AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

Novel Gene Encoding 5-Aminosalicylate 1,2-Dioxygenase from *Comamonas* sp. Strain QT12 and Catalytic Properties of the Purified Enzyme

Hao Yu,[a] Shuxue Zhao,[a] Lizhong Guo[a]

[a]Shandong Provincial Key Laboratory of Applied Mycology, College of Life Sciences, Qingdao Agricultural University, Qingdao, People's Republic of China

ABSTRACT The 1,125-bp *mabB* gene encoding 5-aminosalicylate (5ASA) 1,2-dioxygenase, a nonheme iron dioxygenase in the bicupin family that catalyzes the cleavage of the 5ASA aromatic ring to form *cis*-4-amino-6-carboxy-2-oxohexa-3,5-dienoate in the biodegradation of 3-aminobenzoate, was cloned from *Comamonas* sp. strain QT12 and characterized. The deduced amino acid sequence of the enzyme has low sequence identity with that of other reported ring-cleaving dioxygenases. MabB was heterologously expressed in *Escherichia coli* cells and purified as a His-tagged enzyme. The optimum pH and temperature for MabB are 8.0 and 10°C, respectively. Fe^{II} is required for the catalytic activity of the purified enzyme. The apparent K_m and V_{max} values of MabB for 5ASA are 52.0 ± 5.6 μM and 850 ± 33.2 U/mg, respectively. The two oxygen atoms incorporated into the product of the MabB-catalyzed reaction are both from the dioxygen molecule. Both 5ASA and gentisate could be converted by MabB; however, the catalytic efficiency of MabB for 5ASA was much higher (~70-fold) than that for gentisate. The *mabB*-disrupted mutant lost the ability to grow on 3-aminobenzoate, and *mabB* expression was higher when strain QT12 was cultivated in the presence of 3-aminobenzoate. Thus, 5ASA is the physiological substrate of MabB.

IMPORTANCE For several decades, 5-aminosalicylate (5ASA) has been advocated as the drug mesalazine to treat human inflammatory bowel disease and considered the key intermediate in the xenobiotic degradation of many aromatic organic pollutants. 5ASA biotransformation research will help us elucidate the microbial degradation of these pollutants. Most studies have reported that gentisate 1,2-dioxygenases (GDOs) can convert 5ASA with significantly high activity; however, the catalytic efficiency of these enzymes for gentisate is much higher than that for 5ASA. This study showed that MabB can convert 5ASA to *cis*-4-amino-6-carboxy-2-oxohexa-3,5-dienoate, incorporating two oxygen atoms from the dioxygen molecule into the product. Unlike GDOs, MabB uses 5ASA instead of gentisate as the primary substrate. *mabB* is the first reported 5-aminosalicylate 1,2-dioxygenase gene.

KEYWORDS 5-aminosalicylate, dioxygenase, 3-aminobenzoate, *Comamonas*, biodegradation

Ring-cleaving dioxygenases, catalyzing the cleavage of the C—C bond of the benzene ring, play an important role in the aerobic microbial degradation of aromatic compounds (1–4). The ring cleavage reaction is the last catalytic step in the conversion of the aromatic substrate to a nonaromatic product. The substrates for these enzymes are first activated by the introduction of substituents, such as the hydroxyl group in most cases. Ring-cleaving dioxygenases are classified as two groups, namely, the intradiol dioxygenases (type I), which cleave the ring between two adjacent hydroxylated carbons, and extradiol dioxygenases (type II and III), which cleave the ring

Received 20 June 2017 **Accepted** 5 October 2017

Accepted manuscript posted online 16 October 2017

Citation Yu H, Zhao S, Guo L. 2018. Novel gene encoding 5-aminosalicylate 1,2-dioxygenase from *Comamonas* sp. strain QT12 and catalytic properties of the purified enzyme. J Bacteriol 200:e00395-17. https://doi.org/10.1128/JB.00395-17.

Editor Anke Becker, Philipps-Universität Marburg

Address correspondence to Hao Yu, yuhaosunshine@163.com, or Lizhong Guo, glz119@126.com.

between one hydroxylated carbon and its adjacent nonhydroxylated carbon (1, 2). Gentisate 1,2-dioxygenases (GDOs) as well as salicylate 1,2-dioxygenase (SDO), a special GDO that can use salicylate as the substrate, are the most investigated extradiol dioxygenases (type III) that cleave the aromatic ring between the hydroxylated carbon and the adjacent carboxylated carbon (1, 5–11). All reported GDOs belong to the ferrous iron-dependent bicupin family of dioxygenases with an extraordinary substrate spectrum; they have the ability to cleave salicylate and various substituted salicylates, such as 5-aminosalicylate (5ASA) (5, 6, 11).

For several decades, 5ASA, a monohydroxylated benzoate, has been used as the drug mesalazine to treat inflammatory bowel disease and is mainly eliminated by *N*-acetylation in mammals (12, 13). In bacteria, 5ASA is considered the key intermediate in the xenobiotic degradation of many aromatic organic pollutants (14–19). It is the breakdown product of azo dyes and substituted naphthalenesulfonates such as Mordant Yellow 3, Mordant Orange 1, and 6-aminonaphthalene-2-sulfonic acid (14, 16–18). In the pathway for aerobic microbial degradation of 3-aminobenzoate, 3-aminobenzoate is first converted to 5ASA, which is further oxidized by a dioxygenase, 5-aminosalicylate 1,2-dioxygenase, to a ring cleavage product (15). Thus, microbial transformation of 5ASA is essential for the detoxification of contaminated water or soils (20). However, little information is available about the microbial transformation of 5ASA. Stolz et al. reported the properties of a 5-aminosalicylate 1,2-dioxygenase, which may use 5ASA as its primary substrate, obtained from the 5ASA-degrading *Pseudomonas* sp. strain BN9 and showed that *cis*-4-amino-6-carboxy-2-oxohexa-3,5-dienoate (*cis*-ACOHDA) is the product of this enzyme (19, 21). However, 5-aminosalicylate 1,2-dioxygenase has not been studied in detail. Instead, the conversion of 5ASA into a ring cleavage product by other dioxygenases in the bicupin family, such as GDOs and SDOs, has been studied extensively. For these enzymes, 5ASA is considered the best alternate substrate. For some GDOs, the V_{max} value with 5ASA is ~63% of that with gentisate (5, 6, 22). With structure information, it is speculated that the Asp and/or Gln in the mechanism center stabilizes the deprotonation of the 5-hydroxy/5-amino groups (23, 24). Although 5ASA is converted by these enzymes with significantly higher V_{max} values than other substrates, the catalytic efficiencies (k_{cat}/K_m values) of these enzymes for 5ASA are much lower than those for gentisate. This information indicates that 5ASA is not the primary substrate for these enzymes.

