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Differential Effects of Homologous Transcriptional Regulators NicR2A, NicR2B1, and NicR2B2 and Endogenous Ectopic Strong Promoters on Nicotine Metabolism in *Pseudomonas* sp. Strain JY-Q

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ABSTRACT Nicotine is a toxic environmental pollutant that widely exists in tobacco wastes. As a natural nicotine-degrading strain, *Pseudomonas* sp. strain JY-Q still has difficulties degrading high concentrations of nicotine. In this study, we investigated the effect of two homologous transcriptional regulators and endogenous ectopic strong promoters on the efficiency of nicotine degradation. Comparative genomics analysis showed that two homologous transcriptional regulators, namely, NicR2A and NicR2Bs (NicR2B1 plus NicR2B2), can repress nicotine degradation gene expression. When both *nicR2A* and *nicR2Bs* were deleted, the resulting mutant JY-Q Δ*nicR2A* Δ*nicR2B1* Δ*nicR2B2* (QΔABs) exhibits a 17% higher nicotine degradation efficiency than wild-type JY-Q. Transcriptome sequencing (RNA-seq) analysis showed that the transcription levels (fragments per kilobase per million [FPKM] value) of six genes were higher than those of the other genes in JY-Q. Based on the genetic organization of these genes, three putative promoters, $P_{RS28250}$, $P_{RS09985}$, and $P_{RS24685}$, were identified. Their promoter activities were evaluated by comparing their expression levels using reverse transcriptase quantitative PCR (RT-qPCR). We found that the transcription levels of *RS28250*, *RS09985*, and *RS24685* were respectively 16.8, 2.6, and 1.6 times higher than that of *hspB2*, encoding 6-hydroxy-3-succinylpyridine hydroxylase, which is involved in nicotine degradation. Thus, two strong endogenous promoters, namely, $P_{RS28250}$ and $P_{RS09985}$, were selected to replace the original promoters of *nic2* gene clusters. The effect of the endogenous ectopic promoter was also related to the position of target gene clusters. When the promoter $P_{RS28250}$ replaced the promoter of *hspB2*, the resultant mutant QΔABs-ΔP_{hspB2}::$P_{RS28250}$ exhibited nicotine-degrading efficiency 69% higher than that of JY-Q. This research suggests a feasible strategy to enhance strains' capacity for nicotine degradation by removal of repressing regulatory proteins and replacing the target promoter with strong endogenous ectopic promoters.

IMPORTANCE This study evaluated the differential effects of homologous NicR2A and NicR2Bs and endogenous ectopic strong promoters on nicotine metabolism in *Pseudomonas* sp. strain JY-Q. Based on our differential analysis, a feasible strategy is presented to modify wild-type (WT) strain JY-Q by removing repressing regulatory proteins NicR2A and NicR2Bs and replacing the target promoter with strong endogenous ectopic promoters. The resulting mutants exhibited high tolerance and degradation of nicotine. These findings should be beneficial for improving the pollutant-degrading capacity of natural strains through genomic modification.

KEYWORDS nicotine degradation, transcriptional repressors, endogenous promoter, *Pseudomonas*

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Nicotine is a hypertoxic *N*-heterocyclic compound. Vast volumes of tobacco waste containing a high concentration of nicotine are produced by tobacco factories, resulting in a major threat to the environment (1). Nicotine has been detected in rivers and groundwater (2). Various efforts have been devoted to degrading this nicotine. Biodegradation has been widely studied as an efficient, low-energy-consumption and pollution-free method to remove nicotine (3, 4).

Nicotine-degrading strains that have been isolated from different environments can utilize nicotine as their sole carbon and nitrogen source for growth (5–7). Tobacco waste extract (TWE) contains substantial amounts of nicotine and includes microbial communities that are an important source for the isolation and screening of nicotine-degrading bacteria (8). Some strains have been studied in depth, such as *Arthrobacter* sp. M2012083 (9), *Pseudomonas putida* S16 (10–12), *Pseudomonas* sp. JY-Q (13–15), *Agrobacterium tumefaciens* S33 (16, 17), and *Ochrobactrum* sp. SJY1 (18, 19). Moreover, the discoveries of bacterial degradation pathways and the elucidation of degradation mechanisms provide the opportunity to modify strains for improving their nicotine-degradation ability (20). The following three nicotine-degradation pathways have been identified in nicotine-degrading microorganisms: pyridine, pyrrolidine, and a variant of the pyridine and pyrrolidine pathways (17). In our previous study we found that *Pseudomonas* sp. strain JY-Q, isolated from tobacco waste extract, uses the pyrrolidine pathways to degrade nicotine, and the final product enters the tricarboxylic acid (TCA) cycle (Fig. 1a) (14, 15). Generally, a catabolic pathway consists of catabolic enzymes and regulatory elements that control the transcription of catabolic operons (21). Four transcriptional regulatory factors involved in nicotine regulation have been described, as follows: transcriptional repressor HdnoR (22) and the transcriptional activator PmfR (23) in *Arthrobacter nicotinovorans*, the transcriptional repressor NicR2 in *Pseudomonas putida* S16 (24), and a putative two-component regulatory system (TCS) in *Shinella* sp. strain HZN7 (25).

Most of the bacterial degraders isolated from the environment can effectively degrade low-concentration pollutants (26). Therefore, many strategies have been used to improve the degradation efficiency, for example, the introduction of effective genes, the overexpression of some crucial genes, and mixed degradation by multiple strains (27). However, the concentration of nicotine in tobacco waste extract is 10 to 15 g/liter (14), making most strains unable to grow well. There are only two studies on improving the degradation ability of nicotine using genetically modified strains. One involved the introduction of the glutamate decarboxylase (GAD)-dependent system and the global regulator (IrrE) of extreme radiation resistance into *Pseudomonas putida* S16 for improving the acid resistance of the strains to enhance biodegrading activities (28); the other involved knocking out the initial glucose metabolism genes in JY-Q to improve the utilization rate of nicotine, but the growth rate of these modified bacteria was poor, ultimately failing to achieve the goal (15).

The current study focuses on the differential effects of homologous NicR2A and NicR2Bs (NicR2B1 plus NicR2B2) functioning as repressors of downstream nicotine metabolism by gene knockout and reverse transcriptase quantitative PCR (RT-qPCR) verification and the differential effects of an endogenous ectopic strong promoter on the nicotine metabolism gene cluster using a variety of insertion positions in the genome. One noteworthy finding is that the repression effect of NicR2Bs is stronger than that of NicR2A. A potential strategy was introduced to enhance the expression of nicotine-degrading genes in JY-Q to improve the nicotine-degradation ability. First, transcriptional repressor genes were knocked out so that the strain could adapt more quickly to a nicotine environment. Then, strong endogenous promoters were screened based on the transcriptomic analysis results using transcriptome sequencing (RNA-seq) (29, 30). Finally, the strongest endogenous promoter was selected to directly replace the original promoter of *nic2* gene clusters in the genome. Improvements in the gene expression level and increases in the nicotine-degrading ability and tolerance capacity of the

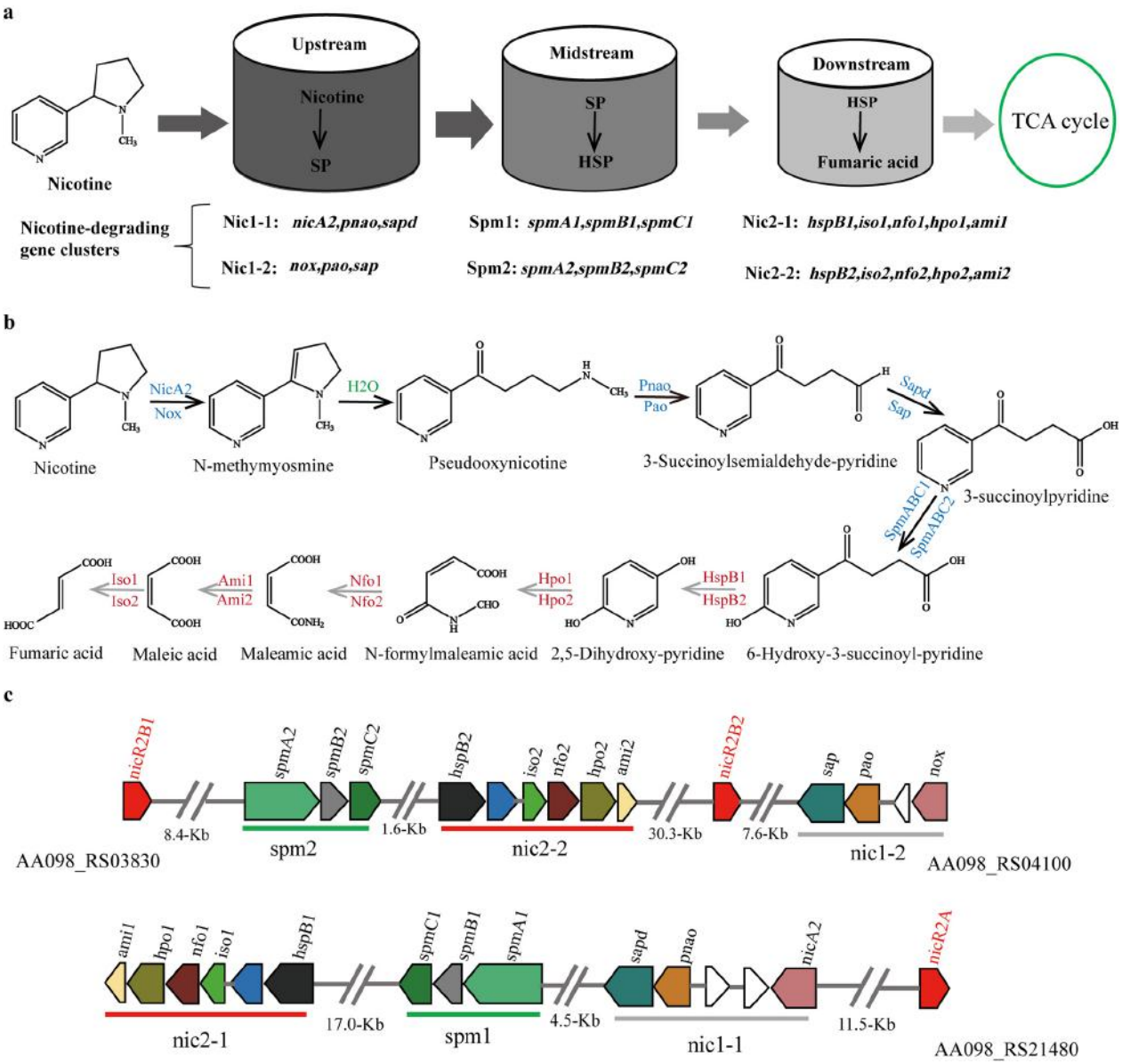


FIG 1 Nicotine metabolism modules (a), pyrrolidine pathway (b), and genetic organization of nicotine-degrading genes (c) in *Pseudomonas* sp. JY-Q. SP, 3-succinoyl-pyridine; HSP, 6-hydroxy-3-succinoyl pyridine.

genomic modified strain were then examined. In this study, we succeeded in using an endogenous ectopic strong promoter to improve microbial nicotine degradation.

