

1 **Discovery of the 1-naphthylamine biodegradation pathway reveals an enzyme that**
2 **catalyzes 1-naphthylamine glutamylation**

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18

19 **Abstract**

20 1-Naphthylamine (1NA), which is harmful to human and aquatic animals, has been
21 used widely in the manufacturing of dyes, pesticides, and rubber antioxidants.
22 Nevertheless, little is known about its environmental behavior and no bacteria have
23 been reported to use it as the growth substrate. Herein, we describe a pathway for 1NA
24 degradation in isolate *Pseudomonas* sp. strain JS3066, determine the structure and
25 mechanism of the enzyme NpaA1 that catalyzes the initial reaction, and reveal how the
26 pathway evolved. From genetic and enzymatic analysis, a cluster of 5 genes encoding
27 a dioxygenase system was determined to be responsible for the initial steps in 1NA
28 degradation through glutamylation of 1NA. The γ -glutamylated 1NA was subsequently
29 oxidized to 1,2-dihydroxynaphthalene which was further degraded by the well-
30 established pathway of naphthalene degradation via catechol. Enzymatic analysis
31 showed that NpaA1 catalyzed conversion of various anilines and naphthylamine
32 derivatives. Structural and biochemical studies of NpaA1 revealed that the broad
33 substrate specificity of NpaA1 is due to a large hydrophobic pocket, which is different
34 from type I glutamine synthetase (GSI). The findings enhance understanding of
35 degrading polycyclic aromatic amines, and will also enable the application of
36 bioremediation at naphthylamine contaminated sites.

37

38 **Introduction**

39 Aromatic amines include monocyclic aromatic amines, such as aniline and
40 chlorinated derivatives, and polycyclic aromatic amines, such as naphthylamine and its

41 derivatives. Aromatic amines have been widely utilized as raw materials for
42 manufacturing dyes, pharmaceuticals, and agrochemicals (Palmiotto et al., 2001).
43 Anthropogenic activities have led to their widespread release into the environment
44 (Akyüz & Ata, 2006; Dupret et al., 2011). The United States produced 1,050 thousand
45 metric tons of aniline in 2013 (Council, 2023). Meanwhile, China's export volume of
46 1-naphthylamine, 2-naphthylamine and their derivatives amounted to 19.8 thousand
47 metric tons in 2013 (Network, 2018). Several aromatic amines are potentially harmful
48 to human health, with both aniline and naphthylamines increasing the risk of bladder
49 tumors (Chung, 2000; Ferraz et al., 2012). These compounds are often found in
50 mixtures in the environment as impurities or by-products (IARC, 1974). Although
51 various aromatic amines cause environmental pollution and threaten human health,
52 their transport and fate are poorly understood.

53 Currently, only a few anilines and their derivatives have been reported to be
54 degraded by microorganisms (Krol et al., 2012; Lee et al., 2008; Qu & Spain, 2011;
55 Takeo et al., 2013). Under aerobic conditions, microbial degradation plays a major role
56 in the elimination of aniline and the molecular basis has been well established in several
57 bacteria (Fukumori & Saint, 1997; Krol et al., 2012; Liang et al., 2005; Takeo et al.,
58 2013). The aniline dioxygenase (AD) enzyme system is responsible for converting
59 aniline to catechol, which is then assimilated via the widespread *meta/ortho*-cleavage
60 pathways. AD comprises a γ -glutamylanilide synthase, a glutamine amidotransferase
61 (GAT)-like enzyme, and a two-component Rieske-type aromatic compound
62 dioxygenase (Takeo et al., 2013). The γ -glutamylanilide synthase catalyzes ATP-

63 dependent ligation of L-glutamate to aniline to generate γ -glutamylanilide (γ -GA). Due
64 to the cytotoxicity of high concentration of γ -GA, the GAT-like enzyme can hydrolyze
65 γ -GA to aniline so as to maintain γ -GA at a proper level inside the cell. Subsequently,
66 the dioxygenase catalyzes the transformation of γ -GA to catechol (Takeo et al., 2013).
67 Previous studies established that the γ -glutamylanilide synthetase and oxygenase of AD
68 play dominant roles in the substrate specificity of the pathway (Ang et al., 2009; Ji et
69 al., 2019). To enhance the engineering potential of AD systems, the structure and
70 mechanism of the oxygenase have been revealed to broaden its substrate range (Ang et
71 al., 2007). However, limited research has been conducted on the γ -glutamylanilide
72 synthetase.

73 In contrast to the well-established degradation of anilines, little is known about the
74 environmental fate and biodegradation of 1NA, and bacteria able to assimilate it have
75 not been reported. Here, we report isolation of an aerobic bacterial strain able to use
76 both 1NA and aniline as growth substrates through selective enrichment with samples
77 from a 1NA-manufacturing site. Detailed investigations established the degradation
78 pathway of 1NA and the genes encoding the enzymes involved. An enzyme system
79 homologous to AD system mentioned above catalyzes glutamylation of 1NA and
80 subsequent oxidation of the product to dihydroxynaphthalene. Previous research has
81 suggested that the substrate specificity of the glutamylating enzyme, which is
82 responsible for the initial glutamylation of aromatic amines, plays a crucial role in
83 determining the range of substrates that can be degraded (Ang et al., 2007; Ji et al.,
84 2019). Therefore, we elucidated the detailed structure and mechanism underlying its

85 substrate specificity. The results will enable us to predict and enhance 1NA
86 biodegradation at contaminated sites and provide the basis for better understanding of
87 the degradation of other polycyclic aromatic amines.

