Purification and characterization of ferredoxin-NADP(+) reductase encoded by Bacillus subtilis yumC

Title:

Purification and characterization of ferredoxin-$NADP^+$ reductase encoded by *Bacillus subtilis yumC* gene

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Abstract

From *Bacillus subtilis* cell extracts, ferredoxin-$NADP^+$ reductase (FNR) was purified to homogeneity and found to be the *yumC* gene product by N-terminal amino acid sequencing. YumC is a ~94 kDa homodimeric protein with one molecule of non-covalently bound FAD per subunit. In a diaphorase assay with 2,6-dichlorophenol-indophenol as an electron acceptor, the affinity to NADPH was much higher than to NADH, with K_m values of 0.57 vs. >200 μM. K_{cat} values of YumC with NADPH were 22.7 and 35.4 s^{-1} in diaphorase and in a ferredoxin-dependent NADPH-cytochrome *c* reduction assay, respectively. The cell extracts contained another diaphorase-active enzyme, the *yfkO* gene product, but its affinity for ferredoxin was very low. The deduced YumC amino acid sequence has high identity to that of the recently identified *Chlorobium tepidum* FNR. A genomic database search indicated that there are more than 20 genes encoding proteins that share a high level of amino acid sequence identity with YumC and annotated variously as NADH oxidase, thioredoxin reductase, thioredoxin reductase-like protein, etc. These genes are found notably in Gram-positive bacteria except for *Clostridia*, and less frequently in archaea and proteobacteria. We propose that YumC and *C. tepidum* FNR constitute a new group of FNR which should be added to the already established plant type, bacteria type, and mitochondria type FNR groups.

Introduction

Ferredoxin (Fd) is a small acidic electron transport protein that contains iron-sulfur cluster(s). The iron-sulfur clusters bound in Fds are [4Fe-4S], [3Fe-4S], and [2Fe-2S] (Beinert et al. 1997). In some cyanobacteria and heterotrophic bacteria, flavodoxins are able to functionally replace Fd. Fds and flavodoxins participate in a variety of redox reaction pathways, including photosynthesis, nitrogen fixation, etc., and some in the reduction of certain cytochrome P450s (Ifuku et al. 1994; Birch et al. 1995; Grinberg et al. 2000). In vertebrate adrenocortical mitochondria, adrenodoxin, a specific Fd, is an electron donor to cytochrome P450.

The enzymes that mediate the redox reactions between $NAD(P)^+$/NAD(P)H and Fd are Fd-$NAD(P)^+$ reductase (FNR) (EC 1.18.1.2 and EC 1.18.1.3 with $NADP^+$ and NAD^+ as the substrate, respectively) (Ceccarelli et al. 2004). The catalytic electron transport function between NAD(P)H and an artificial electron acceptor such as methyl viologen and 2,6-dichlorophenol-indophenol (DPIP) is called diaphorase activity and is shown by FNRs. FNRs contain FAD as a prosthetic group and are present in chloroplasts, mitochondria, and some bacteria.

FNRs are often divided into several groups. The first includes the plant-type FNRs that are monomeric enzymes also found in eukaryotic algae as well as in cyanobacteria, and have been intensively reviewed (Arakaki et al. 1997). The second group is sometimes called the bacteria type and is composed of monomeric FNRs from some bacteria such as *Escherichia coli* (Bianchi et al. 1993) and *Azotobacter vinelandii* (Isas et al. 1995). There are significant similarities between these two groups of FNRs in 3-D structure (Prasad et al. 1998) as well as in amino acid sequence (Arakaki

et al. 1997, Ceccarelli et al. 2004). These two FNR groups can be united to a plant type in a broader sense. The third group is sometimes called the adrenodoxin reductase or mitochondria type FNR and includes enzymes from mammalian mitochondria (Lin et al. 1990), yeast mitochondria (Lacour et al. 1998), the bacterium *Mycobacterium tuberculosis* (Fischer et al. 2002), etc. This type of FNR catalyzes essentially the same reaction as do the plant and bacteria types and there are certain amino acid sequence similarities among them. However, X-ray crystallographic studies indicate that the 3-D structure of adrenodoxin reductase is more similar to those of glutathione reductase and disulfide reductase in a broader sense than to the plant-type FNRs (Ziegler et al. 1999). A bovine adrenodoxin reductase and its homolog from *M. tuberculosis* (FprA) exist as monomeric proteins (Fischer et al. 2002), while glutathione reductases exist as homodimer.

With the entire genome sequences of many bacterial species now determined, several examples have been found, including Gram-positive bacteria and some archaea, whose genomes contain no genes coding for proteins with a high level of sequence identity to known FNRs. Recently, the occurrence of a novel type of FNR in the green sulfur bacterium *Chlorobium tepidum* was reported (Seo and Sakurai 2002). The enzyme shares much higher level of amino acid sequence identity with NAD(P)H-thioredoxin reductases than with plant-type, bacteria-type, or mitochondria-type FNRs. The *C. tepidum* enzyme is homodimeric and contains non-covalently bound FAD as do thioredoxin reductase, but has no di-cysteine motif (CXXC) essential for the catalytic function of the latter enzyme (Ronchi and Williams 1972). *C. tepidum* has another gene (CT0842, Eisen et al. 2002) that encodes a protein containing the di-cysteine motif which shares high levels of amino acid sequence

identity with thioredoxin reductases from various organisms, and which appears to be a genuine thioredoxin reductase gene. BLAST homology searches (Altschul et al. 1997) with the deduced amino acid sequence of *C. tepidum* FNR identified a number of genes from Gram-positive bacteria and archaea that encode proteins which share a high level of amino acid sequence identity with that of *C. tepidum*. These genes are variously annotated as encoding thioredoxin reductases, thioredoxin reductase-related proteins, NADH oxidase, etc. The di-cysteine motif essential for thioredoxin reductase is also absent in all of these proteins.

Bacillus subtilis is a gram-positive bacterium whose whole genome sequence has been elucidated (Kunst et al. 1997). Although the Fd was purified from *E. coli* cells overexpressing *B. subtilis* Fd gene (*fer*) and shown to support reduction of cytochrome P450 (BioI), the enzymes responsible for reduction of Fd have not yet been identified (Green et al. 2003). In this work, we show that the *yumC* gene of *B. subtilis* annotated as coding for a thioredoxin reductase-related protein (Kobayashi et al. 2003), is actually an FNR gene. To the best of our knowledge, this is the first report of an FNR from Gram-positive bacteria.