A 5-aminosalicylate 1,2-dioxygenase has been reported before; however, the gene sequence is still unknown (19, 21). In the present study, a 5-aminosalicylate 1,2-dioxygenase gene, *mabB*, was identified to be involved in the degradation of 3-aminobenzoate in *Comamonas* sp. strain QT12. The catalytic properties of MabB were investigated.

RESULTS

Isolation and characterization of 3-aminobenzoate-degrading strain QT12. Strain QT12, isolated from activated sludge and characterized in our laboratory, can utilize 3-aminobenzoate as the sole source of carbon, nitrogen, and energy. Strain QT12 also grew with gentisate or 5ASA as the sole source of carbon, but it was not able to grow with phenanthrene, 1-hydroxy-2-naphthoate, or salicylate. The 16S rRNA sequence of strain QT12 exhibited nucleotide sequence similarity with the type strains of the *Comamonas* genus (Fig. 1). Therefore, QT12 should be classified under the *Comamonas* genus. Strain QT12 has been deposited at the China Center for Type Culture Collection (CCTCC) under the accession number AB2016309. High-performance liquid chromatography (HPLC) analysis of the products of 3-aminobenzoate degradation by resting cells of strain QT12 indicated that the concentrations of the new intermediate gradually increased with the decrease of 3-aminobenzoate (Fig. 2A). The new intermediate was identified as 5ASA based on the retention time (Fig. 2B) and the spectrum of standard compounds (data not shown). The absorbance at 350 nm increased when 250 μM 5ASA was mixed with 0.1 ml of the cellular extract of strain QT12 in a 1-ml reaction

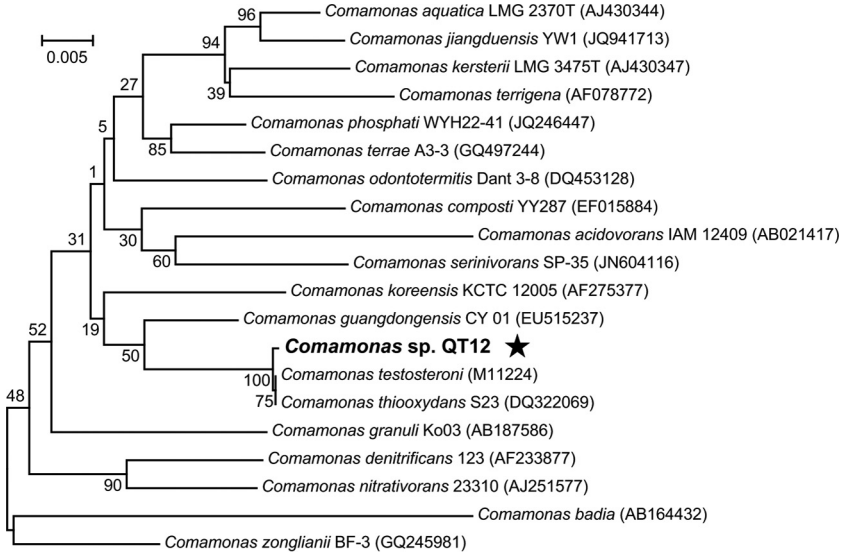

FIG 1 Phylogenetic tree of *Comamonas* sp. QT12 in relation to other type strains within the *Comamonas* family based on the 16S rRNA gene sequences. GenBank accession numbers are indicated in parentheses. Sequence alignment was performed using ClustalW, and phylogenetic inferences were made by MEGA 6.0 software using neighbor-joining methods. Bootstrap values (from 1,000 replicates) are shown at nodes. Bar, evolutionary distance of 5 replacements per 1,000 nucleotide positions.

mixture prepared in 20 mM Tris-HCl buffer (pH 8.0), thus revealing the 5-aminosalicylate 1,2-dioxygenase activity.

Identification of *mabB* gene. A 1,125-bp gene, designated *mabB*, was identified by mining the genome sequence of *Comamonas* sp. QT12. The GDOs and predicted proteins in strain QT12 were aligned using BLAST software. Among all proteins, a protein with two cupin motifs and ~30% amino acid sequence identity with GDOs was found in the genome of strain QT12. Then, several proteins homologous to MabB with

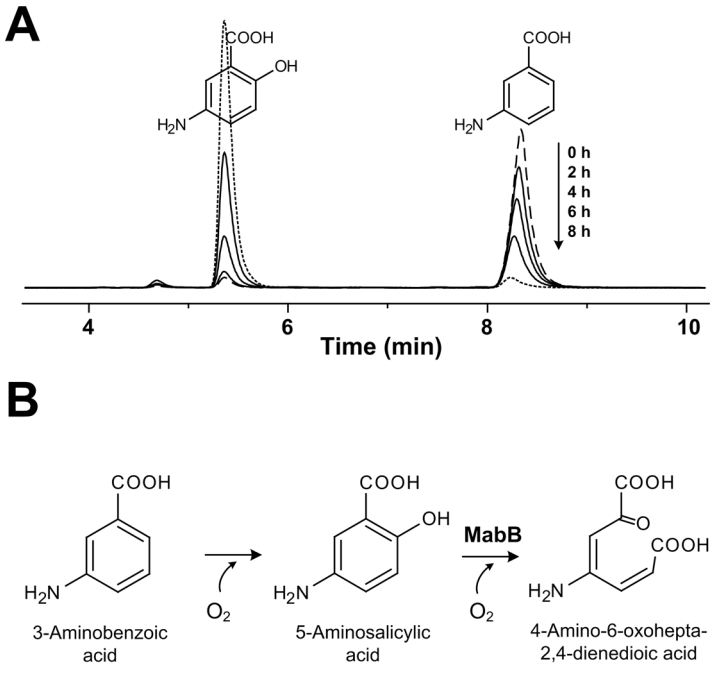

FIG 2 3-Aminobenzoate degradation in *Comamonas* sp. QT12. (A) HPLC analysis of 3-aminobenzoate biotransformation by resting cells of QT12. The signal was monitored at 210 nm. (B) Proposed pathway for the degradation of 3-aminobenzoate in strain QT12.