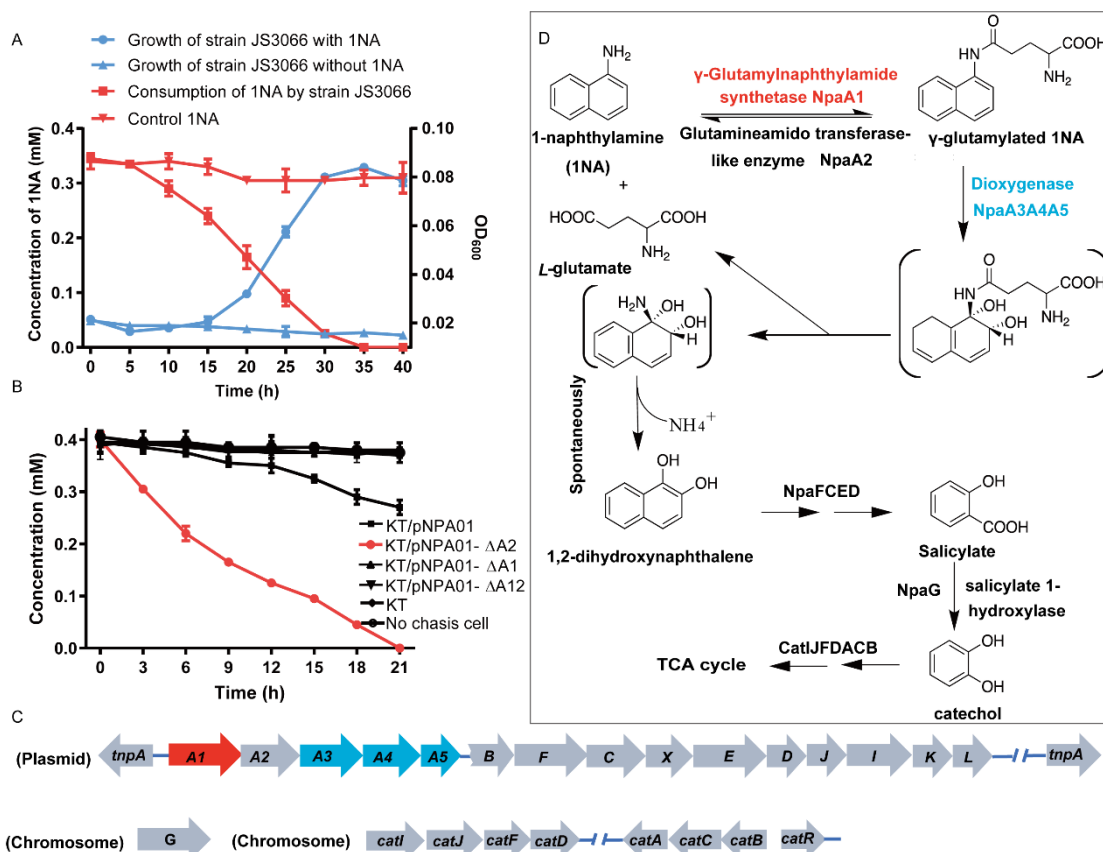
88 **Results**

89 ***Pseudomonas* sp. strain JS3066 is a 1NA degrader.** Selective enrichment with 1NA
90 as the growth substrate yielded an isolate that grew aerobically on 1NA as the sole
91 carbon and nitrogen source (Figure 1A). It could also utilize aniline for growth (data
92 not shown). A BLASTN search against the sequences on the National Center for
93 Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/>) revealed
94 that its 16S rRNA gene sequence shows 99.93% and 99.74% identity with
95 *Pseudomonas* sp. DY-1 (Genbank accession number: CP032616.1) and *Pseudomonas*
96 sp. TCU-HL1 (Genbank accession number: CP015992.1), respectively. Thus, the 1NA
97 degrader was identified as *Pseudomonas* sp. strain JS3066.

98

99 **A proposed 1NA conversion cluster locates on a plasmid.** The genome of strain
100 JS3066 comprises two replicons, one circular chromosome (6,093,500 bp, G+C content
101 of 62.94%) and one circular plasmid designated pJS3066 (109,408 bp, G+C content of
102 63.15%). The replication initiator protein TrfA of plasmid pJS3066 shares 100% amino
103 acid sequence identity with that of plasmid pTP6, which indicates that pJS3066 likely
104 belongs to the IncP-1 β subgroup of plasmids (Stenger & Lee, 2011; Thorsted et al.,
105 1996). Collectively, the whole genome of strain JS3066 is 6,202,908 bp with an average
106 G+C content of 62.95%.

107 The ability of the isolate to degrade aniline coupled with the fact that 1NA is an
108 analog of aniline prompted the hypothesis that the initial reactions of 1NA and aniline
109 catabolism might be mediated by the similar genetic determinants within strain JS3066.
110 Therefore, the well-studied aniline dioxygenase-encoding gene set (*atdA1A2A3A4A5*;
111 accession number: D86080.1) from *Acinetobacter* sp. YAA was used as the query to
112 search the genome of strain JS3066. A cluster of genes closely related to those encoding
113 aniline dioxygenase are located on plasmid pJS3066. The putative naphthylamine
114 dioxygenase-encoding genes, designated *npa* (1-naphthylamine), are encoded in the
115 order *npaA1A2A3A4A5* (Figure 1C). BLASTp analyses of the deduced amino acid
116 sequences of NpaA1A2A3A4A5 against the NCBI database revealed high identities
117 with the γ -glutamylanilide synthase (AtdA1), the GAT-like enzyme (AtdA2), the two-
118 component Rieske-type aromatic compound dioxygenase (AtdA3A4), and the
119 reductase component (AtdA5) in *Acinetobacter* sp. YAA (Supplementary File 1). No
120 other potential aniline dioxygenase homologs were detected in the genome.



121

122 **Figure 1** Degradation of 1NA by *Pseudomonas* sp. strain JS3066. (A) Growth with
 123 1NA as the sole carbon and nitrogen source. (B) 1NA conversion by cell suspensions
 124 of *P. putida* KT2440- Δ *catA* Δ *ggt* harboring pNPA01 (*npaA1* to *npaA5*), pNPA01- Δ A1
 125 (*npaA2* to *npaA5*), pNPA01- Δ A2 (*npaA1* and *npaA3* to *npaA5*), and pNPA01- Δ A12
 126 (*npaA3* to *npaA5*). The ability of *P. putida* KT2440- Δ *catA* Δ *ggt* to convert 1NA together
 127 with spontaneous decomposition of 1NA were also determined as controls. The results
 128 are shown as averages \pm standard deviations from two or more independent
 129 measurements. (C) Organization of genes involved in 1NA degradation by strain
 130 JS3066. Gene *tnpA* encodes a transposase. Proposed functions of the rest of genes
 131 are presented in Table 1. (D) Proposed pathway of 1NA catabolism in *Pseudomonas* sp.
 132 strain JS3066. TCA, tricarboxylic acid. Unstable compounds are enclosed in brackets.

