Rid Enhances the 6-Hydroxypseudooxynicotine Dehydrogenase Reaction in Nicotine Degradation by *Agrobacterium tumefaciens* S33

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**ABSTRACT** *Agrobacterium tumefaciens* S33 degrades nicotine through a hybrid of the pyridine and pyrrolidine pathways. The oxidation of 6-hydroxypseudooxynicotine to 6-hydroxy-3-succinoyl-semialdehyde-pyridine by 6-hydroxypseudooxynicotine dehydrogenase (Pno) is an important step in the breakdown of the *N*-heterocycle in this pathway. Although Pno has been characterized, the reaction is not fully understood; what is known is that it starts at a high speed followed by a rapid drop in the reaction rate, leading to the formation of a very small amount of product. In this study, we speculated that an unstable imine intermediate that is toxic with regard to the metabolism is produced in the reaction. We found that a Rid protein (designated Rid-NC) encoded by a gene in the nicotine-degrading gene cluster enhanced the reaction. Rid is a widely distributed family of small proteins with various functions, and some subfamilies have deaminase activity to eliminate the toxicity of the reactive intermediate, imine. Biochemical analyses showed that Rid-NC relieved the toxicity of the presumed imine intermediate produced in the Pno reaction and that, in the presence of Rid-NC, Pno maintained a high level of activity and the amount of the reaction product was increase by at least 5-fold. Disruption of the rid-NC gene led to slower growth of strain S33 on nicotine. The mechanism of Rid-NC-mediated detoxification of the imine intermediate was discussed. A phylogenetic analysis indicated that Rid-NC belongs to the rarely studied Rid6 subfamily. These results further our understanding of the biochemical mechanism of nicotine degradation and provide new insights into the function of the Rid6 subfamily proteins.

**IMPORTANCE** Rid is a family of proteins that participate in metabolite damage repair and is widely distributed in different organisms. In this study, we found that Rid-NC, which belongs to the Rid6 subfamily, promoted the 6-hydroxypseudooxynicotine dehydrogenase (Pno) reaction in the hybrid of the pyridine and pyrrolidine pathways for nicotine degradation by *Agrobacterium tumefaciens* S33. Rid-NC hydrolyzed the presumed reactive imine intermediate produced in the reaction to remove its toxicity on Pno. The finding further our understanding of the metabolic process of the toxic *N*-heterocyclic aromatic compounds in microorganisms. This study demonstrated that the Rid family of proteins also functions in the metabolism of *N*-heterocyclic aromatic alkaloids, in addition to the amino acid metabolism, and that Rid6-subfamily proteins also have deaminase activity, similar to the RidA subfamily. The ability of reactive imines to damage a non-pyridoxal-5′-phosphate-dependent enzyme was reported. This study provides new insights into the function of the Rid family of proteins.

**KEYWORDS** nicotine degradation, Rid, imine, enamine, deaminase, 6-hydroxypseudooxynicotine dehydrogenation, *Agrobacterium tumefaciens*
the cells. To date, a variety of reactions have been found to produce this kind of toxic reactive compound, which can cause metabolic damage in cells (1–3). For example, the amino acid metabolism (mainly serine, threonine, and cysteine) produces a highly active and toxic enamine intermediate, 2-aminoacrylate, which can covalently bind to pyridoxal 5′-phosphate (PLP) to inactivate PLP-dependent enzymes, such as threonine/serine deaminase (IlvA/TdcB) (4), cysteine desulfurase (CdsH) (5), aspartate aminotransferase (AST) (6), alanine racemase (Alr) (7), transaminase B (IlvE) (1), and serine hydroxymethyltransferase (GlyA) (8). The side reaction of L-malate dehydrogenase in the tricarboxylic acid cycle can reduce α-ketoglutarate to L-2-hydroxyglutaric acid, the accumulation of which causes the neurological disease L-2-hydroxyglutaric aciduria (9).

RutA, RutF, and RutB, the three key enzymes that mediate the catabolic pathways of uracil, produce the toxic compounds peroxyaminoacrylate, aminoacrylate, and malonic semialdehyde, which affect the normal decomposition of uracil (10). The accumulation of the reactive succinic semialdehyde, an intermediate of the nitrogen metabolism in bacteria, hampers the cell quorum-sensing signal attenuation to respond to host regulation signals (11).

To protect cells from the attack of these reactive intermediates, various metabolite damage repair systems have evolved in nature (3). In particular, some members of the RidA/YjgF/YER057c/UK114 protein family (Rid family) have been reported to have deaminase activity and can preemptively counteract the damage caused by the enamine compounds, such as 2-aminoacrylate, produced by the amino acid metabolism (8). The Rid family is a group of proteins with small sequence diversity that is composed of eight subfamilies and is widely distributed in different organisms (12). The typical RidA subfamily exists in all organisms, while the Rid1 to Rid7 (Rid1-7) subfamily exists only in prokaryotes. Most of the proteins in the RidA subfamily can preempt the damage of enamine compounds via their deaminase activity. Enamine is a type of reactive and unstable compound that can be isomerized to imine. These two compounds can covalently bind to the prosthetic PLP group of PLP-dependent enzymes, such as serine/threonine dehydrase and alanine racemase, to affect their activity (13). Enamine and imine can also be spontaneously hydrolyzed to produce harmless ketoacid compounds; however, this reaction is very slow because of the small amount of free water molecules (low water activity) in cells, and RidA proteins can remove them quickly to avoid their accumulation, preempting the damaging effects on the related enzymes. The functions reported for the RidA subfamily of proteins, in addition to their deaminase activity, also include photosynthesis repair in Arabidopsis thaliana (14), endonuclease activities (15), and molecular chaperone activities (16). Moreover, their related functions in mitochondrial metabolism and immunomodulatory pathways were studied in eukaryotes such as yeast, goats, and humans (17–20). The proteins in the Rid1-3 subfamilies also exhibited different degrees of deaminase activity (21), while the functions of the Rid4-7 subfamilies have been rarely reported to date.

