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Enzyme Catalysis and Regulation:

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Characterization of YqjM, an Old Yellow Enzyme Homolog from *Bacillus subtilis* Involved in the Oxidative Stress Response*

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In this paper, we demonstrate that a protein from *Bacillus subtilis* (YqjM) shares many characteristic biochemical properties with the homologous yeast Old Yellow Enzyme (OYE); the enzyme binds FMN tightly but noncovalently, preferentially uses NADPH as a source of reducing equivalents, and forms charge transfer complexes with phenolic compounds such as *p*-hydroxybenzaldehyde. Like yeast OYE and other members of the family, YqjM catalyzes the reduction of the double bond of an array of α,β-unsaturated aldehydes and ketones including nitroester and nitroaromatic compounds. Although yeast OYE was the first member of this family to be discovered in 1933 and was the first flavoenzyme ever to be isolated, the physiological role of the family still remains obscure. The finding that α,β-unsaturated compounds are substrates provoked speculation that the OYE family might be involved in reductive degradation of xenobiotics or lipid peroxidation products. Here, for the first time, we demonstrate on the protein level that whereas YqjM shows a basal level of expression in *B. subtilis*, the addition of the toxic xenobiotic, trinitrotoluene, leads to a rapid induction of the protein *in vivo* denoting a role in detoxification. Moreover, we show that YqjM is rapidly induced in response to oxidative stress as exerted by hydrogen peroxide, demonstrating a potential physiological role for this enigmatic class of proteins.

Old Yellow Enzyme (EC 1.6.99.1), originally isolated from brewers' bottom yeast, was the first enzyme shown to contain a bound flavin (FMN) (1, 2), a cofactor typically found to participate in biological redox reactions. In these reactions, enzyme-bound flavin (as FMN or FAD) serves as a temporary sink of electrons, which are then passed on to an electron-accepting protein or substrate species. In the case of OYE,[1] it has been well established that NADPH serves as the physiological reductant for the enzyme-bound flavin (3, 4). Intriguingly, however, several compounds can act as oxidants such as quinones and many α,β-unsaturated aldehydes and ketones (albeit a lot of which are not naturally occurring); in each it is the olefinic bond that is reduced (5, 6). Molecular oxygen can also serve as a substrate, but it is generally considered to be a fortuitous one because of the slow rate of catalysis and the biological nonproductivity of such a reaction. Therefore, despite decades of extensive biochemical and spectroscopic characterization, the identification of the physiological oxidant for almost all members of the OYE family of enzymes remains elusive. The exception to this is 12-oxophytodienoate reductase 3 from higher plants, which catalyzes the NADPH-dependent reduction of 12-oxophytodienoate to 3-oxo-2(2′[*Z*]-pentenyl)-cyclopentane-1-octanoate, an intermediate in the biosynthesis of the signaling molecule, jasmonic acid (7). For the remainder of the family, the most recent hypothesis based on the interactions of OYE with electrophilic substrates is that the enzyme may serve as a detoxification enzyme in antioxidant defense systems (4). In particular, it is thought that α,β-unsaturated carbonyl compounds that are not capable of resonance stabilization such as the breakdown products of lipid peroxidation are prime candidates as a physiological substrate for the OYE family (4). In such instances, one would expect an up-regulation of the enzyme under conditions of oxidative stress.

Currently, the family of OYE proteins is growing rapidly as homologous genes are discovered both in the course of genomic sequencing projects (in both prokaryotes and eukaryotes) and during characterization of novel enzyme activities. Interestingly, some members of this family, which have been classified as xenobiotic reductases, have recently been shown to be capable of reducing certain xenobiotic compounds containing nitro functional groups (8–12). The production, use, and disposal of these compounds, which are employed in explosives, agricultural chemicals, pharmaceuticals, dyes, and plastics has led to wide scale contamination of the environment and is now of international concern. Due to the current lack of efficient cleanup approaches, the discovery of biological systems capable of providing a novel approach for remediation of sites contaminated by such environmentally toxic compounds is being pursued (13, 14). In this respect, the identification of enzymes capable of using such compounds as substrates is of biotechnological importance.

In this paper, the characteristics of an OYE homolog designated *yqj*M from *Bacillus subtilis* are described. *B. subtilis* is a Gram-positive, aerobic, endospore-forming, rod-shaped bacterium commonly found in soil, in water sources, and in association with plants. The bacterium is well known as an industrial workhorse for the production of heterologous proteins such as amylases and proteases (15). This study is the first of its kind for the description of an OYE homolog from a Gram-positive bacterium and was facilitated by the ability to obtain a homogenous preparation of the recombinant protein in a very high yield in its authentic form. We examine and compare biochemical properties of YqjM that are characteristic of OYE itself

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This work is dedicated to the late Prof. Vincent Massey (deceased August 26, 2002), a pioneer of enzymology who shaped flavoprotein research for over 40 years.

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[1] The abbreviations used are: OYE, Old Yellow Enzyme; NG, nitroglycerin; TNT, 2,4,6-trinitrotoluene; HPLC, high pressure liquid chromatography.

with important revelations for the intriguing heterogeneity that occurs in this family of proteins with respect to spectral and kinetic properties. Most importantly, we show for the first time how the expression of the protein can be induced in the cell, providing important insight into the physiological properties of this enigmatic class of proteins.

