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BIODEGRADATION

Molecular Basis and Evolutionary Origin of 1-Nitronaphthalene Catabolism in *Sphingobium* sp. Strain JS3065

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ABSTRACT Nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) enter the environment from natural sources and anthropogenic activities. To date, microorganisms able to mineralize nitro-PAHs have not been reported. Here, *Sphingobium* sp. strain JS3065 was isolated by selective enrichment for its ability to grow on 1-nitronaphthalene as the sole carbon, nitrogen, and energy source. Analysis of the complete genome of strain JS3065 indicated that the gene cluster encoding 1-nitronaphthalene catabolism (*nin*) is located on a plasmid. Based on the genetic and biochemical evidence, the *nin* genes share an origin with the *nag*-like genes encoding naphthalene degradation in *Ralstonia* sp. strain U2. The initial step in degradation of 1-nitronaphthalene is catalyzed by a three-component dioxygenase, NinAaAbAcAd, resulting in formation of 1,2-dihydroxynaphthalene which is also an early intermediate in the naphthalene degradation pathway. Introduction of the *ninAaAbAcAd* genes into strain U2 enabled its growth on 1-nitronaphthalene. Phylogenic analysis of NinAc suggested that an ancestral 1-nitronaphthalene dioxygenase was an early step in the evolution of nitroarene dioxygenases. Based on bioinformatic analysis and enzyme assays, the subsequent assimilation of 1,2-dihydroxynaphthalene seems to follow the well-established pathway for naphthalene degradation by *Ralstonia* sp. strain U2. This is the first report of catabolic pathway for 1-nitronaphthalene and is another example of how expanding the substrate range of Rieske type dioxygenase enables bacteria to grow on recalcitrant nitroaromatic compounds.

IMPORTANCE Nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) have been widely detected in the environment and they are more toxic than their corresponding parent PAHs. Although biodegradation of many PAHs has been extensively described at genetic and biochemical levels, little is known about the microbial degradation of nitro-PAHs. This work reports the isolation of a *Sphingobium* strain growing on 1-nitronaphthalene and the genetic basis for the catabolic pathway. The pathway evolved from an ancestral naphthalene catabolic pathway by a remarkably small modification in the specificity of the initial dioxygenase. Data presented here not only shed light on the biochemical processes involved in the microbial degradation of globally important nitrated polycyclic aromatic hydrocarbons, but also provide an evolutionary paradigm for how bacteria evolve a novel catabolic pathway with minimal alteration of preexisting pathways for natural organic compounds.

KEYWORDS biodegradation, dioxygenase, naphthalene, nitro-PAHs, nitronaphthalene

Nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) are derivatives of polycyclic aromatic hydrocarbons (PAHs) containing at least one nitro group. Incomplete combustion of fossil fuels and biomass, conversion of environmental PAHs with oxidants and industrial synthesis have led to their frequent occurrence in the biosphere

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(1, 2). Although nitro-PAHs in the atmosphere are readily degraded by photolysis, they are persistent in water and soils (2). The environmental concentrations of nitro-PAHs are usually lower than the corresponding parent PAHs, but their mutagenic potential can be much higher (3, 4) because nitro-PAHs can be activated to more toxic products in the human body through reduction of the nitro group by intestinal microflora (5, 6), or oxidation of the aromatic ring by cytochrome P450 (5, 7). Some fungi can transform nitro-PAHs by the action of nonspecific enzymes, resulting in the formation of ring hydroxylated nitro-PAHs which can be conjugated subsequently with glucose or sulfate (8, 9). In addition, both aerobic and anaerobic bacteria can transform nitro-PAHs by both oxidative and reductive pathways (10–12), but mineralization of nitro-PAHs has not been reported to our knowledge.

In contrast to nitro-PAHs, biochemistry, genetics, and physiology have been extensively investigated in a number of bacterial isolates that are able to grow on nitro-substituted benzenes (13), including nitrobenzene (14), nitrophenols (15), nitrotoluenes (16–18), nitrobenzoates (19, 20), and halogenated nitrobenzenes (21–23). In general, the aerobic degradation pathways of the above compounds are initiated via reductive or oxidative attack at the nitro group followed by ring cleavage and further assimilation. Nitroreductase-catalyzed reduction of nitroaromatics leads to the formation of hydroxylamine or amine derivatives, which are subjected to subsequent oxidation of the aromatic ring. In the oxidative pathways, monooxygenases or dioxygenases convert the initial nitroaromatic substrates to phenols, quinones, or catechols prior to ring-fission and assimilation.

Rieske non-heme iron-dependent dioxygenases are a group of versatile enzymes catalyzing the addition of molecular oxygen to the rings of a variety of aromatic compounds (24). For example, naphthalene dioxygenase (NDO) catalyzes the stereospecific oxidation of naphthalene to *cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene. In bacteria such as *Pseudomonas putida* G7 (25) and *P. putida* NCIB 9816 (26) the naphthalene dioxygenase, NahAaAbAcAd, initiates a catabolic pathway in which naphthalene is catabolized to central metabolites via catechol. In other naphthalene degraders such as *Ralstonia* sp. strain U2 (27, 28) and *Polaromonas naphthalenivorans* CJ2 (29), naphthalene dioxygenases, NagAaAbAcAd, show obvious divergence in sequence identity and operon organization from those of NahAaAbAcAd and naphthalene is catabolized to central metabolites via gentisate. Naphthalene dioxygenase homologues (called nitroarene dioxygenases, NADOs) initiate biodegradation of nitrobenzene (14), halogenated nitrobenzenes (30–32), and nitrotoluenes (16, 17) resulting in the formation of the corresponding catechols and nitrite, thereby enabling bacteria to utilize these simple nitroaromatic compounds as sole source of carbon, nitrogen, and energy for growth.

Nitronaphthalenes have been synthesized commercially for use as intermediates in chemical synthesis of dyes, pesticides, and explosives since the 19th century (33). 1-Nitronaphthalene (1-NN) is listed as high production volume chemical (greater than one million pounds per year) by the United States Environmental Protection Agency (34). Nitronaphthalenes also have been detected in Antarctic airborne particulate matter (35). Although nitronaphthalenes are not evidently carcinogenic, they exhibit significant cytotoxicity (36) and ecotoxicity (37).