Nicotine is a toxic N-heterocyclic aromatic alkaloid produced in tobacco. It can be catabolized by bacteria in various pathways, e.g., the pyridine pathway represented by Arthrobacter (22) and the pyrrolidine pathway represented by Pseudomonas (23). Previously, we found that a novel hybrid of the pyridine and pyrrolidine pathways (also known as the VPP pathway) is responsible for nicotine catabolism in Agrobacterium tumefaciens S33 (24). In this pathway (Fig. 1A), nicotine is first degraded to 6-hydroxypseudooxynicotine by the pyridine pathway through 6-hydroxynicotine and 6-hydroxy-N-methylmyosmine. 6-Hydroxypseudooxynicotine then is catalyzed to 6-hydroxy-3-succinoyl-semialdehyde-pyridine, and the aldehyde compound is further oxidized to 6-hydroxy-3-succinoylpyridine (HSP). Finally, HSP is decomposed through 2,5-dihydroxyppyridine along the pyrrolidine pathway. A great amount of work has been performed to study the biochemical and molecular mechanisms of the hybrid pathway, including genomics and transcriptomics analysis, the purification and characterization of key enzymes in the pathway, and the investigation of the electron transport involved in nicotine oxidation (24–30). Our previous genomics and transcriptomics studies have
shown that the genes related to nicotine degradation form a large gene cluster on a genomic island located on the circular chromosome of *A. tumefaciens* S33 (28) (Fig. 1B). By purifying and characterizing the enzyme, knocking out the gene, and determining the reaction products, the *pno* gene in the cluster was identified to encode 6-hydroxypseudooxynicotine dehydrogenase (Pno; firmly thought of as an oxidase), which catalyzes 6-hydroxypseudooxynicotine oxidation to generate 6-hydroxy-3-succinoyl-semialdehyde-pyridine (29). Because no commercial 6-hydroxypseudooxynicotine is available, the Pno-catalyzed reaction was determined by coupling with the oxidation of 6-hydroxynicotine to 6-hydroxy-N-methylmyosmine and 6-hydroxy-pseudooxynicotine by 6-hydroxynicotine oxidase (Hno). The product 6-hydroxy-3-succinoyl-semialdehyde-pyridine was predicted to be further oxidized by aldehyde dehydrogenase (Ald). Fortunately, we found that Pno could catalyze pseudooxynicotine instead of 6-hydroxypseudooxynicotine. It presented an activity of 52.8 U/mg and an apparent $K_{m}$ value for pseudooxynicotine of 0.42 mM using 2,6-dichlorophenolindophenol (DCPIP) as the electron acceptor and phenazine methosulfate (PMS) as the electron mediator, which are similar to the enzyme activity (32.3 U/mg) with 6-hydroxypseudooxynicotine as the substrate and the apparent $K_{m}$ value (0.37 mM) for 6-hydroxypseudooxynicotine (25). However, during the measurement, it was observed that the Pno-catalyzed reaction, despite the addition of the excessive substrates, electron mediator, and electron acceptor, could not proceed thoroughly under the best conditions obtained by us, as the enzyme activity rapidly decreased and the final amount of the product was far lower than the theoretical value. Considering that 6-hydroxypseudooxynicotine/pseudooxynicotine contains a secondary amine group in the side chain, we hypothesized that a toxic and reactive enamine/imine intermediate is produced during the oxidative reaction.
Interestingly, the open reading frame (ORF) (AWN88_01335) located upstream of the ald gene in the nicotine degradation gene cluster is annotated to encode a Rid protein (designated Rid-NC, with NC stemming from the observation that the gene is located in the nicotine-degrading gene cluster) (Fig. 1B). According to our previous transcriptomics study, the transcription level of rid-NC in the cells grown on nicotine is 7.56 times higher than that obtained in glucose-ammonium medium, which showed a consistent upregulation pattern similar to that of the core nicotine-degrading genes, e.g., pno is upregulated as much as 4.43 times (28). It is well known that some Rid subfamilies have deaminase activity and can hydrolyze the toxic imine intermediate produced by FAD-dependent amine oxidase/dehydrogenase (12, 21). Therefore, the transcriptomics analysis suggests that rid-NC is involved in the oxidation reaction of nicotine degradation. In this study, we identified the function of Rid-NC in the nicotine degradation by A. tumefaciens S33. Based on the results of biochemical assays, we hypothesized that a reactive enamine/imine intermediate is produced in the Pno-catalyzed reaction and confirmed its toxic effect on Pno. Our results indicate that Rid-NC can catalyze the deamination of the reactive enamine/imine intermediates to remove their toxic effects and promote the Pno-catalyzed reaction.

RESULTS

The addition of Rid-NC increased the initial rate of the Pno-catalyzed reaction. rid-NC was heterologously expressed in Escherichia coli C41(DE3) cells. The encoded protein was purified using a HisTrap column and analyzed by SDS-PAGE, which revealed the presence of a band of 17 kDa (Fig. 2A), consistent with the calculated mass (16.6 kDa) of the protein and the molecular mass of 15 to 20 kDa reported in other organisms (12). Gel filtration showed that the purified protein was a homotrimer and that the complex was stable (see Fig. S1A and B in the supplemental material).

