Characterization of the anthranilate degradation pathway in *Geobacillus thermodenitrificans* NG80-2

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Anthranilate is an important intermediate of tryptophan metabolism. In this study, a hydroxylase system consisting of an FADH₂-utilizing monooxygenase (GTNG_3160) and an FAD reductase (GTNG_3158), as well as a bifunctional riboflavin kinase/FMN adenylyltransferase (GTNG_3159), encoded in the anthranilate degradation gene cluster in *Geobacillus thermodenitrificans* NG80-2 were functionally characterized *in vitro*. GTNG_3159 produces FAD to be reduced by GTNG_3158 and the reduced FAD (FADH₂) is utilized by GTNG_3160 to convert anthranilate to 3-hydroxyanthranilate (3-HAA), which is further degraded to acetyl-CoA through a *meta*-cleavage pathway also encoded in the gene cluster. Utilization of this pathway for the degradation of anthranilate and tryptophan by NG80-2 under physiological conditions was confirmed by real-time RT-PCR analysis of representative genes. This is believed to be the first time that the degradation pathway of anthranilate via 3-HAA has been characterized in a bacterium. This pathway is likely to play an important role in the survival of *G. thermodenitrificans* in the oil reservoir conditions from which strain NG80-2 was isolated.

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INTRODUCTION

Anthranilate is an important intermediate of tryptophan metabolism in both eukaryotes and prokaryotes (Hayaishi & Stanier, 1951; Yanofsky, 1956). It is also an intermediate of the biodegradation of many other aromatic compounds, such as 2-nitrobenzoate (2-NBA) (Muraki *et al.*, 2003), quinaldine (Fetzner, 2000), carbazole (Nojiri *et al.*, 2001) and indole (Kamath & Vaidyanathan, 1990). All known anthranilate degradation pathways in bacteria involve a dioxygenase attack to convert anthranilate to catechol (Chang *et al.*, 2003). Early studies in animals and plants showed that anthranilate may be converted to 3-hydroxy-

anthranilate (Jequier *et al.*, 1969; Nair & Vaidyanathan, 1965) by anthranilate hydroxylase, but the enzyme has never been characterized in bacteria.

Examples of different types of known monooxygenases are (i) single-component NAD(P)H-dependent flavoproteins, such as *p*-hydroxybenzoate hydroxylase from *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* (Entsch *et al.*, 2005) and 1-naphthol-2-hydroxylase from *Pseudomonas* sp. strain C4 (Swetha *et al.*, 2007); (ii) two-component enzymes consisting of a flavin reductase and an FADH₂- and/or FMNH₂-utilizing monooxygenase such as *p*-hydroxyphenylacetate-3-hydroxylase from *Escherichia coli* W (Louie *et al.*, 2003), alkanesulfonate monooxygenase from *E. coli* MC4100 (Eichhorn *et al.*, 1999) and *p*-hydroxyphenylacetate-3-hydroxylase from *Acinetobacter baumannii* (Chaiyen *et al.*, 2001); (iii) three-component enzymes consisting of a ferredoxin, a ferredoxin reductase and a terminal oxygenase, such as salicylate oxygenase from *Sphingomonas yanoikuyae* B1 (Cho *et al.*, 2005) and salicylate 1-hydroxylase from *Sphingomonas* sp. strain

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Abbreviation: CE, capillary electrophoresis (abbreviations for chemicals and enzymes are defined in the legend of Fig. 3).

A supplementary figure showing the results of the real-time RT-PCR analysis is available with the online version of this paper.

CHY-1 (Jouanneau *et al.*, 2007); and (iv) multicomponent toluene monooxygenases (Friemann *et al.*, 2009; Notomista *et al.*, 2009).

