdhAa*	Pyruvate dehydrogenase E1 component, alpha subunit	-0.7	yes
RSP_4049	*pdhAb*	dihydrolipoamide acetyltransferase	-1.0	yes
RSP_4050	*pdhB*	Dihydrolipoamide acetyltransferase component (E2)		
		of pyruvate dehydrogenase complex	-1.0	yes
RSP_0108	*nuoJ*	NADH dehydrogenase I chain J	0.7	
RSP_0545		hypothetical protein	0.7	
RSP_0557		hypothetical protein	1.4	
RSP_0743		Putative translation factor	0.6	
RSP_0904	*sitA*	ABC Mn+2/Fe+2 transporter,		
		periplasmic substrate-binding protein SitA	1.1	
RSP_0905	*sitB*	ABC Mn+2/Fe+2 transporter, ATPase subunit SitB	0.8	
RSP_0906	*sitC*	ABC Mn+2/Fe+2 transporter,		
		inner membrane subunit SitC	1.2	
RSP_0908	*sitD*	ABC Mn+2/Fe+2 transporter,		
		inner membrane subunit SitD	1.0	
RSP_1082	*fdsD*	NAD-dependent formate dehydrogenase, delta subunit	0.6	
RSP_1119		ABC protein exporter,		
		fused ATPase and inner membrane subunits	0.7	
RSP_1471		hypothetical protein	0.7	
RSP_2037		hypothetical protein	0.7	
RSP_2308		hypothetical protein	0.7	
RSP_2511		enoyl-CoA hydratase	0.6	
RSP_3308		possible transporter, DME family, DMT superfamily	0.6	
RSP_3509	*expE1*	Hemolysin-type calcium-binding region, RTX	0.7	
RSP_3517		ABC polyamine/opine transporter,		
		inner membrane subunit	0.6	
RSP_3535		enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase		
		bifunctional enzyme	0.7	
RSP_3639		hypothetical protein	0.6	
RSP_3908		conserved hypothetical protein	0.8	
RSP_3940		hypothetical protein	0.6	
RSP_3966		ABC branched chain amino acid transporter,		
		inner membrane subunit	0.7	
RSP_3972		short chain dehydrogenase	0.7	
RSP_4108	*wcaG*	putative nucleotide di-P-sugar epimerase or dehydratase	0.7	
RSP_6076		hypothetical protein	0.7	
	sorX	small RNA	2.0	
	ccsR1	small RNA	1.7	
	sorY	small RNA	2.1	

* It is indicated whether the corresponding genes were detected as potential CcsR/SorY targets.

4.2.5.2 Effect of SorX on the expression levels of CcsR1 and SorY

According to the microarray analysis (Table 4.1), the sRNAs CcsR1 and SorY are induced by pre-SorX overexpression. CcsR1 is one of the four homologous sRNAs described in the first chapter. SorY is another sRNA induced by singlet oxygen (Adnan *et al.*, 2015). CcsR1 and SorY were not detectable in the SorX deletion strain and expression levels of CcsR1 and SorY were increased by pre-SorX overexpression confirmed by Northern blot (Fig 4.14B). A previous study showed that CcsR1-4 are transcribed together with an upstream gene RSP_6037 (Berghoff *et al.*, 2009). The mRNA level of RSP_6037 was decreased in the SorX deletion strain (Fig 4.14A). The intracellular GSH level was affected by CcsR1-4 (Billenkamp *et al*., 2015). It is also true that SorX affected the intracellular GSH level (Fig 4.14C). All these data prove that SorX influences CcsR1 and SorY expressions in *R. sphaeroides*.

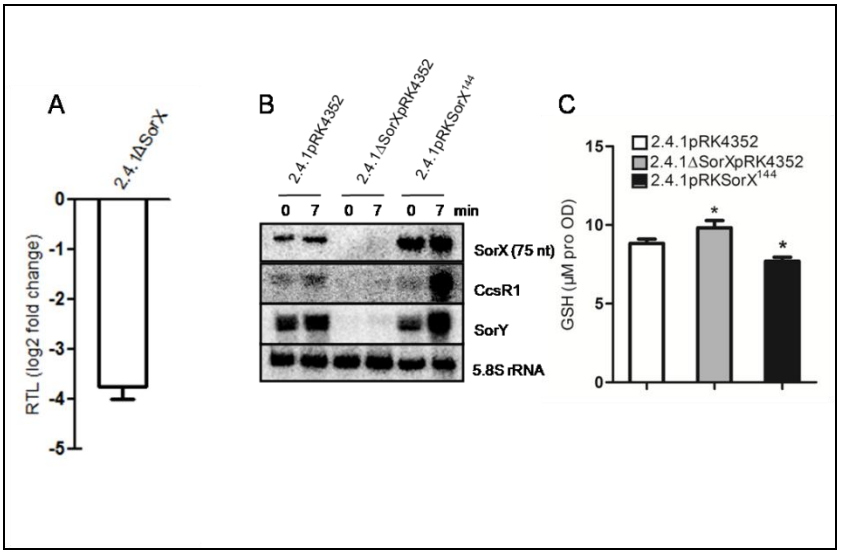

Figure 4.14 (A) Effect of SorX on the mRNA level of RSP_6037 determined by real time RT-PCR. The samples of the SorX mutant and wild type 2.4.1 were taken after 7 min treatment with 100 mM tBOOH. The gene *rpoZ* was used as standard gene for normalization The error bars indicate the standard deviation from the mean of two biological replicates with three technical replicates. The relative transcript level (RTL) is given as $\log_2$ fold change. (B) Expression levels of CcsR1 and SorY affected by SorX were shown by Northern blot. Total RNA was isolated from each strain before and 7 min after adding 100 mM tBOOH and used for Northern blot analysis. 5.8S rRNA was used as a loading control. (C) Effect of SorX on intracellular glutathione (GSH) level. The error bars indicate the standard deviation from the mean of two biological replicates with three technical replicates. Asterisks indicate a statistically significant change ($p \leq 0.05$).

4.2.5.3. Effect of SorX on the promoter activities of RSP_6037 and SorY

To investigate the effect of SorX on promoter activities of RSP_6037-CcsR1-4 and SorY. a fluorescence based *in vivo* reporter system was constructed as follows: A 192

bp fragment containing the promoter region of RSP_6037-CcsR1-4 was amplified by PCR using primers 6037P-F-SacII and 6037P-R-BamHI. Afterwards, the fragment containing positions -192 to -1 with respect to the start codon was subcloned to pJET1.2. The fragment was restricted from pJET1.2 and ligated into pBE4352::eCFP:eCFP containing a 16S promoter (Remes *et al.*, 2015) after restriction with the corresponding enzymes. This yielded the plasmid pBE::P_{RSP_6037}:eCFP (Fig 4.15). The same procedure was used to produce a SorY reporter plasmid. A 201 bp fragment containing the promoter region of *sorY* was amplified by PCR using primers SorYP-F-XbaI and SorYP-R-BamHI. The fragment containing positions -201 to -1 with respect to the start codon was introduced to pBE4352::eCFP:eCFP after restriction with the corresponding enzymes, resulting in the plasmid pBE::P_{SorY}:eCFP.

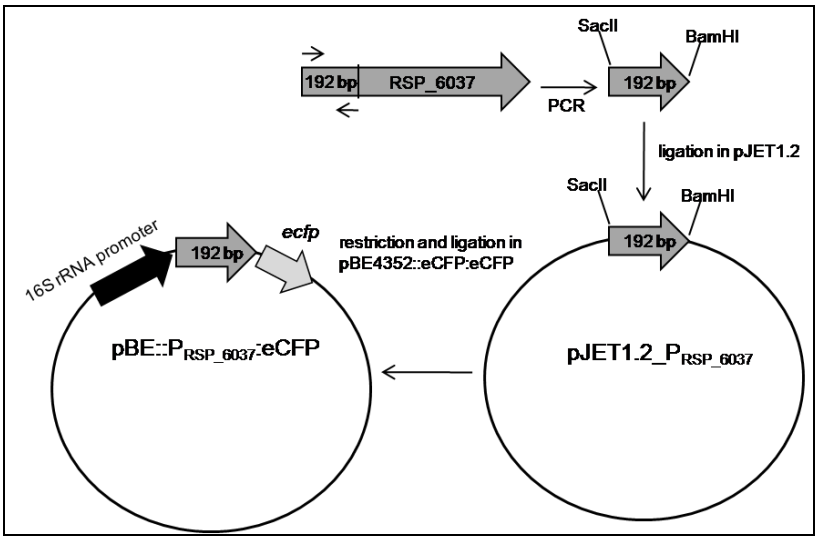

Figure 4.15 Construction of fluorescence reporter plasmid pBE::P_{RSP_6037}:eCFP.

The reporter plasmid pBE::P_{RSP_6037}:eCFP or pBE::P_{SorY}:eCFP was transferred to the SorX mutant (2.4.1ΔSorXpRK4352) and the pre-SorX overexpression strain (2.4.1pRKSorX[144]) to measure the fluorescence activities. The promoter activities of RSP_6037-CcsR1-4 and SorY were up-regulated by pre-SorX overexpression (Fig 4.16). There is no possible binding between SorX and the 5' UTR of RSP_6037-CcsR1-4 or *sorY* mRNA predicted by IntaRNA software tool. This indicates that this regulation is likely an indirect effect.

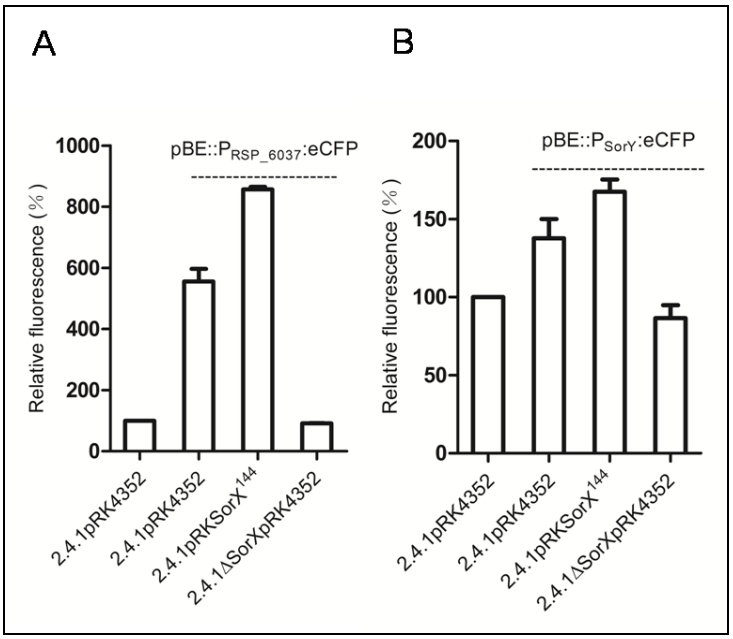

Figure 4.16 Effect of SorX on the promoter activity of RSP_6037 (A) and SorY (B). The promoter regions were transcriptionally fused to *ecfp* to determine promoter activities. Fluorescence intensities were calculated relative to wild type 2.4.1 carrying the empty vector pRK4352 (set to 100%). The error bars indicate the standard deviation from the mean of biological triplicates with three technical replicates.

To test which region before the start codon of RSP_6037-CcsR1-4 is important for this regulation, the 192 bp fragment was shortened to 105 bp including the promoter region (-103 to -76 with respect to the start codon). This shorter fragment containing -105 to -1 with respect to the start codon was fused to *ecfp* to obtain reporter plasmid pBE::SP$_{RSP_6037}$:eCFP. Afterwards, the plasmid was transferred to the strain lacking SorX (2.4.1ΔSorXpRK4352) and the pre-SorX overexpression strain (2.4.1pRKSorX^{144}) to measure the fluorescence activities. The result showed that SorX failed to up-regulate the promoter activity of RSP_6037-CcsR1-4 (Fig 4.17) indicating that the -192 to -104 bp with respect to the start codon region is necessary for this regulation.

