Genome Mining in *Sorangium cellulosum* So ce56

**IDENTIFICATION AND CHARACTERIZATION OF THE HOMOLOGOUS ELECTRON TRANSFER PROTEINS OF A MYXOBACTERIAL CYTOCHROME P450**

KERSTIN MARIA EWEN, FRANK HANNEMANN, YOGAN KHATRI, OLENA PERLOVA, REINHARD KAPPL, DANIEL KRUG, JÜRGEN HÜTTERMANN, ROLF Müller, AND RITA BERNHARDT

**From the Departments of Biochemistry and Pharmaceutical Biotechnology, Saarland University, D-66041 Saarbrücken and the Department of Biophysics, Saarland University, D-66421 Homburg, Germany**

Myxobacteria, especially members of the genus *Sorangium*, are known for their biotechnological potential as producers of pharmaceutically valuable secondary metabolites. The biosynthesis of several of those myxobacterial compounds includes cytochrome P450 activity. Although class I cytochrome P450 enzymes occur wide-spread in bacteria and rely on ferredoxins and ferredoxin reductases as essential electron mediators, the study of these proteins is often neglected. Therefore, we decided to search in the *Sorangium cellulosum* So ce56 genome for putative interaction partners of cytochromes P450. In this work we report the investigation of eight myxobacterial ferredoxins and two ferredoxin reductases with respect to their activity in cytochrome P450 systems. Intriguingly, we found not only one, but two ferredoxins whose ability to sustain an endogenous So ce56 cytochrome P450 was demonstrated by CYP260A1-dependent conversion of nootkatone. Moreover, we could demonstrate that the two ferredoxins were able to receive electrons from both ferredoxin reductases. These findings indicate that *S. cellulosum* can alternate between different electron transport pathways to sustain cytochrome P450 activity.

The cytochrome P450 (CYP) enzymes constitute a superfamily of external monooxygenases. The catalytic versatility of the family members explains their involvement in such diverse biological processes as biosynthesis of steroid hormones, carbon source assimilation, and metabolism of xenobiotics. In addition, cytochrome P450 enzymes have been reported to be involved in the biosynthesis of many pharmaceutically interesting secondary metabolites from a variety of microorganisms (1–4). Cytochromes P450 are usually dependent on an external electron donor. With respect to their electron transport system they can be divided into several classes, with class I (the mitochondrial/bacterial cytochrome P450 systems) being the predominant form in prokaryotes (5). In this system the electrons required for the enzymatic reaction originate from NAD(P)H and are delivered to the cytochrome P450 via a ferredoxin reductase and a ferredoxin. In a number of examples, the heterologous reconstitution of the electron transfer chain has been shown to be ineffective, if possible at all (5). Thus, it is desirable to identify the natural redox partners, especially if genomic sequence information is available. However, even then the identification of the correct interaction partners remains challenging because the encoding genes are frequently located at genomic loci distant to the cytochrome P450 genes (6, 7). Interestingly, members of both the [2Fe-2S] and the non-[2Fe-2S] ferredoxins have been reported to sustain cytochrome P450 catalyzed reactions. The latter group is further subdivided into mono- and dicluster ferredoxins (i.e. the [3Fe-4S] or [4Fe-4S] and the [3Fe-4S] + [4Fe-4S] or [4Fe-4S] + [4Fe-4S] ferredoxins). Remarkably, cytochrome P450 systems depending on non-[2Fe-2S] ferredoxins have been found exclusively in bacteria to date (8, 9).

To fulfill the role as electron mediator, the ferredoxin component of any given cytochrome P450 system has to be reduced. This reduction is achieved by a ferredoxin reductase, which in turn takes up electrons from NAD(P)H. The ferredoxin reductase is often the least characterized constituent of the cytochrome P450 system because these flavoproteins may be unstable (i.e. easily lose their cofactor) and usually show a relatively low level of expression (10).

*Sorangium cellulosum* So ce56 is a genome-sequenced myxobacterial model strain. Because of their biotechnological potential as producers of secondary metabolites, the myxobacteria attract attention from both the academic community and the pharmaceutical industry. To date, more than 100 new basic structures and some 500 derivatives have been reported (11), with almost half of the newly discovered natural products being isolated from the genus *Sorangium* (11, 12). The potent anticancer agent epothilone, for example, was discovered from *S. cellulosum* So ce90 (13, 14). Epothilone is one of so far seven known myxobacterial compounds, the biosynthesis of which involves cytochromes P450 (15). Besides the epothilones, these are the antifungal leupyrrins (16) and the cytotoxic spirangienes (17) (also from *S. cellulosum*), the antibiotic myxovirescin from *Myxococcus* (18), the electron transport inhibitor stigmatellin (19) and the antibiotic aurafuron (20) from *Stigmatella aurantiaca*, and the antifungal ajudazols from *Chondromyces crocatus* (21).
The recently genome-sequenced myxobacterium *S. cellulosum* So ce56 (12) shows great potential for biotechnological applications, as judged on the basis of its capacity for the production of secondary metabolites. Three biologically active compounds have been described so far, namely the fungicidal chivosazoles, the macrolide antibiotic etnangien, and the iron chelator myxochelin (12). Moreover, the bioinformatic analysis of the So ce56 genome has revealed numerous biosynthetic gene clusters of yet unknown function (11, 12). With a size of more than 13 Mbp, the genome of *S. cellulosum* So ce56 is to date the largest sequenced prokaryotic genome (12). It has been shown to harbor 21 cytochrome P450 genes. In light of the significance of *S. cellulosum* as a viable source of bioactive secondary metabolites (14) and the role of cytochromes P450 in the synthesis of natural products (2), it is of great interest to elucidate the function of these enzymes.

Therefore, the investigation of the *S. cellulosum* So ce56 cytochrome P450 systems opens a fascinating field not only with regard to basic research but also to exploit the biotechnological potential of this model strain. To achieve this goal it is important to provide a functional electron transport chain. Thus, the main objective of this work was to identify a myxobacterial ferredoxin/ferredoxin reductase couple able to support reactions catalyzed by *S. cellulosum* So ce56 cytochromes P450.

