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Constitutive Expression of a Nag-Like Dioxygenase Gene through an Internal Promoter in the 2-Chloronitrobenzene Catabolism Gene Cluster of *Pseudomonas stutzeri* ZWLR2-1

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ABSTRACT

The gene cluster encoding the 2-chloronitrobenzene (2CNB) catabolism pathway in *Pseudomonas stutzeri* ZWLR2-1 is a patchwork assembly of a Nag-like dioxygenase (dioxygenase belonging to the naphthalene dioxygenase NagAaAbAcAd family from *Ralstonia* sp. strain U2) gene cluster and a chlorocatechol catabolism cluster. However, the transcriptional regulator gene usually present in the Nag-like dioxygenase gene cluster is missing, leaving it unclear how this cluster is expressed. The pattern of expression of the 2CNB catabolism cluster was investigated here. The results demonstrate that the expression was constitutive and not induced by its substrate 2CNB or salicylate, the usual inducer of expression in the Nag-like dioxygenase family. Reverse transcription-PCR indicated the presence of at least one transcript containing all the structural genes for 2CNB degradation. Among the three promoters verified in the gene cluster, P1 served as the promoter for the entire catabolism operon, but the internal promoters P2 and P3 also enhanced the transcription of the genes downstream. The P3 promoter, which was not previously defined as a promoter sequence, was the strongest of these three promoters. It drove the expression of *cnbAcAd* encoding the dioxygenase that catalyzes the initial reaction in the 2CNB catabolism pathway. Bioinformatics and mutation analyses suggested that this P3 promoter evolved through the duplication of an 18-bp fragment and introduction of an extra 132-bp fragment.

IMPORTANCE

The release of many synthetic compounds into the environment places selective pressure on bacteria to develop their ability to utilize these chemicals to grow. One of the problems that a bacterium must surmount is to evolve a regulatory device for expression of the corresponding catabolism genes. Considering that 2CNB is a xenobiotic that has existed only since the onset of synthetic chemistry, it may be a good example for studying the molecular mechanisms underlying rapid evolution in regulatory networks for the catabolism of synthetic compounds. The 2CNB utilizer *Pseudomonas stutzeri* ZWLR2-1 in this study has adapted itself to the new pollutant by evolving the always-inducible Nag-like dioxygenase into a constitutively expressed enzyme, and its expression has escaped the influence of salicylate. This may facilitate an understanding of how bacteria can rapidly adapt to the new synthetic compounds by evolving its expression system for key enzymes involved in the degradation of a xenobiotic.

Evolutionary changes in a regulated gene promoter have been heavily studied, particularly changes in promoter sequences in the deployment of the transcriptional factors (1). Horizontal gene transfer can significantly alter the composition of bacterial regulons (2). The promoter sequence in a regulatory system can be changed by point mutation (3) or insertion of a transposable element (4) in order to allow the organism to adapt to environmental change. With the rapid development of the chemical industry, many synthetic compounds have been released into the environment in large quantities. This places selective pressure on microorganisms to develop their abilities to utilize these chemicals to grow. In general, a bacterium that has evolved pathways to metabolize toxic compounds as its sole source of carbon and energy must surmount three problems: (i) shift the specificities of key enzymes to run a complete pathway producing central intermediate metabolites, (ii) acquire or evolve a regulatory device for expression of the corresponding catabolism genes, and (iii) avoid the stress caused by the substrate itself or by its degradation of intermediates (5, 6).

In *Ralstonia* sp. strain U2, the catabolism of naphthalene is initiated by naphthalene dioxygenase NagAaAbAcAd (7). This Nag dioxygenase is thought to be the progenitor of nitroarene dioxygenases (collectively called Nag-like dioxygenase) described so far (8), including nitrobenzene dioxygenase (9), 2-nitrotoluene dioxygenase (10), 3-nitrotoluene dioxygenase (11), and 2,4-dinitrotoluene dioxygenase (12, 13). The synthetic nitro- and chloroaromatic compound, 2-chloronitrobenzene (2CNB), has been in the environment only for a short period of time. *Pseudomonas stutzeri* ZWLR2-1 utilizes 2CNB as a sole carbon and energy source (14). In this strain, a 2CNB catabolism cluster was formed

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TABLE 1 Bacterial strains and plasmids in this study

Bacterial strain or plasmid	Genotype or phenotype[a]	Reference(s) or source
Strains		
Pseudomonas stutzeri ZWLR2-1	2-Chloronitrobenzene utilizer, wild type	8, 14
Pseudomonas putida PaW340	Str^r Trp^-	44, 45
Escherichia coli DH5α	*supE44 lacU169* (ϕ80d*lacZ*ΔM15) *recA1 endA1 hsdR17 thi-1 gyrA96 relA1*	46
Plasmids		
pCM130	IncP and *colE1* origin, promoter probe vector, Tc^r	23
pCM*gfp-lacZ*	pCM130 containing *gfp* and new MCS	31
pCM130-P1	pCM130 carrying sequence between positions 2579 and 3869	This study
pCM130-P2	pCM130 carrying sequence between positions 8474 and 8969	This study
pCM130-P3	pCM130 carrying sequence between positions 10420 and 10760	This study
pCM*gfp*-P3*lacZ*	pCM*gfp-lacZ* carrying sequence between positions 10420 and 10760	This study
pCM*gfp*-P32*lacZ*	pCM*gfp-lacZ* carrying sequence between positions 10552 and 10760	This study
pCM*gfp*-P3M1*lacZ*	GG→AA mutation at position 10630 on foundation of pCM*gfp*-P3*lacZ*	This study
pCM*gfp*-P3M2*lacZ*	AC→GT mutation at position 10632 on foundation of pCM*gfp*-P3*lacZ*	This study
pCM*gfp*-P3M3*lacZ*	TA→CG mutation at position 10634 on foundation of pCM*gfp*-P3*lacZ*	This study
pCM*gfp*-P3M4*lacZ*	AA→GG mutation at position 10636 on foundation of pCM*gfp*-P3*lacZ*	This study
pCM*gfp*-P3M5*lacZ*	TG→CA mutation at position 10638 on foundation of pCM*gfp*-P3*lacZ*	This study

[a] Str^r, streptomycin resistant; Tc^r, ticarcillin resistant; MCS, multiple-cloning site; Trp^-, tryptophan auxotroph.

by a patchwork assembly of a Nag-like 2CNB dioxygenase (catalyzing the conversion of 2CNB to 3-chlorocatechol) gene cluster and a 3-chlorocatechol degradation cluster, in which several transposable elements are also present (8), as shown in Fig. 2. Among the 2CNB catabolism cluster, *cnbA* encodes 2CNB dioxygenase (*cnbAa* encodes ferredoxin reductase, *cnbAb* encodes ferredoxin, and *cnbAc* and *cnbAd* encode the α- and β-subunits of oxygenase, respectively), catalyzing the conversion of 2CNB to 3-chlorocatechol with the release of nitrite. CnbC is 3-chlorocatechol 1,2-dioxygenase catalyzing the ring cleavage of 3-chlorocatechol to 2-chloro-*cis*,*cis*-muconate. The 2CNB dioxygenase, together with other nitroarene dioxygenases, is thought to originate from the naphthalene dioxygenase NagAcAd in *Ralstonia* sp. strain U2, which is activated by the LysR transcriptional regulator NagR, with salicylate as an inducer (9, 13, 15–17). It has been speculated that horizontal gene transfer is involved in the rapid evolution of 2CNB catabolism pathway in strain ZWLR2-1 (8). However, the transcriptional regulator gene usually present in the Nag-like nitroarene dioxygenase gene cluster is absent from the 2CNB degradation cluster, leaving it unclear how this cluster is expressed under these circumstances. Considering that 2CNB is a xenobiotic that has existed on earth only since the onset of synthetic chemistry, it may well be a good example for studying the molecular mechanisms underlying rapid evolution in regulatory networks for the catabolism of synthetic compounds in the face of environmental challenges.

This study demonstrates that the 2CNB dioxygenase, unlike all its previously found homologs, is constitutively expressed, and an unusual internal promoter significantly increases the transcription of genes encoding the dioxygenase catalyzing the initial oxidation in 2CNB catabolism. This finding helps us understand how bacteria can rapidly adapt to new synthetic compounds through evolution of their expression systems for key enzymes involved in the degradation of a xenobiotic.

