SIMB Society for Industrial Microbiology and Biotechnology

Journal of Industrial Microbiology and Biotechnology, 2021, 48, kuab064

https://doi.org/10.1093/jimb/kuab064
Advance access publication date: 28 August 2021
Biotechnology Methods – Original Paper

Rapid evaluation of the substrate specificity of 3-nitrobenzoic acid dioxygenase MnbAB via colorimetric detection using Saltzman reagent

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Abstract: Nitroaromatic compounds are essential materials for chemical industry, but they are also potentially toxic environmental pollutants. Therefore, their sensitive detection and degradation are important concerns. The microbial degradation pathways of nitroaromatic compounds have been studied in detail, but their usefulness needs to be evaluated to understand their potential applications in bioremediation. Here, we developed a rapid and relatively sensitive assay system to evaluate the activities and substrate specificities of nitroaromatic dioxygenases involved in the oxidative biodegradation of nitroaromatic compounds. In this system, nitrous acid, which was released from the nitroaromatic compounds by the dioxygenases, was detected and quantified using the Saltzman reagent. *Escherichia coli* producing the 3-nitrobenzoic acid dioxygenase complex MnbAB from *Comamonas* sp. JS46 clearly showed the apparent substrate specificity of MnbAB as follows. MnbAB accepted not only 3-nitrobenzoic acid but also several other *p*- and *m*-nitrobenzoic acid derivatives as substrates, although it much preferred 3-nitrobenzoic acid to others. Furthermore, the presence of a hydroxy or an amino group at the *ortho* position of the nitro group decreased the activity of MnbAB. In addition, MnbAB accepted 2-(4-nitrophenyl)acetic acid as a substrate, which has one additional methylene group between the aromatic ring and the carboxy group of 3-nitrobenzoic acid. This is the first report about the detailed substrate specificity of MnbAB. Our system can be used for other nitroaromatic dioxygenases and contribute to their characterization.

Keywords: Nitroaromatic compounds, 3-Nitrobenzoic acid dioxygenase, Nitrous acid, Biodegradation, Saltzman reagent

Introduction

Nitroaromatic compounds have been widely used as important industrial materials for making diverse nitrogen-containing aromatic compounds. For example, aniline (aminobenzene) is traditionally produced by the reduction (hydrogenation) of nitrobenzene (Driessen et al., 2017) and is used for the synthesis of many commercial products including polyurethane (Akindoyo et al., 2016), rubber, dyes, and pharmaceuticals (Kahl et al., 2012). Nitroaromatic compounds have also been found as natural products in bacteria, fungi, and plants (Winkler & Hertweck, 2007). Examples are antibiotics with a nitroaromatic moiety produced by the genus *Streptomyces*, such as chloramphenicol, aureothin, thaxtomins, and rufomycins (Parry et al., 2011; Winkler & Hertweck, 2007). However, nitroaromatic compounds are rather rare in nature, and mainly exist as anthropogenic pollutants (Peres & Agathos, 2000). Because they are potentially toxic, mutagenic, and carcinogenic, their sensitive detection and degradation are important issues (Peres & Agathos, 2000; Kovacic & Somanathan, 2014).

Many microorganisms have evolved their biodegradation pathways to catabolize nitroaromatic compounds, including nitrobenzene, nitrotoluene, nitrophenol, and nitrobenzoic acid, as carbon, nitrogen, and energy sources (Marvin-Sikkema & de Bont, 1994; Ju & Parales, 2010). These biodegradation pathways can be roughly divided into two routes: the oxidative pathway and the reductive pathway (Ju & Parales, 2010). The nitrobenzene biodegradation pathways are shown in Fig. 1a. In the oxidative pathway, nitrobenzene dioxygenase catalyzes the introduction of vicinal diol onto the nitrated carbon and its adjacent carbon atoms. Then, catechol is generated by the spontaneous release of nitrous acid and further catabolized via ring cleavage. This pathway was identified in *Comamonas* sp. JS765 (Ju & Parales, 2010; Nishino & Spain, 1995). In contrast, in the reductive pathway, nitrobenzene is reduced to hydroxyaminobenzene via nitrosobenzene by nitrobenzene nitroreductase. During further degradation of hydroxyaminobenzene, ammonia is released. This pathway was identified in *Pseudomonas pseudoalcaligenes* JS45 (Ju & Parales, 2010; Nishino & Spain, 1993). These pathways and enzymes can be applied to bioremediation to detoxify environmental nitroaromatic contaminants (Peres & Agathos, 2000).

The *mnbA* and *mnbB* genes (NCBI accession number AY639949) encode the 3-nitrobenzoic acid (3NBA) dioxygenase complex in *Comamonas* sp. JS46. This strain was originally isolated as a 3NBA-degrading bacterium (Goodall et al., 1998), and MnbA and MnbB are required for 3NBA catabolism (Providenti et al., 2006). MnbA is a Rieske-type non-heme iron-dependent oxygenase (Ferraro et al., 2005; Bugg & Ramaswamy, 2008; Wackett, 2002), and MnbB is considered as an oxidoreductase that supplies reducing equivalents to MnbA (Providenti et al., 2006). Similar to the

Received: August 8, 2021. **Accepted:** August 22, 2021.