RESULTS

Differential effect of homologous genes *nicR2A* and *nicR2Bs* (*nicR2B1* plus *nicR2B2*) in *Pseudomonas* sp. JY-Q. There are duplicate homologous nicotine-degrading gene clusters in JY-Q (14). Based on their location in the pyrrolidine pathway of nicotine degradation in JY-Q, these gene clusters are classified into the following three function modules: upstream module (Nic1), midstream module (Spm), and downstream module (Nic2) (Fig. 1a). Based on the genomics and comparative genomics analysis, two different putative transcriptional regulatory proteins were identified in JY-Q, namely, NicR2A and NicR2Bs (with two gene copies, *nicR2B1* and *nicR2B2*). At the

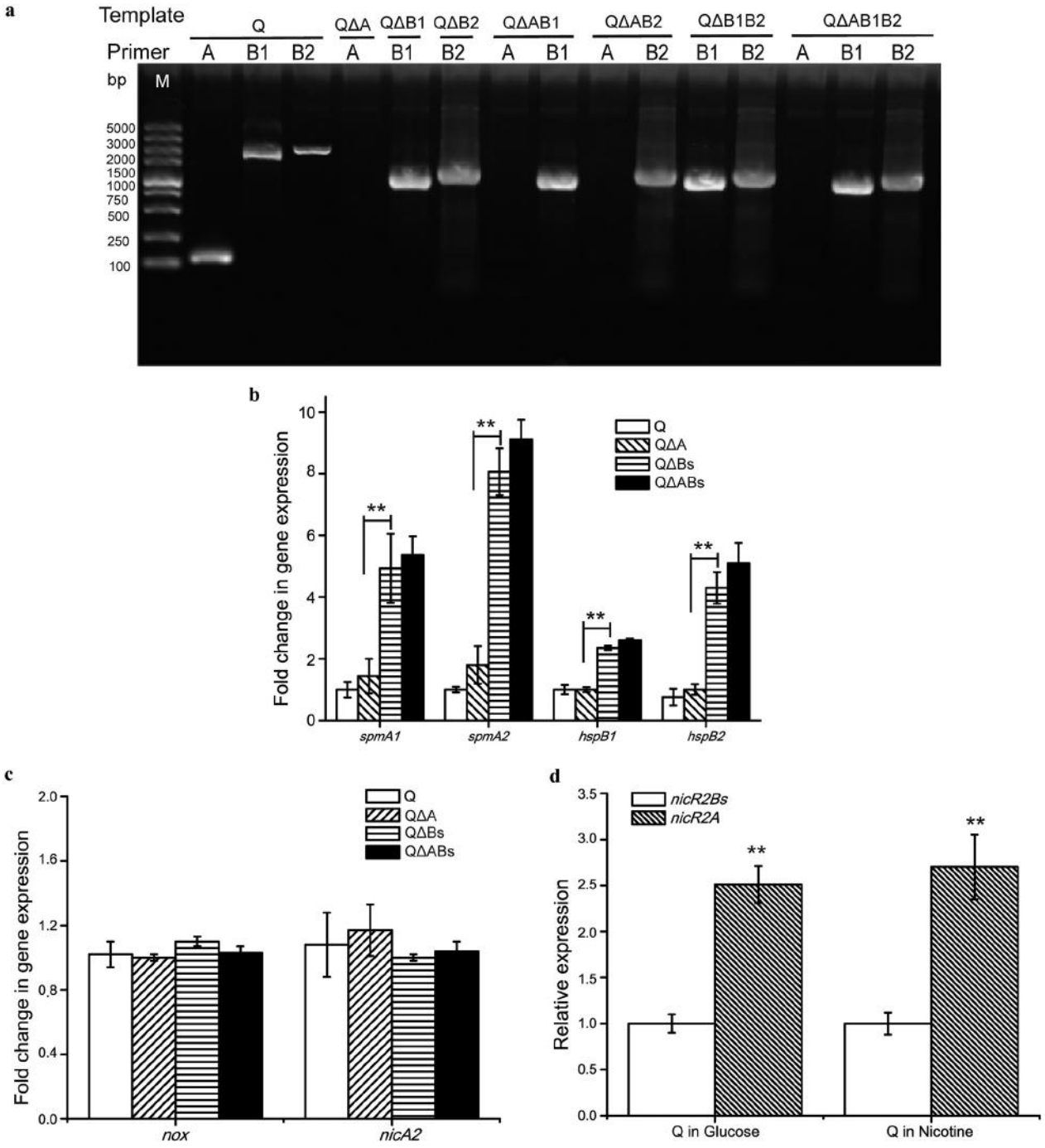


FIG 2 RT-qPCR analysis of nicotine degradation gene transcripts produced in JY-Q and its mutants with a knockout of the transcriptional regulator gene grown in BSM containing 4 g/liter glucose. (a) PCR verified the deletion of *nicR2A*, *nicR2B1*, and *nicR2B2* in mutants. A, the inner primer pair of *nicR2A*; B1, the primer pair outside *nicR2B1*; B2, the primer pair outside *nicR2B2*. (b) mRNA expression levels of *spmA1*, *spmA2*, *hspB1*, and *hspB2*. (c) mRNA expression levels of *nox* and *nicA2*. (d) mRNA expression levels of *nicR2A* and *nicR2Bs*. The data are the means of three independent experiments, and error bars indicate the standard deviations. *t* test was used for significance analysis; *, $P < 0.05$ (significant); **, $P < 0.01$ (extremely significant).

protein sequence level, NicR2A and NicR2Bs showed 100% and 70% similarity, respectively, to NicR2 in *Pseudomonas putida* S16 (see Fig. S1 in the supplemental material).

A cumulative knockout of *nicR2A*, *nicR2B1*, and *nicR2B2* was performed in JY-Q to produce single, double, and triple mutants for testing the potential repressing effect of

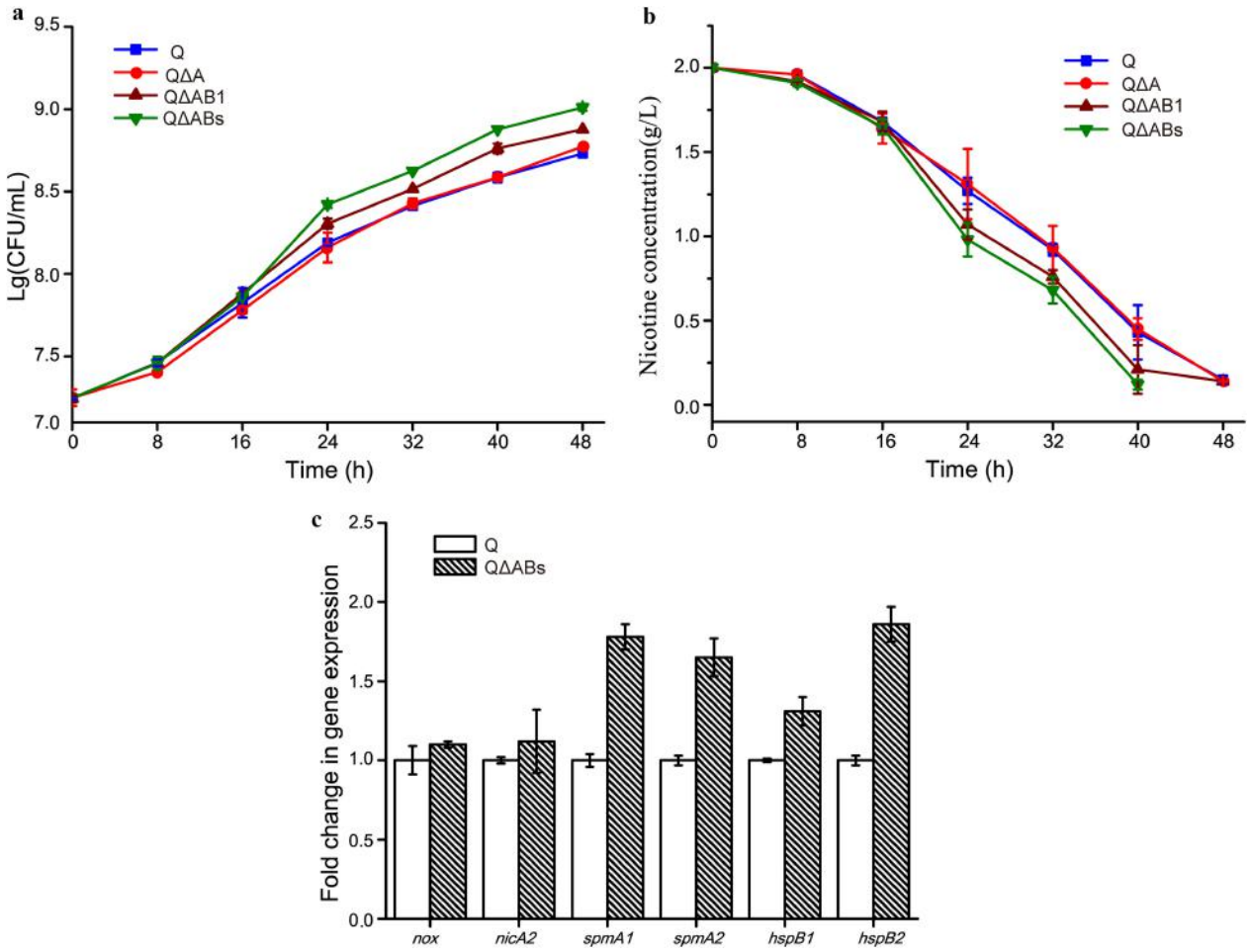


FIG 3 Growth (a); nicotine degradation (b); and expression levels of genes *nox*, *nicA2*, *spmA1*, *spmA2*, *hspB1*, and *hspB2* (c) of WT strain JY-Q and its mutant strains QΔA, *QΔnicR2AΔnicR2B1*, and QΔABs in BSM containing 2 g/liter nicotine. The data are the means of three independent experiments, and error bars indicate the standard deviations. *t* test was used for significance analysis; *, $P < 0.05$ (significant); **, $P < 0.01$ (extremely significant).

