NADPH-related processes studied with a SoxR-based biosensor
in *Escherichia coli*

Alina Spielmann, Meike Baumgart, Michael Bott*
IBG-1: Biotechnology, Institute of Bio- and Geosciences, Forschungszentrum Jülich, Jülich, Germany

*Corresponding author:
Phone: +49 (0)2461 613294
Fax: +49 (0)2461 612710
Email: m.bott@fz-juelich.de

Running title: Studies with the *E. coli* NADPH biosensor pSenSox
Abstract

NADPH plays a crucial role in cellular metabolism for biosynthesis and oxidative stress responses. We previously developed the genetically encoded NADPH biosensor pSenSox based on the transcriptional regulator SoxR of *Escherichia coli*, its target promoter $P_{soxS}$ and eYFP as fluorescent reporter. Here we used pSenSox to study the influence of various parameters on the sensor output in *E. coli* during reductive biotransformation of methylacetacacetate (MAA) to $(R)$-methyl 3-hydroxybutyrate (MHB) by the strictly NADPH-dependent alcohol dehydrogenase of *Lactobacillus brevis* (*LbAdh*). Redox cycling drugs such as paraquat and menadione strongly activated the NADPH biosensor and mechanisms responsible for this effect are discussed. Absence of the RsxABCDGE complex and/or RseC caused an enhanced biosensor response, supporting a function as SoxR reducing system. Absence of the membrane-bound transhydrogenase PntAB caused an increased biosensor response, whereas the lack of the soluble transhydrogenase SthA or of SthA and PntAB was associated with a strongly decreased response. These data support the opposing functions of PntAB in NADP$^+$ reduction and of SthA in NADPH oxidation. In summary, the NADPH biosensor pSenSox proved to be a useful tool to study NADPH-related processes in *E. coli*.

Keywords

NADPH biosensor, RsxABCDGE complex, RseC, transhydrogenases PntAB and SthA
Introduction

Genetically encoded biosensors based on transcriptional regulators (TRs) and using fluorescent proteins as reporters are highly useful tools for monitoring physiological responses at the single-cell level and for enabling high-throughput screening in strain and enzyme development (Dietrich et al., 2010; Eggeling et al., 2015; Mahr and Frunzke, 2016; Rogers et al., 2016). In recent years, we developed TR-based biosensors for amino acids and used them e.g. to identify novel mutations for overproduction of L-lysine, L-arginine, and L-histidine by FACS-based screening of genome-wide, gene-specific, and codon-specific mutant libraries (Binder et al., 2012; Binder et al., 2013; Schendzielorz et al., 2014) or for increasing L-valine production by adaptive laboratory evolution (Mahr et al., 2015).

Besides the sensors for amino acids, we also established a TR-based biosensor responding to the intracellular availability of NADPH. It is based on the SoxRS regulatory system of *Escherichia coli*, which governs the expression of oxidative stress response genes by a regulatory cascade, in which synthesis of SoxS depends on transcriptional activation of soxS expression by SoxR (Greenberg et al., 1990; Tsaneva and Weiss, 1990). In the genome, the soxR gene is located divergently to the soxS gene. Whereas initial studies indicated that the promoter of soxR is located within the soxS coding region (Wu and Weiss, 1991), a subsequent analysis revealed that the promoters of the two genes overlap (Hidalgo et al., 1998). The transcription factor SoxR is a homodimer with each subunit containing an [2Fe-2S] cluster (Hidalgo and Demple, 1994; Watanabe et al., 2008). SoxR activity is controlled by a change of the redox state of its [2Fe−2S] clusters, which is associated with conformational changes: only in the oxidized [2Fe−2S]^{2+} state, but not in the reduced [2Fe−2S]^{+} state, SoxR activates soxS expression (Ding et al., 1996; Gaudu and Weiss, 1996). SoxR binds to its target site, which is located between the -10 and -35 regions of the soxS promoter and downstream of the -10 region of the soxR promoter, both in the oxidized and in the reduced state with high affinity (Hidalgo and Demple, 1994; Gaudu and Weiss, 1996). Besides activating soxS expression in the oxidized state, SoxR simultaneously represses expression of its own gene, both in the oxidized and the reduced state (Hidalgo et al., 1998). SoxR was previously
considered to activate expression of soxS only, but recent studies uncovered further direct SoxR target genes (Seo et al., 2015).

SoxS functions as a transcriptional activator of genes, many but not all of which are responsible for coping with damage caused by oxygen radicals, such as sodA for superoxide dismutase, zwf for the NADPH-generating glucose 6-phosphate dehydrogenase, or fumC for fumarase C (Blanchard et al., 2007; Seo et al., 2015). It has been shown that the intrinsic instability of SoxS (t_{1/2} ~ 2 min) and the degradation of SoxS, primarily through the Lon protease, are responsible for the rapid return of the SoxRS system to the inactive state when the stimuli activating the system are no longer present (Griffith et al., 2004).

Current evidence indicates that there are multiple ways how the conversion of inactive, reduced SoxR into active, oxidized SoxR can be triggered. These include direct oxidation of SoxR by superoxide (Liochev and Fridovich, 2011; Fujikawa et al., 2012) and by redox-cycling drugs (Gu and Imlay, 2011), nitrosylation of SoxR (Ding and Demple, 2000), and conditions leading to a diminished NADPH/NADP⁺ ratio within cells (Liochev and Fridovich, 1992; Krapp et al., 2011). The responsiveness to the NADPH availability is presumably due to the fact that SoxR is subject to permanent autoxidation under aerobic conditions, but is kept in the reduced state by NADPH-dependent reductases (Koo et al., 2003).

In a previous study we made use of the NADPH-responsiveness of the SoxRS system to construct the biosensor pSenSox, in which the SoxR-activated soxS promoter controls expression of the eyfp gene, allowing detection of SoxR activation at the single cell level (Siedler et al., 2014). Using the reduction of methyl acetocacetate (MAA) to R-methyl 3-hydroxybutyrate (MHB) by the strictly NADPH-dependent alcohol dehydrogenase of Lactobacillus brevis (LbAdh) as model reaction, we could show that the specific eYFP fluorescence of E. coli cells correlated not only with the MAA concentration added to the cells, but also with the specific LbAdh activity when a fixed MAA concentration was provided. The latter property enabled high-throughput screening of an LbAdh mutant library by fluorescence-activated cell sorting (FACS) for variants with improved activity for the alternative substrate 4-methyl-2-pentanone (Siedler et al., 2014).
In this study, we employed pSenSox to test various conditions, including growth media, redox cycling drugs, mutants lacking SoxR reductases, and mutants lacking transhydrogenases for their influence on SoxR activity.