EXPERIMENTAL PROCEDURES

Molecular Techniques—Basic molecular techniques were adopted from Ausubel *et al.* (16) or Sambrook *et al.* (17).

B. subtilis Strain, Culture Medium, and Preparation of Cell Extracts—*B. subtilis* strain 168 obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (GmbH) was used. The bacteria were grown in an air lift 300-liter batch fermentor (Bioengineering) containing 200 liters of either Spizizen's minimal salt medium (18) supplemented with 0.5% D-glucose, 0.05% vitamin-free casamino acids (Difco), 0.05% yeast extract or in LB medium. Both types of medium were supplemented with 50 μg/ml L-tryptophan. The cells were grown with strong aeration at 37 °C to the mid-logarithmic phase of growth. They were then harvested by centrifugation and subsequently stored at −80 °C until required. For the induction studies, a 5-ml stock culture was used to inoculate 50 ml of LB medium in a 250-ml Erlenmeyer flask. After growth for 1 h at 37 °C, the compound/condition being tested was applied to the culture, and 1-ml samples were taken at the indicated time intervals for analysis.

Cloning and Expression of YqjM from B. subtilis—The DNA sequence of the *yqj*M gene of *B. subtilis* was obtained from SubtiList (available on the World Wide Web at genolist.pasteur.fr/SubtiList/help/credits.html). Genomic DNA was isolated from *B. subtilis* cell paste (1 g) using the QIAamp Tissue kit (Qiagen) according to the instructions described by the manufacturer. The open reading frame of *B. subtilis yqj*M was amplified by the polymerase chain reaction (PerkinElmer Life Sciences), using the isolated genomic DNA as template while incorporating appropriate restriction sites, and cloned into the *Nde*I and *Xho*I restriction sites of pET21a (Novagen). This allows expression of authentic *B. subtilis* YqjM under control of the isopropyl-1-thio-β-D-galactopyranoside-inducible T7 promoter. The construct was verified by sequence analysis and was transformed into *Escherichia coli* BL21-CodonPlus™-(DE3)-RIL cells (Stratagene) for expression. Induction was carried out for 4 h at 30 °C using 0.1 mM isopropyl-1-thio-β-D-galactopyranoside.

Generation of Antisera/Immunochemical Methods—Antisera used in this study were generated in rabbits (Eurogentec) from recombinant protein that accumulated in inclusion bodies. The inclusion bodies were purified from bacterial extracts, in the first instance, as described by Harlow and Lane (19), and were further refined by 10% SDS-PAGE (20). The gel slice containing the protein band of interest was used to raise a polyclonal antiserum. The antibodies produced were affinity-purified before use, and Western blot analysis was carried out essentially as described in Ref. 21. Briefly, for each experimental condition tested and for each sample, 500 ng of total *B. subtilis* protein extract was separated by 10% SDS-PAGE and transferred to nitrocellulose. YqjM and chorismate synthase were immunodecorated using the corresponding antibodies at a dilution of 1:1000 and 1:2000, respectively, and detected using a sheep anti-rabbit peroxidase conjugate and the chemiluminescent system supplied by Roche Applied Science. Changes in protein expression were estimated from the relative intensity of the observed bands using the purified protein as a standard. Protein concentration was determined using the Bradford assay (Pierce).

Purification of Recombinant YqjM—For the isolation of the authentic YqjM, cell paste from 4 liters of culture medium (~20 g) was resuspended in 80 ml of Buffer A (50 mM Tris-HCl, pH 7.5, containing 1.3 mM phenylmethylsulfonyl fluoride). Lysozyme was added to a final concentration of 1 mg/ml, and after 20 min at 4 °C the cells were further lysed by sonication. DNase I (0.1 mg/ml) was added to help clarify the resulting sonicated suspension. The cell debris was removed by centrifugation at 25,000 × *g* for 30 min at 4 °C, and the supernatant was subjected to ion exchange chromatography on a DEAE-Sephacel column (2.5 × 15 cm) freshly equilibrated in Buffer A. After extensive washing with Buffer A, bound protein was eluted with a linear gradient of 250 ml each of Buffer A and Buffer B (same as Buffer A but containing 400 mM KCl). The yellow fractions containing YqjM were pooled, concentrated, and dialyzed overnight in Buffer A. The dialysate was brought to 30% ammonium sulfate saturation and applied to a phenyl-Sepharose column (2.5 × 15 cm) freshly equilibrated in Buffer C (Buffer A containing 1.5 M ammonium sulfate). After washing, bound protein was eluted with a linear gradient of 250 ml of Buffer C and 250 ml of Buffer A. Fractions containing YqjM were pooled, concentrated into 50 mM Tris-HCl, pH 7.5, and stored at −80 °C. The progress of protein purification was monitored by 10% SDS-PAGE using the buffer system described by Laemmli (20).

Ligand Binding Titrations—Titrations were performed in 100 mM Tris-HCl, pH 7.4, by systematically varying the ligand concentration. The spectra were recorded between 300 and 800 nm with a Uvikon 933 spectrophotometer (BioTek Kontron Instruments AG). Dissociation constants were calculated from the spectral perturbations observed upon the addition of ligand up to saturating concentrations using the software Sigma Plot version 4.14 (Jandel Scientific).