This study aimed to explore the microbial degradation of nitronaphthalenes by a newly isolated 1-NN utilizer *Sphingobium* sp. strain JS3065. The pathway and genes involved were identified by whole genome sequencing, bioinformatic mining and biochemical analyses. A plasmid-borne gene cluster, closely related to the naphthalene degrading gene cluster in *Ralstonia* sp. strain U2, was responsible for the catabolism of 1-nitronaphthalene to central metabolites via gentisate. This study reveals the genetic determinants of the 1-NN degradation pathway, and provides insight about a facile mechanism for bacterial adaption to a recalcitrant pollutant.

RESULTS

Isolation of 1-nitronaphthalene utilizer. Bacteria able to grow with 1-NN were isolated from soil at a former chemical manufacturing plant (Fig. 1). Phylogenetic analysis

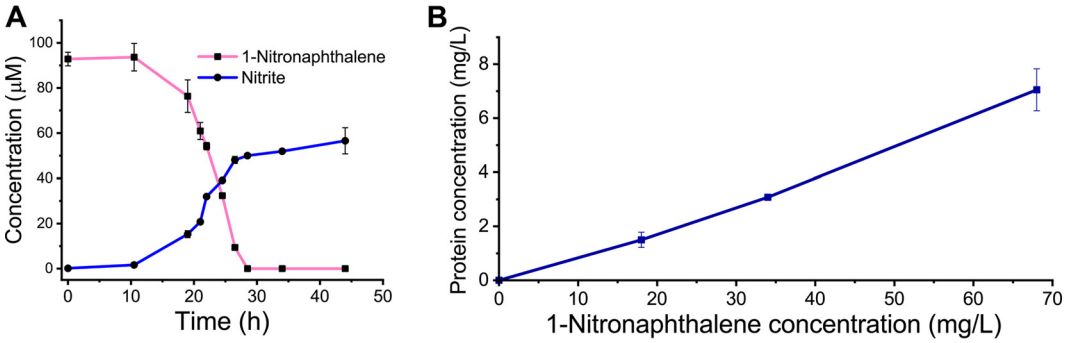
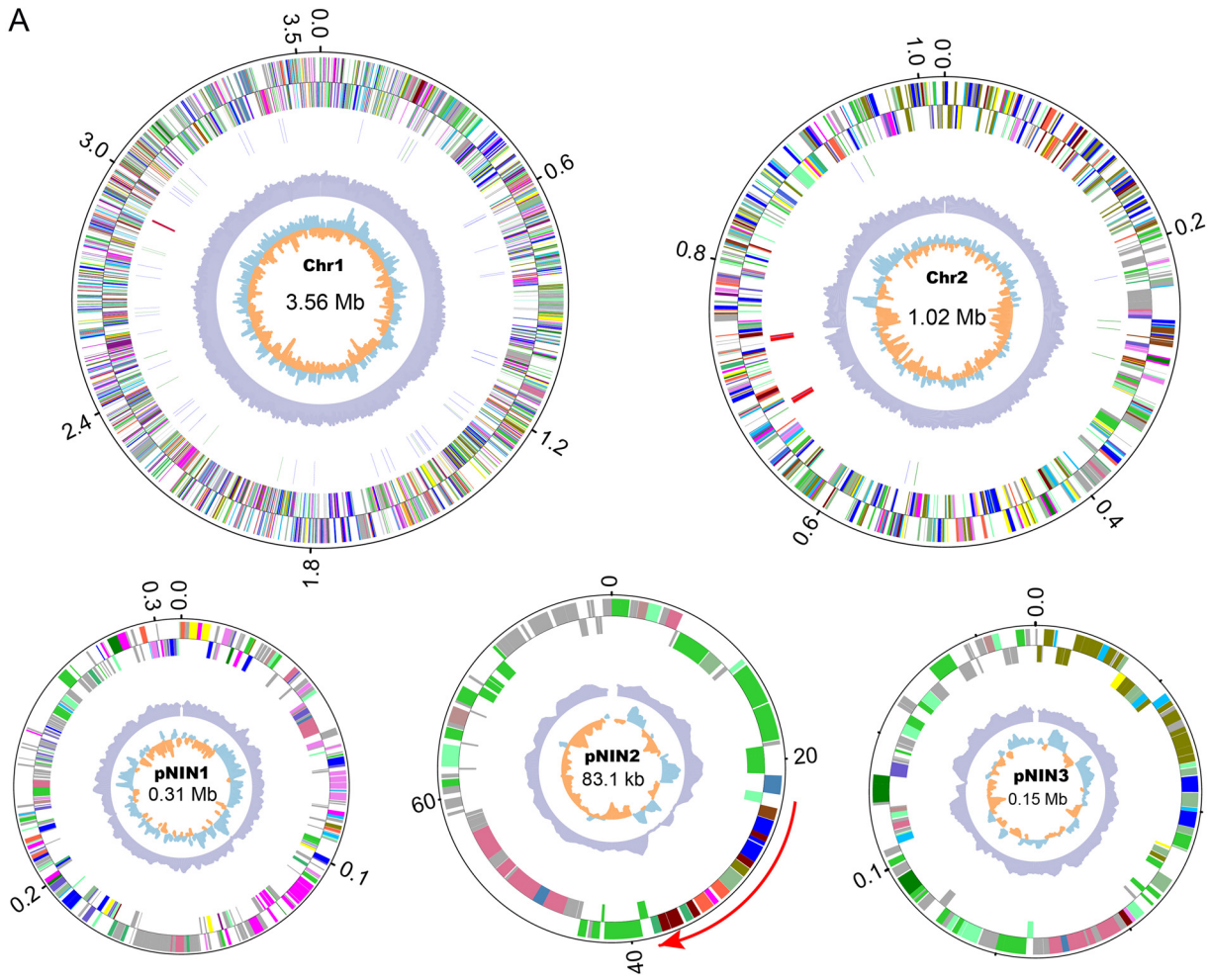


FIG 1 Growth of *Sphingobium* sp. strain JS3065 utilizing 1-nitronaphthalene (1-NN) as the sole source of carbon, nitrogen and energy. (A) 1-Nitronaphthalene disappearance and nitrite accumulation. (B) Growth on minimal mineral medium supplemented with various concentrations of 1-nitronaphthalene. The protein concentrations representing the biomass accumulation were measured at stationary phase.

based on the 16S rRNA gene sequence indicated that the strain belongs to the genus *Sphingobium*, therefore it was designated as *Sphingobium* sp. strain JS3065. During growth on 1-NN, excess nitrogen was released as nitrite (Fig. 1A). The biomass accumulation resulting from utilizing 1-NN as sole carbon, nitrogen and energy sources was indicated by the increase of final protein concentrations with increasing initial 1-NN concentrations (Fig. 1B). Strain JS3065 was unable to grow on 2-nitronaphthalene or naphthalene under the same conditions. In separate experiments, *Ralstonia* sp. strain U2, a well-characterized naphthalene degrader, was unable to grow on 1-NN (Fig. S1).