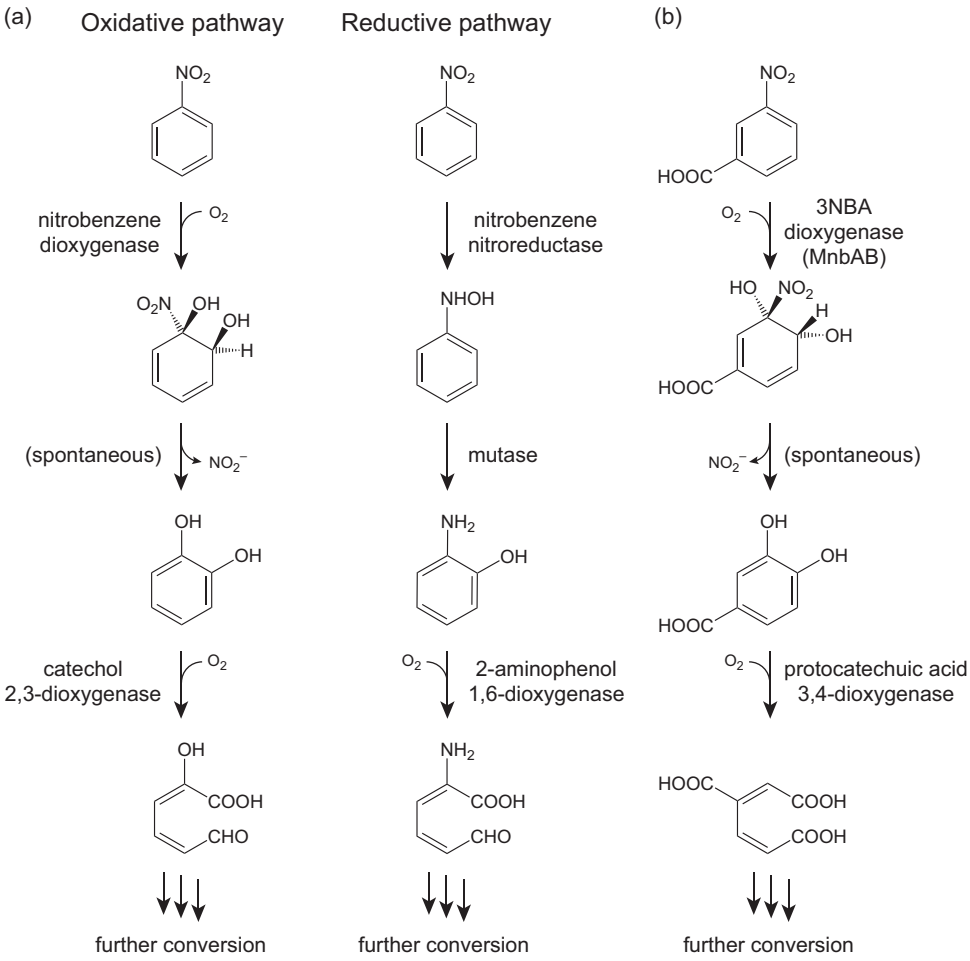

Fig. 1 Biodegradation pathways of nitroaromatic compounds. (**a**) The oxidative and reductive nitrobenzene biodegradation pathways. In the oxidative pathway (*left*), nitrobenzene dioxygenase introduces 1,2-vicinal diol onto the benzene ring. After the spontaneous release of nitrous acid to form catechol, the ring is oxidatively cleaved by catechol 2,3-dioxygenase. This pathway was identified in *Comamonas* sp. JS765. In the reductive pathway (*right*), nitrobenzene is reduced to hydroxyaminobenzene via nitrosobenzene by a nitrobenzene nitroreductase. A mutase converts the compound into 2-aminophenol via the Bamberger rearrangement. The ring is then cleaved by a 2-aminophenol 1,6-dioxygenase. This pathway was identified in *P. pseudoalcaligenes* JS45. (**b**) The oxidative 3NBA biodegradation pathway. MnbAB introduces 3,4-vicinal diol in the first step.

nitrobenzene dioxygenase (Ju & Parales, 2010; Nishino & Spain, 1995), MnbAB introduces two hydroxy groups into the C-3 and C-4 of 3NBA using molecular oxygen as a substrate (Fig. 1b). Then, through the spontaneous release of nitrous acid, protocatechuic acid (3,4-dihydroxybenzoic acid) is produced (Fig. 1b). In the 3NBA degradation pathway, the resulting protocatechuic acid is further metabolized (Fig. 1b). Elucidation of the substrate specificity of MnbAB is important for the application of MnbAB in bioremediation, but it has not been well studied until now.