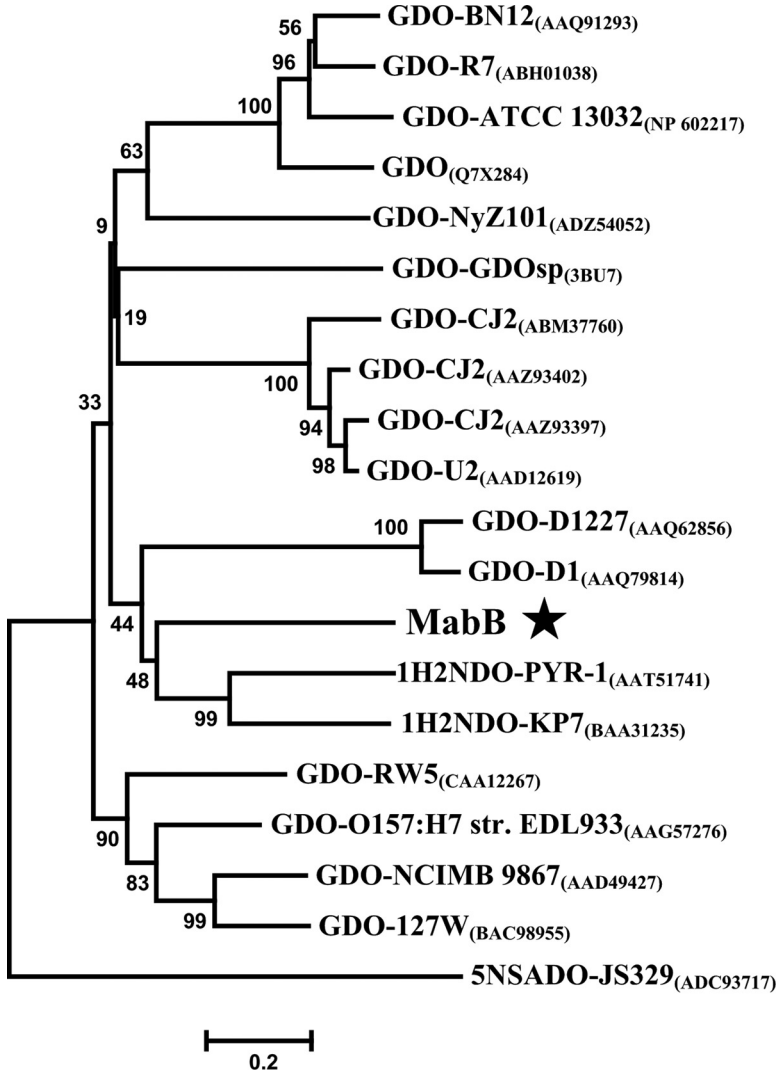

FIG 3 Phylogenetic analysis of MabB. The tree was constructed for MabB and several orthologous representative of dioxygenases of the bicupin family by using the neighbor-joining methods with a bootstrap of 1,000. The lengths of the lines are proportional to the genetic distance between proteins. The bar represents 0.2 amino acid substitution per site. 5NSADO, 5-nitrosalicylic acid 1,2-dioxygenase. GenBank accession numbers or protein identifications are listed at the end of each name.

known functions were identified by sequence alignment with a nonredundant protein sequence database by using BLAST software. Phylogenetic analysis of MabB and homologous proteins showed that MabB exhibits a maximum sequence identity, which is only ~36.1%, with 1-hydroxy-2-naphthoic acid dioxygenase (1H2NDO) from *Mycobacterium vanbaalenii* strain PYR-1 among the selected dioxygenases (Fig. 3).

Genes involved in catabolism of aromatic compounds are usually upregulated when the substrate is present in the growth medium (25). To prove the association between 3-aminobenzoate degradation and *mabB*, the mRNA levels of *mabB* in the presence or absence of 3-aminobenzoate were studied using reverse transcription-PCR (RT-PCR) and reverse transcription-quantitative PCR (RT-qPCR). The transcription level of *mabB* increased by ~6.7-fold in the presence of 3-aminobenzoate compared with that in the absence of 3-aminobenzoate (Fig. 4A and B). Thus, *mabB*, induced by 3-aminobenzoate or its degradation intermediates, plays an important physiological role in 3-aminobenzoate degradation in *Comamonas* sp. strain QT12.

Function of *mabB* on 3-aminobenzoate degradation in QT12. To confirm that *mabB* is essential for 3-aminobenzoate degradation in strain QT12, it was disrupted by

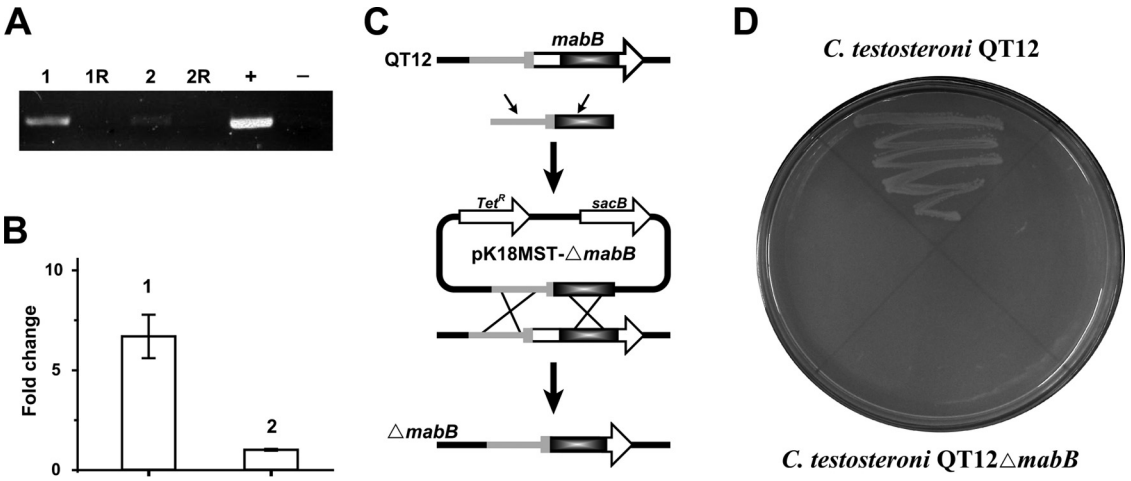

FIG 4 Identification of the *mabB* gene in *Comamonas* sp. QT12. (A) RT-PCR analysis of *mabB* expression with cDNA (lane 1) or RNA (lane 1R) from cells in the presence of 3-aminobenzoate, cDNA (lane 2) or RNA (lane 2R) from cells in the absence of 3-aminobenzoate, genomic DNA (lane +), and ddH_2O (lane −) as the templates. (B) The expression level of *mabB* in the presence (bar 1) or absence (bar 2) of 3-aminobenzoate. Each value is the mean from three parallel replicates ± SD. (C) Construction of *mabB*-disrupted mutant strain *Comamonas* sp. QT12 Δ*mabB*. (D) Strain QT12 and *mabB*-disrupted mutant grown on a solid MSM plate containing 3-aminobenzoate as the sole carbon and nitrogen source.