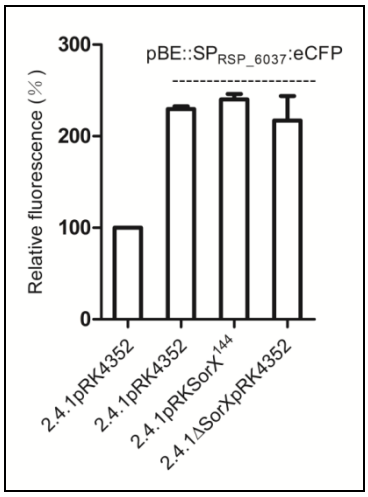

Figure 4.17 Effect of SorX on the promoter activity of RSP_6037. The short promoter region was transcriptionally fused to *ecfp* to determine promoter activity. Fluorescence intensities were calculated relative to wild type 2.4.1 carrying the empty vector pRK4352 (set to 100%). The error bars indicate the standard deviation from the mean of biological triplicates with three technical replicates.

4.2.6 The *potA* mRNA is a putative target of SorX

53 mRNAs with a $\log_2$ fold change of > 0.6 or < -0.6 were detected according to microarray analysis (Table 4.1). Among all the genes regulated by SorX, there are also some genes regulated by CcsR1-4 or SorY according to the microarray data (Table 4.1). This indicates that these genes are likely indirectly regulated by SorX. Besides these genes, the mRNA of RSP_1882 (*potA*) which encodes a subunit of a putative polyamine transporter was down-regulated according to the microarray data. An interaction of SorX and the 5' UTR of the *potA* mRNA was predicted by the IntaRNA software tool (Fig 4.20A). Moreover, RSP_1883 (*potD*) encoding another subunit of the putative polyamine transporter showed decreased synthesis rate according to the proteomes analysis (data not shown). The *potD* gene is located in the same operon with *potA* and transcribed with *potA*. Based on these data, the further analysis was focused on *potA* mRNA.

The mRNA level of *potA* was decreased after 7 min of treatment with tBOOH in the wild type strain (Fig 4.18A), while the mRNA level of *potA* was slightly increased in the SorX mutant under the same condition. Moreover, the level of *potA* mRNA was increased in the SorX deletion strain compared to that in wild type (Fig 4.17A). This proved that SorX affects the mRNA level of *potA*. The same is the case for *potD* (RSP_1883) (Fig 4.18B). Next, the half-life of *potA* mRNA in the wild type and SorX deletion strain was determined, because the stability of target mRNAs are often affected by most of the sRNAs (Storz *et.al.*, 2011; Beisel *et.al.*, 2010). The half-life of *potA* was increased in the SorX deletion strain compared to that in wild type (Fig 18C) which supported a direct interaction between SorX and *potA* mRNA.

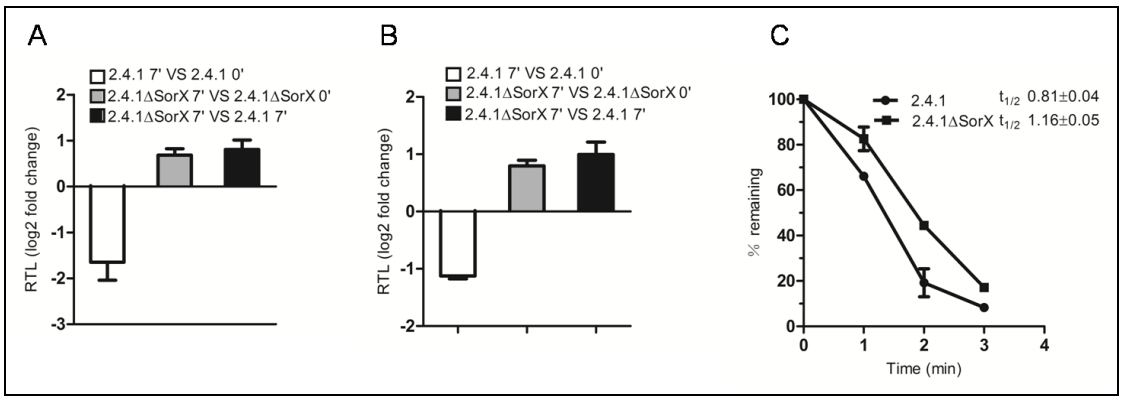

Figure 4.18 Effect of SorX on *potA* (A) and *potD* (B) mRNA levels as quantified by real time RT-PCR. Total RNA was isolated from wild type 2.4.1 and the SorX deletion strain (2.4.1ΔSorX) before (0 min) and 7 min after treatment with tBOOH. The relative transcript level (RTL) is given as $\log_2$ fold change. (C) Effect of SorX on *potA* mRNA stability. The half-life of *potA* mRNA in wild type 2.4.1 and the SorX deletion strain (2.4.1ΔSorX) was calculated by real time RT-PCR (expression values were normalized to 16S rRNA). Initiation of transcription was inhibited by rifampicin. The error bars indicate the standard deviation from the mean of biological triplicates with two technical replicates.

4.2.7 Direct base pairing between SorX and the *potA* mRNA

By using a software tool IntaRNA, a direct interaction between SorX and *potA* mRNA was predicted (Fig 4.20A). In order to verify this interaction, a *lacZ* based *in vivo* reporter system was constructed as follows: a 87 bp fragment of *potA* containing the seed region -12 - -6 (Fig 4.20A) was amplified by PCR using primers 1882RE-F-BamHI and 1882RE-R-HindIII. The fragment was then introduced into a *lacZ* reporter plasmid pPHU4352 containing the 16S promoter (Mank *et al*., 2012), resulting in reporter plasmid pPHU*potA* (Fig 4.19).

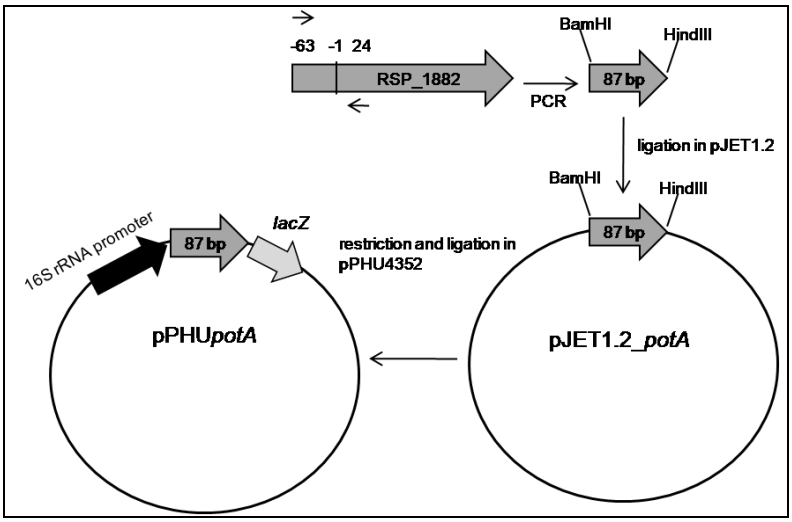

Figure 4.19 Construction of reporter plasmid pPHU*potA*.

The upstream region of *potA* containing the predicted seed region was translationally fused to the *lacZ* gene. The β-galactosidase activity was measured in a SorX deletion strain harboring the empty vector (pBBR4352) and the reporter plasmid was used as control (set to 100%). When the pre-SorX overexpression plasmid (pBBRRSorX^{144}) instead the empty vector pBBR4352 was present, the β-galactosidase activity decreased to about 50-60% (Fig 4.20B). The β-galactosidase activity also decreased if the SorX overexpression plasmid (pBBRSorX^{76}) was present in the cell (Fig 4.20C) indicating that the small SorX fragment has the ability to target *potA* mRNA. However there was no effect on β-galactosidase activity of pre-SorX overexpression on a *potD-lacZ* reporter indicating that *potD* is indirectly regulated by SorX (Fig 4.20D).

To further confirm the interaction between SorX and *potA* mRNA. the three nucleotides CTC in the putative seed region of SorX were mutated to GAG (Fig 4.20A) as follows: The plasmid pBBRSorX^{144} was used as template, the triple mutation (CTC → GAG) in *sorX* at positions +34-36 with respect to the sRNA 5' end was introduced by inverse PCR using primers SorXMU-F and SorXMU-R. If three nucleotides were mutated to weaken the predicted interaction with *potA*, pre-SorX overexpression failed to affect the β-galactosidase activity of the *potA-lacZ* reporter. Furthermore, the mutated pre-SorX overexpression in wild type failed to enhance resistance to organic hydroperoxides (Fig 21A) indicating the seed region is important for the function of SorX for enhancing the resistance to orgnic hydroperoxides. If the compensatory mutations (GAG mutated to CTC) were introduced into *potA* to recover the interaction (Fig 20A), a strong decrease of β-galactosidase activity was observed if the mutated pre-SorX overexpression plasmid was present (Fig 20B), however, pre-SorX overexpression had no effect on the mutated *potA-lacZ* reporter (Fig.4.20B). All these data proved there is a direct interaction between SorX and 5' UTR of *potA* mRNA. Moreover, pre-SorX overexpression had no effect on β-galactosidase activity in the RNA chaperon Hfq deletion strain (Fig 4.20E) indicating that Hfq is necessary for SorX-*potA* interaction. It is also true that SorX overexpression in *hfq* deletion strain had no effect on resistance to organic hydroperoxides (Fig 4.21B). This supports the view that SorX enhances resistance to organic hydroperoxides dependent on Hfq protein.