Results and Discussion

Influence of different media on the NADPH biosensor response

To test the influence of different media on the response of the pSenSox-based NADPH biosensor, the biotransformation of MAA to MHB, catalyzed by the NADPH-dependent LbAdh, was performed in three complex media (TB, 2xTY, or LB) and in a defined minimal medium (M9) with glucose as carbon source using the experimental setup shown in Fig. 1 and described in the methods section. The experiments with the different media, including control cultures in which MAA was omitted or in which pSenNeg, encoding a defective LbAdh, was used, are shown in Fig. 2. MAA itself had a negative influence on growth, even in the absence of LbAdh, and this negative influence was further enhanced in the presence of LbAdh activity, when MAA was reduced to MHB with NADPH as reductant. Regarding the response of the SoxRS-based NADPH biosensor, the experiments shown in Fig. 2 confirmed that expression of the eyfp gene is dependent on the biotransformation of MAA to MHB by LbAdh. In the absence of either MAA or LbAdh activity, eYFP synthesis was not induced.

When comparing the different media, it became obvious that TB allowed by far the best growth, followed by 2xTY and LB medium, in which the cells grew comparably, and M9 glucose medium, in which almost no growth occurred (Appendix 1). When comparing the different media with respect to eYFP synthesis, the highest fluorescence after 24 h was obtained in 2xTY medium and LB medium, whereas it was much lower and comparable for TB and M9-glucose medium (Appendix 1). The almost complete lack of growth in M9-glucose was due to the biotransformation of MAA to MHB, as growth was observed in the absence of MAA or of LbAdh activity (Fig. 2). In this medium, cells have to synthesize all cellular components, in particular amino acids, from glucose, whereas in the other media the presence of yeast extract and tryptone provides amino acids and other cellular components that do not need to be
synthesized by the cell but can be imported from the medium. Nevertheless, also in these media the NADPH-dependent reduction of MAA to MHB had a strong negative effect on growth, presumably due to a lack of NADPH for biosynthetic purposes. An interesting case is TB medium. Although this medium allowed the best growth, the biosensor response was much lower compared to 2xTY or LB and similar to that in M9-glucose medium. Besides a higher concentration of yeast extract and phosphate buffering, the major difference of TB medium to 2xTY and LB is the presence of glycerol as additional carbon source.

In conclusion, media without a separately added carbohydrate as carbon source, such as 2xTY and LB, led to a higher biosensor response than media containing an added carbohydrate, such as M9-glucose or TB, which contains 4 mL/L glycerol. This is probably due to a higher NADPH availability by carbohydrate catabolism. M9 glucose medium can in principle be used to monitor the SoxR-based NADPH biosensor response, which can be necessary or advantageous for experiments in which components of yeast extract or tryptone are disturbing. Overall, the strongest biosensor signal was observed in 2xTY medium, which was therefore chosen for the following experiments.

Influence of redox cycling drugs on the NADPH biosensor response

Paraquat (1, 1′-dimethyl-4, 4′-bipyridinium dichloride) and menadione (2-methyl-1,4-naphthoquinone) have been reported to induce the soxRS regulon in E. coli (Greenberg et al., 1990; Wu and Weiss, 1991; Seo et al., 2015). We therefore monitored the response of E. coli BL21(DE3)/pSenSox to different paraquat concentrations (0 µM, 1 µM, 5 µM) and different menadione concentrations (0 µM, 5 µM, 10 µM) using the experimental setup shown in Fig. 1, except that paraquat and menadione were added instead of MAA. At the concentrations used, both compounds had only minor effects on growth (data not shown), but clearly triggered a concentration-dependent activation of the SoxRS-based biosensor response (Fig. 3). These results confirm the strong responsiveness of the SoxRS system to paraquat and menadione. Higher concentrations of paraquat (0.01, 0.1, 1, 5, 10 mM) and menadione (15, 20, 25, 50 µM)
were tested, but did not lead to further increased fluorescence (data not shown). Addition of up to 5 mM H$_2$O$_2$ did not elicit a SoxR response (data not shown).

Paraquat and menadione are redox-cycling drugs, which mediate the transfer of electrons from NADPH to oxygen, leading to the continuous generation of superoxide (Kappus and Sies, 1981). Several possibilities exist how redox cycling drugs activate the SoxRS response: (i) the superoxide radical has been shown to directly oxidize the [2Fe-2S] cluster of SoxR (Fujikawa et al., 2012), thus forming active SoxR; (ii) the redox-cycling drug might directly interact with reduced SoxR and oxidize it, leading to active SoxR (Gu and Imlay, 2011); (iii) the reduction of the redox cycling agent by NADPH might lead to a decreased NADPH/NADP$^+$ ratio, thereby interfering with the NADPH-dependent reduction of SoxR and causing an increased level of oxidized, active SoxR; (iv) the redox-cycling drugs might be directly reduced by the SoxR-reducing system(s) of the cell, thereby inhibiting SoxR reduction and causing increased levels of oxidized active SoxR. It is possible that several of these mechanisms contribute to the activation of SoxR by paraquat and menadione.

_Influence of rseC and rsxABCDGE deletion on the NADPH biosensor response_

By screening an _E. coli_ mutant library, mutations in the _rseC_ gene and in the _rsxABCDGE_ operon were found to cause constitutive expression of a _P_$_{soxS}$-_lacZ_ reporter gene in a SoxR-dependent manner (Koo et al., 2003). Further studies led to the conclusion that the membrane-integral RsxABCDGE complex and the membrane protein RseC constitute a SoxR-reducing system (Koo et al., 2003). The statement that purified RsxC exhibits NADPH-dependent cytochrome c reduction activity (Koo et al., 2003) suggests that NADPH serves as electron donor of the Rsx complex. To test the influence of this reducing system on the NADPH biosensor response, we constructed deletion mutants of _E. coli_ BL21(DE3) lacking either _rseC_ (ΔrseC), or _rsxABCDGE_ (Δrsx), or all of these genes (ΔrseCΔrsx).

