Coinducible Catabolism of 1-Naphthol via Synergistic Regulation of the Initial Hydroxylase Genes in *Sphingobium* sp. Strain B2

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ABSTRACT 1-Naphthol, a widely used raw material for organic synthesis, is also a well-known organic pollutant. Due to its high toxicity, 1-naphthol is rarely used by microorganisms as the sole carbon source for growth. In this study, catabolism of 1-naphthol by *Sphingobium* sp. strain B2 was found to be greatly enhanced by additional supplementation with primary carbon sources (e.g., glucose, maltose, and sucrose), and 1-naphthol was even used as the carbon source for growth when strain B2 cells had been preinduced by both 1-naphthol and glucose. A distinct two-component flavin-dependent monooxygenase, NdcA1A2, was found to be responsible for the initial hydroxylation of 1-naphthol to 1,2-dihydroxynaphthalene, a more toxic compound. Transcriptional levels of *ndcA1A2* genes were significantly upregulated when strain B2 cells were cultured with both 1-naphthol and glucose compared to cells cultured with only 1-naphthol or glucose. Two transcriptional regulators, the activator NdcS and the inhibitor NdcR, were found to play key roles in the synergistic regulation of the transcription of the 1-naphthol initial catabolism genes *ndcA1A2*.

IMPORTANCE Cometabolism is a widely observed phenomenon, especially in the field of microbial catabolism of highly toxic xenobiotics. However, the mechanisms of cometabolism are ambiguous, and the roles of the obligately coexisting growth substrates remain largely unknown. In this study, we revealed that the roles of the coexisting primary carbon sources (e.g., glucose) in the enhanced catabolism of the toxic compound 1-naphthol in *Sphingobium* sp. strain B2 were not solely because they were used as growth substrates to support cell growth but, more importantly, because they acted as coinducers to interact with two transcriptional regulators, the activator NdcS and the inhibitor NdcR, to synergistically regulate the transcription of the 1-naphthol initial catabolism genes *ndcA1A2*. Our findings provide new insights into the cometabolic mechanism of highly toxic compounds in microorganisms.

KEYWORDS 1-naphthol, NdcA1A2, coinducible catabolism, cometabolism, synergistic regulation

Many organic compounds with high toxicity are recalcitrant to biodegradation and very difficult for microorganisms to use as the sole carbon and energy sources for growth. Sometimes, they can be partially metabolized by microorganisms in the obligate presence of a growth substrate (e.g., glucose and acetate), and this phenomenon is commonly called cometabolism (1–3). Several explanations, including the broad substrate specificity of catabolic enzymes, imperfect coupling between enzyme specificity and gene regulation, and metabolic interdependence of the catabolic

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cosubstrate, have been used to elucidate the mechanism of cometabolism (2). However, the specific mechanism behind cometabolism in individual cases is not always clear. In particular, the roles of obligately coexisting growth substrates in cometabolism remain largely unclear.

1-Naphthol is an important raw material for the organic synthesis of pharmaceuticals, pesticides, and dyestuffs (4, 5) and is also the major intermediate during the degradation of carbaryl (6), napropamide (7, 8), and polycyclic aromatic hydrocarbons (PAHs) (9). Because of its common use, 1-naphthol is frequently detected in soil, water, and even the ocean (10, 11). 1-Naphthol has been reported to be more toxic than its parent compound, naphthalene, and to inhibit the growth, motility, and biofilm dispersion of *Pseudomonas aeruginosa* and *P. putida* (12). Due to its high toxicity, 1-naphthol is resistant to biodegradation by microorganisms, and only a few fungi (*Fusarium solarii*, *Cunninghamella elegans*, and *Heliscus lugdunensis*) (13, 14) and bacteria (*Pseudomonas*, *Arthrobacter*, and *Rhodococcus* species) are reported to partially degrade 1-naphthol (10). To the best of our knowledge, rare strains have been reported to be capable of utilizing 1-naphthol as the sole carbon source for growth (15).

In our previous study, 1-naphthol was found to accumulate as the intermediate during the catabolism of (*S*)-napropamide [(*S*)-NAP] in *Sphingobium* sp. strain B2 (see Fig. S1A in the supplemental material), when (*S*)-NAP was added as the sole carbon source in minimal salt medium (MSM) (7, 8). 1-Naphthol was very slowly degraded in MSM (without other carbon sources) by strain B2, and no cell growth of strain B2 was observed. However, the catabolism of 1-naphthol by strain B2 was greatly enhanced when glucose was also added (7), which is similar to the phenomenon of cometabolism. In this study, the roles of the obligately coexisting primary carbon sources in the enhanced catabolism of 1-naphthol in strain B2 were revealed. They not only support cell growth but also, more importantly, act as coinducers to synergistically regulate the transcription of the 1-naphthol initial hydroxylase genes *ndcA1BA2*. Our findings will enhance our understanding of the cometabolism mechanism of toxic compounds in microorganisms.

RESULTS

Cometabolism of 1-naphthol by strain B2 in the presence of glucose. It was found that approximately 0.1 mM 1-naphthol accumulated at 12 h when 0.1 mM (*S*)-NAP was used as the sole carbon source by strain B2 (inoculated at an optical density at 600 nm [OD₆₀₀] of 0.25) in MSM (see Fig. S1B in the supplemental material). The 1-naphthol remained undegraded until 24 h (Fig. S1C) and was degraded to an undetectable level at 108 h, with the OD₆₀₀ of strain B2 cells decreasing from the initial 0.25 to 0.18 (Fig. 1A). In contrast, when 0.8 mM glucose was additionally supplemented in the MSM, 0.1 mM 1-naphthol was quickly degraded to an undetectable level (within 6 h), and the cell OD₆₀₀ increased from the initial 0.25 to 0.34 (Fig. 1A), showing that glucose significantly promoted 1-naphthol catabolism (Fig. 1A and Fig. S1C).

Role of the primary carbon sources in the catabolism of 1-naphthol. The effects of different primary carbon sources (at 0.8 mM) on cell growth and 1-naphthol degradation by strain B2 were investigated. Strain B2 utilized sucrose as the preferential carbon source for growth, followed by maltose, glucose, xylose, and sodium pyruvate (Fig. S2A). However, glucose and maltose, instead of sucrose, best promoted the degradation of 1-naphthol (Fig. S2B). We found that the degradation efficiency of 1-naphthol by treatment with inoculum at an OD₆₀₀ of 0.6 (the maximum OD₆₀₀ value when strain B2 cells grew on 0.8 mM sucrose with the initial OD₆₀₀ of 0.25) in MSM was far lower than that of the treatment with inoculum at an OD₆₀₀ of 0.25, with addition of 0.8 mM glucose (Fig. S2B). All these data suggested that the promotion of 1-naphthol degradation by glucose was not solely due to the cell growth effect.

