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A system for the heterologous expression of complex redox proteins in *Rhodobacter capsulatus*: characterisation of recombinant sulphite:cytochrome *c* oxidoreductase from *Starkeya novella*

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Abstract **The phototrophic purple non-sulfur bacterium *Rhodobacter capsulatus* expresses a wide variety of complex redox proteins in response to changing environmental conditions. Here we report the construction and evaluation of an expression system for recombinant proteins in that organism which makes use of the *dor* promoter from the same organism. A generic expression vector, pDorEX, was constructed and used to express sulphite:cytochrome *c* oxidoreductase from *Starkeya novella*, a heterodimeric protein containing both molybdenum and haem *c*. The recombinant protein was secreted to the periplasm and its biochemical properties were very similar to those of the native enzyme. The pDorEX system therefore seems to be potentially useful for heterologous expression of multi-subunit proteins containing complex redox cofactors.**

Key words: Redox protein; Molybdoenzyme; Protein expression; Haem *c*

1. Introduction

Respiratory and photosynthetic electron transfer pathways are characterised by the presence of enzymes with multiple redox centres. This is particularly true with respect to prokaryotic systems, where an array of redox complexes underpins the diversity of lithotrophic and respiratory metabolism [1,2]. Many of these redox complexes are located in the periplasm or on the outer face of the cytoplasmic membrane. Examples of prosthetic groups found in periplasmic redox proteins include the molybdenum pterin cofactor (Moco), iron–sulfur clusters and *c*-type cytochromes [1]. It has been established that polypeptides containing prosthetic groups such as the molybdenum pterin cofactor and [Fe–S] clusters are translocated to the periplasm via a novel pathway known as the 'Tat' system [3–5]. The prosthetic group is bound in the cytoplasm and the polypeptide is secreted in a folded form. In contrast, haem-containing proteins are secreted via the Sec system and different forms of haem are acquired in the periplasm [6]. Understanding the mechanistic aspects of the biogenesis of complex periplasmic redox proteins is a major challenge in microbial biochemistry. An additional problem is finding a suitable expression system for producing recombinant forms of such proteins. *Escherichia coli*, the organism of choice for the expression of most proteins, may be a poor system for the expression of a number of complex redox proteins, as has been recently shown, for example, for two flavocytochromes *c* [7].

The α(3)-Proteobacterium [8] *Rhodobacter capsulatus* is particularly suited for the expression of periplasmic redox proteins. Unlike *E. coli*, it produces an abundance of *c*-type cytochromes during anaerobic (phototrophic) growth. Moreover, both the unmodified and the dinucleotide form of molybdopterin (MPT) is found in enzymes containing Moco in *R. capsulatus* [9,10]. In this paper we describe the construction of a system for the expression of complex redox proteins in *R. capsulatus*. This expression system makes use of the promoter for the dimethyl sulfoxide reductase (*dor*) operon from the same organism [11]. We have used this system to express the SorAB protein (sulphite:cytochrome *c* oxidoreductase) from *Starkeya novella*, an organism which belongs to the α(2)-subgroup of the Proteobacteria [12]. The SorAB protein contains both molybdenum, coordinated by a mono-MPT Mo form of Moco, and a *c*-type haem [13]. These cofactors are located on different subunits of the enzyme, which, as indicated by the nature of the respective leader sequences encoded by the *sorAB* genes [13], are translocated to the periplasm by the Tat and Sec systems, respectively. An expression system for such a protein has to overcome the difficulties posed by the nature of the two redox groups present and the mechanism of secretion.

2. Materials and methods

2.1. Strains, media and plasmids

Bacterial strains and plasmids used in this study are specified in Table 1. *E. coli* was routinely cultivated at 37°C using liquid or solidified Luria–Bertani medium [14]. *R. capsulatus* strains were cultivated on RCV [15] or TYS medium [16], under aerobic or phototrophic conditions. Where necessary, antibiotics were added to the growth media in the following concentrations: ampicillin *E.c.* 100 μg/ml; tetracycline *E.c.* 10 μg/ml, *R.c.* 1 μg/ml; kanamycin *R.c.* 10 μg/ml; gentamicin *R.c.* 4 μg/ml. *S. novella* (DSMZ 506^T) was cultivated on DSMZ medium no. 68 as in [13].