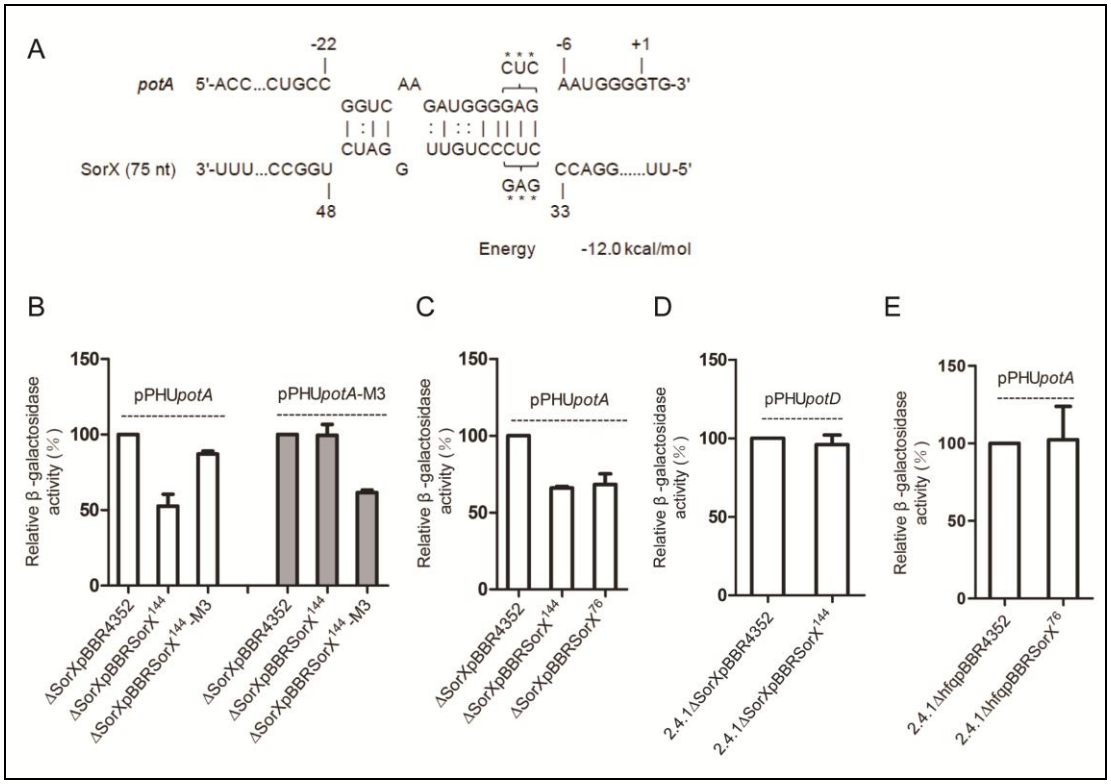

Figure 4.20 (A) Interaction of SorX-*potA* as predicted by IntaRNA. Position +1 of the mRNA represents the translational start site. The seed region in *potA* is from –21 to –5 with respect to the start codon. A triple mutation (M3) in SorX (CUC → GAG) and a compensatory mutation in *potA* (GAG → CUC) are marked by asterisks. (B) Relative β-galactosidase activities for *lacZ* fusions with wild-type *potA* or mutated *potA*. Wild-type SorX or mutated SorX were overexpressed in the SorX deletion background (2.4.1ΔSorXpBBRSorX^{144}, 2.4.1ΔSorXpBBRSorX^{144}-M3). (C) Relative β-galactosidase activities for a *lacZ* fusion with *potA*. Pre-SorX (pBBRSorX^{144}) or SorX (pBBRSorX^{76}) was overexpressed in the SorX deletion strain. (D) Relative β-galactosidase activities for *lacZ* fusions with *potD*. Wild-type SorX was overexpressed in the SorX deletion background (2.4.1ΔSorXpBBRSorX^{144}). (E) Relative β-galactosidase activities for a *lacZ* fusion with *potA*. SorX (pBBRSorX^{76}) was overexpressed in an *hfq* deletion strain. β-galactosidase activities in an empty vector control strain were set to 100%. The error bars indicate the standard deviation from the mean of biological triplicates with two technical replicates.

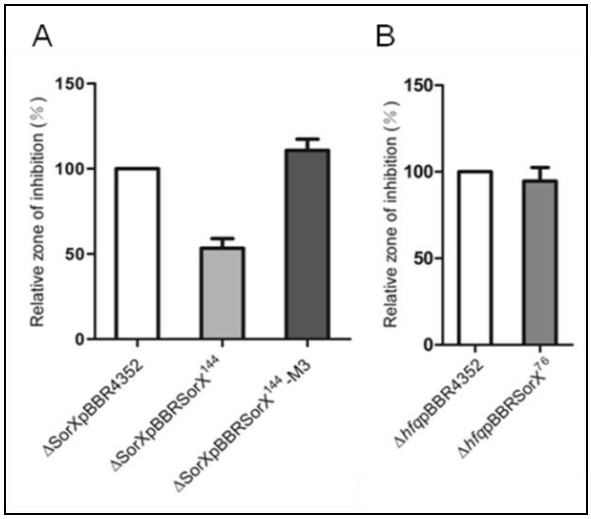

Figure 4.21 Effect of SorX on resistance to organic hydroperoxides in the SorX deletion background (A) or the *hfq* deletion strain (B) as shown by zone inhibition assays. Strains harboring the empty vector pBBR4352 served as controls (set to 100%). 1 M tBOOH was applied for the assay.

4.2.8 Effect of *potA* on resistance to singlet oxygen and organic hydroperoxides

The *potA* mRNA was identified as the direct target of SorX. To uncover the function of *potA* in *R. sphaeroides*, a *potA* deletion strain was constructed as follows: a ~ 400 bp upstream and a ~ 600 bp downstream fragment of *potA* were amplified by PCR using primers 1882KO-UP-F-XbaI and 1882KO-UP-R-PstI, 1882KO-DO-F-PstI and 1882KO-DO-R-HindIII. Afterwards, the fragments were ligated into pPHU281(Hübner *et al*., 1991) after restriction with the corresponding enzymes. The kanamycin resistance cassette stemming from pUC4K plasmid which has no transcriptional terminator was introduced into the PstI site to produce the plasmid pPHUΔ*potA*_up_Km_down (Fig 4.22). The suicide vector was transferred to *R. sphaeroides* 2.4.1 by conjugation. The conjugants were selected ON agar plates containing kanamycin. Correct double crossover was confirmed by PCR analysis of chromosomal DNA isolated from kanamycin-resistant and tetracycline-sensitive conjugants with primers 1882OUT-F and 1882OUT-R.

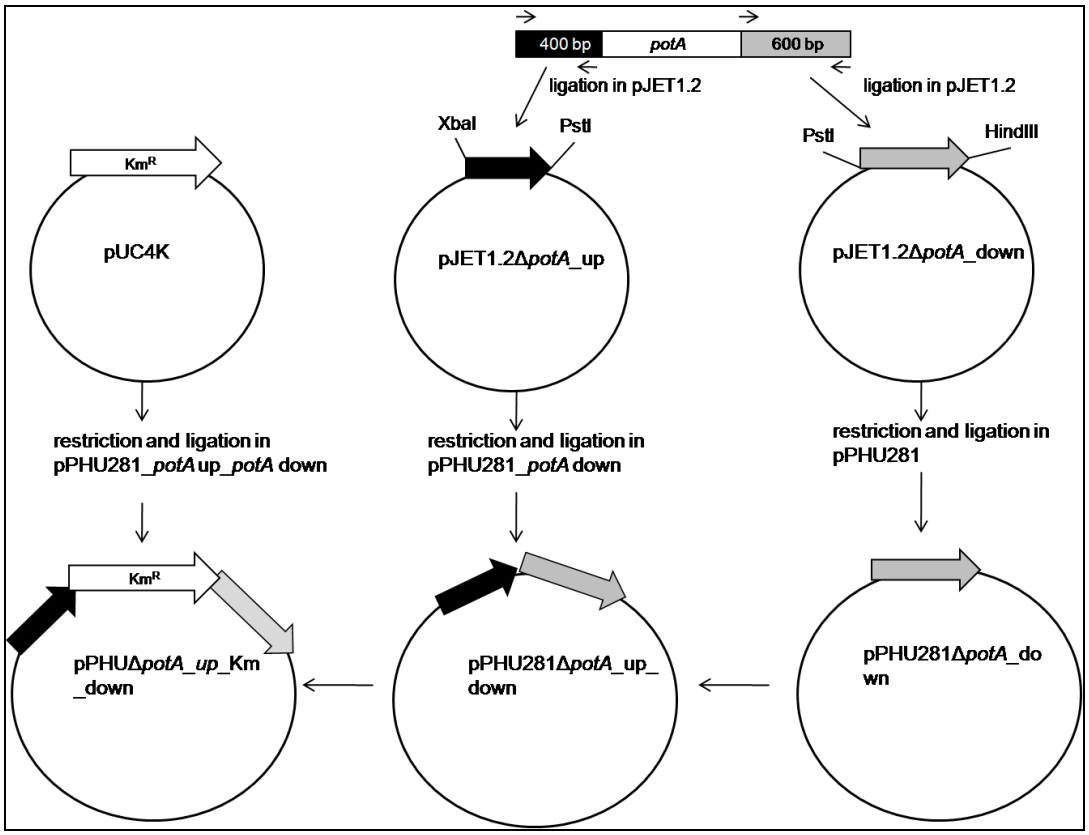

Figure 4.22 Construction of suicide vector pPHUΔ*potA*_up_Km_down for *potA* deletion strain.

Deletion of *potA* increased resistance to singlet oxygen and organic hydroperoxides compared to wild type as shown by zone inhibition assays (Fig 4.23A). This indicates that lower expression level of *potA* is beneficial to the cell under oxidative stress condition. Moreover, pre-SorX overexpression in a *potA* deletion strain had no effect on resistance to singlet oxygen and organic hydroperoxides (Fig 4.23B) supporting the view that SorX overexpression enhances resistance to singlet oxygen and organic hydroperoxides by targeting *potA* mRNA.

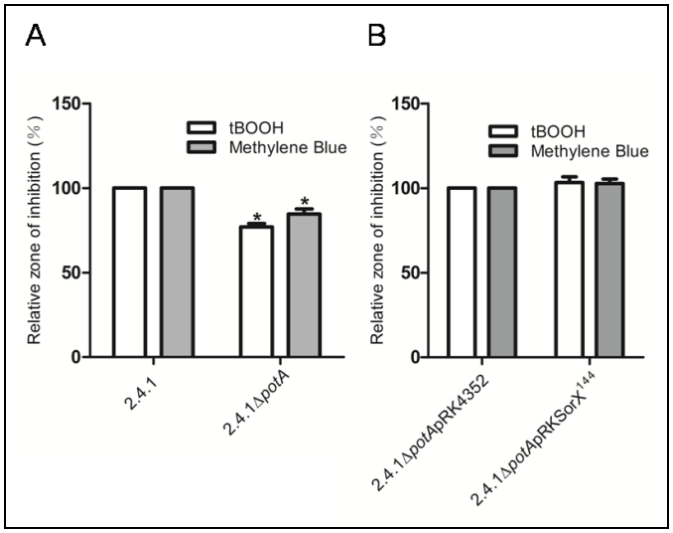

Figure 4.23 (A) Effect of *potA* deletion on resistance to singlet oxygen and organic hydroperoxides shown by zone inhibition assays. Asterisks indicate a statistically significant change in inhibition zones ($p \leq 0.05$). The wild type served as control (set to 100%). (B) Effect of SorX overexpression in the *potA* deletion strain on resistance to singlet oxygen and organic hydroperoxides. The error bars indicate the standard deviation from the mean of biological triplicates. The *potA* deletion strain harboring a empty vector (pRK4352) is used as control (set to 100%).

4.2.9 Effect of spermidine on resistance to organic hydroperoxides

The PotABCD transporter can specifically uptake spermidine in *E. coli* (Kashiwagi *et al*., 1993). It likely serves a similar function in *R. sphaeroides*. *R. sphaeroides* strains were cultured with 10 mM spermidine under aerobic condition. Zone inhibition assays showed that the addition of spermidine caused a higher sensitivity to organic hydroperoxides in the wild type, the SorX deletion strain and the *potA* deletion strain (Fig 4.24A). For all three strains, an increase of ROS level after the addition of spermidine to the culture was observed (Fig 4.24B). The ROS level was increased stronger in the SorX deletion strain in which the *potA* gene showed a higher expression level. However, the ROS level was still increased in the *potA* deletion strain, may due to the existence of other polyamine transporters (e.g. RSP_3515-RSP_3518, RSP_3393-3397) in *R. sphaeroides*. The GSH level was decreased in the wild type after adding spermidine, however, this decrease was not significant in the *potA* deletion strain (Fig 4.24C). These data indicate that high spermidine level causes oxidative stress and lower expression level of *potA* induced by SorX decreases the ROS level in the cell.