Interestingly, strain B2 cells that had been simultaneously preinduced by both glucose and 1-naphthol quickly degraded 0.1 mM 1-naphthol even in the absence of glucose (Fig. 1B and Fig. S1D). In contrast, strain B2 cells individually preinduced by glucose or 1-naphthol did not exhibit an obvious degradation of 1-naphthol within

FIG 1 1-Naphthol degradation by strain B2 and identification of the metabolite produced from 1-naphthol. (A) Degradation of 1-naphthol (0.1 mM) and growth of strain B2 in the presence or absence glucose. Glu (+), in the presence of 0.8 mM glucose; Glu (-), in the absence of 0.8 mM glucose. (B) Degradation of 1-naphthol and growth of strain B2 using different preinduced cells in the absence of glucose. Solid blue triangle, growth (glucose preinduced); solid black square, growth (1-naphthol preinduced); solid red circle, growth (1-naphthol + glucose preinduced); empty blue triangle, degradation (glucose preinduced); empty black square, degradation (1-naphthol preinduced); empty red circle, degradation (1-naphthol + glucose preinduced). (C and D) Degradation of 1-naphthol by the wild-type strain B2, $\Delta ndcA1$ mutant, and $B2\Delta ndcA1$ -pBBRndcA1 complemented strain in the absence (C) or presence (D) of glucose. (E) HPLC spectra of the metabolites catalytically produced from 1-naphthol by NdcA1A2 in the presence of 1.2 mM sodium borohydride. (F) Tandem mass spectrometry analysis of the metabolite (1,2-dihydroxynaphthalene; RT = 5.467 min) produced from 1-naphthol.

24 h in MSM (Fig. S1D), indicating that the catabolism of 1-naphthol by strain B2 was coincuded by both glucose and 1-naphthol. Compared to the cell number decrease of strain B2 individually preinduced by 1-naphthol or glucose, a cell number increase from 1.3×10^8 CFU/ml to 1.5×10^8 CFU/ml was observed for strain B2 simultaneously preinduced by both glucose and 1-naphthol during the degradation of the sequentially added 0.4 mM 1-naphthol in MSM (without glucose) (Fig. 1B). All these data showed that 1-naphthol could be used by strain B2 as the sole carbon source for growth if the cells had been preinduced by both 1-naphthol and glucose. The phenomenon observed in our study is somewhat different from the traditional cometabolism, where xenobiotics are thought to be partially metabolized and not utilized as carbon and energy sources (16).

FIG 2 Proposed catabolic pathway of 1-naphthol and the catabolic genes involved. (A) Proposed catabolic pathway of 1-naphthol in strains B2 and C5pp. (B) Phylogenetic analysis of NdcA1 and McbC revealing their relationships to other flavin-dependent monooxygenase family members from subgroup D and subgroup A. (C) Genetic organization of the 1-naphthol catabolic gene clusters *ndc* and *mcb* in strains B2 and C5pp.

The *ndcA1BA2* gene cluster is responsible for 1-naphthol catabolism. 1-Naphthol has been reported first to be hydroxylated to 1,2-dihydroxynaphthalene and then enter the tricarboxylic acid (TCA) cycle via the salicylate pathway in *Pseudomonas* sp. strain C5pp (Fig. 2A) (17). To clone the genes involved in 1-naphthol catabolism in strain B2, sequences of previously reported enzymes related to downstream catabolism of 1-naphthol were subjected to local BLAST searches against the genome sequence of strain B2 (GenBank accession number [VDBZ000000000](https://www.ncbi.nlm.nih.gov/nuccore/VDBZ000000000)) using the tblastn program in BioEdit, because we supposed that enzymes for the downstream catabolism of 1-naphthol are conserved. Fortunately, a gene cluster containing the downstream catabolic genes for 1-naphthol was found in the genome of strain B2 (Fig. 2C and Table S1). Upstream of these catabolic genes, three genes (*ndcA1*, *ndcB*, and *ndcA2*), which were originally annotated to encode 4-nitrophenol 2-monooxygenase (57.9% identity; Swiss-Prot database), bifunctional riboflavin kinase/flavin mononucleotide (FMN) adenylyltransferase (44.2% identity), and NADH-dependent flavin reductase (46.1% identity), respectively, were found and supposed to be involved in 1-naphthol hydroxylation (Table S1). In accordance with this, the comparative transcriptome data also showed that the *ndcA1BA2* genes were significantly upregulated in strain B2 cells

TABLE 1 Comparison of the transcriptional levels of some catabolic related genes by comparative transcriptome sequencing

Culture conditions ^a	Fold change for:						
	16S rRNA gene	<i>rpoD</i>	<i>ndcA1</i>	<i>ndcB</i>	<i>ndcA2</i>	<i>ndcR</i>	<i>ndcS</i>
TRD1/control	1.00	0.83	7.35	6.74	5.52	0.71	0.58
TRD2/control	1.01	0.70	0.99	1.77	2.31	0.52	1.80
TRD3/control	1.00	0.99	456.49	614.31	563.10	1.33	2.09
TRD4/control	1.02	0.84	0.99	1.95	2.24	0.68	3.03

^aControl, no substrate; TRD1, cultured with 0.1 mM 1-naphthol; TRD2, cultured with 0.8 mM glucose; TRD3, cultured with 0.1 mM 1-naphthol + 0.8 mM glucose; TRD4, cultured with 1.6 mM glucose.

that were cultured with both 1-naphthol and glucose compared to those solely cultured with 1-naphthol or glucose (Table 1).

As we expected, knockout of *ndcA1* (Fig. S3) resulted in the complete loss of the capacity to degrade 1-naphthol in the $\Delta ndcA1$ mutant, and the complementation of *ndcA1* (pBBR*ndcA1*) restored the degradation in the B2 $\Delta ndcA1$ -pBBR*ndcA1* complemented strain whether glucose was present or not (Fig. 1C and D). These data confirmed that *ndcA1* is the key gene for 1-naphthol catabolism. In the absence of glucose, the OD₆₀₀ values of B2, B2 $\Delta ndcA1$, and B2 $\Delta ndcA1$ -pBBR*ndcA1* strains all decreased when cultured with 0.1 mM 1-naphthol. In the presence of 0.8 mM glucose, the OD₆₀₀ values of B2 and B2 $\Delta ndcA1$ -pBBR*ndcA1* strains increased much more than that of the B2 $\Delta ndcA1$ strain during the degradation of 0.1 mM 1-naphthol, further confirming that 1-naphthol could be used by strain B2 as the carbon source for growth (Fig. S4A and B).