2.2. Molecular biological and genetic methods

All molecular techniques were performed according to standard procedures [14,17]. The inserts of all plasmids generated were con-

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Abbreviations: CV, column volume; Moco, molybdenum pterin cofactor; rSorAB, recombinant SorAB

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firmed by sequencing [18] using BigDye Terminator v.2 (Applied Biosystems). *Pfu* turbo Polymerase (Stratagene) was used for PCR amplification. Diparental matings were performed according to Simon et al. [19] with mating conditions as set out in [20].

2.3. Construction of pDorEX and derivatives

The *dorC–dorR* intergenic region was amplified by PCR from pALS4 [21] (EXDORPRF aaa aaa gct tCG TCT TCA TCA CGG CCC C; EXDORPRR aaa agg atc cCC AGA TTT TCC TGA TCA C) as primers. The purified PCR product was cloned into pBluescriptII using the *Hin*dIII and *Bam*HI restriction sites, creating pDorEX. A 1.6-kb fragment carrying the entire *sorAB* genes was amplified from pTNSOR32 [13] (EXSORAF aaa agg atc cAT GCT CAA CAG ACG CCA AAT C; EXSORBR aaa atc tag cTC AAT AGG TCT TTG CCA GAT A). The amplicon was purified using the Qiaquick Gel Purification Kit (Qiagen) and cloned into pDorEX using the *Bam*HI and *Xba*I restriction sites, resulting in pSorEX. For transfer into *R. capsulatus* strains, pSorEX was digested with *Hin*dIII and *Xba*I, the insert was isolated, purified and subsequently ligated to a *Hin*dIII/*Xba*I digested pRK415 vector [22], forming pRK-sorex (Fig. 1).

The DNA sequences used for the construction of pDorEX and pSorEX have the accession numbers U49506 [21] and AF154565 [13].

2.4. Expression of the SorAB protein in R. capsulatus and preparation of periplasmic and whole cell extracts

For SorAB expression *R. capsulatus* strains harbouring pRK-sorex were grown phototrophically for 18–20 h on RCV medium supplemented with the necessary antibiotics, 60 mM dimethyl sulfoxide (DMSO) and 1 mM sodium molybdate. The cells were harvested by centrifugation (5000×*g*, 20 min, 4°C) and periplasmic extracts prepared as in [23]. Extracts of total soluble protein were prepared from 30 ml expression cultures. The harvested cells were resuspended in 10 ml 20 mM potassium phosphate buffer, pH 7.6, 1 mM EDTA and broken by two passages through a French Pressure Cell (1000 p.s.i., Aminco). Unbroken cells and debris were removed by centrifugation (15 000×*g*, 30 min, 4°C), followed by ultracentrifugation of the resulting supernatant (145 000×*g*, 90 min, 4°C) to remove the membrane fraction.

2.5. Purification of native and recombinant SorAB protein

The native SorAB protein from *S. novella* was purified as in [13]. The recombinant SorAB (rSorAB) protein was purified from a periplasmic extract which was loaded onto a DEAE–Sephacel column (1.6 cm×17 cm) equilibrated in 20 mM Tris–HCl, pH 7.8, (buffer A). The column was washed with three column volumes (CV) of 20 mM Tris–HCl, pH 7.8, followed by a linear gradient from 0 to 250 mM NaCl in buffer A (15 CV). Active fractions were concentrated by ultrafiltration (cut-off 10 kDa, Amicon) and solid ammonium sulphate added to a concentration of 15% (w/v). The sample was applied to a Phenyl Sepharose FF column (1.6 cm×19 cm) equilibrated in 15% ammonium sulphate (w/v) in buffer A. A step gradient was used in eluting the protein: 15–11.5% ammonium sulphate, 1 CV, 11.5% ammonium sulphate, 2 CV, 11.5% ammonium sulphate to 0% ammonium sulphate in 12 CV. The sample was reconcentrated, dialysed against buffer A with 150 mM NaCl and loaded onto a HiLoad Superdex75 prepgrade (16/60) column equilibrated in the same buffer.