We first prepared the true substrate of Pno, 6-hydroxypseudooxynicotine, from 6-hydroxynicotine via the catalysis of Hno, which was identified using liquid chromatography-mass spectrometry (LC-MS) analysis (Fig. S2). The prepared 6-hydroxypseudooxynicotine sample, which also contained some 6-hydroxy-N-methylmyosmine, was catalyzed by Pno to 6-hydroxy-3-succinoyl-semialdehyde-pyridine using DCPIP as the electron acceptor, as determined by LC-MS (Fig. S2B). Subsequently, we attempted to add Rid-NC to the reaction mixture to test its effects on Pno enzyme activity. The addition of purified Rid-NC to the reaction mixture led to an increase in the initial reduction rate of DCPIP (Fig. 2B), and its increase was proportional to the increase in Rid-NC concentration (Fig. 2C), indicating that Rid-NC can promote the oxidation reaction.
catalyzed by Pno using 6-hydroxypseudooxynicotine as the substrate. However, the promotion effect of Rid-NC was not very obvious. The reason may be that 6-hydroxypseudooxynicotine was not purified from the Hno reaction mixture due to its poor stability and low concentration, and the mixture was directly used in the Pno reaction tests. As the true substrate (6-hydroxypseudooxynicotine) is difficult to obtain, we then tested the reaction using pseudooxynicotine instead of 6-hydroxypseudooxynicotine as the substrate of Pno.

In our previous studies, it was observed that, in the absence of PMS, purified Pno can slowly catalyze the oxidation of pseudooxynicotine to reduce the artificial electron acceptor DCPIP (25). This was further confirmed by determining the reaction product using LC-MS analysis (Fig. S3). Here, we increased the amount of Pno in the reaction mixture to more clearly observe the effect of Rid-NC on the Pno-catalyzed reaction. Figure 3A shows the effects of different concentrations of Rid-NC on the reduction of DCPIP by Pno. The addition of purified Rid-NC to the reaction mixture led to a great increase in the initial reduction rate of DCPIP, and its increase was proportional to the increase in Rid-NC concentration (Fig. 3B), indicating that Rid-NC can also effectively promote the pseudooxynicotine oxidation reaction catalyzed by Pno. Thus, for convenience, here we used pseudooxynicotine instead of 6-hydroxypseudooxynicotine to perform subsequent tests.

Because the artificial electron mediator PMS was not added to the reaction mixture, the reaction proceeded slowly (Fig. 3A). Thus, 0.05 mM PMS was added to the reaction mixture in the subsequent experiments to ensure that the reaction proceeded more quickly, correspondingly reducing the amount of Pno added to make the total activity in each reaction consistent. Figure 3C clearly shows that Rid-NC increased the initial reaction rate in the presence of PMS. In addition, the total amount of DCPIP reduced by Pno was significantly different in the presence or absence of the addition of Rid-NC at the end of the reactions. In the reaction without Rid-NC, DCPIP was not completely reduced, while the amount of reduced DCPIP increased after adding Rid-NC, which was proportionally related to the amount of Rid-NC added (inset in Fig. 3C).

The genome of *A. tumefaciens* S33 contains a second *rid* gene (AWN88_01150), which is located upstream of the gene annotated as a d-amino acid oxidase and encodes a protein named Rid-AA (with AA stemming from the prediction that it functions in amino acid metabolism) in this study, with 60% identity to Rid-NC. We tested its activity in the Pno-catalyzed reaction and investigated the activity of RidA from *Salmonella enterica*, which is a typical protein of the RidA subfamily that hydrolyzes reactive enamine/imine compounds (31). The two Rid proteins were heterologously expressed in *E. coli* and purified using a procedure similar to that used for Rid-NC (Fig. S1C). Figure 3D shows that Rid-AA from strain S33 and RidA from *S. enterica* also promoted the reduction of DCPIP by Pno, similar to Rid-NC. Their enhancements of the Pno activity were almost identical when the same amounts of Rid proteins were added. This result suggests that Rid-AA and RidA have a function similar to that of Rid-NC in this reaction, and that, most likely, a reactive enamine/imine is produced as the reaction intermediate, similar to those produced in amino acid metabolism.

**The addition of Rid-NC helped maintain the activity of Pno.** It is assumed that the activity of Pno would greatly decrease after it meets the toxic enamine/imine intermediate during the reaction. Therefore, we compared its activity change in the presence/absence of Rid-NC. As shown in Fig. 3E, the reaction rate decreased significantly at the end of the first stage of the reaction (420 s); subsequently, the substrate, electron mediator, and electron acceptor were supplemented (440 s), starting the second stage of the reaction, in which DCPIP continued to be reduced by Pno. In the presence of Rid-NC (which was added at the beginning of the first stage), the reactions of both stages were quicker than that observed in the absence of Rid-NC. In the second stage of the reaction, Pno activity (14.2 U/mg) in the reaction in which Rid-NC was added in advance, as calculated according to the initial reaction rate, was decreased by 25.7%
compared with that recorded in the first stage (19.1 U/mg), while the enzymatic activity observed without adding Rid-NC was decreased by about 51.1%, from 15.3 U/mg to 7.4 U/mg (Fig. 3E). These results suggest that Rid-NC helps maintain the activity of Pno and that Pno is affected by some toxic intermediate produced in the reaction, which can be eliminated by Rid-NC.