### EXPERIMENTAL PROCEDURES

**Bioinformatic Analysis—**Putative ferredoxin and ferredoxin reductase genes were identified by searching the genome of *S. cellulosum* So ce56 for sequences similar to those of various known ferredoxins and flavoproteins. Translation of the DNA sequences was performed using the ExPASy Translate tool. Prediction of molecular mass and theoretical pl values was performed using Compute pl/Mw(22). Prediction of the ferredoxin cluster type and checking for the presence of NAD(P)H and flavin binding domains in the flavoproteins was done via Pfam Version 21.0 (23).

**Materials—**Phusion™ High Fidelity DNA polymerase was purchased from Finnzymes (Espoo, Finland), Fast-Link™ DNA Ligation kit was from EPICENTRE Biotechnologies (Madison, WI), restriction enzymes were from Promega (Madison, WI), PreScission Protease was from GE Healthcare, and glucose-6-phosphate dehydrogenase was from Roche Diagnostics. Oligonucleotides were purchased from BioTeZ Berlin-Buch GmbH (Berlin, Germany) or Eurofins-MWG (Ebersberg, Germany). The Escherichia coli strains used were from Novagen (affiliate of Merck KGaA, Darmstadt, Germany), nutrient broth medium was from Sifin (Berlin, Germany), antibiotics were from Carl Roth GmbH (Karlsruhe, Germany), and isopropyl-β-d-thiogalactopyranoside was from Acros Organics. FAD, FMN, and phenylmethylsulfonyl fluoride were obtained from Serva Electrophoresis GmbH (Heidelberg, Germany), NADH and NADPH were from GERBU Biochemicals GmbH (Gaiaberg, Germany), Glucose 6-phosphate was obtained from Carl Roth GmbH, Nootkatone and sodium dithionite were purchased from Fluka Analytical/Sigma-Aldrich Chemie GmbH (Steinheim, Germany); dithiothreitol and reduced glutathione were likewise from Sigma-Aldrich. Chromatography media for protein purification were purchased from GE Healthcare for glutathione-Sepharose 4 Fast Flow and Superdex 75 pg and Clontech Laboratories for Talon Metal Affinity Resin. Other standard chemicals and reagents used were of the highest purity commercially available.

**Isolation of Genomic DNA and Plasmid Purification—**Genomic DNA from *S. cellulosum* So ce56 was isolated using the Puregene® Genomic DNA purification kit (Gentra, Minneapolis, MN) as per the manufacturer's instructions. Plasmid DNA purification was performed using either the NucleoSpin Plasmid QuickPure kit or the Nucleobond Plasmid Purification AX 100 kit (Macherey-Nagel, Düren, Germany).

**Amplification of Genes and Cloning into Expression Vectors—**DNA fragments encoding the proteins of interest were prepared by polymerase chain reaction using genomic DNA of *S. cellulosum* So ce56 as template. The PCR primers were designed to introduce an NcoI restriction site at the 5′-end of the fragment and an EcoRI and/or HindIII restriction site at the 3′-end, respectively. Apart from these modifications, fragments with and without a sequence stretch encoding a C-terminal His_tag were generated. For expression in *E. coli*, the plasmids pKKHC and pGEX6P1_nco were used. Plasmid pKKHC (24) was a gift from Todd Porter (Ann-Arbor, Michigan, MI). Plasmid pGEX6P1_nco was derived from pGEX6P1 (Amersham Biosciences) by introducing an NcoI recognition sequence in the multiple cloning site via site-directed mutagenesis. The fidelity of the PCR amplification was verified by DNA sequencing (performed by Eurofins-MWG, Ebersberg, Germany) after either the subcloning step or in the final expression vector.

**Expression of Recombinant Myxobacterial Ferredoxins and Ferredoxin Reductases—**Precultures of *E. coli* BL21 harboring a ferredoxin expression plasmid were grown in nutrient broth medium containing 100 μg/ml ampicillin overnight. They were used to inoculate the 1-liter main culture containing 100 μg/ml ampicillin and incubated at 37 °C until absorbance at 600 nm reached ~1.0. At this point expression was induced by adding 1 mM isopropyl-β-d-thiogalactopyranoside. The incubation was continued for a minimum of 10 h (up to 48 h) at 25 or 37 °C, depending on the protein to be expressed. At the end of the expression period the *E. coli* cells were harvested by centrifugation at 4000 × g for 20 min, and the cell pellet was stored at −20 °C until further use. All of the following procedures were carried out at 4 °C. In the case of the ferredoxin reductases, overnight cultures of *E. coli* Rosetta(DE3)pLysS transformed with the adequate expression plasmid were used to inoculate 1 liter medium containing 100 μg/ml ampicillin and 50 μg/liter chloramphenicol. The bacteria were grown at 37 °C under constant shaking until the absorbance at 600 nm reached 1.0. Induction was done as described above; afterward, the cultures were cultivated for another 24–48 h at 37 °C. The bacteria were harvested, and the cell pellet was stored as described above.

**Purification of Glutathione S-transferase-tagged Ferredoxin Fdx1—**The bacterial pellet was resuspended in phosphate-buffered saline (25) containing 1 mM EDTA. After the addition of phenylmethylsulfonyl fluoride (50 μg/ml), cells were sonicated, the suspension was centrifuged, and the ferredoxin-containing supernatant was loaded onto a column packed with glutathione-Sepharose. Affinity chromatography and cleavage of the
glutathione S-transferase tag was performed according to the manufacturer’s recommendations. The cleaved-off ferredoxin was collected and concentrated for size exclusion chromatography, which was performed using Superdex 75 pg and 50 mM potassium phosphate buffer, pH 7.4, with a constant flow rate of 0.1 ml/min. Suitable fractions were collected, concentrated, and stored at −20 °C.

Purification of His-tagged Ferredoxins Fdx2, Fdx3, Fdx5, Fdx6, and Fdx8 and Ferredoxin Reductases FdR_A and FdR_B and CYP260A1—In the case of the His-tagged proteins, the bacteria were resuspended in 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 50 μg/ml phenylmethylsulfonyl fluoride and disrupted by sonication. After centrifugation the supernatant was loaded onto an IMAC column (TALON™ Resin). Purification was carried out following the manufacturer’s instructions. Fractions containing the heterologously expressed protein were collected and concentrated for size exclusion chromatography, which was performed using a Superdex 75 pg column and 50 mM potassium phosphate buffer, pH 7.4, with a constant flow rate of 0.1 ml/min. Suitable fractions were collected, concentrated, and stored at −20 °C.