MATERIALS AND METHODS

Bacterial strains, chemicals, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in lysogeny broth (LB) medium at 37°C. *Pseudomonas putida* PaW340 strains were grown in LB medium at 30°C with 50 μg/ml streptomycin. *Pseudomonas stutzeri* ZWLR2-1 was grown at 30°C in LB or M9 medium (14) supplemented with 0.5 mM 2CNB or 10 mM succinate. For solid media, agar was added to a final concentration of 12 g/liter. When required, antibiotics were used at the following concentrations: ampicillin, 100 mg/liter; kanamycin, 50 mg/liter; and tetracycline hydrochloride, 20 mg/liter. Substrate 2CNB was purchased from Fluka Chemical Co. (Buchs, Switzerland), and 3-chlorocatechol was from TCI (Tokyo, Japan); catechol and *o*-nitrophenyl β-D-galactopyranoside (ONPG) were purchased from Sigma (St. Louis, MO). All primers used are listed in Table 2.

Plasmid construction. All DNA manipulation was performed according to standard procedures (18). Restriction enzymes, DNA polymerases, and T4 DNA ligase were purchased from New England BioLabs (Ipswich, MA), and TaKaRa (Dalian, China). A NOVA SLIC kit purchased from LCP Biomed Co. (Lianyungang, China) was used for ligation-independent cloning. The plasmid DNA preparation, DNA purification kit, and RNA preparation kit were purchased from Omega (Norcross, GA) and Tiangen (Beijing, China) and used according to the manufacturers' specifications. Plasmid DNA was transferred into *E. coli* and *Pseudomonas* species strains by chemical transformation or electroporation. All cloning procedures were verified by DNA sequencing (Tsingke, Wuhan, China).

Assays of 2CNB dioxygenase and chlorocatechol 1,2-dioxygenase from strain ZWLR2-1. Strain ZWLR2-1 was inoculated into 5 ml of M9 medium supplemented with 10 mM succinate and 0.5 g/liter $(NH_4)_2SO_4$ and cultured at 30°C for 12 h. Then, 4 ml of the cultures was inoculated into 400 ml of the same medium for another 12 h to build biomass. The cells were harvested by centrifugation and washed before being equally distributed into 5 portions and resuspended in 100 ml of M9 medium. While nothing was added to samples 3 and 4, 0.5 mM 2CNB was added to samples 1 and 2, and 1 mM salicylate was added to sample 5. After 4 h of incubation at 30°C, all five treated cell samples were harvested by centrifugation, washed, and resuspended in M9 medium (8).

For quantitative analysis of 2CNB dioxygenase, samples 1, 3, and 5 were resuspended in 5 ml of M9 medium containing 0.5 mM 2CNB. The supernatant of the reaction mixture was collected at appropriate intervals for dioxygenase activity analysis by measuring the nitrite released from the substrate. Nitrite was assayed as described elsewhere (9), using sodium nitrite as a standard.

Samples 2 and 4 were used for quantitative analysis of chlorocatechol

TABLE 2 Primers used in this study

Primer purpose and name	Sequence (5′ to 3′)	PCR-amplified product
RT-PCR		
PP1 cnb1603 s	GATTTCTACCTGTCGCCGACCCGC	PP1
PP1 cnb2165 a	TGGAGGACAAGCTGGGGGAAGATCG	
PP2 cnb2117 s	CCGTGACGACCAATCACATACAGGC	PP2
PP2 cnb2712 a	GATCGCGCAGCATGTGCTTATTTTC	
PP3 cnb2645 s	GGAAAATGTGGATTTCGATCTGCCG	PP3
PP3 cnb3153 a	CCTGGCGAATTCCGTTCACAATGTC	
PP4 cnb3590 s	TCCCTACAAGATCCCGGATCAAGGC	PP4
PP4 cnb4145 a	CTCGCGTATGCTCTGCCAGCTCTCC	
PP5 cnb3946 s	TGCGCTGTTCCACGACGGGGAGATC	PP5
PP5 cnb5633 a	CGGATCGCGTCTGGCTCCAACGGAC	
PP6 cnb5630 s	TCCGCGCTTTGCTCGAAAACATCTG	PP6
PP6 cnb6105 a	GCCCACGACGACAAAGACCTCATCC	
PP7 cnb7023 s	ATACGCCGATCTGCCCTGGCTATGG	PP7
PP7 cnb7439 a	CTGGTGGGTGAGCACCGCGCGACTG	
PP8 cnb8417 s	CGATGAAGCCAAGCTGCGCCACTAC	PP8
PP8 cnb8997 a	AAATCCGTCATCGTTCCGTCCGTCC	
PP9 cnb8827 s	CGCCGATCAGTGGCAACGGAATTAT	PP9
PP9 cnb9833 a	ACAGGGCGCGTGATTTGACGTGG	
PP10 cnb9659 s	GTGCGCCTTGTGGAGAGAGCTTTTG	PP10
PP10 cnb10164 a	ATGGCCCGGTGAGCCCTCGGTGATG	
PP11 cnb10467s	CTTCCTGTCCCAGGTCTACGCAAGC	PP11
PP11 cnb11065a	GGAGCCATAGCCCCAGCCGTGGTAA	
PP12 cnb11185s	CTGTTTTGATGCAGAAGCTCCCCCG	PP12
PP12 cnb12715a	GCAACAGAGCCCAGGCAGGCATCCC	
qRT-PCR[a]		
QCnbC3070s	GGACAAGAAGCGTTAGGAGCG	
QCnbC3225a	GACATCGAGCATAAGCGGAATC	
QrealE4253s	GGCGGTCTGTTCGTCTATG	
QrealE4353a	TCAAGCACCTCCTGATTGG	
QCnbF4772s	GCTTCGATCTCCGACCTCTTC	
QCnbF4885a	GAGACCATCCGCGTTTGCTTC	
QCnbAb5959s	GCCTTTTCGACATCGAGACAG	
QCnbAb6066a	TTCCCAGCATCACCTCCAAGC	
QCnbAa 6058 s	TGCTGGGAAGTCAGGCCGATG	
QCnbAa 6217 a	ATAGGGGCGGGCGCTCATAGG	
QCnbD 7296 s	ATGAGCGCAGAGGCCGCACTTC	
QCnbD 7454 a	GCGAACGATCAGGTAGCTCTGGTGG	
QcnbAc10812s	CTTTTCCAGCACGAGATGAAGACC	
QcnbAc10918a	GACGCCCATTTTGGCTGTGAC	
QCnbAd 12253 s	GCTTGGCTTGAACACTTCGTTGC	
QCnbAd 12374 a	TAGTTCTCGTTGTAGATGTTCACCG	
RT2085s	ACCTCTGACTGCCGCCAATCTTTGC	
RT5565s	CCGGGCCGTCGAGCTGATCTTCAAG	
RT7812a	GCTTCTCCTGCGCTTCGGCGATATC	
16S s1	CATGCTGAGAACCTGCCAGAG	
16S a1	CTTAGAGTGCCCACCATAACG	
Promoter screening		
PA13s EcoR I	CTGAATTCGTTCCCGAGTCGGTGTCGCCGAAC	PA
PA495a BamH I	CGGGATCCTCACAGATGCAGGCGGTCCTGG	
PB 1252s EcoR I	CGGAATTCAAGCTTGAGCATGCTTGGCG	PB
PB 1498a BamH I	CGCGGATCCAGCGCAGCCGCTTCTCCATC	
PC 2868s PstI	AAAACTGCAGGACGCTATGTCGATCGC	PC
PC 3175a HindIII	GCCCAAGCTTGCCTGACCAGGACCTGGC	
PD 10752s EcoR I	CGGAATTCGTGAGTGAAGCAGGGCTGAC	PD
PD 11285a BamH I	CGCGGATCCCCAGGCCACCAGAGTGCTTG	
PE 11120s EcoR I	CGGAATTCATGCCTGGGCTTGAAAGAAG	PE
PE 11504a BamH I	CGCGGATCCACATTACGCTCATTCCACTG	