**FIG 3** Effects of Rid-NC, Rid-AA, and RidA on the Pno-catalyzed reaction. (A) Effect of different concentrations of Rid-NC (0 to 156.8 μM) on the reduction of DCPIP with pseudooxynicotine by Pno (no PMS was added). The reaction mixture contained 50 mM Tris-HCl (pH 8.5), 1 mM pseudooxynicotine, 0.1 mM DCPIP, and 0.5 U of Pno (standard activity). The DCPIP reduction was monitored at 600 nm. (B) Relationship between the concentration of Rid-NC and the DCPIP reduction activity (U/mg) catalyzed by Pno. Data from panel A were used. (C) Different amounts of Rid-NC were added to the reaction mixture (0.05 mM PMS was added); the inset shows the relationship between the concentration of Rid-NC and the DCPIP reduction activity (U/mg) catalyzed by Pno. (D) Different Rid homologs (12 μM for each Rid protein) were added to the reaction mixture. All reaction mixtures in the tests depicted in panels C and D contained 50 mM Tris-HCl (pH 8.5), 1 mM pseudooxynicotine, 0.1 mM DCPIP, 0.05 mM PMS, 7.8 mU of Pno (standard activity), and Rid protein as indicated. (E) Effect of the presumed imine intermediate on the activity of Pno in the presence or absence of Rid-NC. The reaction proceeded in two stages. Rid-NC (64 μM) was added at the beginning of the first stage, and the second stage was started by supplementing the substrates at 440 s. The initial reaction mixture was as mentioned above, with the exception of the addition of 10.0 μM of Pno (standard activity). The reaction mixture in the second stage contained 50 mM Tris-HCl (pH 8.5), 0.83 mM pseudooxynicotine (PN), 0.083 mM DCPIP, and 0.017 mM PMS. (F) Production of 3-succinoyl-semialdehyde-pyridine (SAP) from pseudooxynicotine dehydrogenation by Pno in the presence of different amounts of Rid-NC. The three controls were as follows: (i) no Pno&no Rid-NC (0 μM Pno and 0 μM Rid-NC [black line]), (ii) Rid-NC (0 μM Pno and 34.0 μM Rid-NC [red line]), and (iii) Pno (7.8 μU of Pno [standard activity] and 0 μM Rid-NC [blue line]). The two experimental groups (Pno+Rid-NC) were as follows: (i) 7.8 μU of Pno and 17.0 μM Rid-NC (green line) and (ii) 7.8 μU of Pno and 34.0 μM Rid-NC (pink line).
The addition of Rid-NC increased the yield of the product. The formation of the 3-succinoyl-semialdehyde-pyridine product in the reaction was determined by high-performance liquid chromatography (HPLC). As shown in Fig. 3F, the yield of the product in the reaction with Rid-NC was twice that observed without adding Rid-NC after 2 min. The amount of the product in the reaction without Rid-NC basically stopped increasing after about 10 min, while the amount of product in the reaction with Rid-NC was still increasing after 2 h, which was 5 times the amount detected without adding Rid-NC. These results clearly indicate that Rid-NC promotes the Pno-catalyzed reaction and suggest that the generation of a reactive intermediate in the reaction affects Pno activity and prevents the reaction (Fig. 3E and F); in contrast, Rid-NC can eliminate this effect and enable the reaction to proceed normally (Fig. 3C to E).

Effects of pH on the function of Rid. The promotion effect of Rid can also be reflected by the difference in the activity of Pno under different pH conditions. As shown in Fig. 4A, the Pno-catalyzed reaction without Rid was optimal at pH 8.5 (around 25 U/mg), whereas the Pno-catalyzed reaction rates in the presence of Rid-NC or Rid-AA increased to a similar high enzymatic activity (33 to 35 U/mg) at pH 8.5 and pH 9.0, with a lower activity (around 32 U/mg) observed in the case of RidA from S. enterica. At pH 7.5, the promotion effects of Rid-NC, Rid-AA, and RidA were not obvious. Moreover, at pH 9.5 and pH 10.0, even though the original Pno activity was very low, Rid still significantly promoted the reaction. According to the curve shown in Fig. 4A, Rid-AA and Rid-NC had similar effects on Pno activity, which is consistent with the high similarity of their protein sequences. RidA had lower effects on Pno activity than did Rid-NC and Rid-AA; however, the activity of the enzyme was still much higher than that detected without adding Rid. The promotion effect of Rid on the reaction can be specifically expressed by the fold changes in the activity of the Pno enzyme in the presence of Rid versus its absence (Fig. 4B). For Rid-NC, it was about 1.5 times at pH 8.5, while it was >2 times at pH 9.0 and pH 9.5. Moreover, the activity was lower for Rid-AA and RidA, but the trend was similar. Thus, the fold changes in the activity of the enzyme in an alkaline environment were higher, i.e., the promotion of Rid was more obvious under this condition (Fig. 4B). Previous reports have mentioned that Rid is more active under alkaline conditions (31), which is consistent with that observed for Rid-NC, Rid-AA, and RidA (Fig. 4A and B) and may be attributed to their conserved protein sequences (21).

Effects of mutation of key amino acid residues on the function of Rid. Sequence alignments were performed for the Rid protein sequences, including RidA, Rid-AA, Rid-NC and their similar sequences (Fig. S4). Compared with the active sites reported in the Rid protein family (12), the active sites of Rid-NC were predicted. The Tyr16, Gly32, and Glu122 residues of Rid-NC are known key sites related to Rid deamination activity; however, Rid-NC does not contain an Arg105 residue, which is an important site for deaminase activity in most Rid proteins. Therefore, we mutated some of the predicted
sites and determined their effects on the activity changes of Pno to explore their roles in the protein structure or substrate binding, which may further affect the hydrolytic efficiency of Rid-NC. In addition to introducing the mutations of Y16A and E122A, the P104A, S105A, and V106A mutations were also considered because the substrate-binding site of most Rid proteins is located at the Arg105 position. As shown in Fig. 5, the overall effects of the Y16A, E122A, and P104A/S105A/V106A mutations on the Pno-catalyzed reaction were negative. The Pno enzyme activity was highest at pH 8.5; as mentioned above, it was increased by 1.5-fold after the addition of wild-type Rid-NC, while this increased enzymatic activity was reduced to about 1.2 times after the addition of the mutant protein (V106A), which was lower than that observed for the Y16A mutation (1.3 times). The Pno activity was relatively low at pH 9.5, and adding wild-type Rid-NC increased the enzymatic activity by up to 2.3 times. In contrast to that observed at pH 8.5, the effect of the mutations at pH 9.5 was most obvious in the case of the E122A mutant, which increased Pno enzyme activity only by about 1.75 times, similar to the result obtained for P104A (1.8 times); this confirmed that Glu122 plays an important role in the alkaline environment. In addition, the V106A mutant caused a decrease in the activity change, from 2.3 to 2.1 times, which may be explained by the fact that a better activity of the Rid protein is observed under alkaline conditions. However, in all mutation experiments, in addition to the known Glu122, the site responsible for the significant activity decrease was Pro104/Val106, suggesting that these residues have a function similar to that of the Arg105 residue in other Rid proteins, which is essential for its deaminase activity and substrate binding.