Materials and methods

Purification of ferredoxin-NADPH reductase from *B. subtilis*

B. subtilis 168 CA (106309 Pasteur Culture Collection) stock culture was kindly provided by Drs. K. Kobayashi and N. Ogasawara of Nara Institute of Science and Technology. Cells were grown in Luria-Bertani medium for 18 h at 37°C, collected by centrifugation at 10,000 × *g* for 20 min and stored at -80°C until use. The cells were suspended in 50 mM 2-amino-2-hydroxymethyl-1,3-propanediol (Tris)-HCl buffer (pH 7.8) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM *n*-caproic acid, and 1mM *p*-aminobenzamidine and disrupted at 0°C using 6 1-min sonication cycles, each followed by a 1-min rest interval (VP-30s, TAITEC, Japan). The sonicate was centrifuged at 20,000 × *g* for 20 min at 4°C, and the supernatant retained. The fraction obtained from the supernatant by precipitating between 35 % and 80 % saturation of ammonium sulfate at 4°C was collected by centrifugation at 20,000 × *g* for 20 min, dissolved in 50 ml of 20 mM 2-morpholinoethanesulfonic acid (MES)-NaOH buffer (pH 6.5), and dialyzed three times against 5 l of the same buffer for 2 h each at 4°C. After removing undissolved proteins by centrifugation at 200,000 × *g* for 60 min, the supernatant was diluted with two volumes of the same buffer and applied to Matrex Blue A (Amicon), a dye ligand affinity gel column (2.8 × 10

cm) equilibrated with 20 mM MES-NaOH buffer (pH 6.5) at 4°C. The column was washed with two column volumes of the equilibration medium and the bound proteins were eluted by a total of 432-ml linear gradient of 20 mM MES-NaOH (pH 6.5) buffer as a starting solution and 0.1 M glycine-NaOH (pH 11.5) buffer containing 1.5 M KCl as a feeding solution. Eluted fractions were assayed for NADPH-DPIP diaphorase activity. High activity fractions were pooled, concentrated by ultrafiltration (YM-10, Amicon) and applied to a gel-permeation column (Sephacryl S-200 HR 26/60, Pharmacia) equilibrated with 50 mM Tris-HCl buffer (pH 7.8) containing 150 mM NaCl at 4°C. The proteins were eluted with the same buffer at a flow rate of 20 ml h^{-1}. Fractions rich in the diaphorase activity were pooled, desalted by ultrafiltration (YM-10, Amicon) and applied to an anion exchange column (Mono Q 10/10, Pharmacia) equilibrated with 50 mM Tris-HCl (pH 7.8) at room temperature. After washing with two column volumes of the same buffer, the proteins were eluted from the column with a 70-ml linear gradient of NaCl concentration ranging from 0 to 350 mM in 50 mM Tris-HCl (pH 7.8) yielding two major peaks of diaphorase activity (Fig. 1). Peak fractions were pooled separately and purified as follows. The buffer was changed to 10 mM MES-NaOH (pH 6.5) by ultrafiltration (YM-10, Amicon), and the concentrate (2 ml) was applied to Matrex Red A (Amicon), a dye affinity column (1 × 10 cm) equilibrated with 20 mM MES-NaOH (pH 6.5). After washing with two column volumes of the same buffer, the activity was eluted as single-peak fractions, with a 24-ml linear gradient of 20 mM MES-NaOH (pH 6.5) buffer and 0.1 M glycine-NaOH (pH

11.5) buffer containing 1.5 M KCl. The buffer was changed to 50 mM Tris-HCl (pH 7.8) by ultrafiltration (Ultrafree 4, Millipore) and the concentrate was applied to an anion exchange column (Mono Q 5/5, Pharmacia) equilibrated with 50 mM Tris-HCl buffer (pH 7.8). The proteins were eluted with a 40-ml linear gradient of NaCl, from 0 to 400 mM in 50 mM Tris-HCl (pH 7.8) and stored at -80^{o}C until use.

Preparation of ferredoxin from *B. subtilis*

Fd was purified from the flow-through fraction from the Matrex Blue A column in FNR purification as described above. The purification procedures were essentially as described by Seo et al. (2001) for purification of Fds from *C. tepidum*. During the initial course of purification, Fd was detected by a cytochrome *c* reductase assay in the presence of spinach FNR that was prepared essentially according to Seo et al. (2001). Because crude preparations were contaminated with Fd-independent NAD(P)H-cytochrome *c* reductase activity, Fd-dependent cytochrome *c* reductase activity was estimated by subtracting the former activity obtained in the absence of spinach FNR. For purification, the flow-through fractions were applied to a diethylaminoethyl (DEAE)-cellulose column (2.6 × 15 cm, DE52, Whatman) equilibrated with 20 mM MES-NaOH buffer (pH 6.5), and after washing with two column volumes of the same buffer, Fd was eluted with a linear gradient of

NaCl concentration from 0 to 1 M. Active fractions were concentrated by ultrafiltration (YM-3, Millipore) and applied to a gel-permeation column (Sephacryl S-200 26/60, Pharmacia). Active fractions were collected and a saturated ammonium sulfate solution in 50 mM Tris-HCl (pH 7.8) was added to 2 M, and after standing for 20 min centrifuged at 20,000 × *g* for 20 min. The supernatant was applied to a hydrophobic column (Phenyl Superose 5/5, Pharmacia) equilibrated with the same buffer containing 2 M ammonium sulfate, and Fd was eluted with 30 ml of a reverse linear gradient of ammonium sulfate, concentration from 2 M to 0.8 M. Fd-rich fractions were collected, desalted by ultrafiltration (YM-3, Amicon) and applied to a Mono Q 5/5 column equilibrated with 20 mM bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (BisTris)-HCl buffer (pH 6.5). Fd was eluted with a 22.5 ml linear gradient of NaCl, concentration from 0 to 0.6 M, in 20 mM BisTris-HCl buffer (pH 6.5), and the final preparation was stored at -80°C until use. Fd concentration was estimated from ε_{390}= 16.0 mM^{-1} cm^{-1} (Green et al. 2003).

Enzyme activity assays

In this report, the turnover rates are expressed in number of molecules of oxidized NAD(P)H or reduced $NAD(P)^+$ by one molecule of native-form enzyme per second.

NAD(P)H-DPIP diaphorase activity was assayed at 24°C by monitoring the reduction of DPIP as

the decrease of absorbance at 600 nm (ε_{600} = 21.8 mM^{-1} cm^{-1}) for 1 min. The reaction mixture (500 µl) contained 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)-NaOH (pH 7.0), 0.1 mM DPIP, 10 mM glucose 6-phosphate (G7250, Sigma), 5 U of recombinant glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides,* G8404, Sigma), NAD(P)H and diaphorase (YumC or YfkO, see Results) as indicated in figure legends and tables. The diaphorase activity was calculated by subtracting the blank rate obtained in the absence of YumC and YfkO from the observed rate.

Cytochrome *c* reduction activity was assayed at 24°C by monitoring the increase in absorbance at 550 nm for 1 or 3 min under aerobic conditions. The reaction mixture (500 µl) contained 50 mM HEPES-NaOH (pH 7.0), 0.1 mM cytochrome *c* (horse heart, ε_{550} = 27.8 $mM^{-1}cm^{-1}$, C2506, Sigma) and either 7.6 nM YumC or 40 nM YfkO with NADPH and Fd as indicated in figure legends and tables.

NAD(P)H oxidase activity was assayed at 24°C by monitoring the decrease of absorption at 340 nm (ε_{340} = 6.22 $mM^{-1}cm^{-1}$) for 3 min. The reaction mixture contained 100 mM potassium phosphate (pH 7.0), 0.15 mM NAD(P)H and either 111 pmol of YumC or 51 pmol of YfkO in the presence or absence of externally added flavin mononucleotide (FMN) or FAD as indicated in the tables.