NicR2A and NicR2Bs on the expression of nicotine metabolism genes (Fig. 2a). Reverse transcriptase quantitative PCR (RT-qPCR) revealed that the transcriptional levels of *spmA1*, *spmA2*, *hspB1*, and *hspB2* significantly increased in triple-mutant QΔABs (JY-Q Δ*nicR2A* Δ*nicR2B1* Δ*nicR2B2*) when grown in basic salt medium (BSM) containing 4 g/liter glucose (Fig. 2b). However, the transcriptional levels of *nox* and *nicA2* in all mutant strains remained similar to those of the wild-type (WT) strain (Fig. 2c). These data suggested that the gene clusters of modules Spm and Nic2 were repressed by the transcriptional regulatory factors NicR2A and NicR2Bs, while the gene clusters of the module Nic1 were not.

The gene expression levels of *spmA1*, *spmA2*, *hspB1*, and *hspB2* in the mutant strain JY-Q Δ*nicR2B1* Δ*nicR2B2* (QΔ*nicR2B1*Δ*nicR2B2*) were significantly higher than those for the mutant strain JY-Q Δ*nicR2A* (QΔ*nicR2A*) in the absence of nicotine (Fig. 2b). Meanwhile, the expression levels of *nicR2A* and *nicR2Bs* (*nicR2B1* and *nicR2B2*) were detected in wild-type strain JY-Q in the presence or absence of nicotine. Unexpectedly, the results (Fig. 2d) showed that the transcriptional level of *nicR2A* was even higher than that of *nicR2Bs* and suggested that the sequence differences, especially the residues of their N-terminal extension, might lead to stronger repression of NicR2Bs than NicR2A.

Nicotine-degradation efficiency of the strain QΔABs. The nicotine-degrading capacity of the mutants QΔ*nicR2A*, JY-Q Δ*nicR2A* Δ*nicR2B1* (QΔ*nicR2A*Δ*nicR2B1*), and

QΔABs (QΔ*nicR2A*Δ*nicR2B1*Δ*nicR2B2*) was evaluated in BSM containing 2 g/liter nicotine. QΔABs exhibited a higher growth rate and degradation efficiency than other mutants (Fig. 3a). At 48 h, the optical density at 600 nm (OD_{600}) of QΔABs reached 1.18, while that of strain JY-Q was 1.06, which indicated that there was a statistically significant difference in growth rate. However, the increase in growth rate was small. Consistent with this result, the nicotine degradation efficiency of QΔABs increased by 17% compared to JY-Q (Fig. 3b).

RT-qPCR results showed that the mRNA levels of *nicA2*, *nox*, *spmA1*, *spmA2*, *hspB1*, and *hspB2* in QΔABs were higher than those in JY-Q in the presence of 2 g/liter nicotine (Fig. 3c). In particular, the transcription levels of four genes in modules Spm and Nic2 significantly increased in QΔABs (*spmA1* increased by 1.7-fold, *spmA2* increased by 1.6-fold, *hspB1* increased by 1.3-fold, and *hspB2* increased by 1.8-fold). However, the increase in the transcriptional level of nicotine-degrading genes in QΔABs was still not sufficiently elevated. Therefore, further research was necessary to improve the gene expression level and efficiency of nicotine metabolism.

Screening of endogenous strong promoters from JY-Q using RNA-seq analysis and RT-qPCR verification. The transcriptomic analysis of JY-Q, in the presence or absence of nicotine as a carbon, nitrogen, and energy source, was performed. From the RNA-seq analysis of JY-Q, transcriptional levels of all genes were ranked from high to low, based on their fragments per kilobase per million (FPKM) values. Six genes with higher FPKM values were first screened (see Table S1 in the supplemental material). Then, three genes, namely, *RS28250*, *RS09985*, and *RS24685*, were further screened out according to whether there was a reverse open reading frame (ORF) upstream of the gene, so as to ensure that there must be a promoter in the intergenic region between the gene and its upstream ORF (see Fig. S2a in the supplemental material).

RT-qPCR was performed to detect the relative expression level of these three genes and the control genes *hspB1* and *hspB2* in JY-Q grown in nicotine medium to verify the reliability of the selected promoter. The results showed that the relative expression levels of *RS28250*, *RS09985*, and *RS24685* were 16.8, 2.6, and 1.6 times higher, respectively, than that of *hspB2* (Fig. S2b). Thus, two putative strong promoter sequences, namely, $P_{RS28250}$ (the intergenic sequence upstream of *RS28250*) and $P_{RS09985}$ (the intergenic sequence upstream of *RS09985*), were selected for replacing the original promoter of nicotine degradation gene clusters.

Effect of endogenous ectopic strong promoters on nicotine metabolism. In our previous studies, viridicatum pigment accumulation was detected in the process of nicotine degradation in both JY-Q and its mutant with *hpo* knocked out (QΔ*hpo*), and the mutant QΔ*hpo* exhibited more viridicatum pigment accumulation than the wild type JY-Q (14). The viridicatum pigment accumulation may result from a lower rate of metabolism due to a lower expression level of the downstream genes than those upstream in the nicotine metabolism pathway, such as *hspB* in module Nic2. Therefore, $P_{RS28250}$ and $P_{RS09985}$ were used to replace the intergenic sequence upstream of Nic2 gene clusters in QΔABs (see Fig. S3 in the supplemental material) to obtain six mutants (Table 1) for improving the expression level of downstream metabolic genes.

The above mutants and JY-Q were cultured in BSM containing 2 g/liter nicotine to determine the growth rates and nicotine degradation efficiency (Fig. 4a). The strains QΔABs-ΔP_{hspB2}::$P_{RS09985}$ (QΔABs-29), QΔABs-ΔP_{hspB1}::$P_{RS09985}$,ΔP_{hspB2}::$P_{RS28250}$ (QΔABs-19-28), and QΔABs-ΔP_{hspB1}::$P_{RS09985}$,ΔP_{hspB2}::$P_{RS09985}$ (QΔABs-19-29) showed severe growth defects compared to QΔABs. The strains QΔABs-ΔP_{hspB1}::$P_{RS28250}$ (QΔABs-18) and QΔABs-ΔP_{hspB1}::$P_{RS09985}$ (QΔABs-19) grew well, and QΔABs-28 (QΔABs-ΔP_{hspB2}::$P_{RS28250}$) grew best, as indicated by its OD_{600} reaching 1.1 at 24 h, while the OD_{600} of QΔABs only reached 0.78. High-performance liquid chromatography (HPLC) was used to detect the nicotine concentration in the medium. The data also showed that the efficiency of nicotine degradation in the three strains QΔABs-29, QΔABs-19-28, and QΔABs-19-29 was very low compared with QΔABs, while the strain QΔABs-28 showed the highest nicotine-degradation ability, degrading all of the nicotine in 24 h, at least 8 h less than that observed for QΔABs (Fig. 4b). Compared with the original nicotine