Genome analysis of strain JS3065. The complete genome of strain JS3065 is 5.1 Mb, comprising 2 circular chromosomes and 3 plasmids (Fig. 2A). The GC content is $\sim$ 64% for the chromosomes and $\sim$ 61% for the plasmids. Annotation of the JS3065 genome revealed the presence of 4885 open reading frames (Table 1). Strain JS3065 showed the highest degree of genomic identity with *Sphingobium japonicum* UT26, which is a gamma- hexachlorocyclohexane degrader (38) that has not been reported to be able to degrade 1-NN. The average nucleotide identity (ANI) of the 2 strains is 87.35%. The backbone of plasmid pNIN2 is similar to that of pLB1 which is a γ-hexachlorocyclohexane degradation plasmid from *S. japonicum* UT26 (39). The predicted replication initiator protein (RepA) of pNIN2 has 98% amino acid sequence identify to that of pLB1 (GenBank No. BAF30426.1) which was reported to represent a new incompatibility group based on the sequence identities of RepA homologs (39).

The observation of nitrite accumulation during the growth of strain JS3065 on 1-NN (Fig. 1A) suggested the involvement of an oxygenase in the catabolic pathway (16). Therefore, the genome sequence of strain JS3065 was searched for the presence of genes similar to those previously reported to encode denitration of nitroaromatic compounds. The amino acid sequences of various characterized monooxygenases acting on nitrophenols (40, 41) or dioxygenases acting on nitrobenzene/nitrotoluenes/chloronitrobenzenes (14, 16, 17, 30, 32) were used as query sequences for BLAST analyses. No plausible homolog (amino acid sequence identity $>$ 30% and coverage $>$ 50%) of the denitrating monooxygenase was found, whereas 4 genes encoding a three-component dioxygenase closely related to nitroarene dioxygenases and naphthalene dioxygenase (NagAaAbAcAd) were located on plasmid pNIN2. No other ring hydroxylating aromatic dioxygenase homolog was found in the genome of strain JS3065. Considering that 1-NN is structurally analogous to naphthalene and some naphthalene dioxygenase homologues exhibit denitration activity toward various nitroaromatic compounds (13, 17, 31), the dioxygenase gene cluster on pNIN2 was analyzed in more detail and designated as the *nin* cluster (nitronaphthalene-degrading gene cluster). The cluster where the 4 genes encoding the putative dioxygenase are located contains 17 genes exhibiting more than 80% amino acid sequence identities and similar gene organization to that of the *nag*-like naphthalene catabolic genes in *Ralstonia* sp. strain U2 (Fig. 2B). Interestingly, the *ninB*



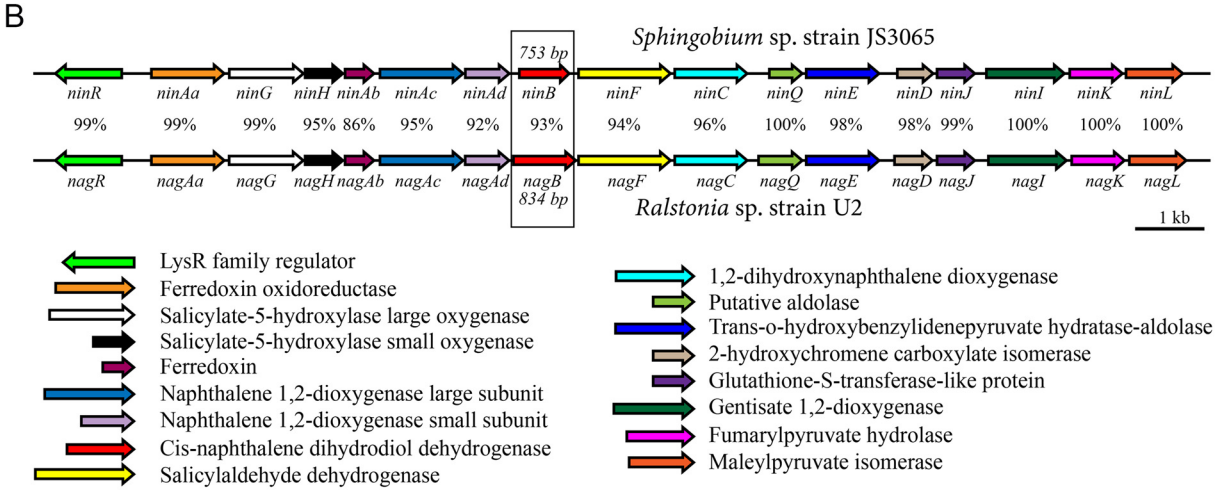


FIG 2 (A) Circular representation and genetic features of the chromosomes (chr1 and chr2) and plasmids (pNINs) of strain JS3065. From outside to center: genes on forward strand (colored by COG categories), genes on reverse strand, noncoding RNA, GC content and GC skew. The red arrow indicates the position of the *nin* cluster on plasmid pNIN2. (B) The predicted 1-nitronaphthalene (*nin*) catabolic genes and their annotations. The *nin* gene cluster from *Sphingobium* sp. strain JS3065 is compared with *nag* genes from the naphthalene degrader *Ralstonia* sp. strain U2. The nucleotide sequence identities of the homologues are shown between them. For the pairwise gene alignments, the coverage is 100% excepting the *ninB* (90%), *ninQ* (71%) and *ninD* (99%). The *ninB* highlighted in the box is truncated compared to the *nagB* encoding the *cis*-naphthalene dihydrodiol dehydrogenase.

TABLE 1 Genomic information of strain JS3065

Name	Size (bp)	ORFs no.	ORFs length (bp)[a]	Avg length (bp)[b]	GC content (%)	Percentage of ORFs length (%)[c]
Chr1	3555142	3429	3200505	933	64.18	90
Chr2	1017992	889	894435	1006	63.73	88
pNIN1	307737	331	256218	774	61.66	83
pNIN2	83075	99	75249	760	60.35	91
pNIN3	148600	137	133890	977	61.73	90

[a]ORFs length means the total length of all ORFs in bp.
[b]Average length means the average length of all ORFs.
[c]Percentage of ORFs length means the ratio of total gene length to genome length.

(753 bp), which seems to be unnecessary for 1-NN catabolism in strain JS3065, is truncated compared to the intact *nagB* (834 bp) that encodes naphthalene dihydrodiol dehydrogenase in strain U2 (Fig. 2B).