Photoreduction of Fd was assayed under strictly anoxic conditions by a heterologous assay system containing *C. tepidum* photochemical reaction center (3.84 µM bacteriochlorophyll *a*), 2 µM *B.*

subtilis Fd, 95 nM YumC or 101 nM YfkO, 0.1 mM $NADP^+$, 20 mM Tris-HCl (pH 7.8), 20 mM NaCl, 5 mM sodium ascorbate, 0.1 mM DPIP, 0.1% Triton X-100, 5 mM D-glucose, 1.25 units glucose-oxidase, 5×10^{-3} units catalase, and 0.25% (v/v) ethanol in a 1 ml mixture (Seo et al. 2001).

Miscellaneous methods

SDS-PAGE analysis was performed as described by Laemmli (1970), and protein bands in the gel were visualized by silver staining (Daiichi Kagaku, Tokyo). The native molecular mass was deduced by gel-permeation chromatography on Superdex 200 10/30 (Pharmacia) with a buffer containing 50 mM Tris-HCl (pH 7.8) and 200 mM NaCl at a flow rate of 0.5 ml min^{-1} with Molecular weight marker kit MW-GF-200 (Sigma) for standards: cytochrome *c* (horse heart, 12.4 kDa), carbonic anhydrase (bovine erythrocytes, 29.0 kDa), albumin (bovine serum, 66.0 kDa), alcohol dehydrogenase (yeast, 150 kDa), beta-amylase (sweet potato, 200 kDa), and blue dextran (2,000 kDa).

For determination of enzyme-bound flavins, the enzymes were treated with trichloroacetic acid (5%, w/v), and the flavins in the neutralized supernatant (pH 7.0) were determined by assuming ε_{450}= 11.3 mM^{-1} cm^{-1} for FAD and ε_{445} = 12.5 mM^{-1} cm^{-1} for FMN.

N-terminal amino acid sequence was analyzed by the Edman degradation method with Procise

491 or Procise cLC 494 sequencer (Applied Biosystems).

Genome sequence data were obtained from the Swissprot (sp), trembl (tr), pir (pir), prf (prf) databases on the GenomeNet server (http://www.genome.ad.jp, Kyoto Univ). Sequence alignments were obtained using CLUSTAL W (Thompson et al. 1994) on the GenomeNet server (Kyoto Univ.). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 (Kumar et al. 2001).

Results

Purification of NADPH diaphorases

Two major NADPH diaphorase activities in the cell extract from *B. subtilis* were purified using the protocol described in Materials and methods (Table 1). The Matrex Blue A column adsorbed considerable amounts of NADPH diaphorase activity while effectively removing bulk proteins as un-adsorbed fractions. NADPH diaphorase activity was subsequently eluted from the column as a broad peak using pH together with KCl concentration gradients (data not shown). The subsequent Sephacryl S-200 column chromatography effectively removed large molecular weight impurities (data not shown). By the first Mono Q column, NADPH diaphorase activities were resolved into two large peak fractions (Fig. 1). Each peak fraction was pooled separately and further purified by a Matrex Red A column, with the second Mono Q chromatography of each fraction yielding two NADPH diaphorase proteins purified to apparent homogeneity on SDS-PAGE analysis (Fig. 2a). The 20 and 17 N-terminal amino acid residues of the purified preparations from both peak fractions derived from the first Mono-Q column (Fig. 1) were determined. The sequences of ADLKTQILDAYNFRHATKEF and MREDTKVYDITIIGGGP agreed completely with the amino acid sequences of proteins deduced from the *yfkO* and *yumC* genes (Kunst et al. 1997), respectively.

Purification of ferredoxin from *B. subtilis*

Starting from the flow-through fraction from the Matrex Blue A column in FNR purification as described above, Fd was eluted from the DEAE-cellulose column as a broad peak at around 400 mM NaCl. The following gel-permeation column chromatography effectively separated Fd from NADPH oxidase activities. The Phenyl Superose column chromatography yielded a major and a minor peak, both active in supporting cytochrome *c* reduction (data not shown). The major peak fraction was further purified by a Mono Q column yielding a single peak (data not shown). The absorption spectrum is typical of the 4Fe-4S type, similar to that of Fd expressed in *E. coli* (Green et al. 2003), with an A_{390}/A_{280} ratio of 0.74. The N-terminal 10-residue sequence of this fraction (AKYTIVDKDT) was found to be identical to the deduced sequence from the *fer* gene in the *B. subtilis* genome database (Kunst et al. 1997), as well as that determined by amino acid sequencing of the expressed *fer* gene in *E. coli* (Green et al. 2003).

Molecular characterization of *YumC* and *YfkO*

The purified YumC migrated as a single band with an apparent molecular mass of 40 kDa on

SDS-PAGE analysis (Fig. 2a. lane 1), which compares favorably with the 36.8 kDa deduced from the *yumC* gene (Kunst et al. 1997) without a bound flavin coenzyme. The apparent molecular mass of the purified native YumC was estimated to be 94 kDa by gel permeation column chromatography (data not shown). These results indicate that YumC exists as a dimer of two identical polypeptides under the experimental conditions used in this study. Purified YumC shows an absorption spectrum typical of a flavoenzyme, with peaks at about 271, 378, and 457 nm (Fig. 2b). In order to determine the particular species of flavin coenzyme, FNR was treated with trichloroacetic acid, and the fluorescence spectrum of the extracted flavin coenzyme was measured. The ratio of pH-dependent fluorescence intensity at pH 7 versus pH 2 (emission at 525 nm, excitation at 366 nm) was 0.534, indicating that the bound flavin coenzyme of YumC is FAD (Brolin and Ågren 1977). From the absorbance of the released FAD at 450 nm at pH 7, the absorption coefficient of YumC was calculated to be 12.3 (mM FAD)$^{-1}$ cm^{-1} or 24.6 (mM of dimeric YumC)$^{-1}$ cm^{-1} at 457 nm. The latter value was used in calculating the molar concentrations and specific activities of the enzyme in the subsequent studies.

The purified YfkO migrated as a single band with an apparent molecular mass of 26 kDa by SDS-PAGE analysis (Fig. 2a, lane 3), which compares favorably with the 25.5 kDa deduced from *yfkO* gene (Kunst et al. 1997) without bound flavin coenzyme. The apparent molecular mass of purified native YfkO was estimated to be 30 kDa by gel permeation chromatography (data not

shown), indicating that YfkO exists as a monomer under the experimental conditions used in this study. Purified YfkO shows an absorption spectrum typical of a flavoenzyme, with peaks at about 273, 357, and 453 nm (Fig. 2c). In order to determine the particular species of flavin coenzyme, YfkO was treated with trichloroacetic acid, and the fluorescence spectrum of the extracted flavin coenzyme was measured. The ratio of pH-dependent fluorescence intensity at pH 7 versus pH 2 (emission at 525 nm, excitation at 366 nm) was 2.49, indicating that the bound flavin coenzyme of YfkO is FMN (Brolin and Ågren 1977). From the absorbance of the released FMN at 445 nm at pH 7, the absorption coefficient of the YfkO was calculated to be 13.0 (mM FMN or mM monomeric YfkO)$^{-1}$ cm^{-1} at 453 nm. This value was used in calculating the molar concentrations and specific activities of the enzyme in the subsequent studies.