Here, we report the development of a rapid and relatively sensitive system to evaluate the enzyme activities and substrate specificities of nitroaromatic dioxygenases that function in the oxidative degradation pathway. With the detection and quantification of the released nitrous acid using the Saltzman reagent (Saltzman, 1954), we elucidated the substrate specificity of MnbAB as a model. This method can be applied to other nitroaromatic dioxygenases to analyze their enzymatic profiles.

Materials and Methods

Strains, Media, and Culture Conditions

Escherichia coli strain JM109 was used for plasmid construction, and it was cultivated at 37°C in Luria-Bertani (LB; 0.5% yeast extract, 1% tryptone, and 1% sodium chloride) medium with ampicillin (100 mg/L). The *E. coli* Δ*nfsAB* strain was used for heterologous gene expression and whole-cell bioconversion, and it was cultivated in LB medium or terrific broth (TB; 1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 0.017 M KH_2PO_4, and 0.072 M K_2HPO_4) with ampicillin (100 mg/L), kanamycin (50 mg/L), and chloramphenicol (34 mg/L). *E. coli* strains BLR(DE3) and Δ*nfsA* were used for gene disruption, and they were cultivated in LB medium or super optimal broth (SOB) medium (2% tryptone, 0.5% yeast extract, 0.05% sodium chloride, 0.24% anhydrous magnesium sulfate, and 0.0186% potassium chloride). *E. coli* strain DGF-298WΔ100::revΔ234::SC was provided by the National Bio-Resource Project (NBRP), MEXT, Japan. This strain was used for whole-cell bioconversion and was cultivated in LB medium or TB medium. Specific modifications in the media are described below. Unless mentioned otherwise, all chemicals were purchased from Tokyo Chemical Industry (Tokyo, Japan), FUJIFILM Wako Pure Chemicals Corporation (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), or Sigma Aldrich (MO, USA).

Plasmid Construction

For heterologous expression of *mnbA* and *mnbB* from *Comamonas* sp. JS46, their coding sequences were optimized for gene

expression in *E. coli* according to its codon usage, and the optimized genes (see Appendix in the Supplementary Materials) were artificially synthesized (Thermo Fisher Scientific, MA, USA). The *mnbA* and *mnbB* genes were amplified by polymerase chain reaction (PCR) using primer sets mnbA-F/R and mnbB-F/R, respectively (Table S1). The *mnbB* fragment was introduced into the NdeI site of pETDuet-1 using the In-Fusion system (Takara Bio, Shiga, Japan). After linearization of the resulting plasmid by PCR amplification using a primer set Duet-F/R (Table S1), the *mnbA* fragment was connected to the *mnbB*-containing pETDuet-1 vector using the In-Fusion system to obtain pETDuet-1-*mnbAB*. The nucleotide sequences of *mnbA* and *mnbB* were confirmed not to harbor unintended PCR mutations by Sanger sequencing.

Gene Disruption of *nfsA* and *nfsB*

The DNA fragments for gene disruption of *nfsA* and *nfsB*, which include their flanking regions for homologous recombination and either kanamycin (*nfsA*) or chloramphenicol (*nfsB*) resistance genes, were prepared using PCR. The primer pair and template were DnfsA-F/R and pK19mobsacB for *nfsA*, and DnfsB-F/R and pACYCDuet-1 for *nfsB*, respectively (Table S1). After introduction of pKD46 into *E. coli* BLR(DE3), the resulting strain BLR/pKD46 was cultivated at 30°C in SOB medium with ampicillin and 10 mM L-arabinose to induce expression of the Red recombinase genes. When the OD_{600} reached ~0.3, the cells were harvested (4°C, 12,000 × g for 2 min), washed twice with water, and resuspended in 10% glycerol to prepare the competent cells. The DNA fragment for *nfsA* disruption was introduced into BLR/pKD46 via electroporation and SOB with catabolite repression (SOC) medium (2% tryptone, 0.5% yeast extract, 20 mM glucose, 0.05% sodium chloride, 0.24% anhydrous magnesium sulfate, and 0.0186% potassium chloride) was added. After incubation at room temperature for 24 hr, the cells were inoculated on LB agar containing ampicillin and kanamycin, and incubated at 37°C for 24 hr. The obtained transformants were cultivated at 37°C in LB medium without ampicillin to remove pKD46. After confirmation of plasmid removal and gene disruption of *nfsA*, the resulting strain Δ*nfsA* was transformed again with pKD46 and the DNA fragment for *nfsB* disruption using the same procedure described above. After removing pKD46, the resulting strain Δ*nfsAB* was confirmed to have lost both *nfsA* and *nfsB* (Fig. S1) and to show resistance to kanamycin and chloramphenicol.

Gene Expression and Whole-Cell Bioconversion Analysis

pETDuet-1-*mnbAB* was introduced into *E. coli* Δ*nfsAB*, and the resulting strain Δ*nfsAB*-*mnbAB* was cultivated at 37°C in TB medium with ampicillin, 0.1 mM $Fe(NH_4)_2(SO_4)_2$, 0.1 mM $FeSO_4$, and 1 mM L-cysteine hydrochloride until the OD_{600} reached ~0.5. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.1 mM to induce gene expression. The cells were further cultivated for 16 hr at 26°C. Afterward, the cells were harvested via centrifugation (4°C, 5,000 × g for 15 min), washed with bioconversion buffer (50 mM potassium phosphate buffer containing 10% glycerol and 150 mM NaCl, pH 7.4), and resuspended in bioconversion buffer to a cell density of 40 g/L (wet weight).