TABLE 1 Strains and plasmids used in this study

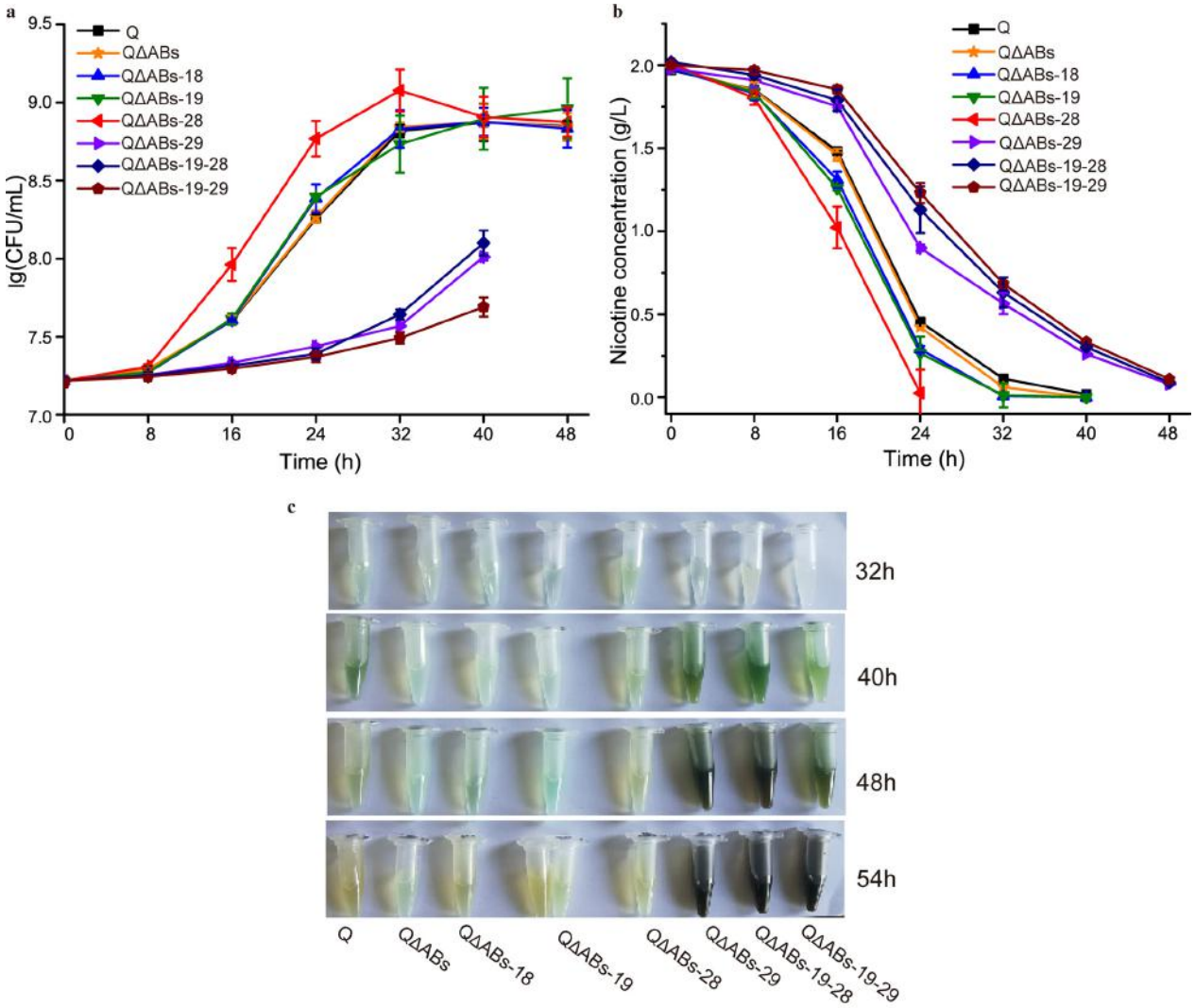
Strain or plasmid	Relevant genotype or characteristic(s)	Reference or source
Strains		
Pseudomonas sp. JY-Q	Wild type	13
QΔ*nicR2A*	*nicR2A* deleted in JY-Q	This study
QΔ*nicR2B1*	*nicR2B1* deleted in JY-Q	This study
QΔ*nicR2B2*	*nicR2B2* deleted in JY-Q	This study
QΔ*nicR2A*Δ*nicR2B1*	*nicR2A* and *nicR2B1* deleted in JY-Q	This study
QΔ*nicR2A*Δ*nicR2B2*	*nicR2A* and *nicR2B2* deleted in JY-Q	This study
QΔ*nicR2B1*Δ*nicR2B*	*nicR2B1* and *nicR2B2* deleted in JY-Q	This study
QΔ*nicR2A*Δ*nicR2B1*Δ*nicR2B2* (QΔABs)	*nicR2A*, *nicR2B1*, and *nicR2B2* deleted in JY-Q	This study
QΔABs-18	QΔABs-ΔP_{hspB1}::$P_{RS28250}$	This study
QΔABs-19	QΔABs-ΔP_{hspB1}::$P_{RS09985}$	This study
QΔABs-28	QΔABs-ΔP_{hspB2}::$P_{RS28250}$	This study
QΔABs-29	QΔABs-ΔP_{hspB2}::$P_{RS09985}$	This study
QΔABs-19-28	QΔABs-ΔP_{hspB1}::$P_{RS09985}$,ΔP_{hspB2}::$P_{RS28250}$	This study
QΔABs-19-29	QΔABs-ΔP_{hspB1}::$P_{RS09985}$,ΔP_{hspB2}::$P_{RS09985}$	This study
E. coli DH5α	Host to replicate plasmids	Our lab
E. coli WM3064	Nutrient-deficient strain for conjugation	Our lab
Plasmid		
pk18mobsacB	Suicide vector, mob$^+$, *sacB*, Km^r	42

content, the nicotine-degradation rates of QΔABs and JY-Q were 0.083 g/liter/h and 0.049 g/liter/h, respectively. The nicotine-degradation efficiency of QΔABs was 69% higher than that of JY-Q. Interestingly, we found that the color of the medium changed during the growth of these strains (Fig. 4c). For the strains that grew poorly, such as QΔABs-29, QΔABs-19-28, and QΔABs-19-29, a green-colored substance appeared in the medium at 40 h, and then the color further converted to a dark-green color. However, for the strain QΔABs-28, a slight green tint appeared in the medium during the growth of the strain, which also indicated that the overexpression of the *nic2* gene clusters could effectively balance the whole metabolism.

RT-qPCR was performed to determine the relative expression level of the crucial genes in these strains grown in BSM plus nicotine for determining whether promoter replacement changed the transcriptional expression level of nicotine-degrading gene clusters. The result showed that there was no difference in the expression of *nox* in all strains (Fig. 5a) and of *spm* except in strain JY-Q (Fig. 5b). In strains QΔABs-19, QΔABs-19-28, and QΔABs-19-29, the expression level of *hspB1* was lower than that of other strains (Fig. 5c), which indicates that $P_{RS09985}$ reduced the transcriptional expression level of the *nic2-1* gene cluster when it replaced the original promoter of this gene cluster. However, the expression level of *hspB2* in QΔABs-28 was significantly higher than that of other strains; it was 1.9 times that of QΔABs and 3.8 times that of JY-Q. In addition, the expression levels of *hspB2* in QΔABs-29 and QΔABs-19-29 strains were even lower than that of QΔABs (Fig. 5d).

In summary, $P_{RS28250}$ most effectively improves the transcriptional level of the *nic2-2* gene cluster, while the effect of $P_{RS09985}$ was worse than the original promoter.

Nicotine-degrading efficiency and tolerance of the mutant QΔABs-28. QΔABs-28 as well as other mutants were grown on a BSM solid plate containing 15 g/liter nicotine to evaluate the growth ability of QΔABs-28 in an environment with a high concentration of nicotine (Fig. 6a). Strains QΔABs-19, QΔABs-18, QΔABs-28, and QΔABs all could grow on the plate, and QΔABs-28 grew best among all the strains. This finding indicates that the overexpression of a nicotine-degradation gene can improve the tolerance of the strain to a high concentration of nicotine. In addition, the effect of different concentrations of nicotine on QΔABs-28 was further evaluated in liquid BSM containing 2 g/liter, 5 g/liter, and 10 g/liter nicotine. The results (Fig. 6b and c) showed that the growth rate and

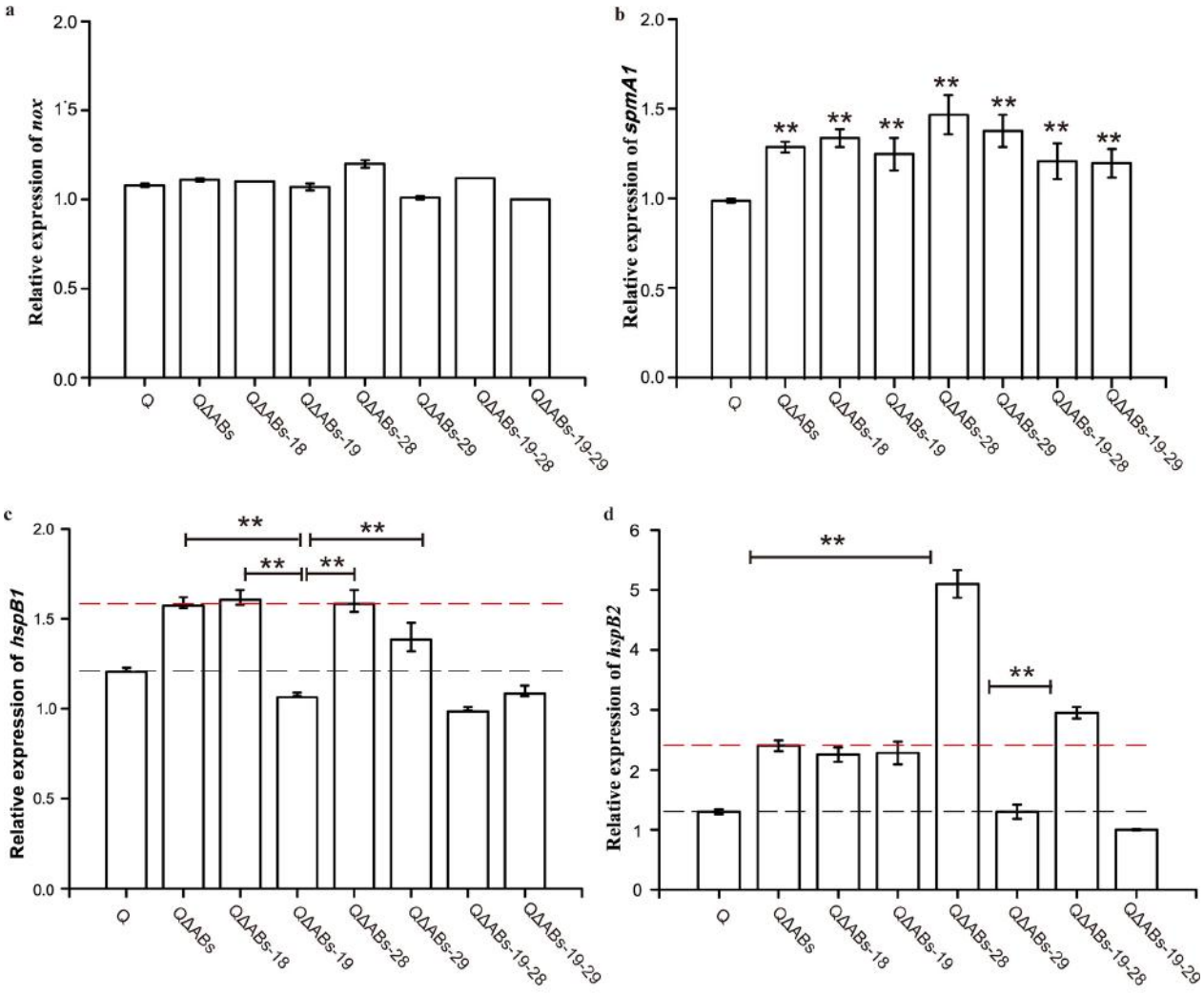


d

Strains	Q	QΔABs	QΔABs-18	QΔABs-19	QΔABs-28	QΔABs-29	QΔABs-19-28	QΔABs-19-29
P_{hspB1}	+	+	-	-	+	+	-	-
$P_{RS28250}$			+					
$P_{RS09985}$				+			+	+
P_{hspB2}	+	+	+	+	-	-	-	-
$P_{RS28250}$					+		+	
$P_{RS09985}$						+		+

FIG 4 Growth (a), nicotine degradation (b), and the broth color change (c) of the promoter replaced mutant and WT strains in BSM containing 2 g/liter nicotine. (d) Data regarding promoter replacement in the strains (+, original or inserted promoter replacing the original one; −, replaced).