Enzymatic activities

Under conditions favoring low NAD(P)H oxidase activity, such as the case with YumC (Table 2), FNR activity can be assayed by Fd-mediated cytochrome *c*. In the presence of *B. subtilis* Fd, YumC efficiently supported cytochrome *c* reduction at a high rate with NADPH as the electron donor and with NADH at a very low rate (Fig. 3, Table 2). The Fd-mediated cytochrome *c* reduction activity of YfkO with either NADPH or NADH, was too low to accurately measure (< 0.01 s^{-1}, Table 2). These

results, based on our enzyme activity determinations, indicate that YumC functions as FNR in *B. subtilis* while YfkO does not.

Both YumC and YfkO supported diaphorase activity with DPIP as an electron acceptor, but the affinities for NADPH were much higher than for NADH (Fig. 4). The diaphorase reaction rates of both enzymes were almost saturated by less than 1 mM NADPH, but not by NADH even at 2 mM (Fig. 4, Table 2).

Several flavoenzymes support O_2 reduction with NAD(P)H in the presence or absence of externally added flavin coenzymes, and are categorized as NAD(P)H oxidases by some authors (Schmidt et al. 1986; Maeda et al. 1992; Nishiyama et al. 2001). NADPH oxidase activity of YumC was less than 1% of NADPH-cytochrome *c* reductase activity in the absence of externally added flavin coenzymes and was considerably increased by the presence of externally added FAD or FMN (Table 2). NADH oxidase activities in the presence and absence of externally added flavin coenzymes were significant, but not very high compared with the NADPH-cytochrome *c* reductase activity. YfkO showed NAD(P)H oxidase activities in the presence and absence of externally added flavin coenzymes, but these activities were not so high compared with NADPH diaphorase activity.

The $NAD(P)^+$ reduction activity was measured with reduced ferredoxin from *B. subtilis* generated by an illuminated *C. tepidum* photochemical reaction center complex (Seo et al. 2001). YumC catalyzed the reduction of $NADP^+$, but the activity of YfkO was too low to measure (Table 2).

Discussion

Two major NADPH-diaphorase active proteins in extract from *B. subtilis* were identified as *yumC* and *yfkO* gene products by N-terminal amino acid sequencing of the purified proteins. YumC is a protein that shares high amino acid sequence identity with FNR from the green sulfur bacterium *C. tepidum*. The latter FNR (CT1512 gene product, Eisen et al. 1997) is a novel homo-dimer type that shares a medium level of amino acid sequence identity with genuine thioredoxin reductases from various organisms (Seo and Sakurai 2002). *B. subtilis* contains two genes, *yumC* and *ycgT*, both of which code for proteins that shares higher amino acid sequence identity with *C. tepidum* FNR (*E* (expectation value) = 1×10^{-60} and $E = 2 \times 10^{-61}$, respectively) than with a protein encoded by a genuine thioredoxin reductase gene ($E = 1 \times 10^{-24}$ and $E = 8 \times 10^{-24}$, respectively) of *B. subtilis*. From NADPH-diaphorase assay, it seems that *ycgT* was expressed to an insignificant extent under our culture conditions (Fig. 1). In addition to a high level of overall amino acid sequence identity, including the lack of the di-cysteine motif essential for catalysis of thioredoxin reductase, purified YumC from *B. subtilis* shares several biochemical properties with FNR from *C. tepidum* (Seo and Sakurai 2002): both proteins are homodimeric, non-covalently bind one FAD per subunit, and efficiently catalyze the electron transport reaction between Fd and NADPH. It is noteworthy that *B. subtilis* YumC has 20-fold lower NADPH oxidase activity than FNR from an anaerobic photosynthetic bacterium *C. tepidum* (Seo and Sakurai 2002) in the absence of externally added

flavin nucleotides. The former activity is about 2-fold lower than that of spinach (Zanetti and Forti 1966), an oxygen-evolving photosynthetic organism.

C. tepidum FNR uses both NADH and NADPH without significant discrimination between them (Seo and Sakurai 2002), but YumC is far more specific to NADPH than to NADH (Fig. 4, Table 2). This difference may be explained by the different biochemical roles of FNR in the two bacteria. Green sulfur bacteria are anaerobic photoautotrophs, and they assimilate CO_2 by the reductive TCA cycle (Evans and Buchanan 1965). For the operation of this cycle, both NADH and NADPH are required: malate dehydrogenase preferentially uses NADH (Dalhus et al. 2002) and isocitrate dehydrogenase NADPH (Lebedeva et al. 2002). For autotrophic growth, the reaction center complex reduces Fd and electrons are channeled to $NAD(P)^+$ via FNR. If FNR reduces both NAD^+ and $NADP^+$, it would be beneficial for green sulfur bacteria.

To heterotrophic *B. subtilis*, a more specific role may be assigned to FNR. In some heterotrophic bacteria, FNRs are more specific for NADPH than for NADH and may be implicated in a response to oxidative stress although an exact molecular mechanism is still not clear. In *E. coli*, FNRs participate in cellular defense against oxidative damage, and FNR-deficient mutants are abnormally sensitive to methyl viologen and hydrogen peroxide (Krapp et al. 1997, 2002). When *E. coli* cells devoid of native FNR gene were transformed to express the mitochondria-type FNR gene from the platyhelminthes *Schistosoma mansoni* (Girardini et al. 2002), they became tolerant to reactive

oxygen species. The *S. mansoni* enzyme is thought to be an NADPH-specific type by Giardini et al. (2002). Krapp et al. (2002) proposed that FNR contributes to NADPH homeostasis in stressed bacteria. It will be worthy to investigate whether *B. subtilis* YumC is also involved in defense against reactive oxygen species.

The turnover number of *B. subtilis* FNR for NADP(H) was 35.4 s^{-1} (Table 2) which is slightly smaller than those of plastidic FNRs, but considerably larger than those from *A. vinelandii* and *E. coli* (Ceccarelli et al. 2004).

In order to obtain an estimate of the minimum number of genes required to sustain a living bacterial cell, Kobayashi et al. (2003) systematically inactivated *B. subtilis* genes and studied the necessity of each for sustained cell growth because the bacterium is one of the best studied bacteria and is a model for low-G + C Gram-positive bacteria. They found that among the approximately 4,100 genes present in the *B. subtilis* genome, only 192 were essential by gene inactivation, with another 79 by prediction. Only 4% of the essential genes encoded proteins with unknown function. They found the *yumC* gene to be essential by gene inactivation and annotated as possibly coding for a thioredoxin reductase-like protein.

BioI belongs to cytochrome P450 of *B. subtilis* and was shown to be involved in pimelic acid production in an NADPH-dependent manner (Stok and De Voss 2000; Green et al. 2001). Recently, *B. subtilis* Fd overexpressed in *E. coli* was purified and its redox potential was reported to be –385 ±

10 mV and found to be the direct electron donor to BioI (Green et al. 2003). However, the biochemical pathway leading to Fd reduction has not been elucidated. We propose that Fd is reduced by YumC with NADPH as an electron donor. The Fd gene (*fer*) is not an indispensable gene (Kobayashi et al. 2003) probably because the bacterium has two putative flavodoxin genes.

