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Pathway for H_2O_2 and O_2 detoxification in *Clostridium acetobutylicum*

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An unusual non-haem diiron protein, reverse rubrerythrin (revRbr), is known to be massively upregulated in response to oxidative stress in the strictly anaerobic bacterium *Clostridium acetobutylicum*. In the present study both *in vivo* and *in vitro* results demonstrate an H_2O_2 and O_2 detoxification pathway in *C. acetobutylicum* involving revRbr, rubredoxin (Rd) and NADH : rubredoxin oxidoreductase (NROR). RevRbr exhibited both NADH peroxidase (NADH : H_2O_2 oxidoreductase) and NADH oxidase (NADH : O_2 oxidoreductase) activities in *in vitro* assays using NROR as the electron-transfer intermediary from NADH to revRbr. Rd increased the NADH consumption rate by serving as an intermediary electron-transfer shuttle between NROR and revRbr. While H_2O_2 was found to be the preferred substrate for revRbr, its relative oxidase activity was found to be significantly higher than that reported for other Rbrs. A revRbr-overexpressing strain of *C. acetobutylicum* showed significantly increased tolerance to H_2O_2 and O_2 exposure. RevRbr thus appears to protect *C. acetobutylicum* against oxidative stress by functioning as the terminal component of an NADH peroxidase and NADH oxidase.

INTRODUCTION

Reactive oxygen species (ROS) are formed when molecular oxygen diffuses into cells and is adventitiously reduced at the active sites of redox enzymes containing flavins or quinones (Valentine *et al.*, 1998; Imlay, 2003). Aerobic micro-organisms scavenge these toxic ROS via the superoxide dismutase (SOD)/catalase system. In anaerobes this system sometimes seems to be absent or incomplete (Imlay, 2002; Dolla *et al.*, 2006). Thus, in the genome of the strict anaerobe *Clostridium acetobutylicum*, no catalase was annotated (Nölling *et al.*, 2001), and a function has not yet been demonstrated for the annotated SODs. Regeneration of molecular oxygen by the SOD/catalase system may be a disadvantage for anaerobes. Nevertheless, *C. acetobutylicum* survives short periods of aeration (O'Brien & Morris, 1971), and therefore an alternative detoxification system must exist. Interestingly, all genes for a superoxide reductase (SOR) and peroxidase-dependent detoxification pathway for ROS proposed for *Desulfovibrio vulgaris* (Lumppio *et al.*, 2001) are also present in clostridial species. The purified *C. acetobutylicum* SOR homologue, desulfoferrodoxin (Dfx), was shown to be capable of functioning in superoxide reduction (Riebe *et al.*, 2007). In other bacteria and archaea, rubrerythrin (Rbr) has been assigned a role as a peroxidase (NAD(P)H : H_2O_2 oxidoreductase) in this alternative pathway (Coulter *et al.*, 1999; Weinberg *et al.*, 2004; Kurtz, 2006). A *Porphyromonas gingivalis rbr* mutant showed increased sensitivity to ROS (Sztukowska *et al.*, 2002) and reduced virulence in mice (Mydel *et al.*, 2006). A putative SOD activity attributed to *Clostridium perfringens* Rbr (Lehmann *et al.*, 1996) may be an artefact (Coulter *et al.*, 1999; Fournier *et al.*, 2003), and, in any case, is much lower than those of classical SODs.

Rbrs are characterized by a unique combination of a non-sulfur, oxo-bridged diiron site similar to that of ferritin and a rubredoxin-like $[Fe(SCys)_4]$ domain (Kurtz, 2006). The evidence indicates that during Rbr's peroxidase function, the reduced diiron site reacts directly with hydrogen peroxide, while the $[Fe(SCys)_4]$ site transfers electrons from an exogenous donor to the oxidized diiron site. The reactivity of Rbrs with dioxygen is typically low relative to that with hydrogen peroxide.

Interestingly, the *C. acetobutylicum* genome encodes four proteins homologous to Rbrs. The arrangement of the domains in two of these Rbrs is the same as that of most other known 'classical' Rbrs, with an N-terminal diiron domain and a C-terminal $[Fe(SCys)_4]$ domain. However, in

Abbreviations: Dfx, desulfoferrodoxin; FNR, ferredoxin-NADP$^+$ reductase; HRP, horseradish peroxidase; NROR, NADH : rubredoxin oxidoreductase; Rd, rubredoxin; revRbr, reverse rubrerythrin; ROS, reactive oxygen species; SOD, superoxide dismutase.

A supplementary figure is available with the online version of this paper.

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the other two *C. acetobutylicum* Rbrs, the positions of these domains are reversed. These latter have, therefore, been named 'reverse' Rbrs (revRbrs) (May *et al.*, 2004). The 99 % identical open reading frames of the two *C. acetobutylicum* revRbrs, *cac3597* (*rbr3B*) and *cac3598* (*rbr3A*), are in adjacent positions in the genome and form an operon. In previous studies, we demonstrated an increased expression of the revRbrs during oxidative or heat stress (May *et al.*, 2004). Kawasaki *et al.* (2007) showed that recombinant, purified, reduced revRbr could be oxidized by hydrogen peroxide and that cell extracts from microxically grown *C. acetobutylicum* could catalyse reduction of as-isolated (partially oxidized) revRbr by NAD(P)H. However, they did not identify the NAD(P)H : revRbr oxidoreductase, nor did they report any *catalytic* activities of revRbr.

We now present new *in vitro* and *in vivo* data demonstrating that revRbr functions as the terminal component of an NADH peroxidase (NADH : H_2O_2 oxidoreductase) and an NADH oxidase (NADH : O_2 oxidoreductase) in *C. acetobutylicum*. We also compare these revRbr activities with those of one of the *C. acetobutylicum* Rbrs, RubY, which has the 'classical' Rbr domain structure, and which is apparently not upregulated upon oxidative stress. These findings demonstrate a key role for revRbr in oxidative stress tolerance of *C. acetobutylicum*, including the aerotolerance of a recently described Δ*perR* mutant (Hillmann *et al.*, 2008).