nicotine degradation ability of the QΔABs-28 were higher than those of QΔABs. Compared with QΔABs, the degradation efficiency of QΔABs-28 increased by 39%, 14%, and 11% in BSM containing 2 g/liter, 5 g/liter, and 10 g/liter nicotine, respectively. Moreover, when the mutant QΔABs-28 grew in 10 g/liter nicotine, the growth rate in the early stage was much lower than that in 2 g/liter and 5 g/liter nicotine. It should be noted that, in 10 g/liter nicotine, QΔABs-28 could not completely



e

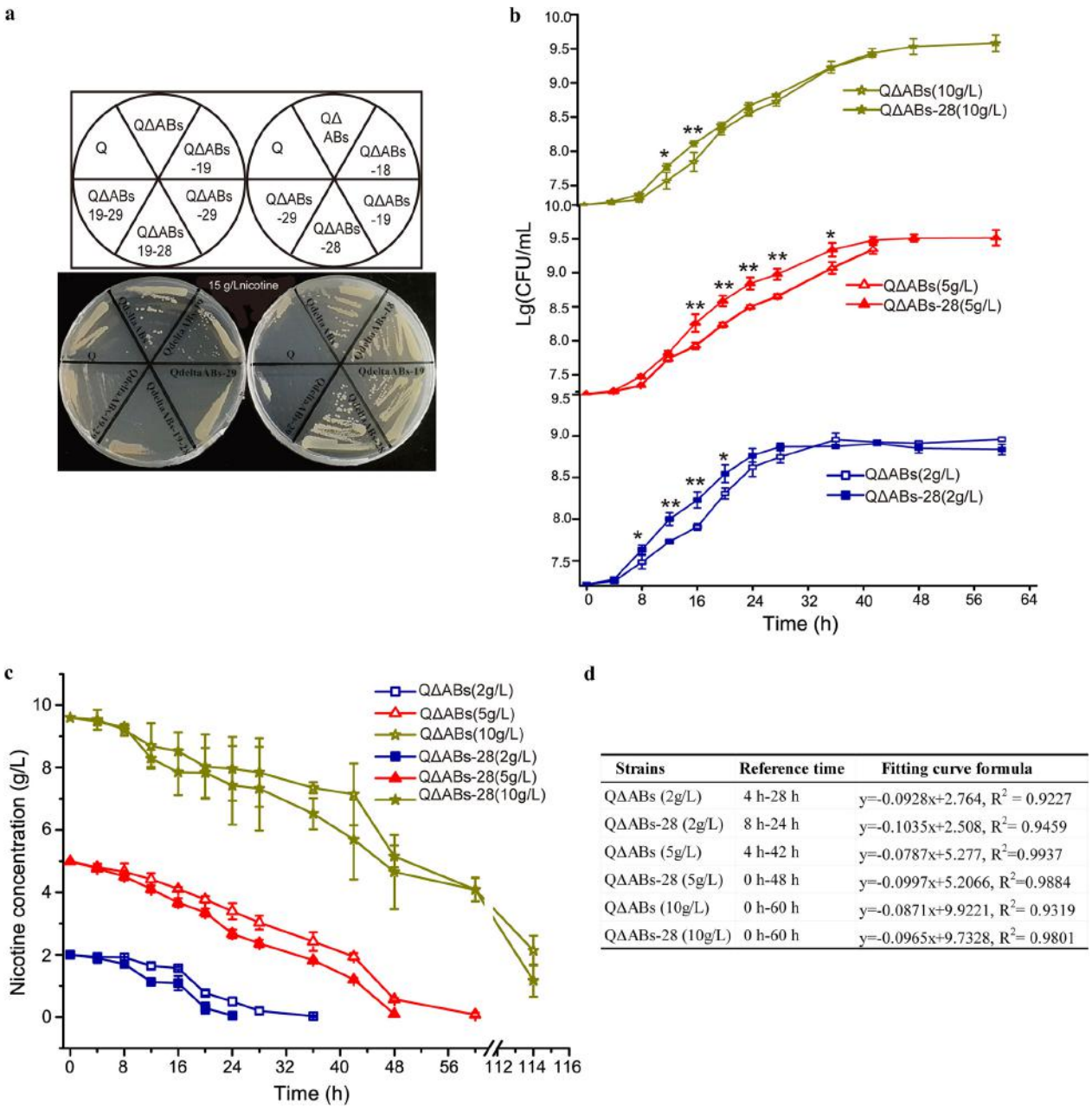
Strains	Q	QΔABs	QΔABs-18	QΔABs-19	QΔABs-28	QΔABs-29	QΔABs-19-28	QΔABs-19-29
PhspB1	+	+	-	-	+	+	-	-
PRS28250			+					
PRS09985				+			+	+
PhspB2	+	+	+	+	-	-	-	-
PRS28250					+		+	
PRS09985						+		+

FIG 5 mRNA expression levels of *nox* (a), *spmA1* (b), *hspB1* (c), and *hspB2* (d) in seven mutants and the WT (Q) grown in BSM containing 2 g/liter nicotine. The data are the means of three independent experiments, and error bars indicate the standard deviations. *t* test was used for significance analysis between the mutant or/and the wild type JY-Q; *, $P < 0.05$ (significant); **, $P < 0.01$ (extremely significant). (e) Data regarding promoter replacement in the strains (+, original or inserted promoter replacing the original one; −, replaced).

degrade all nicotine at 114 h and that the residual nicotine concentration was 1.54 g/liter (Fig. 6c).

DISCUSSION

During past decades, an increasing number of nicotine degraders have been isolated from the natural environment or from nicotine-polluted samples. However, most



Strains	Reference time	Fitting curve formula
QΔABs (2g/L)	4 h-28 h	y=-0.0928x+2.764, R^2 = 0.9227
QΔABs-28 (2g/L)	8 h-24 h	y=-0.1035x+2.508, R^2= 0.9459
QΔABs (5g/L)	4 h-42 h	y=-0.0787x+5.277, R^2=0.9937
QΔABs-28 (5g/L)	0 h-48 h	y=-0.0997x+5.2066, R^2=0.9884
QΔABs (10g/L)	0 h-60 h	y=-0.0871x+9.9221, R^2= 0.9319
QΔABs-28 (10g/L)	0 h-60 h	y=-0.0965x+9.7328, R^2= 0.9801

FIG 6 Growth profile of WT and mutant strains after 40 h on solid BSM plate containing 15 g/liter nicotine (a), growth (b), and nicotine degradation curves (c) of the mutant QΔABs-28 and the control QΔABs in liquid BSM containing 2, 5, and 10 g/liter nicotine. It should be noted that, after 42 h, there was a large amount of green substance accumulation in 5 g/liter and 10 g/liter nicotine medium of strain QΔABs; thus, its OD_{600} was not measured. The data are the means of three independent experiments, and error bars indicate the standard deviations. *t* test was used for significance analysis; *, $P < 0.05$ (significant); **, $P < 0.01$ (extremely significant). (d) Strain data.

strains showed no growth when the nicotine concentration increased to 5 to 6 g/liter (3–7). Compared with the reported strains, *Pseudomonas* sp. JY-Q exhibited a higher tolerance to nicotine because it was isolated from a high-nicotine-content sample. Its native nicotine-degradation ability is greater than most other reported bacterial strains (13–15). However, when it was used to degrade nicotine in a real environment with a high nicotine content, such as TWE, the degrading efficiency of JY-Q was still insufficient. In this study, inducible promoters were screened by transcriptomics analysis.

Mutant QΔABs-28 was then constructed using a combined strategy of a repressor removal and promoter replacement of the downstream gene *hspB*, which exhibited significant improvement of nicotine removal efficiency in both BSM and TWE. The nicotine-degradation efficiency of strain QΔABs-28 increased by 69% compared with that of the WT. In particular, QΔABs-28 grew well on a 15-g/liter nicotine solid plate and in 10 g/liter nicotine liquid medium, exhibiting a nicotine-degrading ability at a much higher concentration than that of other reported strains. This finding suggests an additional strategy for strain modification to improve the ability of biodegradation using inducible promoters.

NicR2 in JY-Q showed a repression effect on the *nic2* cluster (including *hspB*), similar to that observed in S16 (24). The difference regarding NicR2 between JY-Q and S16 is that there are homologous regulating proteins, NicR2A and NicR2Bs, that show different effects on nicotine metabolism in JY-Q (Fig. 2b). NicR2Bs exhibited a greater repression effect on the expression of *hspB1*, *hspB2*, *spmA1*, and *spmA2* than NicR2A, while NicR2A and NicR2Bs both show no effect on the expression of *nic1*. These results present additional insights into the effects of NicR2 on nicotine degradation, and all the duplicate homologous repressors needed to be mined and deleted for strain improvement in nicotine metabolism.

In promoter engineering, the promoter involved in heavy constitutive expression is usually chosen as a strong promoter. In this study, only the promoters of nicotine-induced genes having higher FPKM values than those of *hspB2* were selected to enhance downstream nicotine metabolism through promoter replacement. The context for the genetic organization of promoters and their flanking genes and RT-qPCR analytical results indicate that the promoter of the nicotine-induced gene *RS28250* was the strongest among all test genes (Fig. S2). The resultant mutant QΔABs-28, in which P_{hspB2} was replaced by $P_{RS28250}$, exhibited an increased expression of genes *spmA1*, *hspB1*, and *hspB2*, but not *nox* (Fig. 5). Meanwhile, QΔABs-28 also showed the highest increase in nicotine-degradation efficiency (Fig. 4). Specifically, $P_{RS28250}$ enhanced *hspB2* expression at a higher level than all other promoters and improved *hspB1* and *spmA1* expression at a level close to that of $P_{RS09985}$. That is why QΔABs-28 showed a higher increase in nicotine-degradation efficiency than $P_{RS09985}$. The enhanced expression of both duplicate genes (such as *hspB1* and *hspB2*) can result in significant improvements in nicotine metabolism.

Generally, genetic manipulation strategies, including gene deletion, overexpression of crucial genes using a strong promoter, ribosome binding site (RBS) engineering, and *de novo* pathway reconstruction, have been widely applied to obtain ideal engineered organisms (31, 32). The degradation of nicotinic acid (NA) could be enhanced by increasing the activity of relevant genes due to the deletion of the transcriptional regulator (33). Similarly, the nicotine degradation ability of QΔABs was improved in this study. Meanwhile, studies have shown that some environmental pollutants, such as nicotine and NA, and their metabolic intermediates usually play the role of inducers, which can effectively release the inhibition of related metabolic genes by combining with the repressors (12, 22, 34). This is consistent with our results. In the absence of nicotine, the expression level of *hspB2* in QΔABs was 6.7 times that in JY-Q (Fig. 2b), while in the presence of nicotine, the relative expression level of *hspB2* was only 1.8 times that in JY-Q (Fig. 3c); *spmA1*, *spmA2*, and *hspB1* showed the same trend. These results also showed that nicotine or its metabolites could remove repression in JY-Q. Although we did not achieve the expected effect of efficient degradation of nicotine, it is meaningful to remove the repressors so that the strain can respond to nicotine quickly and reduce unnecessary protein synthesis.