The gene *ndcA1* encodes the oxygenase component, which determines substrate recognition. The gene *ndcA2* encodes the NADH-dependent flavin reductase, constituting the two-component monooxygenase system together with NdcA1. The *ndcB* gene encodes the bifunctional riboflavin kinase/FMN adenylyltransferase that catalyzes the following two-step reactions: the conversion of riboflavin to FMN and the adenylylation of FMN to FAD (18), which is the required cofactor for flavin-dependent monooxygenases. Phylogenetic analysis showed that NdcA1, together with the oxygenase components of 4-hydroxyphenylacetate 3-hydroxylase (HpaB) and phenol 2-hydroxylase (PheA1), belong to the flavin-dependent subgroup D monooxygenases, which usually catalyze aromatic ring hydroxylation or *N*-hydroxylation reactions (Fig. 2B).

NdcA1A2 hydroxylates 1-naphthol to generate 1,2-dihydroxynaphthalene.

NdcA1 and NdcA2 were expressed as fusion proteins in *Escherichia coli* BL21(DE3) with an N-terminal His tag and purified by Ni²⁺ chelation affinity chromatography (Fig. S5A and B). The mixture of NdcA1 and NdcA2 (1:10 molar ratio of NdcA1 to NdcA2) showed hydroxylation activity toward 1-naphthol. Because the standard compound 1,2-dihydroxynaphthalene (retention time [RT], 5.43 min) could be spontaneously oxidized to the red compound 1,2-naphthoquinone (RT, 4.47 min) (19, 20), sodium borohydride was added to our reaction system to inhibit this kind of oxidation. The metabolite catalytically produced from 1-naphthol by NdcA1A2 showed an RT of 5.46 min in high-performance liquid chromatography (HPLC) (Fig. 1E), almost equal to that of the standard compound 1,2-dihydroxynaphthalene. With a molecular ion peak [M-H]⁻ at *m/z* 159.04 in liquid chromatography-mass spectrometry (LC-MS) (Fig. 1F), the metabolite was finally identified to be 1,2-dihydroxynaphthalene. These results confirmed that NdcA1A2 catalyzed the *ortho*-hydroxylation at the 2-position of 1-naphthol to generate 1,2-dihydroxynaphthalene.

The kinetic parameters and the catalytic efficiencies (k_{cat}/K_m) of NdcA1A2 (1:10 molar ratio of NdcA1 to NdcA2) against 1-naphthol are a K_m of $49.0 \pm 6.4 \mu\text{M}$, a k_{cat} of $1.6 \pm 0.1 \text{ s}^{-1}$, and a k_{cat}/K_m value of $(3.2 \pm 0.1) \times 10^{-2} \text{ s}^{-1} \mu\text{M}^{-1}$ (means $\pm$ standard deviations from triplicate assays). The optimal temperature and pH for the activity of NdcA1A2 toward 1-naphthol were 30°C and 5.0, respectively (see Fig. S6A and B in the supplemental material). SDS (10%, mass/vol) and cetyltrimethylammonium bromide

FIG 3 Transcriptional levels of *ndcA1BA2* genes in strain B2 cells under different culture conditions. (A, B, and C) Relative transcriptional levels of *ndcA1*, *ndcA2*, and *ndcB* genes in strain B2 cells under different culture conditions. a, 0.1 mM 1-naphthol; b, 0.8 mM glucose; c, 0.1 mM 1-naphthol plus 0.8 mM glucose; d, 1.6 mM glucose; e, 3.2 mM glucose; f, 1.6 mM sucrose; g, 1.6 mM maltose; h, 1.6 mM xylose; i, 1.6 mM sodium pyruvate; j, blank. The *trfA* gene was used as the internal reference, and the data in each column were calculated by the $2^{-\Delta\Delta CT}$ method. (D) The transcriptional levels of the genes under different treatments, as revealed by comparative transcriptome analysis. FPKM, fragments per kilobase per million; control, no substrate; TRD1, 0.1 mM 1-naphthol; TRD2, 0.8 mM glucose; TRD3, 0.1 mM 1-naphthol plus 0.8 mM glucose; TRD4, 1.6 mM glucose.

(CTAB) (10%, mass/vol) inhibited approximately 10 to 16% of the activity of NdcA1A2, but phenylmethylsulfonyl fluoride (PMSF) (100 mM), Tween 20 (10%, mass/vol), and Tween 80 (10%, mass/vol) inhibited approximately 77 to 82% of the activity (Table S2).

Coinducible transcription of *ndcA1BA2*. The six segments overlapping each other, covering *ndcA1*, *ndcB*, and *ndcA2* genes, could be amplified from the cDNA transcribed from the RNA of strain B2, showing that the gene cluster *ndcA1BA2* was cotranscribed (Fig. S7A). The gene cluster *ndcA1BA2* was found to be located on a plasmid according to the genome sequencing data. To avoid the disturbance of plasmid copy number on transcriptional data, the gene *trfA* (the initial replication gene of plasmid) was used as the internal reference. When strain B2 cells were cultured with both 0.1 mM 1-naphthol and 0.8 mM glucose, reverse transcription-quantitative PCR (RT-qPCR) data showed that the transcriptional levels of *ndcA1*, *ndcA2*, and *ndcB* at 2 h were 6.6- to 65.7-, 1.4- to 9.6-, and 2.2- to 21.3-fold of that when cells were cultured with only 1-naphthol or glucose (Fig. 3A to C). Other primary carbon sources (e.g., sucrose, maltose, xylose, and sodium pyruvate) alone or high concentrations of glucose (1.6 to 3.2 mM) did not promote the transcriptions of these catabolic genes. If the 16S rRNA gene was used as the internal reference, the transcriptional data agreed with that using *trfA* as the internal reference, while the transcriptional levels of catabolic genes increased even more in the 1-naphthol plus glucose treatments (Fig. S8A to C). When strain B2 cells had been preinduced by both 1-naphthol and glucose, the transcriptional levels of *ndcA1*, *ndcA2*, and *ndcB* at 2 h were 22.5- to 48.7-, 14.2- to 32.5-, and 19.6- to 32.7-fold of that individually preinduced by 1-naphthol or glucose (using 16S rRNA gene as the internal reference) (Fig. S9A to C).

