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 mM). 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 mM 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′-aaaacgacggccagtGAATTCTCACAGGAAGATTAGCAGGTTGTC-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* DH5*α* cells expressing recombinant 1-nitronaphthalene dioxygenase from plasmid pUC19-*ninA* were cultured in lysogeny broth (LB) containing ampicillin (100 *μ*g/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* DH5*α* cells harboring the pUC19 vector were used as control. The reaction mixtures containing 1-NN (50 mM) 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 donor *E. coli* S17-1 *λ*-*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 × *g*, 40 min). All assays were performed in 0.5 mL of 50 mM Tris-HCl buffer (pH 7.6) containing 30 *μ*g of protein from the crude extracts and gentisate (0.1 mM). Gentisate 1,2-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 *μ*mol 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 *μ*m, 4.6 × 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 × 30 m, Agilent Technologies). The conditions were as follows: injection volume, 1 *μ*L; 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: 35–350 m/z mass range at an

electron energy of 70 eV, EI energy source. After the aromatic substrates were biotransformed, the ethyl acetate 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 and BioSample accession SAMN30184334.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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

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