Microbial Degradation of Nicotinamide by a Strain *Alcaligenes* sp. P156

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A novel *Alcaligenes* sp. strain P156, which can utilize nicotinamide as its sole source of carbon, nitrogen and energy, was enriched and isolated from soil in a solid waste treatment plant. Aerobic growth and degradation with nicotinamide were characterized. Seven nicotinamide degradation-related genes were obtained by sequence alignment from the genome sequence of strain P156. Four genes, designated *naaA*, *naaD*, *naaE* and *naaF*, were cloned and heterologously expressed. Nicotinamide degradation is initiated by deamination to form nicotinic acid catalyzed by the nicotinamidase NaaA, which shares highest amino acid sequence identity (27.2%) with nicotinamidase from *Arabidopsis thaliana*. Nicotinic acid is converted to 6-hydroxynicotinic acid, which is further oxidized to 2,5-dihydroxyhydrindine (2,5-DHP). 2,5-DHP is then transformed to a ring-cleavage product, N-formylmaleamic acid, by an Fe2+ dependent dioxygenase NaaD. N-formylmaleamic acid is transformed to fumaric acid through maleamic acid and maleic acid by NaaE and NaaF, respectively. To our knowledge, this is the first report of the complete microbial degradation of nicotinamide in bacteria. Nicotinamide is considered as a model compound for the study of microbial degradation of pyridinic compounds, and the nicotinamide degrading related genes in strain P156 were distributed differently from the reported similar gene clusters. Therefore, this study contribute to the knowledge on the degradation of pyridinic compounds.

Pyridine and its derivatives compose one of the largest classes of known organic chemicals. The nitrogen atom makes pyridine ring more electrophilic, which gives pyridinic compounds outstanding biological activities. Therefore, pyridinic compounds are widely used in chemical, agricultural, pharmaceutical, and food industries. In nature, pyridine ring can be found in coenzymes, plant alkaloids, and the secondary products generated by microorganisms1–3. Just like its homocyclic analogs, pyridinic compounds are considered persistent pollutants and most of them are harmful to human and other organisms and classified as priority pollutants by United States Environmental Protection Agency4–6. Pyridine and its derivatives are easy to spread in environment due to the water solubility property7. Therefore it is necessary to remove pyridinic compounds from the environment. Pyridinic compounds can be removed by physical8,9, chemical or biological methods. Microbial biodegradation could remove these compounds efficiently without secondary pollution8,5,7, therefore, microorganisms play significant roles in degradation of pyridinic derivatives5,10.

Nicotinamide, also known as a form of vitamin B3, is a part of the coenzyme nicotinamide adenine dinucleotide (NADH/NAD+) and is crucial to life. Nicotinamide is the most distributed and commonly used pyridinic compounds found in food, dietary supplement, cosmetics and medication11. Although nicotinamide was widespread in the environment, information of microbial degradation of nicotinamide is quite limited. The study of microbial transformation of nicotinamide mainly focus on the first deamination step12,13. In the deamination step, nicotinamide was transformed to nicotinic acid by nicotinamidase releasing NH4+. This reaction is not only involved in NAD+ biosynthesis, but in many other important physiological processes. The product, nicotinic acid, does not seem to influence these physiological processes, therefore, the fate of nicotinic acid in these organisms were not reported11,14. Nicotinamide is ubiquitous in all living organisms, thus, degradation of nicotinamide could be considered as model systems for the degradation of pyridinic compounds. Microbial degradation of

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nicotinamide will help us to understand the degradation processes of other pyridinic pollutants, such as picloram and diquat.1,2

In this study, we have isolated a nicotinamide degrading bacterium, *Alcaligenes* sp. P156, from the soil in solid waste treatment plant. The genome sequence of strain P156 was determined, and a DNA fragment was predicted to be involved in nicotinamide degradation. The functions of four genes were characterized in detail. The objective of this work is to demonstrate the nicotinamide catabolism in bacteria.