METHODS

Reagents and proteins. Reagents and buffers were at least analytical grade. Ferredoxin-NADP$^+$ reductase (FNR) from spinach, horseradish peroxidase (HRP), glucose oxidase and *E. coli* iron superoxide dismutase (Fe-SOD) were purchased from Sigma. Concentrations were assumed to be those provided by Sigma. 10-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red) was from Synchem OHG. Reagents were used without further purification.

Construction of Rd, NROR, RubY and revRbr expression plasmids and transformations. *Clostridium acetobutylicum* ATCC 824 genomic DNA was isolated according to Bertram & Dürre (1989). *C. acetobutylicum* Rd was cloned and purified as described in our previous work (Riebe *et al.*, 2007). The *rbr3B* (*cac3597*, encoding revRbr), *rubY* (*cac2575*) and *nror* (*cac2448*) genes were amplified using the following oligonucleotides: Rbr_Fw_*Bam*HI (5′-GAAAGGATCCATGAAAAAATTTAAATGTGTTG-3′), Rbr_Rev_*Xma*I (5′-TTATCCCGGGTTTGAAATATCTGTTTAATAAAC-3′), RubY_Fw_*Bam*HI (5′-GAGGGGATCCATGAAATCACTTAAAGG-3′), RubY_Rev_*Xma*I (5′-CTTATCCCGGGATAGTTTTCACTTAATATTTC-3′), NROR_Fw_*Bam*HI (5′-GATGGGATCCATGAAAAGCACAAAAATTTT-3′) and NROR_Rev_*Xma*I (5′-TAAATTCCCGGGTAAATTATTTAATATTGCATC-3′), introducing a *Bam*HI or a *Xma*I restriction site. The *rbr3B*, *rubY* and *nror* fragments were cloned into a pThyd*A* vector (Girbal *et al.*, 2005). The resulting plasmids pTrbr3B, pTrubY and pTnror were transformed into *Escherichia coli* DH5α. These strains were used for overexpression of the respective proteins.

These same plasmids could also be used for overexpression of the proteins in *C. acetobutylicum*. For transformation in *C. acetobutylicum*, plasmids were reisolated and subcloned into a methylation strain, *E. coli* ER2275 (Bermejo *et al.*, 1998)/pANI (Mermelstein & Papoutsakis, 1993). The methylated plasmids were electroporated into *C. acetobutylicum* ATCC 824 as follows. Cultures (100 ml) were grown in clostridial growth medium (CGM, Roos *et al.*, 1985) at 37 °C to OD_{600} 0.8–1.0 in anaerobic serum flasks and then harvested with SS34 tubes in an anaerobic chamber. Cells were centrifuged at 5000 ***g*** for 1 min and then washed once with 10 ml electroporation buffer containing 270 mM sucrose and 200 mM phosphate at pH 7.4. After a second centrifugation step the pellet was suspended in 2 ml prechilled electroporation buffer. One microgram of DNA was used for anaerobic electroporation of 400 µl competent cells in a 4 mm electroporation cuvette. Electroporation conditions were 2 kV, 50 µF, 600 Ω. After electroporation, 600 µl fresh medium was added and cells were regenerated for 3 h at 37 °C. Then 200 µl aliquots were plated on reinforced clostridium agar (RCA) plates supplemented with erythromycin (20 mg l^{-1}).

Protein expression and purification. The thiolase (*thl*) promoter in the pThyd*A* vector is active in both Gram-positive and Gram-negative organisms. Proteins could, therefore, be expressed in either *E. coli* DH5α or *C. acetobutylicum* strains using the same vector constructs.

E. coli DH5α overexpression cultures were grown aerobically in 500 ml Luria–Bertani broth supplemented with 100 µg ampicillin ml^{-1} at 30 °C under continuous shaking for 18 h. A higher level of iron incorporation into revRbr and Rd was achieved by addition of 40 µg $FeSO_4.7H_2O$ ml^{-1} to the cultures (Coulter & Kurtz, 2001). Cultures were harvested at 8000 ***g*** for 10 min and pellets were suspended in 5 ml buffer W (100 mM Tris/HCl, 150 mM NaCl, pH 8.0) without EDTA. Disruption of the cells was achieved by ultrasonication under anaerobic conditions in a MAC 5000 anaerobic chamber. The lysates were centrifuged for at least 30 min at 12 000 ***g*** at 4 °C in anaerobic SS34 tubes. The resulting crude extract was loaded onto columns containing a Strep-Tactin-Sepharose matrix. Further purification steps were carried out as described in the IBA standard protocol under anaerobic conditions (Schmidt & Skerra, 2007). Protein concentrations were determined using the Bradford assay (Bradford, 1976). Purity of the elution fractions was analysed by SDS-PAGE (Laemmli, 1970).

Analytical gel filtration. Analytical gel filtration was performed using a Superose 12 10/300 GL (GE Healthcare) by loading 100 µl revRbr (10 mg ml^{-1}) and developed using a 0.5 ml min^{-1} isocratic flow of buffer containing 50 mM MOPS and 250 mM NaCl pH 7.3. Molecular mass was estimated by comparing retention time to that of known standards in the LMW Gel Filtration Calibration kit (GE Healthcare).