2.6. Enzyme assay and analytical techniques

Sulphite:cytochrome *c* oxidoreductase activity was measured spectrophotometrically in a Hitachi double-beam UV-3000 UV/VIS spectrophotometer. Standard assays were performed as in [13]. For determination of K_M values, the sulphite concentration was varied between 2.5 and 0.004 mM, cytochrome *c* concentration between 0.02 and 0.001 mM (absorbance was monitored at 417 nm in this case). An assay using 1 mM ferricyanide as electron acceptor was used in deriving K_{cat} values. Protein content was determined with the 2-D Quant Kit (Amersham Biosciences). Denaturing and non-denaturing PAGE were carried out by the method of [24], activity stains were performed as in [25]. Haem type and content of the recombinant enzyme were determined in alkaline pyridine solution [26]. Molybdenum content was measured by ICP–MS analysis (CSIRO Division of Livestock Industry, Long Pocket Laboratories, Indooroopilly, Australia).

CD spectra of the native and the recombinant protein were recorded between 190 and 260 nm on a Jasco J-710 Spectropolarimeter. Samples were either reduced by addition of 2 mM sulphite or oxidised by dialysis against 0.05 mM potassium ferricyanide in 10 mM Tris–HCl, pH 8.0, for 1 h. Both treatments were followed by exhaustive dialysis against 10 mM Tris–HCl, pH 8.0. Analytical ultracentrifugation was carried out in a Beckman XL-1 analytical centrifuge (AN60Ti rotor) at 13 000 rpm for 16 h at 20°C. Absorption was monitored at 280 and 360 nm against a buffer sample.

2.7. Materials

All chemicals were purchased in analytical reagent or corresponding quality.