Results

Isolation and Identification of *Alcaligenes* sp. strain P156. The bacterial strains were isolated from contaminated soil in a solid waste treatment plant. The strain, which could use picolinic acid (PIA) and nicotinamide as the sole source of carbon, nitrogen, and energy, was obtained by plate streaking with enrichment cultivation and designated as P156 (Table 1). The 16S rRNA gene of strain P156 exhibits high sequence similarity with those of *Alcaligenes* strains, having the highest similarity of ~97% with *Alcaligenes faecalis* subsp. *paraealis*. Strain P156 was identified as a Gram-negative, aerobic strain and classified as *Alcaligenes* sp. Isolated P156 strain has been deposited in the China Center For Type Culture Collection, Wuhan under the accession numbers CCTCC AB 2016309.

Co-degradation of PIA and nicotinamide by strain P156. Strain P156 was cultivated with PIA, nicotinamide and a mixture of PIA and nicotinamide. As we can see from Fig. 1, the growth of strain P156 reached a maximum with the mixture substrates compared with the single substrate (Fig. 1a). Strain P156 has the shortest lag phase when grow with PIA; however, the final biomass of strain P156 with PIA is the smallest. PIA and nicotinamide degraded simultaneously in the culture with mixed substrates. PIA biodegradation seemed to be slightly slowed down in the presence of nicotinamide; however, the degradation rate of nicotinamide was basically the same in the presence or absence of PIA (Fig. 1b).

PIA and nicotinamide degradation reactions were performed by resting cells of strain P156 prepared from MSM + PIA/nicotinamide or MSM + NH4Cl + sodium citrate, respectively. PIA was completely transformed within 6 hours by PIA-induced resting cells of strain P156; however, PIA concentration in reaction with non-PIA induced cells was basically unchanged (Fig. 2a). Unlike PIA, the nicotinamide was completely transformed within 8 hours by resting cells of strain cultivated with nicotinamide or NH4Cl + sodium citrate (Fig. 2b). Besides, no nicotinic acid was accumulated in the samples from resting cell reactions. The results indicated that the expression of enzyme responsible for PIA conversion was induced by PIA. However, the enzyme responsible for nicotinamide conversion was expressed constitutively.

Growth kinetics with nicotinamide. In the batch experiment, the growth of strain P156 increased with the increase of nicotinamide concentration. The lag phase was basically unchanged with different concentration of nicotinamide (Fig. 3a). The specific growth rate (μ) and initial concentration of nicotinamide have been plotted in Fig. 3b. Kinetic constants were estimated by specific growth rate fitted to Monod's model and Haldane inhibitory growth model. It has been found that Monod’s model can represent the date of entire region (Fig. 3b), which indicates that nicotinamide have no inhibitory effect on growth of strain P156 in this range. The maximum specific growth rate (μmax) and half saturation (Ks) are 0.32 ± 0.06 h⁻¹ and 1.27 ± 0.53 mg/mL, respectively.

Nicotinamide degradation by *Alcaligenes* sp. P156. Strain P156 could use nicotinamide as the sole source of carbon, nitrogen and energy. To confirm the degradation pathway of nicotinamide in strain P156, time course biotransformation of nicotinamide by resting cells of *Alcaligenes* sp. P156 was analyzed by HPLC and LC-MS. When the signal representing nicotinamide decreased, two new peaks emerged in the HPLC signal. The retention time and spectra of the new products resemble those of 6-hydroxynicotinic acid and 2,5-dihydroxypyridine (2,5-DHP), respectively (Figs 4a, S1 and S2). When use 6-hydroxynicotinic acid as the substrate, 2,5-DHP was detected by HPLC (data not shown). Chromatograms of resting cell reaction samples on thin-layer chromatography (TLC) are presented in Fig. 4b. It can be seen that the spots representing nicotinamide shallowed, and the spots representing nicotinic acid and 6-hydroxynicotinic acid emerged and deepened.