Comparative transcriptome analysis also confirmed the coinducible transcription of *ndcA1BA2* genes (Fig. 3D). Compared to TRD1 (cultured with 0.1 mM 1-naphthol), 3,205 genes were upregulated, and 16 genes, including *ndcA1BA2*, were upregulated by

FIG 4 Degradation of 0.1 mM 1-naphthol by wild-type strain B2 (WT), regulator mutants, and complemented strains in MSM (in the absence of glucose). (A) Degradation of 1-naphthol by $\Delta ndcR$ mutant, $\Delta ndcS$ mutant, and $\Delta ndcR \Delta ndcS$ double mutant. (B) Degradation of 1-naphthol by mutants complemented with *ndcR* (pBBR*ndcR*). (C) Degradation of 1-naphthol by mutants complemented with *ndcS* (pBBR*ndcS*). The cells were inoculated at an initial OD₆₀₀ of 0.25 in all treatments.

more than 50 times in TRD3 (cultured with 0.1 mM 1-naphthol plus 0.8 mM glucose). Compared to TRD2 (cultured with 0.8 mM glucose), 2,991 genes were upregulated, and 11 genes, including *ndcA1BA2*, were upregulated by more than 100 times in TRD3. Compared to no substrate treatment (control), *ndcA1*, *ndcB*, and *ndcA2* were 456.49-, 614.31-, and 563.10-fold upregulated, respectively, in TRD3 (Fig. 3D and Table 1). The *rpoD* gene and 16S rRNA genes were not found to be significantly upregulated in TRD3 (Table 1). In brief, the RT-qPCR and comparative transcriptional data further confirmed that the catabolism of 1-naphthol in strain B2 is coinducible. The phenomenon found in our study had never been mentioned before in the field of cometabolism, and we propose that glucose and other primary carbon sources (or their metabolic intermediates) play important roles in the transcriptional regulation of the initial catabolic genes *ndcA1BA2*.

Synergistic regulation of the transcription of *ndcA1BA2* by transcriptional activator NdcS and inhibitor NdcR. Two regulatory genes, *ndcS* and *ndcR*, located upstream of the *ndcA1BA2* cluster, were found (Fig. 2C). In addition, a catabolite regulator/repressor binding site was also found near the promoter region of *ndcA1BA2* (Fig. S7B). Based on the conserved domain analysis, NdcS and NdcR belong to LysR and IclR family transcriptional regulators, which are commonly reported to be involved in the regulation of microbial catabolic genes for xenobiotics (21, 22).

When *ndcR* was knocked out, the $\Delta ndcR$ mutant could degrade 0.1 mM 1-naphthol to an undetectable level within 3 h even in the absence of glucose (Fig. 4A and Fig. S10A). The complementation of *ndcR* (pBBR*ndcR*) to the $\Delta ndcR$ mutant delayed 1-naphthol degradation again, and 24 h was needed to degrade 0.1 mM 1-naphthol (Fig. 4B). These results showed that NdcR acted as a negative regulatory protein (transcriptional inhibitor) for 1-naphthol catabolism in strain B2.

Interestingly, the $\Delta ndcS$ mutant (Fig. S10B) lost the ability to degrade 1-naphthol, while the $\Delta ndcS \Delta ndcR$ double mutant restored weak degradation ability toward 1-naphthol (Fig. 4C). The complementation of *ndcS* (pBBR*ndcS*) in both the $\Delta ndcS$ mutant and the $\Delta ndcS \Delta ndcR$ double mutant significantly promoted 1-naphthol degradation, showing that NdcS acted as a positive regulatory protein (transcriptional activator) for 1-naphthol catabolism in strain B2.

Moreover, the transcriptional levels of *ndcA1*, *ndcA2*, and *ndcB* in the $\Delta ndcR$ mutant were about 10-fold higher than that of the wild-type strain B2 under no inducer conditions. When 1-naphthol was used as the inducer alone, the transcriptional levels in the $\Delta ndcR$ mutant were increased about 100- to 200-fold compared to the wild type (Fig. 5A to C), while glucose alone did not significantly promote the transcription levels in the $\Delta ndcR$ mutant. All these data indicated that NdcR constitutively bound to the promoter area and inhibited the transcription of *ndcA1BA2*, and 1-naphthol instead of glucose (or their metabolites) activated the promotion function of NdcS.

In the $\Delta ndcS$ mutant, the transcriptional levels of *ndcA1*, *ndcA2*, and *ndcB* were low under any inducing conditions. Similar phenomena were observed in the $\Delta ndcS \Delta ndcR$

FIG 5 Transcriptional levels of *ndcA1* (A), *ndcA2* (B), and *ndcB* (C) in strain B2 (WT), $\Delta ndcR$ mutant, $\Delta ndcS$ mutant, and $\Delta ndcR \Delta ndcS$ double mutant, cultured with 0.1 mM 1-naphthol, 0.8 mM glucose, or 0.1 mM 1-naphthol plus 0.8 mM glucose. Cells with neither 1-naphthol nor glucose were used as the control (Φ). The transcriptional levels were determined at 2 h. The 16S rRNA gene was used as an internal standard. The data in each column were calculated by the $2^{-\Delta\Delta CT}$ method using 3 replicates.

double mutant (Fig. 5A to C), showing that NdcS was the prerequisite for high-level transcription. The simultaneous supplementation of glucose and 1-naphthol could not promote the transcriptional levels in the $\Delta ndcS$ mutant, indicating that the relief of the inhibition function of NdcR by glucose (or its metabolites) was not enough for the high-level transcription of *ndcA1BA2* (Fig. 5A to C).

DISCUSSION

Cometabolism is usually defined as a process by which a normally nonbiodegradable substance is biodegraded only in the presence of an additional carbon source, and it is a widely observed phenomenon in various fields, including wastewater treatment, bioelectricity generation, and especially the biodegradation of highly toxic xenobiotics (23–26). However, few studies on the mechanisms underlying cometabolism have been carried out.

In our study, the catabolism of 1-naphthol by strain B2 in MSM was found to be greatly enhanced by the supplementation with glucose or other primary carbon sources. This kind of metabolic phenomenon should fall into the category of cometabolism

FIG 6 Proposed model of the coinducible catabolism of 1-naphthol in *Spingobium* sp. strain B2. The transcription of the 1-naphthol initial catabolic gene cluster *ndcA1BA2* is proposed to be synergistically coregulated by two signal molecules, 1-naphthol and the primary carbon source (e.g., glucose), via two regulators, the activator NdcS and the inhibitor NdcR. NdcR might constitutively bind to the promoter area of *ndcA1BA2* and inhibit the transcription of these catabolic genes, preventing the sudden production of the more toxic compound 1,2-dihydroxynaphthalene. When glucose is present, it is partially used as the carbon source by strain B2 to cope with the toxic effects of 1-naphthol and 1,2-dihydroxynaphthalene. Glucose or its metabolites then might act as inducers to relieve the inhibition function of NdcR. At this time, the transcription of *ndcA1BA2* is at a low level. On the basis of the relief of NdcR, the additional presence of 1-naphthol will significantly promote the transcription of *ndcA1BA2* by activating the function of NdcS. When *ndcA1BA2* genes are normally transcribed, 1-naphthol is finally mineralized, providing carbon and energy sources for strain B2 cells.