Identification of a 1-nitronaphthalene dioxygenase (NinAaAbAcAd). A 5.2 kbp fragment (*ninAaGHAbAcAd*) with the putative 1-NN dioxygenase was inserted into PUC19 and heterologously expressed in *E. coli* DH5α for functional analysis. Cells expressing 1-NN dioxygenase transformed 1-NN and released stoichiometric amounts of nitrite (Fig. 3A). No nitrite was detected upon incubation of 1-NN with the *E. coli* cells containing PUC19 only. The formation of another major product, 1,2-dihydroxynaphthalene, was confirmed by GC-MS (Fig. 3B). The product shows a molecular ion at *m/z* 304, which is consistent with that of authentic 1,2-dioxynaphthalene (Fig. 3B). However, attempts to quantify the production of 1,2-dihydroxynaphthalene were not successful, likely due to its instability under the assay conditions (42). The results established that NinAaAbAcAd catalyzes the denitration of 1-NN with the formation of 1,2-dihydroxynaphthalene and nitrite.

Growth of a naphthalene degrader carrying *ninAaAbAcAd* on 1-NN. In order to confirm the role of NinAaAbAcAd in the utilization of 1-NN by strain JS3065, efforts were made to construct a knockout mutant of *ninAaAbAcAd*, but without success. Therefore, the putative 1-NN dioxygenase genes *ninAaAbAcAd* were introduced into a naphthalene degrader to test their activity in supporting growth on 1-NN. Although the naphthalene degrader *Ralstonia* sp. strain U2 does not grow on 1-NN and *Sphingobium* sp. strain JS3065 does not grow on naphthalene, the catabolism of naphthalene in strain U2 and the proposed catabolism of 1-NN in strain JS3065 produce the common intermediate, 1,2-dihydroxynaphthalene (Fig. 4A). Both naphthalene dioxygenase (NagA) and

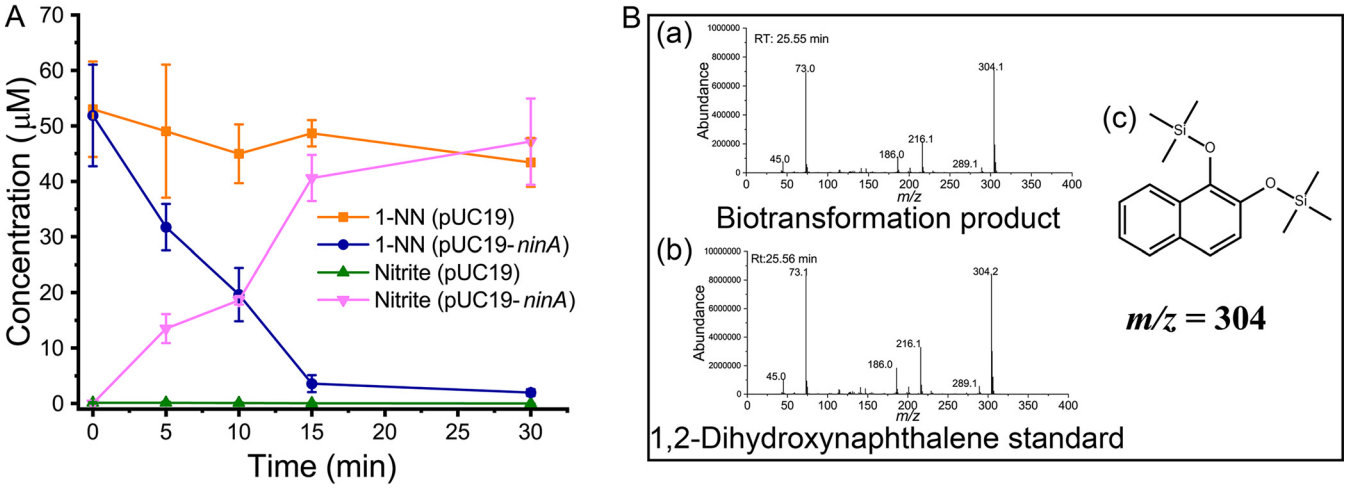


FIG 3 Functional identification of 1-nitronaphthalene dioxygenase NinAaAbAcAd. (A) Time course analysis of 1-nitronaphthalene biotransformation by *E. coli* cells carrying pUC19-*ninA*. Plasmid pUC19 was used as the negative control. (B) Identification of the product of 1-nitronaphthalene oxidation catalyzed by nitronaphthalene dioxygenase. Biotransformation of 1-nitronaphthalene was catalyzed by *E. coli* cells carrying the *ninAaAbAcAd*. (a) Mass spectrum of biotransformation product. (b) Mass spectrum of synthetic 1,2-dihydroxynaphthalene. (c) Trimethylsilyl (TMS) derivative of 1,2-dihydroxynaphthalene.

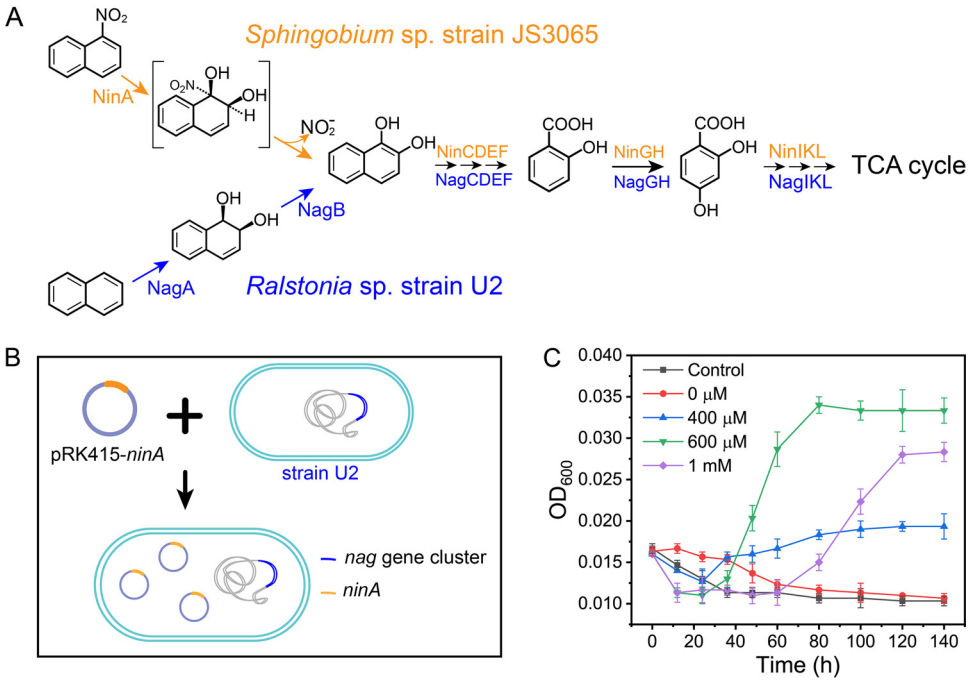
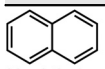
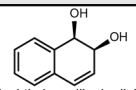
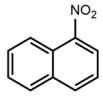
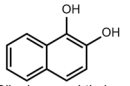
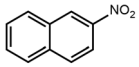
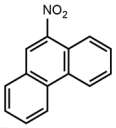
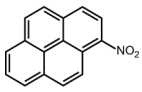
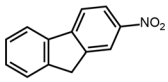
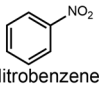
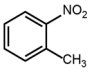
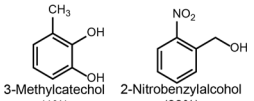
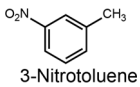
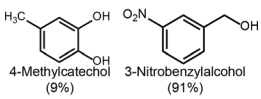
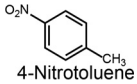
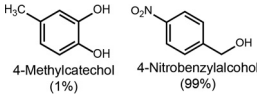