133 Figure 1-Source data 1. Raw data for the Figure 1A and Figure 1B

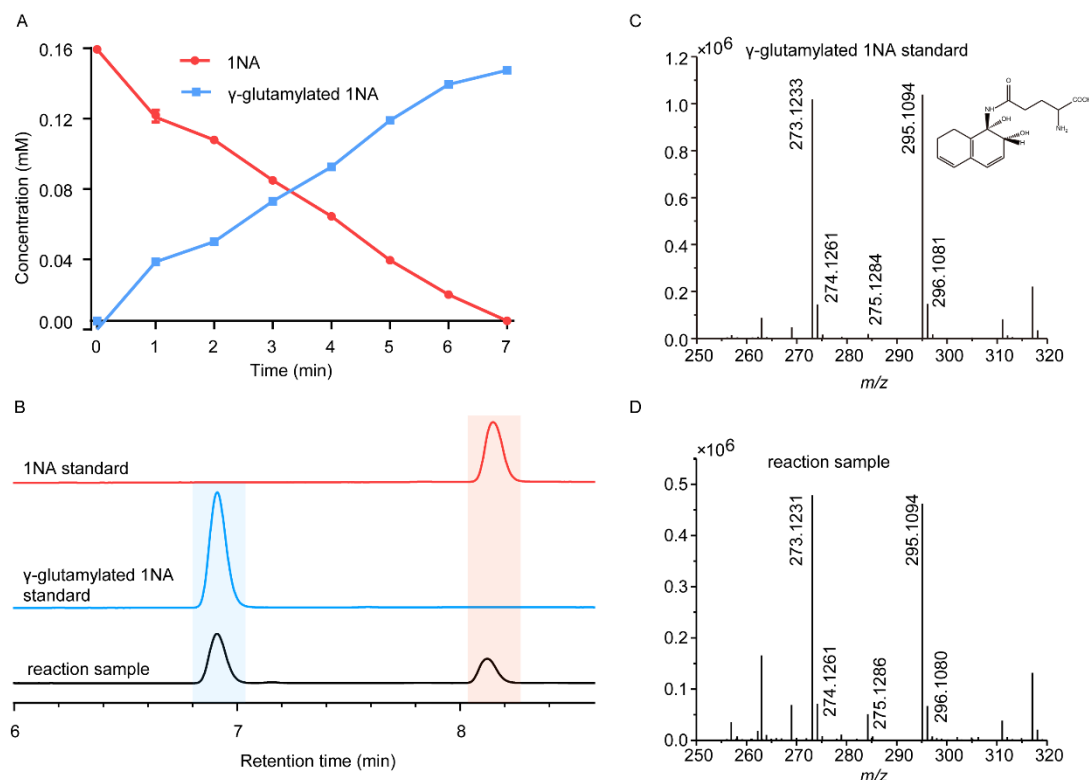
134

135 **NpaA1A3A4A5 convert 1NA to 1,2-dihydroxynaphthylene.** To investigate the roles
 136 of *npaA1A2A3A4A5* genes in the initial oxidation of 1NA, plasmids carrying different
 137 combinations of the five genes were constructed and introduced into *P. putida* KT2440-
 138 Δ *catA* Δ *ggt* which is unable to catabolize catechol. Then the abilities of recombinant

139 KT/pNPA01 (including *npaA1A2A3A4A5* genes) and its derivatives to metabolize 1NA
140 were analyzed. Recombinant KT/pNPA01 and KT/pNPA01- Δ A2 (including *npaA1*,
141 *npaA3* to *npaA5*) were able to transform 1NA (Figure 1B). However, cells harboring
142 either *npaA2A3A4A5* genes (recombinant KT/pNPA01- Δ A1) or *npaA3A4A5* genes
143 (KT/pNPA01- Δ A12) were unable to transform 1NA. Gas chromatography-mass
144 spectrometry (GC-MS) analysis revealed that the main product formed during the
145 transformation of 1NA by recombinant KT/pNPA01 was 1,2-dihydroxynaphthalene
146 (Figure 1—figure supplement 1). Notably, recombinant KT/pNPA01 retained the ability
147 to transform aniline into catechol (Figure 1—figure supplement 2).

148 To enable further characterization, NpaA1 was expressed and purified as an N-
149 terminal Strep II-tagged fusion protein. 1NA conversion by NpaA1 was investigated in
150 a reaction mixture similar to that used previously for transformation of aniline to γ -
151 glutamylated aniline (Ji et al., 2019). 1NA (λ_{\max} , 310 nm) was transformed by purified
152 NpaA1 to a new product with maximum absorption at around 287 nm (Figure 2—figure
153 supplement 1). The HPLC retention time of the product was equal to that of the
154 authentic γ -glutamylated 1NA and ultra-performance liquid chromatography-
155 quadrupole-time of flight-mass spectrometry (UPLC-QTOF MS) analysis showed that
156 the product had a molecular ion at m/z 273.1231 [M+H]⁺, which is identical to that of
157 authentic γ -glutamylated 1NA (Figure 2C and 2D). During γ -glutamylation of 1NA,
158 the 1NA consumption (0.16 mmol) was almost equivalent to the total accumulation of
159 γ -glutamylated 1NA (0.157 mmol) (Figure 2A and 2B). In view of the above analyses,
160 NpaA1 was established as a γ -glutamyl-naphthylamide synthase catalyzing ligation of

161 1NA and L-glutamate to form γ -glutamylated 1NA.



162

163 **Figure 2** Conversion of 1NA by recombinant protein NpaA1. (A) The time course of
164 1NA degradation and γ -glutamylated 1NA accumulation. The results are shown as
165 averages \pm standard deviations from two or more independent measurements. (B)
166 HPLC profiles of 1NA standard, γ -glutamylated 1NA standard, and the reaction
167 products of 1NA conversion catalyzed by NpaA1. The detection wavelength was 280
168 nm. (C-D) UPLC/QTOF-MS analysis of the intermediate captured during 1NA
169 transformation by NpaA1. The mass spectra of authentic γ -glutamylated 1NA (C) and
170 reaction product γ -glutamylated 1NA (D).

171 Figure 2-Source data 1. Raw data of Figure 2A and 2C-D.

172

173 Although recombinant KT/pNPA01- Δ A12 failed to transform 1NA, it was able to
174 catalyze conversion of γ -glutamylated 1NA into 1,2-dihydroxynaphthalene, indicating
175 that γ -glutamylated 1NA is an intermediate and a direct substrate for the dioxygenase
176 NpaA3A4A5 (Figure 1—figure supplement 3). Experiments with different
177 combinations of *npaA1A2A3A4A5* genes have established that only four Npa proteins,
178 namely, NpaA1 (GS-like enzyme), NpaA3 (large subunit of oxygenase component of

179 dioxygenase), NpaA4 (small subunit of oxygenase component of dioxygenase), and
180 NpaA5 (reductase component of dioxygenase) are essential for the conversion of 1NA
181 to 1,2-dihydroxynaphthylene (Figure 1B and 1D).