Response to oxidative stress. All experiments were carried out at 37 °C. Ten-millilitre cultures of either wild-type or pTrbr3B-transformed (revRbr-overexpressing) *C. acetobutylicum* were grown anaerobically to OD_{600} 0.8–1.0 in CGM. Medium for the revRbr overexpression strain was supplemented with 20 mg erythromycin l^{-1}. Aliquots (1 ml) of each culture were treated anaerobically with H_2O_2 (at concentrations of 0.125, 0.25, 0.375, 0.5, 0.625, 0.75, 0.875 or 1.0 mM) for 30 min, then plated. Another set of 1 ml aliquots were flushed with air for 15 min, then returned to the anaerobic chamber and plated after an additional 15 min. A further 1 ml aliquot of each culture was treated anaerobically only with 50 µl 50 mM Tris/HCl pH 7.5. Aliquots (25 µl) of the treated 1 ml cultures were plated on RCA containing 2.5 µg erythromycin ml^{-1} for the revRbr overexpression strain or no antibiotic for the wild-type strain. The plates were incubated for 2–4 days at 37 °C and c.f.u. were then counted. C.f.u. from plates of the Tris/HCl buffer-treated cultures were taken as the 100 % survival reference.

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Redox-dependent spectral changes of revRbr. Reduction of oxidized (as-isolated) revRbr by a mixture of NROR, Rd and NAD(P)H was monitored spectrophotometrically. Conditions are listed in the figure legends.

For oxidation of reduced revRbr, the purified revRbr was reduced by an equimolar amount of sodium dithionite under anaerobic conditions. This pretreated revRbr (0.1 mM) was then incubated in anaerobic 50 mM MOPS and 0.1 mM EDTA pH 7.0 with 0.5 mM H_2O_2 or diluted in aerobic MOPS buffer.

NAD(P)H peroxidase/oxidase activities. These assays were modified from that described by Coulter & Kurtz (2001) using either *C. acetobutylicum* NROR or FNR from spinach plus Rd as electron donors to revRbr. In some cases direct reaction of NROR with revRbr was tested without addition of Rd. The activities were measured at room temperature by rates of either NADH (with NROR) or NADPH (with FNR) consumption as the decrease in absorbance at 340 nm (ε_{340} 6220 M^{-1} cm^{-1}). All reagents and proteins were added from anaerobic stock solutions into a 400 μl cuvette. The reaction mix contained 0.25 mM H_2O_2, 0.1 mM NAD(P)H, 1 μM FNR or NROR in 50 mM MOPS and 0.1 mM EDTA at pH 7.0. After 30 s *C. acetobutylicum* Rd (1 μM, Bradford) and after a further 30 s revRbr was added at various concentrations (Bradford) to this premix. NAD(P)H oxidase activities of revRbr were measured analogously, but in aerobic buffer without added H_2O_2. Further variations are described in the text and figure legends. All reactions were also tested using RubY instead of revRbr.

H_2O_2 consumption. In the presence of H_2O_2, HRP oxidizes 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) to the coloured resorufin. The assay procedure was adapted from that described by Seaver & Imlay (2001) as follows. One hundred microlitres of a 0.2 mM stock solution of Amplex Red in 50 mM potassium phosphate pH 7.8 was added to 250 μl of the sample mix. In the assay a calibrated flux of H_2O_2 was produced by the glucose/glucose oxidase reaction (as used for a peroxidase assay described by Smith *et al.*, 1974). The composition of the sample mix was as follows: 200 μl 50 mM Tris/HCl buffer at pH 7.0 containing 2 μg glucose oxidase ml^{-1}, 0.1 mM NADPH and 1 μM FNR. After 30 s, 10 μl of a 0.2 mg ml^{-1} HRP stock solution in a buffer of 50 mM MOPS and 0.1 mM EDTA at pH 7.0 was added, and after a further 30 s, 1 μM Rd. After addition of HRP a small increase of absorbance occurred. Purified revRbr was added to various concentrations after a constant baseline was obtained. To start the H_2O_2 generation, 10 μl of a glucose stock solution containing 0.15 M glucose, 0.1 M NH_4Cl and 0.1 M $CaCl_2$ was added to the reaction mix. H_2O_2 consumption by the peroxidase activity of revRbr was measured as a decreased H_2O_2 generation by the glucose/glucose oxidase reaction. In some cases the reaction was started by addition of HRP. Production of resorufin was followed by the increase in absorbance at 560 nm.

O_2 consumption. O_2 consumption during the revRbr reactions was measured at room temperature using a Fibox 3 Single Channel Fiber Optic oxygen meter (PreSens Precision Sensing). The premix contained all components listed above for the NAD(P)H peroxidase assay except H_2O_2. Protein components were added at 2 min intervals with revRbr added last. The reaction buffer was aerobic 50 mM MOPS with 0.1 mM EDTA at pH 7.0. The total volume of the mix was 4 ml. In some cases 0.3 mM H_2O_2 was also added to the premix prior to addition of revRbr.

O_2 consumption rates were measured for whole-cell suspensions of the revRbr overexpression and wild-type strains of *C. acetobutylicum* as follows. Ten-millilitre cultures were grown as described above, and 450 μl aliquots were suspended with 50 μl 37 % formaldehyde for cell counting. Then 4.5 ml aliquots were flushed with air for 5 min and loaded into the cuvette of an optode. The cuvette was sealed, and O_2 consumption was followed at room temperature using the Fiber Optic oxygen meter. Fresh CGM was used as a control. O_2 concentrations of 280–320 μM were taken as saturated solutions. This concentration was reached 1–2 min after start of the measurement due to the breadboard construction. Consumption rates were calculated as fmol h^{-1} per cell.

RESULTS

Isolation and purification of *C. acetobutylicum* revRbr, RubY, NROR and Rd

Rd, revRbr, RubY and NROR were expressed in *E. coli* or in *C. acetobutylicum* and purified via Strep-Tactin column chromatography (Fig. 1); 5 mg revRbr, 1 mg RubY, 3.5 mg Rd and up to 1.5 mg NROR were obtained from 500 ml *E. coli* culture volumes.