FIG 4 (A) The proposed catabolic pathway of 1-nitronaphthalene in strain JS3065 and the naphthalene catabolic pathway via gentisate (27, 28). (B) Schematic of constructing an artificial 1-nitronaphthalene degrader based on the naphthalene degrader *Ralstonia* sp. strain U2. (C) Growth of *Ralstonia* sp. strain U2 carrying pRK415-*ninA* on various concentrations of 1-nitronaphthalene. Control: strain U2 carrying pRK415 incubated with (nominal) 0.6 mM 1-nitronaphthalene.

cis-naphthalene dihydrodiol dehydrogenase (NagB) are required for converting naphthalene into 1,2-dihydroxynaphthalene in strain U2, whereas 1-NN dioxygenase (NinA) alone can catalyze transformation of 1-NN to 1,2-dihydroxynaphthalene (Fig. 3). Therefore, 1-NN could theoretically be catabolized through a classic naphthalene catabolic pathway with addition of the initial 1-NN dioxygenase (Fig. 4B). The naphthalene degrader U2 containing the 5.2 kbp fragment (*ninAaGHAbAcAd*) from strain JS3065 grew on 1-NN (Fig. 4C). Although XAD-7 was included to minimize toxicity nominal concentrations of 1000 μM exceeded the capacity of the resin and exerted inhibition on the *Ralstonia* cells, which were not previously adapted to growth on nitronaphthalene. The result not only confirms the role of *ninA in vivo*, but also establishes the ability of the naphthalene downstream pathway to accommodate the product of 1-NN oxidation. The result firmly supports the hypothesis that alteration of the substrate range of naphthalene dioxygenase would be necessary and sufficient to allow a naphthalene degrader to grow on 1-NN.

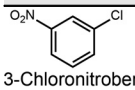
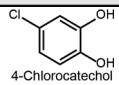
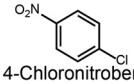
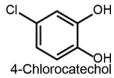
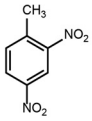
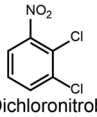
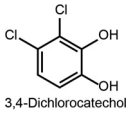
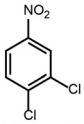
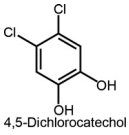
Substrate specificity of 1-NN dioxygenase. Whole-cell biotransformation assays based on nitrite release were performed in *E. coli* carrying the pUC19-*ninA* to test the substrate range of the 1-NN dioxygenase toward a variety of nitroaromatic substrates. The specific activity of NinAaAbAcAd toward 1-nitronaphthalene was 2.85 U/mg protein, whereas weak activity was observed with 2-nitronaphthalene. This result was consistent with the observation that strain JS3065 was unable to grow on 2-nitronaphthalene. Failure to detect the transformation product of 2-nitronaphthalene was probably due to low activity and the instability of its product. NinAaAbAcAd had no denitrating activity toward other tested nitro-PAHs including 9-nitrophenanthrene, 1-nitropyrene, and 2-nitrofluorene (Table 2). In contrast, it exhibited denitrating activities toward a variety of nitro-substituted monoaromatic substrates, including 2,3-dichloronitrobenzene, 3-chloronitrobenze, and 3-nitrotoluene. The products identified by GC-MS were consistent with the expected denitration reactions (Table 2). In addition, 1-NN dioxygenase transformed naphthalene into naphthalene dihydrodiol (Table 2), which was also observed previously for other nitroarene dioxygenases (14, 43).

TABLE 2 Substrate specificity of 1-nitronaphthalene dioxygenase

Substrates	Relative activities[a]	Products[b]
 Naphthalene	–[d]	 Naphthalene dihydrodiol
 1-Nitronaphthalene	100%	 1,2-Dihydroxynaphthalene
 2-Nitronaphthalene	4%	ND[c]
 9-Nitrophenanthrene	ND	ND
 1-Nitropyrene	ND	ND
 2-Nitrofluorene	ND	ND
 Nitrobenzene	<1%	 Catechol
 2-Nitrotoluene	2%	 3-Methylcatechol (1%) 2-Nitrobenzylalcohol (99%)
 3-Nitrotoluene	14%	 4-Methylcatechol (9%) 3-Nitrobenzylalcohol (91%)
 4-Nitrotoluene	<1%	 4-Methylcatechol (1%) 4-Nitrobenzylalcohol (99%)
 2-Chloronitrobenzene	4%	 3-Chlorocatechol

(Continued on next page)

TABLE 2 (Continued)

Substrates	Relative activities[a]	Products[b]
 3-Chloronitrobenzene	42%	 4-Chlorocatechol
 4-Chloronitrobenzene	<1%	 4-Chlorocatechol
 2,4-Dinitrotoluene	ND	ND
 2,3-Dichloronitrobenzene	65%	 3,4-Dichlorocatechol
 3,4-Dichloronitrobenzene	8%	 4,5-Dichlorocatechol

[a]Relative denitration activities were calculated by specific activities, which were determined in whole-cell biotransformation assays based on the production of nitrite. The specific activity toward 1-nitronaphthalene (2.85 μmol min^{-1}mg protein^{-1}) was considered as 100%. All biological replicates ($n > 3$) gave comparable results and a representative assay is shown.
[b]The products were identified by GC-MS comparison with authentic compounds. The ratio of products was calculated by peak area of each product.
[c]ND, the hydroxylated products were not detected.
[d]–, not determined.