182

183 **Proposed pathway for 1,2-dihydroxynaphthalene degradation in strain JS3066.**

184 Downstream of the *npaA1A2A3A4A5* genes lie *npaBFCXEDJIKL* genes (Figure 1C and
185 Supplement File 1), whose products, except for *napX* encoding a transposase, are
186 closely related to those involved in naphthalene degradation by *Ralstonia* sp. strain U2
187 and related strains (Fuenmayor et al., 1998; Zhou et al., 2001). In strain U2, a putative
188 aldolase-encoding gene (*nagQ*) lies between the 1,2-dihydroxynaphthalene
189 dioxygenase-encoding gene (*nagC*) and the *trans*-o-hydroxybenzylidenepyruvate
190 hydratase-aldolase-encoding gene (*nagE*); in strain JS3066, however, *npaX*, a putative
191 transposase-encoding gene, lies there instead.

192 Under oxic conditions, naphthalene is often first oxidized to salicylate, which is
193 further channeled to tricarboxylic acid (TCA) cycle intermediates either via gentisate
194 or catechol catabolic pathways. The metabolism of naphthalene via catechol has been
195 studied in *P. putida* G7 (bearing the catabolic plasmid NAH7) (Sota et al., 2006) and in
196 *P. putida* NCIB 9816-4 (bearing the catabolic plasmid pDTG1) (Dennis & Zylstra, 2004)
197 at the genetic level. Likewise, the gentisate pathway has been found in *Ralstonia* sp.
198 strain U2 (Fuenmayor et al., 1998; Zhou et al., 2001) and *Polaromonas*
199 *naphthalenivorans* CJ2 (Jeon et al., 2006). Based on the above bioinformatic analysis
200 and analogy with the established pathways for naphthalene degradation, the putative

201 genes sufficient to encode the lower degradation pathway of INA would be complete,
202 except for the absence of a gene responsible for salicylate conversion. Given the fact
203 the gentisate-catabolic genes are intact in strain JS3066 (Figure 1C and 1D), we
204 searched for a gene which would catalyze conversion of salicylate to gentisate. Contrary
205 to expectation, no putative salicylate 5-hydroxylase-encoding gene was found either
206 upstream or downstream of *npaAIA2A3A4A5BFCXEDJIKL* genes. We therefore
207 searched the whole genome for genes encoding putative enzymes capable of catalyzing
208 (i) transformation of salicylate to catechol (Dennis & Zylstra, 2004; Jouanneau et al.,
209 2007; Sota et al., 2006), (ii) conversion of salicylate to gentisate via salicylyl-CoA and
210 gentisyl-CoA (Zhou et al., 2021), and (iii) direct ring fission of salicylate to 2-oxohepta-
211 3,5-dienedioic acid (Hintner et al., 2001). The search revealed a putative salicylate 1-
212 hydroxylase-encoding gene, designated *npaG* and located on the chromosome of strain
213 JS3066. The product of *npaG* exhibits 77% identity with NahG of *P. putida* G7 (Sota
214 et al., 2006). NpaG was functionally expressed and found to catalyze conversion of
215 salicylate to catechol with specific activity of 15.0 ± 1.3 U mg^{-1} (Figure 1—figure
216 supplement 4). The bioinformatic analysis did not reveal candidate genes encoding
217 enzymes that catalyze any of the known alternative routes of salicylate metabolism. The
218 above results supported the hypothesis that *npaBFCXEDJIKL-G* genes, products of
219 which catalyze conversion of 1,2-dihydroxynaphthalene to catechol, are involved in
220 INA degradation in strain JS3066. The putative genes responsible for encoding the
221 well-defined *ortho*-cleavage pathway of catechol degradation are located on the
222 chromosome in strain JS3066 (Figure 1C). The genes show high similarities to their

223 counterparts in other strains that degrade catechol (Supplementary File 1).

224

225 **NpaA1 converts multiple aromatic amine substrates to γ -glutamylated aromatic**

226 **amines.** Conversion to the corresponding γ -glutamylated amines is an essential step for

227 aromatic amines oxidation, because the dioxygenase is unable to directly act on the

228 amines. Furthermore, the glutamylating enzyme plays an essential role in the substrate

229 specificity of the pathway (Ji et al., 2019). Enzyme assays with purified NpaA1 revealed

230 its activity not only against polycyclic aromatic amines such as 1NA and 2-

231 naphthylamine, but also monocyclic aromatic amines and their chlorinated derivatives

232 such as aniline and 3,4-dichloroaniline (Table 1). In contrast, AtdA1 from an aniline-

233 degrading strain YAA as well as other reported glutamylating enzymes acting on

234 chloroaniline only exhibited activity against monocyclic aniline derivatives (Takeo et

235 al., 2013). It is worth noting that the in vitro activity of NpaA1 and AtdA1

236 heterologously expressed in *Escherichia coli* is significantly lower than their activity in

237 their wild-type hosts. This issue was encountered in previous reports on AtdA1 (Takeo

238 et al., 2013)(Ji et al., 2019), though the reasons for the discrepancy remain unexplained.

239 Here, the optimal pH of NpaA1 for enzymatic activity was 8.0 (Figure 3—figure

240 supplement 1A), and the optimal temperature was 50°C (Figure 3—figure supplement

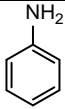
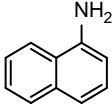
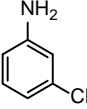
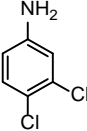
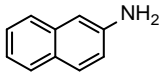
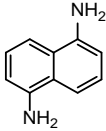
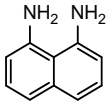
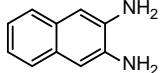
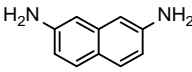
241 1B). The broad substrate specificity and high optimal temperature of NpaA1 indicate a

242 potential for its biotechnological applications by protein engineering.

243

244

245 **Table 1 Specific activities of NpaA1 and AtdA1 against different substrates**

Substrate	NpaA1 Relative activity	AtdA1 Relative activity
 (Aniline)	100%	100%
 (1-naphthylamine)	100%	0
 (3-chloroaniline)	76.5%	29.4%
 (3,4-dichloroaniline)	31.4%	0
 (2-naphthylamine)	23.5%	0
 (1,5-naphthalenediamine)	5.9%	0
 (1,8-naphthalenediamine)	0	0
 (2,3-naphthalenediamine)	7.8%	0
 (2,7-naphthalenediamine)	0	0

246 The specific activities of NpaA1 ($25.5 \pm 0.7 \text{ U g}^{-1}$) and AtdA1 ($8.5 \pm 2.1 \text{ U g}^{-1}$) against
 247 aniline were defined as 100%. The results are shown as averages \pm standard deviations
 248 from two or more independent measurements.