Photoreduction—Photoreduction was performed under anaerobic conditions in argon-saturated 100 mM potassium phosphate buffer, pH 7.6, using EDTA (1 mM) as the electron donor and a slide projector for illumination (750 μmol·m^{-2}·s^{-1}).

Substrate Specificity—Apparent steady state kinetic constants were obtained by following the oxidation of β-NADPH/β-NADH at 340 nm in a Uvikon spectrophotometer while systematically varying the substrate concentration. The assays were performed in the presence of an oxygen-consuming system (20 mM glucose, 10 units of glucose oxidase) at 25 °C in 1 ml of 100 mM Tris buffer, pH 7.5, containing either 100 μM β-NADPH or 100 μM β-NADH, and by adding YqjM at concentrations ranging from 3.89 to 19.4 nM depending on the reaction rate being observed with the particular substrate. The concentration of *B. subtilis* YqjM was determined using a molar extinction coefficient of 11,600 M^{-1}·cm^{-1} at 455 nm, deduced by a comparison of the absorption maximum at 455 nm of the purified protein with that of the flavin released upon denaturation in 2% SDS. Whenever possible, stock solutions of the substrates were prepared in the assay buffer. However, some substrates had to be dissolved in ethanol, in which case the final concentration of ethanol in the assay mixture was kept constant over the entire range of substrate concentrations. The software Sigma Plot version 4.14 (Jandel Scientific) was used for the evaluation of the data.

RESULTS

Expression and Purification of YqjM—*B. subtilis yqj*M was cloned into the expression vector pET21a, and expression of the YqjM protein (37.4 kDa) was achieved in *E. coli* BL21-CodonPlus™(DE3)-RIL cells (Stratagene) (Fig. 1*i*, *lane 1*). The protein was expressed to a high degree, constituting ~50% of the total cellular protein, and was found to be almost equally distributed between the soluble and insoluble bacterial crude extracts (Fig. 1*i*, compare *lanes 1* and *2*). A simple two-step purification strategy combining ion exchange and hydrophobic chromatography resulted in a preparation of the protein that had a purity of >98% (Fig. 1*i*, *lanes 3* and *4*). The protein was obtained in a particularly high yield (161 mg/liter of *E. coli* culture), and the identity of the purified protein as *B. subtilis* YqjM was confirmed by N-terminal sequencing analysis (10 cycles of 50 pmol of protein resolved the amino acids ARKLFTPITI, which are identical to the data base entry for this protein lacking the N-terminal methionine residue, accession number P54550). In an attempt to determine the quaternary structure, the isolated protein was subjected to gel filtration on a Superose 12 column (Amersham Biosciences). A comparison of the elution volume of the single protein peak obtained with calibration standards revealed a molecular mass of 147 kDa, indicating that the native protein is tetrameric in solution (data not shown).

Cofactor Content—Spectrum A in Fig. 1*ii* shows the UV-visible absorbance spectrum of the recombinant YqjM as isolated. The spectrum is typical for a flavin-containing protein, showing maxima at 372 and 455 nm with well resolved shoulders at about 430 and 490 nm (22). Upon denaturation with SDS, the flavin was released from the purified enzyme, indicating that the cofactor is not covalently bound to the protein. A comparison of the HPLC elution time of the released flavin (23) with those of FMN and FAD standards identified the flavin cofactor as FMN (data not shown). The ratio of absorbances at 277 and 455 nm of protein *versus* bound FMN, respectively, was 20.7 indicating that the protein as isolated was not fully saturated with the flavin cofactor. Therefore, the protein was