For 3NBA reduction analysis, 1 mM (final concentration) of 3NBA was added to 3 mL of the cell suspension in a 15-mL tube, and the cell suspension was mixed with gentle rotation at room temperature for 12 hr. After centrifugation (room temperature, 5,000 × g for 15 min), the supernatants were analyzed by high-performance liquid chromatography (HPLC) using a 1,100 series spectrometer (Agilent Technologies, CA, USA) equipped with a COSMOCORE $2.6C_{18}$ column (2.1-mm ID × 150 mm, Nacalai Tesque). Compounds were separated with a linear gradient of water and acetonitrile containing formic acid (0.1%) at a flow rate of 0.4 mL/min.

To confirm the expression of *mnbA* and *mnbB*, 95 μL of the cell suspension was placed into each well of a 96-well plate (Thermo Fisher Scientific). After adding 5 μL of 3NBA solution in DMSO to each well, the plates were incubated at 28°C with rotational shaking (600 rpm) for various periods of time. After centrifugation (room temperature, 1,570 × g for 10 min), 50 μL of each supernatant was transferred to a new 96-well plate. After 50 μL of methanol was added into each well to denature proteins, the plate was centrifuged again and 50 μL of each supernatant was transferred to a new 96-well plate. Then, 50 μL of Saltzman reagent was added to each well. Absorbance at 545 nm was measured using a microplate reader (SpectraMax M2, Molecular Device, CA, USA).

For substrate specificity analysis, 95 μL of the cell suspension was added into each well of a 96-well plate. Then, 5 μL of the substrate solution in DMSO or methanol was added to each well, and the plate was incubated at 28°C for 1 hr with rotational shaking (600 rpm). After centrifugation (room temperature, 1,570 × g for 10 min), 50 μL of each supernatant was transferred to a new 96-well plate. After 50 μL of methanol was added into each well to denature proteins, the plate was centrifuged again and 50 μL of each supernatant was transferred to a new 96-well plate. Then, 50 μL of Saltzman reagent was added to each well, and absorbance at 545 nm was measured using the microplate reader. The experiments were performed in triplicate. Saltzman reagent (Saltzman, 1954) consisted of solution A (80 g/L sulfanilamide, 20% (vol/vol) phosphoric acid) and solution B (5.6 g/L *N*-(1-naphthyl)ethylenediamine dihydrochloride). Solutions A and B were mixed at a volume ratio of 10:1 immediately before use. The concentrations of nitrous acid were determined using a standard curve with sodium nitrite as the standard.

Results

Double Disruption of Two Nitroreductase Genes and Observation of Its Influence on the Ability to Reduce 3-Nitrobenzoic Acid in *Escherichia coli*

In the oxidative degradation pathway, the nitro group is released as nitrous acid from the aromatic ring by nitroaromatic dioxygenases. Therefore, for the rapid detection of oxygenation activity, we decided to combine an *E. coli* bioconversion system and a detection method using the Saltzman reagent, which is commonly used for the colorimetric detection of nitrous acid (Saltzman, 1954). However, *E. coli* is capable of reducing various kinds of nitroaromatic compounds using promiscuous nitroreductases (Zenno et al., 1996a; Zenno et al., 1996b; Mercier et al., 2013; Rau & Stolz, 2003). Two major nitroreductases, NfsA and NfsB, have broad substrate specificities in reducing the nitro groups of various nitrobenzene derivatives, including 4-nitroacetophenone and 4-nitrobenzoic acid (Zenno et al., 1996a; Zenno et al., 1996b; Mercier et al., 2013; Rau & Stolz, 2003). The disruption of both the *nfsA* and *nfsB* genes drastically decreased the reduction activity of nitroaromatic compounds, 7-nitrocoumarin-3-carboxylic acid (Mercier et al., 2013) and nitrofurazone (Rau & Stolz, 2003). However, reduction of 3NBA by *E. coli* has not yet been tested.