Geobacillus thermodenitrificans NG80-2 was isolated from a deep-subsurface oil reservoir in Northern China (Wang *et al.*, 2006). In our previous study to sequence the whole genome of NG80-2 (Feng *et al.*, 2007), a putative anthranilate degradation gene cluster (*GTNG_3150*–*GTNG_3164*) encoding proteins for the degradation of anthranilate via a 3-hydroxyanthranilate (3-HAA) *meta*-cleavage pathway was found adjacent to the tryptophan degradation gene cluster (*GTNG_3165*–*GTNG_3169*). *GTNG_3158*, *GTNG_3159* and *GTNG_3160* show 39, 32 and 43 % identity to the NADH-dependent FMN reductase (SsuE) from *E. coli* MC4100 (accession no. P80644), the FAD synthetase (RibF) from *Corynebacterium ammoniagenes* (accession no. Q59263) and the 4-hydroxyphenylacetate-3-hydroxylase oxygenase component (HpaB) from *Thermus thermophilus* HB8 (accession no. 2YYG_A), respectively. *GTNG_3158* and *GTNG_3160* are proposed to constitute a hydroxylase system for the conversion of anthranilate to 3-HAA, and *GTNG_3159* to produce FAD as the cofactor for the hydroxylase system. *GTNG_3150*–*GTNG_3157*, showing 23–55 % identity to Nba proteins, are predicted to be 3-HAA *meta*-cleavage pathway enzymes based on the previous study on the homologous proteins in *P. fluorescens* KU-7 (Muraki *et al.*, 2003).

In this study, *GTNG_3158* as an FAD reductase, *GTNG_3159* as a bifunctional riboflavin kinase/FMN adenylyltransferase and *GTNG_3160* as an FADH₂-utilizing monooxygenase were characterized *in vitro*. The *GTNG_3158* and *GTNG_3160* proteins were shown to constitute a hydroxylase system to convert anthranilate to 3-HAA in the initial reaction of the degradation pathway. The functions of two key enzymes in the 3-HAA *meta*-cleavage pathway, NbaC for the conversion of 3-HAA to 2-amino-3-carboxymuconate-6-semialdehyde (ACMS) and NbaD for the conversion of ACMS to 2-aminomuconate-6-semialdehyde (2-AMS), were also confirmed. The physiological role of the anthranilate degradation pathway in NG80-2 was investigated by mRNA analysis of the genes encoding the key pathway enzymes.

METHODS

Materials. Restriction enzymes, rTaq DNA polymerase and the PrimeScript RT-PCR kit were purchased from TaKaRa. T4 DNA ligase was from Promega, and DNase I from Roche. PMSF, FMN, FAD, anthranilate and 3-HAA were purchased from Sigma-Aldrich. The Chelating Sepharose Fast Flow column and LMW-SDS Marker kit were purchased from GE Healthcare. SYBR Green PCR master mix was purchased from Applied Biosystems. Other chemicals and reagents were from Shanghai Sangon.

Bacterial strains and growth conditions. *G. thermodenitrificans* NG80-2 and *E. coli* BL21(DE3) (Novagen) were grown in Luria-Bertani (LB) medium at 60 and 37 °C, respectively, with shaking. When necessary, 50 mg kanamycin l⁻¹ was added to the medium.

Real-time RT-PCR. NG80-2 cells were grown in a mineral medium (Wang *et al.*, 2006) supplemented with 1 % (w/v) sucrose, anthranilate or tryptophan as the sole carbon source at 60 °C with shaking for 12 h, and the pellet was collected by centrifugation. Total RNA was extracted with Trizol reagent according to the manufacturer's protocol. RNA (500 ng) was denatured at 80 °C for 5 min and reverse transcribed at 42 °C for 20 min using the PrimeScript RT-PCR kit. Real-time PCR for quantification of cDNA was performed using the SYBR Green PCR master mix and the ABI 7300 Real-time PCR System according to the manufacturer's instructions. PCR was performed as follows: 50 °C for 2 min; 95 °C for 10 min; 95 °C for 15 s and 60 °C for 30 s, 40 cycles. 16S RNA was used as an internal control for normalization. Primer pairs used were 5'-AGACAC-GGCCCAGACTCCTAC-3'/5'-CTCCTTCGTCCCTCACAACAG-3' for 16S RNA, 5'-CGTATCCGTCGTGACTGC-3'/5'-ACAAACCC-CATCACTTTGTGC-3' for *GTNG_3158*, 5'-GCATGTTGACGACCTTGACG-3'/5'-GGTTCAAAGCGGTTCAGTTCG-3' for *GTNG_3159*, 5'-TGCTTGAGACGATTCGTGGTC-3'/5'-CTCCATGTCTGCCGCT-AGTTC-3' for *GTNG_3160*, 5'-AACCGATCAATCTGTGGAAGTTC-3'/5'-AAGTCACGCCGTTTGTAGGAC-3' for *nbaC*, 5'-GGAATG-TGCCACTGTTTGTCC-3'/5'-GCCTCCAAGGATCAGGCTG-3' for *nbaD*. The average cycle number at the threshold (C_T) was normalized against 16S RNA. The fold change of expression level of each gene was calculated using the comparative C_T method (User's Manual for ABI 7300 Real-time PCR System) based on the change in the cycle numbers between the anthranilate or tryptophan and sucrose samples.