When monitoring the growth behavior of the deletion mutants and the parental strain in shake flask experiments with 2xTY medium, all strains exhibited the same growth behavior, showing that under these conditions RseC and the Rsx complex are dispensable (Appendix
The influence of ΔrseC, Δrsx, and ΔrseCΔrsx mutations on the NADPH biosensor signal was analyzed according to the standard experimental setup shown in Fig. 1 with 30 mM MAA as substrate for the NADPH-dependent LbAdh. As shown in Fig. 4, all deletion mutants showed an increased fluorescence signal compared to the parental strain, with the strongest response in the ΔrseC mutant, followed by the ΔrseCΔrsx mutant and the Δrsx mutant.

To confirm that the observed effects were due to the gene deletions, plasmids pACYC-rseC, pACYC-rsx, and pACYC-rseC-rsx were constructed and transferred into the corresponding deletion mutants. The parent vector pACYCDuet-1 served as control. Basal expression of rseC and/or rsxABCDGE in the respective deletion mutants without addition of IPTG resulted in decreased biosensor signals compared to the ones obtained in the mutants carrying the control plasmid pACYCDuet-1, but the response was still higher than in the parental strain (Appendix 3). Although complementation was only partial, which could be due to an inadequate expression level of the plasmid-encoded genes, it confirmed that the rseC and rsx deletions were responsible for the increased biosensor response.

The results described above are in agreement with previous data showing a function of RsxABCDGE and RseC in SoxR reduction (Koo et al., 2003). An interesting observation made by Koo and coworkers and by us was that the deletion of both rseC and the rsx cluster had no additive effect and expression of the reporter gene was even somewhat lower in the ΔrseCΔrsx mutant than in the ΔrseC single mutant. This indicates that the Rsx complex and RseC do not function independently to reduce SoxR, but work together, as proposed previously (Koo et al., 2003). The RsxABCDGE complex belongs to the family of Rnf complexes, enzymes that drive the endergonic reduction of ferredoxin (E₀' = −420 mV) with NAD(P)H (E₀' = −320 mV) by the proton- or sodium-motive force via import of H⁺ or Na⁺, or, in the reverse reaction, the exergonic reduction of NAD(P)⁺ with reduced ferredoxin coupled to the export of H⁺ or Na⁺ (Biegel et al., 2011). The redox potential of SoxR in its DNA-free and its DNA-bound state was reported to be -293 mV and -320 mV (Kobayashi et al., 2015), respectively, i.e. in the same range as the one of NAD(P)H. Consumption of proton- or sodium-motive force via the Rsx complex to drive reduction of SoxR by NADPH allows the cell to keep most SoxR in the reduced state in the
absence of inducing conditions. In fact, it was reported that in wild-type cells overproducing SoxR almost all of the protein was present in the reduced state, but in rsxC and rseC mutants only about 60% and 56% (Koo et al., 2003). The final steps of electron transfer to SoxR are unknown at present. In the Rnf complexes, RnfB is suggested as electron donor for ferredoxin (Biegel et al., 2011). Therefore, the homologous RsxB protein could serve to reduce SoxR, or alternatively, RseC might transfer electrons from RsxB to SoxR, as a conserved cysteine motif in the N-terminal region of RseC could be part of an iron-sulfur cluster. Further studies are required to solve this issue. The observation that in rsxC and rseC mutants still about 60% and 56% of SoxR was in the reduced state (Koo et al., 2003) suggests that further enzymes for SoxR reduction exist, which need to be identified.

We also tested the response of the rsx and rseC mutants in the presence of 5 µM paraquat instead of MAA. Although the parental strain already showed a strong response to paraquat, that of the mutants was still further increased. Again, the ΔrseC mutant showed the highest specific fluorescence followed by the ΔrseCΔrsx mutant and the ΔrsxABCDGE mutant (Fig. 4C, D). The observation that paraquat and rsx and/or rseC deletion showed an additive effect on the biosensor response confirms that a fraction of SoxR must still be in the reduced state in the mutants and suggests that paraquat-based activation of SoxR is not due to interference with SoxR reduction by the Rsx/RseC system.

Influence of the transhydrogenase deletions ΔpntAB, ΔsthA and ΔsthAΔpntAB on the NADPH biosensor response

Transhydrogenases catalyze the reversible interconversion of NADH and NADPH. E. coli possesses two transhydrogenases, the membrane-bound, proton-translocating transhydrogenase PntAB and the soluble, energy-independent transhydrogenase SthA (also called UdhA) (Sauer et al., 2004). Due to the relevance of transhydrogenases in the regulation of cellular NADPH levels, we studied the influence of these enzymes on the NADPH biosensor response by constructing deletion mutants of E. coli BL21(DE3) lacking either sthA, or pntAB, or both.
The growth behavior of the transhydrogenase mutants was tested in shake flask experiments using 2xTY medium. Whereas the ΔpntAB mutant grew like the parental strain, the ΔsthA mutant and the ΔsthAΔpntAB double mutant showed a growth defect that became apparent during the exponential growth phase (Appendix 4). Presumably, an excess of NADPH is formed in this growth phase, which cannot be readily diminished in the absence of SthA. The defect could be largely abolished by plasmid-based expression of sthA (Appendix 5), confirming that it was caused by the sthA deletion.

The influence of SthA and PntAB on the NADPH biosensor signal was analyzed according to the standard experimental setup shown in Fig. 1 with 30 mM MAA as substrate for the NADPH-dependent LbAdh. Whereas the ΔpntAB mutant displayed a slightly increased biosensor signal, it was decreased by more than 60% in the ΔsthA mutant and also in the ΔsthAΔpntAB double mutant (Fig. 5). The latter result showed that the sthA deletion was dominant over the pntAB deletion.

To confirm that the observed effects were due to the gene deletions, plasmids pACYC-pntAB and pACYC-sthA were constructed and transferred into the corresponding deletion mutants. The parent vector pACYCDuet-1 served as control. Plasmid-based expression of pntAB in the ΔpntAB mutant completely prevented the increase in the biosensor signal and even reduced it to a small extent. Vice versa, plasmid-based expression of sthA in the ΔsthA mutant completely prevented the decrease in the sensor response and even increased it (Appendix 5). The presence of a second plasmid reduced the differences in the biosensor signal between the ΔsthA mutant and the parent strain, which might be due to a negative effect on the copy number of pSenSsox and thus LbAdh activity.