With the rapid development of bioinformatics and the wide application of sequencing technology, RNA-seq technology provides an opportunity to detect the expression level of all genes in an organism, allowing us to screen for endogenous promoters having different strengths (35). In this study, endogenous strong promoters were also screened to replace the promoter and improve the expression level of the downstream

gene cluster of nicotine. The resultant mutant QΔABs successfully grew without a green-tint appearance, resulting from the accumulation intermediate metabolites in the process of nicotine degradation, indicating the fine-tuned metabolic flux of *nic2*. However, the expression level of a gene in prokaryotes is usually coregulated by promoters, enhancers, and attenuators (36). In this study, we also found that the strength of a selected promoter, identified by RT-qPCR, decreased when it was inserted at the replacing location. These phenomena may be related to the sequence around the promoter and the adjacent ORF (29, 30). Based on this situation, more reliable assessment methods, such as reporter gene characterization (29), RBS engineering (37), and removing redundant sequences in the intergenic region (38), should be used to screen and obtain promoters of different strengths, which is likely to achieve precise fine-tuned metabolic networks.

In addition, we aimed at constructing a mutant strain that could efficiently metabolize nicotine in high nicotine environments. Thus, we chose only strong promoters, which serve the genes with significantly higher FPKM values than those of *hspB*. Fortunately, the resultant mutant with promoter replacement did exhibit enhanced nicotine metabolism. However, in future studies, it is important for us to compare the difference between the three promoters in this study and the strong constitutive promoters without nicotine induction.

We also found that QΔABs-28 still had a long lag phase at high concentrations of nicotine, which may indicate that nicotine is still toxic to cells, or perhaps is due to other causes, such as a slow induction of nicotine-degradation genes (Fig. 6b). We further conclude that the increased expression level of nicotine degradation genes in these strains and their tolerance to nicotine are not exclusively associated. Using glutathione *S*-transferases and toxin eflux pumps, such as ATP-binding cassette (ABC) transporters, may be a potential way to reduce nicotine toxicity (39, 40).

In summary, two aspects are presented as limiting factors for nicotine in the wild-type strain JY-Q. First, the two homologous proteins NicR2A and NicR2Bs have different abilities to repress nicotine metabolism genes. Second, endogenous inducible promoters also have different transcriptional abilities when in different positions of the genome, requiring further studies to understand the role of endogenous promoters in the strain. In addition, the strategy of knocking out the transcriptional regulatory genes and replacing the original promoter of the whole gene cluster in the genome in an effort to regulate metabolic flux is feasible. Moreover, these strategies may be helpful for engineering other microorganisms for degrading environmental pollutants.

MATERIALS AND METHODS

Chemicals and media. Nicotine (99%) was purchased from Flukachemie GmbH (Buchs, Switzerland); the enzyme required for the DNA molecular manipulation experiment was purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). All other chemicals and solvents used in this study were analytical grade and commercially available.

The Luria-Bertani (LB) medium (pH 7.0) composition (g/liter) was as follows: peptone (10.0), yeast extract (5.0), and NaCl (10.0). Basic salt medium (BSM) composition (pH 7.0) (g/liter) was as follows: Na_2HPO_4 (5.57), KH_2PO_4 (2.44), K_2SO_4 (1.0), $MgCl_2{\cdot}6H_2O$ (0.2), $CaCl_2$ (0.001), $FeCl_3{\cdot}6H_2O$ (0.001), and $MgCl_2{\cdot}4H_2O$ (0.0004).

Bacterial strains, plasmids, and culture conditions. *Pseudomonas* sp. JY-Q was isolated from tobacco waste extract of Hangzhou Liqun Environment Protecting Paper Co., Ltd., Hangzhou, China, and has been deposited in the China Center for Type Culture Collection (CCTCC; number M2013236).

Escherichia coli WM3064 is a mutant with nutritional deficiency; in the experiment, 2,6-diaminodicarboxylic acid (DAP) was added to the medium to revive the growth of the strain. JY-Q was cultured in LB medium or BSM supplemented with nicotine or glucose at 30°C, and *E. coli* WM3064 was cultured in LB broth supplemented with 57 mg/liter DAP at 37°C. If necessary, kanamycin (50 μg/ml) was added to the medium.

The plasmid pK18mobsacB is a *Pseudomonas* suicide plasmid in which the *sacB* gene is a transformation selective marker and is responsible for the suicide of the plasmid on an LB agar plate containing 15% (wt/vol) sucrose. All the strains and plasmids used in this study are listed in Table 1.

RNA extraction and quantitative RT-PCR. In the early exponential stage, the cells were centrifuged at 10,000 $\times$ *g* for 5 min at 4°C. The total RNA was extracted by using RNA Isolator (Vazyme Biotech Co., Ltd., Nanjing, China). Subsequently, the HiScript II Q RT SuperMix qPCR kit (purchased from Vazyme

TABLE 2 All gene knockout, promoter replacement, and RT-qPCR primers

Primer	Sequence (5′–3′)[a]	Production length (bp)	Description
Gene knockout primers			
nicR2A-F	CCGCAGTTGGGCCTAAA	115	Verify the knockout of *nicR2A*
nicR2A-R	CCTAGGAACCCGTGCATAG		
nicR2B1-UF	AATTCGAGCTCGGTACCCGGGGATCCATGCGATGTCATAACCTTGGG	579	Knockout of *nicR2B1*
nicR2B1-UR	CGGTACCTCAGGCGCGCT ACTGCACCCTGGCTGAACACTTAA		
nicR2B1-DF	TTAAGTGTTCAGCCAGGGTGCAGTAGCGCGCCTGAGGTACCG	575	
nicR2B1-DR	TAAAACGACGGCCAGTGCCAAGCTTGGGTCAACAACAGCGTAATGC		
nicR2B2-UF	AATTCGAGCTCGGTACCCGGGGATCCACACGCCGAGATCACCCAAAG	615	Knockout of *nicR2B2*
nicR2B2-UR	TCGGTACATCAGGCGCGCTACTGCACCCTAGCTGAACACTTAAC		
nicR2B2-DF	GTTAAGTGTTCAGCTAGGGTGCAGTAGCGCGCCTGATGTACCGA	673	
nicR2B2-DR	TAAAACGACGGCCAGTGCCAAGCTTGCTCTGTCCGCTGGATCGTGT		
Primers for promoter replacement and verification			
1*RS09985*-UF	AATTCGAGCTCGGTACCCGGGGATCCCGTTCGTCGGCAACAACTCTT	816	$P_{RS09985}$ replaces P_{hspB1}
1*RS09985*-UR	AAGTCCTTGAAGGGGAACACGATGAGCATGAAACAGCGCGTAA		
1*RS09985*-PF	TTACGCGCTGTTTCATGCTCATCGT GTTCCCCTTCAAGGACTT	551	
1*RS09985*-PR	CGGAAAAGCTCATCTGGCTCATCACTACTCCTCGCAGGTCTTCTC		
1*RS09985*-DF	GAGAAGACCTGCGAGGAGTAGTGATGAGCCAGATGAGCTTTTCCG	803	
1*RS09985*-DR	TAAAACGACGGCCAGTGCCAAGCTTGTTCAGCTTGGAATAGGTGCTGC		
1UUF	CTCCAGTTGGGTTGGTTACGTG	1,418	Verify the recombination
09985PR	CGGAAAAGCTCATCTGGCTCATCACTACTCCTCGCAGGTCTTCTC		
1UUF	CTCCAGTTGGGTTGGTTACGTG		
1DDR	CGAACAGCGTGGTCAACTGAG		
2UUF	TGCTCCAAGGTTTCGCTCAC	1,462	Verify the recombination
09985PR	CGGAAAAGCTCATCTGGCTCATCACTACTCCTCGCAGGTCTTCTC		
2UUF	TGCTCCAAGGTTTCGCTCAC		
2DDR	TTAACGCAAACGCATCAAACA		
1*RS28250*-UF	AATTCGAGCTCGGTACCCGGGGATCCCGTTCGTCGGCAACAACTCTT	816	$P_{RS28250}$ replaces P_{hspB1}
1*RS28250*-UR	AACAAGTGAGGGCAACACCCTATGAGCATGAAACAGCGCG		
1*RS28250*-PF	CGCGCTGTTTCATGCTCATAGGGTGTTGCCCTCACTTGTT	603	
1*RS28250*-PR	CGGAAAAGCTCATCTGGCTCATGAGTATTCCAGCGCCTTGG		
1*RS28250*-DF	CCAAGGCGCTGGAATACTCATGAGCCAGATGAGCTTTTCCG	802	
1*RS28250*-DR	TAAAACGACGGCCAGTGCCAAGCTTGTTCAGCTTGGAATAGGTGCTGC		
2*RS09985*-UF	AATTCGAGCTCGGTACCCGGGGATCCGAAACACCTAGGGAATGGACTAGC	662	$P_{RS09985}$ replaces P_{hspB2}
2*RS09985*-UR	CGAGAAGACCTGCGAGGAGTAGTGTTAACCGACATAACGGAGATCAA		
2*RS09985*-PF	TTGATCTCCGTTATGTCGGTTAACACTACTCCTCGCAGGTCTTCTCG	556	
2*RS09985*-PR	ATGACGCGCTTATCAATACTCATCGTGTTCCCCTTCAAGGACTT		
2*RS09985*-DF	AAGTCCTTGAAGGGGAACACGATGAGTATTGATAAGCGCGTCAT	676	
2*RS09985*-DR	TAAAACGACGGCCAGTGCCAAGCTTCCACCTGCATGGTGGTTTTAC		
2*RS28250*-UF	AATTCGAGCTCGGTACCCGGGGATCCGAAACACCTAGGGAATGGACTAGC	657	$P_{RS28250}$ replaces P_{hspB2}
2*RS28250*-UR	CCAAGGCGCTGGAATACTCTTAACCGACATAACGGAGATCAA		
2*RS28250*-PF	TTGATCTCCGTTATGTCGGTTAAGAGTATTCCAGCGCCTTGG	608	
2*RS28250*-PR	ATGACGCGCTTATCAATACTCATAGGGTGTTGCCCTCACTTGTT		
2*RS28250*-DF	AACAAGTGAGGGCAACACCCTATGAGTATTGATAAGCGCGTCAT	626	
2*RS28250*-DR	TAAAACGACGGCCAGTGCCAAGCTTCCACCTGCATGGTGGTTTTAC		
1UUF	CTCCAGTTGGGTTGGTTACGTG	1,473	Verify the recombination
28250-PR	ATGACGCGCTTATCAATACTCATAGGGTGTTGCCCTCACTTGTT		
2UUF	TGCTCCAAGGTTTCGCTCAC	1,517	Verify the recombination
28250-PR	ATGACGCGCTTATCAATACTCATAGGGTGTTGCCCTCACTTGTT		
RT-qPCR primers			
q nox-F	CCGATAGTGAGTCTGCGTGT	143	RT-qPCR for *nic1-2*
q nox-R	CCCAGCGAGCAGAGCTTAAT		
q nicA2-F	CCCGTCCGAGATTCTGCTTA	145	RT-qPCR for *nic1-1*
q nicA2-R	GTCGTAATGACAGTTCCACTCA		
q hspB1-F	TCGCCCCCAAGAACTACCC	118	RT-qPCR for *nic2-1*
q hspB1-R	CAGGATACTAATCGAGCATCTC		
q hspB2-F	GTCGTAGCGAAGAAGTCCTGG	132	RT-qPCR for *nic2-2*
q hspB2-R	CCACTCGTCTTTGCCTACACC		