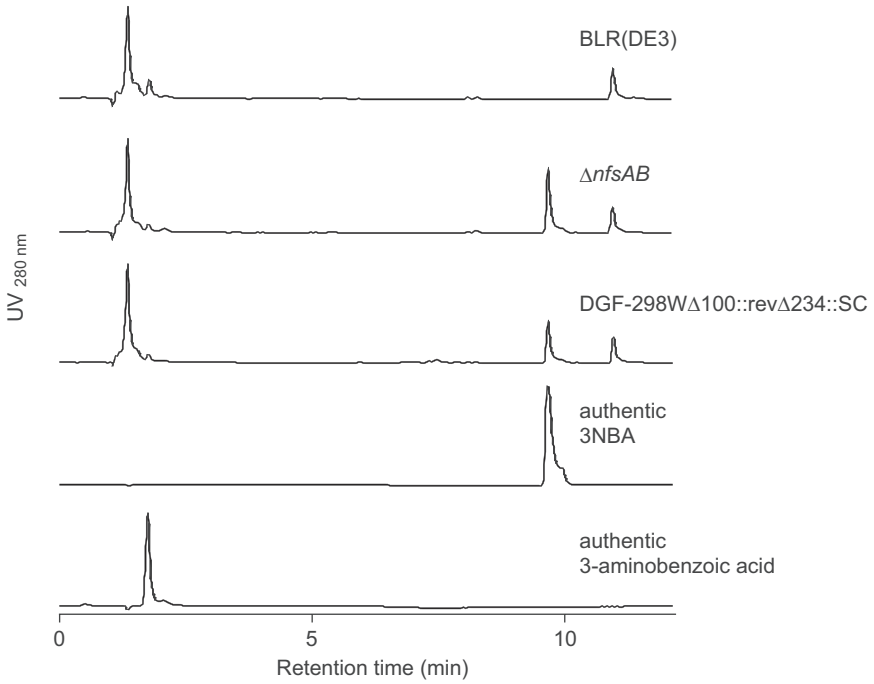

Fig. 2 Reduction of 3NBA by *E. coli* strains. 3NBA (1 mM) was added to the cell suspension, and the mixture was gently mixed with rotation for 12 hr. Supernatants were analyzed by HPLC. From the top, BLR(DE3), Δ*nfsAB*, DGF-298WΔ100::revΔ234::SC, authentic 3NBA, and authentic 3-aminobenzoic acid.

We first examined the ability of *E. coli* to reduce 3NBA. 3NBA was added to a cell suspension of *E. coli* BLR(DE3), and the mixture was incubated for 12 hr at room temperature. The supernatants of the culture were analyzed by HPLC. As a result, 3NBA was not detected, indicating that 3NBA was converted to other compounds by *E. coli* BLR(DE3) (Fig. 2). 3-Aminobenzoic acid, which should be synthesized from the reduction of 3NBA, was also not detected (Fig. 2). 3-Aminobenzoic acid may have been further metabolized by endogenous enzymes in *E. coli*.

To construct an *in vivo* evaluation system for 3NBA dioxygenases, we disrupted the *nfsA* and *nfsB* genes of *E. coli* BLR(DE3) to prevent the unintended reduction of nitroaromatic compounds. A plasmid encoding the Red recombinase under the arabinose-inducible promoter, pKD46, was used to facilitate gene recombination (Datsenko & Wanner, 2000). The *nfsA* and *nfsB* genes were substituted with the kanamycin and chloramphenicol resistance genes, respectively, resulting in the Δ*nfsAB* strain (Fig. S1). As a control, we also prepared another *E. coli* strain DGF-298WΔ100::revΔ234::SC harboring a reduced genome that lacks both *nfsA* and *nfsB* (Mizoguchi et al., 2008). When 3NBA was incubated with the cell suspensions of the Δ*nfsAB* and DGF-298WΔ100::revΔ234::SC strains, a considerable amount of 3NBA remained intact (Fig. 2). We assumed that some cryptic nitroreductases other than NfsA/NfsB may exist and contribute to the reduction of 3NBA to some extent in *E. coli*, and that such nitroreductases could be further removed in the DGF-298WΔ100::revΔ234::SC strain. Contrary to our expectation, however, the amount of 3NBA remaining in the culture of the DGF-298WΔ100::revΔ234::SC strain did not increase compared with that of the Δ*nfsAB* strain (Fig. 2). Therefore, we chose the Δ*nfsAB* strain as the host in subsequent experiments. In the Δ*nfsAB* strain, the reduction of 3NBA was suppressed enough not to hamper the measurement of the activity of a 3NBA dioxygenase heterologously produced (see below).

Examination of the *In Vivo* Dioxygenation Activity of MnbA and MnbB in *Escherichia coli*