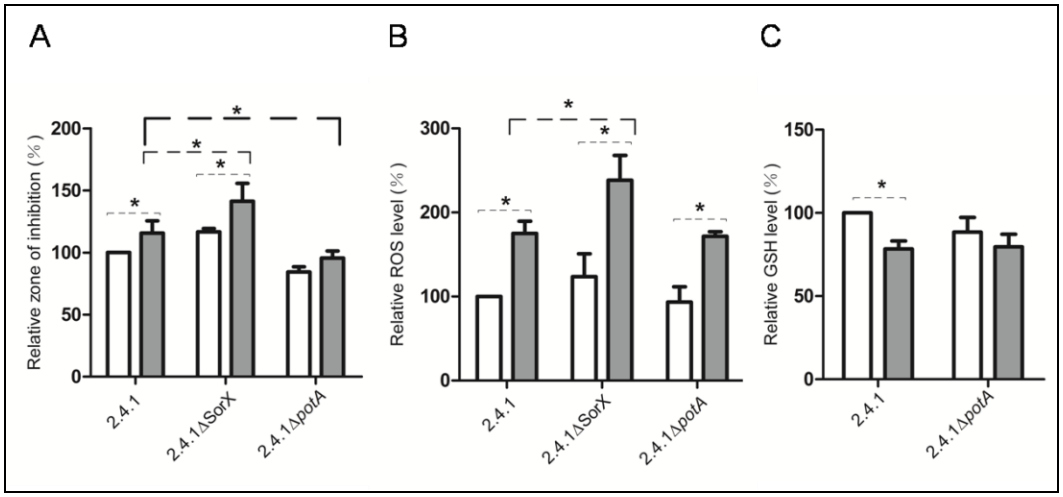

Figure 4.24 (A) Effect of spermidine on cellular reactive oxygen species (ROS) levels. Cultures were grown under aerobic condition and harvested at an OD_{660} of 0.2 to quantify cellular reactive oxygen species (ROS) levels using the oxidation-sensitive fluorescent probe 2,7-dihydrodichlorofluorescein diacetate. The ROS level in wild type 2.4.1 without spermidine served as a control and was set to 100%. (B) Effect of spermidine on resistance to organic hydroperoxides was monitored by zone inhibition assays. The inhibition zone diameter from wild type 2.4.1 without spermidine served as a control and was set to 100%. (C) Effect of spermidine on intracellular GSH levels. The GSH level in wild type 2.4.1 without spermidine served as a control and was set to 100%. The error bars indicate the standard deviation from the mean of biological triplicates with technical replicates. Asterisks indicate a statistically significant change ($p \leq 0.05$). Cultures were untreated (white bars) or treated with 10 mM spermidine (grey bars).

4.3 OmpR increases resistance to organic hydroperoxides

4.3.1 Expression of *ompR* is induced by various stress conditions

The *ompR* (RSP_0847) gene is transcribed from a RpoHI/HII dependent promoter and induced by singlet oxygen (Glaeser *et al*., 2007). It is known that RpoHI/HII dependent promoters are induced by several stress conditions like heat stress, hydrogen peroxide, superoxide, organic hydroperoxides and cadmium chloride (Billenkamp *et al*., 2015). Western blot was used to monitor the levels of the OmpR protein in response to several stress conditions. An antibody raised against the OmpR protein was used for western blot. According to the result, *ompR* expression was moderately induced by organic hdyroperoxides, diamide, heat stress and SDS+EDTA (Fig 4.25). Moreover, the *ompR* mRNA showed higher levels under these stress conditions as determerined by the real time RT-PCR (Fig 4.26). These data suggest that OmpR likely plays an important role in the oxidative stress response in *R. sphaeroides*. In order to analyze the function of OmpR in *R. sphaeroides*, it was attempted to construct an *ompR* deletion strain and a constitutive overexpression strain. Several methods were tried to delete *ompR* from the chromosome in *R. sphaeroides*. However, no *ompR* deletion strain was obtained. This indicates that the *ompR* gene is essential to the cell.

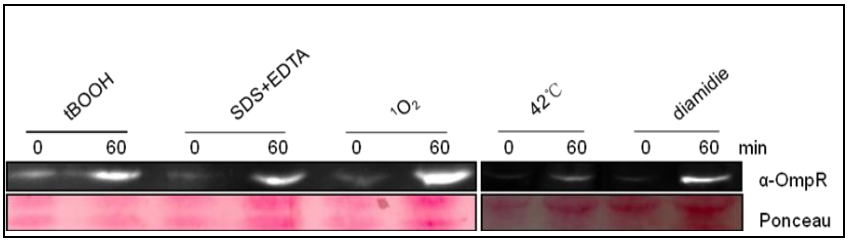

Figure 4.25 OmpR protein levels under various stress conditions as determined by western blot using an OmpR specific antibody. The samples were taken before (0 min) and after 60 min treatment with agents as indicated or after applying heat stress. 50 µg of total protein were applied in each lane. Ponceau staining were used as a loading control.

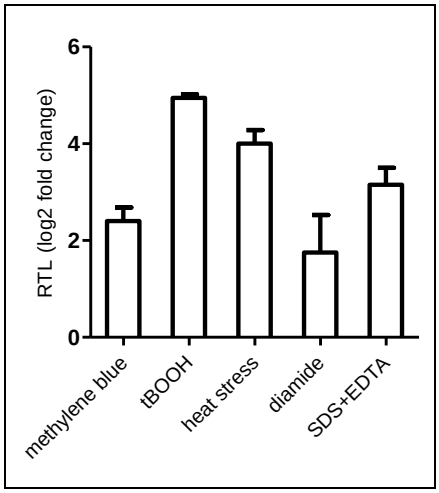

Figure 4.26 *ompR* mRNA levels under various stress conditions as determined by real time RT-PCR. Samples were taken before (0 min) and 7 min after treatment with agents as indicated or after applying heat stress. The gene *rpoZ* was used as internal standard gene for normalization. The relative transcript level (RTL) is given as $\log_2$ fold change after 7 min compared to 0 min. The error bars indicate the standard deviation from the mean of biological triplicates with technical replicates.

4.3.2 Effect of OmpR on resistance to organic hydroperoxides

Since no *ompR* deletion strain was obtained, a flag-tagged OmpR overexpression strain was construced for further co-immunoprecipitation experinment as follows: the *ompR* gene was amplified by PCR using primers 0847flagfbam and 0847flagReco, resulting in a 779 bp fragment. The stop codon of *ompR* was replaced by a sequence encoding 3xFLAG (Uzzau *et al*., 2001) and a newly introduced TGA. The fragment was then subcloned to pJET1.2. Afterwards, the fragment was restricted from pJET1.2 and ligated with pRK4352 containing a 16S promoter. This yielded the plasmid pRKOmpRflag (Fig 4.27). Afterwards, the plasmid was transferred to *R. sphaeroides* 2.4.1 by conjugation.

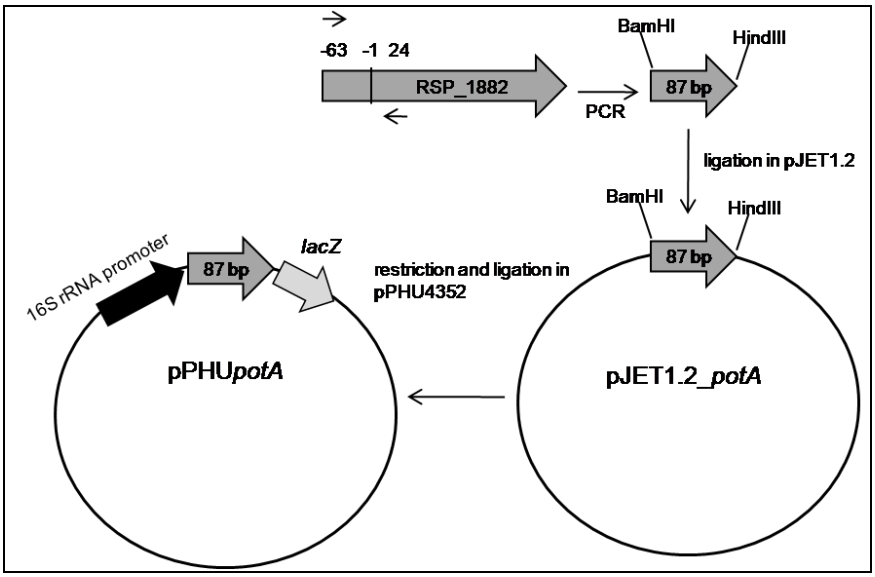

Fig 4.27 Construction of a flag-tagged OmpR overexpression plasmid pRKOmpRflag.

A flag-tagged OmpR overexpression strain (2.4.1pRKOmpRflag) and a wild type strain 2.4.1 containing an empty vector (pRK415) were used for zone inhibition

assays. The results demonstrated that the flag-tagged OmpR overexpression increased resistance to organic hydroperoxides, but had no significant effect on resistance to singlet oxygen (Fig 4.28). This suggests that OmpR is likely involved in response to organic hydroperoxides in *R. sphaeroides*.

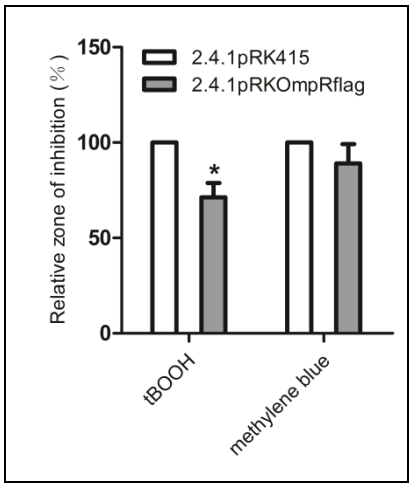

Figure 4.28 Effect of OmpR on resistance to oxiditive stress shown by zone inhibition assays. The wild type harboring an empty vector (pRK415) served as a control and the inhibition zones of control were set to 100%. The error bars indicate the standard deviation from the mean of biological triplicates with technical replicates. Asterisk indicates a statistically significant change ($p \leq 0.05$).

4.3.3 Effect of OmpR on global gene expression

The global transcriptome analysis between the wild type strain containing an empty vector (2.4.1pRK415) and the strain overexpressing OmpR together with pre-SorX strain (2.4.1pRKOmpR-pre-SorX) was performed (performed by Johannes Schwarz). According to the microarray data, 67 mRNAs with a $\log_2$ fold change of >0.6 or < -0.6 are detected (Table 4.2).

Table 4.2 Genes with a $\log_2$ fold change of > 0.6 or < -0.6 were shown according to microarray data.

Gene no	Name	Log2 fold change ratio	Gene Description
RSP_6132	*pqqA*	-2.17	putative coenzyme PQQ synthesis protein A
RSP_4050	*pdhB*	-1.79	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex
RSP_4049	*pdhAb*	-1.50	dihydrolipoamide acetyltransferase
RSP_1020	*glcD*	-1.42	putative glycolate oxidase subunit protein
RSP_0161		-1.21	Spermidine/putrescine-binding periplasmic protein
RSP_1980	*glcB*	-1.21	malate synthase
RSP_2877	*coxL*	-1.10	Putative carbon monoxide dehydrogenase large chain
RSP_0910	*dctP*	-1.10	TRAP-T family transporter, C4-dicarboxylate-binding protein DctP
RSP_2878	*coxS*	-1.04	Putative carbon-monoxide dehydrogenase small chain
RSP_0911	*dctQ*	-1.01	TRAP-T family C4-dicarboxylate transporter, DctQ (4TMs) subunit
RSP_0097	*smoM*	-1.00	TRAP-T family sorbitol/mannitol transporter, periplasmic binding protein, SmoM
RSP_3106		-0.98	Cation efflux transporter, CDF family
RSP_2585		-0.96	hypothetical protein
RSP_0912	*dctM*	-0.92	TRAP-T family C4-dicarboxylate transporter, DctM (12TMs) subunit
RSP_2876		-0.92	putative carbon monoxide dehydrogenase medium chain