Table 1. Growth and degradation of strain P156 with different substrates. All the experiments were performed in MSM with 1 mg/mL corresponding substrate. *ND*, not detectable.

| Substrates   | Growth | Degradable |
|--------------|--------|------------|
| 2-hydroxypridine | ND     | ND         |
| 3-hydroxypridine | ND     | ND         |
| 4-hydroxypridine | ND     | ND         |
| picolinic acid   | +      | +          |
| nicotinic acid   | +      | +          |
| isonicotinic acid | ND    | ND         |
| nicotinamide     | +      | +          |
| 6-hydroxynicotinic acid | + | + |
| 2,3-dihydroxypridine | ND     | ND         |
| 2,5-dihydroxypridine | ND     | +          |
| 2,6-dihydroxypridine | ND     | ND         |

In the batch experiment, the growth of strain P156 increased with the increase of nicotinamide concentration. The lag phase was basically unchanged with different concentration of nicotinamide (Fig. 3a). The specific growth rate (μ) and initial concentration of nicotinamide have been plotted in Fig. 3b. Kinetic constants were estimated by specific growth rate fitted to Monod’s model and Haldane inhibitory growth model. It has been found that Monod’s model can represent the date of entire region (Fig. 3b), which indicates that nicotinamide have no inhibitory effect on growth of strain P156 in this range. The maximum specific growth rate (μmax) and half saturation (Ks) are 0.32 ± 0.06 h⁻¹ and 1.27 ± 0.53 mg/mL, respectively.
gradually. In LC-MS results, signal representing the molecular mass of nicotinamide ([M-H]−, m/z 123.1) disappeared at 4 h, the new signals representing nicotinic acid ([M-H]−, m/z 124.1) and 6-hydroxynicotinic acid ([M-H]−, m/z 140.1) were observed (Fig. 4b).

Based on HPLC and LC-MS results, it can be concluded that nicotinamide was initially transformed into nicotinic acid, then the nicotinic acid was further transformed through 6-hydroxynicotinic acid and 2,5-DHP (Fig. 5a). To accelerate the study of molecular mechanism, the genome of strain P156 was sequenced. Several genes were found in the genome of strain P156 that encode proteins showing amino acids sequence identity with reported nicotinic acid or nicotine degradation proteins from *P. putida* KT2440 or *P. putida* S1611,16,17. ALFP_1623 shows 83.3% sequence identity with 6-hydroxynicotinic acid (NicC) from *P. putida* KT2440, and is predicted to catalyze the hydroxylation of 6-hydroxynicotinate. The deduced amino acid sequence of the gene *naaD* shares 54.4% sequence identity with NicX from strain KT2440, indicating that the gene may be responsible for the conversion of 2,5-DHP to N-formylmaleamic acid. Proteins from NaaE to NaaG were supposed to catalyze the conversion of 2,5-DHP to fumaric acid (Fig. 5a,b).

**The naaA gene encodes the nicotinamidase.** The first enzymatic step in nicotinamide degradation is nicotinamide deamination. This step was catalyzed by nicotinamidase, which has not been studied in pyridinic compound degradation strains. A gene, *naaA*, was identified in the genome of strain P156 by sequence alignment. NaaA showed low amino acid sequence identity with functionally reported nicotinamidase. It showed the highest sequence identity (27.2%) with nicotinamidase from *Arabidopsis thaliana* (Fig. 6a). To confirm the function of *naaA*, it was cloned into pET28a, expressed and the His-tagged enzyme was purified. NaaA could transform nicotinamide into nicotinic acid without adding other coenzyme (Fig. 6b). The optimum pH and temperature for NaaA catalyzed reaction were 7.0 and 40 °C, respectively (Fig. 6c,d).
The **naaD** gene encodes the 2,5-DHP 5,6-dioxygenase. The ring-cleavage reaction from 2,5-DHP to N-formylmaleamic acid is one of the key steps in pyridinic compounds degradation, and this is due to that 2,5-DHP is the key metabolic intermediate of many pyridine derivatives. To confirm its function, the **naaD** gene was cloned, heterologously expressed in *E. coli* cells and purified as a homogeneity form. The molecular mass of the purified band on SDS-PAGE was approximately ~40 kDa. Oxidative activity of the purified NaaD was measured at 320 nm, and the results indicated that NaaD is responsible for the 2,5-DHP conversion to N-formylmaleamic acid (Fig. 7). The metal cheater binding amino acids are conserved among the aligned sequences (Fig. S3). The addition of Fe$^{2+}$ significantly increased the enzyme activity of NaaD, indicating that Fe$^{2+}$ is required for the oxidative activity of NaaD.