Characterization of S. cellulosum So ce56 Ferredoxins and Ferredoxin Reductases by UV-visible Spectroscopy—UV-visible spectra from 250 to 700 nm were recorded in 50 mM potassium phosphate buffer, pH 7.4, with a constant flow rate of 0.1 ml/min. Suitable fractions were collected, concentrated, and stored at −20 °C.

Characterization of S. cellulosum So ce56 Ferredoxins and Ferredoxin Reductase FdR_B by EPR—EPR spectra were recorded on a Bruker ESP300 spectrometer (X-band, 9.5 GHz) equipped with a continuous flow helium cryostat ESR 900 and ITC 4 temperature controller (Oxford Instruments) to allow measurements down to 5 K. Parameters in standard measurements were: modulation frequency, 100 kHz; microwave power, 2 milliwatts; modulation amplitude, 5 G; time constant, 41 ms. The microwave frequency was measured by a HP 53550B frequency counter. Other parameters of measurements are given in the legend for Fig. 2. The samples (150 μM) in oxidized form or after reduction with dithionite were transferred in EPR quartz tubes (Wilms) and frozen in liquid nitrogen. The spectral parameters (g-tensor, line width) of the FeS centers were obtained by simulation of the spectra with the programs Simfonia or Xsophe (Bruker). Partial reduction of FdR_A and FdR_B was achieved by titration with NADH or NADPH.

Identification of the Ferredoxin Reductase Flavin Cofactor—For LC-MS analysis of the flavin the purified flavoproteins were diluted to a final concentration of 10 μM with methanol at 23 °C and centrifuged for 10 min at 15,000 × g. In the case of FdR_B, this treatment resulted in the denaturation of the protein and release of the flavin cofactor to the supernatant. The flavin cofactor of FdR_A, however, remained bound to the precipitated protein. To remove the prosthetic group of FdR_A, the protein was diluted in water and denatured by incubation at 100 °C for 10 min in the dark (26). The resulting flavin-containing supernatant was concentrated by evaporation and afterwards diluted with methanol to a final concentration of 10 μM. Five μl of the flavin solution were analyzed by LC-MS (Agilent 1100 series, Bruker HCT plus Ion Trap mass spectrometer). Chromatography and identification of the flavin species was performed as described previously (27).

Ferricyanide Assay—K_m values of FdR_A and FdR_B for NADPH were obtained by spectrophotometrically observing the reduction of ferricyanide at 420 nm (ε420 = 1020 M⁻¹ cm⁻¹) (28). The reduction of ferricyanide was assayed in 50 mM Tris-HCl, pH 8.0, at 23 °C using 1 μM FdR, 2 mM potassium ferricyanide, and different concentrations of NAD(P)H. The reaction was initiated by the addition of NAD(P)H, and the reaction progress was followed over 60 s. Reactions were performed in triplicate. The obtained reaction rates were fitted using Origin software (Microcal) to derive K_m values.

CYP260A1-dependent Substrate Conversion Assay—CYP260A1-dependent conversion of nootkatone was used to investigate the ability of different ferredoxin-ferredoxin reductase combinations to deliver electrons to an actual S. cellulosum So ce56 cytochrome P450. For each combination we prepared at least triplicate reactions. The reaction mixture contained 2 μM FdR, 20 μM ferredoxin, 0.25 μM CYP260A1, 100 μM nootkatone, and a NADPH-regenerating system consisting of 5 mM glucose 6-phosphate, 1 mM MgCl_2, and 1 unit of glucose-6-phosphate dehydrogenase in 10 mM potassium phosphate buffer, pH 7.4. The total reaction volume was 500 μl. The reaction was started by the addition of NADPH to a final concentration of 200 μM and incubated at 30 °C for 90 min. Samples were extracted two times with chloroform, and the extracts were dried and resuspended in 200 μl of acetonitrile. HPLC analysis was performed employing a reversed phase column (Waters 3.9 × 150-mm column WAT036975) and an isotropic solvent system containing acetonitrile/water (40:60). Substrate and products were detected at 240 nm. Assuming the absorbance properties of the products are the same as those of the substrate, the peak areas were used to determine the overall product formation.

LC-MS Analysis of Nootkatone Conversion Products by LC-MS—The products of the CYP260A1-dependent conversion of nootkatone were analyzed by LC-MS (Agilent 1100 series solvent delivery system coupled to a Bruker HCT plus ion trap mass spectrometer). Separation was performed on a RP-C18 column (Luna HST, 100 × 2 mm, 2.5-μm particle size; Phenomenex) at 32 °C using a mobile phase gradient from 5 to 95% B at a flow rate of 0.4 ml/min. Solvent A (water) and solvent B (acetonitrile) each contained 0.1% formic acid. Mass spectrometric detection was carried out in positive ionization mode ([M + H]^+ = 235 m/z or 251 m/z for mono- or dihydroxylated products, respectively).

NADPH Consumption Assay—In the NADPH consumption assay the four combinations, Fdx2-FdR_B, Fdx8-FdR_B, Fdx2-FdR_A, and Fdx8-FdR_A, shown to support the catalytic activity of CYP260A1 were further investigated. The reaction mixture contained 2 μM FdR, 20 μM ferredoxin, and 0.25 μM CYP260A1 in 10 mM potassium phosphate buffer, pH 7.4. The substrate (nootkatone) concentration was varied from 0 to 100 μM. After the addition of NADPH to a final concentration of 200 μM, the absorbance at 340 nm was monitored for 5 min.
Results

Bioinformatic Analysis—Searching the genome of S. cellulosum So ce56 for possible interaction partners of cytochrome P450 systems revealed the presence of eight putative ferredoxin genes and two putative ferredoxin reductase genes. Neither flavodoxin-like nor cytochrome P450 reductase-like genes were found.