The results obtained with the ΔpntAB mutant indicate that, under the conditions used, PntAB catalyzes NADP⁺ reduction by NADH, leading to an increased NADPH availability. Absence of pntAB therefore results in a lowered NADPH availability and thus an increased biosensor signal. This conclusion is in agreement with previous studies showing that PntAB is involved in NADPH formation in *E. coli* (Sauer et al., 2004) and that overexpression of pntAB enhanced conversion of acetophenone to (R)-phenylethanol by the NADPH-dependent alcohol
dehydrogenase of *Lactobacillus kefir* (Weckbecker and Hummel, 2004) and improved the biosynthesis of 3-hydroxypropionic acid from its precursor malonyl-CoA by an NADPH-dependent malonyl-CoA reductase (Rathnasingh et al., 2012). Moreover, heterologous overexpression of the *E. coli* pntAB genes in *C. glutamicum* was shown to enhance production of L-lysine, whose biosynthesis is strongly NADPH-dependent (Kabus et al., 2007).

In contrast to PntAB, our results obtained with the ΔsthA mutant indicate that under the conditions employed SthA catalyzes NADPH oxidation. Absence of sthA thus results in an increased NADPH availability, which is reflected by a decreased biosensor signal. These data are in agreement with previous studies showing that SthA is required under conditions leading to excess NADPH formation (Sauer et al., 2004). The observation that pntAB deletion in the ΔsthA mutant did not reverse the decrease in the biosensor signal suggests that SthA activity is much higher than PntAB activity, which can be expected based on the fact that PntAB catalyzes a reaction coupled to proton transfer across the membrane. In conclusion, our data confirm that PntAB and SthA play important and opposite functions for NADPH availability in *E. coli*.

**Concluding remarks**

In this study, we employed the NADPH biosensor pSenSox to study various processes expected to influence the NADPH availability in *E. coli*. We could confirm that the lack of rsxABCDGE and rseC activated the pSenSox-based response. Paraquat and the Δrsx/ΔrseC deletion had an additive effect on the sensor response, indicating that further SoxR-reducing systems exist besides Rsx/RseC and that the paraquat-induced response is presumably not due to interference of paraquat with SoxR reduction by Rsx/RseC. The transhydrogenases PntAB and SthA had opposite effects on the biosensor response, in agreement with PntAB being involved in NADP⁺ reduction and SthA catalyzing NADPH oxidation. In conclusion, the pSenSox-based NADPH biosensor is a useful tool not only for HT-screening of the activity of NADPH-dependent alcohol dehydrogenases (Siedler et al., 2014), but also for analyzing conditions and proteins influencing NADPH availability or SoxR reduction. Recently, another
NADPH biosensor was described, which is based on the specific oxygen-independent amplification of the intrinsic fluorescence of NADPH by the mBFP protein (Hwang et al., 2012). The mBFP protein was shown to be well suited to study the dynamics of intracellular NADPH availability with a resolution of seconds and to allow the quantitation of NADPH (Goldbeck et al., 2018). This is not possible with the pSenSox NADPH biosensor, which requires transcription, translation, and oxygen-dependent maturation of eYFP. However, pSenSox allows to preserve changed NADPH levels as a stable fluorescence signal, which is a prerequisite e.g. for FACS-based screening of mutant libraries of NADPH-dependent enzymes, and it allows to specifically analyze the effects of enzymes that are involved in SoxR reduction or oxidation, which is presumably not possible with the mBFP sensor. Thus, mBFP and pSenSox represent two different types of NADPH biosensors which both have their specific advantages and application fields.

Materials and Methods

Bacterial strains, plasmids and growth conditions

All strains and plasmids used in this work are listed in Table 1. *E. coli* BL21(DE3) (Invitrogen, Karlsruhe, Germany) and its derivatives were used for all studies with the NADPH biosensor. *E. coli* NEB5α was employed for cloning purposes. Transformation of *E. coli* cells was performed as described (Hanahan, 1983). Cells were cultivated at 30°C or 37°C in lysogeny broth (LB) (Miller, 1972), in 2xTY (16 g L⁻¹ tryptone, 10 g L⁻¹ yeast extract, 5 g L⁻¹ sodium chloride), in M9 mineral medium (33.7 mM Na₂HPO₄×2H₂O, 22.0 mM KH₂PO₄, 8.55 mM NaCl, 9.35 mM NH₄Cl, 1 mM MgSO₄×7H₂O, 0.3 mM CaCl₂, 1 µg mL⁻¹ biotin, 1 µg mL⁻¹ thiamin, trace elements) supplemented with 0.4% (w/v) glucose (Sambrook and Russell, 2001), or in terrific broth (TB) medium (12 g L⁻¹ tryptone, 24 g L⁻¹ yeast extract, 4 mL glycerol, 12.54 g L⁻¹ K₂HPO₄, 2.31 g L⁻¹ KH₂PO₄; pH 7.0). Plasmids were selected by adding antibiotics to the medium to a final concentration of 100 µg mL⁻¹ carbenicillin, 34 µg mL⁻¹ chloramphenicol, or 50 µg mL⁻¹ kanamycin.
Recombinant DNA work and construction of deletion mutants

Standard methods such as PCR and DNA restriction enzyme digestion were carried out according to established protocols (Sambrook and Russell, 2001). Oligonucleotides were synthesized by Eurofins Genomics (Ebersberg, Germany) and are listed in Appendix 6. Construction of pACYC-rseC, pACYC-rsx, pACYC-rseC-rsx, pACYC-sthA, and pACYC-pntAB was performed by Gibson assembly (Gibson et al., 2009). All plasmids were sequenced by Eurofins Genomics, Ebersberg, Germany. The construction of *E. coli* deletion mutants was performed with the lambda Red recombinase method (Datsenko and Wanner, 2000) using a recent protocol (Jensen et al., 2015). For the markerless deletion of genes, the arabinose-inducible lambda Red recombineering genes (*exo*, *bet*, and *gam*) and the rhamnose-inducible flippase (FLP) recombinase were introduced into *E. coli* BL21(DE3) using the temperature-sensitive plasmid pSIJ8 (Jensen et al., 2015). The FRT-flanked kanamycin cassette, which was integrated into the *E. coli* genome at the locus of the gene to be deleted, was encoded by plasmid pKD4. For amplification of the FRT-flanked kanamycin cassette of pKD4, oligonucleotides that carried homology regions to the up- and downstream regions of the genes to be deleted were used. All gene deletions were verified by colony PCR using DreamTaq Master Mix 2X (Thermo Scientific, Schwerte, Germany) and the oligonucleotides listed in Appendix 6.