The **naaE** and **naaF** genes are responsible for the conversion from N-formylmaleamic acid to maleic acid. Two genes (**naaE** and **naaF**), which are located on each side of the **naaD** gene, are predicted for the conversion from N-formylmaleamic acid to maleic acid. To confirm the function of these two genes, **naaE** and **naaF** was cloned and heterologously expressed in *E. coli* cells. As shown in Fig. 8a, the product of NaaD catalyzed reaction was used as the substrate for NaaE. The pyridine ring of 2,5-DHP was opened to form N-formylmaleamic acid within 30 seconds, and the absorbance at 340 nm gradually increased in the following 5.5 min. When NaaE was added at 3 min, the absorbance at 340 nm decreased indicating that N-formylmaleamic acid was converted by NaaE. To identified the product of NaaE catalyzed reaction, formate dehydrogenase and NAD$^+$ were added 3 min after adding NaaE. The absorbance at 340 nm increased, indicating that formic acid was the product of NaaE catalyzed reaction (Fig. 8a). NaaE is a N-formylmaleamate deformylase, which transforms N-formylmaleamic acid to maleamic acid and formic acid.

To determine the function of NaaF, it was mixed with maleamic acid. After 3 h incubation, the product was detected by LC-MS. The result indicated that the signal representing maleamic acid ([m/z 114.0] disappeared, while a new signal with [m/z 115.0] was observed (Fig. 8b). The results indicated that maleamic acid was transformed into maleic acid by NaaE. The gene **naaF** encodes a maleamate amidase.

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**Figure 2.** PIA and nicotinamide degradation by resting cells of strain P156. (a) PIA degradation by resting cells of strain P156 prepared from MSM with PIA (green line) and citrate/NH$_4$Cl media (black line). (b) Nicotinamide degradation by resting cells of strain P156 prepared from MSM with Nicotinamide (red line) and citrate/NH$_4$Cl media (black line). Each value is the mean of results from three parallel replicates ± the SD.
Discussion

Pyridinic compounds have significant biological activities, the substitution groups on pyridine ring play important roles for their biological physiology\(^\text{19}\). The production of pyridine derivatives by organic synthesis requires strict conditions for high yield, resulting in high cost\(^\text{20,21}\). Biocatalysis, with high selectivity and mild reaction conditions, is a useful supplementary technology for the chemical industry in these reactions\(^\text{22,23}\). However, biocatalysis technology in pyridinic compounds production is limited by the lack of enzymes. Microorganisms, which are able to degrade pyridine and its derivatives, are important bio-resources to provide useful elements for biocatalysis of pyridinic compounds\(^\text{22}\). In this study, *Alcaligenes* sp. strain P156, which could use nicotinamide as its sole source of carbon, nitrogen and energy, was isolated and characterized. The first step of nicotinamide degradation in strain P156 is deamination to form nicotinic acid (Fig. 5). The following degradation pathway of nicotinic acid is similar with that in *P. putida* KT2440. Microbial degradation of nicotinamide has been reported previously, of which nicotinamide was transformed into nicotinic acid\(^\text{12,13}\). However, the further degradation of nicotinic acid in these microorganisms has not been reported. In this study, we revealed that strain P156 could transform nicotinamide to nicotinic acid, and nicotinic acid could be further degraded through 2,5-DHP pathway. For the first time, the complete nicotinamide degradation pathway is reported in one bacterium.

![Image of growth and specific growth rate](image)

Figure 3. Growth and specific growth rate of *Alcaligenes* sp. P156. (a) Growth of strain P156 with different concentrations of nicotinamide at 30 °C pH 7.0. (b) Monod’s growth model fitted to results of batch growth experimental data to determine the growth kinetics parameters of strain P156. Each value is the mean of results from three parallel replicates the SD.
Figure 4. HPLC and ESI-MS analysis of nicotinamide degradation by *Alcaligenes* sp. P156. (a) The reaction samples at 0 h (black line), 1 h (blue line), 4 h (green line) and 8 h (red line) for the degradation of nicotinamide by resting cells of *Alcaligenes* sp. P156. (b) LC-MS analysis of the reaction samples at 0 h and 4 h. The structure of the compounds have been indicated in the figure. The inset is the photograph of TLC plate (visualized in UV light) with reaction samples at 0 h, 1 h, 2 h and 3 h. The compounds, represented by the spots, were indicated on the right of TLC plate (full length spots can be observed in Supplementary Information).