RSP_0829	*lctB*	-0.91	Lactate dehydrogenase
RSP_2968		-0.91	dihydrolipoamide dehydrogenase
RSP_2400		-0.88	ABC putrescine transporter, ATPase subunit
RSP_3622		-0.87	hypothetical protein
RSP_2410	*rpoH1*	-0.85	RNA polymerase sigma factor
RSP_6025		-0.83	hypothetical protein
RSP_1951		-0.80	hypothetical protein
RSP_1140	*ilvE*	-0.79	branched-chain amino acid aminotransferase
RSP_1613		-0.78	TRAP-T family transporter, DctP subunit
RSP_3372		-0.78	TRAP-T family transporter, periplasmic binding protein
RSP_0346		-0.78	ABC transporter, substrate binding protein
RSP_1593		-0.77	phosphate acetyltransferase
RSP_3323		-0.76	Putative flavoprotein containing monooxygenase involved in K^+ transport
RSP_2397		-0.75	ABC putrescine transporter, periplasmic substrate-binding subunit
RSP_2578	*xoxF*	-0.74	putative pqq dehydrogenase protein
RSP_2879		-0.74	hypothetical protein
RSP_3732		-0.73	ABC sugar transporter, periplasmic binding protein
RSP_0381		-0.72	hypothetical protein
RSP_3620		-0.71	Cold-shock DNA-binding protein
RSP_2579	*cycB*	-0.71	cytochrome c553i
RSP_0372		-0.69	ABC basic amino acid transporter, solute-binding protein
RSP_1883		-0.69	ABC polyamine/opine transporter, periplasmic substrate-binding protein
RSP_1747	*bztA*	-0.69	ABC glutamate/glutamine/aspartate/asparagine transporter, periplasmic substrate-binding protein
RSP_2401		-0.68	putative 6-aminohexanoate-cyclic-dimer hydrolase
RSP_0091	*takP*	-0.68	ABC sorbitol/mannitol transporter, periplasmic binding protein
RSP_4047	*pdhAa*	-0.66	Pyruvate dehydrogenase E1 component, alpha subunit
RSP_0842		-0.66	putative porin
RSP_3297		-0.66	ABC branched-chain amino acid transporter, periplasmic binding protein
RSP_1889		-0.66	hypothetical protein
RSP_1019		-0.66	putative glycolate oxidase subunit protein
RSP_2399		-0.65	ABC putrescine transporter, inner membrane subunit
RSP_3295		-0.65	ABC branched-chain amino acid transporter, ATPase subunit
RSP_1913		-0.64	3-oxoadipate CoA-transferase, beta subunit
RSP_3621		-0.63	Cold-shock DNA-binding protein
RSP_3105		-0.61	PAS sensor protein
RSP_0995	*phaE*	-0.60	pH adaption potassium efflux system, PhaE subunit
RSP_4193		0.61	hypothetical protein
RSP_2359		0.61	hypothetical protein
RSP_6108	*pufB*	0.61	LHI beta, Light-harvesting B875 subunit
RSP_1352		0.61	D-3-phosphoglycerate dehydrogenase
RSP_2805	*nemA*	0.65	Putative NADH-flavin oxidoreductase
RSP_0906	*sitC*	0.65	ABC Mn^{+2}/Fe^{+2} transporter, inner membrane subunit SitC
RSP_1943		0.68	hypothetical protein
RSP_2357		0.70	hypothetical protein
RSP_1944		0.74	Uroporphiryn-III C-methyltransferase/siroheme synthase

RSP_2358		0.75	possible phage phi-C31 gp36-like protein/Major capsid protein, HK97 family
RSP_1351	*serC*	0.77	phosphoserine aminotransferase
RSP_4209		0.83	Acyltransferase 3 family
RSP_3117		0.83	hypothetical protein
RSP_6037		0.84	hypothetical protein
RSP_0904	*sitA*	0.96	ABC Mn^{+2}/Fe^{+2} transporter, periplasmic substrate-binding protein SitA
RSP_1409		0.97	Beta-Ig-H3/Fasciclin
RSP_3509	*expE1*	1.11	Hemolysin-type calcium-binding region, RTX
RSP_0557		1.96	hypothetical protein

In order to identify the genes regulated by OmpR, the $\log_2$ fold changes in transcript levels between overexpression of OmpR together with pre-SorX (2.4.1pRKOmpR-pre-SorX) and overexpression of pre-SorX (pRKSorX^{144}) were compared. Based on the $\log_2$ fold changes shown in two microarray data, the genes under comparison were divided into three groups (Fig 4.28). If the genes with $\log_2$ fold change of > 0.6 or < -0.6 are both detected by overexpressing OmpR together with pre-SorX and overexpressing pre-SorX, this indicates that these genes are only regulated by SorX, not by OmpR (Fig 4.28A). If the genes with $\log_2$ fold change of > 0.6 or < -0.6 are only detected by overexpressing pre-SorX, this indicates these genes are regulated by SorX, but expression levels of these genes are not affected by overexpressing OmpR together with pre-SorX indicating that OmpR likely has an opposite effect on expressions of these genes compared to SorX. This supports the view that the genes are both regulated by SorX and OmpR (Fig 4.28B). If the genes with $\log_2$ fold change of > 0.6 or < -0.6 are only detected by overexpressing OmpR together with pre-SorX, this indicates that the genes are only regulated by OmpR (Fig 4.28C). To prove whether this strategy is conceivable, some genes putatively regulated by OmpR (Fig 4.28C) were selected and the mRNA levels of these genes were qualified by real time RT-PCR in the flag-tagged OmpR overexpression strain (Fig 4.29). The result showed that the mRNA level of RSP_6037 was increased by the flag-tagged OmpR overexpression, while RSP_2410 (*rpoHI*), RSP_4209, RSP_3106 and RSP_1409 showed lower expression levels in the flag-tagged OmpR overexpression strain. The real time RT-PCR confirmed that these genes are regulated by OmpR. Among these genes, we were mostly interested in RSP_6037 and *rpoHI*, since RSP_6037 is transcribed with CcsR1-4 and *rpoHI* encodes a sigma factor involved in stress response in *R. sphaeroides* (Nuss *et al*., 2010).

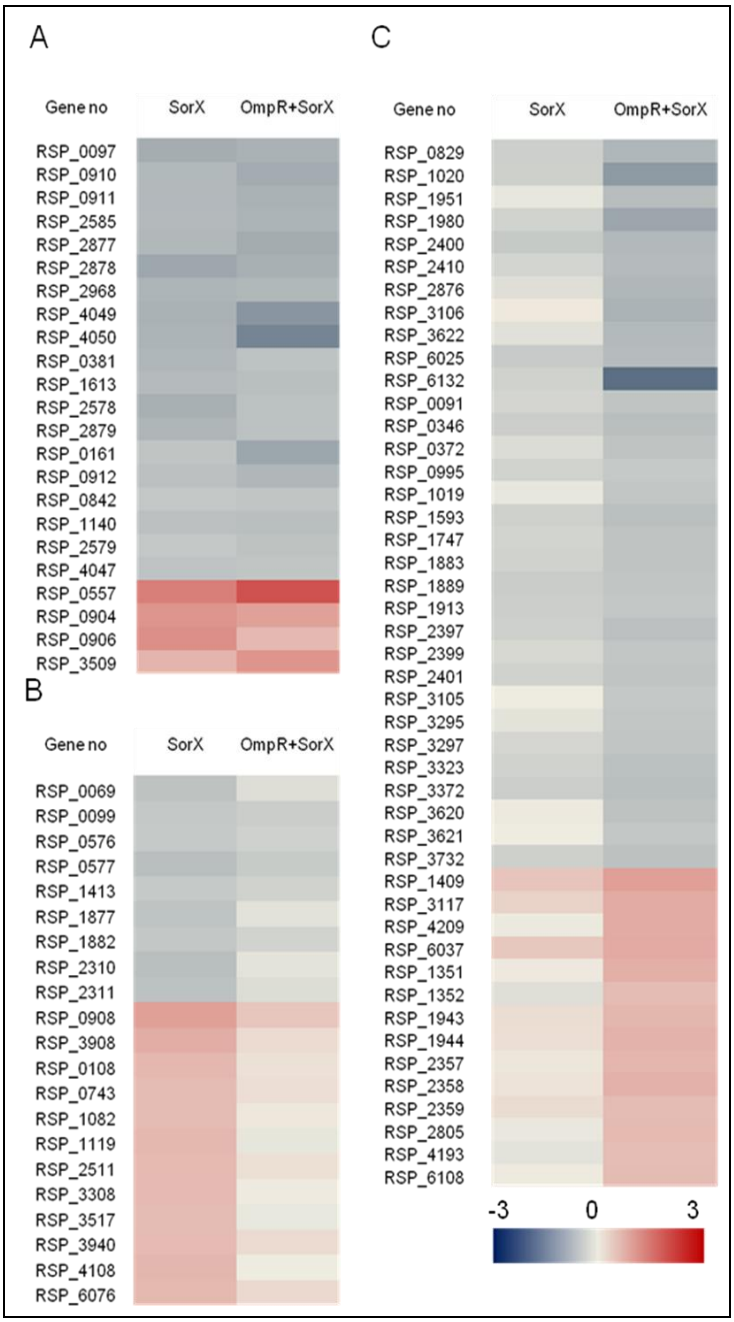

Figure 4.28 Comparison of log_2 fold changes in transcript levels between OmpR together with pre-SorX overexpression (pRKOmpR-pre-SorX) and pre-SorX overexpression (pRKSorX^{144}) in *R. sphaeroides*. The genes selected under comparison were divided to three groups according to the log_2 fold change. (A) The genes with log_2 fold change of > 0.6 or < -0.6 are detected both by overexpressing OmpR together with pre-SorX and overexpressing pre-SorX. (B) The genes with log_2 fold change of > 0.6 or < -0.6 are only detected by overexpression of pre-SorX. (C) The genes with log_2 fold change of > 0.6 or < -0.6 are only detected by overexpressing OmpR together with pre-SorX.

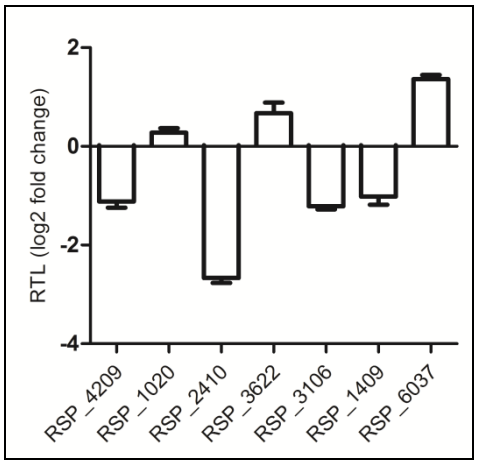

Figure 4.29 The mRNA levels of genes regulated by OmpR were determined by real time RT-PCR. Samples were taken at 7 min after treatment with 100 mM tBOOH in the flag-tagged OmpR overexpression strain and compared to that in wild type containing an empty vector (pRK415) under the same condition. The gene *rpoZ* was used as standard gene for normalization. The error bars indicate the standard deviation from the mean of biological triplicates with technical replicates.

4.3.4 Effect of OmpR on the promoter activities of RSP_6037 and *rpoHI*

RSP_6037 and *rpoHI* are putative targets of OmpR according to comparison of the microarray data and to real time RT-PCR results. The first question is whether OmpR is able to regulate the promoter activities of RSP_6037 and *rpoHI*. A ~200 bp fragment before the start codon of RSP_6037 or *rpoHI* (RSP_2410) was fused to *ecfp* to obtain the transcriptional reporter plasmid pBE::P_{RSP_6037}:eCFP or pBE::P_{RpoHI}:eCFP. The reporter plasmid was transferred to the flag-tagged OmpR overexpression (2.4.1pRKOmpRflag) strain and to wild type 2.4.1 containing an empty vector control (pRK415) to measure the fluorescence activities. We could prove that the promoter activity of RSP_6037 was up-regulated by the flag-tagged OmpR overexpression (Fig 4.30A). Moreover, the sRNA CcsR1 transcribed with the RSP_6037 gene was accumulated by overexpressing the flag-tagged OmpR (Fig 4.31). While the *rpoHI* promoter activity was decreased (Fig 4.30B). These data support that RSP_6037 and *rpoHI* are the targets of OmpR.

Moreover, the promoter of *ompR* was fused to *ecfp* to obtain the reporter plasmid pBE::P_{OmpR}:eCFP, then the plasmid was transferred to the flag-tagged OmpR overexpression (2.4.1pRKOmpRflag) strain and to wild type 2.4.1 containing an empty vector control (pRK415) to measure the fluorescence activities. OmpR promopter activity was down-regulated by the flag-tagged OmpR overexpression (Fig 4.30C). This supports the view that OmpR is able to regulate its own expression.