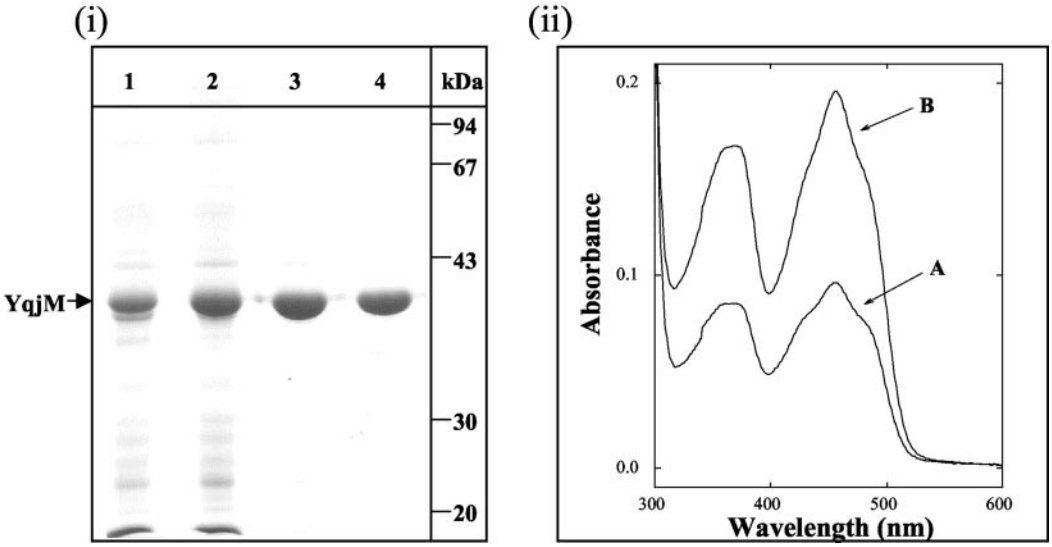

FIG. 1. **Purification and UV-visible absorbance properties of recombinant *B. subtilis* YqjM.** *i*, analysis of the purification of YqjM by SDS-PAGE. The protein was visualized by Coomassie Blue staining. *Lane 1*, pellet of crude cell extract after centrifugation; *lane 2*, supernatant of crude cell extract after centrifugation; *lane 3*, pooled fractions after DEAE-Sephacel chromatography; *lane 4*, pooled fractions after phenyl-Sepharose chromatography. The protein mass standards are as indicated. *ii*, UV-visible absorbance spectra of YqjM. Spectrum A indicates the absorbance spectrum between 300 and 600 nm of the protein as isolated, whereas spectrum B is the absorbance spectrum recorded after reconstitution with FMN.

incubated with an excess of free FMN in an attempt to reconstitute as much of the active holoenzyme as possible. After removing the unbound FMN by dialysis, the UV-visible spectrum revealed that there was an approximate 2-fold increase in the ratio of the absorbance at 455 nm relative to that at 277 nm, reflecting the increase in protein-bound flavin (Fig. 1*ii*, *spectrum B*). It was also observed that after reconstitution there was a 1.6-fold increase in the activity of the protein using *N*-ethylmaleimide (see below) as substrate, substantiating the partial reconstitution of the holoenzyme.

Formation of Charge Transfer Complexes between YqjM and para-Substituted Phenols—One of the most notable features of OYE is its ability to form charge transfer complexes between the enzyme bound flavin and a variety of phenolic compounds (24). YqjM was titrated with a series of *para*-substituted phenolic compounds to test the propensity of the protein to form charge transfer complexes similar to those reported for OYE (24). A representative titration experiment is shown in Fig. 2. Upon the addition of *para*-hydroxybenzaldehyde to YqjM, a long wavelength hyperchromic effect on the absorbance developed as a function of ligand concentration. Simultaneously, there was an obvious hypochromic effect observed in the 400–500-nm region of the flavin absorption spectrum. These reciprocal absorbance changes due to the interacting chromophores are typical of charge transfer transitions (24). The dissociation constants for a range of *para*-substituted phenols were determined and are summarized in Table I. All compounds produced similar long wavelength hyperchromic effects, and there appeared to be a trend in that the lower the pK_a of the phenolic compound the lower the observed dissociation constant (Table I). Such phenolic compounds are known to bind in the deprotonated form (24), thereby indicating the ability of the protein to lower the pK_a of the free phenol. From previous detailed studies (25), it seems reasonable to assume that it is the phenolate that binds to the enzyme and that the phenolate and the flavin act as the charge donor and acceptor, respectively (24).

Photoreduction—Reduction of the YqjM-bound FMN was achieved under anaerobic conditions by irradiation with white incandescent light in the presence of EDTA as a source of electrons (Fig. 3). The reduction of the flavin cofactor proceeded with a decrease in the absorbance at 455 nm (Fig. 3) and

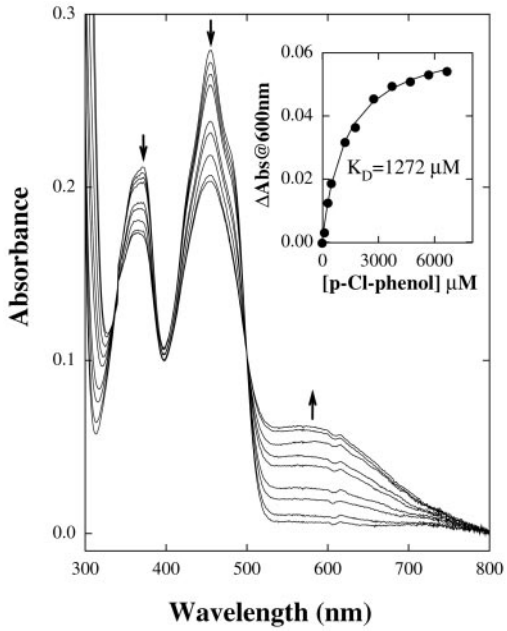

FIG. 2. **Titration of YqjM with *p*-chlorophenol.** The protein (25.4 μM) was titrated with *p*-chlorophenol (0–6682 μM), and the absorbance spectra were recorded between 300 and 800 nm. The *inset* shows the change in absorbance at 600 nm due to the formation of the YqjM/ligand complex as a function of the concentration of ligand. The data points (*solid circles*) were fitted to a hyperbolic equation represented by the *solid line*, from which the dissociation constant (K_D) for the ligand was obtained, using the software Sigma Plot.

contrasts markedly with that observed for the related OYE (26). Photoreduction of yeast OYE proceeds in a two-step process, initially producing the red anionic semiquinone with its characteristic spectral peak at about 380 nm, before further reduction produces the two-electron reduced dihydroflavin form of the enzyme-bound flavin. During photoreduction of YqjM, spectral changes characteristic of the red anionic semiquinone or the blue neutral semiquinone were not observed, and the enzyme-bound flavin was converted directly to the dihydroflavin form (Fig. 3). Following the admission of

TABLE I
Titration of YqjM with para-substituted phenolic compounds

The absorbance spectra of YqjM (26.7 μM) were recorded upon titration with the indicated *para*-substituted phenols in 100 mM Tris, pH 7.4. The pK_a of the free phenol for each unbound ligand is indicated as are the dissociation constants (K_D) derived from the titration data (*cf.* Fig. 2).