Monitoring the NADPH biosensor response

The NADPH biosensor response during the whole-cell biotransformation of MAA to MHB by the strictly NADPH-dependent *LbAdh* was measured as described (Siedler et al., 2014). The specific fluorescence is defined as the ratio of fluorescence and backscatter. The biotransformations were performed with *E. coli* BL21(DE3) and its derivatives, such as deletion mutants lacking the genes coding for the SoxR-reducing system or transhydrogenases, and mutant strains carrying expression plasmids for the deleted genes. The *E. coli* BL21(DE3) cells were transformed either with plasmid pSenSox carrying the SoxR-based NADPH biosensor
and an intact *LbAdh* gene or with plasmid pSenNeg, in which a part of the *Lbadh* gene was deleted to prevent synthesis of an active *LbAdh* (Siedler et al., 2014).

The experimental setup used for the biotransformation experiments is shown in Fig. 1. Pre-cultures of three biological replicates of the desired strains were incubated overnight at 37°C and 130 rpm in 5 mL medium containing the appropriate antibiotic(s) as selection marker(s). These pre-cultures were used for the inoculation of the main cultures to an optical density at 600 nm (OD<sub>600</sub>) of 0.05. The main cultures were grown in 50 mL medium in the presence of the appropriate antibiotics at 37°C and 130 rpm using 500 mL shake flasks. For complementation experiments the cells were incubated at 30°C. Basal expression of *Lbadh* by the non-induced *tac* promoter allowed for sufficient *LbAdh* activity in the biotransformation experiments (Siedler et al., 2014). When a higher *LbAdh* activity was required, IPTG was added to a final concentration of 0.1 mM when the cultures had reached an OD<sub>600</sub> between 0.6-0.8. To ensure that enough biomass is present for the biotransformation, the cultures were further incubated for at least 5 h, until an OD<sub>600</sub> of 5 or higher was reached. Then the cells were harvested by centrifugation (4°C, 4713 g and 15 min) and resuspended in fresh medium supplemented with the corresponding antibiotic to a final OD<sub>600</sub> of 5. 800 µL of these suspensions were transferred into 48-well microtiter Flowerplates Germany) and cultivated in a Biolector microcultivation system (m2p-labs, Baesweiler, Germany). When analyzing the biosensor response under conditions of reductive biotransformation, 100 µL MAA dissolved in ddH<sub>2</sub>O at the desired concentration was added to the 800 µL cultures or 100 µL ddH<sub>2</sub>O as negative control. To study the effect of redox-cycling drugs and hydrogen peroxide on the biosensor response, the following additions were made to the 800 µL cultures in the Flowerplates: (i) 100 µL paraquat (1, 1’-dimethyl-4, 4’-bipyridinium dichloride; Sigma-Aldrich) dissolved in ddH<sub>2</sub>O to final concentrations of 1 µM and 5 µM; (ii) 100 µL menadione (2-methyl-1,4-naphthoquinone; Sigma-Aldrich) dissolved in DMSO to final concentrations of 5 µM and 10 µM; (iii) 100 µL H<sub>2</sub>O<sub>2</sub> to final concentrations of 0.05 mM or 5 mM. For the negative controls, 100 µL ddH<sub>2</sub>O or 100 µL DMSO was added.
After the desired additions, the Flowerplates were incubated in a BioLector microcultivation system (m2p-laps, Baesweiler, Germany) at 30°C and 1200 rpm (shaking diameter 3 mm), which allows online monitoring of cell density (as backscattered light at 620 nm) and of eYFP fluorescence (excitation wavelength 485 nm, emission wavelength of 520 nm). For the different experiments, the values were normalized to the maximal backscatter and the maximal specific fluorescence observed, which were set as 1.0.

**Acknowledgements**
This work was funded by the German Federal Ministry of Education and Research (BMBF), funding code 031A095B, as part of the project “Molecular Interaction Engineering: From Nature’s Toolbox to Hybrid Technical Systems (MIE)”.

**Author contributions**
A.S., M.Ba., and M.Bo. designed research; A.S. performed all experiments; A.S., M.Ba., and M.Bo. analyzed the data; A.S. prepared the figures; M.Bo. wrote the manuscript.

**Conflict of interest**
The authors declare no conflict of interest.

**Ethics statement.**
None required.

**Data accessibility**
All data can be found within in the manuscript and in the appendices.
References

Biegel, E., Schmidt, S., Gonzalez, J.M., and Müller, V. (2011) Biochemistry, evolution and physiological function of the Rnf complex, a novel ion-motive electron transport complex in prokaryotes. *Cell Mol Life Sci* **68**: 613-634.

Binder, S., Siedler, S., Marienhagen, J., Bott, M., and Eggeling, L. (2013) Recombineering in *Corynebacterium glutamicum* combined with optical nanosensors: a general strategy for fast producer strain generation. *Nucleic Acids Res* **41**: 6360-6369.

Binder, S., Schendzielorz, G., Stäbler, N., Krumbach, K., Hoffmann, K., Bott, M., and Eggeling, L. (2012) A high-throughput approach to identify genomic variants of bacterial metabolite producers at the single-cell level. *Genome Biol* **13**: R40.

Blanchard, J.L., Wholey, W.Y., Conlon, E.M., and Pomposiello, P.J. (2007) Rapid changes in gene expression dynamics in response to superoxide reveal SoxRS-dependent and independent transcriptional networks. *PLoS One* **2**: e1186.

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97**: 6640-6645.

Dietrich, J.A., McKee, A.E., and Keasling, J.D. (2010) High-throughput metabolic engineering: advances in small-molecule screening and selection. *Annu Rev Biochem* **79**: 563-590.

Ding, H., and Demple, B. (2000) Direct nitric oxide signal transduction via nitrosylation of iron-sulfur centers in the SoxR transcription activator. *Proc Natl Acad Sci USA* **97**: 5146-5150.

Ding, H., Hidalgo, E., and Demple, B. (1996) The redox state of the [2Fe-2S] clusters in SoxR protein regulates its activity as a transcription factor. *J Biol Chem* **271**: 33173-33175.

Eggeling, L., Bott, M., and Marienhagen, J. (2015) Novel screening methods-biosensors. *Curr Opin Biotechnol* **35**: 30-36.

Fujikawa, M., Kobayashi, K., and Kozawa, T. (2012) Direct oxidation of the [2Fe-2S] cluster in SoxR protein by superoxide: distinct differential sensitivity to superoxide-mediated signal transduction. *J Biol Chem* **287**: 35702-35708.

Gaudu, P., and Weiss, B. (1996) SoxR, a [2Fe-2S] transcription factor, is active only in its oxidized form. *Proc Natl Acad Sci USA* **93**: 10094-10098.
Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A., and Smith, H.O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Meth* **6**: 343-345.