**Disruption of the rid-NC gene negatively affected the growth of A. tumefaciens S33 on nicotine.** To confirm the function of Rid-NC, the rid-NC gene of strain S33 was deleted through homologous recombination, and the mutant was then complemented by introducing a full-length rid-NC gene (Fig. S1D). The wild-type strain and the S33-Δrid-NC mutant strain were inoculated in HSP medium and nicotine medium, respectively, to test their growth. As shown in Fig. 6, the S33-Δrid-NC mutant strain grew almost as well as the wild-type strain in HSP medium (Fig. 6A); however, its exponential growth on nicotine as the substrate was significantly slower than that of the wild-type strain (Fig. 6B). After complementing the disruption of the gene, the S33-Δrid-NC-C complementation strain was inoculated in nicotine medium, which presented a growth rate similar to that of the wild-type strain (Fig. 6C). This indicates that Rid-NC plays an important role in the oxidative degradation of nicotine by *A. tumefaciens* S33 and functions in the step before HSP formation in the hybrid pathway.

**DISCUSSION**

In previous studies, we experimentally verified that Pno from *A. tumefaciens* S33 acts as a dehydrogenase that catalyzes the oxidation of 6-hydroxypseudoxylnicotine...
or pseudooxynicotine to 6-hydroxy-3-succinoyl-semialdehyde-pyridine or 3-succinoyl-
semialdehyde-pyridine (25, 29). However, the amount of product did not increase with
the increase in the concentration of the reactants, and the reaction rate decreased rap-
 idly. Here, we found that Rid-NC played an important role in promoting the Pno-cata-
lyzed reaction: (i) both Rid-NC and Rid-AA significantly relieved the effect of the pre-
sumably reactive reaction intermediate enamine/imine on Pno activity, and the
addition of Rid-NC even increased the reaction rate by 2.3 times and the amount of
product by at least 5 times; and (ii) mutations of the key amino acid residues of the
Rid-NC protein decreased its enhancement of the Pno-catalyzed reaction.

The reactive intermediate enamine/imine usually has very poor stability in a large
amount of free water in vitro and is spontaneously hydrolyzed with a short half-life
(32). To confirm the existence of the enamine/imine intermediate in the Pno-catalyzed
reaction, we attempted to detect it in the reaction mixture directly by LC-MS or to
determine it after the addition of semicarbazide or methyl iodide to transform it into a
stable intermediate, where the Pno-catalyzed reaction was performed in water or a
mixture of water and organic solvents or even directly in organic solvents (see Fig. S2
and S3 in the supplemental material). Unfortunately, the expected imine intermediate
or its derivative could not be detected. Based on the knowledge of the enamine/imine
intermediates and the Rid family of proteins (12, 13, 31, 32) and our biochemical
assays, we speculated on the existence of the enamine/imine intermediate in the Pno-
catalyzed reaction and proposed a mechanism for its hydrolysis by Rid-NC in vivo (Fig.
7A). The substrate 6-hydroxypseudooxynicotine is dehydrogenated to an imine com-
pound by Pno, which can spontaneously isomerize to an enamine compound, as indi-
cated. Because of the molecular crowding and the shortage of free water molecules
that occur in cells, these reactive intermediates may be more stable and may be largely
accumulated, resulting in a toxic/inhibitory effect on Pno. Thus, Rid-NC is required to
hydrolyze it rapidly. A similar scenario has been reported in the study of the nicotine
degradation in Pseudomonas putida S16 (33), where pseudooxynicotine is proposed to
be oxidized to an imine compound by Pnao. Moreover, the existence of imine inter-
mediates has also been confirmed for the FAD-dependent l-amino acid oxidase and o-
arginine dehydrogenase (21). Pno is a dehydrogenase harboring an FMN and a [4Fe4S]
cluster (25, 29) and may have a similar mechanism. Moreover, another question
remains unanswered, i.e., how does the enamine/imine intermediate inhibit Pno?
According to our previous determination on the purified Pno and its protein sequence
analysis (25, 29), no PLP is used as a cofactor by Pno. Recently, the crystal structure of
6-hydroxypseudooxynicotine amine oxidase (HisD; 80.9% identity to Pno of strain S33
at the protein sequence level) from Pseudomonas geniculata N1 was determined (34),
and it does not contain a PLP, confirming that HisD/Pno is not a PLP-dependent
enzyme. Thus, the inhibition mechanism of enamine/imine on Pno should be distinct
Further studies are needed to elucidate this issue.

Because the true imine substrate of the Rid protein could not be obtained, we could not directly determine the activity of Rid-NC. Its function was indirectly verified by the well-designed coupling experiments with the Pno-catalyzed reaction performed in this study. When 6-hydroxypseudooxynicotine, which was prepared via 6-hydroxynicotine oxidation by Hno, was used as the true substrate of the Pno-catalyzed reaction, the promotion effect of Rid on the Pno-catalyzed reaction was obviously detected (Fig. 2B and C). As 6-hydroxypseudooxynicotine is difficult to obtain and quantify, we used pseudooxynicotine instead in all other tests. It was confirmed that, in the reaction, the enzymatic activity of Pno was significantly increased by adding Rid-NC regardless of whether the electron mediator PMS was present and was proportional to the amount of Rid-NC added (Fig. 3A to C). This indicates that Rid-NC promotes the Pno-catalyzed reaction. In the absence of Rid-NC, the Pno enzymatic activity was greatly inhibited by the reactive enamine/imine produced in the reaction (Fig. 3E and F); in addition, after the addition of Rid-NC, the Pno enzymatic activity was well maintained (Fig. 3E). Moreover, the consumption of DCPIP increased in the reaction with addition of Rid-NC (Fig. 3C to E), and the yield of the 3-succinyl-semialdehyde-pyridine product also increased significantly (Fig. 3F), which further proved that Pno is inhibited during the