As a representative of nitroaromatic dioxygenases, we chose MnbAB (Providenti et al., 2006; Basu et al., 2016; Nadeau & Spain, 1995) as the target of this study. For heterologous expression in *E. coli*, the artificial genes were synthesized, in which the codon usage of *mnbA* and *mnbB* was optimized for *E. coli*. The genes were cloned into pETDuet-1 and introduced into the *E. coli* Δ*nfsAB* strain to obtain the Δ*nfsAB-mnbAB* strain. After cultivation and coexpression of *mnbA* and *mnbB*, 3NBA (final concentration: 0, 1, 5, 10, 25, 50, 100, 250, 500, 750, and 1,000 μM) was added to the cell suspension, and whole-cell bioconversion was carried out (incubation period: 0, 10, 30, 60, 90, and 120 min). After centrifugation to remove the cells, methanol was added to the supernatants to denature and precipitate the proteins present. Proteins in the supernatants hampered the analysis by precipitating and absorbing the dye synthesized after the addition of the Saltzman reagent. Therefore, removing the proteins from the solution prior to the addition of the Saltzman reagent was important. After additional centrifugation to remove protein aggregates, Saltzman reagent was added to the supernatants to investigate the release of nitrous acid, and absorbance at 545 nm was monitored to quantify it. As a result, the color of the solution became clear purple, where the intensity of the color correlated with the concentration of 3NBA (Fig. 3a). Considering that there are no reports on the 3NBA dioxygenase activity in *E. coli* (Ju & Parales, 2010; Díaz et al., 2001), this result indicates that *mnbA* and *mnbB* were correctly expressed to produce active MnbAB complex in *E. coli* cells. When the concentration of 3NBA was more than 750 μM, the concentration of nitrous acid detected was saturated within 30–60 min, and then it decreased after 60 min, probably due to its instability (Fig. 3b). In contrast, when the concentration of 3NBA was less than 500 μM, the highest concentration of nitrous acid was detected at 10-min incubation. Thus, the optimal reaction time seemed to be

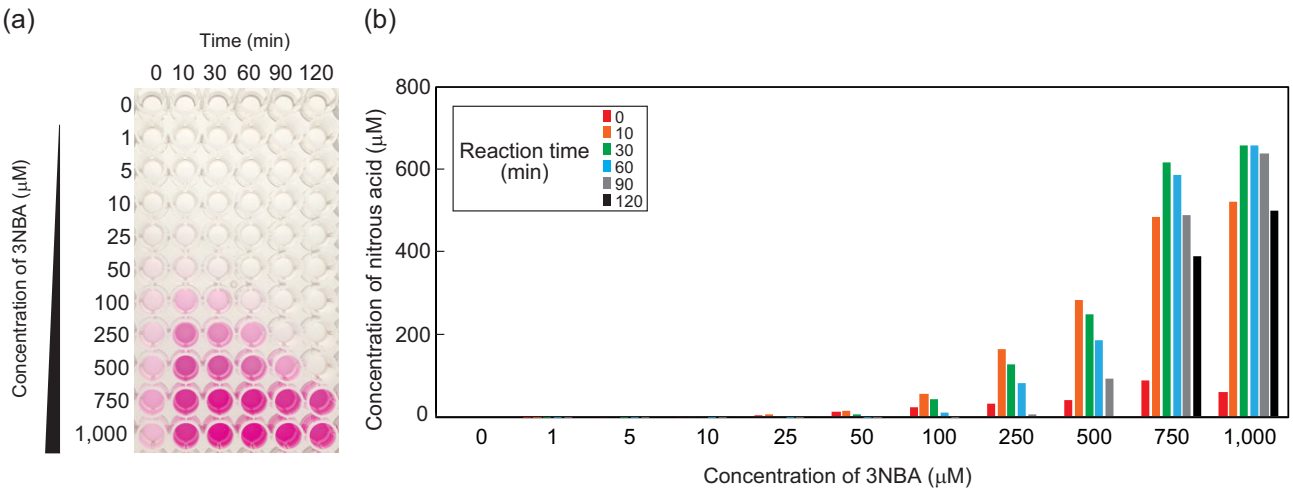

Fig. 3 Conversion of 3NBA with *E. coli* Δ*nfsAB-mnbAB* whole cells and detection of nitrous acid using Saltzman reagent. Various concentrations of 3NBA were added to the cell suspension of *E. coli* Δ*nfsAB-mnbAB* and the samples were incubated for various periods of time. Nitrous acid in the supernatants was detected with the Saltzman reagent (**a**) and quantified by absorbance at 545 nm using a standard curve shown in Fig. S2 (**b**). Although the result of a single assay is shown, we can understand the trend in the detection of nitrous acid in the supernatant of reaction mixtures incubated under different conditions.

different according to the concentration of 3NBA. However, we thought that reaction time is not so critical, because the difference was not so great in general. It is notable that our system was able to detect enzyme activity even at low substrate concentrations (≥50 μM of 3NBA).