Figure 5. Proposed pathway of nicotinamide degradation in *Alcaligenes* sp. P156. (a) Proposed nicotinamide degradation pathway in *Alcaligenes* sp. strain P156. (b) Physical map of the DNA fragment containing genes involved in nicotinamide degradation in strain P156. The arrows indicates the location, direction and size of the transcription of the ORFs.
than with nicotinic acid (Fig. S4). Growth kinetics study revealed that nicotinamide has no inhibitory effect on the growth of strain P156 at concentration range from 0 to 3 mg/mL, therefore, it can be used as growth substrate for bacteria in the environment. Nicotinamide degradation for growth purpose is one of the physiological roles of nicotinamidase. We have compared all the *Alcaligenes* from GenBank database with the nicotinamide degradation genes in P156, it turned out that the nicotinic acid degradation cluster are highly conserved in all of these 30 strains. However, 9 of these strains do not have the NaaA gene. The results indicated that nicotinic acid...
degradation cluster is belong to the core genome of *Alcaligenes*, however, nicotinamidase is belong to the variable genome. The *naaA* gene was introduced into the genome of P156 and give the capacity of growing with nicotinamide for strain P156.

Several pyridine ring α-hydroxylases have been reported, all of them are molybdate containing enzymes including nicotinate dehydrogenase in *P. putida* KT2440[11]. This kind of hydroxylases contain a large subunit with molybdenum-containing domain, a small subunit with [2Fe-2S] cluster-binding domains, and some of them also have a middle FAD-binding subunit. In spite of the middle subunit, all the reported enzymes has only one molybdenum-containing large subunit. However, in *A. faecalis* JQ135 Zhang et al. has proved that the nicotinate dehydrogenase is a three components hydroxylase, including two molybdenum-containing subunit[30].

The *alfp_2914* to *alfp_2916* genes show high sequence identity with the related genes in strain JQ135, the function of these *orf*s involved in nicotinate transformation should be further confirmed. AlFP_1623 has the highest sequence identity with 6-hydroxynicotinate 3-monooxygenase in strain KT2440. We cloned this gene and purified the His-tagged enzyme; however, enzyme activity was not detected. Therefore, further investigation should be done to elucidate the complete molecular mechanism of nicotinamide degradation.

Methods

Chemicals and media. Nicotinamide, nicotinic acid, 6-hydroxynicotinic acid and other pyridinic compounds were purchased from Aladdin (Shanghai, China). 2,5-DHP was purchased from SynChem OHG (Kassel Corp., Kassel, Germany). FAD, NADH were obtained from Sigma-aldrich company. All other reagents and solvents were of analytical or chromatographic grade and were commercially available. The mineral salt medium (MSM) was used for enrichments and isolation as previously described[11]. Soluble pyridinic compounds were added into MSM before inoculation from aqueous stock solutions after sterilized by 0.22 μM filtration.

Strain and cultivation conditions. The enrichment cultivation was carried out by adding 5 g contaminated soil from a solid waste treatment plant (Qingdao, China) into 50 mL MSM with 1 mg/mL PIA. The mixture was cultivated aerobically in a shaker at 30 °C 120 rpm for a week. The culture was then transferred into new
Domestication processes lasted for 2 months before strain P156 was isolated using several times of streaking inoculations. The 16S rRNA gene of strain P156 was amplified using universal primers 27F (5′-GAGTTTGATCATGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGATC-3′). PCR amplification was performed with 30 μL reaction mixtures containing primers, genome of strain P156 and 15 μL 2× Taq Mix (Novoprotein). PCR was carried out as follows: 5 min at 95 °C and then 35 cycles of 30 s at 95 °C, 30 s at 60 °C, and 90 s at 72 °C. The PCR product was sequenced in Sangon Biotech (Shanghai). Sequences alignment and phylogenetic inferences were performed using Clustal W, and the tree was constructed by MEGA6 software obtained using the neighbor-joining method with a bootstrap of 1,000. The 16S rRNA gene sequences of typical species in Alcaligenes were obtained from list of prokaryotic names (LSPN) database.