Ligand	pK_a[a]	K_D
		mM
p-Chlorophenol	9.34	1.3 ± 0.060
p-Cresol	10.30	1.2 ± 0.052
p-Hydroxyacetophenone	8.05	0.058 ± 0.004
p-Nitrophenol	7.15	0.033 ± 0.002
p-Hydroxybenzaldehyde	7.62	0.024 ± 0.002

[a] pK_a of the free phenol as reported by Abramovitz and Massey (24).

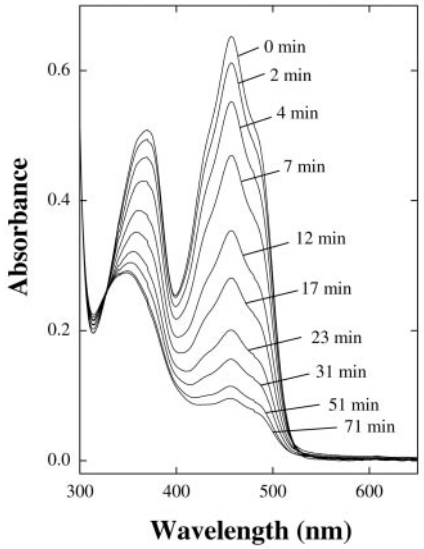

FIG. 3. **Photoreduction of YqjM.** The protein (49 μM) was illuminated with 750 μmol m^{-2}·s^{-1} of white light under anaerobic conditions in the presence of EDTA (1 mM) as a source of electrons. Absorbance spectra were recorded between 300 and 650 nm before (0 min) and after illumination with white light at the times indicated.

oxygen to the tonometer, the reduced enzyme was converted directly to the oxidized form; again, no intermediate was observed.

Catalytic Activity of YqjM—The isolated YqjM was found to catalyze the oxidation of both β−NADH and β−NADPH in the presence of molecular oxygen (35.9 and 49.9 mol of β-NADH/NADPH oxidized·min^{-1}·mol^{-1} of enzyme flavin, respectively). The rate with β-NADPH is very similar to the rate that has been observed for yeast OYE under equivalent conditions (51 mol of β-NADPH oxidized·min^{-1}·mol^{-1} of enzyme flavin) (5). However, as had also been found previously for OYE (5, 6), we found an accelerated rate of NADPH oxidation in the presence of α,β-unsaturated carbonyl compounds (*e.g.* 484 mol of β-NADPH oxidized·min^{-1}·mol^{-1} of enzyme flavin in the presence of *N*-ethylmaleimide). Incidentally, when β-NADH rather than NADPH was used as reductant under these conditions, the rate of oxidation only increased 2-fold upon the addition of *N*-ethylmaleimide as the substrate, indicating a preference for the phosphorylated pyridine nucleotide. We went on to determine the apparent kinetic constants k_{cat} and K_m for a selection of α,β-unsaturated carbonyl compounds in addition to nitrate ester and nitro aromatic compounds as listed in Table II. However, since it is difficult to estimate the relative contribution of both molecular oxygen and the alternative substrate to the oxidation of NADPH, an oxygen-consuming system (glucose/glucose oxidase) was included in the steady state measurements to exclude molecular oxygen as a potential electron acceptor. While YqjM was found to accept structurally different α,β-unsaturated carbonyls, the highest catalytic efficiency was observed with *N*-ethylmaleimide (k_{cat}/K_m = >8.07). Since saturating kinetics were observed with *N*-ethylmaleimide at even the lowest concentration used (1 μM), the K_m value could only be estimated under these conditions. In contrast to *trans*-hex-2-enal (Table II), we did not observe any activity with *trans*-dodec-2-enal, fumaric acid, maleic acid, or maleamic acid, indicating that the size and nature of the β-C substituent group are relevant for substrate recognition.

TABLE II
Steady state kinetic properties of B. subtilis YqjM

Apparent kinetic constants were derived from steady state kinetic analyses. The standard assay was performed at 25 °C in 1 ml of 100 mM Tris, pH 7.4, containing 100 mM β-NADPH, 20 mM glucose, and 10 units of glucose oxidase. The compounds *trans*-dodec-2-enal, fumaric acid, maleic acid, maleamic acid, and 2-nitrothiophene were also tested, but no activity was observed.

Substrate	k_{cat}	K_M	k_{cat}/K_M
	s^{-1}	μM	μM^{-1}·s^{-1}
N-Ethylmaleimide[a]	8.07	<1.0	>8.07
Nitroglycerin[b]	3.72	109	0.0341
Cyclohex-2-enone[b]	4.38	293	0.0150
2,4,6-Trinitrotoluene[b]	3.19	705	0.0045
trans-Hex-2-enal[b]	4.68	2602	0.0018

[a] The assay contained YqjM at a concentration of 3.89 nM.
[b] 19.4 nM YqjM.

The ability to utilize nitroglycerin (NG) as a substrate (Table II) displays the propensity of the enzyme to act as a nitrate ester reductase in common with many of the other members of the Old Yellow Enzyme family (8–11, 27). Additionally, the observation of activity with the nitroaromatic compound 2,4,6-trinitrotoluene (TNT) indicates an ability to act as a nitroreductase (Table II), an activity also observed with members of this family of enzymes (8, 28). Incidentally, a more detailed analysis of the individual reductive and oxidative half-reactions is currently under way in our laboratory and will be published elsewhere.