Substrate Specificity of MnbAB

The substrate specificity of MnbAB was examined using 25 nitroaromatic compounds (Fig. 4a). After preparing a cell suspension of *E. coli* Δ*nfsAB-mnbAB*, each nitro compound (final concentration: 10, 50, 100, and 500 μM) was added and the samples were incubated for 60 min. Relatively long incubation time was set, because we assumed that MnbAB should have much lower activities toward most of the nitroaromatic compounds other than 3NBA, and that this should require longer incubation time to achieve detectable amount of bioconversions. Subsequently, proteins present in the supernatants were removed using methanol, Saltzman reagent was added, and absorbance at 545 nm was monitored as described above. As a result, MnbAB showed the highest activity toward 3NBA (**12**) (Fig. 4b). Moderate activity was detected in 4-nitrobenzoic acid (**13**), 2-(4-nitrophenyl)acetic acid (**16**), 3-hydroxy-4-nitrobenzoic acid (**19**), and 4-amino-3-nitrobenzoic acid (**21**) (Fig. 4c). MnbAB showed no detectable activity toward the compounds that had no carboxy groups (**1**–**9**, Fig. 4c), except for 4-nitrophenol (**10**); MnbAB showed a weak activity toward 4-nitrophenol (**10**), which was comparable to that toward 2-nitrobenzoic acid (**11**, Fig. 4c). These results suggest that MnbAB can accept *m*- and *p*-nitro benzoic acid derivatives, but it much prefers 3NBA (**12**) to others. Interestingly, MnbAB could accept some 2-phenylacetic acid derivatives as a substrate (see **14**–**16** and **18**, Fig. 4c), but not any 3-phenylpropionic acid derivatives (see **17**, **22**, and **23**, Fig. 4c) or *trans*-cinnamic acid derivatives (see **24** and **25**, Fig. 4c), which indicated that one additional methylene group is allowed between the aromatic ring and the carboxy group, but two additional carbon atoms are not (Fig. 4c). Our experiments also indicate that the insertion of a hydroxy or an amino group to the *ortho* position of the nitro group tends to decrease the activity of MnbAB (**20** or **21** vs. **12**; **19** vs. **13**; **18** vs. **15**, Fig. 4c). Although the membrane permeability of the tested compounds should affect the conversion rate, we considered that all the tested substrates have sufficient membrane permeability because aromatic compounds can enter the cells via passive diffusion (Wu et al., 2018; Dıáz et al., 2001) and that bioconversion of various aromatic compounds using recombinant *E. coli* cells have been reported (Otomatsu et al., 2010; Kubota et al., 2005; Ju & Parales, 2011).

Discussion

In this study, we developed a colorimetric detection method to evaluate nitroaromatic dioxygenase activity. Using this method, we clarified the apparent substrate specificity of MnbAB from *Comamonas* sp. JS46. MnbA is a member of the Rieske-type non-heme iron-dependent dioxygenases, which contain a [2Fe-2S] cluster (Ferraro et al., 2005; Bugg & Ramaswamy, 2008; Wackett, 2002). In general, it is difficult to reconstitute the enzymatic reactions that are catalyzed by Rieske-type oxygenases *in vitro* because iron–sulfur clusters are oxygen-sensitive and unstable. In our system, the conversion analysis was carried out within whole, intact cells, therefore protein purification was not necessary. This bioconversion system was effective because the iron–sulfur clusters as well as their reducing equivalents are constantly supplied from the endogenous system in *E. coli*.

Biodegradation enzymes with broad substrate specificities are expected to provide advantages for producer microorganisms to survive in nature. Indeed, the nitrobenzene dioxygenase of *Comamonas* sp. JS765 and 3-nitrotoluene dioxygenase of *Diaphorobacter* sp. DS2 showed broad substrate specificities (Lessner et al., 2002; Singh et al., 2014). In contrast, MnbAB showed a relatively narrow substrate specificity with a strong preference for 3NBA (Fig. 4). To explain the differences in their substrate specificities, structural analysis is necessary. In addition, our method can be used in high-throughput screening for the directed evolution-based engineering of MnbAB to efficiently accept non-native substrates and/or to enhance enzymatic activity.

The *E. coli* Δ*nfsAB-mnbAB* strain can be used as a sensor strain to detect 3NBA. In fact, we have applied this system for the screening of artificially evolved AurF that acquired a new substrate specificity to accept 3-aminobenzoic acid to produce 3NBA