homologous recombination (Fig. 4C). The 3-aminobenzoate degradation ability of *Comamonas* sp. strain QT12 Δ*mabB* was investigated. It turned out that the *Comamonas* sp. QT12 Δ*mabB* strain could not grow with 3-aminobenzoate or 5ASA (Fig. 4D). 5ASA was found to have accumulated in the resting cell reaction by *Comamonas* sp. QT12 Δ*mabB* when 3-aminobenzoate was used as the substrate. The *mabB*-disrupted mutant therefore appeared to have lost the ability to degrade 5ASA. The results suggest that *mabB* is involved in 3-aminobenzoate degradation and is essential for the degradation of 5ASA by QT12.

***mabB* encodes 5-aminosalicylate 1,2-dioxygenase.** The *mabB* gene was cloned into the expression vector pET28a and the *N*-terminal His-tagged MabB was purified to ~95% homogeneity in *Escherichia coli* cells (Fig. 5A). SDS-PAGE of the purified MabB showed a major protein band with a molecular mass of 45 kDa. The conversion of 5ASA by MabB shifted the absorption maximum from 330 to 352 nm, along with a significant increase in the absorption maximum (Fig. 5B). These results clearly resembled the changes occurring in the UV-visible spectrum during the formation of *cis*-ACOHDA, as previously described by Stolz et al. (21).

Characterization of 5-aminosalicylate 1,2-dioxygenase. The optimum pH for purified MabB was found to be 8.0 when the activity was measured in 50 mM citrate-sodium citrate buffer (pH 5.0 to 6.0), potassium phosphate buffer (pH 6.0 to 9.0),

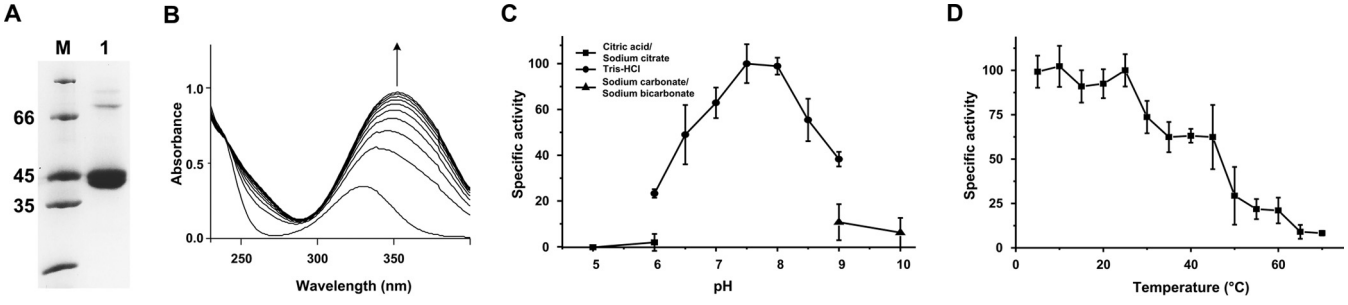

FIG 5 Characterization of MabB. (A) SDS-PAGE of purified MabB. Lanes: M, protein markers (in kilodaltons); 1, MabB. (B) Spectrophotometric changes during the transformation of 5ASA by MabB. The reactions were initiated by the addition of 100 μM 5ASA and MabB, and the spectra were recorded every 1 min. Arrows indicate the direction of spectral changes. (C) pH optimization of MabB. (D) Temperature-dependent enzyme activity of MabB. Each value is the mean from three parallel replicates ± SD.

TABLE 1 Effects of inhibitors on the 5ASA dioxygenase activity[a]

Inhibitor	Concn	Relative activity (%)
EDTA	1 mM	69.1 ± 8.2
	10 mM	27.8 ± 2.5
o-Phenanthroline	10 μM	2.3 ± 0.37
	100 μM	0
SDS	1 mM	0
Control		100

[a]MabB (0.3 μg/ml in reaction mixture) was incubated with different concentrations of inhibitors in 1.9 ml of 20 mM Tris-HCl buffer (pH 8.0) at 25°C for 10 min. The enzyme reaction was started by adding 100 μl of 2 mM 5ASA. Absorbance at 350 nm was monitored.

and $NaHCO_3$-Na_2CO_3 buffer (pH 9.0 to 11.0) at 25°C. The optimum temperature for purified MabB was 10°C when the activity was measured in 20 mM Tris-HCl (pH 8.0) (Fig. 5C and D).

The enzyme activity of MabB increased ~2.3-fold with the addition of 0.25 mM ferrous iron, and only 74% enzyme activity was observed when 0.25 mM ferric iron was added to the reaction catalyzed by MabB. No other metal ion increased the enzyme activity of MabB. Iron concentrations were measured using an inductive coupling plasma emission spectrometer (Optima 8x00; PerkinElmer, Norwalk, CT). The iron/enzyme ratio was 0.1 ± 0.02 mol of ferrous iron per mole of MabB. The effects of various inhibitors on MabB activity were examined. 5-Aminosalicylate dioxygenase was inactivated by 1 mM SDS and 0.1 mM *o*-phenanthroline, a ferrous iron chelator (Table 1), but EDTA had a much lesser influence on MabB activity than *o*-phenanthroline. The results indicated that Fe^{II} is the cofactor of MabB.

Product analysis and ^{18}O isotope-based experiment. The reaction catalyzed by MabB was analyzed by HPLC. A new product, with absorbance at 350 nm, was observed in the HPLC signal, and the peak representative of 5ASA was decreased (Fig. 6A and B). The compound, with the same retention time as the new product, has a molecular weight of 184.06 observed by electrospray ionization-mass spectrometry (ESI-MS) analysis; the molecular weight is consistent with that of *cis*-ACOHDA (Fig. 6C).