Immunological Analysis of B. subtilis YqjM Expression in the Presence of Nitro Compounds—In order to assess expression of YqjM in *B. subtilis*, a polyclonal antiserum was raised against the recombinant protein in rabbit. The antibodies obtained were used to probe Western blots of *B. subtilis* (strain 168) crude bacterial extracts under different growth conditions. In the first instance, the ability of the protein to utilize TNT and NG as substrates prompted examination of whether expression of the protein is induced in the presence of such compounds. *B. subtilis* cultures were grown under standard conditions for 1 h at 37 °C, after which either TNT or NG was added to a final concentration of 0.2 mM, whereas a culture to which no additions were made served as a control. The bacteria were grown for a further 3 h, and samples taken at time intervals were tested for expression of the YqjM protein. Firstly, in Fig. 4*A* it can be seen that in the control culture there is a band labeled by the antibody at 38 kDa corresponding to the calculated molecular mass of *B. subtilis* YqjM (37.4 kDa). Initially, the band appears quite faint, indicating a basal level of expression of the protein under normal growth conditions. However, by 180 min there is a substantial increase (~4-fold) in the amount of protein (Fig. 4*A*), indicating up-regulation in response to general stress as the cells enter stationary phase (Fig. 4*B*). Conversely, upon the addition of TNT, the expression of the protein has already increased ~15-fold by the 15-min time interval, indicating strong induction of the protein under these conditions (Fig. 4*A*). A similar effect appears to occur with NG

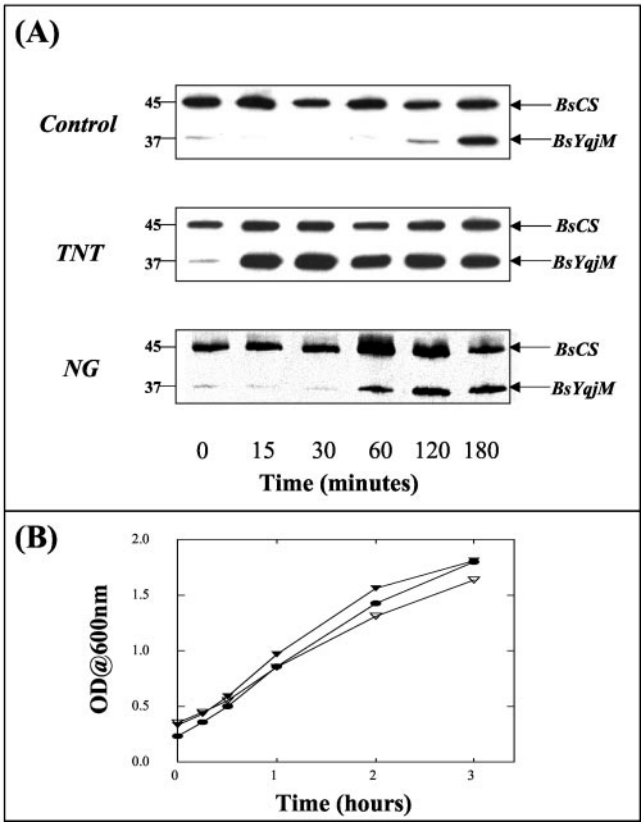

FIG. 4. **Analysis of YqjM expression in *B. subtilis* 168 in the presence of nitro compounds.** *A*, Western blots of crude extracts of *B. subtilis* 168 at the time intervals indicated, grown either in the absence (*Control*) or in the presence of 0.2 mM TNT or 0.2 mM NG and probed with affinity-purified antisera against YqjM (*BsYqjM*, 37.4 kDa) and *B. subtilis* chorismate synthase (*BsCS*, 45 kDa). The *numbers* on the *left* indicate the molecular mass of protein standards in kilodaltons. *B*, growth of *B. subtilis* 168 over time as measured by the optical density at 600 nm. *Filled circles*, control; *open triangles*, in the presence of 0.2 mM trinitrotoluene; *filled triangles*, in the presence of 0.2 mM nitroglycerin.