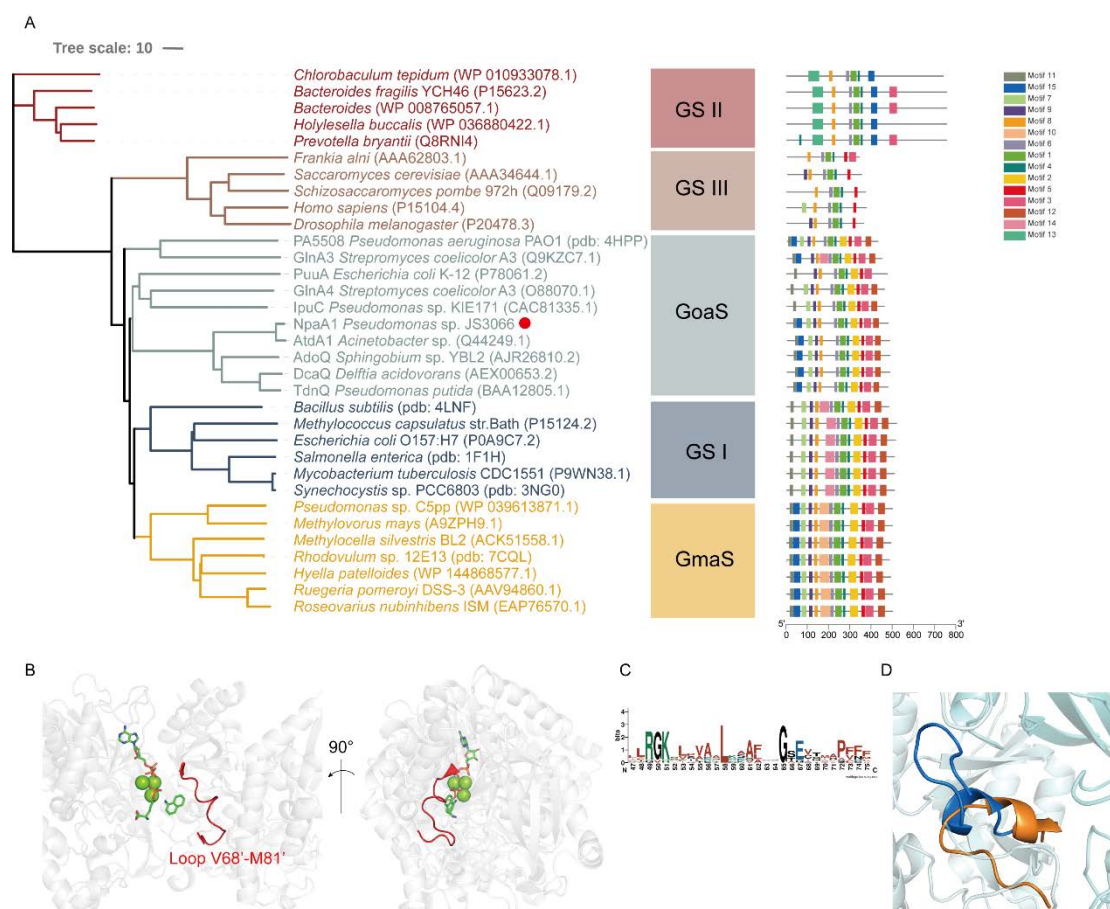
249 Table 1-Source data 1. Raw data for the specific activity of NpaA1 shown in Table 1.

250

251 **NpaA1 is a GoaS protein.** Glutamine synthetase (GS), catalyzing the ATP-dependent
252 synthesis of glutamine from glutamate and ammonium (Harper et al., 2010), is a
253 member of an ancient and ubiquitous family of enzymes. GS enzymes can be divided
254 into three distinct types, GSI, GSII, and GSIII (Brown et al., 1994; de Carvalho
255 Fernandes et al., 2022). Similar to the nomenclature of GS based on functional
256 characteristics, as various organic amine glutamine synthetases have been functionally
257 identified, such as NpaA1, γ -glutamylanilide synthase, and γ -glutamylpolyamine
258 synthetase (Gln3), we propose naming this enzyme class as glutamylorganicamide
259 synthetases (GoaS). To investigate the relationships between GoaS and GS proteins,
260 phylogenetic analysis of functionally identified GoaS and GS was conducted, revealing
261 that NpaA1 is part of a well-separated branch containing 9 other GoaS proteins (Figure
262 3A). Among these GoaS proteins, besides the well-known AtdA1 for aniline
263 degradation and newly identified NpaA1 for 1NA degradation, other enzymes are
264 involved in isopropylamine degradation (IpuA) (de Azevedo Wäsch et al., 2002),
265 chloroaniline degradation (DcaQ, TdnQ) (Król et al., 2012), and putrescine utilization
266 (PuuA) (Kurihara et al., 2008), etc. Although all members are involved in the
267 degradation of organic amine compounds, none of them were reported to catalyze
268 ammonium conversion (de Azevedo Wäsch et al., 2002; Ladner et al., 2012; Rexer et
269 al., 2006; Takeo et al., 2013).

270 GoaS enzymes including NpaA1 showed a closer relationship and more similar
271 motif arrangement to GSI than other types of GS (Figure 3A). There are 11 conserved
272 motifs between GSI and GoaS enzymes, but most members in GoaS have an additional

273 15th motif located in the N-terminal domain, which is absent in GSI (Figure 3A and
 274 Figure 3—figure supplement 2). In GS enzymes, the N-terminal domain contributes to
 275 the substrate binding of the enzyme (Almassy et al., 1986). The presence of the 15th
 276 motif in NpaA1 suggests a distinct substrate binding mode for GoaS compared to GSI.
 277 This may allow NpaA1 to catalyze glutamylation reactions with a broad range of
 278 substrates.