(Continued on following page)

TABLE 2 (Continued)

Primer purpose and name	Sequence (5′ to 3′)	PCR-amplified product
PF 12197s PstI	AAAACTGCAGACTCCTGACCCGAGAAGCAC	PF
PF 12710a HindIII	ACCCAAGCTTCAGGCATCCCCAGGGTTCAC	
PG 879s EcoR I	GCGAATTCTGCGGGCACTTTGGTGCCGCGTG	PG
PG 1433a BamH I	CGGGATCCGACCGAAATGCCGCGTTCCGCC	
PH1601s EcoR I	TCGAATTCTACCTGTCGCCGACCCGC	PH
PH2075a BamH I	CGCGGATCCGATCAGGCGGCTGCTGCGAAATG	
PI 2027s PstI	AAAACTGCAGTGGGCATGCTCAACCACCATTTCG	PI
PI 2524a HindIII	GCCCAAGCTTATTGCCTGGTTCATTGGACATCC	
P1 2578 PstI	AAAACTGCAGACAACGACAATCTGGAAGCATG	P1
P13052 HindIII a	GCCGAAGCTTCCCTCACCGGGCATACCGA	
P2 8473 EcoRIs	CCGGAATTCTTTCGTCGCCACCATCAAGAACAAG	P2
P2 8968 PstI a	AAAACTGCAGTCCGCCAAGCATGCTCAAGC	
P3 10419EcoRIs	CCGGAATTCCATCGATGGCACCAGAGTG	P3
P3 10759PstIa	GGAACTGCAGTAAGTTTTGGTAACTCATAATTG	
5′ RACE		
cnbC R2	GGGCGCATTCGGCAGATAATAGGG	
cnbC R3	AATCTCGCCCGCTTCACCCGTTT	
cnbAc R2	GCAAAATAGATGCGTGCGTCCAACC	
cnbAc R3	CGTTCTGGCGGGAGACGATGACTT	
pCM*gfplacZ*-related vector construction		
pCM*gfplacZ* P3 s	CTCCACTGACAGAAAGCATGCCATCGATGGCACCAGAGTG	
pCM*gfplacZ* P3 a	TCATGGTCATGGATCCTAAGTTTTGGTAACTCATAATTG	
pCM*gfplacZ* P32 s	CTCCACTGACAGAAAGCATGCAGCCTTGTGCACACCCCTG	
pCM*gfplacZ* P32.5 s	CTCCACTGACAGAAAGCATGCATGCTCAAGCTGGACTAAATG	
−35 region mutation		
1 M ggaa a	CATTTAGTTTAGCTTGAGCATCACGCGCATG	
1 M ggaa s	CTCAAGCTAAACTAAATGCTCAAGCTGGACTAAAAC	
2 M acgt a	TTTAACCCAGCTTGAGCATCAC	
2 M acgt s	TGCTCAAGCTGGGTTAAATGCTCAAGCTG	
3 M tagc a	CATTGCGTCCAGCTTGAGCATCAC	
3 M tagc s2	CAAGCTGGACGCAATGCTCAAGCTGGACTAAAAC	
4 M aagg a	CACCTAGTCCAGCTTGAGCATCACG	
4 M aagg s2	CTCAAGCTGGACTAGGTGCTCAAGCTGGACTAAAAC	
5 M tgca a	TGTTTAGTCCAGCTTGAGCATCAC	
5 M tgca s	CTCAAGCTGGACTAAACACTCAAGCTGGACTAAAAC	

[a] qRT-PCR, reverse transcription-quantitative PCR.

1,2-dioxygenase. Cell extracts were prepared by resuspending bacterial pellets in ice-cold phosphate buffer (40 mM [pH 7.4]) and sonicating them in an ice-water bath at 3.0 s on and 7.0 s off for 20 min. Cell debris was removed by two rounds of centrifugation at 17,000 × *g* for 30 min at 4°C. Chlorocatechol 1,2-dioxygenase activity was determined by a spectrophotometric assay, using the method described by Spain and Nishino (19), with a Lambda 25 spectrophotometer (PerkinElmer/Cetus, Norwalk, CT). The reaction mixtures contained 50 mM phosphate buffer (pH 7.4), 50 μM 3-chlorocatechol, and cell extracts, and the reactions were initiated by the addition of substrate. The activity against 3-chlorocatechol was assayed by measuring the increase in absorbance at 260 nm due to the formation of 2-chloro-*cis*,*cis*-muconate. The molar extinction coefficient of 2-chloro-*cis*,*cis*-muconate is 17,100 M^{-1} cm^{-1}, according to Dorn and Knackmuss (20). One unit of enzyme activity is defined as the amount of activity required for the production of 1 μmol product per minute (8). Specific activity is expressed here as units per milligram of protein. Protein concentrations were determined using the Bradford method, with bovine serum albumin as the standard (21).

Stability assay for 2CNB degradation cluster. Considering that the 2CNB degradation genes are located on an unstable large plasmid in strain ZWLR2-1 (14), the following procedure was used to calculate the relative presence of the 2CNB degradation genes in individual colonies from samples 1 and 3 described above. The cells of strain ZWLR2-1 suspensions were serially diluted and spread on LB plates. After 2 days of incubation, the primer pair PE 11120s EcoR I and PE 11504a BamHI was used to PCR amplify a fragment of *cnbAc* to detect the presence of the 2CNB degradation gene cluster in colonies. At least 100 clones were randomly selected for PCR analysis in each case.

Reverse transcription-PCR and real-time PCR. Total RNA of strain ZWLR2-1 was extracted with an RNAprep pure kit for bacteria (Tiangen Biotech, Beijing, China). For reverse transcription-PCR, the RNA was incubated with 1.5 U of RQ1 RNase-free DNase (Promega, Madison, WI) and 20 U of RNase inhibitor (TaKaRa) at 37°C for 30 min to remove any DNA contamination. Reverse transcription-PCR (RT-PCR) was performed by using Moloney murine leukemia virus reverse transcriptase (Promega) and reverse primer PP12 cnb12715a. Negative controls were established without reverse transcriptase. Then, the reverse transcription products (cDNAs) were amplified for 30 cycles using appropriate primer pairs, as shown in Table 2.

For relative quantification, RNA was extracted from strain ZWLR2-1 cells incubated in the presence or absence of 2CNB and then reverse transcribed into cDNA with a PrimeScript RT reagent kit with the genomic

DNA (gDNA) Eraser (TaKaRa, Dalian, China). Real-time quantitative PCR (qPCR) was performed with a CFX Connect real-time PCR detection system (Bio-Rad, Hercules, CA) with iQ SYBR green Supermix (Bio-Rad, Hercules, CA), using the primers described in Table 2. A 160-bp fragment of the 16S rRNA gene of strain ZWLR2-1 was used as the reference to evaluate the relative difference in integrity between individual RNA samples. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in gene expression (22).

For absolute quantification, PCR was operated using primer pairs RT2085s-PP5 cnb5633a, RT5565s-RT7812a, and PP11 cnb10467s-PP12 cnb12715a. Three fragments of 3,548 bp, 2,247 bp, and 2,248 bp in size were recovered as templates for the standard curve. The concentration of the three fragments was determined by a NanoDrop 1000 (Thermo Scientific) to calculate the copy number of cDNA.

Assays of promoter activities by a reporter gene. Promoter screening was performed using Neural Network Promoter Prediction software (http://www.fruitfly.org/seq_tools/promoter.html). Plasmid pCM130 (23) and its derivatives containing possible promoter regions and a report gene *xylE* were transferred into surrogate host *Pseudomonas putida* PaW340 (24, 25) by electroporation at 1,800 V (Electroporator 2510; Eppendorf, Hamburg, Germany). For the catechol 2,3-dioxygenase assay, strain PaW340 carrying pCM130-derived plasmids was grown in 5 ml of LB medium overnight at 30°C, supplemented with streptomycin and tetracycline hydrochloride, and subsequently subcultured into 100 ml to grow for 4 h. Cells were washed with 20 mM phosphate buffer (pH 7.2), suspended in AP buffer (10% acetone [vol/vol] in 100 mM phosphate buffer [pH 7.5]), and broken by sonication for intervals of 3.0 s on and 7.0 s off for 20 min in an ice-water bath.