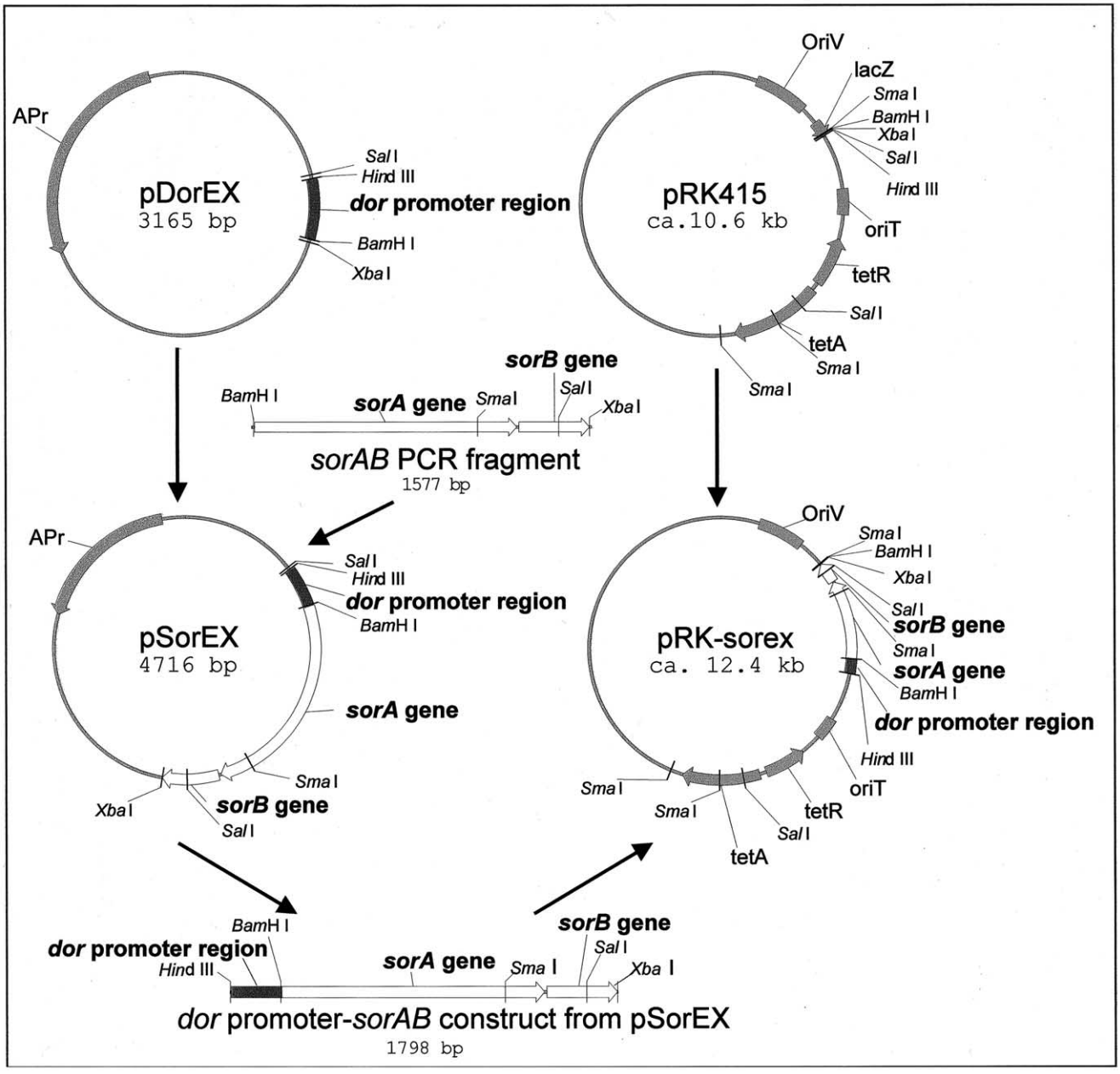
3. Results

3.1. Construction of expression plasmids

The *dor* promoter of *R. capsulatus* was selected for the construction of the basic expression plasmid pDorEX (Fig. 1) because it is a strong promoter in *R. capsulatus* but is, at the same time, tightly regulated by a variety of environmental factors, including anaerobiosis, the presence of DMSO and molybdate [20,21,27]. Induction of *dor* operon expression in *R. capsulatus* is mainly controlled by the DorR response regulator which binds to four conserved boxes found in the promoter region. The entire intergenic region between the *dorR* and *dorC* genes [21] was amplified by PCR and cloned into pBluescript II, generating pDorEX. This plasmid allows the

Table 1
Bacterial strains and plasmids used in this study

Bacterial strain/vectors	Genetic markers/phenotype	References
Rhodobacter capsulatus strains		
37B4	wild type	
37B4 Δ*dorA*	*dorA*::Gm^r, DMSO reductase$^-$	[21]
H123	Km^r::Tn5	[39]
Starkeya novella		
DSMZ 506^T	wild type	[40]
Escherichia coli strains		
DH5α	F$^-$ Φ80d*lacZ*ΔM15(*lacZ*YA-*arg*F)*U169 recA1 endA1 hsdR17* ($r_K^- m_K^+$) *supE44*λ *thi-1 gyrA relA1*	Invitrogen
GM2163	F$^-$ *ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 GalK2 galT22 mcrA dcm-6 hisG4 rfbD1 rpsL136 Dam13::Tn9 xylA5 mtl-1 thi-1 mcrB1 hsdR2*	New England Biolabs
S17-1	Tp^r Sm^r *hsdR pro recA* RP4-2 Tc^r::Mu Km^r::Tn7 in chromosome	[19]
pBluescript II	Amp^r, lacZ′	Stratagene
pRK415	Tc^r, mob	[22]
pALS4	Km^r, Neo^r, lacZ′, part. *dorSRdorCDA* genes, *R. capsulatus*	[21]
pTNSOR32	Km^r, Neo^r, lacZ′, *S. novella sigE sorAB* gene region	[13]
pDorEX	Amp^r, lacZ′, *R. capsulatus dor* promoter	this study
pSorEX	Amp^r, lacZ′, pDorEX-*sorAB* genes from *S. novella*	this study
pRK-sorex	Tc^r, mob, pRK415, containing pSorEX insert	this study



promoter region of pDorEX

SpeI XbaI EagI NotI BstXI SacII SacI

cgtggcaagaccggaggcgaaagtgatcaggaaaatctgggatccactagttctagagcggccgccaccgcggtggagctc

rbs

BamHI

promoter region after insertion of *sorAB genes*

sorA →

gaccggaggcgaaagtgatcaggaaaatctgggatccatgctcaacagacgccaaatcct

M L N R R Q ....