All of the ferredoxin genes are distributed over the whole chromosome and are not in the neighborhood of either one of the putative ferredoxin reductase genes or one of the 21 cytochrome P450 genes in S. cellulosum So ce56 (data not shown). Protein domain analysis of the deduced amino acid sequences of Fdx1-8 using the Pfam Data base indicated that ferredoxins 1 and 5 might harbor a [2Fe-2S] cluster, whereas ferredoxins 2, 4, 6, 7, and 8 were predicted to belong to the [4Fe-4S] cluster ferredoxins with Fdx2, -6, and -8 being the monomer type and Fdx4 and -7 having two clusters (see Table 1). A Pfam match of Fdx3 declared this protein as a “sucrase/ferredoxin-like” protein. As the name indicates, proteins of this type resemble ferredoxins, albeit they are usually considerably larger in size and display sucrolytic activity.

Expression and Purification—As it was not possible to deduce the ferredoxin(s) involved in S. cellulosum cytochrome P450 systems by bioinformatic analysis, we set out to heterologously express and characterize the respective proteins. Using the conditions described, we were able to express six of the eight identified ferredoxins in a soluble form. Whereas Fdx4 could not be expressed at all, Fdx7 was expressed in high yield but was found solely in inclusion bodies. Neither lowering the expression temperature nor coexpression of chaperones nor supplementing the medium with ethanol in varying concentrations before induction (29, 30) afforded the expression of Fdx7 in a soluble form (data not shown). Fdx6 could be expressed in a soluble form, but it proved to be highly unstable, as it precipitated after the initial purification steps after approximately 2 h on ice. By adding 400 mM arginine to the buffer, the protein could be stabilized for a few hours; however, the frozen partly purified Fdx6 precipitated upon thawing even in the presence of arginine or other stabilizing agents such as glycerol or sucrose (31). As Fdx6 could not be sufficiently stabilized to be more than partly purified, we excluded this protein from our studies. The remaining five ferredoxins could be obtained in high purity and sufficient yield to carry out both spectroscopic and functional studies (see Table 2). In addition, the two ferredoxin reductases could be overexpressed in a codon-optimized E. coli strain in excellent yields (see Table 2). Both were found to be soluble and stable during the purification procedure. A loss of the flavin cofactor was not observed.

The purity of the heterologously expressed myxobacterial proteins was monitored by SDS-PAGE. In SDS-PAGE (see Supplement 2) ferredoxins 1 and 5 migrated at the expected size, whereas Fdx3 migrated slightly differently from expected on

| Protein name | Gene name | UniProt entry | No. amino acids | Predicted molecular mass | Theoretical pl | Pfam matches (over residues) |
|--------------|-----------|---------------|-----------------|--------------------------|---------------|----------------------------|
| Fdx1         | sce7334   | A9YE18        | 118             | 12.8                     | 4.94          | Fer2 (6–89)                |
| Fdx2         | sce8005   | A9FH21        | 101             | 11.2                     | 4.29          | Fer2 (2–25)                |
| Fdx3         | sce9225   | A9GDT7        | 127             | 13.6                     | 8.34          | Sucrase/Ferredoxin-like (71–102) |
| Fdx4         | sce3039   | A9GFT9        | 111             | 11.9                     | 4.62          | Fer2 (3–26), Fer4 (34–57)  |
| Fdx5         | sce7304   | A9EXQ0        | 103             | 10.9                     | 5.47          | Fer2 (5–88)                |
| Fdx6         | sce5732   | A9G7R4        | 241             | 25.7                     | 8.16          | Fer4 (155–180)             |
| Fdx7         | sce2755   | A9GAV0        | 94              | 10.4                     | 5.61          | Fer4 (31–55), Fer4 (63–86) |
| Fdx8         | sce5549   | A9G317        | 107             | 11.9                     | 4.32          | Fer4 (52–75)               |
| FdxR_A       | sce7188   | A9E5S5        | 278             | 30.5                     | 6.01          | FAD Binding_6 (7–97)       |
| FdxR_B       | sce5135   | A9FR0         | 244             | 26.7                     | 7.96          | NAD Binding_1 (112–231)    |

| Protein name | Yield mmol/liter | Maximum absorbance maxima (shoulder) | Molar extinction coefficient | g factor |
|--------------|------------------|--------------------------------------|-----------------------------|---------|
| Fdx1         | >600             | 321                                   | \(\varepsilon_{411} = 8320\) | \(g_1 = 2.022\) |
|              |                  |                                      |                             | \(g_2 = 1.937\) |
| Fdx2         | >350             | 390                                   | \(\varepsilon_{457} = 6181\) | \(g_1 = 1.928\) |
|              |                  |                                      |                             | \(g_2 = 1.998\) |
| Fdx3         | >750             | 330                                   | \(\varepsilon_{454} = 6589\) | \(g_1 = 1.963\) |
|              |                  |                                      |                             | \(g_2 = 2.000\) |
| Fdx4         | ND               | 462                                   | \(\varepsilon_{414} = 11704\) | \(g_1 = 1.960\) |
|              |                  |                                      |                             | \(g_2 = 1.918\) |
| Fdx5         | 400              | 418                                   | \(\varepsilon_{454} = 6181\) | \(g_1 = 1.954\) |
|              |                  |                                      |                             | \(g_2 = 1.890\) |
| Fdx6         | ND               | 454                                   | ND                          | ND       |
| Fdx7         | ND               | 487                                   | ND                          | ND       |
| Fdx8         | >250             | 400                                   | \(\varepsilon_{448} = 7970\) | \(g_1 = 2.021\) |
|              |                  |                                      |                             | \(g_2 = 1.990\) |
| FdxR_A       | 400              | 369                                   | \(\varepsilon_{448} = 8760\) | \(g = 2.004\) |
| FdxR_B       | 400              | 388                                   | \(\varepsilon_{448} = 8730\) | \(g = 2.004\) |
the basis of its calculated molecular mass (−17.0 versus 14.4 kDa). Very surprising is the behavior of Fdx2 and Fdx8, which are found at −24.0 or 19.0 kDa instead of 12.0 or 12.7 kDa, respectively. It has been previously reported that ferredoxins might migrate with an apparent molecular mass different from the calculated value (32, 33). The ferredoxin reductases FdR_A and FdR_B migrate between 25 and 35 kDa, which is consistent with the bioinformatic prediction.