Cell debris was removed by centrifugation twice at 17,000 × *g* for 30 min at 4°C. Supernatants were diluted with AP buffer, if necessary. *xylE*-encoded catechol 2,3-dioxygenase was measured by monitoring the formation of 2-hydroxymuconic semialdehyde from catechol. The molar extinction coefficient of 2-hydroxymuconic semialdehyde is 33,000 M^{-1} cm^{-1} at 375 nm (26). One milliunit here corresponds to the formation at 30°C of 1 nmol 2-hydroxymuconic semialdehyde per minute (27).

β-Galactosidase assays were used to examine the expression of promoter-*lacZ* fusions in strain PaW340, which cannot degrade 2CNB. Preinocula of strain PaW340 harboring the relevant constructs were grown overnight in 5 ml of LB medium containing streptomycin and tetracycline hydrochloride at 30°C, and then 50 μl of the cells was inoculated into 5 ml of the same medium at 30°C for another 4 h. Finally, 1 ml of cells was harvested and resuspended in 50 mM PB buffer (pH 7.4). β-Galactosidase activity was determined in SDS- and chloroform-permeabilized cells, as described previously (28).

5′ RACE. The transcriptional start sites (TSSs) of the *cnbC* and *cnbAc* genes were determined by rapid amplification of cDNA ends (RACE), using a SMARTer RACE cDNA amplification kit (Clontech, Mountain View, CA). Total RNA was isolated from LB-grown strain ZWLR2-1. First-strand cDNA was synthesized with a random primer using SMARTScribe reverse transcriptase and linked to the anchor sequence in the same step. The tailed cDNA was then amplified using the primer pairs UPM-cnbCR2 and UPM-cnbAcR2. Finally, two nested-PCR products were obtained with the primer pairs NUP-cnbCR3 and NUP-cnbAcR3, respectively, using the first-round PCR products listed above as templates, before being cloned into pMD18-T (TaKaRa) for sequencing.

RESULTS

Constitutive expression of the Nag-family nitroarene dioxygenase in 2CNB degradation cluster. Because the expression of all reported Nag-like nitroarene dioxygenases was induced by salicylate (5, 17, 29), whole-cell biotransformation was then carried out to determine whether the expression of *cnbAcAd*-encoded 2CNB dioxygenase (also a Nag-like member) in strain ZWLR2-1 is induced by its substrate 2CNB or salicylate, a usual inducer for Nag-like operons. From the assays, the specific activity of 2CNB dioxygenase, the enzyme catalyzing the initial oxidation during 2CNB degradation, was found to be 0.168 ± 0.019 μmol · mg^{-1} · h^{-1} in a 2CNB-incubated cell. In contrast, cells without 2CNB and salicylate-incubated cells also exhibited evident 2CNB dioxygenase activity, with specific activities of 0.116 ± 0.002 and 0.109 ± 0.005 μmol · mg^{-1} · h^{-1}, respectively, from whole-cell biotransformation. However, the activity of ring cleavage enzyme chlorocatechol 1,2-dioxygenase in the cell extracts was 0.056 ± 0.002 U/mg in 2CNB-incubated cells and 0.022 ± 0.001 U/mg in cells without 2CNB.

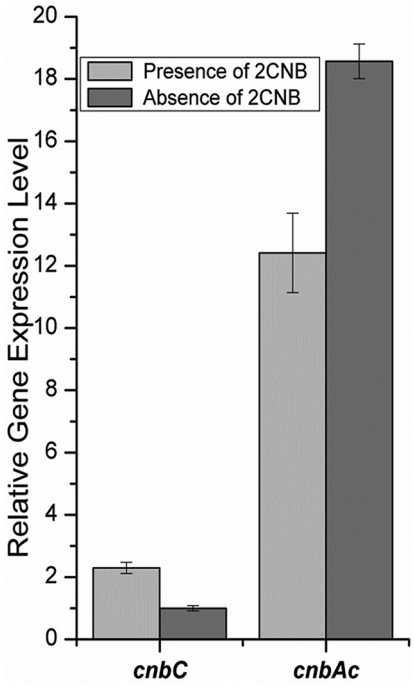

FIG 1 Real-time PCR for quantification of the expression of 2CNB catabolism genes under different conditions. In the 2CNB cluster, *cnbC* codes for 3-chlorocatechol 1,2-dioxygenase, and *cnbAc* codes for the large subunit of 2CNB dioxygenase. The data are derived from three replicates, and the error bars indicate standard deviations.

Because the 2CNB degradation cluster is located on an unstable large plasmid in strain ZWLR2-1 (8), the presence of this cluster in individual colonies from the biotransformation samples was determined using PCR. In the 2CNB-incubated sample, the 2CNB catabolism cluster was present in 82% of the cells. In the samples without 2CNB, the cluster was present in only 68% of the cells. This ratio (82/68) is similar to that of 2CNB-incubated cells to cells without 2CNB (0.168 ± 0.019/0.116 ± 0.002 μmol · mg^{-1} · h^{-1}) in 2CNB dioxygenase activity, suggesting that the difference in activity was due to a higher proportion of 2CNB catabolism cluster-containing cells in 2CNB-incubated samples, and that the expression of the 2CNB catabolism cluster is likely constitutively expressed in this strain.

In addition, the relative quantification of gene transcription by real-time PCR showed that the transcription levels of *cnbAc* and *cnbC* did not differ significantly between cells incubated in the absence and presence of 2CNB, as shown in Fig. 1, also suggesting the constitutive expression of these two critical enzymes encoded by *cnbAcAd* and *cnbC*.

Expression pattern of 2CNB catabolism cluster. In order to determine the pattern of expression of the 2CNB catabolism clus-

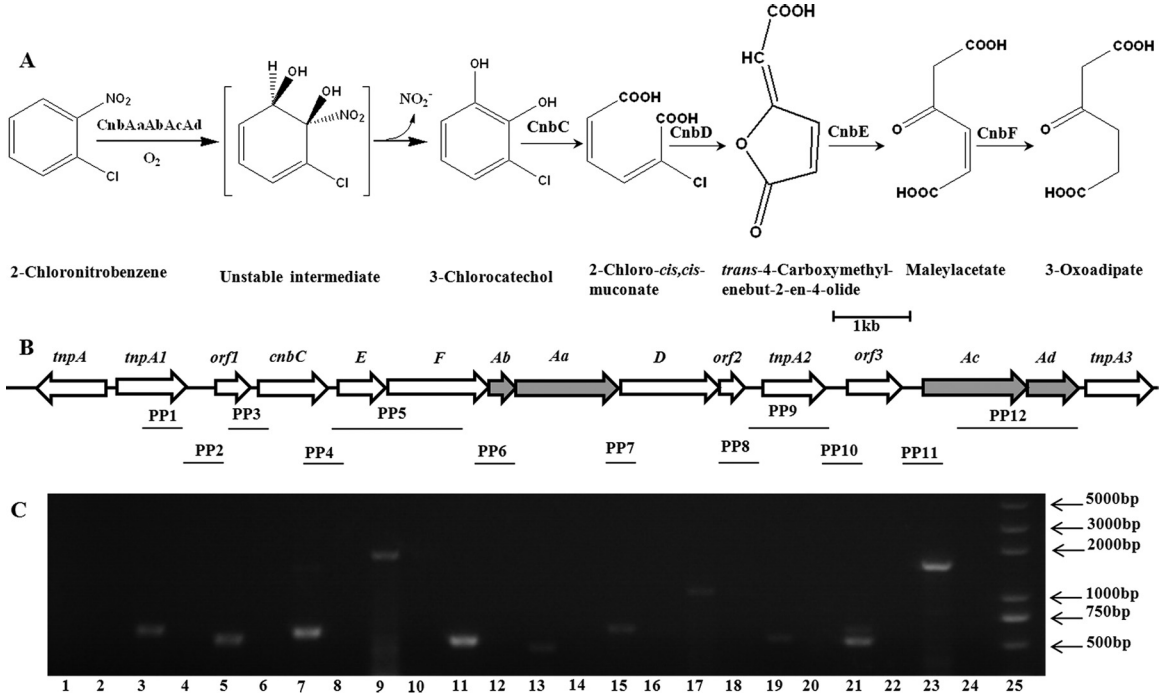

FIG 2 Transcription analysis of 2CNB-metabolic-pathway-encoding gene. (A) Catabolism pathway of 2CNB degradation in strain ZWLR2-1. (B) Genes involved in the 2CNB metabolic pathway (8). The gray boxes represent genes encoding 2CNB dioxygenase. The lines below the gene cluster show the location of the PCR fragments in panel C. (C) Electrophoretic analysis of 2CNB catabolism gene transcription by reverse transcription-PCR. Lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, and 23 show amplified products PP1, PP2, PP3, PP4, PP5, PP6, PP7, PP8, PP9, PP10, PP11, and PP12, respectively, using the corresponding primer pair sets listed in Table 2. Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 show corresponding negative controls that do not have reverse transcriptase. Lane 25 shows a molecular size marker.