Visible absorption spectra (330–800 nm) of the purified revRbr, Rd and NROR are shown in Fig. 2. Spectra of the completely air-oxidized forms of revRbr and Rd showed similar spectral features, with absorption maxima at 350, 375, 492 and 565 nm (Fig. 2, spectra a and b). The peak at 492 nm and shoulder at 565 nm are characteristic of the $[Fe(SCys)_4]$ sites in Rds and in Rbrs (Gupta *et al.*, 1995; Jin *et al.*, 2004a, b). The higher absorption in the range 330–400 nm of revRbr compared to that of Rd is due to the additional oxo-bridged diferric site in the former protein, as can be seen in the difference absorption spectrum (Fig. 2, inset II). The absorption spectra thus indicated that the respective centres of the purified proteins are occupied with iron and should, therefore, be functional. Both

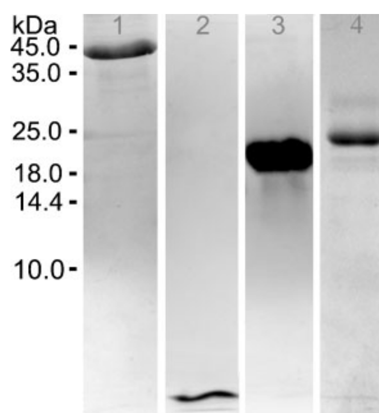

Fig. 1. SDS-PAGE of purified proteins. The proteins were overexpressed in *E. coli* DH5α and purified via Strep-Tactin-affinity chromatography. The purified proteins were run on a 20 % SDS-PAGE gel, which was then stained with Coomassie brilliant blue. Lanes: 1, 10 μg NROR (calculated mol. mass 42.175 kDa, estimated 44 kDa); 2, 10 μg Rd (calculated mol. mass 6.819 kDa, estimated 7 kDa); 3, 20 μg revRbr (calculated mol. mass 21.576 kDa, estimated 21 kDa); 4, 10 μg RubY (calculated mol. mass 23.047 kDa, estimated 23 kDa).

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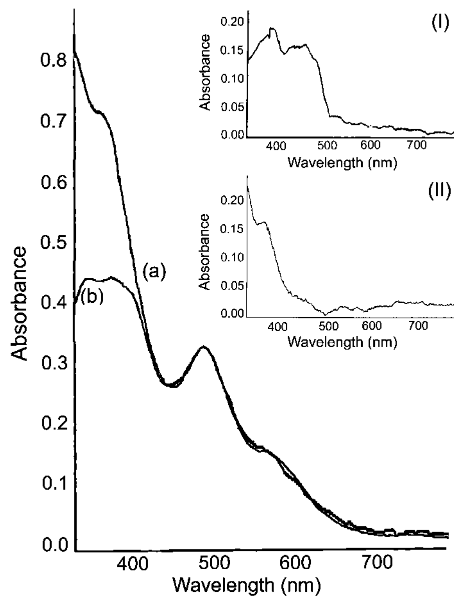

Fig. 2. Absorption spectra of *C. acetobutylicum* revRbr, Rd and NROR. (a) Oxidized *C. acetobutylicum* revRbr; (b) *C. acetobutylicum* Rd. Insets: (I) spectrum of *C. acetobutylicum* NROR; (II) revRbr (a) minus Rd (b) difference spectrum. Spectra were normalized to equal absorbance at 492 nm prior to subtraction.

recombinant proteins, Rd and revRbr, were completely reducible by addition of equimolar sodium dithionite under anaerobic conditions, as indicated by the disappearance of their visible absorption features (data not shown). Cell lysates of *E. coli* or *C. acetobutylicum* overexpressing these proteins as well as purified protein fractions showed a red colour characteristic of the oxidized [Fe(SCys)$_4$] sites. The colour intensity increased after exposure to air and, in cell lysates, decreased again after a few minutes. This latter colour regeneration decreased after repeated mixing of the cell lysates with air.

Protein fractions containing NROR had a yellow colour, and showed absorption maxima at 380 and 450 nm and a shoulder at 490 nm (Fig. 2, inset I) characteristic of a flavin. These results are in agreement with previous studies on NROR (Guedon & Petitdemange, 2001).

Analytical gel filtration experiments indicated that the native molecular mass of as-isolated revRbr is ~80 kDa (data not shown). Based on the calculated monomer molecular mass for the revRbr construct, 21 576.5, these results are consistent with a tetrameric oligomer for revRbr.

NAD(P)H peroxidase/oxidase activities of *C. acetobutylicum* revRbr

According to the activities of other Rbrs (Coulter *et al.*, 1999; Coulter & Kurtz, 2001; Weinberg *et al.*, 2004), *C. acetobutylicum* revRbr should be able to function as the terminal component of an NAD(P)H peroxidase and/or oxidase, transferring electrons to H_2O_2 and/or O_2 via an NAD(P)H oxidoreductase. We have reconstituted such a pathway using heterologously expressed and purified *C. acetobutylicum* revRbr, Rd and NROR. NADH consumption during the reaction was monitored via decreased absorption at 340 nm. In the reconstituted pathway NADH consumption was obtained with O_2, as well as with H_2O_2 as a substrate (Fig. 3, time-courses a and c). In both cases NROR alone was able to transfer the electrons to revRbr (time-courses b and c). Addition of 1 μM Rd doubled the reaction rates of both H_2O_2 and O_2 consumptions (Fig. 3, time-courses b and c). Addition of H_2O_2 to an aerobic reaction slightly enhanced the reaction rate (Fig. 3, time-course c). No consumption of NADH occurred when either NROR or revRbr was omitted from an otherwise complete assay mixture. The specific NADH peroxidase and NADH oxidase activities of revRbr of *C. acetobutylicum* are given in Table 1.