Also not an indispensable gene (Kobayashi et al. 2003), *yfkO* encodes a protein that shares a high level of amino acid sequence identity ($E<10^{-50}$) with more than 20 gene products from a variety of bacteria, some of which are considered to be NAD(P)H-flavin oxidoreductase or putative NAD(P)H nitroreductase homologs. Because YfkO catalyzes Fd-mediated cytochrome *c* reduction by NAD(P)H at only marginal rates (Table 2), we concluded that it is not FNR and did not further characterize its biochemical properties.

A BLAST search (Altschul et al. 1997) with *B. subtilis* YumC as a query sequence revealed that there are more than 30 genes in prokaryotes coding for similar proteins variously annotated as thioredoxin reductase, thioredoxin reductase-like protein, NAD(P)H oxidase, etc., with *E* values $<10^{-34}$: 26 genes found in Gram-positive bacteria, 2 archaea, 3 gamma-proteobacteria, 5 alpha-proteobacteria, and 1 beta-proteobacterium.

All of these genes are devoid of the di-cysteine motif essential for thioredoxin reductase as *C. tepidum* and *B. subtilis* FNRs. Each of these bacterial species contains another gene with high identity to genuine thioredoxin reductase genes conserving the characteristic di-cysteine motif. No

genes coding for a protein with significant homology to YumC ($E<10^{-30}$) are found in the 15 enterobacteria whose complete genome sequences are available. It is noteworthy that, of 27 Gram-positive bacteria whose whole genome sequences are available, 23 contain genes coding for proteins with high similarity to YumC. Those that do not contain this type of gene belong to the genus *Clostridia* that vigorously reduce Fd by a pyruvate-ferredoxin reductase (EC 1.2.7.1) reaction (Uyeda and Rabinowitz 1971). There are no reports on purification of FNR from *Clostridia*, although the presence of FNR activities were reported in cell free extracts from some clostridial strains many years ago (Jungermann et al. 1973).

The archaeon *Sulfolobus solfataricus* contains a gene (pir:2606382A) that codes for a protein with significant homology to *B. subtilis* YumC. Arcari et al. (2000) purified the protein (*Ss*NOX38) that is a homodimer with Mr of 38 kDa per subunit containing one non-covalently bound FAD per subunit and devoid of the di-cysteine motif (CXXC) essential for thioredoxin reductase. The protein was reported to have very low diaphorase activities with DPIP or 5,5'-dithiobis-(2-nitrobenzoic) acid (DTNB) as an electron acceptor and measurable oxidase activities in the presence of either FMN or FAD. The reaction with Fd was not reported. The oxidase activity with NADH in the presence of FAD was twice as large as that with NADPH. However, the oxidase activities were not very high with a K_{obs} value of about 4.5 sec^{-1} that is comparable to the NADPH oxidase activity of YumC in

the presence of FMN with a K_{obs} value of about 3.35 sec^{-1}, and considerably lower than the Fd-mediated cytochrome *c* reduction activity of YumC (Table 2).

Amino acid sequences of FNR, thioredoxin reductase, and glutathione reductase, including related putative proteins deduced from genomic information have been aligned using CLUSTRAL (Thompson et al. 1994) and a phylogenetic tree with bootstrap values constructed using MEGA2 (Kumar et al. 2001) (Fig. 5). From these results we propose that FNR can be divided into 4 types: 1) a plant type which also includes cyanobacterial FNR, 2) a bacteria type represented by *E. coli* FNR, 3) a mitochondria type or adrenodoxin reductase type, and 4) a novel type represented by the FNRs of *B. subtilis* and *C. tepidum* (Fig. 5). This type may be referred to as the Gram-positive bacteria type, although *Clostridia* are apparently devoid of it, and the Gram-negative green sulfur bacterium *C. tepidum* also contains this type of FNR (Seo and Sakurai 2002, Eisen et al. 2002). This last type of FNR is more closely related to thioredoxin reductase than to any other type of FNR.

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Figure legends

Fig. 1. Elution profile of NADPH diaphorase activities from *B. subtilis* from Mono Q column (10/10, Amersham). Fractionation volume: 2 ml, flow rate: 1 ml/min. (□) NADPH diaphorase activity ([NADPH] = 0.1 mM, 10 μl sample in 500 μl reaction mixture); (●) A_{280}; upper bars indicate active fractions pooled for the next purification steps.

Fig. 2. Proteins were analyzed on 12% SDS-PAGE and silver-stained (Panel a). *Lane 1*: 340 ng of purified YumC. *Lane 2*: Molecular mass (numbers on the left side) standards (Silver stain SDS-PAGE standard, BioRad). *Lane 3*: 87 ng of purified YfkO. Protein amount was determined by absorption coefficients described in experimental procedures.The ultra violet–visible absorption spectra of purified YumC (Panel b) and Yfk O. (Panel c). Samples were dissolved in 50 mM Tris-HCl Buffer (pH 7.8) at 24 ^{o}C. Panel C: SDS-PAGE analysis of purified YumC and YfkO.

Fig. 3. Dependence of NADH- and NADPH-dependent cytochrome *c* reduction activity of YumC on Fd concentrations. (●) NADH and (○) NADPH. The reaction mixture contained 3.8–38 pmol YumC, and 10 μM NADPH or NADH in 500 μl of reaction mixture containing indicated concentrations of *B. subtilis* Fd. The reaction was initiated by the addition of YumC. The optical cells containing the reaction mixtures were put into the photometer and the absorbance change at 550 nm was measured

for 1 min. K_{obs}: number of oxidized NAD(P)H (dimeric YumC)$^{-1}$ sec^{-1}. The rate without Fd was < 0.158 and 0.521 s^{-1} for NADPH and NADH, respectively, and these values were subtracted from the observed ones.

Fig. 4 Dependence of DPIP-diaphorase activity on NADH and NADPH concentrations. (●) NADH and (○) NADPH; a: YumC. b: Yfk O. The reaction mixture contained either 2.0 pmol YfkO or 5.6 pmol YumC in 500 μl of reaction mixture containing indicated concentrations of NAD(P)H. The reaction was initiated by the addition of YfkO or YumC. The optical cells containing the reaction mixtures were put into the photometer and the absorbance change at 600 nm was measured for 1 min. K_{obs}: moles of oxidized NAD(P)H (moles of enzyme)$^{-1}$ s^{-1}.

Fig. 5. Phylogenetic tree of amino acid sequences of FNR, adrenodoxin reductases, glutathione reductase, NAD(P)H-thioredoxin reductases, and NADPH-thoredoxin reductase-like protein (lacking disulfide reduction motif) (TRLP). The sequences were aligned using CLUSTALW 1.8 (Thompson et al. 1994) and a tree was constructed using the Neighbor-joining method on MEGA version 2.1 (Kumar et al. 2001). Bootstrap values after 1000 resampling are indicating as percentages at the corresponding branches. Lower scale indicates the relative distances between nodes. *A. thaliana* (*Arabidopsis thaliana*) NAD(P)H-thioredoxin reductase (pir:S44027) and glutathione

reductase (sp:P48641); *A. vinelandii* (*Azotobacter vinelandii*) FNR (pir:A57432); *B. halodurans* (*Bacillus halodurans*) TRLP (pir:H84075); *B. subtilis YumC* (pir:B70015)*, ycgT* (pir:G69759) and TR (pir:A69727); *C. reinhardtii* (*Chlamydomonas reinhardtii)* FNR; *C. tepidum* (*Chlorobium tepidum*) FNR (sptr:Q8KCB2) and TR (sptr:Q8KE48); *D. radiodurans* (*Deinococcus radiodurans*) FNR (sptr:Q9RX19); *E. coli* (*Esherichia coli* K-12) TR (pir:A28074), GR (pir:A24409) and FNR (pir:S40867); *H. sapiens* (*Homo sapiens*) AR (pir:A40487) and GR (sp:P00390); *M. leprae* (*Mycobacterium leprae*) FNR (pir:T45351); *R. prowazekii* (*Rickettsia prowazekii*) TRLP (pir:D71655); *S. cerevisiae* (*Saccharomyces cerevisiae*) TRLP (pir:S61171); *S. oleracea* (*Spinacia oleracea*) FNR (pir:S00438); *S. solfataricus* (*Sulforobus solfataricus*) NADH oxidase (prf:2606382A); *S. pyogenes* (*Streptococcus pyogenes*) SF370 (serotype M1) TRLP (sptr:Q9A0B5); *S. PCC6803* (*Synechocystis PCC6803*) FNR (pir:S76200).