Fig. 1. Construction of pDorEX and derived plasmids pSorEX and pRK-sorex. Upper panel: schematic representation of the cloning steps used in vector construction. Lower panel: Sequence of the relevant cloning site prior to and after insertion of the *sorAB* target genes. rbs = ribosome binding site.

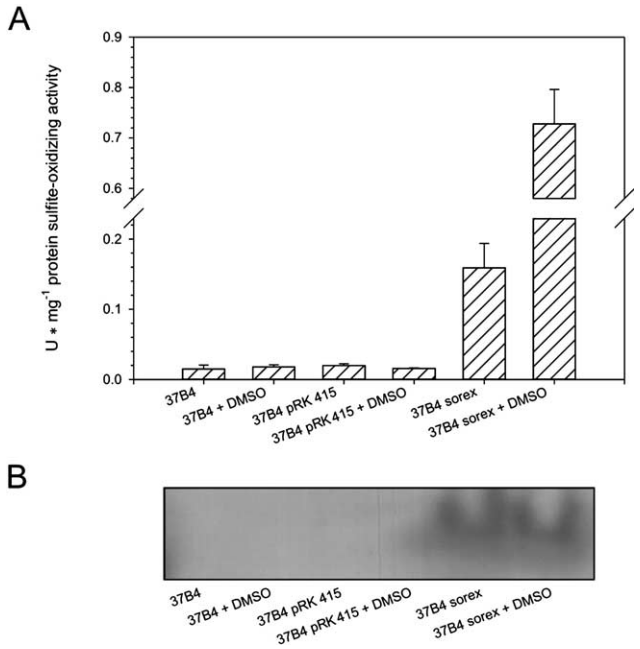


Fig. 2. Sulphite:cytochrome *c* oxidoreductase activity in cell extracts of *R. capsulatus* 37B4 under non-inducing and inducing (+60 mM DMSO) conditions. Panel A: Sulphite:cytochrome *c* oxidoreductase activities in total soluble protein extracts. Panel B: Sulphite:acceptor oxidoreductase activity stain of periplasmic cell extracts (10% native PAGE). Both experiments used wild-type 37B4, and 37B4 harbouring either pRK415 or pRK-sorex. Cells were cultivated phototrophically on RCV medium without added molybdenum. Errors are given as 95% confidence intervals.

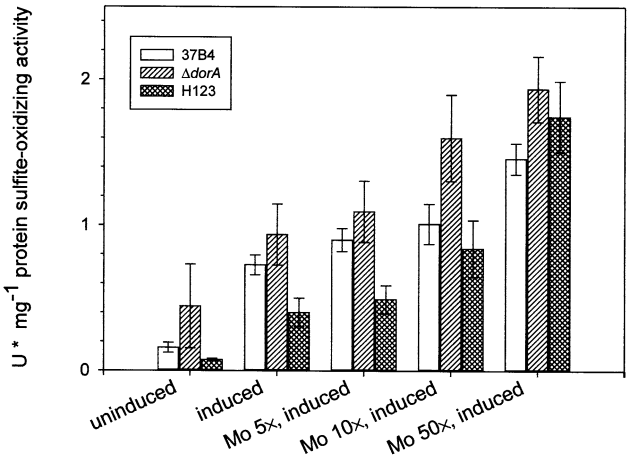


Fig. 3. Optimisation of rSorAB expression in *R. capsulatus* strains 37B4, 37B4 Δ*dorA* and H123 harbouring pRK-sorex. Activities were measured in induced (+60 mM DMSO) and non-induced expression cultures with or without the addition of molybdenum to 5×, 10× and 50× the original levels. Error bars represent 95% confidence levels.

construction of fusions of the *dor* promoter to genes or operons of interest by cloning these into the *Bam*HI site of pDorEX. The fusions can be used for protein expression in *R. capsulatus*. The *Bam*HI site on the prospective insert has to be located immediately upstream of the start codon of the gene/operon of interest (Fig. 1). Other restriction sites further downstream of the *Bam*HI site of pDorEX can be used for directed cloning (e.g. *Spe*I, *Xba*I, *Not*I, *Sac*I, *Sac*II). Under aerobic growth conditions, toxic effects on *E. coli* host cells caused by basal transcription from the *dor* promoter should be minimal due to its control by the DorR protein.