Two oxygen atoms were added to the product of the MabB-catalyzed reaction. To determine the source of these oxygen atoms, the reaction was performed in the presence of $^{18}O_2$. The reaction products were analyzed using the negative ion modes of ESI-MS. A new signal, at *m/z* 188.05, was observed in the $^{18}O_2$ experiments (Fig. 6D), which is consistent with the incorporation of two ^{18}O atoms into *cis*-ACOHDA. On the basis of this finding as well as that of the previous study (21), we concluded that MabB catalyzes the ring cleavage reaction of 5ASA to form *cis*-ACOHDA and that two oxygen atoms from the dioxygen molecule are incorporated into the ring cleavage product. The results confirmed that the 1,125-bp-long sequence encoded the 5-aminosalicylate 1,2-dioxygenase.

Substrate specificity and catalytic kinetics of MabB. The oxidation of gentisate, 1-hydroxy-2-naphthoate, and salicylate by MabB was spectrophotometrically examined at a concentration of 100 μM in 20 mM Tris-HCl buffer (pH 8.0) at 25°C. The results showed that MabB was able to convert gentisate, which led to a new absorption maximum at 335 nm (Fig. 7A). Gentisate conversion was also confirmed by HPLC (Fig. 7B) and liquid chromatography-mass spectrometry (LC-MS); a product at *m/z* 185.02 was observed (data not shown).

The Michaelis-Menten kinetic constants of MabB for 5ASA and gentisate were determined. The apparent K_m and V_{max} values for 5ASA were 52.0 ± 5.6 μM and 850 ± 33.2 U/mg, respectively, and those for gentisate were 823 ± 212 μM and 185 ± 13.1 U/mg, respectively (Table 2). MabB is able to catalyze the ring cleavage of 1-hydroxy-2-naphthoate. However, 1-hydroxy-2-naphthoate is oxidized by MabB at a rate less than 1% of that observed for 5ASA. In addition, no activity was detected in reaction with salicylate.

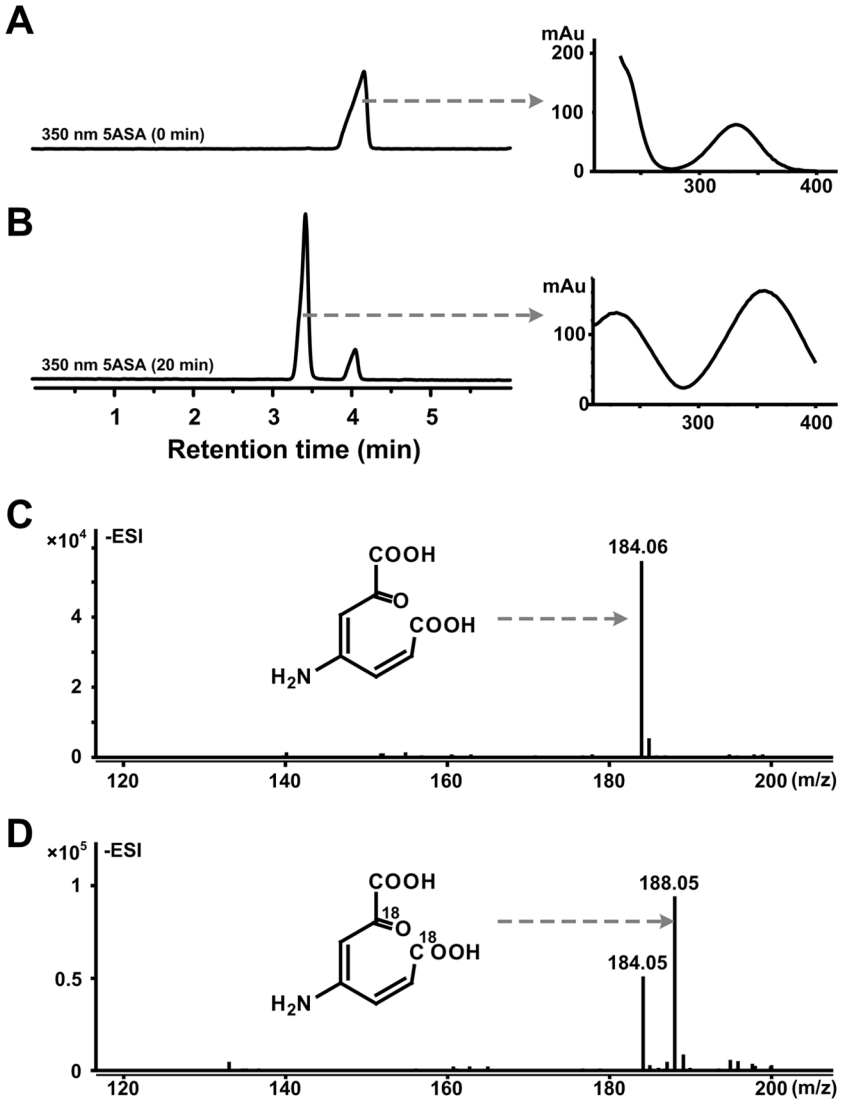

FIG 6 HPLC and ESI-MS analysis of MabB-catalyzing reactions. (A and B) HPLC analysis of the reactions of MabB at 0 min (A) and 20 min (B). (C) ESI-MS analysis of MabB catalyzing reaction; 200 μM 5ASA was mixed with MabB in 20 mM phosphate buffer (pH 8.0). After 20 min, 2 volumes of ethanol were added to the mixture to terminate the reaction. The product *cis*-ACOHDA is indicated. (D) ESI-MS analysis of ^{18}O-labeling MabB-catalyzing experiments. The ^{18}O-labeled *cis*-ACOHDA is indicated.

Site-directed mutagenesis. Three His residues are responsible for the ferrous iron binding in aromatic ring cleavage dioxygenases, as previously reported. Multiple alignment of MabB and related dioxygenases by ClustalW revealed that the three His residues involved in ferrous iron binding in GDOs were highly conserved in MabB at positions 107, 109, and 150 (26). Site-directed mutants involving these amino acids were generated and expressed in *E. coli* cells. No enzymatic activity was observed in the three mutant proteins compared with that of the wild type. In addition, in the GDO from *Corynebacterium glutamicum*, the substrate range has been reported to expand upon the introduction of a site-directed mutation (Ala to Gly) (6). The amino acid Ala, which is essential for substrate selectivity, is also conserved in MabB at position 93. The mutant $MabB_{G93A}$ showed the ability to oxidize salicylate. The K_m and V_{max} values of $MabB_{G93A}$ for salicylate were 559 $\pm$ 36 μM and 28.0 $\pm$ 1.3 U/mg, respectively.