NADH and *C. acetobutylicum* NROR could be replaced by NADPH and spinach FNR for the revRbr peroxidase/oxidase activities. FNR has been previously used in comparable systems of other bacteria (Coulter *et al.*, 1999; Coulter & Kurtz, 2001; Riebe *et al.*, 2007). Thus, *C. acetobutylicum* Rd could be reduced by spinach FNR using NADPH as an electron donor. However, in contrast to NROR, Rd was absolutely necessary for the peroxidase/oxidase activity when using FNR. As shown in Fig. 4, NADPH consumption was observed only when FNR, Rd and revRbr were all present in the reaction mix; no consumption of NADPH occurred in either the aerobic oxidase (time-course a) or the anaerobic peroxidase (time-

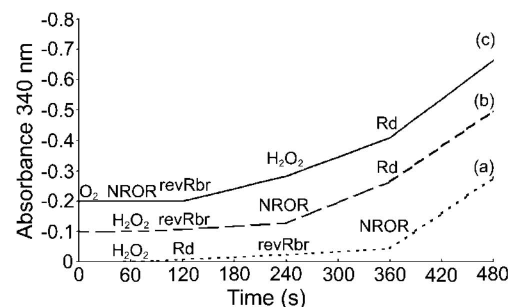

Fig. 3. NADH oxidase/peroxidase activity of revRbr using Rd and NROR. Activities were monitored at room temperature as the decrease in absorbance at 340 nm due to NADH consumption. In the premix 0.1 mM NADH was supplemented to the anaerobic reaction buffer of 50 mM MOPS and 0.1 mM EDTA at pH 7.0. All proteins (1 μM) and H_2O_2 (0.25 mM) were added from anaerobic stock solutions at the indicated time points. Absorbance spikes caused by addition of the various components are omitted. (a) Anaerobic NADH peroxidase activity initiated by addition of NROR; (b) doubling of anaerobic NADH peroxidase activity by addition of Rd; (c) aerobic NADH oxidase and peroxidase activities.

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Table 1. NADH peroxidase and NADH oxidase activities of *C. acetobutylicum* and purified proteins

Enzyme activities were measured at room temperature in 50 mM MOPS buffer supplemented with 0.1 mM EDTA at pH 7.0 containing 0.1 mM NADH and also 1 μM of the respective proteins or 100 μg of the cell lysates. One unit was defined as the amount of protein that causes the oxidation of 1 μmol NADH in 1 min.

Source/activity	NADH oxidase* [mU (mg protein)$^{-1}$]	NADH peroxidase† [mU (mg protein)$^{-1}$]
C. acetobutylicum wild-type cell lysate	16 ± 2	17 ± 2
C. acetobutylicum pTrbr3B cell lysate	24 ± 3	24 ± 2
NROR/RubY	17 ± 2	647 ± 6
NROR/Rd/RubY	137 ± 10	952 ± 18
NROR/revRbr	420 ± 21	660 ± 22
NROR/Rd/revRbr	644 ± 46	993 ± 16

*NADH oxidase activity was measured in aerobic buffer and is per mg of total protein (cell lysates) or of revRbr (purified proteins).

†NADH peroxidase activity was measured in anaerobic buffer containing 0.25 mM H_2O_2 and is per mg of total protein (cell lysates) or of revRbr (purified proteins).

course c) activities if any one of the proteins was omitted from the assay mixture. Generation of H_2O_2 by glucose/glucose oxidase reaction in aerobic solutions increased the NADPH consumption rate over that of the O_2-only reactions (Fig. 4, time-course b). Time-course (c) in Fig. 4 shows that when a small amount (25 μM) of H_2O_2 was added to the anaerobic reaction mixture, NADPH consumption ceased when the H_2O_2 was consumed. A second anaerobic addition of the same amount of H_2O_2 resulted in the same rate and extent of NADPH consumption as did the first addition. Subsequent aeration of the reaction mix by vortexing resulted in further consumption of NADPH, confirming the oxidase and peroxidase activities under these conditions. The NAD(P)H peroxidase activity of revRbr was found to be in the same order of magnitude as reported for other Rbrs (Coulter *et al.*, 1999; Coulter & Kurtz, 2001).

NADH peroxidase and NADH oxidase activities of *C. acetobutylicum* RubY

The two *C. acetobutylicum* genes that encode Rbr homologues with the 'classical' diiron and [Fe(SCys)$_4$] domain structure (*rubY* and *cac3018*) are not induced under oxidative stress conditions (Hillmann *et al.*, 2006). We nevertheless overexpressed and purified one of these classical Rbrs, RubY, and tested its oxidase and peroxidase activities. The purified RubY was found to be a homodimer under non-denaturing conditions (data not shown) and exhibited peroxidase activity comparable to that of revRbr (Table 1). As was the case for revRbr, Rd was not necessary for RubY's peroxidase activity, but the presence of Rd increased this activity. However, the oxidase activity of RubY was very low compared to that of revRbr. With NROR in the absence or presence of Rd the NADH oxidase activity of RubY was only ~4 % or ~21 %, respectively, of the corresponding revRbr NADH oxidase activities.

H_2O_2 is the preferred substrate of revRbr

In addition to the consumption of NAD(P)H due to the activity of revRbr, the consumption of O_2 and H_2O_2 was also followed. HRP catalyses oxidation of Amplex Red to the dye resorufin, by an H_2O_2 flux generated via glucose/glucose oxidase (Seaver & Imlay, 2001; Smith *et al.*, 1974). As shown in Fig. 5, this oxidation was inhibited when revRbr was added to an assay mixture also containing NADPH, FNR and Rd, consistent with consumption of

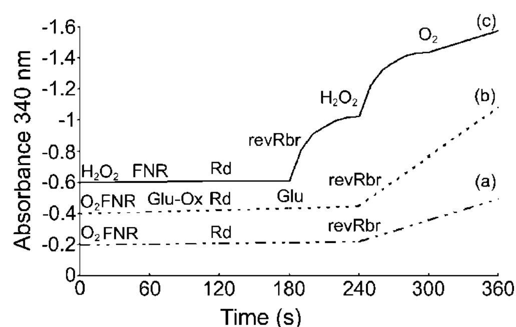

Fig. 4. NADPH oxidase/peroxidase activity of revRbr using Rd and FNR. The activities were monitored at room temperature as the decrease in absorbance at 340 nm due to NADPH consumption. In the premix 0.1 mM NADPH was supplemented to the anaerobic reaction buffer of 50 mM MOPS and 0.1 mM EDTA at pH 7.0. All proteins (1 μM) were added from anaerobic stock solutions at indicated time points. Absorbance spikes caused by mixing and addition of the various components are omitted. (a) NADPH oxidase activity; (b) NADPH consumption in aerobic buffer under constant H_2O_2 flux produced by glucose/glucose oxidase; (c) NADPH consumption in anaerobic buffer with addition of low concentrations (25 μM) of H_2O_2 and air (by mixing) at indicated time points. Glu-Ox, glucose oxidase; Glu, glucose.