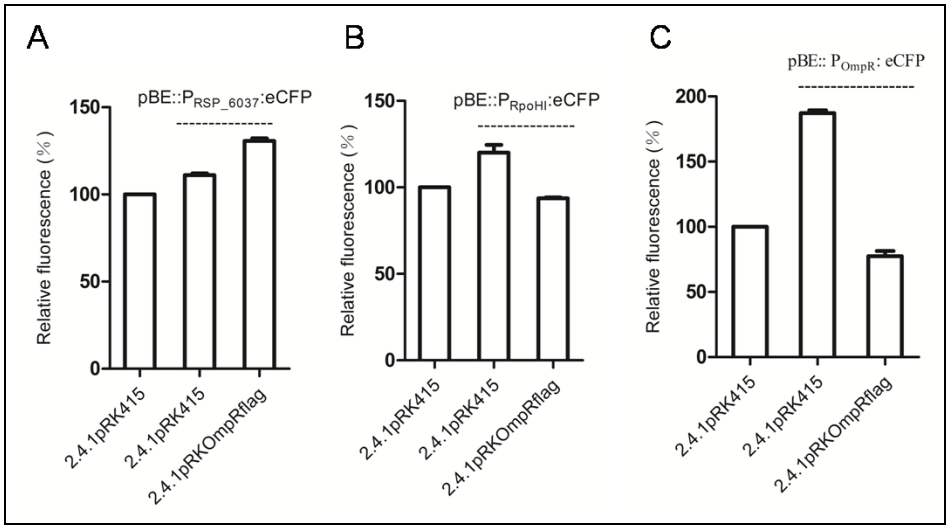

Figure 4.30 Effect of OmpR on the promoter activities of RSP_6037 (A), *rpoHI* (B) and *ompR* (C). The promoter regions were transcriptionally fused to *ecfp* to determine promoter activities. Fluorescence intensities were calculated relative to wild type 2.4.1 carrying the empty vector pRK4352 (set to 100%). The error bars indicate the standard deviation from the mean of biological triplicates with three technical replicates.

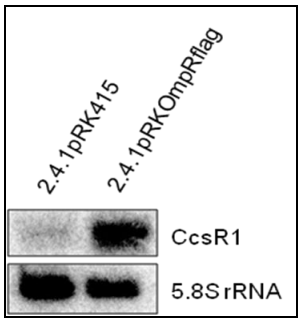

Figure 4.31 Effect of OmpR on expression level of CcsR1. Total RNA was isolated from each strain 7 min after adding 100 mM tBOOH and used for Northern blot analysis. 5.8S rRNA was used as a loading control.

5 Discussion

5.1 The role of CcsR1-4 in response to oxidative stress

Non-coding sRNAs are regulators which mostly influence gene expression at the post-transcriptional level. Most bacterial sRNAs are involved in stress responses. Since *R. sphaeroides* has been the model for studying the singlet oxygen stress response, several sRNAs have been identified under singlet oxygen stress (Berghoff *et al*., 2009). CcsR1–4, which are transcribed with RSP_6037 encoding a hypothetical protein, are four homologous sRNAs. Several tandem duplicate sRNAs are also found in other bacteria, such as PrrF1 and PrrF2 which are involved in iron homeostasis in *Pseudomonas aeruginosa* (Wilderman *et al*., 2004). However, for CcsR1–4, the sRNAs are derived from the 3' UTR of the upstream gene RSP_6037. 3' UTR-derived sRNAs are also identified in other bacteria (Chao *et al*., 2012; Kawano *et al*., 2005). Based on their biogenesis, 3' UTR derived sRNAs have been divided into two major types: Type I in which the sRNAs are expressed from a promoter inside the mRNA coding sequence or the 3′ UTR of the mRNA; Type II in which sRNAs are processed from the parental mRNA (Miyakoshi *et al*., 2015). CcsR1-4 belong to the Type II 3' UTR derived sRNAs.

CcsR1-4 are transcribed from a RpoHI/RpoHII dependent promoter and induced by ROS, such as, singlet oxygen, organic hdyroperoxides, superoxide and hdyroperoxides. Overexpression of CcsR1-4 enhanced resistance to oxidative stresses. This suggests that CcsR1-4 are likely involved in response to oxidative stresses in *R. sphaeroides*. A single copy of CcsR is sufficient for enhancing resistance to oxidative stresses, but all four sRNAs are needed for maximum effect indicating that the four copies of CcsR1-4 possibly show functional redundancy. They can bind to the same target mRNA and thereby increase the efficiency of regulation. This is also true for the sRNAs OmrA and OmrB which have the similar sequence at their 5′ and 3′ ends. They both can regulate several genes encoding multiple outer membrane proteins in *E. coli* (Guillier and Gottesman, 2006).

In order to search for the target genes of CcsR1-4, a transcriptome analysis was performed. Most affected genes showed lower expression levels in the CcsR1-4 overexpression strain. This is in agreement with the fact that most of sRNAs negatively regulate gene expression in the cell (Waters and Storz, 2009). Most genes regulated by CcsR1-4 are related to C1 metabolism, contribute to C1 metabolism by

cofactor synthesis or encode subunits of the pyruvate dehydrogenase complex. Combining the transcriptome analysis with the prediction software tool IntaRNA, the *flhR* mRNA was identified as a putative target and proved to directly interact with CcsR1-4 by an *in vivo lacZ* reporter system and gel shift assay. Two stem-loops each with a CCUCCUCCC sequence are located in the each CcsR sRNA (Fig 5.1). It was shown that CcsR1-4 use the aSD sequence CCUCC to target an AG-rich sequence in the upstream of the actual SD sequence within the *flhR* mRNA (Billenkamp *et al.*, 2015). CcsR1-4 belong to the "cuckoo family" of RNAs which consist of 2 to 4 adjacent hairpins in the secondary structure. Each hairpin loop carries the same motif, CCUCCUCCC. The sRNAs of the "cuckoo family" are highly conserved among Alphaproteobacteria (Reinkensmeier and Giegerich, 2015). The "cuckoo family" sRNAs identified in *Sinorhizobium meliloti* are involved in heat stress response (Schlüter *et al.*, 2010).

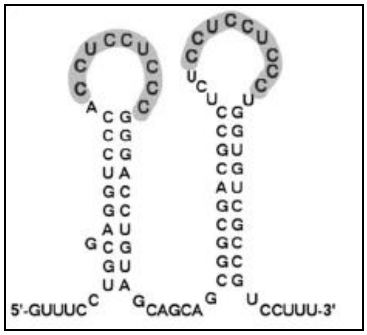

Figure 5.1 The secondary structure of CcsR1 (Billenkamp *et al.*, 2015)**.** The CCUCCUCCC sequences are highlighted.

The *flhR* mRNA encodes a transcriptional activator belonging to the LuxR family. It can activate the transcription of *adhI* and *cycI* which have important functions in GSH-dependent formaldehyde detoxification upon exposure to formaldehyde and methylated compounds. It was known that AdhI like class III alcohol dehydrogenases are involved in oxidative stress response in *E. coli* (Echave *et al.*, 2003). Moreover, the *cycB-xoxJ* operon and *fdhA* mRNA which are involved in methanol and formaldehyde oxidation are under regulation of a FlhR homologue in *Paracoccus denitrificans* (Harms *et al*., 2001).

Most genes regulated by CcsR1-4 are related to C1 metabolism and C1 metabolism was indirectly affected by CcsR1-4 by targeting *flhR*. Interestingly, it was shown that all of the subunits of the pyruvate dehydrogenase complex were down-regulated by overexpression of CcsR1–4 and deletion of *flhR*, however, there is no direct interaction between the *pdhB* mRNA and CcsR1–4. This indicates that the pyruvate dehydrogenase complex gene *pdhB* is under control of FlhR.

C1 metabolism is repressed by CcsR1-4, but how is C1 metabolism related to oxidative stress? First, a *pqqA* gene which encodes a peptide as the precursor of PQQ is regulated by CcsR1-4. PQQ is an important redox cofactor in Gram-negative bacteria and is functionally related to C1 metabolism (Anthony, 2001). Down-regulation of *pqqA*, results in decreased stimulation of C1 metabolism. C1 metabolism is also repressed by CcsR1-4 directly binding to *flhR* mRNA. Since the respiratory electron transport chain is the main source of ROS, down-regulation of PqqA and FlhR by CcsR1-4 resulting in decrease stimulation of C1 metabolism is beneficial to the cell under oxidative stress conditions (Fig 5.1A). Secondly, a GSH dependent AdhI pathway is affected by CcsR1–4. The GSH-dependent AdhI pathway can detoxify the reactive aldehydes produced by oxidative protein carbonylation under oxidative stresses. The *flhR* gene expression is induced by reactive aldehydes and the FlhR protein activates the expression of genes related to the AdhI pathway. Since GSH is used to detoxify the aldehydes, less GSH is available for the protein oxidation repair mechanism. CcsR1–4 are induced by oxidative stress. They target the *flhR* mRNA and repress its translation, resulting in repression of the AdhI pathway. The repression of the GSH-dependent AdhI pathway by CcsR1-4 leads to allocation of GSH to protein repair in concert with the thioredoxin system, which can prevent the toxic effect of accumulating aldehydes. Therefore, under oxidative stress condition, expression of *flhR* regulated by CcsR1–4 causes allocation of GSH to different cellular mechanisms which protect the oxidative damage (Fig 5.1B).