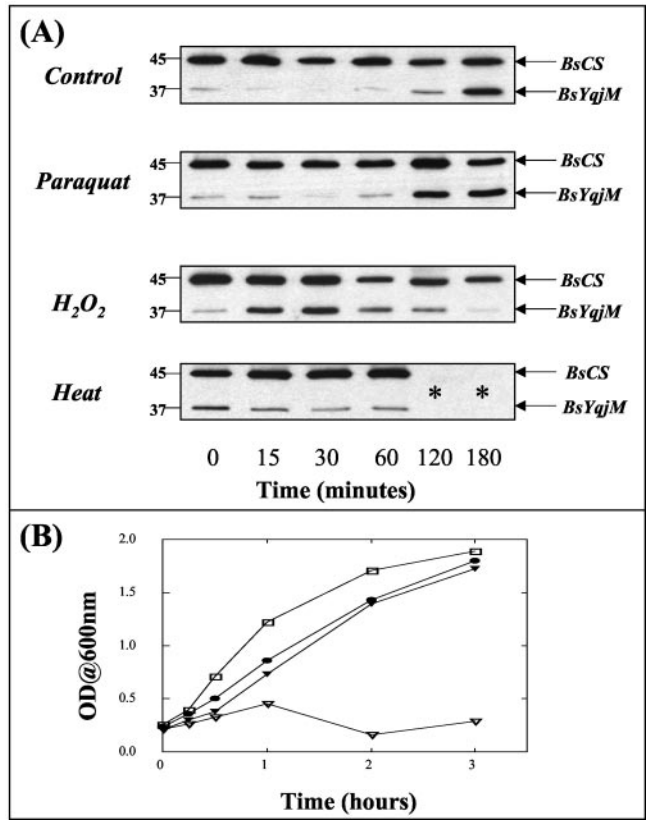

FIG. 5. **Analysis of YqjM expression in *B. subtilis* 168 under conditions of oxidative stress.** *A*, Western blots of crude extracts of *B. subtilis* 168 at the time intervals indicated, grown either in the absence (*Control*, as shown in Fig. 4) or in the presence of 0.2 mM paraquat, 0.2 mM hydrogen peroxide or at 48 °C (*, no protein extracted for these samples) and probed as outlined for Fig. 4. The *numbers* on the *left* indicate the molecular mass of protein standards in kilodaltons. *B*, growth of *B. subtilis* 168 over time as measured by the optical density at 600 nm. *Filled circles*, control; *open triangles*, in the presence of 0.2 mM hydrogen peroxide; *filled triangles*, in the presence of 0.2 mM paraquat.

(Fig. 4*A*), but it is not nearly as pronounced as that observed with TNT (~3-fold induction by the 60-min time interval). An antibody raised against *B. subtilis* chorismate synthase (mobility corresponding to 45 kDa), which is constitutively expressed, served as a protein loading and transfer control. In addition, preimmune sera did not cross-react with any protein bands under the conditions used (data not shown).

Induction of B. subtilis YqjM under Conditions of Oxidative Stress—TNT is thought to induce oxidative stress conditions in the cell by enhancing superoxide and hydrogen peroxide production (29–32). This therefore prompted us to investigate whether expression of the YqjM protein is induced directly under conditions of oxidative stress. *B. subtilis* cultures were grown as before, and after 1 h of growth at 37 °C either paraquat (which induces production of superoxide), hydrogen peroxide, or heat shock (48 °C) was applied to the cultures, and samples taken at time intervals were tested for expression of YqjM. The results obtained are shown in Fig. 5. Firstly, the control culture for this study is as before, where YqjM is induced ~4-fold at the 3-h time interval, indicating up-regulation in response to general stress as the cells enter stationary phase (Fig. 5*B*). However, in the presence of paraquat (0.2 mM), YqjM expression is induced at an earlier stage of growth (~4-fold at the 2-h interval). This effect becomes even more pronounced in the presence of hydrogen peroxide (0.2 mM), where expression of the protein is already enhanced ~4-fold at the first time interval assessed (15 min). This provides strong evidence not only for the involvement of this protein in a general stress response but its direct involvement in the oxidative stress response. The expression of chorismate synthase, a protein that is constitutively expressed throughout the growth cycle and would not be expected to be involved in the stress response of the cell, acts as a control (Fig. 5*A*). Conversely, it appears that expression of YqjM is not rapidly induced by heat shock treatment, at least under the conditions used here (Fig. 5*A*). It should be noted that the concentration of hydrogen peroxide used in Fig. 5 is sublethal for the cells, and growth is impaired after 30 min (Fig. 5*B*), and hence total protein production declines (Fig. 5*A*).

DISCUSSION

This is the first time an OYE homolog from a Gram-positive bacterium has been studied with respect to its biochemical properties and its physiological role. The biochemical properties of *B. subtilis* YqjM presented herein confirm that this protein belongs to the family of flavoprotein oxidoreductases exemplified by yeast OYE. The pieces of evidence include (i) that YqjM binds FMN tightly but noncovalently and utilizes NADPH as the preferred reductant, (ii) the protein forms charge transfer complexes with phenolic compounds similar to those characteristic of OYE (33), and (iii) it can utilize compounds such as *N*-ethylmaleimide or cyclohex-2-enone as substrates. Taken together, YqjM shares salient biochemical features of the OYE family of proteins; hence, it can be concluded

that it is the first representative of this family in Gram- positive bacteria.

In addition to the biochemical evidence, the similarity of YqjM to other members of the OYE family derives from amino acid sequence alignments (not shown). YqjM shows an average of 33% identity and 50% similarity to OYE1 from *Saccharomyces carlsbergensis*, OYE1 and -2 from *Saccharomyces cerevisiae*, the three isozymes from *Arabidopsis thaliana*, *N*-ethylmaleimide reductase from *E. coli*, and pentaerythritol tetranitrate reductase from *Enterobacter cloacae*, with highest identity to *P. putida* XenA (40%), a xenobiotic oxidoreductase. Due to this high degree of similarity, it can be safely predicted that YqjM also folds into an $(\beta\alpha)_8$-barrel structure as found for other members of the family (3, 34, 35). Attempts to crystallize YqjM have been successful, and an x-ray crystallographic study of the crystals is currently under way in our laboratory.