FIG 7 Proposed function of Rid-NC in the oxidation reaction of 6-hydroxypseudooxynicotine to 6-hydroxy-3-succinyl-semialdehyde-pyridine catalyzed by Pno in A. tumefaciens S33 (A) and the predicted mechanism of detoxification of the imine intermediate by Rid-NC (B). In vitro, the imine intermediate can be spontaneously hydrolyzed; however, Rid-NC is required to process this intermediate in vivo because of the shortage of free water molecules.

from that of the PLP-dependent enzymes (1, 7). Further studies are needed to elucidate this issue.
reaction process and that this inhibition can be greatly weakened by Rid-NC. The use of the typical RidA instead of Rid-NC also promoted the Pno-catalyzed reaction (Fig. 3D). Therefore, we concluded that Rid-NC is similar to RidA and functions as a deaminase (hydrolysis of enamine/imine compound).

Homologous proteins of Rid-NC exist in many nicotine-degrading bacteria, such as Shinella sp. strain HZN7 (71.9% identity to the Rid-NC protein sequence) (35), Ochrobactrum sp. strain SJY1 (76.6% identity) (36), Pseudomonas sp. strain JY-Q (42.5% identity) (37), and P. putida J5 (NdaE, 41.1% identity) (38). Moreover, the genes that encode all of these proteins are located in the nicotine-degrading gene cluster in these strains. Knockout of the gene encoding NdaE in the P. putida J5 caused the strain to lose the ability to use nicotine as the sole carbon and nitrogen source (38). However, no further study of these Rid proteins was performed, especially regarding their biochemical function. In this study, we deleted the rid-NC gene from A. tumefaciens S33 by homologous recombination. The S33-Δrid-NC mutant exhibited a significantly slower exponential growth than did the wild-type strain on nicotine, although it finally reached the same amount of biomass as the wild-type strain (Fig. 6B). We predict that its homologous gene, rid-AA, can partially compensate for its disruption in the mutant. The transcriptomics data have shown that the rid-AA gene has a similar transcription level when the strain is grown in both nicotine medium (fragments per kilobase value [FPKM] value of 62.7) and glucose-ammonium medium (FPKM value of 27.3) (28). After complementing the mutant strain, the complementation strain S33-Δrid-NC-C grew almost as well as the wild-type strain in nicotine medium (Fig. 6C). These results suggest that Rid plays a key role in nicotine degradation.

We further explored the biochemical mechanism via which Rid-NC catalyzes the hydrolysis of the reactive enamine/imine produced in the Pno-catalyzed reaction. We aligned the protein sequences of the typical RidA, Rid-NC, Rid-AA, and similar sequences (with an identity higher than 45%) that showed the conserved amino acid residues in Rid-NC (Fig. S4). According to the biochemical mechanism of the Rid family and their protein structures (13, 14, 31, 39, 40), the typical Rid protein forms a barrel shape through hydrogen bonding of triple symmetric monomers. Each monomer consists of five to six β-sheets and two α-helices. The Rid-NC and Rid-AA proteins analyzed in this study also contained five β-sheets and two α-helices (Fig. S4). The purified trimeric Rid-NC protein was very stable (Fig. S1A and B), which provided structural support for Rid-NC for us to understand its activity. In Rid proteins, the reactive position of the binding imine is located in the cavity (crack) structure formed by the terminal β-sheets of two monomers. Combined with the results of previous reports (12, 13), the conserved amino acid sites of the Rid family in this cavity were summarized. Glu120 and Cys107 can interact with water molecules in the environment via hydrogen bonding, which greatly shortens the distance between the water molecule and the substrate, promoting the hydrolysis reaction. Arg105 is an important amino acid residue in the RidA and Rid1-3 subfamilies with deaminase activity. The side chain can form a stable salt bridge with the enamine/imine substrate, and the carbonyl oxygen of the skeleton also forms a hydrogen bond with a hydrogen atom on the imine. The carbonyl oxygen of the Gly31 skeleton forms a hydrogen bond with another hydrogen atom on the imine, and the hydroxyl group of Tyr17 forms a hydrogen bond with the imine nitrogen of the substrate. Thus, the structure arrangement stabilizes the tight binding of the substrates and the protein and determines the exact position of the substrates in the protein to facilitate rapid hydrolysis. We predict that the Rid-NC reaction analyzed in the present study is similar to the reaction described above. Through conserved amino acid analysis, we predicted that Ser105/Val106 is a new site of binding to the substrate or forms a stable cavity structure together with Pro104, so that the substrate can reasonably bind to rid-NC (Fig. 7B). To verify this hypothesis, we generated mutations of these sites. The results showed that the mutations caused a decrease in the Rid-NC activity, confirming that they are critical for catalysis (Fig. 5 and 7B). In addition, the Tyr16 and Glu122 mutations also affected the activity of Rid-NC. Rid-AA and RidA
had similar cavity structures and active sites of Glu122 and Tyr16; therefore, they also had the function of catalyzing the hydrolysis of the imine compound, but their activity was low in the Pno-catalyzed reaction, mainly because of their low compatibility with the substrate. Based on our results and knowledge of the Rid family of proteins, we propose a mechanism for the detoxification of the imine intermediate in the Pno reaction catalyzed by Rid-NC (Fig. 7B). Rid family proteins can be divided into eight subfamilies, i.e., the RidA and Rid1-7 subfamilies (12). Based on the sequence alignment of Rid proteins from multiple sources, we constructed an evolutionary tree (Fig. S5). In the tree, Rid-NC and Rid-AA were determined to belong to the Rid6 subfamily. Moreover, some Rid proteins from eukaryotes were independently grouped into eu1 and eu2. The Rid4-7 subfamilies were rarely studied; thus, this was a very important example of the identification of deaminase activity in the Rid6 subfamily.