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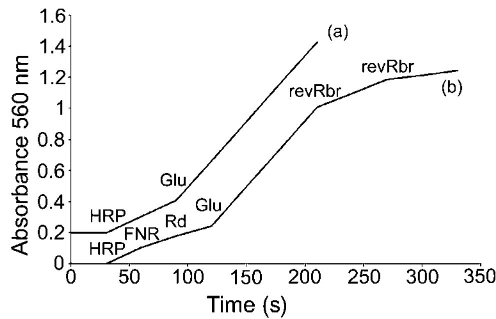

Fig. 5. H_2O_2 generation is inhibited by revRbr. H_2O_2 generation due to glucose/glucose oxidase reaction was measured by the Amplex Red/HRP system. Generation of resorufin was measured at 560 nm. (a) Control reaction containing only Amplex Red/HRP and glucose/glucose oxidase; (b) generation of H_2O_2 is inhibited by addition of revRbr to a reaction mix containing also Rd, FNR and NADPH. Glu, glucose.

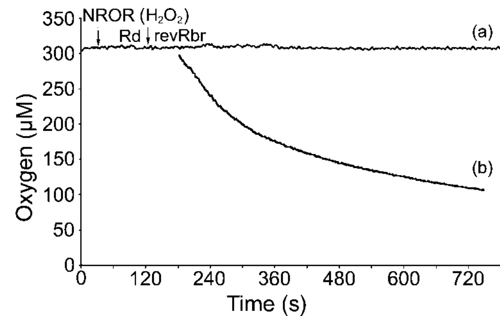

Fig. 6. H_2O_2 inhibits O_2 consumption by revRbr. O_2 consumption rates were measured directly using an O_2 consumption meter in aerobic reaction mixtures containing 0.1 mM NADH, 1 µM NROR and 0.1 mM EDTA in 50 mM MOPS, pH 7.0. Rd and revRbr (both dithionite reduced and 1 µM) were added at the indicated time points. (a) H_2O_2 was added to a concentration of 0.25 mM at the time indicated by the arrow; (b) no H_2O_2 was added to the reaction mixture.

H_2O_2. This inhibition was proportional to the concentration of revRbr (Fig. 5, time-course b).

Addition of revRbr (1 µM) to an aerobic premix containing NROR (1 µM), NADH (0.1 mM) and Rd (1 µM) caused a decrease of the dissolved O_2 with a maximal activity of 12.5 µM O_2 per µM revRbr per min (Fig. 6, time-course b). However, addition of 0.25 mM H_2O_2 to an aerobic assay mixture followed by addition of revRbr resulted in no measurable O_2 consumption (Fig. 6, time-course a). This inhibition of O_2 consumption by H_2O_2 coupled with the increase in NADH consumption when H_2O_2 is generated in the presence of O_2 (Fig. 4, time-courses a vs b) means that H_2O_2 outcompetes O_2 as a substrate for revRbr.

Reduction of revRbr by NAD(P)H and reoxidation by H_2O_2

Fig. 7 represents a reductive titration of revRbr. Oxidized revRbr showed a characteristic absorbance peak at 492 nm (line a). After addition of NROR this peak disappeared, indicating a NROR-catalysed reduction of revRbr by NADH (line c). Addition of an excess of H_2O_2 rapidly reoxidized the NADH-reduced revRbr under anaerobic assay conditions (line d). Analogous spectral titrations (Supplementary Fig. S1, available with the online version of this paper) showed that FNR and Rd together catalyse reduction of revRbr by NADPH under anaerobic conditions, and the resulting reduced revRbr can then be fully reoxidized by H_2O_2. These results are fully consistent with the NAD(P)H peroxidase activity of revRbr described above.

Effect of revRbr overexpression on oxidative stress resistance of *C. acetobutylicum*

To analyse the effect of revRbr on oxidative stress tolerance *in vivo*, one of the two (99 % identical) revRbr genes, *rbr3B*, was cloned into an expression vector, pTrbr3B, and overexpressed in *C. acetobutylicum.* In survival experiments (Fig. 8), the revRbr overexpression strain exhibited a greatly increased tolerance to added H_2O_2 compared to the wild-type strain. The revRbr overexpression strain survived 30 min exposure to concentrations up to 0.3 mM added H_2O_2 at least as well as the non-H_2O_2-treated control, whereas corresponding survival of the wild-type strain

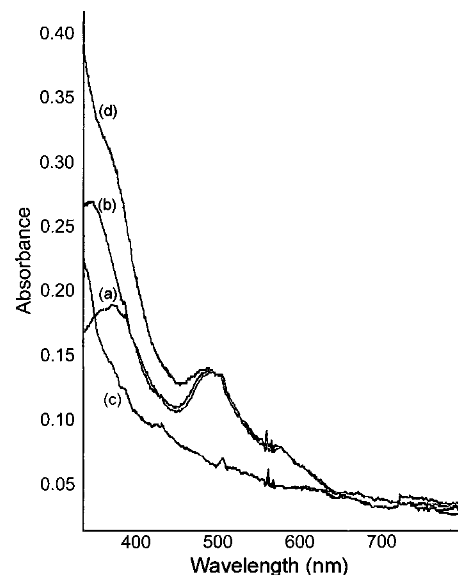

Fig. 7. Reductive titrations of revRbr with NADH/NROR and reoxidation by H_2O_2. UV-vis absorption spectra are shown for (a) 100 µM as-isolated revRbr and (b) 100 µM as-isolated revRbr plus 125 µM NADH. (c) Same sample as in (b) after addition of 1 µM NROR; (d) same sample as in (c) after addition of 100 mM H_2O_2. All solutions were in 50 mM MOPS, 0.1 mM EDTA pH 7.0.