Goldbeck, O., Eck, A.W., and Seibold, G.M. (2018) Real time monitoring of NADPH concentrations in *Corynebacterium glutamicum* and *Escherichia coli* via the genetically encoded sensor mBFP. *Front Microbiol* **9**: 2564.

Greenberg, J.T., Monach, P., Chou, J.H., Josephy, P.D., and Demple, B. (1990) Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. *Proc Natl Acad Sci USA* **87**: 6181-6185.

Griffith, K.L., Shah, I.M., and Wolf, R.E., Jr. (2004) Proteolytic degradation of *Escherichia coli* transcription activators SoxS and MarA as the mechanism for reversing the induction of the superoxide (SoxRS) and multiple antibiotic resistance (Mar) regulons. *Mol Microbiol* **51**: 1801-1816.

Gu, M., and Imlay, J.A. (2011) The SoxRS response of *Escherichia coli* is directly activated by redox-cycling drugs rather than by superoxide. *Mol Microbiol* **79**: 1136-1150.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**: 557-580.

Hidalgo, E., and Demple, B. (1994) An iron-sulfur center essential for transcriptional activation by the redox-sensing SoxR protein. *EMBO J* **13**: 138-146.

Hidalgo, E., Leautaud, V., and Demple, B. (1998) The redox-regulated SoxR protein acts from a single DNA site as a repressor and an allosteric activator. *EMBO J* **17**: 2629-2636.

Hwang, C.S., Choi, E.S., Han, S.S., and Kim, G.J. (2012) Screening of a highly soluble and oxygen-independent blue fluorescent protein from metagenome. *Biochem Biophys Res Commun* **419**: 676-681.

Jensen, S.I., Lennen, R.M., Herrgard, M.J., and Nielsen, A.T. (2015) Seven gene deletions in seven days: Fast generation of *Escherichia coli* strains tolerant to acetate and osmotic stress. *Sci Rep* **5**: 17874.
Kabus, A., Georgi, T., Wendisch, V.F., and Bott, M. (2007) Expression of the *Escherichia coli* pntAB genes encoding a membrane-bound transhydrogenase in *Corynebacterium glutamicum* improves L-lysine formation. *Appl Microbiol Biotechnol* **75**: 47-53.

Kappus, H., and Sies, H. (1981) Toxic drug effects associated with oxygen metabolism: redox cycling and lipid peroxidation. *Experientia* **37**: 1233-1241.

Kensy, F., Zang, E., Faulhammer, C., Tan, R.K., and Büchs, J. (2009) Validation of a high-throughput fermentation system based on online monitoring of biomass and fluorescence in continuously shaken microtiter plates. *Microb Cell Fact* **8**: 31.

Kobayashi, K., Fujikawa, M., and Kozawa, T. (2015) Binding of promoter DNA to SoxR protein decreases the reduction potential of the [2Fe-2S] cluster. *Biochemistry* **54**: 334-339.

Koo, M.S., Lee, J.H., Rah, S.Y., Yeo, W.S., Lee, J.W., Lee, K.L. et al. (2003) A reducing system of the superoxide sensor SoxR in *Escherichia coli*. *EMBO J* **22**: 2614-2622.

Krapp, A.R., Humbert, M.V., and Carrillo, N. (2011) The soxRS response of *Escherichia coli* can be induced in the absence of oxidative stress and oxygen by modulation of NADPH content. *Microbiology* **157**: 957-965.

Liochev, S.I., and Fridovich, I. (1992) Fumarase C, the stable fumarase of *Escherichia coli*, is controlled by the soxRS regulon. *Proc Natl Acad Sci USA* **89**: 5892-5896.

Liochev, S.I., and Fridovich, I. (2011) Is superoxide able to induce SoxRS? *Free Radic Biol Med* **50**: 1813.

Mahr, R., and Frunzke, J. (2016) Transcription factor-based biosensors in biotechnology: current state and future prospects. *Appl Microbiol Biotechnol* **100**: 79-90.

Mahr, R., Gätgens, C., Gätgens, J., Polen, T., Kalinowski, J., and Frunzke, J. (2015) Biosensor-driven adaptive laboratory evolution of L-valine production in *Corynebacterium glutamicum*. *Metab Eng* **32**: 184-194.

Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
Rathnasingh, C., Raj, S.M., Lee, Y., Catherine, C., Ashok, S., and Park, S. (2012) Production of 3-hydroxypropionic acid via malonyl-CoA pathway using recombinant *Escherichia coli* strains. *J Biotechnol* **157**: 633-640.

Rogers, J.K., Taylor, N.D., and Church, G.M. (2016) Biosensor-based engineering of biosynthetic pathways. *Curr Opin Biotechnol* **42**: 84-91.

Sambrook, J., and Russell, D. (2001) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Sauer, U., Canonaco, F., Heri, S., Perrenoud, A., and Fischer, E. (2004) The soluble and membrane-bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of *Escherichia coli*. *J Biol Chem* **279**: 6613-6639.

Schendzielorz, G., Dippong, M., Grünberger, A., Kohlheyer, D., Yoshida, A., Binder, S. et al. (2014) Taking control over control: Use of product sensing in single cells to remove flux control at key enzymes in biosynthesis pathways. *ACS Synth Biol* **3**: 21-29.

Seo, S.W., Kim, D., Szubin, R., and Palsson, B.O. (2015) Genome-wide reconstruction of OxyR and SoxRS transcriptional regulatory networks under oxidative Stress in *Escherichia coli K-12* MG1655. *Cell Rep* **12**: 1289-1299.

Siedler, S., Schendzielorz, G., Binder, S., Eggeling, L., Bringer, S., and Bott, M. (2014) SoxR as a single-cell biosensor for NADPH-consuming enzymes in *Escherichia coli*. *ACS Synth Biol* **3**: 41-47.

Studier, F.W., and Moffatt, B.A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* **189**: 113-130.

Tsaneva, I.R., and Weiss, B. (1990) *soxR*, a locus governing a superoxide response regulon in *Escherichia coli K-12*. *J Bacteriol* **172**: 4197-41205.

Watanabe, S., Kita, A., Kobayashi, K., and Miki, K. (2008) Crystal structure of the [2Fe-2S] oxidative-stress sensor SoxR bound to DNA. *Proc Natl Acad Sci USA* **105**: 4121-4126.

Weckbecker, A., and Hummel, W. (2004) Improved synthesis of chiral alcohols with *Escherichia coli* cells co-expressing pyridine nucleotide transhydrogenase, NADP+-
dependent alcohol dehydrogenase and NAD⁺-dependent formate dehydrogenase.