**UV-visible Spectroscopy**—Ferredoxins 1 and 5 were reddish-brown in color and displayed characteristic spectral features of [2Fe-2S] ferredoxins (see Table 2 and Fig. 1a) known from [2Fe-2S] ferredoxins like adrenodoxin (414 nm, 455 nm) (34) or putidaredoxin (415 nm, 455 nm) (35). Pfam analysis of Fdx3 did not reveal the nature of the iron-sulfur cluster in this protein, but phylogenetic analysis suggested similarity to the [2Fe-2S] ferredoxin of Clostridium pasteurianum. Indeed, the UV-visible spectrum of Fdx3 shows maxima at 330, 462, and 545 nm which is reminiscent of the spectra of the [2Fe-2S] ferredoxins of C. pasteurianum (32) and Azotobacter vinelandii (36), both of which belong to the thioredoxin-like ferredoxins. However, in the case of Fdx3 we observed no local maximum at 420 nm, which was described for the other two proteins. Ferredoxins 2 and 8 were dark green-brown colored and showed visible absorption bands between 390 and 400 nm (see Table 2 and Fig. 1b), which confirms them to be non-[2Fe-2S] ferredoxins, although it allows no distinction between [3Fe-4S] or [4Fe-4S] as well as dicluster types (i.e. 7Fe or 8Fe ferredoxins) (8, 35).

Both ferredoxin reductases were bright yellow in color as expected for flavoproteins. The spectral features of FdR_A (Table 2, Fig. 1c) are typical for ferredoxin reductases with local maxima at 369 and 448 nm as well as a pronounced shoulder at 473 nm. These values correspond very well with those reported for ferredoxin reductases employed in cytochrome P450 systems such as adrenodoxin reductase (maxima 376 and 450 nm, shoulder at 475 nm (37)) or putidaredoxin reductase (maxima at 379 and 455 nm, shoulder at 480 nm (38)). In the case of FdR_B, we observed a slight bathochromic shift in the spectrum; it displayed local maxima at 388 and 457 nm as well as a shoulder at 490 nm (Table 2, Fig. 1c).

**EPR Spectroscopy**—Because [3Fe-4S] and [4Fe-4S] as well as the dicluster non-[2Fe-2S] ferredoxins cannot be distinguished by UV-visible spectroscopy, we performed EPR measurements. In addition, for the [2Fe-2S] ferredoxins, valuable information regarding possible functions can be obtained by EPR spectroscopy because [2Fe-2S] ferredoxins involved in cytochrome P450 systems usually have axial-type EPR spectra (e.g. adrenodoxin or putidaredoxin (35)). Indeed, reduced Fdx1 shows an axial-type EPR spectrum (Fig. 2a), with the g factors given in Table 2 indicating a slightly more rhombic g symmetry for Fdx1 than for adrenodoxin. The values agree very well with those obtained from typical cytochrome P450 class I ferredoxins like adrenodoxin and putidaredoxin (35). In contrast, reduced Fdx3 and Fdx5 display rhombic EPR spectra (Fig. 2b). The spectrum of Fdx3, and its g factors (Table 2) resemble those of the [2Fe-2S] ferredoxin of C. pasteurianum (g1 = 2.00, g2 = 1.95, g3 = 1.92 (32)) and A. vinelandii (g1 = 2.01, g2 = 1.94, g3 = 1.92 (36)). The spectral parameters of Fdx5 (Table 2) on the other hand are more reminiscent of plant-type ferredoxins like spinach ferredoxin (g1 = 2.05, g2 = 1.96, g3 = 1.89) or parsley ferredoxin (g1 = 2.05, g2 = 1.96, g3 = 1.90) (35). The asymmetry observed in the EPR lines of Fdx5 indicates the presence of a minority species (<20% of total intensity) with a slightly increased rhombicity (g1 ≈ 2.06, g2 ≈ 1.97, g3 ≈ 1.87).

![Figure 1. UV-visible spectra of oxidized S. cellulosum So ce56 ferredoxins and ferredoxin reductases.](image-url)

*FIGURE 1. UV-visible spectra of oxidized S. cellulosum So ce56 ferredoxins and ferredoxin reductases.* In panel a the spectra of the [2Fe-2S] ferredoxins Fdx1 (black line), Fdx3 (light gray line) and Fdx5 (gray line) are shown. The UV-visible spectra of the non-[2Fe-2S] ferredoxins Fdx2 (black line) and Fdx8 (gray line) are depicted in panel b. The ferredoxin reductases FdRA (black line) and FdRB (gray line) are found in panel c. The maxima of the oxidized protein spectra are given in Table 2. ~20 μM protein solutions in 50 mM potassium phosphate buffer, pH 7.4, were used.
Both non-[2Fe-2S] ferredoxins from *S. cellulosum* So ce56 gave unexpected results during EPR measurements. In the case of Fdx2 only a very weak signal with the characteristic features of a [3Fe-4S] cluster was detected at 10 K in the oxidized protein. This signal disappeared upon reduction, but no additional signal of a FeS cluster was found. In contrast, oxidation of Fdx2 with increasing amounts of K₃[Fe(CN)₆] led to a considerable increase in the intensity of the [3Fe-4S] cluster signal, which displayed g factors of $g_1 = 2.020$, $g_2 = 1.998$, and $g_3 = 1.963$. Under oxidizing conditions Fdx8 gave a signal at very low temperature (10 K) with a narrow line at $g$ of 1 G, and a time constant of 20 ms. For all measurements 150 μM protein solutions in 100 mM potassium phosphate buffer, pH 7.4, were used. 