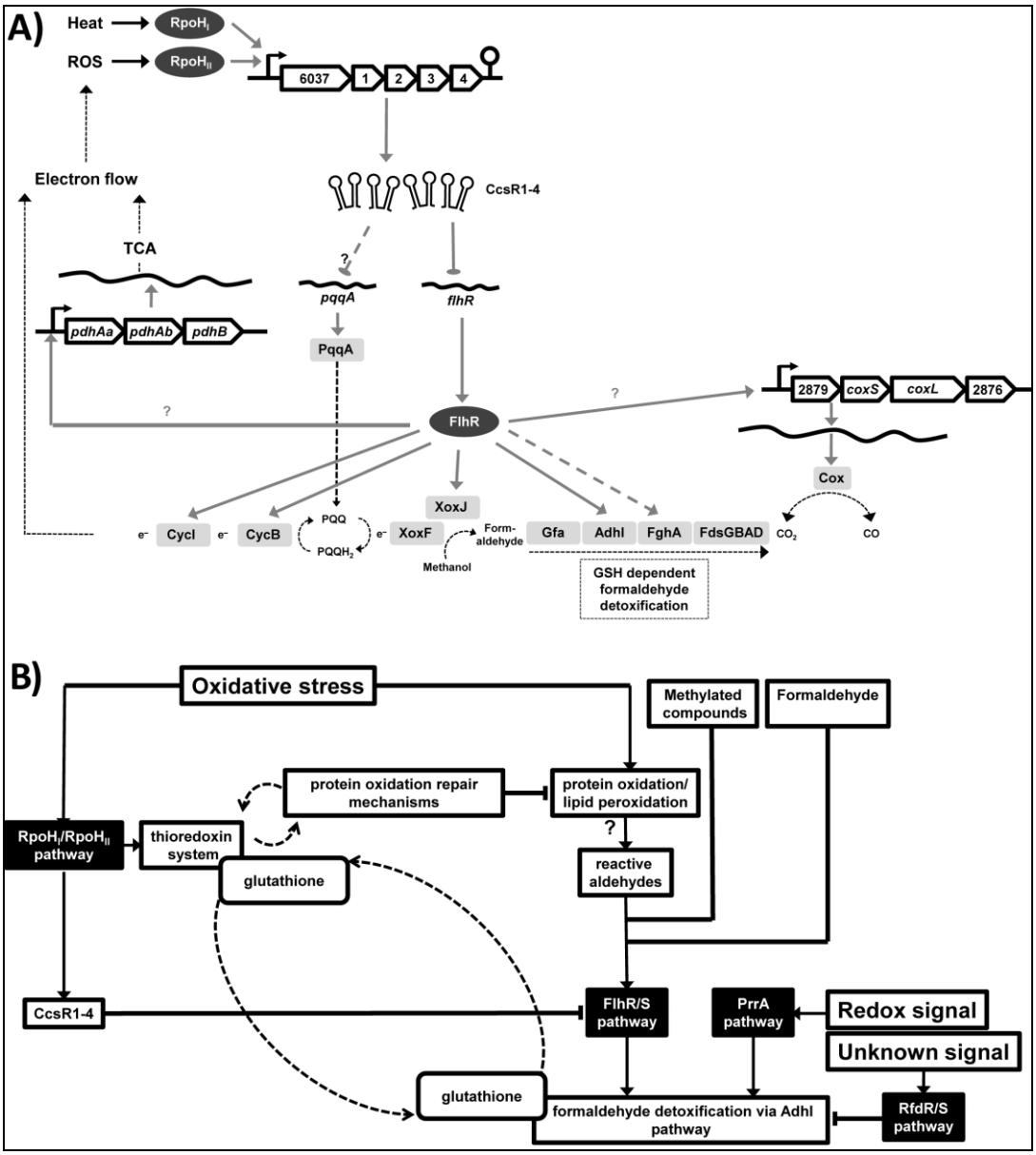

Figure 5. 2 Metabolic network of CcsR1-4-dependent targets and effect on C1 metabolism from: Billenkamp *et al*., 2015**.** (A) CcsR1-4 are induced by oxidative stersses and repress *flhR* expression directly, resulting in repression of several genes related to C1 metabolism and electron transport. Moreover, *pqqA* expression is regulated by CcsR1–4 through an unknown mechanism. FlhR regulates *adhI* and *cycI* in *R. sphaeroides*. *cycB* and *fghA* are regulated by FlhR. Moreover, *xoxJ* is located in an operon together with *cycB* in *R. sphaeroides*. Furthermore, *xoxJ* and *cycB* also show FlhR dependence in *R. sphaeroides* and a dependence of the pyruvate dehydrogenase complex gene *pdhB* is under control of FlhR. (B) Under oxidative stress condition, the protein oxidation repair mechanism can prevent the toxic effect of accumulating aldehydes, since reactive aldehydes are produced by carbonylation of oxidative protein. Reactive aldehydes activate *flhR* expression. Moreover, the AdhI pathway is also regulated by PrrA and the response regulator RfdR (repressor of formaldehyde dehydrogenase). CcsR1-4 are induced by oxidative stresses and they can target *flhR* mRNA, resulting in repression of GSH-dependent AdhI pathway. Through the effects of the CcsR1-4, fine-turning of GSH allocation is achieved.

5.2 The role of SorX in response to oxidative stress

SorX is transcribed with the upstream gene RSP_0847 which encodes a homologue of the response regulator OmpR from a RpoHI/RpoHII dependent promoter. SorX is derived from the 3' UTR of the *ompR* transcript by RNAse E cleavage. This is in agreement with the fact that RNAse E is often involved in sRNAs processing and decay (Moll *et al.*, 2003). SorX is induced by oxidative stresses, caused by singlet oxygen and orgnic hydroperoxides. Overexpression and deletion of SorX affected resistance to organic hydroperoxides and singlet oxygen. The results showed that the 75 nt fragment SorX is sufficient for enhanced resistance to organic hydroperoxides and singlet oxygen. The *sorX* gene is considerably conserved among the *Rhodobacteraceae* family. Moreover, synteny analysis further demonstrated that *sorX* is generally located at 3' UTR of the gene which encodes a two-component transcriptional regulator (Fig 5.3). One example is the OmpR like regulator RSP_0847 in *R. sphaeroides*. A SorX homologous sRNA was found in *Roseobacter denitrificans*. It was shown that the 71 nt SorX homologue is induced by singlet oxygen and co-transcribed with the upstream gene *mtrA* which encodes a OmpR homologous protein (Berghoff *et al.*, 2011b). This indicates that the RSP_0847-SorX locus likely has a conserved function in Alphaproteobacteria.

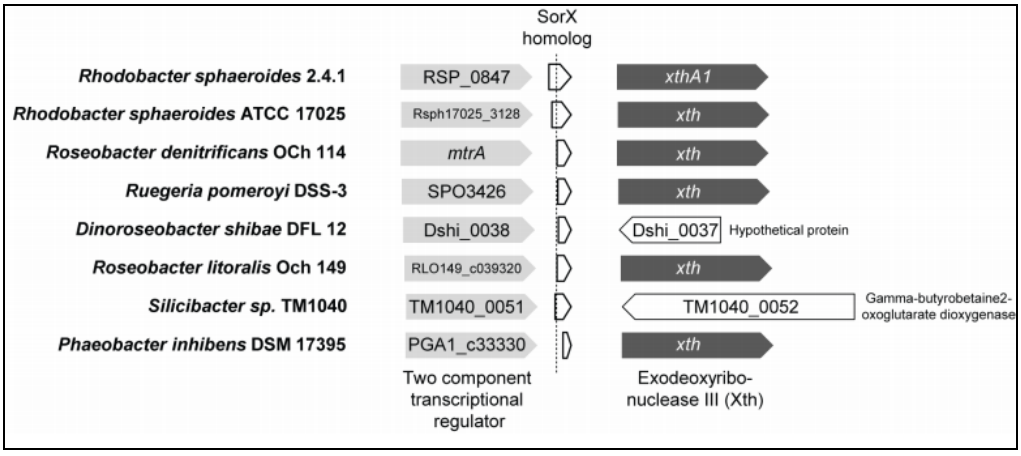

Figure 5.3 Synteny analysis of the *sorX* locus within the *Rhodobacteraceae* family was provided by Bork A. Berghoff (Peng *et al.*, 2016). Light grey arrows imply the gene encoding a two-component transcriptional regulator which is located in the upstream of *sorX*. The dashed line implies the RNase E-dependent processing site of SorX in *R. sphaeroides* 2.4.1. The gene encoding an Exodeoxyribonuclease III (dark grey arrows) is located in the downstream of *sorX* in several cases.

It was predicted that there is a FnrL binding site partly internal to RSP_0847 based on ChIP-seq data (Imam *et al.*, 2014). Oxygen shift assays confirmed that FnrL has a

clear effect on the induction of SorX. This indicates that SorX likely has more than one biogenesis pathway. Since FnrL is an anaerobic regulator and regulates photosynthesis gene expression, it is also possible that SorX is involved in the regulation of photosynthesis genes in *R. sphaeroides*.

There are two sRNAs SorY and CcsR1-4 which have a reported role of responding to oxidative stresses (Billenkamp *et al.*, 2015; Adnan *et al.*, 2015). They were up-regulated by overexpression of SorX. Several sRNAs working together in a regulatory network have also been found in other bacteria (Beisel and Storz, 2010). Our data revealed that SorX indeed affects the rate of transcription from the RpoHI/HII promoters upstream of RSP_6037-CcsR1-4 and SorY, but there are no direct interactions between the promoter regions and SorX indicating they are likely indirectly regulated by SorX. The exact mechanism needs further elucidation.

In order to search for the direct target of SorX, combining the transcriptome data with the bioinformatic predictions software tool IntaRNA, a *potA* mRNA encoding a subunit of polyamine transporter was identified and proved to directly bind to SorX. An anti-SD (aSD) sequence is located between the two stem-loops in the 3' fragment of SorX (Fig 5.4) and the aSD is used to target the SD sequence of *potA* mRNA. This mechanism is also found in other bacteria (Storz and Vogel, 2011). In *R. sphaeroides*, SorY can use the aSD sequence to target the SD sequence of *takP* encoding a TRAP-T transporter (Adnan *et al.*, 2015). Interestingly, there are some homologous features shared by SorX and SorY. They share almost identical aSD sequences and some neighbouring nucleotides (Fig 5.5). It was assumed that they may have an overlapping regulon.

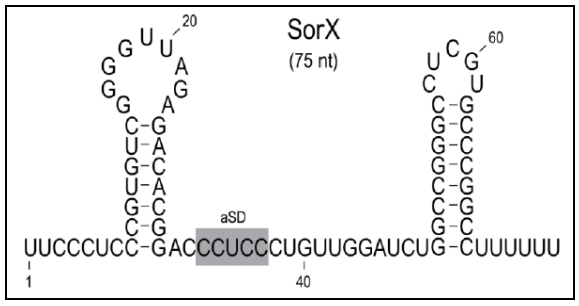

Figure 5.4 The secondary structure of SorX was provided by Bork A. Berghoff (Peng *et al.*, 2016). The aSD sequence is highlighted.

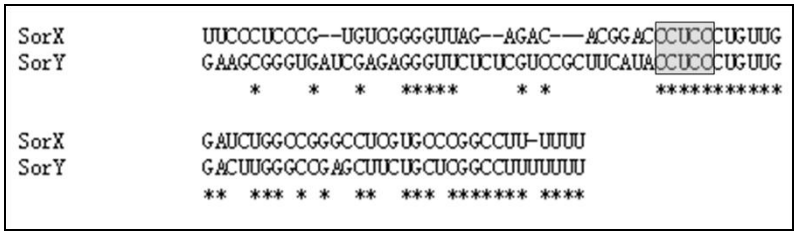

Figure 5.5 Alignment of the SorX and SorY. The aSD sequence is highlighted.

Here the *potA* mRNA was verified as a direct target of SorX. It is the first gene located in the *potADBC* operon. Furthermore, *potD* is also regulated by SorX, but there is no direct interaction between SorX and *potD*. This indicates that the whole transcript is affected by regulating *potA*. In *E. coli*, the proteins encoded by the *pot* operon are related to the uptake of polyamines (Igarashi and Kashiwagi, 1999). Polyamines are necessary for cell growth. In the cell, the polyamine content is regulated by biosynthesis, degradation and transport. Polyamine uptake is energy-dependent and several polyamine uptake systems exist in *E. coli* (Igarashi and Kashiwagi, 1999).

The *potA* mRNA showed a lower expression level in *R. sphaeroides* wild type under singlet oxygen stress. Moreover, it was shown that the *potA* mRNA was down-regulated under metal stress condition in *R. sphaeroides* (Volpicella *et al*., 2014). Interestingly. the *potA* deletion strain showed increased resistance to singlet oxygen and organic hydroperoxides when compared to the parental strain. These data suggest that less *potA* expression under stress condition is beneficial to the cell. PotABCD can uptake the polyamine, especially the spermidine in *E. coli* (Igarashi and Kashiwagi, 1999). Several functions of polyamine have been revealed. Polyamines are used as ROS scavengers, acid tolerance factor and chemical chaperone in the cell. Polyamines can influence gene regulation by stimulating translation of various mRNAs which contain weak or inefficient SD sequences in *E. coli* (Yoshida *et al*., 2004). Polyamines activate the expression of the SOS response of the ColE7 operon and repress expression of genes which are essential for colicin uptake. This causes a survival advantage on colicin-producing *E. coli* under stress conditions (Pan *et al*., 2006). Furthermore, in *E. coli*, an increased paraquat toxicity was observed in the strain defective in the biosynthesis of spermidine. This indicates that polyamines can protect *E. coli* cells from the toxic effect of the superoxide generated by paraquat (Minton *et al*., 1990). Moreover, glutathionylspermidine (Gsp) is synthesized from GSH and spermidine by Gsp synthetase/amidase. GSP is used for protein modification. The level of Gsp-modified proteins increases by oxidative stress

in *E. coli* (Chiang *et al.*, 2010). Furthermore, Gsp is used as thiol cofactor to convert toxic methylglyoxal produced under oxidative stress to non-toxic D-lactate in presence of Ni^{2+} ions by glyoxalase I and II in *E. coli* (Kadia *et al.*, 2012). When *Acinetobacter baumannii* was grown with polyamines, glutathione export was induced by polyamines, resulting in decreased level of intracellular glutathione (Kwon *et al.*, 2013).