Construction of recombinant plasmids. Genomic DNA from *G. thermodenitrificans* NG80-2 was extracted as described previously (Wang *et al.*, 2006). *GTNG_3158*, *GTNG_3159*, *GTNG_3160*, *nbaC* and *nbaD* were amplified by PCR using the primer pairs 5'-CCGGAATTCGTGAAGTTGTAGGTATCTC-3'/5'-CCCAAGCTT-TCAACGATGGGCATGGACAC-3', 5'-GGAATTCATATGAAGGTG-CATGAGGCAAATCA-3'/5'-CCCAAGCTTTACGAGCTAAGCGGCC-ACC-3', 5'-GGAATTCATATGAGGATGGGGATCCGCACAGG-3'/5'-CCCAAGCTTTTCAGGCATTACAACTTTCT-3', 5'-AGAGCCATGGT-TACGAAGAGTATCCAAAACCG-3'/5'-GGAAGCTTCTCGCATTT-CCATTCACCTCGCTTC-3', and 5'-CGAGCATATGGAATGCGAGTA-GACTTCCACACC-3'/5'-GCAAAGCTTTTTCCTACTCACCTTTAC-CGGAC-3', respectively (restriction sites are underlined). PCR was performed as follows: 95 °C for 5 min; 95 °C for 30 s, 56 °C for 45 s and 72 °C for 2 min, 25 cycles; and 72 °C for 5 min. Amplified products were cloned into the *EcoRI/HindIII* (*GTNG_3158*) or the *NdeI/HindIII* (*GTNG_3159* or *GTNG_3160*) site of pET-28a (Novagen), the *NcoI/HindIII* (*nbaC*) site of pET30a (Novagen), or the *NdeI/HindIII* (*nbaD*) site of pET41a (Novagen) to construct plasmid pLW1318 (containing *GTNG_3158*), pLW1279 (containing *GTNG_3159*), pLW1259 (containing *GTNG_3160*), pLW1081 (containing *nbaC*) or pLW1082 (containing *nbaD*). The presence of inserts in the plasmids was confirmed by sequencing using an ABI3730 automated DNA sequencer at Tianjin Biochip Corporation.

Expression and purification of recombinant *GTNG_3158*, *GTNG_3159* and *GTNG_3160*. *E. coli* BL21(DE3) carrying plasmid pLW1318, pLW1279 or pLW1259 was grown in LB supplemented with 50 mg kanamycin l⁻¹ to an OD₆₀₀ of 0.6. Expression of protein was induced by 0.01 mM IPTG at 45 °C for 2.5 h (*GTNG_3158*), or at 37 °C for 3 h (*GTNG_3159* and *GTNG_3160*).

Protein purification was carried out at 4 °C using the following procedure. *E. coli* cells were harvested by centrifugation at 10 000 g for 10 min, washed with binding buffer (20 mM potassium phosphate, pH 7.6, 100 mM NaCl, 10 mM imidazole), and then resuspended in the same buffer supplemented with PMSF (1 mM) and lysozyme (1 g l⁻¹). The resuspended cells were disrupted by sonication (UP200s Ultraschallprozessor, Dr Hielscher, 0.5 cycle, 90 % amplitude). After treatment with RNase A (10 mg l⁻¹) and DNase I (5 mg

l^{-1}), the lysate was incubated on ice for 15 min, and the cell debris was removed by centrifugation at 18 000 g for 30 min. The crude extract was applied to a Chelating Sepharose Fast Flow column according to the manufacturer's instructions. Unbound proteins were washed out with wash buffer (20 mM potassium phosphate, pH 7.6, 100 mM NaCl, 20 mM imidazole). Fusion proteins were eluted with elution buffer (20 mM potassium phosphate, pH 7.6, 100 mM NaCl, 250 mM imidazole) and dialysed against 20 mM potassium phosphate (pH 7.6) containing 20 % glycerol.