The lower catabolic pathway via gentisate. In addition to the 1-naphthalene dioxygenase, the full-length salicylate 5-hydroxylase and gentisate 1,2-dioxygenase genes are also present in the *nin* cluster (Fig. 2B). Thus, the *nin* cluster has the full set of genes encoding the 1,2-dihydroxynaphthalene catabolic pathway via gentisate rather than catechol. Ring-fission enzyme assays with extracts prepared from 1-nitronaphthalene-grown JS3065 cells showed gentisate 1,2-dioxygenase activity (0.15 U/mg protein), but no activity toward catechol. Surprisingly, the extracts prepared from succinate-grown JS3065 cells also exhibited activity (0.07 U/mg protein) toward gentisate. Additional analysis revealed 5 putative gentisate 1,2-dioxygenase genes in the genome of JS3065. Remarkably, 2 chromosomal gentisate 1,2-dioxygenase genes are contiguous and no identifiable regulator genes were found adjacent to them. The multiple copies of gentisate 1,2-dioxygenase genes and relaxed regulation likely contribute to the constitutive expression of gentisate 1,2-dioxygenase in strain JS3065.

DISCUSSION

This study describes the isolation and characterization of *Sphingobium* sp. strain JS3065, the first strain capable of utilizing 1-NN as sole source of carbon, nitrogen, and energy for growth under aerobic conditions. Two lines of evidence indicate that the 1-nitronaphthalene pathway in strain JS3065 is derived from the naphthalene pathway. First, the *nin* cluster, responsible for 1-nitronaphthalene catabolism in strain JS3065, is closely related to the *nag*-like naphthalene catabolic gene cluster from *Ralstonia* sp.

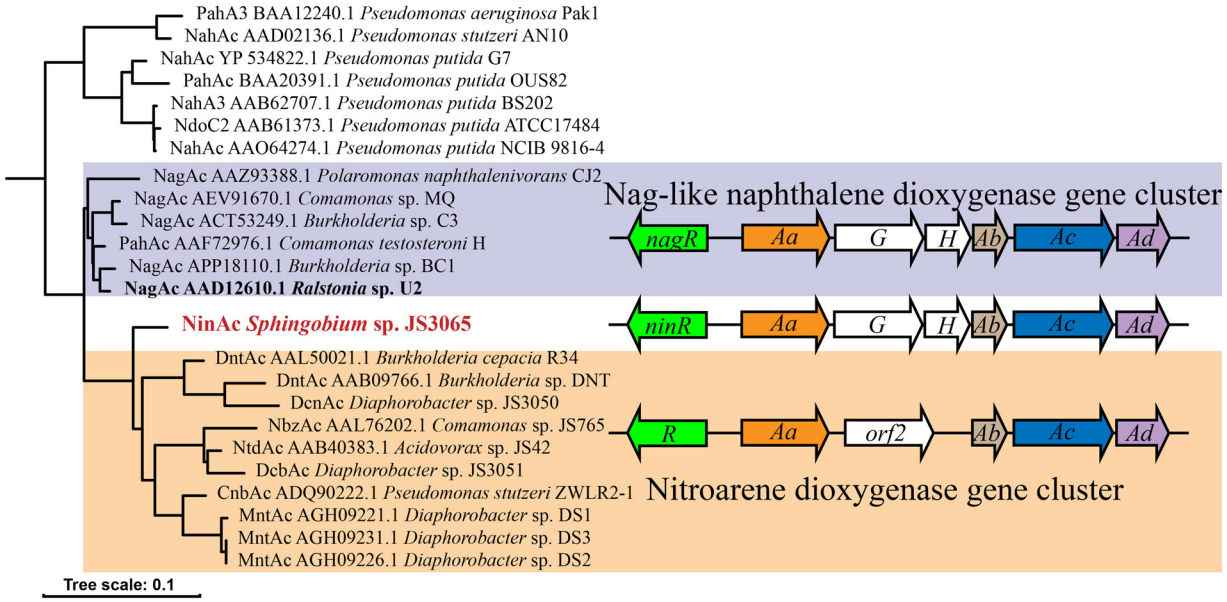


FIG 5 Phylogenetic tree of NinAc homologs. The tree was constructed by the neighbor-joining method. The clades in the brown box are the Nag-like nitroarene dioxygenases and the clades in the violet box are the Nag-like naphthalene dioxygenases. 1-Nitronaphthalene dioxygenase is shown in red. The organization of the gene clusters corresponding to dioxygenases is shown on the right.

strain U2. It is clear that minimal modification of the substrate specificity of the initial dioxygenase would be necessary and sufficient to enable a naphthalene pathway to accommodate 1-NN. Second, the 1-nitronaphthalene dioxygenase retains the ability to catalyze the dioxygenation of naphthalene to form naphthalene dihydrodiol (Table 2).

During the evolutionary process, the naphthalene utilizing capacity in strain JS3065 seems to have been lost, as indicated by the failure to grow on naphthalene despite the activity of NinA with naphthalene (Table 2). The *nin* cluster has all of the full-length genes required for naphthalene utilization except for the gene encoding the *cis*-naphthalene dihydrodiol dehydrogenase (NinB) which is unnecessary for the catabolism of 1-NN. There is a 54 bp truncation on the 5′ end of *ninB*, and a frameshift mutation at position 701 bp corresponding to the intact *nagB* in strain U2. Based on the comparison with the structure of *cis*-naphthalene dihydrodiol dehydrogenase (44), the N-terminal truncated sequence of NinB is in the substrate binding site and contributes to the binding of NAD^+. Because no other *cis*-naphthalene dihydrodiol dehydrogenase homolog is evident in the genome of strain JS3065, the inactivation of NinB would preclude naphthalene utilization without affecting 1-NN utilization. The putative aldolases (NagQ and NinQ) are unnecessary for the catabolism of both naphthalene and 1-NN (28). Therefore, the apparent truncation (186 bp) on the 5′ end of *ninQ* compared to *nagQ* (Fig. 2B) is not expected to affected the metabolic flux. These remanent genes in the *nin* cluster support the argument that the *nin* genes share a recent common ancestor with the *nag* genes.