**FIGURE 2.** EPR spectra of *S. cellulosum* So ce56 ferredoxins and ferredoxin reductase FdR_B. Panel a shows the axial-type EPR spectrum of Fdx1 measured with a microwave power of 6 milliwatts. In panel b the spectra of the rhombic iron-sulfur cluster ferredoxins Fdx3 (black line) and Fdx5 (gray line) are given. In panel c the EPR spectra of oxidized Fdx2 (black line) and Fdx8 (gray line) are depicted (measured at 10 K). The signal of Fdx2 was obtained with a 2-fold molar excess of K₃[Fe(CN)₆] added to the sample. In panel d the EPR spectra of FdR_A (black line) and FdR_B (gray line) semiquinones obtained by partial reduction with NADPH are given. These measurements were carried out at 70 K with a microwave power of 0.02 milliwatts, a modulation amplitude of 1 G, and a time constant of 20 ms. For all measurements 150 μM protein solutions in 100 mM potassium phosphate buffer, pH 7.4, were used. B [mT], magnetic field times millitesla.

The line width of the FdR_A semiquinone was with 17.5 G slightly smaller but is still in the range of a neutral semiquinone (typical line width of 19 G as opposed to the anionic semiquinone with a typical line width of 14–15 G (43)). Interestingly, not only spinach ferredoxin reductase (line width 20 G) but also the mammalian adrenodoxin reductase (line width 18 G) and its mycobacterial homologue FpR_A, which are both known to be components of class I cytochrome P450 systems, were reported to form neutral semiquinones (28, 41, 44).

It is also noteworthy that FdR_A and FdR_B differed in their reduction behavior. Although both could be reduced by NADH and NADPH, the concentrations necessary to observe the semiquinone signal were rather different. In the case of FdR_B, equimolar concentrations of the pyridine nucleotides sufficed to obtain an EPR signal, although its intensity could be increased by further addition of the reductant. In contrast, we were not able to obtain a similar response from FdR_A until we employed an excess of NAD(P)H. A very weak signal could be seen at a 4-fold molar excess of NADPH over FdR_A, which increased with further addition of NADPH. In contrast, even with an 11-fold molar excess of NADH there was barely a signal detectable. Only after the addition of solid NADH in large excess was an EPR spectrum of the FdR_A semiquinone obtained.

**Identification of the Flavin Cofactors**—To determine whether the flavin incorporated into FdR_A and FdR_B was FAD or FMN, we performed LC-MS analyses. For both putative ferredoxin reductases we obtained a single peak with a molecular mass of 784.2 at 8.0 min, which agrees with the theoretical molecular mass of FAD ($C_{27}H_{31}N_6O_5P_2$, 783.5). Also, the retention time for this signal was identical to the one obtained from the reference FAD solution. Moreover, FAD was easily removed from the proteins excluding a covalent linkage.

**Ferricyanide Assay**—To determine the affinity of the putative ferredoxin reductases for NADH and NADPH, we monitored the NAD(P)H-dependent reduction of the artificial electron acceptor potassium ferricyanide in a steady-state assay. For NADPH we determined the $K_m$ values to be 474 ± 83 μM and 120 ± 17 μM for FdR_A and FdR_B, respectively. In the case of NADH we were not able to determine $K_m$ values as both proteins were still not saturated at a NADH concentration of 10 mM. Therefore, we concluded that although NADH is able to reduce both FdR_A and FdR_B (as seen in EPR measurements), the interaction seems to be unspecific.

**Cytochrome P450-dependent Substrate Conversion Assay**—To test the ability of the ferredoxins to receive electrons from either FdR_A or FdR_B and to transfer them to mycobacterial cytochromes P450, we decided to use a genuine So ce56 cytochrome P450. We chose CYP260A1, which is the first identified member of a new cytochrome P450 family. We found that two of the ferredoxins, Fdx2 and Fdx8, were able to efficiently support the catalytic activity of CYP260A1. As depicted in Fig. 3a, we observed a conversion of the sesquiterpenoid compound nootkatone to at least five more hydrophilic products. There is one predominant product (Fig. 3a, peak 2), which eluted at ∼4.4 min. This main product is flanked by two byproducts which show a retention time of 3.6 min (Fig. 3a, peak 3) and 6.2 min (Fig. 3a, peak 1). We found two more products in smaller...
DISCUSSION

Because of the complexity of the bacterial cytochrome P450 systems there is only a small number of prokaryotic class I systems whose electron transport pathways have been investigated and well understood (45). In the present work we characterized the electron transport system of a cytochrome P450 from S. cellulosum So ce56.

Altogether, we identified eight putative ferredoxin genes and two putative ferredoxin reductase genes in the genome of S. cellulosum So ce56. As neither the genomic context nor the comparison with other ferredoxins as well as the according placement in a dendrogram (see Supplement 1) gave evidence for a ferredoxin, which might be more likely involved in cytochrome P450 systems than the others, we aimed at heterologous overexpression and *in vitro* characterization of all identified ferredoxins as well as the two ferredoxin reductases. We succeeded
in overexpressing and purifying five ferredoxins and both ferredoxin reductases.

In steady-state kinetic experiments we determined that both ferredoxin reductases prefer NADPH to NADH. In the case of NADPH, the apparent $K_m$ values were 474 $\mu$M (FdR_A) and 120 $\mu$M (FdR_B), whereas with NADH a saturation of the proteins could not be achieved even with redundant concentrations up to 10 mM. Compared with other ferredoxin reductases ($K_m$ NADPH, e.g. 3.9 $\mu$M for E. coli Flavodoxin reductase (46), 1.82 $\mu$M for bovine adrenodoxin reductase (47), 4.1 $\mu$M for M. tuberculosis FprA (28), and 11.3 $\mu$M for Arabidopsis thaliana mitochondrial Fdr (48)) the $K_m$ values obtained for the mycobacterial proteins are clearly higher, but their preference of NADPH over NADH is consistent with the properties of the other ferredoxin reductases mentioned above.