If *R. sphaeroides* was cultured with spermidine, an increased affinity of the transcriptional regulator PrrA to DNA was shown (Eraso and Kaplan, 2009). PrrA is involved in regulation of photosynthesis gene expression, thus an induction of the photosynthetic apparatus by spermidine under aerobic conditions was oberseved. However, in this study, the effect of spermidine on the formation of photosynthetic complexes was not observed (data not shown). However it was shown that the ROS level was increased in *R. sphaeroides* strains after adding the spermidine to cultures grown in aerobic condition. This suggests that high spermidine level causes stress to the cell. Considering this, less spermidine uptake under oxidative stress conditions would help to decrease ROS level due to down-regulation of the Pot system by SorX (Fig 5.6).

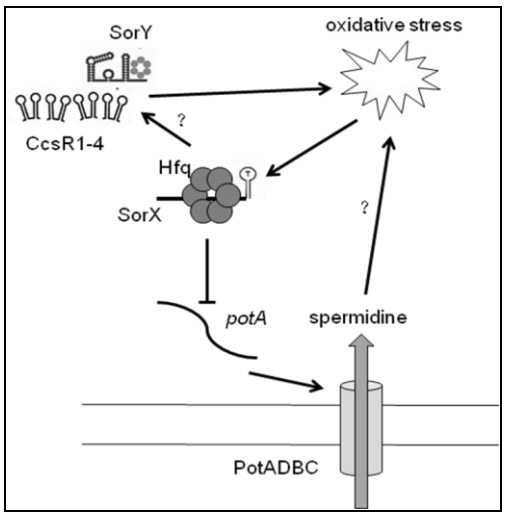

Figure 5.6 The function of SorX under oxidative stress condition. SorX is induced by oxidative stresses. Two sRNAs SorY and CcsR1-4 which have the function to defend oxidative stress are up-regulated by SorX by an unknown mechanism. Moreover, the polyamine transporter PotA is negatively regulated by SorX. This decreses spermidine level in the cell in case high spermidine level increses the ROS level.

5.3 The role of an OmpR like response regulator in the oxidative stress response

Two component systems are signal pathways used to adapt to the changing environmental conditions in bacteria. A homologue of the OmpR response regulator was indentified in *R. sphaeroides* (Glasser *et al*., 2007). In *E.coli*, the OmpR protein regulates the outer membrane proteins OmpC and OmpF (Mattison *et al*., 2002). However, no OmpC and OmpF homologues are identified in *R. sphaeroides*. This suggests that OmpR likely has a different function in *R. sphaeroides*. In the pathogen *Yersinia enterocolitica*, an OmpR like response regulator regulates a variety of virulence genes (Brzóstkowska *et al.,* 2012).

ompR is transcribed from a promoter dependent on RpoHI/HII and is induced by oxidative stresses. Overexpression of a flag-tagged OmpR enhanced resistance to organic hydroperoxides. This indicates that OmpR likely plays an important role in response to organic hydroperoxides in *R. sphaeroides*. To serch for the genes regulated by OmpR, the putative genes were selected by comparison of the $\log_2$ fold changes on transcript levels (microarrays) between a strain overexpressing OmpR together with pre-SorX (2.4.1pRKOmpR-pre-SorX) and a strain only overexpressing pre-SorX (pRKSorX^{144}). Among all the genes regulated by OmpR, a *rpoHI* mRNA encoding a sigma factor is down-regulated by OmpR. RpoHI is involved in stress response in *R. sphaeroides* and has an important function in heat stress response (Nuss *et al*., 2010). Moreover, *ompR* is down-regulated by itself. Interestingly, some genes encoding another Pot polyamine uptake system (RSP_2397 and RSP_2399) are down-regulated by OmpR. It was shown in this study that *potA* mRNA encoding a subunit of polyamine transporter is down-regulated by SorX under oxidative stress conditions. Less polyamine uptake decreases the ROS level in *R. sphaeroides*. It is possible that OmpR down-regulates another polyamine transporter to decrease the ROS level under stress conditions.

SorX is generated from the 3' UTR of the *ompR-sorX* transcript. It was shown that a 3' UTR small RNA CpxQ is derived from the coding arm *cpxP* which is involved in the Cpx response in enterobacteria (Chao and Vogel, 2016). For the *ompR-sorX* transcript, the coding arm *ompR* encodes a response regulator. Overexpression of OmpR enhanced resistance to organic hydroperoxides. Some genes are regulated by OmpR including the genes encoding another polyamine uptake system which may be involved in oxidative stress response (Fig 5.7). The non-coding arm SorX is also

involved in response to oxidative stresses by regulating the *potA* mRNA and the sRNAs CcsR1-4 and SorY. Moreover, there may be an overlap between the SorX regulon and the SorY regulon (Fig 5.7). Interestingly, SorX and OmpR share an overlapping regulon, such as the RSP_6037-CcsR1-4 operon is regulated both by OmpR and SorX (Fig 5.7). All these data indicate that the coding arm *ompR* and the non-coding arm SorX cooperate together to respond to oxidative stresses in *R. sphaeroides.*

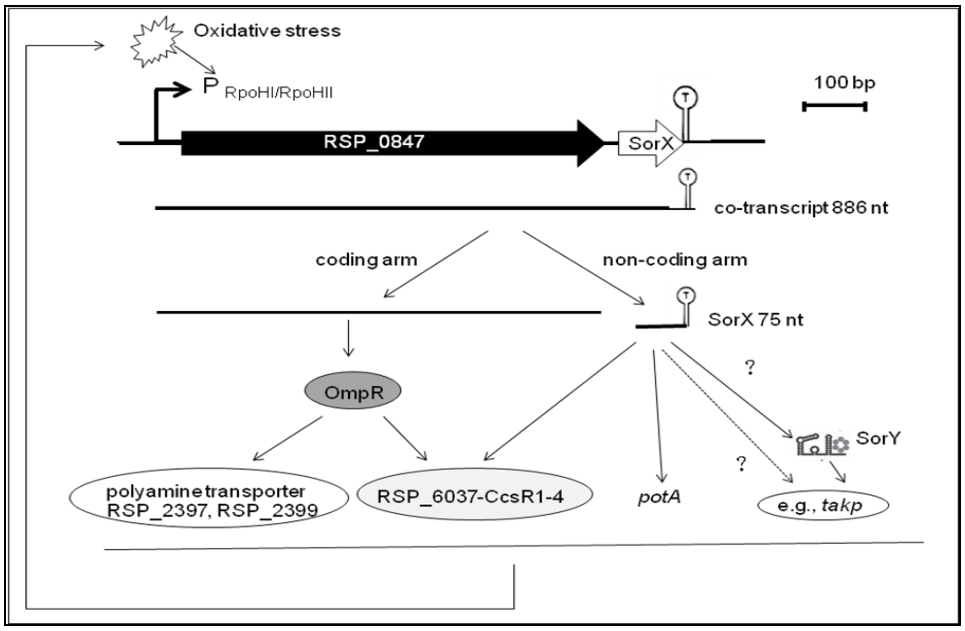

Figure 5.7 The function of the *ompR-sorX* transcript under oxidative stress condition. Under oxidative stress condition, the *ompR-sorX* transcript is induced from a RpoHI/II dependent promoter. SorX post-transcriptionally reduces the expression of the *potA* mRNA. The *ompR* mRNA as coding arm is translated to a response regulator. OmpR is able to regulate multiple genes expression including another Pot polyamine transporter. There is an overlap between the OmpR regulon and the SorX regulon including the RSP_6037-CcsR1-4 operon.

5.4 Perspectives

It was shown that SorX is able to indirectly up-regulate the promoter activities of RSP_6037-CcsR1-4 and SorY, but the mechanism is still unknown. The -200 to -105 region in respect to the start codon of RSP_6037-CcsR1-4 is essential for this regulation. In vitro, the fragment of -200 to -105 region could be used as a bait to search for the putative proteins binding to this fragment.

The *potA* mRNA was verified as the target of SorX, however, more target genes may exist. Moreover, there is an overlap between the SorX regulon and the SorY regulon, since SorX and SorY share the similar aSD sequence. One example is the *takP* mRNA verified as a direct target of SorY. It is also regulated by overexpression of SorX

according to the microarray data. There is a possible direct interaction between SorX and *takP* mRNA predicted by the software tool IntaRNA. Therefore, the *vivo lacZ* reporter system could be used to prove whether there is a direct interaction between SorX and *takP* mRNA.

RSP_6037 and *rpoHI* are target genes of OmpR. Gel shift assays can reveal whether there are direct bindings between OmpR protein and the promoter regions of RSP_6037 and *rpoHI*.

6 Summary

A previous study showed that several sRNAs are induced by singlet oxygen in *R. sphaeroides*. The function of sRNAs CcsR1-4 and SorX were in the focus of this study. Overexpression of CcsR1-4 enhanced resistance to superoxide and organic hydroperoxides. Based on transcriptome analysis, several mRNAs of genes related to C1 metabolism or encoding components of the pyruvate dehydrogenase complex showed lower expression levels in CcsR1-4 overexpression strain. By using an *in vivo lacZ* based reporter system, the *flhR* mRNA was proven as a direct interaction partner of CcsR1-4. The *flhR* mRNA is known to encode a putative transcriptional activator of glutathione (GSH)-dependent methanol/formaldehyde metabolism (Hickman *et al.*, 2004). Down-regulation of *flhR* mRNA represses the glutathione-dependent pathway, which increases the pool of glutathione. More glutathione would be used to counteract oxidative stress. The FlhR-dependent down-regulation of C1 metabolism reduces the main source of reactive oxygen species.

SorX is derived from the 3' UTR of the *ompR* mRNA which encodes a response regulator. Deletion of *sorX* caused a higher sensitivity to singlet oxygen and organic hydroperoxides. The 75 nt SorX is generated from the 3' UTR of the *ompR* transcript by RNase E cleavage and considerably conserved among related species. The 75-nt SorX is sufficient for enhancing resistance to singlet oxygen and organic hydroperoxides. The sRNAs CcsR1-4 and SorY which have a reported role in the oxidative stress response are affected by SorX. A *potA* mRNA encoding a subunit of a polyamine transporter was identified as a direct target of SorX. Deletion of *potA* increased resistance to singlet oxygen and organic hydroperoxides. Moreover, SorX enhances resistance by targeting *potA*. The PotABCD transporter can specifically uptake spermidine in *E. coli* (Kashiwagi *et al.*, 1993). High spermidine level significantly increases ROS level and causes increased sensitivity of *R. sphaeroides* to organic hydroperoxides. It was assumed that less uptake of spermidine counteracts oxidative stress, due to down-regulation of *potA* by SorX in *R. sphaeroides*.

Besides sRNAs induced by singlet oxygen, some proteins were also identified by proteome analysis including a response regulator OmpR homologous protein. Western blot and real time RT-PCR showed that various stress conditions can induce OmpR protein levels and *ompR* expression. Overexpression of a flag-tagged OmpR enhanced resistance to organic hydroperoxides. The flag-tagged OmpR protein is able to up-regulate promoter activity of RSP_6037 which encods a hypothetical protein and

the mRNA level of RSP_6037, however, the OmpR protein down-regulates the promoter activity of *rpoHI* and its mRNA level. These data confirmed that RSP_6037 and *rpoHI* are regulated by OmpR. Furthermore, CcsR1, which is transcribed with the upstream gene RSP_6037, was also accumulated by overexpression of the flag-tagged OmpR protein.

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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 01. August. 2016

Tao Peng