There are indications that the *nin* cluster can be horizontally transferred. The pNIN2 plasmid has a putative 190 bp *oriT* sequence identical to that of the plasmid pLB1 and the genes for conjugative transfer are closely related in these 2 plasmids, indicating that pNIN2 is likely self-transmissible as the plasmid pLB1 (39). Moreover, the presence of transposon-associated elements including Tn*3* and IS*21* transposases (Fig. S2 and Table S1) adjacent to the *nin* cluster indicates potential for lateral transfer of the 1-nitronaphthalene catabolic pathway.

Nitroarene dioxygenases have long been known to share an ancestor with Nag-like naphthalene dioxygenases (17). The catalytic α subunit NinAc of the 1-nitronaphthalene dioxygenase described here shared a common ancestor with the extant naphthalene dioxygenases more recently than any of the other known Nag-like nitroarene dioxygenases (Fig. 5). The observations support the hypothesis that an ancestral 1-nitronaphthalene

dioxygenase was an early step in the evolutionary trajectory from naphthalene dioxygenase to nitroarene dioxygenases. The differences in the extant sequences are clear, but the details of the events that led to the conversion of naphthalene dioxygenase to nitroarene dioxygenase are a mystery. The nature of the ancestral nitroarene dioxygenase that seems to have been the progenitor not only of nitronaphthalene dioxygenase but also the other known *nag*-like nitroarene dioxygenases requires further investigation.

In many cases involving patchwork assembly of catabolic pathways for nitroaromatic compounds, it is difficult to see how the evolutionarily intermediate steps conferred a selective advantage (17, 30). Here, in contrast, a minimal evolutionary change would convert an ancestral naphthalene pathway to a nitronaphthalene pathway without introduction of additional genes. In an environment with substantial availability of nitronaphthalene, a small change in the initial dioxygenase would confer tremendous selective advantage by allowing full use of the molecule for carbon, nitrogen, and energy. Nitronaphthalene might even be a better growth substrate than naphthalene because it contains nitrogen that can be assimilated.

Naphthalene dioxygenases are known to catalyze the dioxygenation of other PAHs such as phenanthrene, anthracene, and fluorene (45, 46), but only monooxygenation is observed for monoaromatic compounds (47). The 1-nitronaphthalene dioxygenase described here readily catalyzes dioxygenation of nitro-substituted benzenes but is unable to act on other nitro-PAHs substrates (Table 2). Previous work has established that evolution of the large subunit controls the substrate specificity of the nitroarene dioxygenases (48–50). The F350T mutation in the large subunit (NagAc) of naphthalene dioxygenase from *Ralstonia* sp. strain U2 enables this enzyme to catalyze denitration of 2,6-dinitrotoluene (51). In contrast, the corresponding residue at position 350 of NinAc is valine, which is identical to that from 2-chloronitrobenzene dioxygenase (CnbAaAbAcAd), 3-nitrotulene dioxygenase (MntAaAbAcAd) and 2,4-dinitrotulene dioxygenase (DntAaAbAcAd). When Val was substituted by Phe at position 350 of MntAc, 3-nitrotulene dioxygenase was no longer active (49), indicating that the valine 350 is likely essential for denitration activities of these enzymes. In addition, alignment of the amino acid sequence of NinAc with its homologs reveals that His258 is unique to NinAc (Fig. S3). For naphthalene dioxygenases, the corresponding residues are valine and for other nitroarene dioxygenases, the corresponding resides are asparagine, valine, or isoleucine. Asn258 of the nitrobenzene dioxygenase catalytic large subunit was reported to be essential for substrate binding and its denitration activity through a hydrogen bond with the nitro group of the substrate (52). Analysis of the structure of nitronaphthalene dioxygenase will be required to evaluate the molecular determinants of substrate specificity and enable development of a strategy for directed evolution for application to other nitro-PAH compounds.

Salicylate and gentisate are 2 key intermediates of the 1-NN catabolic pathway in strain JS3065. Although gentisate 1,2-dioxygenase activity was observed with the extracts of JS3065 cells, it is noteworthy that strain JS3065 failed to grow on gentisate or salicylate. *Ralstonia* sp. strain U2 is also unable to utilize gentisate as growth substrate, but introduction of a gentisate transporter (GenK) from *Corynebacterium glutamicum* ATCC13032 into U2 cells converts it to a gentisate utilizer (53, 54). In strain JS3065, no plausible homologue of the gentisate transporter gene is found in the genome. Therefore, it is likely that transport is a limiting step in gentisate utilization by strain JS3065. On the other hand, strain U2 exhibits robust growth using salicylate as carbon and energy sources, whereas strain JS3065 was unable to grow on salicylate. Transport inefficiency would be a plausible explanation for this phenotype rather than failed induction of the catabolic pathway. Salicylate is the inducer of the *nag* operon from strain U2 as well as the *nag*-like nitroarene dioxygenase gene clusters. All these *nag*-like gene clusters contain a divergently transcribed gene encoding LysR family transcriptional regulators capable of responding to salicylate (55–57). It seems that the induction of the *nin* gene cluster in strain JS3065 is also by salicylate due to the presence of a LysR regulator (NinR) homologous to NagR and salicylate is on the 1-nitronaphthalene pathway.

MATERIALS AND METHODS

Isolation and growth of 1-NN-degrading bacteria. The bacterium *Sphingobium* sp. strain JS3065 was isolated from soil collected at a former chemical manufacturing site in NJ, USA, where environmental contaminants include 1-NN. Enrichments for isolation of 1-NN degraders were conducted in nitrogen-free 1/2 strength minimal salts broth (MSB) (58), pH 7.0, supplemented with 1-NN (100-200 μM). The enrichments were incubated at 30°C in a shaking incubator at 180 rpm in Erlenmeyer flasks. Isolation and growth of the pure culture was done on agar plates containing 1/2 strength MSB with 1-NN crystals in the lids as sole carbon and nitrogen source. Taxonomic identification of the isolate was performed with the universal 16S rRNA gene primers, 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′).

Growth of pure 1-NN degrading bacteria was conducted in 1/2 strength MSB containing 100–200 μM 1-NN as sole carbon, nitrogen and, energy sources. Utilization of the substrate was indicated by HPLC analysis and by nitrite accumulation in the culture. Biomass accumulation was estimated by measurement of protein after completion of growth at various concentrations of 1-NN. Protein concentration was estimated by the bicinchoninic acid (BCA) method (59). Nitrite concentration was quantified by the Griess method (60).

Genome sequencing and analysis. Total genomic DNA was extracted from 1-NN-grown cells of strain JS3065 using an UltraClean microbial DNA isolation kit (Mo Bio Laboratories, Inc.). DNA Sequencing was performed with the Pacific Bioscience (PacBio) RS technology by the Shanghai OE Biotech Co., Ltd. The raw reads were initially assembled with Falcon (61), and the resultant consensus sequences were processed with Circulator (62) for circularization, resulting in the closed genome sequence. The gene annotation was performed with RAST Artemis online (https://rast.nmpdr.org/).