ter, reverse transcription-PCR was carried out to analyze the transcription of the 2CNB catabolism genes. Initially, cDNA was produced by a single specific primer, PP12 cnb12715a, derived from *cnbAd* on the rear of the gene cluster, rather than using random primers. As shown in Fig. 2C, each primer pair produced desirable PCR products using the cDNA from reverse transcription, indicating at least one large mRNA transcript containing all of the 2CNB catabolism genes was present in this 2CNB catabolism cluster. The absence of a product in lane 1 during reverse transcription-PCR suggested that this locus of *tnpA1* is not cotranscribed with the *cnb* cluster, or that the reverse transcriptase used was unable to reverse transcribe the locus at the far end, although it may be part of the operon.

As shown in Fig. 1, the level of transcription of *cnbAc* was higher than that of *cnbC* in the relative quantification analysis. Considering that the entire gene cluster is on a single operon, it is somewhat difficult to understand why the transcription level of the *cnbAc* gene downstream of this operon would be higher than that of *cnbC* located upstream. Subsequent absolute quantification in the transcription of all genes in this cluster showed that the transcription of 6 genes, from upstream *cnbC* to middlestream *cnbD*, gradually became weaker and weaker. The level of transcription of gene *cnbAcAd* downstream was suddenly increased dramatically (Fig. 3A). This unusual phenomenon strongly indicated the presence of possible internal promoters between genes *cnbD* and *cnbAc* to enhance the transcription of *cnbAcAd*, which encode the oxygenase component of 2CNB dioxygenase involved in the initial reaction of 2CNB catabolism in this strain.

Promoter screening. Considering the expression pattern of 2CNB catabolism genes, the presence of possible suboperonic promoters within the 2CNB catabolism operon was searched. After promoter screening, as described in Materials and Methods, the 12 DNA fragments shown in Table 2 in the 2CNB catabolism cluster were deduced to be promoters, and their promoter activity was determined using the reporter gene *xylE* of promoterless vector pCM130 in strain PaW340. Among these, fragments P1, P2, and P3 exhibited promoter activities in comparison with negative-control pCM130, as shown in Fig. 3B. Notably, the activity of promoter P3, which is located directly upstream of the *cnbAcAd* genes, was 60 times higher than that of P1 and 30 times higher than that of P2.

Transcriptional start sites of *cnbC* and *cnbAc*. Transcription start sites (TSSs) of two critical genes, *cnbC* and *cnbAc*, were determined by 5′ rapid amplification of cDNA ends (5′ RACE) using total RNA extracted from strain ZWLR2-1. A guanine (G) base was located 394 bp upstream of the start codon ATG of *cnbC*. The adenine (A) base located 78 bp upstream of ATG was the TSS of *cnbAc*. The locations of these two TSSs matched those of the P1 and P3 promoters, which were determined in promoter screening described above.

Mutation analysis of promoter P3. Truncated and mutated P3 promoters were constructed to determine the minimum length of P3 promoter needed for the maximum activity. According to a previous report, and in the current study, the plasmid pCM130 with reporter gene *xylE* was insufficient to determine slight differences in promoter strength (30). For this reason, another plasmid, pCM*gfp-lacZ* (31), with reporter gene *lacZ*, was used to analyze the activities of the truncated and mutated promoters.

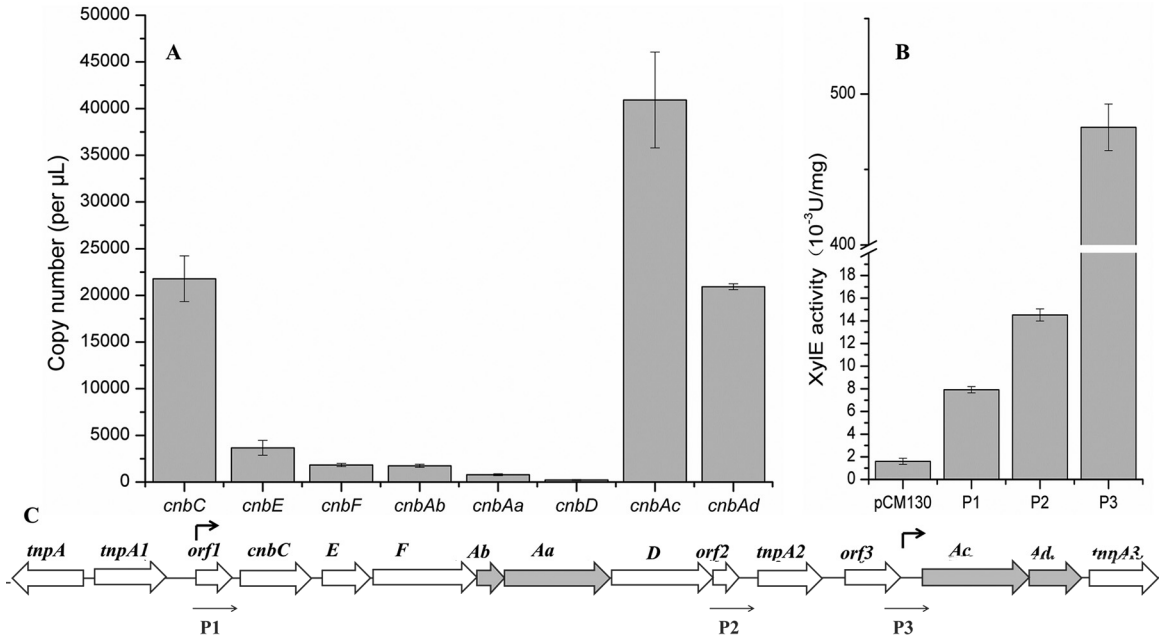

FIG 3 Expression pattern of *cnb* cluster and location of promoters. (A) Absolute quantification using real-time PCR to analyze 2CNB metabolic gene expression in *Pseudomonas stutzeri* ZWLR2-1. (B) Promoter activity assays of DNA fragments P1, P2, and P3 from 2CNB gene cluster in surrogate host *Pseudomonas putida* PaW340, using *xylE* as the reporter gene. The data are derived from at least three replicates, and the error bars indicate standard deviations. (C) Location of the coding genes and three promoter sequences on 2CNB metabolic gene cluster. The gray boxes represent genes encoding 2CNB dioxygenase. The transcription start sites are indicated with bent arrows.

From the comparison shown in Fig. 4A between the P3 sequence and its homologous counterpart from the 2-nitrotoluene utilizer *Acidovorax* sp. strain JS42, a 132-bp fragment between positions 10419 and 10550 in the 2CNB catabolism gene cluster was considered likely to have originated from plasmid pAOVO02 (GenBank accession no. CP000541.1, with 100% identity) in strain JS42, but the rest of the promoter sequence was almost identical to the upstream sequence of the chromosome-encoded Nag-like dioxygenase genes *ntdAcAd* of the 2-nitrotoluene catabolism cluster in strain JS42 (10). When this 132-bp fragment was removed from P3 to produce P32, the promoter activity for P32 was 328.2 ± 36.1 Miller units versus 433.7 ± 31.6 Miller units for the complete P3 sequence. This suggested that the complete P3 sequence was necessary for the maximum promoter activity and it then was selected for mutation analysis.