In summary, we found that Rid-NC, a small protein belonging to the function-unknown Rid6 subfamily, played a role as a deaminase in the Pno-catalyzed reaction and promoted the reaction by hydrolyzing the toxic enamine/imine intermediate. Although most reaction tests were performed using pseudoxynicotine as the substrate in the Pno reaction, the conclusion should be similar to that for its true substrate, 6-hydroxypseudoxynicotine. This study provides new insights on the oxidation process in nicotine degradation, helping to understand the biochemical mechanism involved in the catabolism of N-heterocyclic aromatic alkaloids in bacteria. The toxic effect of the enamine/imine intermediate on non-PLP-dependent enzymes has also been discovered, which raises a new question regarding the toxicity of imines on the metabolism and function of the Rid6 subfamily of proteins.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and biochemicals. Agrobacterium tumefaciens S33 was deposited under accession number CCTCC AB 2016054 (originally CCTCC M 206131) at the China Type Culture Collection (CCTCC). Nicotine or HSP was used as the sole carbon and nitrogen source to grow strain S33 at 30°C. E. coli DH5α and E. coli C41(DE3) cells harboring pCodonPlus were cultured in LB medium at 37°C. PMS and DCPIP were purchased from Sigma-Aldrich (St. Louis, MO), and pseudoxynicotine and 3-succinoyl-semialdehyde-pyridine were purchased from Toronto Research Chemical Inc. (Toronto, Canada). HSP was prepared from nicotine transformation using whole cells of Pseudomonas putida S16 as described previously (41). Chloramphenicol (25 mg/liter) and kanamycin (50 mg/liter) were required to culture the E. coli cells.

Heterologous expression and purification of Rid-NC, Rid-AA, and RidA and mutation of Rid-NC.

Using the genomic DNA of A. tumefaciens S33 as a template, the rid-NC gene (locus_tag AWN88_01335, 408 bp) and rid-AA gene (locus_tag AWN88_01150, 474 bp; the gene was reannotated in GenBank when the manuscript was submitted, where the 63 bp at the 3’ end were excluded) were amplified. The rid-NC gene was cloned into the pET28b(+) plasmid, and the construct was transformed into E. coli C41 (DE3) cells. The cells were cultured at 37°C in LB medium containing 12.5 mg/liter chloramphenicol and 25 mg/liter kanamycin to an optical density of 0.6 at 600 nm. Subsequently, 0.3 mM isopropyl-β-o-thio-galactopyranoside (IPTG) was added to the culture, and the cells were grown at 25°C for 10 h. The cells were then collected by centrifugation and resuspended in lysis buffer (20 mM sodium phosphate containing 0.5 M NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10% glycerol, pH 7.4). After destroying the cells through a low-temperature and ultra-high-pressure continuous flow cell using JN-02C (JNBIo, Guangzhou, China), the sample was centrifuged at 35,328 × g for 1 h at 4°C. The supernatant was loaded onto a 5-ml HisTrap column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) that had been previously equilibrated with binding buffer (20 mM sodium phosphate containing 0.5 M NaCl, pH 7.4). The target protein was eluted at a concentration of 0.5 M imidazole using a stepwise elution gradient. The protein was washed with 50 mM Tris-HCl (pH 7.4) and stored at 4°C after concentration. The ridA gene (locus_tag B4507_20440) from Salmonella enterica (42) was synthesized by the GENEWIZ Corporation (Suzhou, China). The mutations of the Rid-NC were achieved by recombinant PCR. The methods used for the expression and purification of the Rid-AA, RidA, and the variant Rid-NC proteins were the same as those described for the rid-NC gene. All primers used in this study are listed in Table 1.

Assay of Pno activity by adding Rid proteins. 6-Hydroxypseudoxynicotine was prepared from 6-hydroxynicotine via Hno catalysis (29), where 6-hydroxy-N-methylmyosmine is also produced because of the hydrolysis equilibrium between 6-hydroxy-N-methylmyosmine and 6-hydroxypseudoxynicotine. Hno was heterologously expressed and purified according to a previous report (26). The heterologous expression and purification of Pno were performed as described previously (29). The standard enzymatic assay for Pno (25) was performed during purification and was used for quantification of the enzyme added in the subsequent tests. Briefly, the mixture for the standard assay contained 50 mM Tris-HCl buffer (pH 8.5), 1 mM pseudoxynicotine, 0.5 mM PMS, and 0.05 mM DCPIP, and Rid protein was not added. The reaction was started by addition of Pno. All the assays were conducted using quartz cuvettes.
TABLE 1 Primers used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers used for cloning the rid genes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rid-NC</td>
<td>F-primer (BamHI)</td>
<td>CGCGGATCCGATGAGGCCGCAATTTTCCAC</td>
</tr>
<tr>
<td></td>
<td>R-primer (HindIII)</td>
<td>CCAAGCTTGGCTGGCGACCGTG</td>
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<tr>
<td>rid-ACA</td>
<td>F-primer (BamHI)</td>
<td>CGCGGATCCGATGCCGCGCTTGC</td>
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<tr>
<td></td>
<td>R-primer (HindIII)</td>
<td>CCAAGCTTGGCAGCGAGAGTTTTC</td>
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<tr>
<td>ridA</td>
<td>F-primer (BamHI)</td>
<td>CGCGGATCCGATGAGCAAACTATGGCAC</td>
</tr>
<tr>
<td></td>
<td>R-primer (HindIII)</td>
<td>CCAAGCTTGGCAGCAAACTATGGCAC</td>
</tr>
</tbody>
</table>