There are, however, also important differences between YqjM and the other members of the OYE family. For one, YqjM forms a tetramer, whereas all other members so far characterized in this regard form either monomers or dimers in solution (3, 34–36). The fact that there is only weak sequence similarity between the amino acids involved in the dimer interface of OYE and YqjM may be one explanation for the different oligomerization states observed (3).

In addition, photoreduction of YqjM proceeds directly to the fully reduced form, whereas the yeast and plant enzymes exhibit (kinetic) stabilization of a red flavin semiquinone (25, 37). This lack of semiquinone formation during the photoreduction indicates that the flavin environment in YqjM features subtle differences compared with the homologous yeast and plant enzymes. Like YqjM, similar findings have been reported for the bacterial enzymes NADH-dependent morphinone reductase from *P. putida* M10 (36) and the NADPH-dependent nitroester reductases isolated from *Pseudomonas putida* II-B and *Pseudomonas fluorescens* I-C (9). These three enzymes are classified as xenobiotic reductases, catalyzing a NAD(P)H-dependent reduction of electrophilic xenobiotics such as NG, pentaerythritol tetranitrate, 2-cyclohexen-1-one, *N*-ethylmaleimide, morphinone, codeinone, or TNT (8). This involves the direct transfer of two electrons to the electrophilic group to facilitate the reaction (*e.g.* group elimination such as that of nitrite from nitroglycerol catalyzed by *P. putida* nitroester reductase) (9). YqjM shares the lack of semiquinone formation during reduction with the xenobiotic reductases; hence, this property appears to distinguish the subfamily of xenobiotic reductases of bacterial origin from the yeast and plant homologs (9, 36).

We have shown in this study that YqjM can catalyze the reduction of a number of xenobiotic compounds with high specificity (*e.g.* NG and TNT) in addition to compounds previously shown to act as substrates for OYE (Table II). Following the biochemical characterization, nitro compound substrates were utilized to explore their effect on YqjM protein levels in *B. subtilis* cultures. This led to the discovery that YqjM is strongly induced by xenobiotic nitro compounds. Induction of the protein is much more pronounced with the aromatic nitro compound TNT than with NG (Fig. 4). In fact, high levels are detected after 15 min of incubation with TNT, whereas lower levels of protein are induced by NG only after incubation for more than 30 min (Fig. 4). This demonstration of inducible YqjM expression appears to suggest a role for detoxification of this family of proteins *in vivo* as has been proposed recently based on biochemical studies (4). On the other hand, nitro organic compounds are rarely found in nature, and unlike the bacteria isolated from polluted environments near munitions manufacturing sites (9, 38), *B. subtilis*, strain 168, has not evolved in a similarly contaminated environment. Hence, the activity of YqjM would not be expected to have adapted to a specialized nitro compound degradation role. This consideration raises the question of whether elevated YqjM protein levels are truly due to a substrate (TNT) induction of the enzyme or caused by an indirect, secondary induction mechanism. In fact, TNT, but not NG, is known to cause the production of hydrogen peroxide, prompting us to investigate its effect on the level of YqjM expression. Although the response is not as pronounced as with TNT, hydrogen peroxide induces a similarly rapid induction of YqjM (Fig. 5). The observed differences in induction level may be attributed to the mode of action of hydrogen peroxide added externally, inflicting damage on the bacterial cell wall as well as eliciting an oxygen stress response, *versus* being generated in the bacterial cytosol after uptake of TNT. In any case, these results demonstrate that YqjM is inducible by the oxygen stressor hydrogen peroxide with a direct substrate induction of the protein by TNT possibly contributing to the level of expression. This finding points to a new direction in the search for the physiological role of the members of the OYE family. Recently, it has been reported that OYE3 from *S. cerevisiae* is part of the Yap1 and Skn7 controlled oxidative stress response circuit (39), supporting our interpretation that these enzymes are part of the oxidative stress response. Furthermore, a genome-wide immunoprecipitation study of yeast proteins followed by mass spectrometric identification of the precipitated proteins revealed the existence of what may actually be a heterogenous protein complex but nevertheless consisting of OYE2, OYE3, and a number of proteins known to be involved in the oxidative stress response such as AHP1, SOD1, and SNO2 (40). We are currently performing our own analysis to identify any interacting partners of YqjM from *B. subtilis* that would give a clue as to the physiological substrate.

At present, the exact role of these enzymes in oxidative stress response is still unclear, but given the broad substrate specificity of YqjM and of the other members of the family *in vitro*, it appears unlikely that the enzyme has a single specific physiological substrate *in vivo*. Rather, the involvement in a general stress response system suggests that these enzymes function in the maintenance of the redox state of the cell, requiring the transfer of redox equivalents from pyrimidine nucleotides to suitable oxidants such as quinones.

Whatever the molecular role of YqjM and its relatives *in vivo* might be, the properties of the enzyme can be useful for biotechnological applications such as "clean-up" operations due to its broad substrate range, its non-pathogenicity, and the well characterized ability of the organism to secrete proteins into its environment. However, this demands a clear understanding of the mechanism of reductive denitration and further elucidation of the mechanism by which this microbe can respond to the challenge presented by such xenobiotics. Hence, future work will focus on the details of the course of the enzymatic reaction in conjunction with the elucidation of the three-dimensional structure of the enzyme in order to pave the way for a deeper understanding of catalysis and the observed differences of the xenobiotic reductases from other members of the family.

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