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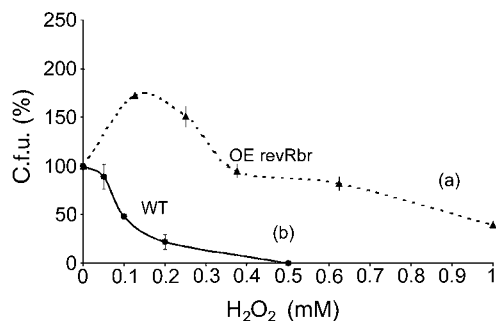

Fig. 8. Survival of *C. acetobutylicum* after exposure to H_2O_2. Anaerobic *C. acetobutylicum* cultures in CGM medium at 37 °C were exposed to various concentrations of added H_2O_2 for 30 min. Then 50 µl aliquots of these exposed cultures were plated on reinforced clostridial agar. Colonies were counted after 2–4 days anaerobic incubation at 37 °C. C.f.u. are expressed as a percentage of the counts of anaerobic Tris/HCl buffer-treated cells of the same culture (means ± SD, n=3). (a) *C. acetobutylicum* revRbr overexpression strain (OE revRbr); (b) *C. acetobutylicum* ATCC 824 wild-type strain.

steadily decreased over the same H_2O_2 concentration range. The wild-type strain did not survive added H_2O_2 concentrations >0.3 mM, whereas the revRbr overexpression strain exhibited 80 % survival up to 0.8 mM H_2O_2. Similarly, compared to the wild-type (18 % c.f.u.), the survival rate of the overexpression strain doubled (44 % c.f.u.) in 10 ml cultures that were flushed with air (500 ml min^{-1}) for 15 min. In agreement with this result the overexpression strain consumed 1.71 fmol O_2 h^{-1} per cell compared to 0.93 fmol O_2 h^{-1} per cell for the wild-type. See also Table 1 for the NADH peroxidase and NADH oxidase activities of *C. acetobutylicum* wild-type and revRbr-overexpressing cells.

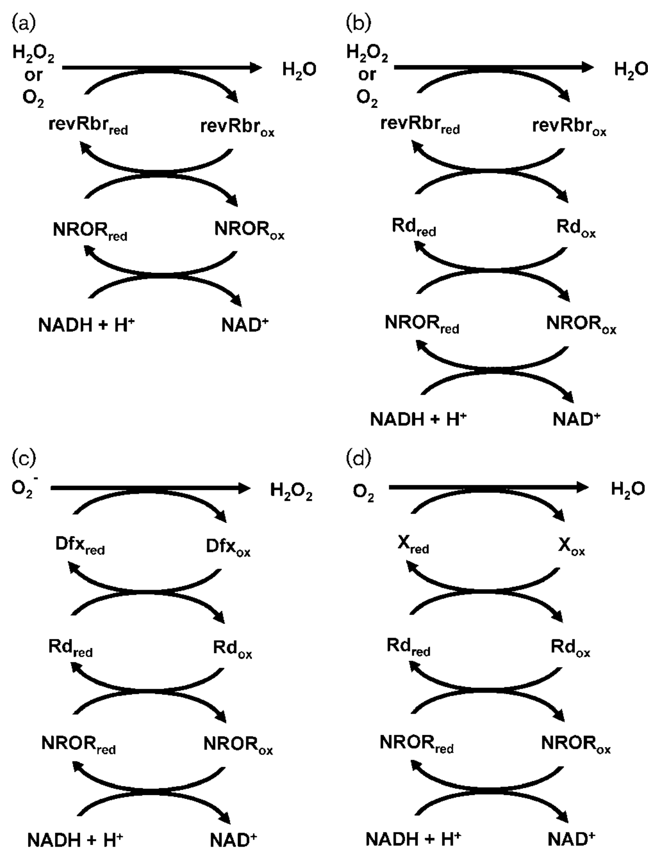

Fig. 9. Detoxification pathways for ROS and oxygen in *C. acetobutylicum*. Rd, rubredoxin; revRbr, reverse rubrerythrin; Dfx, desulfoferrodoxin; X, unknown protein; red, reduced; ox, oxidized. The stoichiometry of the reactions has not been observed. (a) and (b), reduction of H_2O_2 and O_2 to water without and with the involvement of Rd, respectively; (c), reduction of O_2^- to water (Riebe *et al.*, 2007); (d), reduction of O_2 to water by an unknown protein, X.

DISCUSSION

Previous results demonstrated increased revRbr expression upon exposure of *C. acetobutylicum* to air or H_2O_2 (Kawasaki *et al.*, 2004; May *et al.*, 2004) and clearly implicated revRbr in oxidative stress protection. The combined *in vitro* and *in vivo* results reported in this work indicate that this protection most likely arises from revRbr's reductive scavenging of H_2O_2 and possibly O_2.