The two NADPH-dependent, FAD-containing functionally active oxidoreductases were used to identify a possible functional interaction, indicating successful electron transfer with the ferredoxin component(s) of S. cellulosum So ce56. We separately tested all the purified ferredoxins in combination with FdR_A and FdR_B, respectively. With CYP260A1 we had a genuine S. cellulosum So ce56 cytochrome P450 available, which we employed to probe the different electron mediator combinations. We found that none of the mycobacterial ferredoxins harboring a [2Fe-2S] cluster was able to support the CYP260A1-dependent conversion of nootkatone. To our surprise even the ferredoxin most similar to the well known cytochrome P450 system ferredoxins adrenodoxin and putidaredoxin, Fdx1, failed to support the CYP260A1-dependent reaction. Because several non-[2Fe-2S] cluster ferredoxins have been previously reported as homologous interaction partners of bacterial cytochromes P450 (8, 9), we also tested those from myxobacteria as potential electron transfer proteins. Recently published examples for cytochrome P450 systems containing ferredoxins of the [3Fe-4S] cluster type are the P450mor system from Mycobacterium sp. strain HE5 (40), the CYP51 system from M. tuberculosis (41), or the CYP260A1-dependent conversion of nootkatone. To our surprise even the ferredoxin most similar to the well known cytochrome P450 system ferredoxins adrenodoxin and putidaredoxin, Fdx1, failed to support the CYP260A1-dependent reaction. Because several non-[2Fe-2S] cluster ferredoxins have been previously reported as homologous interaction partners of bacterial cytochromes P450 (8, 9), we also tested those from myxobacteria as potential electron transfer proteins. Recently published examples for cytochrome P450 systems containing ferredoxins of the [3Fe-4S] cluster type are the P450mor system from Mycobacterium sp. strain HE5 (40), the CYP51 system from M. tuberculosis (41), or the CYP105D system from Streptomyces coelicolor A3(2) (45). Indeed we found the two non-[2Fe-2S] cluster ferredoxins Fdx2 and Fdx8 capable to deliver electrons from NADPH via FdR_A or FdR_B to CYP260A1 and to sustain the conversion of nootkatone to hydroxylated products. Both ferredoxins work with both reductases, but they do show a preference for ferredoxin reductase FdR_B compared with FdR_A. To consolidate our results and further investigate the ability of the four ferredoxin-ferredoxin reductase combinations to act as efficient electron transport systems to CYP260A1, we determined NADPH oxidation assays and estimated the kinetic constants for nootkatone binding. We found that under the conditions used only the turnovers containing FdR_B displayed NADPH turnover rates high enough to allow the determination of apparent $k_{cat}$ and $K_m$ values for nootkatone. This observation supports our prior findings that the main electron transfer pathways to CYP260A1 consist of FdR_B and either Fdx2 or Fdx8. Regarding the combinations FdR_B-Fdx2-CYP260A1 and FdR_B-Fdx8-CYP260A1, the kinetic parameters were on the same order of magnitude. Nevertheless, the system containing Fdx8 showed a higher $K_m$ value than the one containing Fdx2. At the same time also the apparent $k_{cat}$ value (i.e. the NADPH consumption) of the Fdx8-depending system (0.96 ± 0.45 s$^{-1}$) was higher than the one of the combination with Fdx2 (0.70 ± 0.02 s$^{-1}$). In contrast, we showed that the amount of product formed after 90 min was similar for both combinations (Fdx2, 76 ± 6% product formation; Fdx8, 65 ± 10% product formation) in the HPLC-based substrate conversion assay. Therefore, the higher NADPH consumption of the Fdx8-containing system may hint at an uncoupling of electron transfer from hydroxylation, which is well known for both class I and class II cytochrome P450 systems (49, 50) and would correlate with an increased $K_m$ value.

Taking together these results, we suggest two alternative main electron transfer pathways to the S. cellulosum So ce56 cytochrome P450 CYP260A1 consisting of NADPH, FdR_B, and either Fdx2 or Fdx8. With regard to biological function and significance, the modulation of cytochrome P450 activity via the use of different Fdx/FdR combinations is conceivable. Previously, such a regulation of enzymatic activity by reductase levels has been suggested in Streptomyces (51), and a similar mechanism might be found in myxobacteria. In this work we report for the first time a functional myxobacterial cytochrome P450, supported in vitro by two endogenous electron transfer pathways composed of a FAD-containing ferredoxin reductase and a ferredoxin. The identification of these two alternative electron transport pathways might prove to be of importance not only for basic research but also for practical application, i.e. to exploit the biotechnological potential of the S. cellulosum So ce56 cytochromes P450.

REFERENCES
1. Bernhardt, R. (1996) Rev. Physiol. Biochem. Pharmacol. 127, 137–221
2. Bernhardt, R. (2006) J. Biotechnol. 124, 128–145
3. Bernhardt, R., and Waterman, M. R. (2007) in The Ubiquitous Roles of Cytochrome P450 Proteins (Sigel, A., Sigel, H., and Sigel, R. K. O., eds) pp. 361–396, John Wiley & Sons, Ltd.
4. Bell, S. G., Hoskins, N., Xu, F., Caprotti, D., Rao, Z., and Wong, L. L. (2006) Biochim. Biophys. Acta 1770, 330–344
5. Meyer, F., Morsmann, S., Muñoz-Dorado, J., Pérez, J., Pradella, S., Rachid, M., Meyer, M., Møller, R., and Müller, R. (2006) J. Ind. Microbiol. Biotechnol. 33, 577–588
6. Schneiker, S., Perlov, O., Kaiser, O., Gerth, K., Alici, A., Altmeyer, M. O., Bartels, D., Bekel, T., Beyer, S., Bode, E., Bode, H. B., Bolten, C. J., Choudhuri, J. V., Doss, S., Elnahkady, Y. A., Frank, B., Gaigalat, L., Goeman, K., Groeger, C., Gross, F., Jelsbak, L., Jelsbak, L., Kalinowski, I., Kegler, C., Knauber, T., Konietzny, S., Kopp, M., Krause, L., Krug, D., Linke, B., Mahmud, T., Martinez-Arias, R., McHardy, A. C., Meier, M., Meyer, M., Møller, R., Mørk, S., Muñoz-Dorado, J., Perez, J., Pradella, S., Rachid, S., Raddatz, G., Rosenau, F., Rücker, C., Sasse, F., Scharfe, M., Schuster, S. C., Suen, G., Treuner-Lange, A., Velicer, G. J., Vorhölter, F. J., Weissmann, K. J., Welch, R. D., Wenzel, S. C., Whitworth, D. E., Wilhelm, S., Wittmann, C., Blöcker, H., Pühler, A., and Müller, R. (2007) Nat. Biotechnol. 25, 1281–1289
7. Altman, K. H., Höfle, G., Moll, R., Muller, J., and Prantz, K. (2008) From Soil to the Clinic: The Epothilones, an Outstanding Family of Anti-Tumor Agents, pp. 16–27, Springer Wien, New York
Genome Mining in S. cellulosum So ce56