in a broad sense. However, the specific metabolic phenomenon we observed is somewhat different from the previously so-called cometabolism. The glucose and some other primary carbon sources in our study not only were used as growth substrates to support cell growth but also played important roles in the synergistic regulation of the transcription of initial catabolic genes. The transcriptional levels of the initial hydroxylase genes *ndcA1BA2* were significantly upregulated only when strain B2 cells were cultured with both 1-naphthol and glucose and are precisely controlled by two transcriptional regulators (NdcR and NdcS) (Fig. 6). The transcriptional inhibitor NdcR might constitutively bind to the promoter area of *ndcA1BA2* and inhibit the transcriptions of these initial catabolic genes. When glucose (or another primary carbon source) is present, it (or its metabolites) might act as an inducer to relieve the inhibition of NdcR. At this point, *ndcA1BA2* are transcribed at a relatively low level. Under the prerequisite of the relief of NdcR, the additional presence of 1-naphthol might interact with the transcriptional activator NdcS, significantly promoting the transcriptions of *ndcA1BA2* at a high level. In general, 1-naphthol and glucose (or its metabolic intermediates), two kinds of signaling molecules, work in coordination in a switch analogous to a two-input logic AND gate (27), precisely triggering the initial catabolic gene transcriptions. This is a new regulatory mechanism that has never been reported in the field of cometabolism. Interestingly, these two regulator genes, *ndcR* and *ndcS*, and the *ndcA1BA2* gene cluster are conserved in various bacteria, including *Spingobium* sp. strain C3 (28, 29),

Sphingobium quisquiliarium DC-2 (30), and *Rhizobium* sp. strain X9 (31), which also showed similar cometabolism phenomena for 1-naphthol (data not shown).

What is the significance of the precise regulation of 1-naphthol catabolism in strain B2? It was found that 1-naphthol caused high toxicity to strain B2 cells, which is in accordance with other reports (17, 32). Unexpectedly, the 1,2-dihydroxynaphthalene catalytically produced from 1-naphthol by NdcA1A2 showed even higher toxicity to strain B2 cells than 1-naphthol (see Fig. S11 in the supplemental material), partially explaining why the catabolic rate of 1-naphthol should be precisely regulated in strain B2. Primary carbon sources have been confirmed to play important roles in stress resistance in bacteria by altering the cell conditions (25, 33, 34). Reducing equivalents produced from carbon sources metabolized via the TCA cycle have also been reported to be capable of enhancing the degradation of the azo dye Reactive Black 5 in *Pseudomonas* and *Burkholderia* species (26). It is reasonable that strain B2 triggers the expression of NdcA1A2 to catabolize 1-naphthol only when it first senses the presence of the primary carbon sources, which are used as the carbon and energy sources by strain B2 to cope with the toxic effects of 1-naphthol and 1,2-dihydroxynaphthalene. Otherwise, the constitutive/sudden production of 1,2-dihydroxynaphthalene from 1-naphthol by NdcA1A2 would cause severe harm to cells. In addition, bacteria such as *Escherichia coli* have been reported to produce acidic by-products (e.g., acetate) during glucose metabolism (35). By coincidence, the optimum pH for NdcA1A2 activity was 5.0, which was different from that of most monooxygenases, with an optimal pH range of 6.0 to 8.0 (36, 37). Nevertheless, the relationships between *ndcA1A2* transcription and primary carbon source metabolism still need further investigation, and the detailed regulatory mechanisms for the precise catabolism of 1-naphthol by the two regulators (NdcR and NdcS) will be elaborated in another study.

MATERIALS AND METHODS

Chemicals. 1-Naphthol (99% purity) and 1,2-dihydroxynaphthalene (98% purity) were purchased from J&K Scientific Ltd. (Shanghai, China). Sodium borohydride (99% purity), methanol (chromatography grade), and acetic acid (chromatography grade) were purchased from Merck Co., Ltd. (Darmstadt, Germany).

Strains, plasmids, primers, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 2, and the primers used are listed in Table 3. *Sphingobium* sp. strain B2 was grown at 30°C in Luria-Bertani (LB) medium or cultured in MSM (pH 7.0) supplemented with 0.1 mM 1-naphthol or additionally supplemented with 0.8 mM primary carbon sources (e.g., glucose). *E. coli* strains were grown at 37°C in LB medium. Antibiotics were added at the following concentrations (mg/liter): ampicillin (Amp), 100; kanamycin (Km), 50; and streptomycin (Str), 100.

Degradation of 1-naphthol with or without primary carbon sources. Strain B2 cells were cultured in LB medium at 30°C until the logarithmic phase. The cells were pelleted by centrifugation at 6,000 rpm for 10 min, washed twice with sterilized MSM, and then inoculated into 25 ml of MSM (containing 0.1 mM 1-naphthol) at an OD₆₀₀ of 0.25. To determine the effect of primary carbon sources on the degradation of 1-naphthol, 0.8 mM glucose, sucrose, maltose, xylose, or sodium pyruvate was added. No supplementation of any primary carbon source was used as the control. For the degradation of 1-naphthol by a large amount of cells, strain B2 cells were inoculated at an initial OD₆₀₀ of 0.6. The cultures were shaken in an incubator at 180 rpm and 30°C, and samples (1 ml) were collected at regular intervals to measure the OD₆₀₀ value and the concentration of 1-naphthol. All experiments were carried out in triplicate.

Degradation of 1-naphthol by preinduced B2 cells. MSM containing 0.8 mM glucose, 0.1 mM 1-naphthol, or 0.1 mM 1-naphthol plus 0.8 mM glucose was inoculated with the washed cells of strain B2 at a final OD₆₀₀ of 0.25. These cultures were shaken at 180 rpm and 30°C for 2 h, and then the cells were recovered and rewashed twice with MSM. The rewashed cells were again inoculated at an OD₆₀₀ of 0.25 into 25 ml of MSM containing 0.1 mM 1-naphthol (without glucose). 1-Naphthol (0.1 mM) was supplemented three times every 2 h. The number of cells of strain B2 and the concentration of 1-naphthol were checked at regular intervals. All experiments were carried out in triplicate.

Toxic effect of 1-naphthol and 1,2-dihydroxynaphthalene on strain B2 cells. To compare the toxicity of 1-naphthol, 1,2-dihydroxynaphthalene, and 1,2-naphthaquinone, strain B2 cells in the mid-log phase were pelleted by centrifugation, washed twice with MSM, and then inoculated into 25 ml of MSM containing 0.1 mM 1-naphthol, 1,2-dihydroxynaphthalene, or 1,2-naphthaquinone at an OD₆₀₀ of 0.25. Samples (100 µl) were taken every 3 h and then spread on LB agar plates containing 100 mg/liter streptomycin after appropriate dilution. The numbers of cells grown on agar were counted to calculate the survival rate of strain B2 cells under different treatments.

