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 (Kahi 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, thaxtomin, 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 hydroxynitrobenzene via nitrosobenzene by nitrobenzene nitroreductase. During further degradation of hydroxynitrobenzene, ammonia is released. This pathway was identified in Pseudomonas pseudocaligenes 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 oxoreductase that supplies reducing equivalents to MnbA (Providenti et al., 2006). Similar to the...
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 nfsA 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 KH2PO4, and 0.072 M K2HPO4) 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-298W Δ100::revΔ234::SC was provided by the National BioResource 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 pk19mobbsacB for nfsA, and DnfsB-F/R and pACYCDuet-1 for nfsB, respectively (Table S1). After introduction of pkKD46 into E. coli BLR(DE3), the resulting strain BLR/pKD46 was cultivated at 30°C in SOB medium with ampicillin and 10 mM l-cysteine hydrochloride until the OD600 reached 0.1. The strain was connected to the cation using a primer set Duet-F/R (Table S1), the 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 Expression and Whole-Cell Bioconversion Analysis

pETDuet-1-mnbAB was introduced into E. coli ΔnfsAB, and the resulting strain ΔnfsAB-mnbAB was cultivated at 37°C in TB medium with ampicillin, 0.1 mM Fe(NH₄)₂(SO₄)₂, 0.1 mM FeSO₄, and 1 mM L-cysteine hydrochloride until the OD600 reached ~0.3. The cells were centrifuged at 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 ΔnfsA was transformed again with pKD46 and the DNA fragment for nfsB disruption using the same procedure described above. After removing pKD46, the resulting strain ΔnfsAB was confirmed to have lost both nfsA and nfsB (Fig. S1) and to show resistance to kanamycin and chloramphenicol.

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 nitrofurazon (Rau & Stolz, 2003). However, reduction of 3NBA by E. coli has not yet been tested.
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). 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, 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).

**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.

**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...
were added to the cell suspension of nitroaromatic compounds (Fig. 4a). After preparing a cell suspension (able to detect enzyme activity even at low substrate concentration was not so great in general. It is notable that our system was thought that reaction time is not so critical, because the difference according to the concentration of 3NBA. However, we incubated under different conditions.

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.

**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 bioconversion. 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-nitrobenzoic 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; Díaz 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. JS76S 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).

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 thioluteus (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.

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

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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.