14. Reichenbach, H. (2001) J. Ind. Microbiol. Biotechnol. 27, 149–156
15. Ogura, H., Nishida, C. R., Hoch, U. R., Perera, R., Dawson, J. H., and Ortiz de Montellano, P. R. (2004) Biochemistry 43, 14712–14721
16. Bode, H. B., Wenzel, S. C., Irschik, H., Höfle, G., and Müller, R. (2004) Angew. Chem. Int. Ed. Engl. 43, 4163–4167
17. Frank, B., Knauber, J., Steinmetz, H., Scharfe, M., Blöcker, H., Beyer, S., and Müller, R. (2007) Chem. Biol. 14, 221–233
18. Simonovics, V., Zapp, J., Rachid, S., Krug, D., Meiser, P., and Müller, R. (2006) Chembiochem. 7, 1206–1220
19. Gaitatzis, N., Silakowski, B., Kunze, B., Nordsiek, G., Blöcker, H., Höfle, G., and Müller, R. (2002) J. Biol. Chem. 277, 13082–13090
20. Frank, B., Wenzel, S. C., Bode, H. B., Scharfe, M., Blöcker, H., and Müller, R. (2007) J. Mol. Biol. 374, 24–38
21. Buntin, K., Rachid, S., Scharfe, M., Blöcker, H., Weissman, K. J., and Müller, R. (2008) Angew. Chem. Int. Ed. Engl. 47, 4595–5499
22. Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M. R., Appel, R. D., and Bairoch, A. (2005) in The Proteomics Protocols Handbook (Walker, J. M., ed) pp. 571–607, Humana Press, Springer, Berlin
23. Finn, R. D., Mistry, J., Schuster-Bohler, B., Griffiths-Jones, S., Hollich, V., Lassmann, T., Moxon, S., Marshall, M., Khana, A., Durbin, R., Eddy, S. R., Sonnhammer, E. L., and Bateman, A. (2006) Nucleic Acids Res. 34, D247–D251
24. Porter, T. D., and Larson, J. R. (1991) Methods Enzymol. 206, 108–116
25. Sambrook, J., and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, vol. 3, p. A1.7, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Aliverti, A., Curti, B., van Vanoni, M. A. (1999) Methods Mol. Biol. 131, 9–23
27. Ewen, K. M., Schiffer, B., Uhlmann-Schiffer, H., Bernhardt, R., and Hanemann, F. (2008) FEBS Yeast Res. 8, 432–441
28. McLean, K. J., Scutt, J. N., and Munro, A. W. (2003) Biochem. J. 372, 317–327
29. Caspers, P., Stieger, M., and Burn, P. (1994) Cell. Mol. Biol. 40, 635–644
30. Thomas, J. G., and Baneys, F. (1997) Protein Expr. Purif. 11, 289–296
31. Arakawa, T., Tsumoto, K., Kita, Y., Chang, B., and Ejima, D. (2007) Amino Acids 33, 587–605
32. Fujinaga, J., and Meyer, J. (1993) Biochem. Biophys. Res. Commun. 192, 1115–1122
33. Schiffer, B., Bureik, M., Reinele, W., Müller, E. C., Hannemann, F., and Bernhardt, R. (2004) J. Inorg. Biochem. 98, 1229–1237
34. Grinberg, A. V., Hannemann, F., Schiffer, B., Müller, J., Heinemann, U., and Bernhardt, R. (2000) Proteins 40, 590–612
35. Lovenberg, W. (ed.) (1973) Iron-sulphur Proteins, Vol. II, Molecular Properties, Academic Press, Inc., New York
36. Chatelet, C., and Meyer, J. (1999) J. Biol. Inorg. Chem. 4, 311–317
37. Sagara, Y., Wada, A., Takata, Y., Waterman, M. R., Sekimizu, K., and Horiiuchi, T. (1993) Biol. Pharm. Bull. 16, 627–630
38. Sevrioukova, I., and Poulos, T. L. (2002) J. Biol. Chem. 277, 25831–25839
39. Guiglarelli, B., and Bertrand, P. (1999) Adv. Inorg. Chem. 47, 421–497
40. Sielaff, B., and Andreeesen, J. R. (2005) FEBS J. 272, 1148–1159
41. McLean, K. J., Warman, A. J., Sedlack, H. E., Marshall, K. R., Girvan, H. M., Cheesman, M. R., Waterman, M. R., and Munro, A. W. (2006) Biochemistry 45, 8427–8443
42. Huang, K., Sun, S. I., and Wang, J. H. (1969) Biochem. Biophys. Res. Commun. 34, 48–52
43. Murataliev, M. B. (1999) Methods Mol. Biol. 131, 97–110
44. Kitagawa, T., Sakamoto, H., Sugiyama, T., and Yamano, T. (1982) J. Biol. Chem. 257, 12075–12080
45. Chun, Y. J., Shimada, T., Sanchez-Ponce, R., Martin, M. V., Lei, L., Zhao, B., Kelly, S. L., Waterman, M. R., Lamb, D. C., and Guengerich, F. P. (2007) J. Biol. Chem. 282, 17486–17500
46. Leadbeater, C., McIver, L., Campopiano, D. J., Webster, S. P., Baxter, R. L., Kelly, S. M., Price, N. C., Lysek, D. A., Noble, M. A., Chapman, S. K., and Munro, A. W. (2000) Biochem. J. 352, 257–266
47. Lambeth, J. D., and Kamion, H. (1976) J. Biol. Chem. 251, 4299–4306
48. Takubo, K., Morikawa, T., Nonaka, Y., Mizutani, M., Takenaka, S., Takabe, K., Takahashi, M. A., and Ohta, D. (2003) Proc. Natl. Acad. Sci. USA. 101, 494–499