DISCUSSION

A few ring-cleaving dioxygenases with bicupin folds, represented by GDO, SDO, and 1H2NDO, catalyze the cleavage of the monohydroxylated aromatic ring between the

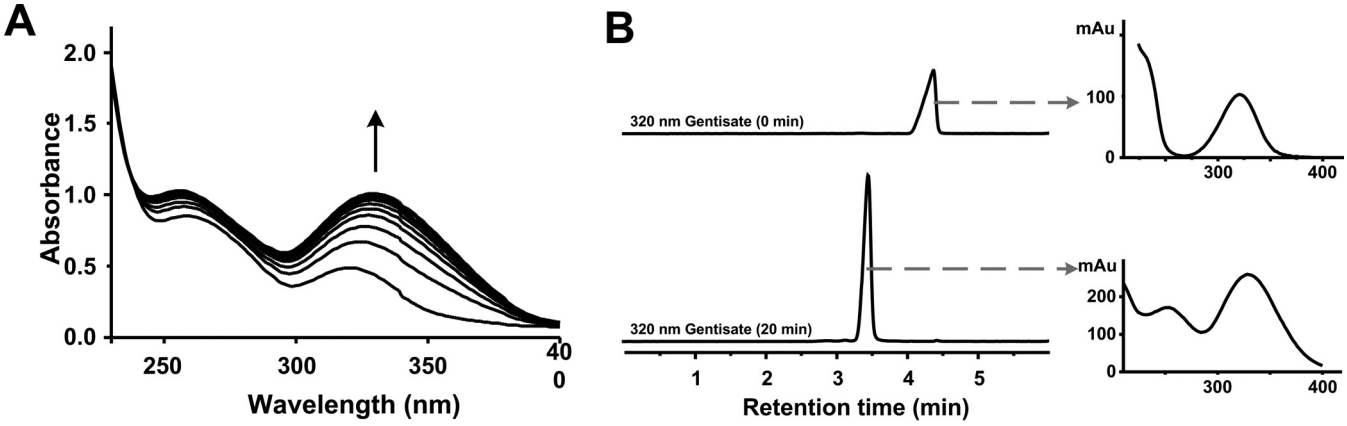

FIG 7 Analysis of MabB-catalyzing reaction with gentisate as the substrate. (A) Spectrophotometric changed during the transformation of gentisate by MabB. The spectra were recorded every 2 min. Arrows indicate the direction of spectral changes. (B) HPLC analysis of gentisate conversion by MabB.

carboxyl and vicinal hydroxyl groups yielding the ring cleavage product (1). Most GDOs and SDOs have broad substrate ranges and can catalyze the ring cleavage reaction of other substituted salicylates except for their primary substrate (5, 27–30). For example, GDOs can convert 5ASA with significantly high activity (5, 22). All 5ASA-utilizing bacteria converted 5ASA through a ring cleavage reaction to produce *cis*-ACOHDA under aerobic conditions (15, 21). It seems that GDO can substitute the function of 5-aminosalicylate 1,2-dioxygenase in 5ASA-utilizing bacteria for 5ASA transformation. However, the reported GDOs have much lesser catalytic efficiency with 5ASA as the substrate compared to that of gentisate (6, 11, 22, 23). Therefore, it is biochemically more economical for these bacteria to possess a 5-aminosalicylate 1,2-dioxygenase, with catalytic properties different from GDOs. A 5-aminosalicylate 1,2-dioxygenase was reported in 1992, which was speculated to use 5ASA as the primary substrate (21). However, the amino acid sequence of this enzyme is still unknown. In this study, a novel gene, *mabB*, was discovered in the genome of *Comamonas* sp. QT12. *mabB* expression is higher in the presence of 3-aminobenzoate, and the *mabB*-disrupted mutant was not able to grow on 3-aminobenzoate or 5-aminobenzoate, indicating its key role in 3-aminobenzoate degradation. The heterologously expressed MabB could incorporate two oxygen atoms from the dioxygen molecule into 5ASA to form the product *cis*-ACOHDA, and it could also transform gentisate or 1-hydroxy-2-naphthoate. However, the low turnover rates indicate that gentisate and 1-hydroxy-2-naphthoate are not the physiological substrates of MabB. The results indicated that 5ASA is not only the primary substrate but also the physiological substrate of MabB. *mabB* is the first sequenced gene that has been shown to encode a functional 5-aminosalicylate 1,2-dioxygenase. The present study adds to the scant knowledge available about 3-aminobenzoate degradation in *Comamonas*.

Although a greater sequence identity (36.1%) was noted between MabB and 1H2NDO than between MabB and GDOs, the biochemical properties of MabB were more closely related to those of GDOs. As 5ASA is a derivative of gentisate, strain QT12 could grow with gentisate but not with 1-hydroxy-2-naphthoate. MabB and GDOs use each other's substrates as the best alternative substrate with relatively high activity (5); however, the rate of conversion of 1-hydroxy-2-naphthoate by MabB is much less than

TABLE 2 Kinetic parameters and substrate specificities of MabB

Substrate	V_{max} (U/mg)	K_m (μM)	k_{cat}/K_m ($s^{-1} \cdot M^{-1} \cdot 10^3$)
5ASA	850 ± 33.2	52.0 ± 5.6	12,303
Gentisate	185 ± 13.1	823 ± 212	169
Salicylate	ND[a]	ND	ND

[a]ND, not detectable.

that observed for gentisate. This may be because both 5ASA and gentisate carry a *para*-electron-donating substituent, which is considered to play a crucial role in directing the oxidative C—C bond cleavage reaction (11, 24). The type and position of salicylate substituents influence the catalytic reaction of GDOs and MabB (23, 24). The 5-hydroxyl group of gentisate fulfils the function of the missing amino group to activate the aromatic ring in the reaction. However, some repulsion exists between the relevant amino group binding part of MabB and the 5-hydroxyl group of gentisate, which may be affected only by a few amino acids. Therefore, it is necessary to determine the structure of MabB. The genome of the 3-aminobenzoate-degrading *Pusillimonas* sp. strain BN9 (previously known as *Pseudomonas* sp. strain BN9) has not been sequenced; therefore, it is not possible to evaluate the sequence homology between MabB and the proteins present in strain BN9. The amino acid sequence identity between MabB and other reported dioxygenases is low. MabB was not found in other reported *Pusillimonas* or *Comamonas* genomes or in any of the other sequenced strains (31, 32). Therefore, it is speculated that *mabB*, 1H2NDO, and GDOs diverged from a common ancestral gene and evolved independently. Further efforts will aim to explore other genes involved in the 3-aminobenzoate degradation pathway in *Comamonas* sp. strain QT12.