Gene knockout and complementation. To knock out the *ndcA1*, *ndcR*, or *ndcS* gene, approximately 1,000-bp upstream and downstream regions flanking the *ndcA1*, *ndcR*, or *ndcS* gene were amplified and then cloned into pAK405 by a ClonExpress II one-step cloning kit (Vazyme, Nanjing, China). The

TABLE 2 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Source or reference
<i>Sphingobium</i> species		
B2	Wild type; Sm ^r	7, 8
B2Δ <i>ndcA1</i>	Strain B2 with <i>ndcA1</i> gene deleted; Sm ^r	This study
B2Δ <i>ndcA1</i> -pBBR <i>ndcA1</i>	B2Δ <i>ndcA1</i> mutant complemented with <i>ndcA1</i> gene	This study
B2Δ <i>ndcS</i>	Strain B2 with <i>ndcS</i> gene deleted; Sm ^r	This study
B2Δ <i>ndcS</i> -pBBR <i>ndcS</i>	B2Δ <i>ndcS</i> mutant complemented with <i>ndcS</i> gene	This study
B2Δ <i>ndcR</i> Δ <i>ndcS</i>	Strain B2 with <i>ndcR</i> and <i>ndcS</i> genes deleted; Sm ^r	This study
B2Δ <i>ndcR</i> Δ <i>ndcS</i> -pBBR <i>ndcS</i>	B2Δ <i>ndcR</i> Δ <i>ndcS</i> mutant complemented with <i>ndcS</i> gene	This study
<i>E. coli</i>		
DH5α	F [−] <i>recA1 endA1 thi-1 supE44 relA1 deoR</i> Δ(<i>lacZYA-argF</i>)U169 ϕ80d/ <i>lacZ</i> ΔM15	TaKaRa
BL21(DE3)	F [−] <i>ompT hsdSB(r_B[−] m_B[−]) gal dcm</i> (DE3); T7 RNA polymerase gene under control of the <i>lacUV5</i> promoter	Invitrogen
Plasmids		
pET-28a(+)	Expression vector, Km ^r	Novagen
pET <i>ncdA1</i>	pET-28a(+) carrying <i>ndcA1</i> , Km ^r	This study
pET <i>ncdA2</i>	pET-28a(+) carrying <i>ndcA2</i> , Km ^r	This study
pAK405	<i>oriT nptII rpsL1 oriV</i> ; Km ^r	47
pAK <i>ndcA1</i>	pAK405 with a 2-kb EcoRI-BamHI PCR-amplified fragment upstream and downstream of <i>ndcA1</i>	This study
pBBR1MCS-2	Broad-host-range cloning vector; Km ^r	48
pBBR <i>ndcA1</i>	pBBR1MCS-2 derivative carrying <i>ndcA1</i> ; Km ^r	This study
pMD19-T	TA cloning plasmid; Amp ^r	TaKaRa

^aSm^r, Km^r, and Amp^r, resistance to streptomycin, kanamycin, and ampicillin, respectively.

corresponding plasmids were electroporated into strain B2, and single-crossover recombinants were selected on LB agar containing Km (50 mg/liter). Individual colonies were selected and cultured in LB medium for 36 h, and then double-crossover recombinants were screened on LB agar containing Str (100 mg/liter). The Δ*ndcA1*, Δ*ndcR*, and Δ*ndcS* mutants were finally confirmed by PCR analysis using the primer pair Δ*ndcA1*-F (5'-ATGAAAGACGATGTCAAGCCCAAC-3')/Δ*ndcA1*-R (5'-TCATTTCTGGAAGCCGCTCGC-3'), Δ*ndcR*-F (5'-ATGAAACGCTTCATAAGGTCTGG-3')/Δ*ndcR*-R (5'-TTAGTTGGGATCGTGATGGCG-3'), and Δ*ndcS*-F (5'-ATGTATCTCAAGCGGTGAAGTATT-3')/Δ*ndcS*-R (5'-TCAAAGTTTGGTGGCAATCT-3'), respectively. Moreover, the Δ*ndcS* Δ*ndcR* double mutant was constructed by knocking out the *ndcS* gene of the Δ*ndcR* mutant using the method described above. The Δ*ndcS* Δ*ndcR* double mutant was confirmed by using the primer pairs Δ*ndcR*-F/Δ*ndcR*-R and Δ*ndcS*-F/Δ*ndcS*-R.

For gene complementation, the *ndcA1*, *ndcR*, or *ndcS* gene was inserted into the EcoRI and BamHI sites of pBBR1MCS-2 using a ClonExpress II one-step cloning kit (Vazyme, Nanjing, China) to generate pBBR*ndcA1*, pBBR*ndcS*, or pBBR*ndcR*. These recombinant plasmids were electroporated into the Δ*ndcA1*, Δ*ndcS*, Δ*ndcR*, and Δ*ndcR* Δ*ndcS* mutants, respectively, and selected on LB agar supplemented with Km (50 mg/liter) and Str (100 mg/liter). The mutant strain transformed with the empty plasmid pBBR1MCS-2 was used as the negative control. The degradation of 1-naphthol by wild-type strain B2, various mutants (Δ*ndcA1*, Δ*ndcR*, and Δ*ndcS* mutants and the Δ*ndcS* Δ*ndcR* double mutant), and complemented strain (Δ*ndcA1*pBBR*ndcA1*, Δ*ndcR*pBBR*ndcR*, Δ*ndcS*pBBR*ndcS*, Δ*ndcR*Δ*ndcS*pBBR*ndcR*, and Δ*ndcR*Δ*ndcS*pBBR*ndcS*) were tested as described above in the presence or absence of 0.8 mM glucose. The initial inoculum was set at an OD₆₀₀ of 0.25.