Substrates specificity for growth and biotransformation of strain P156. Different substrates were added to MSM at a final concentration of 1 mg/mL, and the cultures were incubated at 30 °C for 72 hours. The growth of cultures was detected according to OD 600 nm. Resting cell reactions were performed to identified the transformation of different pyridine derivatives by strain P156. Cells cultivated with PIA in the late exponential phase were centrifuged at 8,000 × g for 15 min at 4 °C. The pellet was washed twice with 50 mM PBS buffer (pH 7.0), and resuspended in the same buffer to OD600 nm = 5.0. The bacterial suspension was designated as resting cell and used immediately in the biotransformation experiments. The experiments were conducted using a series of 250 mL Erlenmeyer flasks, each containing 25 mL resting cell and 0.5 mg/mL substrate. Samples were collected at interval times and centrifuged. Then the supernatants were analyzed by UV-visible spectrophotometer.

Growth kinetics study. In a batch system, the specific growth rate (μ) was defined as

\[
\frac{dX}{dt} = \mu X - K_d X 
\]

(1)

X is cell concentration (OD600 nm), μ is specific growth rate of bacteria (h⁻¹), and K_d is endogenous decay coefficient (h⁻¹). At exponential growth phase the K_d could be neglected, and the equation (1) was reduced to

\[
\frac{dX}{dt} = \mu X 
\]

(2)

The value of μ was determined at the exponential phase of the growth curve according to equation (2).

Two models were used to fit to the experimental data obtained from the batch experiments. Monod model represents bacterial growth under substrate-limited and non-inhibitory conditions as equation (3).

\[
\mu = \frac{\mu_{max} S}{K_s + S} 
\]

(3)

Haldane inhibitory growth model represents the growth kinetics of an inhibitory compound such as pollutants as equation (4)

\[
\mu = \frac{\mu_{app} S_0}{K_s + S_0 + \left[ \frac{S_0}{K_i} \right]^2} 
\]

(4)

The values of the kinetic parameters were obtained from non-linear fitting using Origin software.

Intermediates identification. Resting cell reactions were performed with 1 mg/mL nicotinamide at 30 °C 120 rpm. Samples were collected at interval times and centrifuged. Fractions of the supernatant were diluted 10 times with methanol, and analyzed by HPLC and LC-MS.

Cloning, expression and purification of four genes. Four gene, naaA, naaD, naaE and naaF, were amplified from genomic DNA of strain P156, using primers shown in Table 2. The PCR product was purified and inserted between the restriction sites of expression vector pET28a containing a N-terminal His₆-tag. The

| Name | Sequence (5′-3′) (restriction sites are underlined) |
|------|--------------------------------------------------|
| naaA-F | CGCGGATCCATGGCTATTCAGATTGAT (GAATTC) |
| naaA-R | CCGCTCTAGAGTTATTGTCTGAGTTTCGT (AAGCTT) |
| naaD-F | CGCGAATTCATGGCACCTTGATTATGATCC (GAATTC) |
| naaD-R | CCGCTCTAGAGTTCTCTGACCTCAACACAGGG (AAGCTT) |
| naaE-F | CGCGGATCCATGGATAGCCTCCGATGATCC (GAATTC) |
| naaE-R | CCGCTCTAGAGTTCTCTGACCTCAACACAGGG (AAGCTT) |
| naaF-F | CGCGGATCCATGGCTATTCAGATTGAT (GAATTC) |
| naaF-R | CCGCTCTAGAGTTCTCTGACCTCAACACAGGG (AAGCTT) |