Table 1 Purification of NADPH-DPIP reductases from *Bacillus subtilis*

Sample	Volume (ml)	Total protein (mg)	Total activity (units)	Purification factor	Yield
Crude extract	115	664	262	1	100
35-80 % SAS ppt.	65.0	608	192	0.80	73
Matrex Blue A	257	174	122	1.78	47
Sephacryl S-200	64	59	92.2	3.9	35
MonoQ 10/10					
*Yum*C	5.0	4.9	39	21	15
*Yfk*O	7.5	5.7	47	21	18
Matrex Red A					
*Yum*C	18	2.6	31	30	12
*Yfk*O	9.3	0.85	32	94	12
MonoQ 5/5					
*Yum*C	0.62	0.65	22	86	8.4
*Yfk*O	0.51	0.51	24	120	9.2

Table 2 Reduction activities from NAD(P)H to several electron acceptors

	YumC	YfkO
Fd reduction activity (by cytochrome *c* reductase assay with *B. subtilis* Fd)		
NADPH		
*K*cat	35.4 ± 0.6 s^{-1}	(0.011 s^{-1})[(a)]
Km (Fd)	0.54 ± 0.2 μM	-
NADH		
*K*cat	0.4 ± 0.2 s^{-1}	(< 0.01 s^{-1})[(a)]
Km (Fd)	< 0.1μM	-
DPIP diaphorase activity		
NADH		
*K*cat	-	-
Km	> 0.2 mM	> 2 mM
NADPH		
*K*cat	22.7 ± 0.6 s^{-1}	190 ± 10 s^{-1}
Km	0.57 ± 0.08 μM	180 ± 30 μM
NAD(P)H oxidase activity		
no flavins		
NADH (0.15 mM)	0.58 s^{-1}	0.13 s^{-1}
NADPH (0.15 mM)	0.13 s^{-1}	0.17 s^{-1}
FAD dependent (0.1 mM)		
NADH (0.1 mM)	0.91 s^{-1}	0.31 s^{-1}
NADPH (0.1 mM)	1.65 s^{-1}	0.33 s^{-1}
FMN dependent (0.1 mM)		
NADH (0.1 mM)	0.91 s^{-1}	0.29 s^{-1}
NADPH (0.1 mM)	3.4 s^{-1}	0.31 s^{-1}
$NADP^+$ reduction activity with *B. subtilis* Fd and illuminated *C. tepidum* reaction center		
K_{obs}	0.28 s^{-1}	(< 0.01s^{-1})[(b)]

All rates and *K*cat values are expressed as NAD(P)H oxidation or reduction

(a) The rate at 10 μM NAD(P)H and 2 μM Fd

(b) The rate at 0.1 mM $NADP^+$ and 2 μM Fd

Figure 1

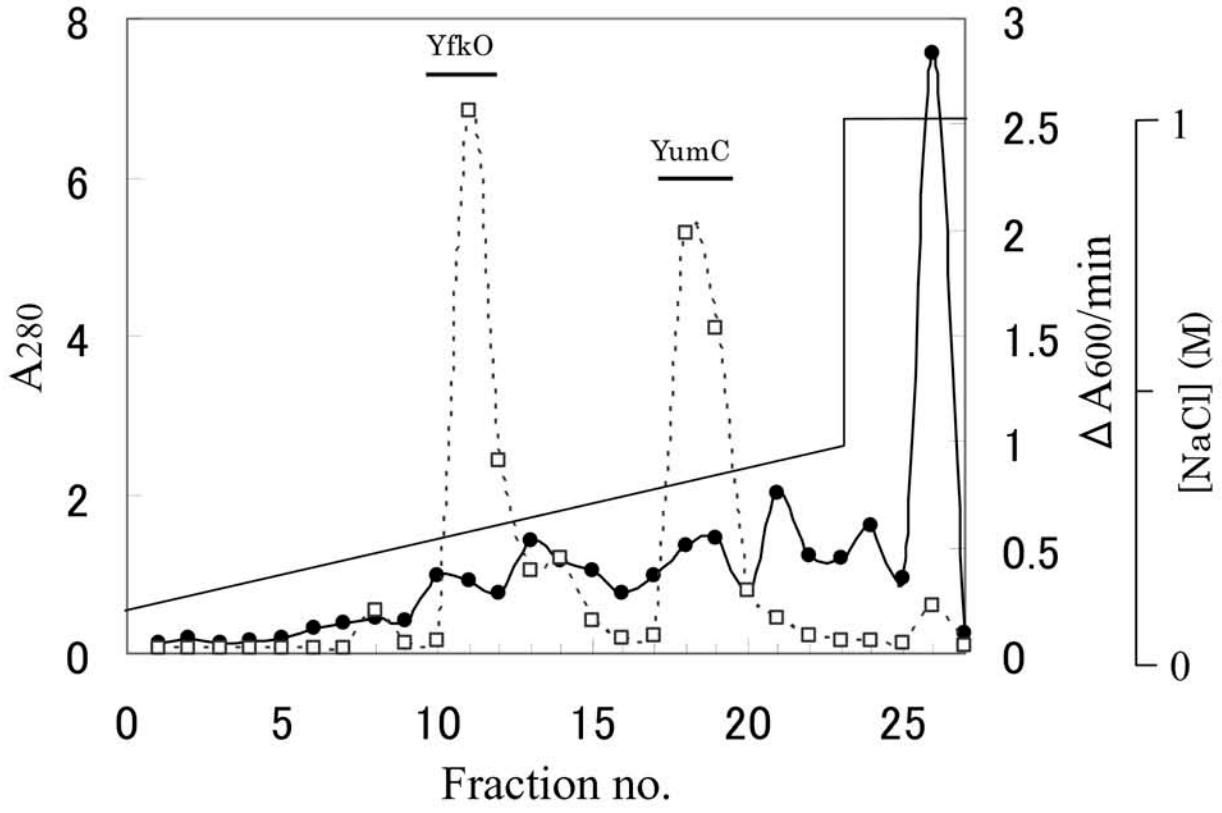

YfkO
YumC
A280
ΔA600/min
[NaCl] (M)
Fraction no.
0
2
4
6
8
0
0.5
1
1.5
2
2.5
3
0
1
0
5
10
15
20
25