Purification of NdcA1 and NdcA2. The *ndcA1* and *ndcA2* genes were expressed in *E. coli* BL21(DE3) and purified as described previously, with minor modifications (38). *E. coli* BL21(DE3) strains harboring the recombinant plasmids [pET-28a(+)-*ndcA1*/*ndcA2*] were cultured in LB medium containing Km (50 mg/liter) at 37°C until the OD₆₀₀ reached 0.6. Protein expression was induced at 16°C with 1.0 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 12 h. The cultures were collected by centrifugation at 10,000 × *g* and 4°C for 10 min and washed twice with MSM. The cells expressing NdcA1 or NdcA2 were suspended in TDA buffer (50 mM Tris-H₂SO₄, 0.5 mM dithiothreitol [DTT], 0.5 mM ascorbic acid, 10% [vol/vol] glycerol, pH 7.4). The cells were then disrupted by sonication (ultrasonic cell crusher; Thermo Fisher Scientific, USA) and centrifuged at 14,000 × *g* at 4°C for 30 min. NdcA1 and NdcA2 were purified using a Ni-nitrilotriacetic acid-Sepharose column (Sangon Biotech, Shanghai, China). NdcA1 and NdcA2 were dialyzed in Tris-H₂SO₄ buffer (pH 7.4) overnight to remove imidazole and Ni²⁺. The purified proteins were analyzed by SDS-PAGE, and the protein concentrations were determined by the Bradford method (39).

Enzymatic activity assays. Enzymatic activity assays of NdcA1A2 were performed in a 500-μl reaction system (acetic acid-sodium acetate buffer, pH 5.0). For kinetic assays, 25, 50, 80, 100, 150, and

TABLE 3 Primers used in this study

Purpose	Plasmid or mutant	Primer	Sequence (5' to 3')
Construction of gene expression plasmids	pET- <i>ndcA1</i>	<i>ndcA1_NdeI</i> <i>ndcA1_XhoI</i>	GTGCCGCGCGGCAGCCATATGAAAGACGATGTCAAGCCCAACG GTGGTGGTGGTGGTCTCGAGTCATTCTGGAAGCCGGCT
	pET- <i>ndcA2</i>	<i>ndcA2_NdeI</i> <i>ndcA2_XhoI</i>	GTGCCGCGCGGCAGCCATATGTCTGGTTTGTACCCGCGC GTGGTGGTGGTGGTCTCGAGTCATTCTCGGCCGTGGC
Construction of gene deletion plasmids	pAK- <i>ndcA1</i>	$\Delta ndcA1_TopF$	CTATGACATGATTACGAATTCAAGTTAATGAGTCTGGAGCCTAGGC
		$\Delta ndcA1_TopR$	GCTCTTTCTCTCTCCAAAGATCCG
		$\Delta ndcA1_BotF$	TTTGGGAGGAGGAAAAGAGCCGCGATGCGGCCGG
		$\Delta ndcA1_BotR$	CAGGTCGACTCTAGAGGATCCTCCGGGATCACCGGCAGT
	pAK- <i>ndcR</i>	$\Delta ndcR_TopF$	CTATGACATGATTACGAATTCTGAAAGATTGGCGGCAG
		$\Delta ndcR_TopR$	TAGGGGCGGTGCGCCTTCTCCCCCGT
		$\Delta ndcR_BotF$	AGGAAGGCGCACCGCCCTAAGCTGGCC
		$\Delta ndcR_BotR$	CAGGTCGACTCTAGAGGATCCTCCAGACTCATTAACTTTAGAGCCAG
	pAK- <i>ndcS</i>	$\Delta ndcS_TopF$	CTATGACATGATTACGAATTCTGCTTACCCAGGCCGCTG
		$\Delta ndcS_TopR$	CTCATAGGGGCGCAAGATCAG
		$\Delta ndcS_BotF$	TGATCTTGCGCCCTATGAGGGTACCCCCCTCCCTCC
		$\Delta ndcS_BotR$	CAGGTCGACTCTAGAGGATCCCATACCAGGCCTTCGCGTT
Confirmation of gene deletions	B2 $\Delta ndcA1$	$\Delta ndcA1$ -F	ATGAAAGACGATGTCAAGCCCAAC
		$\Delta ndcA1$ -R	TCATTTCTGGAAGCCGGCTCGC
	B2 $\Delta ndcR$ $\Delta ndcS$	$\Delta ndcR$	ATGAAAACGCTTCATAAGGTCCG
		$\Delta ndcS$ -F	
	B2 $\Delta ndcS$	$\Delta ndcR \Delta ndcS$ -R	TTAGTTGGGATCGTGATGGCG
		$\Delta ndcS$ -F	ATGTATCTCAAGCGTTGAAGTATTT
Construction of gene complementation plasmids	pBBR <i>ndcA1</i>	pBBR- <i>ndcA1</i> -F	GATAAGCTTGATATCGAATTCGGGTACCCCCCTCCCTCC
		pBBR- <i>ndcA1</i> -R	CGCTCTAGAACTAGTGGATCCTCATTCTGGAAGCCGGCT
	pBBR <i>ndcS</i>	pBBR- <i>ndcS</i> -F	GATAAGCTTGATATCGAATTCGCTCAAGCTTCACGATTTTTCG
		pBBR- <i>ndcS</i> -R	CGCTCTAGAACTAGTGGATCCTCAAAGTTTTCGCTGCGAATCT
	pBBR <i>ndcR</i>	pBBR- <i>ndcR</i> -F	GATAAGCTTGATATCGAATTTCTTCTCCTCCCAAAGATCCG
		pBBR- <i>ndcR</i> -R	CGCTCTAGAACTAGTGGATCCTAGTTGGGATCGTGATGGCG
Identifying transcription units		<i>ndc</i> -F1	CAAGCCCAACGCGGGGGTCA
		<i>ndc</i> -R1	AAGGCCGCTTCATAGGCAC
		<i>ndc</i> -F2	TCAAGGCAGCCAAGACGGCC
		<i>ndc</i> -R2	GTTGCGCCTGATCTTACCG
		<i>ndc</i> -F3	GGTGGTCGTCTCGGGCGCCA
		<i>ndc</i> -R3	CGCTTACCATGCACGGCAG
		<i>ndc</i> -F4	CGCGATCCTGGTGTTCGACA
		<i>ndc</i> -R4	CCCGGTGCCGTGGGTGCGGA
		<i>ndc</i> -F5	TTGACGCGACCGGGGTGTCG
		<i>ndc</i> -R5	CGCCTCGGGGCTGATCTATC
		<i>ndc</i> -F6	GACCGACGGCTACGTCCATC
		<i>ndc</i> -R6	TACGCCGGCAGCTACGAGGC
RT-qPCR		A1RT-F	TATCGCCGAGACGACGCGCG
		A1RT-R	CGAGAAGGAAACCGATGCCG
		A2RT-F	GGGGTCTTTCCGAGCGTGA
		A2RT-R	GTCTTCAGCGGCGGAAAATT
		BRT-F	TCGGCCACAGCTATGCGGCG
		BRT-R	CGAGGCGGCCTCGCTCCAAG
		16SRT-F	GCTTTCGACCTCAGCGTCAATG
		16SRT-R	AGCGCACGTAGGCGGCGATCTAA
		Trf-F/	ATTGGGTCTATCGCTCGGTT
		Trf-R	GCTTAGCCCGATCTTCCAT

200 μ M 1-naphthol was used in a reaction system containing NADH (1 mM), FAD (10 μ M), 6 $\times$ -His-NdcA1 (3 μ g), and 6 $\times$ -His-NdcA2 (9 μ g). The reaction mixture was incubated at 30°C for 5 min, the reaction was stopped by boiling for 1 min, and an equal volume of methanol was added. All data were collected from three independent determinations. One unit of enzymatic activity was defined as the amount of enzyme required to catalyze the transformation of 1 μ mol substrate per second at its optimal reaction temperature.