Based on the location of the transcriptional start site of *cnbAc* mentioned above, the −35 region of the promoter was located in a duplicated 18-bp direct repeat, as shown in Fig. 4A. Interestingly, this 18-bp sequence was present but as a single copy in the same location in the *ntd* cluster of strain JS42. This implies that addition of the 18-bp sequence has formed a new locus for gene transcription of *cnbAcAd* in the 2CNB catabolism cluster in strain ZLWR2-1. To elucidate the critical roles of this newly evolved −35 region for promoter activity, five clones with a 2-base mutation in this region were constructed. The activities of promoters (P3M1 to P3M4) with mutations in this −35 region were decreased 30 to 71% in comparison with the activity of wild-type P3, as shown in Fig. 5.

DISCUSSION

In naphthalene utilizer *Ralstonia* sp. strain U2, the expression of naphthalene dioxygenase NagAcAd is activated by the transcriptional regulator NagR in the presence of the catabolism intermediate salicylate (17). This Nag system was thought to be the archetype of nitroarene dioxygenases catalyzing the initial oxidation of the corresponding substrates in a number of nitroarene utilizers, including dinitrotoluene utilizer *Burkholderia* sp. strain DNT (12, 32), nitrobenzene utilizer *Comamonas* sp. strain JS765 (9), and 2-nitrotoluene utilizer *Acidovorax* sp. JS42 (10, 32). Although salicylate is no longer the catabolism intermediate during the degradation of these compounds, it still serves as an inducer for NagR-like transcriptional regulators to activate the transcription of Nag-like dioxygenase genes in these strains (5, 29, 33). However, in the 2CNB degrader *Pseudomonas stutzeri* ZWLR2-1, no NagR-like transcriptional regulator was present in the catabolism cluster containing the genes encoding the Nag-like 2CNB dioxygenase (8), and the expression of 2CNB dioxygenase is constitutive rather than inducible in all other Nag-like nitroarene dioxygenases previously reported. In this way, this strain has adapted itself to the new pollutant by evolving the always-inducible Nag-like dioxygenase into a constitutive expressed enzyme, and its expression has escaped the influence of salicylate.

The degradation pathway of 2CNB was regarded as a patchwork assembly of Nag-like nitroarene dioxygenase genes and the 3-chlorocatechol degradation cluster (8). Because 2CNB is a toxic and recalcitrant chemical, the first step in its degradation is crucial, and there must be enough expression of the enzymes involved in the initial reactions to deal with this substrate. In the 2CNB catabolism cluster in this study, since the location of the genes *cnbAcAd* encoding 2CNB dioxygenase are downstream of the operon in this catabolism cluster, the weak expression of *cnbAcAd* may not satisfy the metabolic flux in the pathway if no extra internal promoters are available to enhance its expression. It is evident

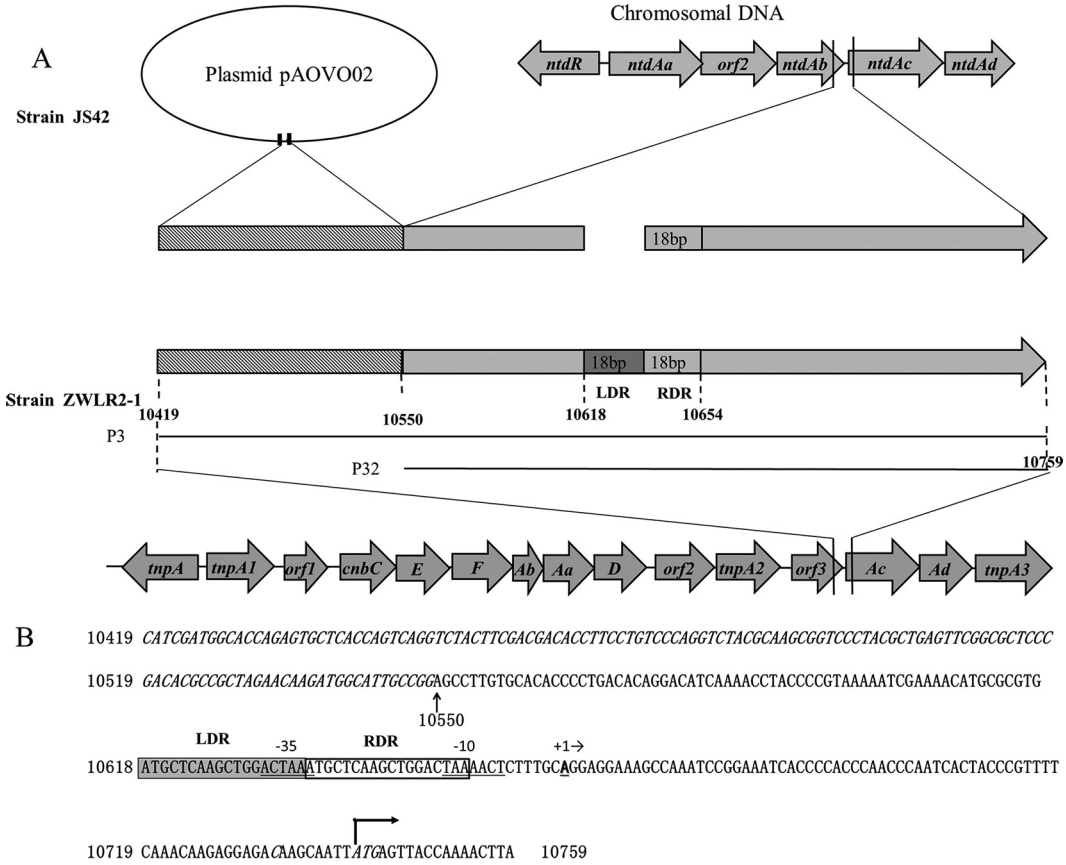

FIG 4 Comparison of homologous sequence of strain JS42 and strain ZWLR2-1. (A) Sketch map of relationship between P3 DNA fragment from strain ZWLR2-1 and homologous sequence in *Acidovorax* sp. JS42. (B) DNA sequence of P3 fragment. Nucleotides 10419 to 10500 from the *cnb* cluster in strain ZWLR2-1 is 100% identical to the 132-bp fragment from plasmid pAOVO02 in strain JS42. Nucleotides 10551 to 10759 are almost identical to the sequence spanning *ntdAb* and *ntdAc* on the *ntd* cluster of strain JS42 chromosomal DNA, except a cytosine (C at nucleotide 10733, italics) in the *cnb* cluster instead of a thymine (T) in the *ntd* cluster, and a duplication of an 18-bp sequence in the *ntd* cluster to form a direct repeat in the *cnb* cluster, where the −10 and −35 regions of the P3 promoter are located. The transcriptional start site is marked +1, and the −10 and −35 regions are underlined. Start codon of the *cnbAc* gene at nucleotide 10743 is indicated with a bent arrow. LDR, left direct repeat; RDR, right direct repeat.

that the expression of the 2CNB gene cluster in strain ZWLR2-1 is unusual, with a strong internal promoter P3 being involved in the high expression of the critical 2CNB dioxygenase in the upper pathway of the 2CNB degradation. Internal promoters in different operons of bacteria have been functionally verified in the biosynthetic pathways of histidine (34), tryptophan (35), alginate (36), structural proteins of photosynthetic apparatus (37), and endospore formation genes (38). However, they are rarely found in the operons for biodegradation, particularly those involved in the degradation of xenobiotics, such as 2CNB. A relevant case was that a mutant strain of *Xanthobacter autotrophicus* GJ10 was developed to grow on higher concentrations (>5 mM) of monobromoacetate, which is toxic to the wild type. This is because the expression of a key enzyme, haloacetate dehalogenase, was activated by the insertion of transposable element IS*1247* to a site proximal to its encoding gene (4).