The significant new *in vivo* result is that a *C. acetobutylicum* strain overexpressing revRbr showed greatly increased tolerance to both air (44 % survival compared to 14 % in case of the wild-type) and H_2O_2 exposure (see Fig. 8). Complementary *in vitro* results show that a reconstituted system consisting of NROR, Rd and revRbr, all from *C. acetobutylicum*, can function as both an NADH peroxidase and an NADH oxidase. The electron-transfer pathway from NADH to O_2 or H_2O_2 via revRbr incorporating these other components is shown diagrammatically in Fig. 9. While this pathway did not absolutely require Rd, its presence increased the NADH peroxidase and oxidase activities. NROR most likely either directly reduces the [Fe(SCys)$_4$] site of revRbr or preferentially reduces the analogous site in Rd, which then transfers its electrons to revRbr. Consistent with this notion, spinach FNR was absolutely dependent on Rd for catalysis of NADPH reduction of revRbr, as was shown earlier for *D. vulgaris* Rbr (Coulter & Kurtz, 2001). The peroxidase activity of other Rbrs requires the [Fe(SCys)$_4$] site (Jin *et al.*, 2004a), presumably to funnel electrons from external donors to the diiron site. The tetrameric oligomer of revRbr could conceivably support either intra- or intersubunit electron transfer between [Fe(SCys)$_4$] and diiron sites. Both cases have been observed in other Rbrs (Jin *et al.*, 2004b; Iyer *et al.*, 2005).

The *in vitro* results show that revRbr can use both O_2 and H_2O_2 as electron acceptors, but that H_2O_2 is the preferred substrate. The inhibition of O_2 consumption in the presence of H_2O_2 (Fig. 6, time-course a) is not due to an

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inactivation of revRbr by H_2O_2, since NADH consumption in the presence of H_2O_2 occurred under both aerobic and anaerobic conditions after addition of H_2O_2 (see Fig. 4, time-courses b and c). The inhibition of O_2 consumption by H_2O_2 is, therefore, probably the result of a higher reactivity of the diiron site of revRbr with H_2O_2 than with O_2, as has been demonstrated for other Rbrs (Coulter & Kurtz, 2001). H_2O_2 probably undergoes a concerted two-electron reduction by abstracting two electrons from the diferrous site of Rbrs, which would minimize Fenton-type chemistry, i.e. the one-electron reduction of H_2O_2 to $OH^{\cdot}$ (Kurtz, 2006). In contrast, four electrons are necessary for the complete reduction of O_2 to water. The two additional electrons are transferred from external donors (NROR or Rd) probably via the [Fe(SCys)$_4$] domain of revRbr. If the reduction of O_2 to water by revRbr occurs via an H_2O_2 intermediate, it apparently is not released from the active site. We did not detect evolution of H_2O_2 during the reaction of revRbr with O_2 (data not shown). We also could not detect any significant catalase activity of revRbr.

While H_2O_2 seems to be the preferred substrate, revRbr uses O_2 as a substrate *in vitro* at a significantly higher relative level than reported for other Rbrs (Coulter *et al.*, 1999, Coulter & Kurtz, 2001). This observation is consistent with the increased consumption of O_2 by the revRbr-overexpressing *C. acetobutylicum* strain. The revRbr oxidase activity could, thus, be relevant when *C. acetobutylicum* is exposed to air, where the intracellular O_2 levels may be higher than the H_2O_2 levels. Exposure of *C. acetobutylicum* to air results in massive increases in transcription (May *et al.*, 2004) and translation (Kawasaki *et al.*, 2004) of revRbr.

In this work we have also shown that one of the two *C. acetobutylicum* Rbrs, RubY, with the 'classical' N-terminal diiron and C-terminal [Fe(SCys)$_4$] domain structure, has peroxidase (H_2O_2 reductase) specific activity comparable to that of the revRbr *in vitro*. It is, therefore, somewhat surprising that the intracellular levels of these classical Rbrs do not seem to be affected by oxidative stress (May *et al.*, 2004; Hillmann *et al.*, 2006), either in *C. acetobutylicum* or in other clostridia (Geissmann *et al.*, 1999). Our recent microarray data (unpublished) confirmed the very low expression level of the *C. acetobutylicum* classical Rbr genes and their lack of upregulation in response to air exposure. This is in contrast to classical Rbrs from sulphate-reducing bacteria (Lumppio *et al.*, 1997, 2001; Dolla *et al.*, 2006) and archaea (Weinberg *et al.*, 2004), which are typically expressed at significant constitutive levels even under anaerobic growth conditions.

The present results explain the observed aerotolerance of a *C. acetobutylicum* deletion mutant of the putative *rbr3AB* repressor, PerR (Hillmann *et al.*, 2008). Deletion of *perR* resulted in massive overproduction of revRbr. Crude cell extracts of this Δ*perR* strain have a red colour due to high amounts of revRbr and show $>$10-fold higher NADH oxidase and peroxidase activities than the wild-type strain. The *C. acetobutylicum* Δ*perR* strain also shows superior O_2 consumption and aerotolerance over the revRbr overexpression strain (16 fmol h^{-1} per cell for Δ*perR* vs 1.71 fmol h^{-1} per cell for the revRbr overexpression strain), consistent with apparent regulation of other O_2 detoxification proteins by *perR* (Hillmann *et al.*, 2008).

The present results clearly demonstrate reductive H_2O_2 and O_2 scavenging by *C. acetobutylicum* revRbr both *in vitro* and *in vivo*. The NADH peroxidase and oxidase activities of revRbr combined with the superoxide reductase activity of Dfx constitute an efficient reductive scavenging system for ROS in *C. acetobutylicum*. Fig. 9 summarizes the proposed ROS and O_2 detoxification pathways involving revRbr, Dfx, and yet to be identified proteins. Candidates for X in Fig. 9 are FprA1 (*cac1027*) and FprA2 (*cac2449*), which are upregulated in response to oxidative stress (Kawasaki *et al.*, 2004, 2005) and homologues of which from other organisms show NAD(P)H oxidase activity (Kurtz, 2007). We have so far been unable to construct an *rbr3AB* deletion strain, which is consistent with a more global stress defence role previously identified for revRbr (Hillmann *et al.*, 2006).

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