Figure 2a

a

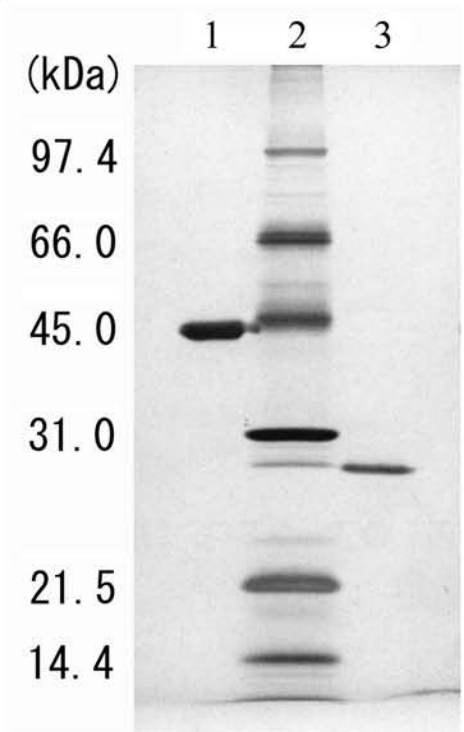
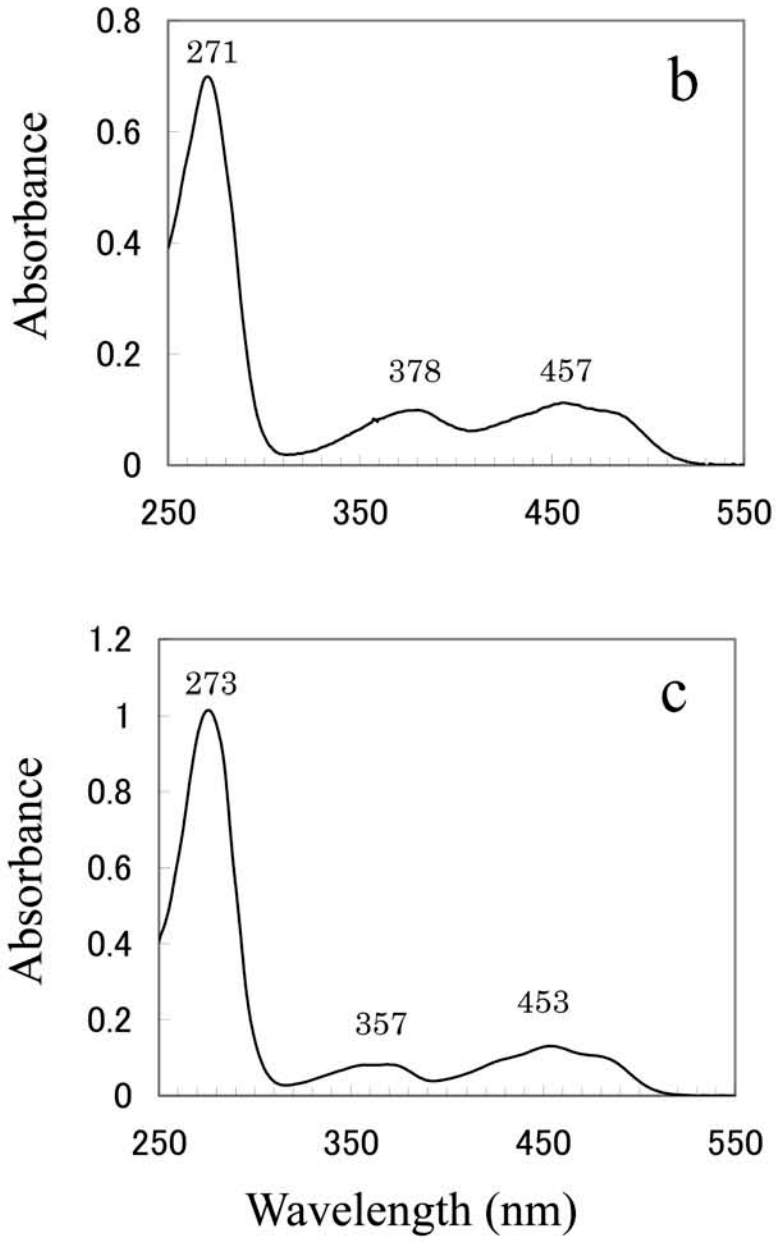


Figure 2b,c


b
271
378
457
0.8
0.6
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Absorbance
250
350
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c
273
357
453
1.2
1
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0.4
0.2
0
Absorbance
250
350
450
550
Wavelength (nm)