Expression and preparation of crude extracts for NbaC and NbaD activity assays. *E. coli* BL21(DE3) carrying plasmid pLW1081 or pLW1082 was grown in LB containing 50 mg kanamycin l^{-1} , with shaking, to an OD_{600} of 0.6. Expression of NbaC and NbaD was induced by 0.05 mM IPTG at 37 °C for 3 h. Cells were harvested by centrifugation (10 000 g for 10 min), washed twice with 50 mM MOPS buffer (pH 6.5) for *E. coli* BL21(DE3) carrying pLW1081 or with 50 mM potassium phosphate buffer (pH 7.0) for *E. coli* BL21(DE3) carrying pLW1082, and resuspended in the same buffer at a concentration of approximately 0.2 g (wet wt) ml^{-1} . Cells were disrupted by sonication and particulates were removed by centrifugation (18 000 g for 30 min at 4 °C).

Protein concentration was determined by the Bradford method (Bradford, 1976). SDS-PAGE and native-PAGE were performed using the methods of Laemmli (1970) and Tulchin *et al.* (1976), respectively.

Riboflavin kinase/FMN adenylyltransferase activity assay. FMN adenylyltransferase activity was assayed by measuring FAD produced, using capillary electrophoresis (CE). The standard reaction mixture (50 μ l) contained 3 mM ATP, 1 mM FMN, 5 mM $MgCl_2$ in 20 mM glycine/NaOH (pH 9) and an appropriate amount of purified GTNG_3159 protein. Reactions were initiated by adding the enzyme, and reaction mixtures were incubated at 60 °C for 10–20 min before being terminated by incubation on ice. Reaction products were analysed by CE. One unit of FMN adenylyltransferase was defined as the amount of GTNG_3159 required to produce 1 nmol FAD min^{-1} . Riboflavin kinase activity was assayed using the same procedure except for the use of 1 mM riboflavin instead of FMN as the substrate.

FAD reductase activity assay. FAD reductase activity was assayed by determining NAD(P)H oxidized spectrophotometrically at 340 nm (Agarwal *et al.*, 2006), using a UV-2550 UV-visible spectrophotometer (Shimadzu). The standard reaction mixture (1 ml) contained 100 μ M FMN or FAD, 150 μ M NAD(P)H in 20 mM glycine/NaOH (pH 9) and an appropriate amount of purified GTNG_3158 protein. Reactions were initiated by adding NAD(P)H, and reaction mixtures were incubated at 60 °C for 10–20 min before being terminated by incubation on ice. One unit of enzyme was defined as the amount of enzyme that catalyses the consumption of 1 μ mol NAD(P)H ($\epsilon_{340}=6220 M^{-1} cm^{-1}$) min^{-1} .

Anthranilate hydroxylase activity assay. Anthranilate hydroxylase activity was assayed by determining 3-HAA produced, using CE. The standard reaction mixture (50 μ l) contained 2 mM anthranilate in 20 mM glycine/NaOH (pH 9), 10 μ l $FMNH_2$ or $FADH_2$ solution (prepared separately), and an appropriate amount of purified GTNG_3160 protein. Reactions were initiated by adding GTNG_3160, and reaction mixtures were incubated at 60 °C for 10–20 min before being terminated by incubation on ice. Reaction products were analysed by CE. To generate $FMNH_2$ or $FADH_2$, a mixture (200 μ l) consisting of 1 mM FAD or FMN, 2 mM NADH in 20 mM glycine/NaOH (pH 9), and an appropriate amount of purified GTNG_3158 protein was incubated at 60 °C, and production of reduced flavin was determined by the increase in A_{340} due to the consumption of NADH.

Anthranilate hydroxylase activity was also determined by performing GTNG_3158, GTNG_3159 and GTNG_3160 reactions simultaneously in a single reaction mixture (50 μ l) containing 2 mM anthranilate, 5 mM $MgCl_2$, 1 mM FMN, 3 mM ATP, 2 mM NADH in 20 mM glycine/NaOH (pH 9) and appropriate amounts of GTNG_3158, GTNG_3159 and GTNG_3160 at 60 °C.