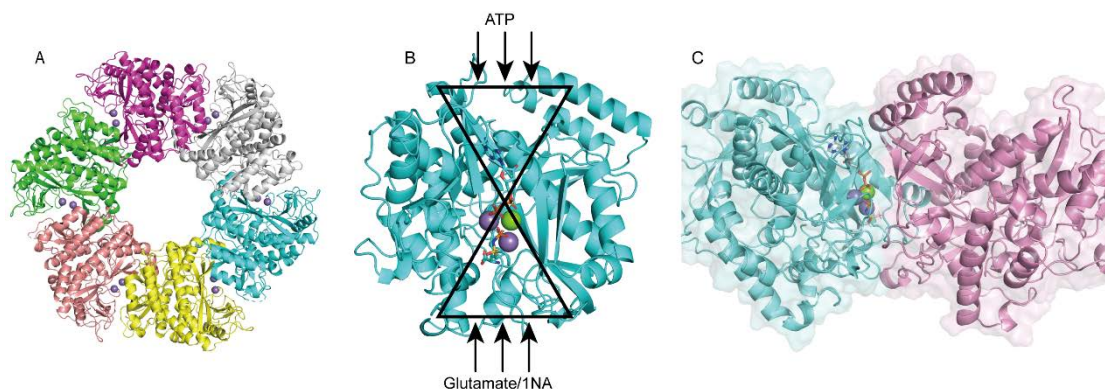


279
 280 **Figure 3** Phylogenetic analysis and motif analysis of GS-like enzymes. (A)
 281 Phylogenetic tree illustrating the evolutionary relationship between amino acid
 282 sequences of NpaA1 from *Pseudomonas* sp. strain JS3066 labelled with red dot and
 283 other related sequences. (B) The loop V68'-M81' (represented as a red cartoon) in
 284 NpaA1—ADP—MetSox-P docking with 1NA complex. Ligands of this complex are
 285 colored in green. (C) Sequence logo of the 15th motif. (D) The overlapping structure of
 286 loop V68'-M81' of NpaA1 (in blue) and corresponding region in StGS (in orange).
 287 GS: glutamine synthetase; GoaS: organic amine glutamine synthetases.
 288 GmaS: γ -Glutamylmethylamide synthetase.

289 **NpaA1 is a hexamer in solution.** The crystal structure of apo-NpaA1 and two
290 complexes with substrates or substrate analogues were obtained (Supplementary File
291 2). The model of NpaA1 built by Alphafold2 (Jumper et al., 2021) was used as template
292 for molecular replacement for apo-NpaA1. Theoretical calculations and SDS-PAGE
293 analysis showed that the molecular weight of the NpaA1 monomer was about 55 kDa.
294 The results of gel filtration show two absorption peaks for purified NpaA1, indicating
295 the presence of two distinct aggregation states in the solution. (Figure 4—figure
296 supplement 1A). Multi-angle light scattering (MALS) analysis showed that NpaA1
297 exists in both monomeric and hexameric states in solution, with hexamers constituting
298 approximately 36.7% of the total population (Figure 4—figure supplement 1C). It has
299 been reported that glutamine synthetase also often exists in different oligomeric states
300 in solution (Joo et al., 2018; Travis et al., 2022). The crystal structure of apo-NpaA1
301 revealed that there are six monomers arranged as a hexamer in an asymmetric unit
302 (Figure 4A). The binding pocket is located at the interface between adjacent subunits
303 (Figure 4A and 4C), and the bifunnel channel similar to GS protein refers to a structural
304 feature characterized by two distinct channels that converge into the common active
305 site. In apo NpaA1, there are two Mn^{2+} ions binding to the active center, coordinated
306 by glutamic acid and histidine residues (Figure 4A and Figure 4—figure supplement
307 2A). When the ATP analog AMPPNP binds to NpaA1, an additional Mg^{2+} binds to the
308 active site, facilitating the binding of the AMPPNP phosphate group (Figure 4B).

309

310



311

312 **Figure 4** Structure analysis of NpaA1. (A) The overall structure of NpaA1. There are
313 six monomers arranged as a hexamer in an asymmetric unit. The six monomers are in
314 different colors. (B) The GS active site is illustrated as a bifunnel with the ATP,
315 glutamate and amine entry and binding site at opposite ends. (C) Active site of NpaA1
316 located at the interface of adjacent subunits. The purple spheres represent the Mn²⁺ ions,
317 and the green spheres represent the Mg²⁺ ions. Ligands are indicated by green sticks.

318

319 **NpaA1 has a large and hydrophobic active pocket.** In order to elucidate the catalytic

320 characteristics of NpaA1 towards organic amines, we conducted a comparative analysis

321 of the differences in the substrate-binding pockets between NpaA1 and GSI family

322 proteins. NpaA1 shows a similar architecture to GSI protein from *Salmonella*

323 *typhimurium* (*StGS*) with a 1.2 Å root mean square deviation (RMSD)(Gill & Eisenberg,

324 2001), and γ -glutamylmethylamide synthetase from *Rhodovulum* sp. 12E13 (*RhGmaS*)

325 with a 1.0 Å RMSD for the aligned C α coordinates (Wang et al., 2021). The

326 coordinating residues involved in ATP, glutamate, and Mn²⁺ binding are highly

327 conserved between NpaA1 and GS (Figure 4—supplement figure 2A and 2B). The

328 structural comparison between NpaA1 and *StGS* indicates a distinction in their

329 ammonium binding sites. In contrast to the small negatively charged pocket for

330 ammonium binding in GSI proteins, docking analysis of NpaA1 with 1NA suggested

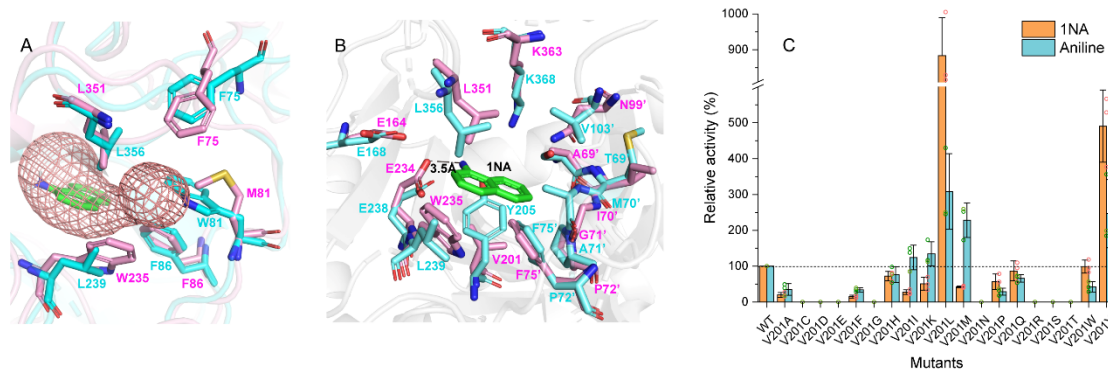
331 that 1NA binds at the interface of the adjacent monomer through electrostatic and

332 hydrophobic interactions (Figure 3B). The positively charged amino group of 1NA