Plasmid construction and DNA manipulation. To express the putative 1-nitronaphthalene dioxygenase, a DNA fragment containing *ninAaAbAcAd* was amplified from *Sphingobium* sp. strain JS3065 genomic DNA using the primer set *ninA*-F-HindIII (5′- gaccatgattacgccAAGCTTATGGAACTGGTAGTAGAACCCCTC-3′) and *ninA*-R-EcoRI (5′-aaaacgacggccagtGAATTCTCACAGGAAGATTAGCAGGTTGTG-3′). The underlined sequences are restriction enzyme recognition sites, and the lowercase sequences are homologous arms used for ligation to convenient vectors by homologous recombination. The above fragment was ligated to the linearized vector pUC19 or pRK415 digested with restriction endonucleases HindIII and EcoRI, resulting in constructs pRK415-*ninA* and pUC19-*ninA*. The *ninAaAbAcAd* was constitutively expressed from the *lac* promoters of the vectors.

Biotransformation of 1-NN. *E. coli* DH5α cells expressing recombinant 1-nitronaphthalene dioxygenase from plasmid pUC19-*ninA* were cultured in lysogeny broth (LB) containing ampicillin (100 μg/mL). The culture was harvested by centrifugation during stationary growth (OD_{600nm} 1.2–1.5), washed twice with 50 mM Tris-HCl buffer (pH 7.4) and suspended in MSB to an OD_{600} of 1.0. The *E. coli* DH5α cells harboring the pUC19 vector were used as control. The reaction mixtures containing 1-NN ($\sim$ 50 μM) were incubated on a rotary shaker at 30°C and 180 rpm. Samples were removed at appropriate intervals for measurement of the concentrations of 1-NN and nitrite in the mixture. The samples collected at 5 min were used for preparation of samples for GC-MS analyses.

Determination of the substrate specificities. The substrate specificity of 1-nitronaphthalene dioxygenase toward a variety of nitroaromatic compounds was investigated by a whole-cell biotransformation method based on the rate of nitrite release. All chemicals were purchased from Sigma-Aldrich with the following exceptions: 1,2-dihydroxynaphthalene, 2-nitrotoluene, 2,3-dichloronitrobenzene, 2,3-dichloronitrobenzene, 2-nitrofluorene and 9-nitrophenanthrene (Macklin, China). Nitroaromatic substrates were used at a final concentration of 0.2 mM. Biotransformation of the substrates by *E. coli* cells carrying pUC19-*ninA* was performed as described above. The denitration activities were calculated based on the nitrite concentrations at 15 min for each substrate. The products for each substrate were analyzed by GC-MS.

Growth of *Ralstonia* sp. strain U2 on 1-NN. Plasmids pRK415 and pRK415-*ninA* were introduced into *Ralstonia* sp. strain U2 by conjugative mating. The donors *E. coli* S17-1 γ-*pir* carrying pRK415 or pRK415-*ninA* and the recipient *Ralstonia* sp. strain U2 were grown in LB media with appropriate antibiotics to an OD_{600nm} of 0.6. Mating procedures were performed according to a method described previously (63). The presence of introduced plasmids in strain U2 was confirmed by PCR and sequencing. The resulting *Ralstonia* strains harboring pRK415 or pRK415-*ninA* were tested for growth on 1-NN at various concentrations in MSB containing 2‰ (wt/vol) XAD-7 beads (Sigma-Aldrich) to minimize the toxicity of the substrate. The flasks inoculated with *Ralstonia* strains were incubated on a rotary shaker (30°C and 180 rpm) and the culture turbidity was monitored over time.

Enzyme assays. Crude extracts were prepared from 1-NN or succinate-grown cells of strain JS3065 by ultrasonication and subsequent centrifugation (16,000 $\times$ *g*, 40 min). All assays were performed in 0.5 mL of 50 mM Tris-HCl buffer (pH 7.6) containing 30 μg of protein from the crude extracts and gentisate (0.1 mM). Gentisate1,2-dioxygenase activity was assayed by measuring the increase in absorbance at 330 nm. The molar extinction coefficient of the product, maleylpyruvate, was taken as 13, 000 $M^{-1}cm^{-1}$ (64). One unit of enzyme activity is expressed as the production of 1 μmol of product per min at 30°C. Specific activities are expressed as units per mg of protein.

Analytical methods. The concentration of 1-NN was quantified by HPLC (Waters e2695 Separation Module) with a C18 reversed-phase column (5 μm, 4.6 $\times$ 250 mm). The eluent was monitored at 280 nm. The mobile phase consisted of water (A) and methanol (B), containing 0.1% (vol/vol) acetic acid in both. The elution gradient was 20% of solvent B for 5 min, then increased to 90% B over 30 min. GC-MS analyses were conducted on a TRACE 1310 gas chromatograph (Thermo Fisher Scientific) using a capillary column HP-5MS (0.25 mm $\times$ 30 m, Agilent Technologies). The conditions were as follows: injection volume, 1 μL; interface temperature, 290°C; source temperature, 230°C; column temperature program: initial temperature 70°C for 2 min, raised to 130°C at 5°C/min, increased to 180°C at 10°C/min, increased to 285°C at 5°C/min, hold for 1 min. Mass spectrometer conditions were: 33–750 *m/z* mass range at an

electron energy of 70 eV, EI energy source. After the aromatic substrates were biotransformed, the clarified supernatants were extracted with 3 volumes of sodium hydroxide-washed ethyl acetate. The fractions containing the products were evaporated to dryness. The dried samples were dissolved in acetonitrile and added to equal volume of *N*,*O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA) for trimethylsilyl (TMS) derivatization at 60°C for 30 min. All the products were analyzed by GC-MS. All proposed products were identified by comparison of retention times and mass spectra with those of authentic standards. The standards were purchased from Sigma with the following exceptions: 3-chlorocatechol (TCI), 1,2-dihydroxynaphthalene (TCI) and 3,4-dichlorocatechol (CFW Laboratories Inc).

Ethical statement. This work does not include any human or animal materials.

Data availability. The complete genome of *Sphingobium* sp. strain JS3065 is available in the NCBI database under BioProject identifier (ID) PRJNA867112 or BioSample accession SAMN30184334.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

ACKNOWLEDGMENTS

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We declare that there are no conflicts of interest in this work.

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