Determination of the substrate specificity and optimum temperature and pH for GTNG_3160 activity. GTNG_3160 activities on anthranilate, 2-hydroxyphenylacetate, 4-hydroxyphenylacetate, salicylate, kynurenine, phenol, 2-NBA, chlorobenzene, naphthalene, naphthol, toluene and ethylbenzene were also assayed in the presence of GTNG_3158 by measuring the decrease in A_{340} due to the consumption of NADH by the hydroxylase system. To determine the temperature optimum, the activity was measured in the standard reaction mixture at pH 9.0 at temperatures ranging from 25 to 100 °C. To determine the pH optimum, the activity was measured at 60 °C at pH ranging from 3.2 to 11.0, using appropriate buffers: citrate/sodium citrate (pH 3.2–5.8), KH_2PO_4/K_2HPO_4 (pH 5.8–8.2), Tris/HCl (pH 8.2–8.4) or glycine/NaOH (pH 8.4–11.0).

Capillary electrophoresis. CE was performed using a Beckman Coulter P/ACE MDQ Capillary Electrophoresis System with a photoelectricity diode array (PDA) detector. The capillary was bare silica (inside diameter, 75 μ m, 57 cm long, with the detector at 50 cm) and conditioned before each run by washing first with 0.1 M NaOH, then with deionized water, and finally with 25 mM borate/sodium hydroxide, pH 10.0 (used as the mobile phase), for 2 min each time. Samples were loaded by pressure injection at 0.5 p.s.i. (3.45 kPa) for 10 s, and separation was carried out at 20 kV. Peak integration and trace alignments were performed using Beckman P/ACE Station software (32 Karat version 5.0). Conversion ratios were calculated by comparing the peak areas of the substrate and product.

HAO activity. 3-Hydroxyanthranilate 3,4-dioxygenase (HAO) activity was measured spectrophotometrically by monitoring the formation of ACMS at 360 nm (Muraki *et al.*, 2003). The assay mixture (1 ml) contained 0.2 mM 3-HAA, 0.1 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 50 mM MOPS buffer (pH 6.5) and an appropriate amount of the cell extract of *E. coli* BL21(DE3) carrying pLW1081. The reaction was started by adding 3-HAA and monitoring the increase in A_{360} for 20 s at 55 °C. One unit was defined as the amount of enzyme that produced 1 μ mol ACMS ($\epsilon_{360}=47500 M^{-1} cm^{-1}$) min^{-1} . The rate of increase in absorbance caused by NbaC was calculated by subtracting the value for the control reaction mixture with cell extract of *E. coli* BL21(DE3) carrying pET30a.

ACMSD activity. ACMS decarboxylase (ACMSD) activity was determined as described by Muraki *et al.* (2003), by measuring the conversion of ACMS to 2-AMS using a pre-assay mixture that consisted of 0.2 mM 3-HAA, 0.1 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, and an appropriate amount of cell extract of *E. coli* BL21(DE3) carrying pLW1081 prepared as described above in 50 mM MOPS buffer (pH 6.5). The reaction mixture was incubated at 55 °C, and the increase in A_{360} due to the formation of ACMS from 3-HAA was monitored. After the reaction was completed, an appropriate amount of the cell extract of *E. coli* BL21(DE3) carrying pLW1082 was added, and the decrease in A_{360} was monitored at 20 s intervals. One unit was defined as the amount of enzyme that converted 1 μ mol ACMS min^{-1} . The rate of decrease in absorbance caused by NbaD was calculated by subtracting the value for the control reaction mixture with the cell extract of *E. coli* BL21(DE3) carrying pET41a.

Bioinformatics methods. Homologous genes were found by NCBI BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST/>).

RESULTS

Protein expression and purification of GTNG_3158, GTNG_3159 and GTNG_3160

GTNG_3158 (FAD reductase), GTNG_3159 (riboflavin kinase/FMN adenylyltransferase) and GTNG_3160 (FADH₂-utilizing monooxygenase) were expressed in *E. coli* BL21(DE3) and purified by nickel ion affinity chromatography. The recombinant GTNG_3158 as prepared was colourless and showed no typical absorption spectrum of flavin-containing flavoproteins (data not shown), indicating no flavin prosthetic groups bound to the protein.