A construct for the expression of the SorAB protein from *S. novella* was generated by PCR amplification of the necessary gene region followed by cloning of the amplicon into the *Bam*HI and *Xba*I sites of pDorEX, forming pSorEX. The *sorAB* amplicon contained the entire gene sequences, including signal sequences and stop codons. After sequence confirmation the complete *dor* promoter–*sorAB* fusion was subcloned into the mobilisable pRK415 plasmid (generating pRK-sorex) (Fig. 1). Problems arising from under-representation of certain tRNAs in the host organism are not expected with the chosen target genes, as DNA from both *R. capsulatus* and *S. novella* has a high GC-content (65.5–66.8 and 67.3–68.4 mol%, respectively) [8,12].

3.2. Expression of SorAB in R. capsulatus

Expression of the SorAB protein from pRK-sorex was assayed in whole cell extracts of *R. capsulatus* 37B4 after phototrophic growth in the presence or absence of DMSO. We were able to show that under both conditions, a sulphite:cytochrome *c* oxidoreductase activity was present in cell extracts from strains that contained pRK-sorex, while there was no activity present in the wild-type strain or in a strain containing pRK415 (Fig. 2). The sulphite-oxidising activity was induced 4.5-fold by the presence of DMSO (Fig. 2a), and was shown to reside in the periplasmic space of the tested strain by a sulphite:acceptor oxidoreductase activity stain carried out on periplasmic extracts of the same *R. capsulatus* strains (Fig. 2b). These results indicate that the recombinant SorAB protein is not only expressed in *R. capsulatus*, but is also targeted to the correct compartment of the cell.

3.3. Optimisation of the system for expression of SorAB

DMSO reductase is expressed to a high level in *R. capsulatus* [20] and it seemed likely that this enzyme would compete with SorAB for Mo. In order to explore this hypothesis, SorAB expression in different strains of *R. capsulatus* (Table 1) and in the presence of added molybdate (1×, i.e. 0.752 mg/l, 5×, 10× and 50×) was investigated. All three strains tested contained similar levels of sulphite-oxidising activity under

Table 2
Purification of recombinant sulphite:cytochrome *c* oxidoreductase from a periplasmic extract of *R. capsulatus*

	Volume ml	Total activity units	Volume activity units/ml	Protein content mg/ml	Specific activity units/mg	Purification factor -fold	Yield %
Periplasm	430	9814.3	22.8	1.4	16.0	1	100
DEAE-Sephacel-Pool, conc.	19	5554.9	292.4	2.9	99.9	6.2	56.6
HIC-Pool, after ultrafiltration	6.5	1641.2	252.5	1.2	214.9	13.4	16.7
Superdex 75 Pool	5	710.9	142.2	0.6	246.4	15.4	7.2

For details see Section 2.