(Continued on next page)

TABLE 2 (Continued)

Primer	Sequence (5′–3′)[a]	Production length (bp)	Description
16S-F	CACACTGGAACTGAGACACG	127	RT-qPCR for 16S rRNA gene
16S-R	TGCTTTACAATCCGAAGACC		
q spmA1-F	AACGTGCCCAGTTTCCGTAT	122	RT-qPCR for *spm1*
q spmA1-R	TGACTCAGGCAATCACCAGT		
q spmA2-F	ACACCACCATGGAAACCGAA	108	RT-qPCR for *spm2*
q spmA2-R	CGGCTGGAATGGAAGGTGAA		
q RS09985-F	TCGTCTGACTGCGACTGAA	162	RT-qPCR for *RS09985*
q RS09985-R	TTGCGGCTGGCTTTGT		
q RS09985-F	TCCGTTGGAAAGAGCATA	186	RT-qPCR for *RS28250*
q RS09985-R	GCGTGATTTTCGTTGAGTC		

[a]Underlining denotes the restriction cleavage site.

Biotech Co., Ltd.) was used to develop reverse transcription reactions. The reaction buffer system of RT-qPCR was prepared with ChamQ SYBR qPCR master mix, and the quantitative PCR was performed with a Bio-Rad CFX real-time PCR system. The expression levels of the 16S rRNA gene were used as internal references. Each reaction was repeated at least three times. The primers used for RT-qPCR are listed in Table 2.

Gene knockout and promoter replacement. For gene knockout, the PCR products of the upstream and downstream homologous arms of the target gene were fused into the suicide plasmid pK18mobsacB. The fusion segment was transformed into *E. coli* DH5α, and the right recombinant plasmids were selected by sequencing. These plasmids were further introduced into *E. coli* WM3064 and then conjugated with JY-Q or its mutant. Subsequently, kanamycin was used to screen out the homologous recombinant strains on the BSM solid plate. Finally, two hybrid transformants were obtained on an LB plate with 15% sucrose (wt/vol). PCR screening and sequencing were used to identify the recombinant strains.

For promoter replacement, PCR was used to clone the intergenic sequence containing the selected promoter and the upstream and downstream homologous arm segments of the replaced promoter from the JY-Q genome. Next, three fragments were linked by overlap and fused into pK18mobsacB. The process of promoter replacement is similar to that of gene knockout, and the specific experimental procedures and screening methods are showed in Fig. S4 in the supplemental material. For example, $P_{RS09985}$ replaced the promoter P_{hspB1} of the *nic2-1* gene cluster, and the constructed promoter replacement vector pK18mobsacb-ΔP_{hspB1}::$P_{RS09985}$ was introduced into WM3064 cells by chemical transformation. Then, the donor strain WM3064/pK18mobsacb-ΔP_{hspB1}::$P_{RS09985}$ and the recipient strain QΔABs were conjugated. Subsequently, the combined bacteria were washed down and coated onto the corresponding resistant plate culture. Next, primers (1UUF and 09985PR) were used to verify the target site of the plasmid integration into the genome (Fig. S3a, left). Finally, the correct strain was screened for second single crossover mutants on a sucrose plate with primers 1UUF and 1DDR. All gene knockout, promoter replacement, and validation primers are listed in Table 2.

Growth phenotype and degradation assays. Cell growth was monitored and recorded by measuring OD_{600}. When the OD_{600} of cells reached 0.6, the bacteria were collected and washed with normal saline three times. Then, the strains were streaked onto the BSM solid plate containing different concentrations of nicotine to observe their growth status.

JY-Q and its mutant strains were cultured in BSM liquid medium with different concentrations of nicotine to detect and evaluate their growth ability and nicotine degradation efficiency. The pH of all BSM media plus nicotine was adjusted with 4 M phosphoric acid to pH 7.0. The OD_{600} of the strain was detected by a spectrophotometer (UV1000; Shanghai Tianmei Instrument Co. Ltd., China) at different times. Then, their cell amounts in $\log_{10}$(CFU/ml) were calculated based on a standard curve between OD_{600} and the cell amount.

If necessary, when there was more pigment accumulation in the medium, the cells were centrifuged and washed/diluted with BSM solution before measurement. Before detection of the nicotine concentration, samples taken at different periods were centrifuged at 10,000 rpm for 10 min, and the supernatant was taken and filtered. High-performance liquid chromatography (HPLC) (Agilent, USA), with column Agilent SB-C_{18} (4.6 mm by 150 mm), was used to examine the degradation ability of JY-Q and its derivatives. The mobile phase included methanol and 0.1 M KH_2PO_4 (pH 3.0), with a volume ratio of 10:90, flow rate of 1 ml/min, wavelength of 254 nm, and detection time of 5 min. All experiments were carried out in three parallel repetitions.

RNA-seq analysis. Strain JY-Q was activated overnight in LB medium and then transferred to LB liquid medium for growth to an OD_{600} of $\approx$0.8. After three sterile washes, JY-Q was transferred to BSM medium in the presence or absence of nicotine. Three parallel cultures were grown at 30°C and 180 rpm. First, RNA was extracted, then the total RNA was tested, and a cDNA library was constructed. The quality control of sequencing reads was analyzed by FastQC software. According to the results of the read comparison, the reads were allocated to the specific transcripts for the read count. This result could not be directly used to characterize the transcriptional expression because it had not been standardized. Thus, the measurement of fragments per kilobase of transcript per million (FPKM) was used to characterize gene expression.

Statistical and bioinformatic methods. Statistical analysis was conducted with IBM SPSS 20.0. All data were shown as the mean ± standard error (SE), with triplicate independent experiments and significance analysis by *t* test. Sequence alignments were conducted by BLASTp (41) and comparison of amino acid multiple sequences with UniProt.

The manuscript does not contain any studies with human participants or animals performed by any of the authors.

Data availability. All RNA-seq data were submitted to SRA with the accession number SRR12678832.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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We declare that we have no conflicts of interest.