The direct repeat sequence has been reported to be important to the expression of chitin metabolism genes in *Streptomyces* (39, 40), some virulence genes in *Salmonella enterica* and *Yersinia pestis* (41), and *Neisseria meningitidis* (42). Genes driven by tandem-repeat-containing promoters also show significantly higher rates of transcriptional divergence in *Saccharomyces cerevisiae* (43). In the P3 promoter evolutionary itinerary of this study, the following two sequence changes apparently occurred to improve promoter strength: (i) a direct repeat of an 18-bp sequence, and (ii) an extended promoter with an extra 132-bp sequence from the plasmid pAOVO02. The 5′-RACE result shows that the −35 region is located in the newly formed 18-bp repeat. Subsequent mutations of the −35 region visibly decreased the P3 promoter strength. The importance of this promoter P3 would be more significant if it was confirmed to be necessary for strain ZWLR2-1 to grow on 2CNB. However, the deletion or mutation of this promoter sequence in the native host was not successful after several attempts. This is likely because the 2CNB catabolism cluster is located on a megaplasmid (8, 14), and this plasmid is unstable, as stated in this study.

The duplication of this 18-bp sequence as a direct repeat is also presented in the corresponding locus in the nitroarene catabolism cluster in DNT (2,4-dinitrotoluene) utilizers *Burkholderia cepacia* R34 (13), *Burkholderia* sp. strain DNT (12), and 3-nitrotoluene-utilizing *Diaphorobacter* species strains (11). Nevertheless, it is not known whether a functional internal promoter containing the 18-bp direct repeat is present for the enhanced expression of catabolism enzymes in these strains, in addition to the expression directed by Nag-like regulators. For the 2CNB utilizer ZWLR2-1

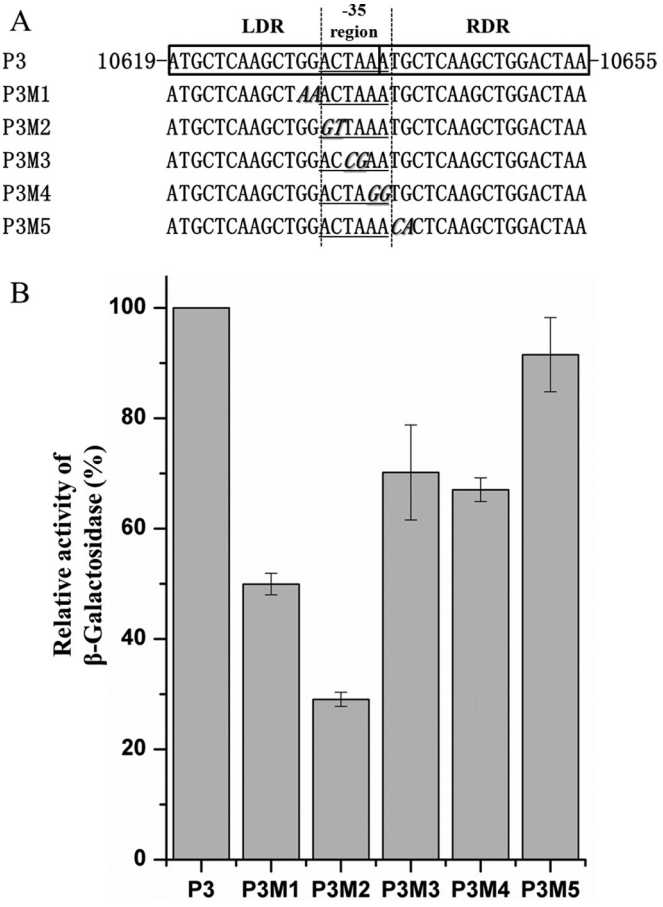

FIG 5 Mutation analysis of promoter activity of the newly formed −35 region at the junction of LDR and RDR. (A) Schematic diagram of mutant construction in the −35 region of the P3 promoter. (B) β-Galactosidase activities of promoter P3 and its mutants P3M1 to P3M5. The data are derived from at least 3 independent measurements, and the error bars indicate standard deviations.

in this study, the strong internal promoter containing the 18-bp direct repeat for expression of the critical enzyme does present an interesting model of promoter evolution during the adaptation to a newly emerging xenobiotic for an environmental organism.

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REFERENCES

1. **Cases I, de Lorenzo V.** 2005. Promoters in the environment: transcriptional regulation in its natural context. Nat Rev Microbiol **3:**105–118. http://dx.doi.org/10.1038/nrmicro1084.
2. **Böhm ME, Huptas C, Krey VM, Scherer S.** 2015. Massive horizontal gene transfer, strictly vertical inheritance and ancient duplications differentially shape the evolution of *Bacillus cereus* enterotoxin operons *hbl*, *cytK* and *nhe*. BMC Evol Biol **15:**17. http://dx.doi.org/10.1186/s12862-015-0282-8.
3. **Youn JW, Jolkver E, Krämer R, Marin K, Wendisch VF.** 2008. Identification and characterization of the dicarboxylate uptake system DccT in *Corynebacterium glutamicum*. J Bacteriol **190:**6458–6466. http://dx.doi.org/10.1128/JB.00780-08.
4. **van der Ploeg J, Willemsen M, van Hall G, Janssen DB.** 1995. Adaptation of *Xanthobacter autotrophicus* GJ10 to bromoacetate due to activation and mobilization of the haloacetate dehalogenase gene by insertion element IS*1247*. J Bacteriol **177:**1348–1356.
5. **de las Heras A, Chavarría M, de Lorenzo V.** 2011. Association of *dnt* genes of *Burkholderia* sp. DNT with the substrate-blind regulator DntR draws the evolutionary itinerary of 2,4-dinitrotoluene biodegradation. Mol Microbiol **82:**287–299. http://dx.doi.org/10.1111/j.1365-2958.2011.07825.x.
6. **de Lorenzo V.** 2008. Systems biology approaches to bioremediation. Curr Opin Biotechnol **19:**579–589. http://dx.doi.org/10.1016/j.copbio.2008.10.004.
7. **Fuenmayor SL, Wild M, Boyes AL, Williams PA.** 1998. A gene cluster encoding steps in conversion of naphthalene to gentisate in *Pseudomonas* sp. strain U2. J Bacteriol **180:**2522–2530.
8. **Liu H, Wang SJ, Zhang JJ, Dai H, Tang HR, Zhou NY.** 2011. Patchwork assembly of *nag*-like nitroarene dioxygenase genes and the 3-chlorocatechol degradation cluster for evolution of the 2-chloronitrobenzene catabolism pathway in *Pseudomonas stutzeri* ZWLR2-1. Appl Environ Microbiol **77:**4547–4552. http://dx.doi.org/10.1128/AEM.02543-10.
9. **Lessner DJ, Johnson GR, Parales RE, Spain JC, Gibson DT.** 2002. Molecular characterization and substrate specificity of nitrobenzene dioxygenase from *Comamonas* sp. strain JS765. Appl Environ Microbiol **68:**634–641. http://dx.doi.org/10.1128/AEM.68.2.634-641.2002.
10. **Parales JV, Kumar A, Parales RE, Gibson DT.** 1996. Cloning and sequencing of the genes encoding 2-nitrotoluene dioxygenase from *Pseudomonas* sp. JS42. Gene **181:**57–61. http://dx.doi.org/10.1016/S0378-1119(96)00462-3.
11. **Singh D, Kumari A, Ramanathan G.** 2014. 3-Nitrotoluene dioxygenase from *Diaphorobacter* sp. strains: cloning, sequencing and evolutionary studies. Biodegradation **25:**479–492. http://dx.doi.org/10.1007/s10532-013-9675-9.
12. **Suen WC, Haigler BE, Spain JC.** 1996. 2,4-Dinitrotoluene dioxygenase from *Burkholderia* sp. strain DNT: similarity to naphthalene dioxygenase. J Bacteriol **178:**4926–4934.
13. **Johnson GR, Jain RK, Spain JC.** 2002. Origins of the 2,4-dinitrotoluene pathway. J Bacteriol **184:**4219–4232. http://dx.doi.org/10.1128/JB.184.15.4219-4232.2002.
14. **Liu H, Wang SJ, Zhou NY.** 2005. A new isolate of *Pseudomonas stutzeri* that degrades 2-chloronitrobenzene. Biotechnol Lett **27:**275–278. http://dx.doi.org/10.1007/s10529-004-8293-3.
15. **Haigler BE, Wallace WH, Spain JC.** 1994. Biodegradation of 2-nitrotoluene by *Pseudomonas* sp. strain JS42. Appl Environ Microbiol **60:**3466–3469.
16. **Suen WC, Spain JC.** 1993. Cloning and characterization of *Pseudomonas* sp. strain DNT genes for 2,4-dinitrotoluene degradation. J Bacteriol **175:**1831–1837.
17. **Jones RM, Britt-Compton B, Williams PA.** 2003. The naphthalene catabolic (*nag*) genes of *Ralstonia* sp. strain U2 are an operon that is regulated by NagR, a LysR-type transcriptional regulator. J Bacteriol **185:**5847–5853. http://dx.doi.org/10.1128/JB.185.19.5847-5853.2003.
18. **Sambrook J, Fritsch EF, Maniatis T.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
19. **Spain JC, Nishino SF.** 1987. Degradation of 1,4-dichlorobenzene by a *Pseudomonas* sp. Appl Environ Microbiol **53:**1010–1019.
20. **Dorn E, Knackmuss HJ.** 1978. Chemical structure and biodegradability of halogenated aromatic compounds substituent effects on 1,2-dioxygenation of catechol. Biochem J **174:**85–94. http://dx.doi.org/10.1042/bj1740085.
21. **Bradford MM.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:**248–254. http://dx.doi.org/10.1016/0003-2697(76)90527-3.
22. **Livak KJ, Schmittgen TD.** 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods **25:**402–408. http://dx.doi.org/10.1006/meth.2001.1262.
23. **Marx CJ, Lidstrom ME.** 2001. Development of improved versatile broad-host-range vectors for use in methylotrophs and other Gram-negative