$V_{\max}$ and K_m values were estimated by fitting the initial rate data to the Michaelis-Menten equation using nonlinear regression with GraphPad Prism software (GraphPad Software Inc., San Diego, CA). All kinetic parameters were reported as the mean and standard deviation values derived from triplicate experiments. The optimum temperatures for the activities of NdcA1A2 were determined by comparing their enzymatic activities at 10 to 70°C. The optimum pH was determined by comparing the enzymatic activities in a pH range from 3.5 to 10.5. The following four buffers were used: 50 mM acetic acid-sodium acetate buffer (pH 3.5 to 6.0), 50 mM citric acid-sodium citrate buffer (pH 5.8 to 8.5), 50 mM Tris-HCl buffer (pH 7.7 to 9.0), and 50 mM glycine-NaOH buffer (pH 9.0 to 10.5).

The effects of inhibitors and detergents on enzymatic activity were studied as described previously (38). Inhibitors or detergents, such as PMSF, ethylenediaminetetraacetic acid (EDTA), SDS, and Triton X-100, were added at a final concentration of 100 mM, and CTAB, Tween 20, and Tween 80 were added at a final concentration of 10% (mass/vol).

Chemical determination and metabolite identification. 1-Naphthol was measured by HPLC (UltiMate 3000 RSLC; Thermo Fisher Scientific, Waltham, MA) (7). The metabolite produced from 1-naphthol was identified by HPLC-MS as described previously (40), with minor modifications. Because the product 1,2-dihydroxynaphthalene was readily oxidized to 1,2-naphthoquinone, 1.2 mM sodium borohydride was added to inhibit oxidation (41).

Transcriptional levels of *ndcA1BA2* revealed by RT-qPCR. To investigate different inducing conditions on transcriptions, strain B2 cells were first cultured and washed twice with sterilized MSM and then inoculated into 25 ml of MSM at an OD_{600} of 0.25. The cells were then cultured with one of the following: a, 0.1 mM 1-naphthol; b, 0.8 mM glucose; c, 0.1 mM 1-naphthol plus 0.8 mM glucose; d, 1.6 mM glucose; e, 3.2 mM glucose; f, 1.6 mM sucrose; g, 1.6 mM maltose; h, 1.6 mM xylose; i, 1.6 mM sodium pyruvate; and j, blank. The cells were incubated for 2 h and then sampled for investigation of the transcriptional levels of *ndcA1BA2* genes.

To investigate the effect of the two regulatory genes *ndcR* and *ndcS* on transcription, strain B2, $\Delta ndcR$ mutant, $\Delta ndcS$ mutant, and the $\Delta ndcS \Delta ndcR$ double mutant were cultured and washed twice with sterilized MSM. The washed cells were inoculated into 25 ml of MSM at a final OD_{600} of 0.25. The cultures were then supplemented with one of the following: a, 0.1 mM 1-naphthol; b, 0.8 mM glucose; c, 0.1 mM 1-naphthol plus 0.8 mM glucose; and d, no inducers. The cells were incubated for 2 h and then sampled for investigation of the transcriptional levels of *ndcA1BA2* genes.

For transcriptional analysis, total RNA of collected cells was isolated using TRIzol reagent (Thermo Fisher Scientific, USA). The isolated RNA was purified, and cDNA was synthesized with the HiScript III first-strand cDNA synthesis kit (+gDNA wiper) according to the manufacturer's instructions. To identify the transcription units along with *ndcA1BA2*, using the cDNA as templates, RT-PCR was performed using the primers listed in Table 3 by a method described previously, with minor modifications (42). To identify the transcriptional levels of *ndcA1*, *ndcB*, and *ndcA2* genes, the primer pairs A1RT-F/A1RT-R, A2RT-F/A2RT-R, BRT-F/BRT-R, 16SRT-F/16SRT-R, and TRT-F/TRT-R were used for the amplification of the *ndcA1*, *ndcA2*, *ndcB*, 16S rRNA, and *trfA* genes (Table 3), respectively, using cDNA as the template. RT-qPCR was performed in an Applied Biosystems 7300 real-time PCR system (Applied Biosystems, USA) with ChamQ SYBR color qPCR master mix (Vazyme Biotech Co., Ltd., Nanjing, China). All analyses were performed in triplicate, and the $2^{-\Delta\Delta CT}$ method was used for the quantitative analysis of the transcription of *ndcA1*, *ndcA2*, and *ndcB* genes in different treatments.

Comparative transcriptome analysis (RNA-seq). Strain B2 was cultured as described previously, inoculated into 100 ml of MSM to a final OD of 0.25, and then supplemented with the following: a, 0.1 mM 1-naphthol; b, 0.8 mM glucose; c, 0.1 mM 1-naphthol plus 0.8 mM glucose; d, 1.6 mM glucose; e, no substrate. The cells were cultured for 4 h and then collected by centrifugation, and total RNA was extracted using the TRIzol (Invitrogen, Carlsbad, CA) method by following the manufacturer's protocol. The isolated RNA was delivered to the Beijing Genomics Institute (BGI; Shenzhen, China) for reverse transcription and sequencing. Constructed mRNA libraries were sequenced using a BGISEQ500 platform. Clean reads were obtained by removing low-quality reads, and reads containing poly-N and adapters were mapped back onto the genome sequence of strain B2 using Bowtie2 (v2.2.5) (43, 44). The gene expression level of each sample was determined using RSEM v1.2.8 (43, 44). Genes with an adjusted *P* value of ≤ 0.05 , found by DESeq2, were considered significantly differentially expressed (45, 46).

Data availability. The data sets generated and analyzed in this study are available from the corresponding author upon request. *Sphingobium* sp. strain B2 has been deposited in the China Centre for Type Culture Collection (CCTCC) under accession number M2018684 (available for scientific study upon request [jiang_jjd@njau.edu.cn]). The sequences for the *ndcA1* (locus WP_145206854) and *ndcA2* (WP_145206855) genes and the genome of *Sphingobium* sp. strain B2 have been submitted to the GenBank databases under accession numbers [MT434350](#), [MT434351](#), and [VDBZ00000000](#), respectively.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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