REFERENCES

1. Piotrowska-Cyplik A, Olejnik A, Cyplik P, Dach J, Czarnecki Z. 2009. The kinetics of nicotine degradation, enzyme activities and genotoxic potential in the characterization of tobacco waste composting. Bioresour Technol 100:5037–5044. https://doi.org/10.1016/j.biortech.2009.05.053.
2. Valcárcel Y, González Alonso S, Rodríguez-Gil JL, Gil A, Catalá M. 2011. Detection of pharmaceutically active compounds in the rivers and tap water of the Madrid Region (Spain) and potential ecotoxicological risk. Chemosphere 84:1336–1348. https://doi.org/10.1016/j.chemosphere.2011.05.014.
3. Liu JL, Ma GH, Chen T, Hou Y, Yang SH, Zhang KQ, Yang JK. 2015. Nicotine-degrading microorganisms and their potential applications. Appl Microbiol Biotechnol 99:3775–3785. https://doi.org/10.1007/s00253-015-6525-1.
4. Zhong W, Zhu C, Shu M, Sun K, Zhao L, Wang C, Ye Z, Chen J. 2010. Degradation of nicotine in tobacco waste extract by newly isolated *Pseudomonas* sp. ZUTSKD. Bioresour Technol 101:6935–6941. https://doi.org/10.1016/j.biortech.2010.03.142.
5. Brandsch R. 2006. Microbiology and biochemistry of nicotine degradation. Appl Microbiol Biotechnol 69:493–498. https://doi.org/10.1007/s00253-005-0226-0.
6. Li H, Li X, Duan Y, Zhang KQ, Yang J. 2010. Biotransformation of nicotine by microorganism: the case of *Pseudomonas* spp. Appl Microbiol Biotechnol 86:11–17. https://doi.org/10.1007/s00253-009-2427-4.
7. Zhao L, Zhu CJ, Gao Y, Wang C, Li XZ, Shu M, Shi YP, Zhong WH. 2012. Nicotine degradation enhancement by *Pseudomonas stutzeri* ZCJ during aging process of tobacco leaves. World J Microbiol Biotechnol 28:2077–2086. https://doi.org/10.1007/s11274-012-1010-9.
8. Liu HG, He HL, Cheng CH, Liu JL, Shu M, Jiao Y, Tao F, Zhong WH. 2015. Diversity analysis of the bacterial community in tobacco waste extract during reconstituted tobacco process. Appl Microbiol Biotechnol 99:469–476. https://doi.org/10.1007/s00253-014-5960-8.
9. Yao YX, Tang HZ, Su F, Xu P. 2015. Comparative genome analysis reveals the molecular basis of nicotine degradation and survival capacities of *Arthrobacter*. Sci Rep 5:8642. https://doi.org/10.1038/srep08642.
10. Yu H, Tang HZ, Wang LJ, Yao YX, Wu G, Xu P. 2011. Complete genome sequence of the nicotine-degrading *Pseudomonas putida* strain S16. J Bacteriol 193:5541–5542. https://doi.org/10.1128/JB.05663-11.
11. Tang HZ, Wang LJ, Meng XZ, Ma LY, Wang SN, He XF, Wu G, Xu P. 2009. Novel nicotine oxidoreductase-encoding gene involved in nicotine degradation by *Pseudomonas putida* strain S16. Appl Environ Microbiol 75:772–778. https://doi.org/10.1128/AEM.02300-08.
12. Hu H, Wang L, Wang W, Wu G, Tao F, Xu P, Deng Z, Tang H. 2019. Regulatory mechanism of nicotine degradation in *Pseudomonas putida*. mBio 10:e00602-19. https://doi.org/10.1128/mBio.00602-19.
13. Li J, Qian SL, Xiong L, Zhu CY, Shu M, Wang J, Jiao Y, He HL, Zhang FM, Linhardt RJ, Zhong WH. 2017. Comparative genomics reveals specific genetic architectures in nicotine metabolism of *Pseudomonas* sp. JY-Q. Front Microbiol 8:2085–2096. https://doi.org/10.3389/fmicb.2017.02085.
14. Li J, Wang J, Li SS, Yi FM, Xu J, Shu M, Shen MJ, Jiao Y, Tao F, Zhu CY, Zhang H, Qian SL, Zhong WH. 2019. Co-occurrence of functional modules derived from nicotine-degrading gene clusters confers additive effects in *Pseudomonas* sp. JY-Q. Appl Microbiol Biotechnol 103:4499–4510. https://doi.org/10.1007/s00253-019-09800-4.
15. Zhang H, Zhao R, Huang CC, Li J, Shao YH, Xu J, Shu M, Zhong WH. 2019. Selective and faster nicotine biodegradation by genetically modified *Pseudomonas* sp. JY-Q in the presence of glucose. Appl Microbiol Biotechnol 103:339–348. https://doi.org/10.1007/s00253-018-9445-z.
16. Wang SN, Liu Z, Xu P. 2009. Biodegradation of nicotine by a newly isolated *Agrobacterium* sp. strain S33. J Appl Microbiol 107:838–847. https://doi.org/10.1111/j.1365-2672.2009.04259.x.
17. Huang H, Yu W, Wang R, Li H, Xie H, Wang S. 2017. Genomic and transcriptomic analyses of *Agrobacterium tumefaciens* S33 reveal the molecular mechanism of a novel hybrid nicotine-degrading pathway. Sci Rep 7:4813. https://doi.org/10.1038/s41598-017-05320-1.
18. Yu H, Tang HZ, Li YY, Xu P. 2015. Molybdenum-containing nicotine hydroxylase genes in a nicotine degradation pathway that is a variant of the pyridine and pyrrolidine pathways. Appl Environ Microbiol 81:8330–8338. https://doi.org/10.1128/AEM.02253-15.
19. Yu H, Tang HZ, Zhu XY, Li YY, Xu P. 2015. Molecular mechanism of nicotine degradation by a newly isolated strain, *Ochrobactrum* sp. strain SJY1. Appl Environ Microbiol 81:272–281. https://doi.org/10.1128/AEM.02265-14.
20. Zhao Q, Yue S, Bilal M, Hu H, Wang W, Zhang X. 2017. Comparative genomic analysis of 26 *Sphingomonas* and *Sphingobium* strains: dissemination of bioremediation capabilities, biodegradation potential and horizontal gene transfer. Sci Total Environ 609:1238–1247. https://doi.org/10.1016/j.scitotenv.2017.07.249.
21. Díaz E, Prieto MA. 2000. Bacterial promoters triggering biodegradation of aromatic pollutants. Curr Opin Biotechnol 11:467–475. https://doi.org/10.1016/s0958-1669(00)00126-9.
22. Sandu C, Chiribau CB, Brandsch R. 2003. Characterization of HdnoR, the transcriptional repressor of the 6-hydroxy-D-nicotine oxidase gene of *Arthrobacter nicotinovorans* pAO1, and its DNA-binding activity in response to L- and D-nicotine derivatives. J Biol Chem 278:51307–51315. https://doi.org/10.1074/jbc.M307797200.
23. Chiribau CB, Sandu C, Igloi GL, Brandsch R. 2005. Characterization of PmfR, the transcriptional activator of the pAO1-Borne purU-mabO-folD operon of *Arthrobacter nicotinovorans*. J Bacteriol 187:3062–3070. https://doi.org/10.1128/JB.187.9.3062-3070.2005.
24. Wang L, Tang H, Yu H, Yao Y, Xu P. 2014. An unusual repressor controls the expression of a crucial nicotine-degrading gene cluster in *Pseudomonas putida* S16. Mol Microbiol 91:1252–1269. https://doi.org/10.1111/mmi.12533.
25. Qiu JG, Liu M, Wen RT, Zhang D, Hong J. 2015. Regulators essential for nicotine degradation in *Shinella* sp. HZN7. Process Biochem 50:1947–1950. https://doi.org/10.1016/j.procbio.2015.07.013.
26. Wang B, Xu J, Gao JJ, Fu XY, Han HJ, Li ZJ, Wang LJ, Tian YS, Peng RH, Yao QH. 2019. Construction of an *Escherichia coli* strain to degrade phenol completely with two modified metabolic modules. J Hazard Mater 373:29–38. https://doi.org/10.1016/j.jhazmat.2019.03.055.
27. Liu LN, Bilal M, Duan X, Iqbal HMN. 2019. Mitigation of environmental pollution by genetically engineered bacteria—current challenges and future

perspectives. Sci Total Environ 667:444–454. https://doi.org/10.1016/j.scitotenv.2019.02.390.
28. Zhou Z, Liu Y, Zanaroli G, Wang Z, Xu P, Tang H. 2019. Enhancing bioremediation potential of *Pseudomonas putida* by developing its acid stress tolerance with glutamate decarboxylase dependent system and global regulator of extreme radiation resistance. Front Microbiol 10:2033. https://doi.org/10.3389/fmicb.2019.02033.
29. Zhao F, Liu X, Kong A, Zhao Y, Fan X, Ma T, Gao W, Wang S, Yang C. 2019. Screening of endogenous strong promoters for enhanced production of medium-chain-length polyhydroxyalkanoates in *Pseudomonas* mendocina NK-01. Sci Rep 9:1798. https://doi.org/10.1038/s41598-019-39321-z.
30. Zhou SH, Ding RP, Chen J, Du GC, Li HZ, Zhou JW. 2017. Obtaining a panel of cascade promoter-5'-UTR complexes in *Escherichia coli*. ACS Synth Biol 6:1065–1075. https://doi.org/10.1021/acssynbio.7b00006.
31. Dong H, Zhao C, Zhang T, Zhu H, Lin Z, Tao W, Zhang Y, Li Y. 2017. A systematically chromosomally engineered *Escherichia coli* efficiently produces butanol. Metab Eng 44:284–292. https://doi.org/10.1016/j.ymben.2017.10.014.
32. Ye J, Hu D, Yin J, Huang W, Xiang R, Zhang L, Wang X, Han J, Chen GQ. 2020. Stimulus response-based fine-tuning of polyhydroxyalkanoate pathway in *Halomonas*. Metab Eng 57:85–95. https://doi.org/10.1016/j.ymben.2019.10.007.
33. Guragain M, Jennings-Gee J, Cattelan N, Finger M, Conover MS, Hollis T, Deora R. 2018. The transcriptional regulator BpsR controls the growth of Bordetella bronchiseptica by repressing genes involved in nicotinic acid degradation. J Bacteriol 200:e00712-17. https://doi.org/10.1128/JB.00712-17.
34. Jimenez JI, Juarez JF, Garcia JL, Diaz E. 2011. A finely tuned regulatory circuit of the nicotinic acid degradation pathway in *Pseudomonas putida*. Environ Microbiol 13:1718–1732. https://doi.org/10.1111/j.1462-2920.2011.02471.x.
35. Luo YZ, Zhang L, Barton KW, Zhao HM. 2015. Systematic identification of a panel of strong constitutive promoters from *Streptomyces albus*. ACS Synth Biol 4:1001–1010. https://doi.org/10.1021/acssynbio.5b00016.
36. Zhou D, Yang R. 2006. Global analysis of gene transcription regulation in prokaryotes. Cell Mol Life Sci 63:2260–2290. https://doi.org/10.1007/s00018-006-6184-6.
37. Salis HM, Mirsky EA, Voigt CA. 2009. Automated design of synthetic ribosome binding sites to control protein expression. Nat Biotechnol 27:946–950. https://doi.org/10.1038/nbt.1568.
38. Gama-Castro S, Salgado H, Santos-Zavaleta A, Ledezma-Tejeida D, Muñiz-Rascado L, García-Sotelo JS, Alquicira-Hernández K, Martínez-Flores I, Pannier L, Castro-Mondragón JA, Medina-Rivera A, Solano-Lira H, Bonavides-Martínez C, Pérez-Rueda E, Alquicira-Hernández S, Porrón-Sotelo L, López-Fuentes A, Hernández-Koutoucheva A, Del Moral-Chávez V, Rinaldi F, Collado-Vides J. 2016. RegulonDB version 9.0: high-level integration of gene regulation, coexpression, motif clustering and beyond. Nucleic Acids Res 44:D133–D143. https://doi.org/10.1093/nar/gkv1156.
39. He C, Huang Y, Liu P, Wei J, Yang Y, Xu L, Xiao M. 2019. Transcriptome analysis of genes and metabolic pathways associated with nicotine degradation in *Aspergillus oryzae* 112822. BMC Genomics 20:86. https://doi.org/10.1186/s12864-019-5446-2.
40. Wang HX, Zhu PP, Zhang Y, Sun KK, Lu ZM. 2018. ndpT encodes a new protein involved in nicotine catabolism by *Sphingomonas melonis* TY. Appl Microbiol Biotechnol 102:10171–10181. https://doi.org/10.1007/s00253-018-9371-0.
41. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL. 2009. BLAST+: architecture and applications. BMC Bioinformatics 10:421. https://doi.org/10.1186/1471-2105-10-421.
42. Schafer A, Tauch A, Jager W, Kalinowski J, Thierbach G, Puhler A. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145:69–73. https://doi.org/10.1016/0378-1119(94)90324-7.