bacteria. Microbiology **147:**2065–2075. http://dx.doi.org/10.1099/00221287-147-8-2065.
24. **Marconi AM, Beltrametti F, Bestetti G, Solinas F, Ruzzi M, Galli E, Zennaro E.** 1996. Cloning and characterization of styrene catabolism genes from *Pseudomonas fluorescens* ST. Appl Environ Microbiol **62:**121–127.
25. **Franklin FCH, Williams PA.** 1980. Construction of a partial diploid for the degradative pathway encoded by the TOL plasmid (pWWO) from *Pseudomonas putida* mt-2: evidence for the positive nature of the regulation by the *xylR* gene. Mol Gen Genet **177:**321–328. http://dx.doi.org/10.1007/BF00267445.
26. **Inouye S, Nakazawa A, Nakazawa T.** 1981. Molecular cloning of TOL genes *xylB* and *xylE* in *Escherichia coli*. J Bacteriol **145:**1137–1143.
27. **Zukowski MM, Gaffney DF, Speck D, Kauffmann M, Findeli A, Wisecup A, Lecocq JP.** 1983. Chromogenic identification of genetic regulatory signals in *Bacillus subtilis* based on expression of a cloned *Pseudomonas* gene. Proc Natl Acad Sci U S A **80:**1101–1105. http://dx.doi.org/10.1073/pnas.80.4.1101.
28. **Griffith KL, Wolf RE, Jr.** 2002. Measuring β-galactosidase activity in bacteria: cell growth, permeabilization, and enzyme assays in 96-well arrays. Biochem Biophys Res Commun **290:**397–402. http://dx.doi.org/10.1006/bbrc.2001.6152.
29. **Ju KS, Parales JV, Parales RE.** 2009. Reconstructing the evolutionary history of nitrotoluene detection in the transcriptional regulator NtdR. Mol Microbiol **74:**826–843. http://dx.doi.org/10.1111/j.1365-2958.2009.06904.x.
30. **Hassett DJ, Ochsner UA, Groce SL, Parvatiyar K, Ma JF, Lipscomb JD.** 2000. Hydrogen peroxide sensitivity of catechol-2,3-dioxygenase: a cautionary note on use of *xylE* reporter fusions under aerobic conditions. Appl Environ Microbiol **66:**4119–4123. http://dx.doi.org/10.1128/AEM.66.9.4119-4123.2000.
31. **Zhang WM, Zhang JJ, Jiang X, Chao HJ, Zhou NY.** 2015. Transcriptional activation of multiple operons involved in *para*-nitrophenol degradation by *Pseudomonas* sp. strain WBC-3. Appl Environ Microbiol **81:**220–230. http://dx.doi.org/10.1128/AEM.02720-14.
32. **Ju KS, Parales RE.** 2010. Nitroaromatic compounds, from synthesis to biodegradation. Microbiol Mol Biol Rev **74:**250–272. http://dx.doi.org/10.1128/MMBR.00006-10.
33. **Lessner DJ, Parales RE, Narayan S, Gibson DT.** 2003. Expression of the nitroarene dioxygenase genes in *Comamonas* sp. strain JS765 and *Acidovorax* sp. strain JS42 is induced by multiple aromatic compounds. J Bacteriol **185:**3895–3904. http://dx.doi.org/10.1128/JB.185.13.3895-3904.2003.
34. **Alifano P, Fani R, Liò P, Lazcano A, Bazzicalupo M, Carlomagno MS, Bruni CB.** 1996. Histidine biosynthetic pathway and genes: structure, regulation, and evolution. Microbiol Mol Biol Rev **60:**44–69.
35. **Horowitz H, Platt T.** 1983. Initiation *in vivo* at the internal *trp* p2 promoter of *Escherichia coli*. J Biol Chem **258:**7890–7893.
36. **Paletta JL, Ohman DE.** 2012. Evidence for two promoters internal to the alginate biosynthesis operon in *Pseudomonas aeruginosa*. Curr Microbiol **65:**770–775. http://dx.doi.org/10.1007/s00284-012-0228-y.
37. **LeBlanc H, Lang AS, Beatty JT.** 1999. Transcript cleavage, attenuation, and an internal promoter in the *Rhodobacter capsulatus puc* operon. J Bacteriol **181:**4955–4960.
38. **Guillot C, Moran CP, Jr.** 2007. Essential internal promoter in the *spoIIIA* locus of *Bacillus subtilis*. J Bacteriol **189:**7181–7189. http://dx.doi.org/10.1128/JB.00915-07.
39. **Delic I, Robbins P, Westpheling J.** 1992. Direct repeat sequences are implicated in the regulation of two *Streptomyces* chitinase promoters that are subject to carbon catabolite control. Proc Natl Acad Sci U S A **89:**1885–1889. http://dx.doi.org/10.1073/pnas.89.5.1885.
40. **Ni X, Westpheling J.** 1997. Direct repeat sequences in the *Streptomyces* chitinase-63 promoter direct both glucose repression and chitin induction. Proc Natl Acad Sci U S A **94:**13116–13121. http://dx.doi.org/10.1073/pnas.94.24.13116.
41. **Perez JC, Groisman EA.** 2009. Transcription factor function and promoter architecture govern the evolution of bacterial regulons. Proc Natl Acad Sci U S A **106:**4319–4324. http://dx.doi.org/10.1073/pnas.0810343106.
42. **Martin P, Makepeace K, Hill SA, Hood DW, Moxon ER.** 2005. Microsatellite instability regulates transcription factor binding and gene expression. Proc Natl Acad Sci U S A **102:**3800–3804. http://dx.doi.org/10.1073/pnas.0406805102.
43. **Vinces MD, Legendre M, Caldara M, Hagihara M, Verstrepen KJ.** 2009. Unstable tandem repeats in promoters confer transcriptional evolvability. Science **324:**1213–1216. http://dx.doi.org/10.1126/science.1170097.
44. **Liu H, Zhang JJ, Wang SJ, Zhang XE, Zhou NY.** 2005. Plasmid-borne catabolism of methyl parathion and *p*-nitrophenol in *Pseudomonas* sp. strain WBC-3. Biochem Biophys Res Commun **334:**1107–1114. http://dx.doi.org/10.1016/j.bbrc.2005.07.006.
45. **Williams PA, Murray K.** 1974. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existence of a TOL plasmid. J Bacteriol **120:**416–423.
46. **Woodcock DM, Crowther PJ, Doherty J, Jefferson S, DeCruz E, Noyer-Weidner M, Smith SS, Michael MZ, Graham MW.** 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. Nucleic Acids Res **17:**3469–3478. http://dx.doi.org/10.1093/nar/17.9.3469.