*Biotechnol Lett* **26**: 1739-1744.

Wu, J., and Weiss, B. (1991) Two divergently transcribed genes, *soxR* and *soxS*, control a superoxide response regulon of *Escherichia coli*. *J Bacteriol* **173**: 2864-2871.
| Strain or plasmid | Relevant characteristics | Source or reference |
|-------------------|-------------------------|---------------------|
| **Escherichia coli** |                         |                     |
| NEB5α             | fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17; strain used for general cloning procedures | New England Biolabs |
| BL21(DE3)         | F- ompT hsdS8 (r-, m-) gal dcm (DE3); parent strain used in this study | (Studier and Moffatt, 1986) |
| BL21(DE3)ΔrseC    | Derivative of BL21(DE3) with an in-frame deletion of rseC, locus-tag ECD_02464 | This study |
| BL21(DE3)Δrsx     | Derivative of BL21(DE3) with an in-frame deletion of rsxABCDGE, locus-tag ECD_01597-ECD_01602 | This study |
| BL21(DE3)ΔrseCΔrsx| Derivative of BL21(DE3) with an in-frame deletion of rseC and rsxABCDGE, locus-tags ECD_02464, and ECD_01597 - ECD_01602 | This study |
| BL21(DE3)ΔsthA    | Derivative of BL21(DE3) with an in-frame deletion of ΔsthA, locus-tag ECD_03847 | This study |
| BL21(DE3)ΔpntAB   | Derivative of BL21(DE3) with an in-frame deletion of ΔpntAB, locus-tags ECD_01571 and ECD_01572 | This study |
| BL21(DE3)ΔsthAΔpntAB | Derivative of BL21(DE3) with an in-frame deletion of ΔsthA and ΔpntAB, locus-tags ECD_03847, ECD_01571 and ECD_01572 | This study |
| **Plasmids**      |                         |                     |
| pSenSox          | AmpR; pBtac-Lbadh derivative containing the soxRS-based NADPH biosensor and the *Lactobacillus brevis* adh gene under control of the tac promoter | (Siedler et al., 2014) |
| pSenNeg          | AmpR; pSenSox derivative with an incomplete *Lbadh* gene preventing synthesis of an active LbAdh | (Siedler et al., 2014) |
| pSIJ8            | AmpR; lambda Red-mediated gene replacement vector expressing lambda Red recombinase and flipase recombinase genes (pKD46, rhaRS-prha-FLP, amp) | Addgene; (Jensen et al., 2015) |
| pKD4             | KanR; template plasmid for FRT-flanked kan cassette needed for lambda Red-mediated gene replacement | Addgene; (Datsenko and Wanner, 2000) |
| pACYC-Duet-1     | CmR; vector for the coexpression of two target genes, each under the control of a separate T7 promoter and associated ribosomal binding site (p15A oriVec 2(P7), lacI) | Merck Millipore |
| pACYC-rseC       | CmR; pACYCDuet-1-derivative for expression of rseC under the control of the T7 promoter | This study |
| pACYC-rsx        | CmR; pACYCDuet-1-derivative for expression of rsxABCDGE under the control of the T7 promoter | This study |
| Plasmid         | Description                                                                 | Reference |
|-----------------|-----------------------------------------------------------------------------|-----------|
| pACYC-rseC-rsx  | Cm<sup>R</sup>; pACYCDuet-1-derivative for expression of *rseC* and *rsxABCDG* under the control of the T7 promoter | This study |
| pACYC-sthA      | Cm<sup>R</sup>; pACYCDuet-1-derivative for expression of *sthA* under the control of the T7 promoter | This study |
| pACYC-pntAB     | Cm<sup>R</sup>; pACYCDuet-1-derivative for expression of *pntAB* under the control of the T7 promoter | This study |
| pACYC-sthA-pntAB| Cm<sup>R</sup>; pACYCDuet-1-derivative for expression of *sthA* and *pntAB* under the control of the T7 promoter | This study |
Figure legends.

Fig. 1. (A) NADPH-dependent reduction of MAA to MHB by the \textit{LbAdh}. (B) Experimental setup used in this work to study the responses of the SoxR-based NADPH biosensor encoded by plasmid pSenSox during whole-cell biotransformation of MAA to MHB. (1) \textit{E. coli} BL21(DE3) carrying pSenSox was cultivated in shake flasks with a starting \textit{OD}_{600} of 0.05. (2) If desired, IPTG was added to the cultures at an \textit{OD}_{600} between 0.6 and 0.8. In the absence of IPTG, basal expression of the \textit{Lbadh} gene by the \textit{tac} promoter is sufficient for \textit{LbAdh} synthesis and biotransformation of MAA to MHB. However, IPTG can be added to maximize \textit{Lbadh} expression. (3) To ensure that enough biomass is formed for the biotransformation, the cultures were grown for at least 5 h until an \textit{OD}_{600} of 5 or higher was reached. (4) For the biotransformation, 800 \textmu L of the culture with an \textit{OD}_{600} adjusted to 5 were transferred into a Flowerplate (m2p-labs, Baesweiler, Germany) and the biotransformation was started by the addition of 100 \textmu L MAA at the desired concentration to these cultures. To study the effect of redox-cycling drugs either 100 \textmu L paraquat or 100 \textmu L menadione were added to the cultures at the desired concentration. (5) The change in eYFP fluorescence and biomass was monitored for around 24 h with a BioLector microcultivation system that enables online recording of eYFP fluorescence (excitation at 485 nm, emission at 520 nm) and biomass gain (change in cell density measured as backscattered light at 620 nm (Kensy et al., 2009). The specific fluorescence over the time course of 24 h corresponds to the ratio of absolute fluorescence/backscatter.

Fig. 2. Influence of different media on the NADPH biosensor response during biotransformation of MAA to MHB using the experimental setup shown in Fig.1. The changes in fluorescence and biomass were followed during the biotransformation of MAA to MHB by the NADPH-dependent alcohol dehydrogenase of \textit{L. brevis} (\textit{LbAdh}) using \textit{E. coli} BL21(DE3)/pSenSox and \textit{E. coli} BL21(DE3)/pSenNeg cultures cultivated either in M9 mineral medium supplemented with 0.4% (w/v) glucose (A and B), or in LB medium (C and D), or in
2xTY medium (E and F), or in TB medium (G and H). Mean values and standard deviations of three independent biological replicates are shown. The values were normalized to the maximal backscatter value measured in TB medium and to the maximal specific fluorescence measured in 2xYT medium, which were set as 1.0.