333 binds in a negatively charged pocket consisting of residues E232 and E234, and forms
334 hydrogen bonds with residue E234 and the phosphate group of the intermediate γ -
335 glutamyl phosphate (Figure 5C). The corresponding entry for 1NA in NpaA1 is
336 surrounded by a long loop V68'-M81' which is located on part of the 15th motif in the
337 N-terminal domain of the adjacent monomer (Figure 3A-3C). The unique loop V68'-
338 M81' seems to form part of the active pocket leading to a larger and more hydrophobic
339 cavity (318.17 Å³) compared to that of the *StGS* protein (PDB ID:1F1H; 163.83 Å³)
340 (Figure 3D). The enriched hydrophobic residues on the 15th motif of loop V68'-M81'
341 (A69', I70', G71', P72', and F75') stabilize the aromatic ring of 1NA through
342 hydrophobic interaction. The volume of the large pocket in NpaA1 is sufficient to
343 accommodate 1NA, which also allows NpaA1 to accommodate a wider range of
344 substrates. The 15th motif is present in the majority of GoaS enzymes responsible for
345 catalyzing the glutamylation of organic amines. Similarly, GmaS, which possesses the
346 15th motif, also acts on organic amines as substrates (Figure 3A). The 15th motif is
347 situated in the vicinity of the active pocket in GmaS(Wang et al., 2021) and GoaS,
348 suggesting that this motif may play a role in facilitating the binding of organic
349 compounds in these GS-like enzymes.

350

351



352

353 **Figure 5** Residues involved in 1NA binding. (A.) The 1NA binding tunnel was
354 predicted by Caver 3.0 (in green). The structures of NpaA1 (in pink) and AtdA1 (in
355 cyan) overlap, with the amino acids contributing to the formation of tunnel bottlenecks
356 highlighted as sticks. (B) The aromatic amine binding pocket of NpaA1 superimposed
357 on AtdA1. 1NA is green, residues for NpaA1 are pink, and residues for AtdA1 are cyan.
358 The black dashed line represents the hydrogen bond. (C) Relative activity of saturation
359 mutants for V201. The activity of wild-type NpaA1 for 1NA is set to 100% (20.8 ± 0.5
360 U/g), and wild-type NpaA1 showed equivalent activity to 1NA and aniline. Error bars
361 represent the standard deviation of three biological replicates.

362 Figure 5-Source data1. Raw data for relative activity of mutants shown in Figure 5.

363

364 **The difference in the organic amine binding tunnels between NpaA1 and AtdA1.**

365 Despite the high identity (90%) between NpaA1 and AtdA1, only NpaA1 is capable of
366 converting both monocyclic and polycyclic aromatic amines. To investigate how the
367 differences in amino acids would affect substrate preferences and conversion rates, a
368 predicted structure of AtdA1 was obtained from the AlphaFold2 protein structure
369 database (Jumper et al., 2021). Structure overlapping of NpaA1 and AtdA1 suggested
370 that the binding pockets in these two proteins are similar, while the variations in tunnel
371 and binding pocket residues between NpaA1 and AtdA1 likely account for their
372 different substrate specificities. We employed CAVER 3.0 software(Chovancova et al.,
373 2012) to analyze potential tunnels for the entry of 1-naphthylamine or aniline into
374 NpaA1 and AtdA1. The prediction of the tunnel in NpaA1 is based on structural
375 docking after the removal of the ligand MetSox-P from the NpaA1—ADP—MetSox-P

376 complex and subsequent docking of phosphorylated glutamate. 1NA docked in NpaA1
377 was selected as a mandatory site. The tunnel analysis in NpaA1 identified eight tunnels.
378 Among the generated tunnels, a tunnel extending from the buried 1NA binding region
379 to the surface of the bifunnel-shaped entry appears to be the most likely candidate for
380 facilitating 1NA diffusion and overall catalysis in NpaA1 (Figure 5A). One of the
381 predicted tunnels in AtdA1 overlaps with this tunnel in NpaA1, therefore likely to be
382 the amine binding channel.

383 Subsequently, we explored tunnel bottlenecks to pinpoint crucial residues
384 influencing substrate specificity, because restricted sections within a tunnel typically
385 play a significant role in regulating ligand mobility (Kaushik et al., 2018). Tunnel radius
386 analysis in NpaA1 revealed a distinct bottleneck region formed by several hydrophobic
387 amino acids (L351, M81', F86', F75', W235) (Figure 5A). These aromatic amino acids,
388 found within the tunnel, likely participate in π - π interactions with substrates or cation-
389 π interactions with the positively charged amino groups of aromatic amines in NpaA1.
390 Their cooperative action should facilitate substrate passage through the channel and
391 subsequent binding to the active center. The amino acid differences in the substrate-
392 binding tunnels may be a key factor contributing to the broader substrate specificity of
393 NpaA1 compared to AtdA1. The amino acids forming the bottleneck of the aromatic
394 amine binding channel in AtdA1 closely resemble their counterparts in NpaA1.
395 However, in NpaA1, M81 and W235 correspond to W81 and L239 in AtdA1. In NpaA1,
396 the indole ring of W235 is oriented parallel to the channel and likely engages in π - π
397 interactions or cation- π interactions with substrates or surrounding aromatic amino

398 acids, assisting in substrate entry. The indole ring of W81 in AtdA1 may constrain the
399 radius of its channel, whereas M81 in NpaA1 offers more space.

400 **V201 is a key site influencing the selectivity of NpaA1 substrates.** The substrate
401 binding tunnel terminates at the active center. Docking analysis of NpaA1 and AtdA1
402 revealed V201 implicated in 1NA binding, in the NpaA1 pocket, leading to a larger
403 naphthylamine-binding volume. In AtdA1, Y205 (conserved in GS proteins) with a
404 larger tryptophan side-chain group may increase steric hindrance for naphthylamine's
405 diphenyl ring (Murray et al., 2013; Wang et al., 2021). Surprisingly, the V201Y mutant
406 in NpaA1 exhibited higher catalytic activity for both 1NA and aniline compared to the
407 wild-type (Figure 5B). Single-site saturation mutagenesis was performed to investigate
408 the effect of V201 for 1NA binding in NpaA1 (Figure 5C). In contrast to V201Y,
409 V201F altered NpaA1's substrate preference, indicating the contribution of hydroxyl
410 groups on tryptophan to enhanced substrate binding. The substitution of Val201 with
411 Leu enhanced activity toward 1NA by 8.8-fold, indicating a more accommodating
412 pocket for naphthylamines. Mutating V201 to hydrophobic amino acids with longer
413 side chains increased the likelihood of catalyzing monocyclic aniline conversion. In
414 summary, V201 is pivotal in determining substrate preference in NpaA1.