Characterization of GTNG_3159 as a bifunctional riboflavin kinase/FMN adenylyltransferase

In the reaction catalysed by GTNG_3159 using either FMN or riboflavin as the substrate, FAD was detected as the sole product by CE analysis (data not shown). Higher activity was obtained with FMN (270.3 ± 18.2 U mg⁻¹) than with riboflavin (170.09 ± 11.34 U mg⁻¹). The absence of FMN intermediate when riboflavin was used as the substrate indicates that GTNG_3159 possesses the activities of both riboflavin kinase for the conversion of riboflavin to FMN and FMN adenylyltransferase for the conversion of FMN to FAD, and the activity for the latter reaction is higher.

Characterization of GTNG_3158 as an FAD/FMN reductase

Both FAD and FMN could be reduced by GTNG_3158. Higher activity was obtained with FAD (58.2 ± 0.382 U mg⁻¹) than with FMN (55.47 ± 0.19 U mg⁻¹) using NADPH as the cofactor. Using FAD as specific substrate, NADH could also be used as electron donor, with 95% activity relative to NADPH.

Characterization of GTNG_3160 as an FADH₂-utilizing monooxygenase

In the reaction catalysed by GTNG_3160 when FADH₂ (produced by the GTNG_3158 reaction) was added,

Fig. 1. CE chromatographs of anthranilate standard (a), 3-HAA standard (b), and the reaction catalysed by GTNG_3160 in the presence of FADH₂ and anthranilate (c).

anthranilate was oxidized to 3-HAA as determined by CE analysis (Fig. 1). When FADH₂ was replaced by FMNH₂, no products were detected (data not shown). Therefore, GTNG_3160 is an FADH₂-utilizing monooxygenase. Anthranilate could also be oxidized to 3-HAA by GTNG_3158 and GTNG_3160 in the presence of FAD, or by GTNG_3158, GTNG_3159 and GTNG_3160 in the presence of FMN and ATP (data not shown). Anthranilate could not be oxidized by GTNG_3160 alone or combined with GTNG_3158 in the presence of FMN (data not shown).

Substrate specificity, temperature and pH optimum of GTNG_3160

GTNG_3160 showed the highest activity towards anthranilate (151.28 ± 11.28 U mg⁻¹), and could also utilize several other aromatic compounds, including 2-hydroxyphenylacetate (62.79% relative activity), 4-hydroxyphenylacetate (42.7% relative activity) and salicylate (49.6% relative activity) as indicated by the consumption of NADH by the hydroxylase system. It could not utilize kynurenine, phenol, 2-NBA, chlorobenzene, naphthalene, naphthol, toluene or ethylbenzene. In our assay system,

Fig. 2. Effects of temperature (a) and pH (b) on the activity of GTNG_3160.

Fig. 3. (a) Organization of the anthranilate degradation gene cluster in *G. thermodenitrificans* NG80-2. Genes and predicted ORFs are indicated by arrows, and the arrowheads indicate the directions of transcription. (b) Degradation pathways of tryptophan and anthranilate in *G. thermodenitrificans* NG80-2. Anthranilate catechol *ortho*-cleavage pathway reported in other bacteria and 2-NBA degradation pathway in *P. fluorescens* KU-7 are also shown. Abbreviations: ACMS, 2-amino-3-carboxymuconate-6-semialdehyde; 2-AM, 2-aminomuconate; 2-AMS, 2-aminomuconate 6-semialdehyde; 3-HAA, 3-hydroxyanthranilate; 2-HABA, 2-hydroxylaminobenzoate; HO, 4-hydroxy-2-oxovalerate; KFA, kynurenine formamidase; KYN, kynureninase; 2-NBA, 2-nitrobenzoate; NbaC, 3-hydroxyanthranilate 3,4-dioxygenase; NbaD, ACMS decarboxylase; NbaE, 2-AMS dehydrogenase; NbaF, 2-aminomuconate deaminase; NbaG, 4-oxalocrotonate decarboxylase; NbaH, 2-oxopent-4-dienoate hydratase; NbaI, 4-hydroxy-2-oxovalerate aldolase; NbaJ, acylating acetaldehyde dehydrogenase; NbaA, 2-nitrobenzoate nitroreductase; NbaB, 2-hydroxylaminobenzoate mutase; OC, 4-oxalocrotonate; OE, 2-oxopent-4-dienoate; TDO, tryptophan 2,3-dioxygenase.