**Fig. 3.** Influence of different concentrations of the redox-cycling drugs paraquat (PQ) and menadione (MD) on the NADPH biosensor response using *E. coli*/pSenSox. The changes in fluorescence were followed after the addition of either PQ (A) or MD (B) over a time period of 24 h. The experimental setup shown in Fig. 1 was used for all cultivations in 2xTY medium containing 100 μg mL\(^{-1}\) carbenicillin. No MAA was added in these experiments. Mean values and standard deviations of three independent biological replicates are shown.

**Fig. 4.** Comparison of the NADPH biosensor response (A, C) and cell density (B, D) of *E. coli* BL21(DE3) and its ΔrseC, Δrsx, and ΔrseCΔrsx mutants during biotransformation of 30 mM MAA to MHB (panels A and B) using the standard experimental setup shown in Fig. 1, and in the presence of 5 μM paraquat instead of MAA (panels C and D). Mean values and standard deviations of three independent biological replicates are shown.

**Fig. 5.** Comparison of the NADPH biosensor response (A) and cell density (B) of *E. coli*/pSenSox and its ΔpntAB, ΔsthA, and ΔsthAΔpntAB mutants during biotransformation of MAA (30 mM) to MHB using the standard experimental setup shown in Fig. 1. Mean values and standard deviations of three independent biological replicates are shown.
Appendix 1

Influence of different media on the NADPH biosensor response (A) and cell density (B) of *E. coli*/*pSenSox* during biotransformation of 70 mM MAA to MHB. The strain was cultivated in either M9 mineral medium with 0.4% (w/v) glucose, or in LB medium, or in 2xTY medium, or in TB medium. The experimental setup shown in Fig. 1 was used for all cultivations in one of the media mentioned above containing 100 μg mL$^{-1}$ carbenicillin. Mean values and standard deviations of three independent biological replicates are shown.

Appendix 2

Growth behavior of the mutants *E. coli* ΔrseC, *E. coli* Δrsx and *E. coli* ΔrseCΔrsx in comparison to the parental strain *E. coli* BL21(DE3). The strains were cultivated in 50 mL 2xTY medium in 500 mL shake flasks at 30°C and 130 rpm and growth was monitored as OD$_{600}$. Mean values and standard deviations of three independent biological replicates are shown.
Appendix 3

Complementation studies of the ΔrseC, Δrsx, and ΔrseCΔrsx mutants carrying pSenSox using plasmids pACYC-rseC, pACYC-rsx, and pACYC-rseC-rsx. For comparison, the parent strain carrying pSenSox and pACYCDuet-1 was used. The experiment was performed according to the standard setup shown in Fig. 1 with 30 mM MAA. IPTG was not added to the cultures as it had a negative effect on growth. Mean values and standard deviations of three independent biological replicates are shown.
Appendix 4

Growth (OD$_{600}$) of the transhydrogenase mutants $\Delta$pntAB, $\Delta$sthA and $\Delta$sthA$\Delta$pntAB compared to the parental strain $E.$ coli BL21(DE3) (A) and complementation of the $\Delta$sthA and $\Delta$sthA$\Delta$pntAB strains (B). The strains were cultivated at 30°C and 130 rpm in 500 mL shake flasks with 50 mL of 2xTY medium. Complementation studies were performed in the presence of 0.1 mM IPTG and 34 μg mL$^{-1}$ chloramphenicol. Mean values and standard deviations of three independent biological replicates are shown.

Appendix 5

Complementation studies of the $\Delta$pntAB and $\Delta$sthA mutants carrying plasmid pSenSox with plasmids pACYC-pntAB or pACYC-sthA. For comparison, the parent strain and the mutants carrying pSenSox and pACYCDuet-1 were used. The experiment was performed according to the standard setup shown in Fig. 1 with 30 mM MAA. Mean values and standard deviations of three independent biological replicates are shown.
### Appendix 6

Oligonucleotides used in this study.

| Oligonucleotide | Sequence (5' → 3') and properties |
|-----------------|-----------------------------------|
| rseC fw          | GAAATGTTCATACCGTATGGATTTGATCTGGAAACGCCTTC GCATTGTGGAAGAAGCACGTCTTC |
| rseC rv          | GCCGAAATCACATTGTCGATGGAAACTCCGCAGCAACACGCG AAACGCATTGGCCAGAGAATATTAAGTTCGGCCAGCGCA ATGGGAATTAGCAGCGGTCTTC |
| rsxABCDGE fw     | GTAAACTACTACGTATAGGAGCTGCTGGCTGCTGCTGACTAGG CAAACCATCATCAAATACCCGGATGGAAGGGAATATC GTGTAGGCGCTGCTGCTTC |
| rsxABCDGE rev    | ATGGGAATTAGCCATGGTCC |
| pntAB fw         | ATTTAGCTGTACGTAGACGGTTGCTGCTGCTGACTAGG CAAACCATCATCAAATACCCGGATGGAAGGGAATATC GTGTAGGCGCTGCTGCTTC |
| pntAB rv         | ATGGGAATTAGCCATGGTCC |
| sthA fw          | TGGACGGGATCAAATGCGCTTACCGCGATTTGACACAGG CTGGTGCTTATATGGTAGCTATACGGCTGCTGCTGCTGCTGCTGCTTC |
| sthA rv          | ATGGGAATTAGCCATGGTCC |

Oligonucleotides used for colony-PCRs and sequencing of plasmids.

| ΔrseC verification fw | GACGGTCGGCGGTATGGAATGGG |
| ΔrseC verification rev | GACGGTCGGCGGTATGGAATGGG |
| ΔrsxABCDGE verification fw | GTTGGAACCCGCAATTGTCGCTTC |
| Delta A | Verification | Sequence |
|---------|--------------|----------|
| ΔrsxABCDGE | rv | CCCGCCAATTGAGTACG |
| ΔsthA | fw | TGCTGCGTGAGGTGAAAGTC |
| ΔsthA | rv | AGTCGCAAGTCCGCAATG |
| ΔpntAB | fw | GCTAAATGACCGTCTATGC |
| ΔpntAB | rv | GATTGCTGGCCTTTCGCTT |
| pACYCDuet-1 MCS_1 | sequencing fw | CAGCAGCCATCACCATCATC |
| pACYCDuet-1 MCS_2 | sequencing fw | CGAAATTAATACGACTCACTATAGG |