Primers used for mutating the rid-NC gene

| rid-NC_Y16A  | B-primer | CATGTCTCAGCAACGTCCTTCATCCTC |
|             | C-primer | GAGAACCCTGACGATGCTTCCTGGC |
| rid-NC_P104A | B-primer | AACCAGCGCTTCTGTGGCCATA |
|             | C-primer | CAAAGGCCTGCTGGGTCATG |
| rid-NC_S105A | B-primer | AAACCGCGGCGGGCTTGTGGC |
|             | C-primer | GGCCCCGCGGTTGTGGCATCAG |
| rid-NC_V106A | B-primer | GCCAACCCGGACGATGTGGC |
|             | C-primer | CCCTCAGCGGTTGGCATGAGG |
| rid-NC_E122A | B-primer | TGGGTGCGACGAAATGGTG |
|             | C-primer | CGTGTCGGGCCACAGCCGGCA |
|             | D-primer | GATCAGGCCGCTGTCGTC |

Primers used for disrupting the rid-NC gene

| rid-NC       | A-primer (BamHI) | CGCGGATCCGATGAGCAAACTATGGCAG |
|             | B-primer | GGGGCTAACGCGCTGCTAGTATTTCTGAC |
|             | C-primer | GAAGTCGAGAATCTAGCAGCGAGT |
|             | D-primer (XhoI) | CGGTGTGCGAGTGGTACG |

Primers used for complementing the rid-NC gene

| rid-NC       | F-primer (XhoI) | CGCGGATCCGATGAGCAAACTATGGCAG |
|             | R-primer (BamHI) | CGCGGATCCGATGAGCAAACTATGGCAG |

aThe recognition site is underlined.

(1-cm light path) filled with 1-mL reaction mixture at 30°C and monitored at 600 nm (DCPIP, <21 mM cm−1). One unit was defined as the reduction of 1 μmol of DCPIP per minute. The activity measured under the standard conditions was called standard activity in this study. When 6-hydroxypseudoxoynicotine was used as the substrate, the reaction mixture contained 50 mM Tris-HCl buffer (pH 8.5), around 0.4 mM 6-hydroxypseudoxoynicotine, around 0.4 mM 6-hydroxy-N-methylmyosmine, 100 mM NaCl, 0.1 mM DCPIP, 0.05 mM PMS, and the Pno enzyme. Rid protein was added as indicated. Instead of 6-hydroxypseudoxoynicotine as a substrate of Pno, pseudoxoynicotine was used in most assays in this study. The reaction mixture contained 50 mM Tris-HCl buffer (pH 8.5), 1 mM pseudoxoynicotine, 0.1 mM DCPIP, no PMS or 0.05 mM PMS (as indicated), and the Pno and Rid enzymes. The amounts of Pno and Rid added were changed to give the best presentation of various experiment purposes. The Rid activity is presented as the fold change of the Pno activity increase after the addition of the Rid protein. The 50 mM Tris-HCl buffer (pH 7.5 to 9.0) and the Gly-NaOH buffer (pH 9.0 to 10.0) were used to measure the optimal pH for the Rid protein.

Analytical methods. Protein concentration was determined via Bradford analysis using bovine serum albumin as a standard (43). Gel filtration was used to determine the relative molecular mass of the proteins on a GE Superdex G200 column (10 by 300 mm). The following protein standards were used for calibration: dextran blue 2000 (2,000 kDa; Sigma), ferritin from equine spleen (440 kDa; Sigma), γ-globulins from bovine blood (150 kDa; Solarbio), bovine serum albumin (66 kDa; Solarbio), albumin from chicken egg white (44.3 kDa; Sigma), myoglobin (16.7 kDa; Sigma), and vitamin B12 (1,360 Da; Sinopharm Chemical Reagent Co., Ltd., China). The buffer used in these experiments was 50 mM Tris-HCl containing 150 mM NaCl (pH 7.4), and the flow rate was 0.5 ml min−1.

The reaction substrates and products were quantified using HPLC (29). After reacting for different time (0 to 2 h), the protein in the reaction mixture was removed by heating at 100°C for 30 s and centrifuging at 10,000×g for 20 min at 4°C, followed by the analysis of the supernatant via HPLC. A ZORBAX Eclipse XDB-C18 column (250 by 4.6 mm; particle size, 5 μm; Agilent) was used. The mobile phase was methanol and Milli-Q-filtered water (10:90, vol/vol), where the water contained 1 mM H2SO4. The flow...
rate was set at 0.6 ml/min, and the injection volume was 20 µl. The detection wavelengths were set at 259 nm for pseudooxynicotine and 263 nm for 3-succinoyl-semialdehyde-pyridine. The column temperature was 30°C. A standard curve of pseudooxynicotine and 3-succinoyl-semialdehyde-pyridine was prepared for quantification, respectively.

The reaction products of Hno and Pno were also analyzed using LC-MS according to a previous report (25), with minor modifications. For determining the products of pseudooxynicotine oxidation by Pno, a mobile phase containing 8 mM formic acid solution (A) and methanol (B) was used, and the following gradient program was applied at a flow rate of 0.7 ml/min: 0 to 50 min for 95% to 5% A plus 5% to 95% B.

**Disruption of the rid-NC gene.** The rid-NC gene in the genome of *A. tumefaciens* S33 was disrupted by homologous recombination using the suicide plasmid vector pBl205SK, and the mutant was then complemented with pBBR1MCS-5 harboring the full-length rid-NC gene as described previously (29). The primer sequences used for rid-NC gene disruption and complementation are listed in Table 1. To evaluate the effects of gene disruption and complementation on cell growth, the S33-Δrid-NC mutant strain and the S33-Δrid-NC-C complementation strain were grown in HSP medium and nicotine medium, respectively, at 30°C. The wild-type strain *A. tumefaciens* S33, which was used as a control, was grown under identical conditions.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**

**ACKNOWLEDGMENTS**

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