GTNG_3160 was active at temperatures ranging from 25 to 70 °C (Fig. 2a), and at pH values ranging from pH 5 to 11 (Fig. 2b), with maximal activity detected at 60 °C, pH 9.

HAO and ACMSD activity

HAO activity in the crude extract of *E. coli* BL21(DE3) carrying pLW1081 (containing *nbaC*) was determined to be 133.4 U mg⁻¹. ACMSD activity in the cell extract of *E. coli* BL21(DE3) carrying pLW1082 (containing *nbaD*) was determined to be 33.4 U mg⁻¹. No activity of HAO or ACMSD was observed in the crude extract from *E. coli* BL21(DE3) carrying pET30a or pET41a.

Real-time RT-PCR analysis of *nbaC*, *nbaD*, *GTNG_3158*, *GTNG_3159* and *GTNG_3160*

The transcription levels of *nbaC*, *nbaD*, *GTNG_3158*, *GTNG_3159* and *GTNG_3160* increased 2695–4575-fold when anthranilate was used as the sole carbon source instead of sucrose for the growth of NG80-2, and 2281–3573-fold when tryptophan was used (see Supplementary Fig. S1, available with the online version of this paper). The result indicates the utilization of the 3-HAA *meta*-cleavage pathway for the degradation of anthranilate and the involvement of this pathway in the degradation of tryptophan in NG80-2 under physiological conditions. The ability of NG80-2 to utilize anthranilate and tryptophan as the sole carbon source for growth was confirmed experimentally (data not shown).

DISCUSSION

To our knowledge, this is the first time that the degradation pathway of anthranilate via 3-HAA has been characterized in a bacterium (Fig. 3). The physiological role of this pathway in the degradation of anthranilate and tryptophan was confirmed by real-time RT-PCR analysis. The 3-HAA *meta*-cleavage pathway in *P. fluorescens* KU-7 is utilized for the degradation of 2-NBA, and two additional genes *nbaAB* (encoding 2-NBA nitroreductase and 2-hydroxylaminobenzoate mutase respectively) located outside the *nba* gene cluster are responsible for the conversion of 2-NBA to 3-HAA (Muraki *et al.*, 2003). NG80-2 contains no *nbaAB* genes and is unable to utilize 2-NBA. No genes encoding anthranilate dioxygenase for the conversion of anthranilate to catechol were found in NG80-2. Therefore, anthranilate is unlikely to be degraded via the catechol *ortho*-cleavage pathway in NG80-2 as reported in other bacteria.

It is likely that the anthranilate degradation gene cluster (*GTNG_3150*–*GTNG_3164*) for the conversion of anthranilate to acetyl-CoA and the adjacent tryptophan degradation gene cluster (*GTNG_3165*–*GTNG_3169*) for the conversion of tryptophan to anthranilate evolved as a single gene cluster, while tryptophan might be an important energy source for NG80-2. The opposite

transcription directions and separate regulators in the present gene clusters indicate that they function independently of each other. This would be advantageous for the survival of *G. thermodenitrificans* under oil reservoir conditions, where aromatic compounds are abundant, and the anthranilate degradation pathway may be utilized as a central downstream metabolic pathway for the degradation of those compounds.

The presence of a riboflavin kinase/FMN adenylyltransferase gene in the anthranilate degradation gene cluster is unexpected, as NG80-2 contains a second riboflavin kinase/FMN adenylyltransferase gene for the synthesis of FAD (*GTNG_1121*, 35 % identity to *GTNG_3159*). In contrast, other reported FADH₂-utilizing monooxygenases only have a co-transcribed FAD reductase to form two-component systems. Presumably, the cellular FAD level is tightly regulated. Therefore, to have a co-transcribed FAD synthetase allows FAD to be readily produced to be reduced by *GTNG_3158*, which uses the flavin as a co-substrate rather than a cofactor. Co-transcription of *GTNG_3158*, *GTNG_3159* and *GTNG_3160* was confirmed by the real-time RT-PCR analysis. Whether *GTNG_3159* is required for or only enhances the activity of *GTNG_3160* under physiological conditions needs to be further investigated.

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