Table 2. Oligonucleotides used in this study. PCR primers naaA-F/-R, naaD-F/-R, naaE-F/-R and naaF-F/-R are used for the construction of expression recombination plasmid pET-28a-naaA, pET28a-naaD, pET28a-naaE, and pET28a-naaF.
recombinant plasmids were verified by sequencing and transformed into E. coli BL21(DE3) cells for recombinant expression, respectively. Each E. coli BL21(DE3) cells carrying recombinant plasmid was grown in LB medium with 50 μg/mL kanamycin at 37 °C to an OD600nm of 0.6–0.8. Protein expression was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the culture was then shaken at 30 °C overnight. Cells were harvested by centrifugation, dissolved in 50 mM Tris-HCl (pH 8.0) buffer, and broken by ultrasonication. His-tagged enzymes were purified in a gravity wash column with Ni2+ NTA His Sefinoose resin (Sagon Biotech, Shanghai). The resin was balanced in 50 mM Tris-HCl (pH 8.0) buffer, and the protein was washed by the same buffer with 50 mM imidazole.

Enzyme assay. The nicotinamidase activity was monitored according to the decrease of substrate nicotinamide. The reaction was terminated by adding 0.1 volume of 1M H2SO4, and, after 3 min, 0.2 volume of 1M NaOH and 0.7 volume of methanol were added. After that, the substrate concentration in the reaction mixture was analyzed by HPLC. The 2,5-DHP dioxygenase activity was monitored according to the absorbance at 320 nm in 50 mM Tris-HCl buffer (pH 8.0) at room temperature (25 °C) in UV2600 UV-Vis spectrophotometer (Shimadzu, Japan). The cuvette contained NaaD and 250 μM 2,5-DHP in the presence or absence of 5μM FeSO4. The 6-hydroxynicotinic acid 3-monoxygenase activity was determined by measuring the decrease in absorbance at 340 nm due to the substrate-dependent oxidation of NADH. Assays were carried out in 50 mM Tris-HCl buffer (pH 8.0) at room temperature. The cuvette contained cell extract of strain P156, 250 μM substrate and 250 μM NADH in a total volume of 800μL. The enzyme assays were initiated by the addition of enzyme.

Analytical methods. Pyridinic compounds were analyzed by TLC, HPLC (Agilent, series 1100) or UV2600 UV-Vis spectrophotometer. The samples were subjected to TLC analysis using silica gel 60 F254. The composition of the eluent was chloroform:methanol:acetic acid = 10:1:0.1 (vol/vol/vol). After the separation process, the plate was dried and observed at 254 nm. The pyridinic compounds in resting cell reactions were determined by HPLC using an Eclipse XBD-C18 reverse-phase column (5 μm; 4.6 × 150 mm; Keystone Scientific, Bellefonte, PA) with a DAD detector. The mobile phase consisted of 85:15 (vol/vol) methanol: 1 mM H2SO4 at a flow rate of 0.5 mL/min at 30 °C. The mobile phase for the samples of NaaA catalyzed reaction consisted of 80% (vol/vol) 20mM ammonium acetate and 20% (vol/vol) methanol at a flow rate of 1.0 mL/min at 30 °C. LC-MS analysis was performed on an Agilent 6460 triple quadrupole system equipped with electrospray ionization (ESI) sources in 20% (vol/vol) methanol and 80% (vol/vol) deionized water (18 MΩ/cm) (0.05% formic acid [vol/vol]) at a flow rate of 0.2 mL/min with the same Eclipse XBD-C18 reverse-phase column. All samples were treated with the addition of 9 volumes of methanol at 4 °C for 10 min followed a centrifugation at 12,000 × g for 2 min. Then the samples were filtered through a 0.22-μm Sartorius filter prior to HPLC and LC-MS analysis.

Nucleotide sequence accession numbers. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession CP021079. The sequences of 16S rRNA gene from strain P156 is available in GenBank under accession numbers KU740245.

Ethical statement. This article does not contain any studies with human participants or animals performed by any of the authors.

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Author Contributions
C.H., K.L. and H.Y. conceived and designed the project. C.H., S.Z. and H.Y. performed the experiment. K.L. and H.Y. contributed reagents and materials. C.H. and H.Y. analyzed data. K.L. and H.Y. wrote the manuscript. All authors have read and approved.

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