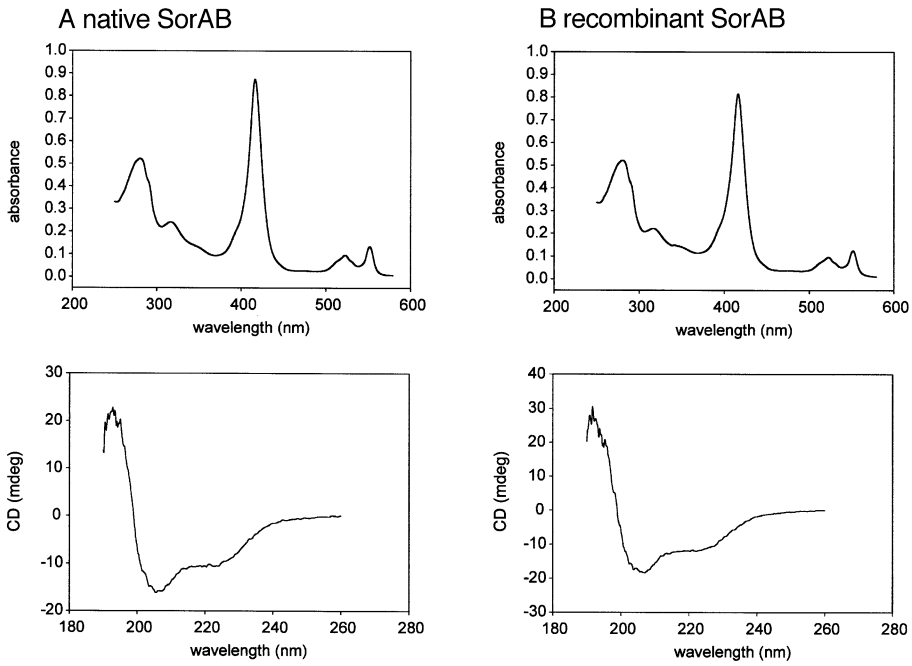


Fig. 4. Spectral properties of sulphite-reduced SorAB (left) and rSorAB (right) protein. Top: UV/Vis spectra. Bottom: CD spectra. CD scan parameter: Pathlength 0.1 cm, sensitivity 100 mdeg, band width 1 nm, response time 1 s, resolution 0.2 nm, scan speed 50 nm/min, accumulation: 10 times. Protein concentration in CD spectra: SorAB 0.234 mg/ml; rSorAB 0.288 mg/ml.

both inducing and non-inducing conditions with the exception of the 37B4 Δ*dorA* strain, which appeared to contain slightly elevated levels of activity under non-inducing conditions (Fig. 3). The measured sulphite-oxidising activity increased with increasing amounts of molybdate present in the medium (Fig. 3), reaching almost twice the original level observed and approaching saturation in the presence of 50× Mo. All expression cultures were therefore supplemented with 1 mM molybdate.

3.4. Purification and characterisation of the recombinant SorAB protein

Initial attempts to purify rSorAB from strain 37B4 had shown that separation of this protein from DMSO reductase is a major problem, and therefore the 37B4 Δ*dorA* strain was selected for further experiments. The recombinant SorAB protein was purified from the periplasmic extract of 12 l of expression culture by a combination of weak anion exchange, hydrophobic interaction and size exclusion chromatography (Table 2). After the size exclusion step the protein was considered to be homogeneous as indicated by SDS–PAGE.

The optical and CD spectra of rSorAB were virtually identical to those of the native enzyme (Fig. 4). Table 3 shows a detailed comparison of the native SorAB and rSorAB. The kinetic properties of the two enzymes were very similar in terms of both K_{cat} and the K_M values for sulphite and cytochrome *c* (horse heart). The high activity of the rSorAB protein was consistent with the presence of both *c*-type haem and Mo in the enzyme. Molecular weight determination of rSorAB showed that, like the native enzyme, it is a heterodimer.

4. Discussion

Periplasmic enzymes with multiple-redox centres are of central importance in bacterial electron transfer. Many of the enzymes of interest come from microorganisms whose genetics are not well-developed and hence there is a need for heterologous expression systems. However, not all redox cofactors

Table 3
Physico-chemical and catalytic properties of native and recombinant SorAB protein

	Native SorAB	Recombinant SorAB
Subunit structure	αβ	αβ
Mol. mass ox. (anal. ultracentrifugation)	42.6 kDa	42 kDa
Mo content/holoenzyme	0.624	0.63
Haem content/holoenzyme	0.8 ± 0.15[a]	0.91
Spectral properties (sulphite-reduced)	α 552 nm	α 552 nm
	β 523 nm	β 523 nm
	γ 416.5 nm	γ 416 nm
	δ 317 nm	δ 317 nm
$E_{Soret\ red}/E_{280}$	1.67	1.56
K_M sulphite	27 μM[a]	32 ± 3.8 μM
K_M cytochrome *c* (horse heart)	4 μM[a]	2.3 ± 0.8 μM
K_{cat}	11769 ± 648 s^{-1}	10875 ± 288 s^{-1}

Errors for catalytic properties of the enzymes are given as 95% confidence intervals.
[a]Reported in [13].