References
Akindoyo, J. O., Beg, M. D. H., Ghazali, S., Islam, M. R., Jeyaratnam, N., & Yuvaraj, A. R. (2016). Polyurethane types, synthesis and applications – a review. RSC Advances, 6(115), 114453–114482.
Basu, S., Chowdhury, P. P., Deb, S., & Dutta, T. K. (2016). Degradation pathways of 2- and 4-Nitrobenzoates in Cupriavidus sp. strain ST-14 and construction of a recombinant strain, ST-14 :3NBA, capable of degrading 3-nitrobenzoate. Applied and Environmental Microbiology, 82(14), 4253–4263.
Bugg, T. D. H. & Ramaswamy, S. (2008). Non-heme iron-dependent dioxygenases: Unravelling catalytic mechanisms for complex enzymatic oxidations. Current Opinion in Chemical Biology, 12(2), 134–140.
Datsenko, K. A. & Warner, B. L. (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proceedings of the National Academy of Sciences, 97(12), 6640–6645.
Díaz, E., Ferrández, A., Prieto, M. A., & García, J. L. (2001). Biodegradation of aromatic compounds by Escherichia coli. Microbiology and Molecular Biology Reviews, 65(4), 523–569.
Driessen, R. T., Kamphuis, P., Mathijsen, L., Zhang, R., van der Ham, L. G. J., van den Berg, H., & Zeeuw, A. J. (2017). Industrial process design for the production of aniline by direct amination. Chemical Engineering & Technology, 40(5), 838–846.
Ferraro, D. J., Oekhar, L., & Ramaswamy, S. (2005). Rieske business: Structure–function of Rieske non-heme oxygenases. Biochemical and Biophysical Research Communications, 338(1), 175–190.
Goodall, J. L., Thomas, S. M., Spain, J. C., & Peretti, S. W. (1998). Operation of mixed-culture immobilized cell reactors for the metabolism of meta- and para-nitrobenzoate by Comamonas sp. JS46 and Comamonas sp. JS47. Biotechnology and Bioengineering, 59(1), 21–27.
He, J. & Hertweck, C. (2004). Biosynthetic origin of the rare nitroaryl moiety of the polyketide antibiotic aureothin: Involvement of an unprecedented N-oxygenase. Journal of the American Chemical Society, 126(12), 3694–3695.
Ju, Kou-San & Parales, R. E. (2010). Nitroaromatic compounds, from synthesis to biodegradation. Microbiology and Molecular Biology Reviews, 74(2), 250–272.
Ju, Kou-San & Parales, R. E. (2011). Evolution of a new bacterial pathway for 4-nitrotoluene degradation. Molecular Microbiology, 82(2), 355–364.
Kahl, T., Schröder, Kai-Wilfrid, Lawrence, F. R., Marshall, W. J., Höke, H., & Jackh, R. (2012). Aniline. In Ullmann’s Encyclopedia of Industrial Chemistry. Wiley-VCH. Weinheim, 3, 465–478.
Kovacic, P. & Somanathan, R. (2014). Nitroaromatic compounds: Environmental toxicity, carcinogenicity, mutagenicity, therapy and mechanism. Journal of Applied Toxicology, 34(8), 810–824.
Kubota, M., Nodate, M., Yasumoto-Hirose, M., Uchiyama, T., Kagami, O., Shizuri, Y., & Misawa, N. (2005). Isolation and functional analysis of cytochrome P450 CYP153A genes from various environments. Bioscience, Biotechnology, and Biochemistry, 69(12), 2421–2430.
Lessner, D. J., Johnson, G. R., Parales, R. E., Spain, J. C., & Gibson, D. T. (2002). Molecular characterization and substrate specificity of nitrobenzene dioxygenase from Comamonas sp. strain JS765. Applied and Environmental Microbiology, 68(2), 634–641.
Marvin-Sikkema, F. D. & de Bont, J. A. M. (1994). Degradation of nitroaromatic compounds by microorganisms. Applied Microbiology and Biotechnology, 42(4), 499–507.
Mercier, C., Chalansonnet, V., Orenge, S., & Gilbert, C. (2013). Characteristics of major Escherichia coli reductases involved in aerobic nitro and azo reduction. Journal of Applied Microbiology, 115(4), 1012–1022.
Mizoguchi, H., Sawano, Y., Kato, J. I., & Mori, H. (2008). Superpositioning of deletions promotes growth of Escherichia coli with a reduced genome. DNA Research, 15(5), 277–284.
Nadeau, L. J. & Spain, J. C. (1995). Bacterial degradation of nitrobenzoic acid. Applied and Environmental Microbiology, 61(2), 840–843.
Nishino, S. F. & Spain, J. C. (1993). Degradation of nitrobenzene by a Pseudomonas pseudoalcaligenes. Applied and Environmental Microbiology, 59(8), 2520–2525.
Nishino, S. F. & Spain, J. C. (1995). Oxidative pathway for the biodegradation of nitrobenzene by Comamonas sp. strain JS765. Applied and Environmental Microbiology, 61(6), 2308–2313.
Otomatsu, T., Bai, L., Fujita, N., Shindo, K., Shimizu, K., & Misawa, N. (2010). Bioconversion of aromatic compounds by Escherichia coli that expresses cytochrome P450 CYP153A13a gene isolated from an alkane-assimilating marine bacterium Alcanivorax borkumensis. Journal of Molecular Catalysis B: Enzymatic, 66(1-2), 234–240.
Parry, R., Nishino, S., & Spain, J. (2011). Naturally occurring nitro compounds. Natural Product Reports, 28, 1, 152–167.
Peres, C. M & Agathos, S. N. (2000). Biodegradation of nitroaromatic pollutants: From pathways to remediation. Biotechnology Annual Review, 6, 197–220.
Providenti, M. A., Shaye, R. E., Lynes, K. D., McKenna, N. T., O’Brien, J. M., Rosolen, S., Campbell Wyndham, R., & Lambert, I. B. (2006). The locus coding for the 3-nitrobenzoate dioxygenase of Comamonas sp. strain JS46 is flanked by IS1071 elements and is subject to deletion and inversion events. Applied and Environmental Microbiology, 72(4), 2651–2660.

Rau, J. & Stolz, A. (2003). Oxygen-insensitive nitroreductases NfsA and NfsB of Escherichia coli function under anaerobic conditions as lawsono-dependent azo reductases. Applied and Environmental Microbiology, 69(6), 3448–3455.

Saltzman, B. E. (1954). Colorimetric microdetermination of nitrogen dioxide in atmosphere. Analytical Chemistry, 26(12), 1949–1955.

Singh, D., Kumari, A., Ramaswamy, S., & Ramanathan, G. (2014). Expression, purification and substrate specificities of 3-nitrotoluene dioxygenase from Diaphorobacter sp. strain DS2. Biochemical and Biophysical Research Communications, 445(1), 36–42.

Wackett, L. P. (2002). Mechanism and applications of Rieske non-heme iron dioxygenases. Enzyme and Microbial Technology, 31(5), 577–587.

Wang, C. & Chen, H. (2017). Convergent theoretical prediction of reactive oxidant structures in diiron arylamine oxygenases AurF and CmlI: Peroxo or hydroperoxo? Journal of the American Chemical Society, 139(37), 13038–13046.

Winkler, R. & Hertweck, C. (2007). Biosynthesis of nitro compounds. ChemBioChem, 8(9), 973–977.

Wu, W., Liu, F., & Singh, S. (2018). Toward engineering E. coli with an autoregulatory system for lignin valorization. Proceedings of the National Academy of Sciences, 115(12), 2970–2975.

Zenno, S., Koike, H., Kumar, A. N., Jayaraman, R., Tanokura, M., & Saigo, K. (1996a). Biochemical characterization of NfsA, the Escherichia coli major nitroreductase exhibiting a high amino acid sequence homology to Frp, a Vibrio harveyi flavin oxidoreductase. Journal of Bacteriology, 178(15), 4508–4514.

Zenno, S., Koike, H., Tanokura, M., & Saigo, K. (1996b). Gene cloning, purification, and characterization of NfsB, a minor oxygen-insensitive nitroreductase from Escherichia coli, similar in biochemical properties to FRase I, the major flavin reductase in Vibrio fischeri. Journal of Biochemistry, 120(4), 736–744.