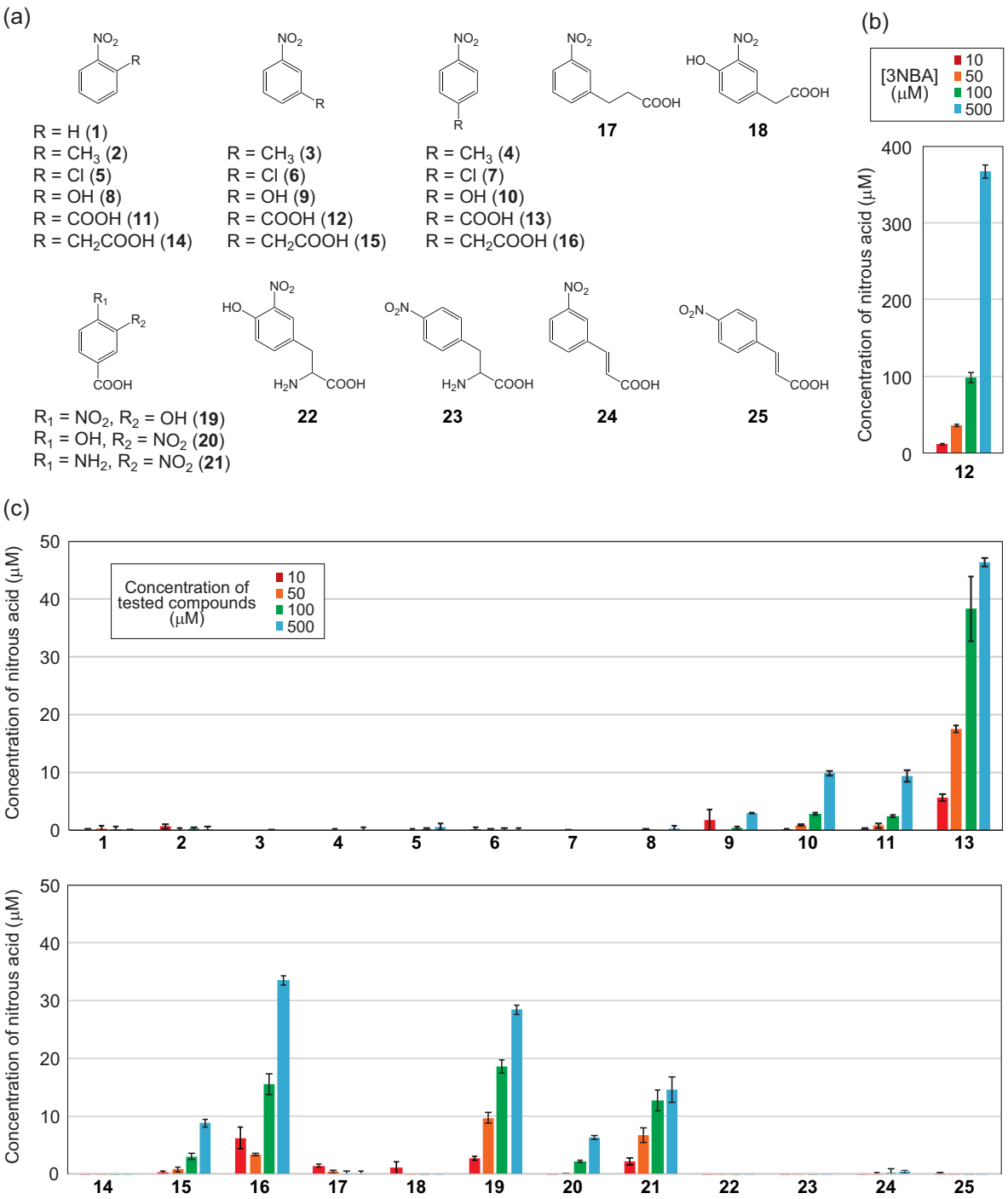

Fig. 4 Substrate specificity of MnbAB evaluated by quantification of nitrous acid. (**a**) Structures of tested compounds. (**b, c**) Concentration of the released nitrous acid after 60 min of the reaction toward 3NBA (**b**) or other compounds (**c**). Data are means and the error bars indicate the standard error ($n = 3$).

(data not shown). AurF is a di-iron arylamine monooxygenase that catalyzes *N*-oxygenation of 4-aminobenzoic acid to yield 4-nitrobenzoic acid in aureothin biosynthesis in *Streptomyces thioletus* (He & Hertweck, 2004; Wang & Chen, 2017). Thus, this study can be applied for the evolution-based engineering of biosynthetic enzymes that have a potential to produce 3NBA.

The method for nitrous acid detection using the Saltzman reagent is very sensitive. Therefore, we were able to detect enzymatic activity even when the bioconversion generated products at micromolar levels, which enabled the effective evaluation of the substrate specificity of the nitroaromatic dioxygenase MnbAB. This system can be used to investigate the substrate specificities of other nitroaromatic dioxygenases that catalyze oxidation accompanied with the release of nitrous acid. Thus, by applying our system to various nitroaromatic dioxygenases, we may be able to detect a broad range of nitroaromatic compounds in environment samples. In other words, a biosensor of nitroaromatic compounds, which are potentially toxic environmental pollutants, could be further developed based on our system. Thus, this study provides a new bio-based detection method for environmental nitro

contaminants and this method can contribute to environmental bioremediation.

Acknowledgments

We dedicate this paper to Dr. Arnold Demain. Dr. Teruhiko Beppu, a former professor in our laboratory, had often talked about his warm and sincere personality as well as his great achievements, so the corresponding author had a great respect for him even before the first encounter with him at IUMS 2011 Sapporo.

Supplementary Material

Supplementary material is available online at *JIMB* (*www.academic.oup.com/jimb*).

Funding

This work was financially supported by CREST, Japan Science and Technology Agency (grant number JPMJCR13B3) to Y.O., A3 Foresight Program from the Japan Society for the Promotion of Science (JSPS) to Y.O., Cross-ministerial Strategic Innovation Promotion Program (SIP), "Technologies for Smart Bio-industry and Agriculture" from the Cabinet Office, Government of Japan (funding agency: Bio-oriented Technology Research Advancement Institution, NARO, grant number 18087978) to Y.O., and Grant-in-Aid for JSPS Research Fellow to H.T. (grant number 17J06071).

Author Contributions

All authors designed the study. H.T. performed the experiments. H.T. and Y.K. analyzed the data. All authors discussed the results, wrote the paper, and approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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