MATERIALS AND METHODS

Chemicals. 5-Aminosalicylic acid, gentisic acid, and salicylic acid were obtained from Sangon Biotech (Shanghai, China). 1-Hydroxy-2-naphthoate was purchased from Aladdin (Shanghai, China). $^{18}O_2$ was obtained from Shanghai Research Institute of Chemical Industry. RNAprep pure cell/bacteria kit was purchased from TianGen (Beijing, China). All-In-One RT master mix (with AccuRT) and EvaGreen 2× PCR master mix was purchased from Abm (Canada). All other chemicals used in this study were of analytical grade and commercially available.

Bacterial plasmids and strains. Active sludge samples were collected from the wastewater treatment plant in Qingdao, China. Approximately 10 g of activated sludge was inoculated in 50 ml of MSM (33) medium containing 1.0 mg/ml 3-aminobenzoate and incubated for 1 week at 30°C with shaking at 150 rpm. Subsequently, 5 ml of the enrichment culture was transferred to the same fresh medium, and this was incubated for 1 week under the same conditions. This procedure was repeated five times to enrich for the 3-aminobenzoate-degrading bacteria. Serial dilutions of the final culture were spread on MSM agar plates containing 1 mg/ml 3-aminobenzoate. The colonies obtained were reinoculated into liquid MSM to confirm 3-aminobenzoate utilization. The isolated strain was observed using a JSM-7500F scanning-electronic microscope (JEOL, Japan). The 16S rRNA gene was amplified from the genome of strain QT12 using primers 27F and 1492R to further identify the isolated strain. The draft genome sequence of strain QT12 was obtained by paired-end sequencing using the Hi-Seq 2500 system (Illumina) with ~120-fold coverage. The reads were assembled with Velvet software to 1,035 contigs (34). The genome sequence was annotated using the Rapid Annotation using Subsystem Technology (RAST) server (35). The draft genome sequence of strain QT12 comprises 5,980,770 bases and has a G+C content of 60.8%.

pET28a was used for the expression of the His-tagged 5-aminosalicylate 1,2-dioxygenase. *E. coli* DH5α was routinely used as a host in cloning experiments. *E. coli* strain BL21(DE3) was used as the host for overexpressing the gene cloned in the pET28a. *E. coli* strains were cultured in Luria-Bertani broth (10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter) at 37°C. *Comamonas* sp. strain QT12 was cultivated in MSM medium containing 1 mg/ml 3-aminobenzoate at 30°C with shaking at 200 rpm. 3-Aminobenzoate was added to the medium before autoclaving.

RT-qPCR. RT-qPCR was used to analyze *mabB* expression. *Comamonas* sp. QT12 was grown in 3-aminobenzoate and citrate-NH_4Cl as its sole sources of carbon and nitrogen, respectively. Total RNA was prepared using an RNAprep pure cell/bacteria kit according to the manufacturer's instructions. The cDNA was synthesized using All-In-One RT master mix (with AccuRT). The primers were *mabB*-q-F/*mabB*-q-R for *mabB* and 16S-q-F/16S-q-R for 16S rRNA. RT-qPCR was performed in a volume of 20 μl on a Stratagene Mx3000P PCR instrument (Agilent Technologies, Palo Alto, CA) with EvaGreen 2× PCR master mix and RT-qPCR primers. The threshold cycle (C_T) values for *mabB* were normalized to the expression level of the 16S rRNA gene. Expression levels were calculated according to the $2^{-\Delta\Delta CT}$ method. Primers for RT-qPCR are listed in Table 3.

Construction of an *mabB* mutant. The construction of an *mabB* disruption mutant was performed using a homologous recombination gene replacement system. Briefly, the upstream and downstream DNA fragments (~700 bp) of *mabB* and the tetracycline (Tet) resistance cassette, amplified from pMK2017, were cloned into the suicide vector pK18*mobsacB* to produce pK18MST-Δ*mabB*. The recombinant plasmids were transformed into *E. coli* S17-1λpir to create a donor strain. *Comamonas* sp. QT12 was used as a recipient strain. In a conjugation experiment, equal numbers of donor and recipient cells were mixed and dropped on LB agar without antibiotics and incubated overnight at 37°C. Single crossover recombinants were obtained by plating the cells on LB agar containing 30 μg/ml Tet and 100 μg/ml ampicillin (Amp). After several rounds of propagation in LB broth without antibiotics, the

TABLE 3 Oligonucleotides used in this study

Name	Sequence (5′–3′)
mabB-28a-F	CGCGGATCCATGAACGCACCGGATTCTT
mabB-28a-R	CCCAAGCTTTCAGAGGGAAGAGAGTTCG
mabB-q-F	GCCGCAGCCATCCGTTTCG
mabB-q-R	AGGTTGACCAGCGGGAAGT
16S-q-F	GTGAGATGTTGGGTTAAG
16S-q-R	GTACCAGCCATTGTATGA
H107A-F	GCCCCTGCGGCCCGCCACGCG
H107A-R	GCGTGGCGGGCCGCAGGGGCC
H109A-F	GCGCACCGCGCCGCGGCCGCAG
H109A-R	GCGGCCGCGGCGCGGTGCGCA
H150A-F	GTACCTGGGCCGAACACGCCA
H150A-R	GCGTGTTCGGCCCAGGTACCG
QmabB-1F[a]	CGCGGATCCGGTGCCCTACAAGTTGGTGA
QmabB-1R[a]	GCCGCCCTTGCTGAGATCGGTTTGGAAAGAA
QmabB-2F[a]	CAAACCGATCTCAGCAAGGGCGGCTCGGTGTA
QmabB-2R[a]	CCGGAATTCGCCGGTGATGAGCTG
TmabB-F[b]	CGAACAGATCCAGCCCTTTG
TmabB-R[b]	TGTTGATGTAAACCTTCAG

[a]Used for the construction of homologous recombination plasmid pK18MST-Δ*mabB*.
[b]Used to confirm the construction of Q*mabB* double crossover mutant strain.

double-crossover recombinants were screened using the sucrose counter-selection method (25). The double-crossover mutant was confirmed by PCR, and the size of the PCR amplicon obtained using the genomic DNA of *Comamonas* sp. QT12 Δ*mabB* was supposed to be 1.6 kb. The primers used for the construction of gene-deficient mutants are listed in Table 3.