can be easily produced in standard expression systems using *E. coli*. The low level of *c*-type cytochrome expression has been enhanced by over-expression of the *ccm* genes, encoding enzymes of *c*-type cytochrome biogenesis [28–30]. On the other hand, molybdoenzymes have been successfully expressed in *E. coli* [31–34]. However, in all cases these enzymes were expressed in the cytoplasm making use of the fact that the molybdenum pterin cofactor is inserted in this compartment, whether or not the protein is secreted. Since *c*-type cytochromes are assembled in the periplasm [35], such an approach could not be used for the expression of an enzyme like SorAB, since it requires that both the Moco-containing subunit (SorA) and the *c*-type cytochrome (SorB) are secreted. Although we did not determine the efficiency of expression and secretion of SorA in *E. coli*, our experience with *R. capsulatus* DMSO reductase is that its level of expression in *E. coli* is very low and secretion was not efficiently performed (Shaw, Huston, McEwan, unpublished observations).

In contrast to *E. coli*, *R. capsulatus* is able to synthesise most types of the metal-containing prosthetic group. This is particularly true for MPT-containing enzymes and *c*-type cytochromes, as shown in this study, but iron–sulfur proteins and copper-containing proteins should also be assembled in this host. The successful expression of SorAB in *R. capsulatus* was also dependent upon both the Tat system for secretion of SorA and the Sec system for secretion of SorB. It is interesting that the heterodimeric SorAB complex was formed in *R. capsulatus*, as in *S. novella*. This indicates that assembly of the complex is either a spontaneous process or that additional information present in the operon structure or gene sequences guides this process.

The correct assembly and secretion of the rSorAB protein also enabled us to purify it from the periplasmic protein fraction, which led to a considerable simplification and shortening of the purification procedure when compared with that for the native enzyme [13]. The properties of the rSorAB protein were very similar to those of the native one, indicating that the host organism was able to induce correct folding of both subunits. Therefore, the *dor* promoter-based expression system seems to be well suited for the expression and secretion of complex redox proteins. Another possible use may be in the expression of membrane-targeted proteins, as *Rhodobacter* contains a substantial intracellular membrane system and, as a consequence, a high surface area-to-volume ratio.

A recently described expression system for flavocytochromes *c* from purple sulfur bacteria [36] that also uses *Rhodobacter* strains as host organisms showed that both subunits of the protein were expressed and had the respective cofactors attached. This underlines the suitability of *Rhodobacter* strains for the expression of multi-subunit proteins with complex cofactors. However, neither the localisation of the recombinant flavocytochromes nor their catalytic activities were reported, and expression only yielded 0.1 mg/l–0.5 mg/l of holoprotein. In comparison, using the *dor* promoter system a yield of ca. 3.5 mg/l (0.25 mg/l purified) rSorAB protein was achieved. The lower expression levels found for the flavocytochromes may be due to the fact that the expression vectors used promoters that did not originate from *Rhodobacter* species.

The pDorEX system relies on a two-plasmid strategy that requires two subcloning steps. As vectors that can be transferred into *R. capsulatus* are generally quite large (>10 kb) and often not fully characterised, this strategy allows for easy manipulation of the initial construct, which is propagated in *E. coli* before it is transferred into pRK415. An alternative to this subcloning step is the transfer of the RP4 *mob* locus and *tet* genes into the pDorEX construct as set out in [21].

An interesting observation in relation to expression control is that, while the chromosomal copy of the *dor* promoter is tightly controlled and DMSO reductase activity is hardly detectable under non-inducing conditions [20,21], the *dor* promoter located in *trans* on the expression plasmid seems to be controlled less stringently than expected. A possible explanation for this may be the lack of an autoregulatory feedback loop, as has been shown for the closely related *tor* operon from *E. coli* [37]. However, this unexpected property will also allow for the expression of recombinant proteins in a DMSO-independent manner, which may be further enhanced by using suitable genetic backgrounds for expression (such as the *regA* regulatory mutant [38]), that will cause constitutive DMSO reductase expression [20]. The pattern of molybdate concentration-dependent induction of protein expression from pRK-sorex was similar to the one found by Solomon and coworkers for DMSO reductase [27], who also showed that this induction is due to changes in the levels of transcription of the *dor* operon controlled by MopB in an unknown manner. As our knowledge of the regulation of *dor* operon expression increases this system may be optimised for different tasks by choosing suitable genetic backgrounds in host *Rhodobacter* strains.

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