Figure 3

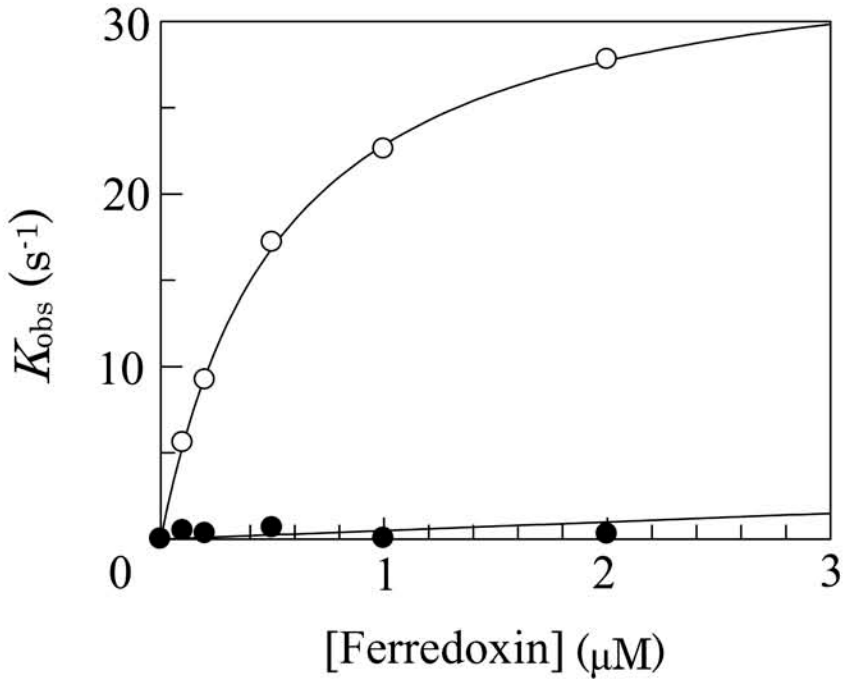


Figure 4

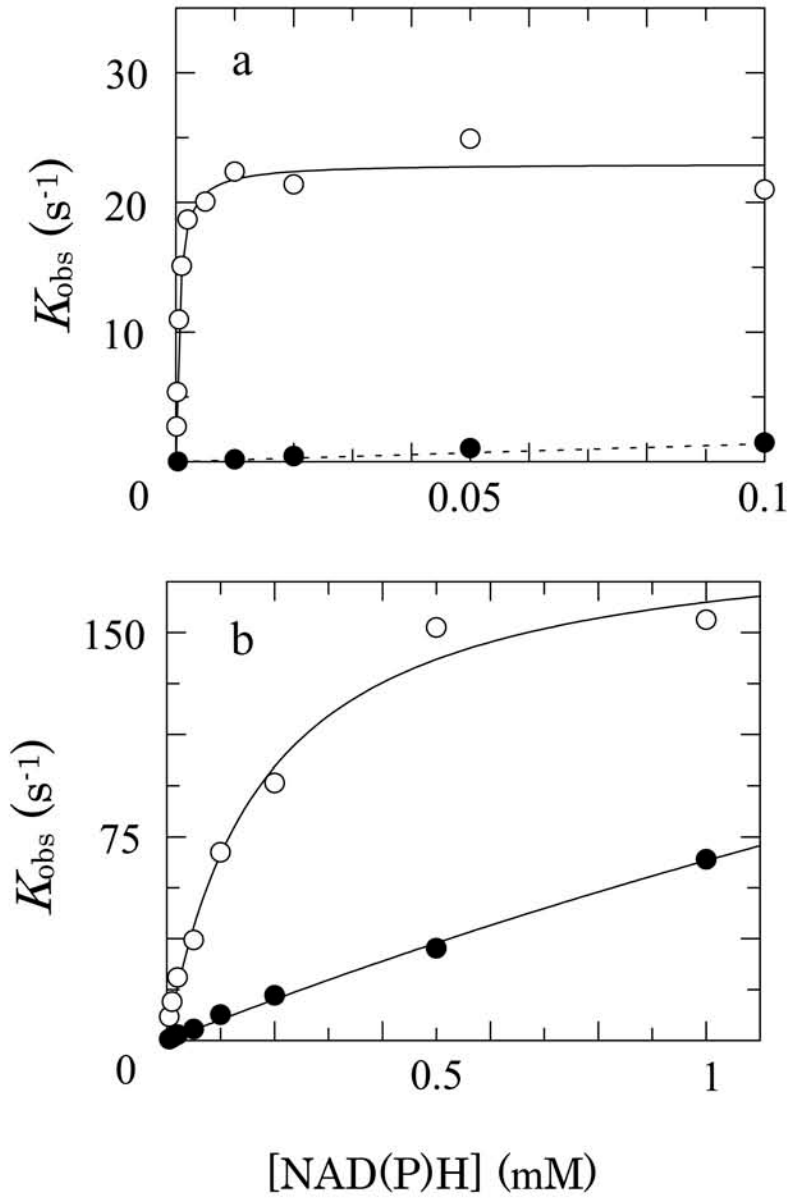
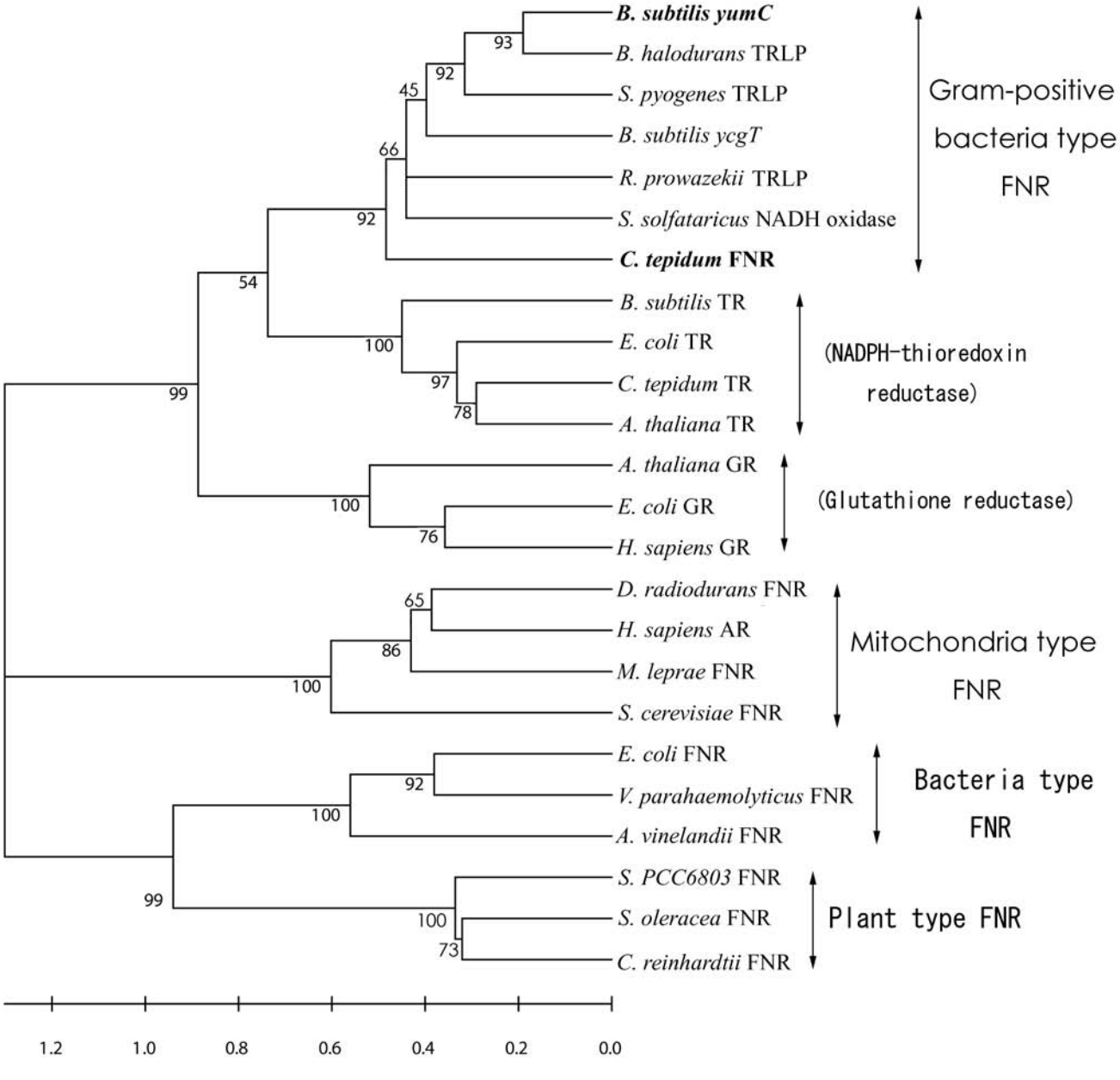


Figure 5


B. subtilis yumC
B. halodurans TRLP
S. pyogenes TRLP
B. subtilis ycgT
R. prowazekii TRLP
S. solfataricus NADH oxidase
C. tepidum FNR
B. subtilis TR
E. coli TR
C. tepidum TR
A. thaliana TR
A. thaliana GR
E. coli GR
H. sapiens GR
D. radiodurans FNR
H. sapiens AR
M. leprae FNR
S. cerevisiae FNR
E. coli FNR
V. parahaemolyticus FNR
A. vinelandii FNR
S. PCC6803 FNR
S. oleracea FNR
C. reinhardtii FNR
93
92
45
66
92
54
100
97
78
99
100
76
65
86
100
92
100
99
100
73
Gram-positive bacteria type FNR
(NADPH-thioredoxin reductase)
(Glutathione reductase)
Mitochondria type FNR
Bacteria type FNR
Plant type FNR
1.2
1.0
0.8
0.6
0.4
0.2
0.0