Cloning the presumed 5-aminosalicylate 1,2-dioxygenase from *Comamonas* sp. QT12. The *mabB* gene was amplified from the genome DNA of *Comamonas* sp. QT12 using the primers *mabB*-28a-F and *mabB*-28a-R. The PCR product was purified and cut with the restriction enzymes BamHI and HindIII. Subsequently, the DNA fragment was ligated into pET28a, which was previously digested with BamHI and HindIII. This led to the formation of plasmid pET28a-*mabB*, which coded for an *N*-terminal His-tagged recombinant MabB. The recombinant plasmid was verified by DNA sequencing.

The *mabB* gene carrying site-directed mutations was amplified from pET28a-*mabB* by overlap extension PCR (36) using the primers H107A-F/H107A-R, H109A-F/H109A-R, H150A-F/H150A-R, and A93G-F/A93G-R for generating the site-directed mutants $MabB_{H107A}$, $MabB_{H109A}$, $MabB_{H150A}$, and $MabB_{A93G}$, respectively. The oligonucleotide primers used are listed in Table 3.

Expression and purification of His-tagged MabB in *E. coli*. The recombinant plasmid pET28a-*mabB* was transformed into *E. coli* BL21(DE3) cells. *E. coli* BL21(DE3) (pET28a-*mabB*) cells were incubated at 37°C in Luria broth with kanamycin (50 μg/ml). The cultures were transferred to 30°C with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) when the optical density at 600 nm (OD_{600}) reached 0.8. Approximately 12 h after IPTG was added, the cells were harvested by centrifugation at 5,000 $\times$ *g* for 5 min and washed twice with 20 mM Tris-HCl buffer (pH 8.0). The induced *E. coli* cells were resuspended in the same buffer and disrupted by ultrasonication in an ice-water bath for 36 min (on for 3 s, off for 6 s). Subsequently, the cell debris was removed by centrifugation at 12,000 $\times$ *g* for 10 min at 4°C. The cell extracts were loaded onto an Ni-nitrilotriacetic acid (Ni-NTA) (GE, USA) column, which was previously equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The column (2 ml) was washed with 20 ml of Tris-HCl buffer (20 mM, pH 8.0) and 10 ml of 10 mM imidazole dissolved in Tris-HCl buffer (20 mM, pH 8.0). Purified MabB was eluted using 100 mM imidazole dissolved in Tris-HCl buffer (20 mM, pH 8.0). The imidazole in the protein was removed by ultracentrifugation. Purified protein was stored at −40°C. Protein concentrations were determined according to the methods of Bradford (37), using bovine serum albumin as the standard.

Enzyme assay of purified His-tagged MabB. The conversion of the substrate by MabB was spectrophotometrically determined at 25°C (UV2310II; Techcomp, Shanghai). 5-Aminosalicylate dioxygenase activity was measured according to the increase in the absorption at 350 nm, with a reaction coefficient of 10,200 liters/mol/cm, as reported by Stolz et al. (21). GDO activity was determined according to the increase in the absorption at 330 nm, with a reaction coefficient of 9,800 liters/mol/cm (8, 11). Salicylate dioxygenase activity was measured according to the increase in the absorption at 283 nm with an apparent reaction coefficient of 13,600 liters/mol/cm. A reaction coefficient was used for the determination of product concentrations according to the change in the absorbances measured at a particular wavelength. All reactions were performed in 20 mM Tris-HCl (pH 8.0) containing 0.25 mM $FeCl_2$. The 5 mM $FeCl_2$ stock solution was mixed with 5 mM vitamin C. One unit of the enzyme activity was defined as the conversion of 1 μmol of substrate per minute. To determine the effects of the metal ions on enzyme activities, 0.25 mM $FeCl_2$, $FeCl_3$, NaCl, KCl, $BaCl_2$, $CuSO_4$, $ZnCl_2$, $MgSO_4$, Li_2SO_4, $Cd(NO_3)_2$, $CoCl_2$, $Na_2(MoO_4)$, $CaCl_2$, and $MnSO_4$ were added into the reaction mixtures of MabB.

^{18}O isotope experiment. Approximately 1.5 ml of 0.5 mM 5ASA (dissolved in 20 mM phosphate buffer, pH 8.0) was added to a 10-ml sealed rubber plug glass bottle. High purity argon gas was pumped into the bottle for 20 min to eliminate dioxygen. Subsequently, 100 ml of $^{18}O_2$ gas was pumped into the

bottle. The reaction was initiated with the addition of 50 μl MabB. The reaction was terminated by adding 2 volumes of ethanol, and then the samples were analyzed by ESI-MS. A reaction performed in air was used as the control.

Sequence analysis of MabB. Searches of the nonredundant protein sequence database were performed using BLAST. Multiple-sequence alignment was performed by ClustalW. The phylogenetic tree was constructed using MEGA 6.0.

Analytical methods. The turnover of the substrates by MabB was analyzed by HPLC with diode array detection, using a reverse-phase column (Welch C18 Xtimate, 4.6 mm by 250 mm) at 30°C. The mobile phase was 85% (vol/vol) 0.6 mM $NH_3{\cdot}H_2O$ and 15% (vol/vol) methanol at a flow rate of 0.5 ml/min. The substrate and product were monitored according to their characteristic absorption peaks. Mass spectrometry (MS) analysis was performed on an Agilent 6460 QQQMS equipped with electrospray ionization (ESI) sources. The mobile phase of ESI-MS consisted of 90% (vol/vol) deionized H_2O (0.1% formic acid [vol/vol]) and 10% (vol/vol) acetonitrile at a flow rate of 0.5 ml/min. Samples for HPLC and ESI-MS were treated with the addition of 2 volumes of ethanol to precipitate the proteins and were then filtered through a 0.22-μm-pore-size filter after centrifugation at 12,000 × *g* for 2 min.

Accession number(s). The sequence of the 16S rRNA gene from strain QT12 is available in GenBank under accession number KY684811. The whole genome shotgun project of *Comamonas* sp. QT12 has been deposited at DDBJ/ENA/GenBank under the accession number MZNW00000000. The version described in this paper is version MZNW01000000. The nucleotide sequence of the 1,125-bp *mabB* gene has been deposited in the GenBank DNA database under accession no. KY399772.

ACKNOWLEDGMENTS

This work was supported by grants from the Chinese National Natural Science Foundation (31600086), the Shandong Provincial Natural Science Foundation (ZR2016CQ06), the Qingdao Agricultural University Scientific Research Foundation (6631115052), and the Open Funding Project of State Key Laboratory of Microbial Metabolism.

We declare that we have no conflicts of interest with the contents of this article.

H.Y. and L.G. designed the study and wrote the paper. H.Y. purified and characterized MabB. S.Z. constructed vectors for expression the enzyme. All authors analyzed the results and approved the final version of the manuscript.

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