415 **Discussion**

416 In contrast to well-studied microbial degradation of monocyclic aromatic amines
417 (Arora, 2015), the mechanisms for microbial degradation of polycyclic aromatic amines
418 have remained unknown until now. *Pseudomonas* sp. strain JS3066 is the first reported
419 isolate with the ability to mineralize 1NA. The fact that enrichment from soil at a former

420 naphthylamine manufacturing site yielded an isolate capable of robust growth
421 (doubling time approximately 6 h) on 1NA suggests strongly that it plays a role in
422 naphthylamine degradation *in situ*.

423 The mechanism involved in degradation of 1NA by strain JS3066 was elucidated
424 both at genetic and biochemical levels. The proposed pathway (Fig. 2D) involves
425 conversion of 1NA to 1,2-dihydroxynaphthalene catalyzed by homologs of the aniline
426 dioxygenase enzyme system. The involvement of *npaA1-npaA5* is supported strongly
427 by both bioinformatics and experimental demonstration of the ability to catalyze the
428 key reactions. Subsequent metabolism to salicylate is catalyzed by enzymes closely
429 related to those of the *nag* pathway for naphthalene degradation (Fuenmayor et al., 1998;
430 Zhou et al., 2001).

431 The proposed pathway for degradation of dihydroxynaphthalene involves well-
432 established enzymes and reactions, and is supported by the close association of the
433 genes encoding the lower pathway for naphthalene degradation with those encoding the
434 initial oxidation of naphthylamine. The evidence for the intermediate steps is supported
435 by bioinformatics and analogy with established systems, but rigorous determination of
436 the details will require additional experimental confirmation.

437 The strong activity of the naphthylamine dioxygenase enzyme system toward
438 aniline as well as the high sequence similarity indicates recent divergence from a
439 common ancestor. The fact that the isolate retains the ability to grow on aniline indicates
440 that the initial enzyme system plays a dual role in degradation of both aniline and
441 naphthylamine.

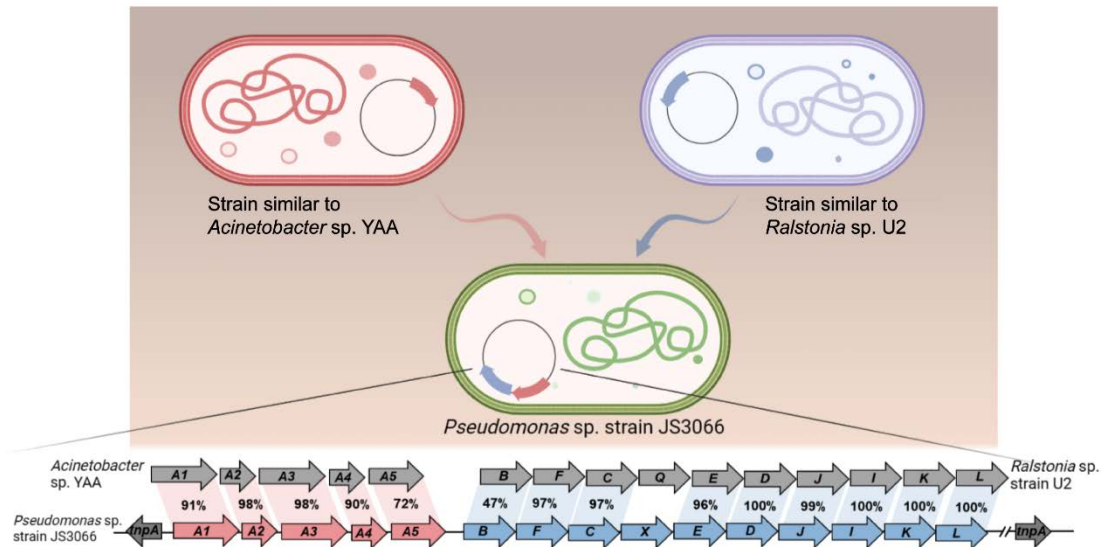


Figure 6 Likely sequence of HGT for recruitment of genes involved in 1NA degradation pathway in strain JS3066.

445

446 Horizontal gene transfer (HGT) has played an important role in the dispersal of

447 pathogenicity-related genes, antibiotic resistance genes, and biodegradative genes.

448 Bacteria respond to selective pressure exerted by anthropogenic chemicals by HGT of

449 genetic determinants that enable the recipient to utilize the compound (van der Meer et

450 al., 1992). For example, naphthalene catabolic genes (i.e., naphthalene dioxygenase)

451 (Herrick et al., 1997), atrazine catabolism genes *atzABC* (de Souza et al., 1998), and

452 2,4-dichlorophenoxyacetic acid degradative genes *tfdA* (McGowan et al., 1998) have

453 been widely disseminated in soil bacteria via HGT. Whether or not hosts acquired

454 specific genes by HGT can be predicted by comparison of the differences in G+C

455 content, codon usage, phylogenies between the candidate genes and the whole genomes,

456 as well as analyzing the presence or absence of mobile genetic elements adjacent to

457 defined genes (Garcia-Vallvé et al., 2000; Syvanen, 1994). The G+C content of the

458 *npaA1A2A3A4A5* gene cluster is 42.60% which is significantly lower than those of the

459 complete genome (62.95%), *npaG* gene (67.90%) and *npaBFCXEDJIKL* genes

460 (58.82%). The *npaA1A2A3A4A5* genes and the *npaBFCXEDJIKL* genes are flanked by
461 transposase-encoding genes. Based on these observations, it is likely that the genes
462 encoding enzymes that make up the 1NA degradation pathway have originated from
463 those involved in biodegradation of aniline and of naphthalene, a process probably
464 mediated by HGT.

465 During this process, the genes (i.e., *nagAaGHAbAcAd* in strain U2 and similar
466 strains) encoding transformation of naphthalene into *cis*-naphthalene dihydrodiol and
467 conversion of salicylate to gentisate within the naphthalene degradation pathway were
468 completely replaced with the *npaA1A2A3A4A5* genes likely derived from aerobic
469 aniline degraders (e.g., *Acinetobacter* sp. YAA and similar strains) (Figure 6). It is
470 worthwhile to note that the *npaB* gene, equivalent to *nagB* in strain U2, which encodes
471 *cis*-naphthalene dihydrodiol dehydrogenase and is located immediately downstream of
472 the *nagAaGHAbAcAd* genes, is truncated in its 5' end probably due to substitution of
473 the *nagAaGHAbAcAd* genes with the *npaA1A2A3A4A5* genes, therefore serving as a
474 tracer for assembly of 1NA degradation pathway. More importantly, the above-
475 mentioned process led to the loss of the *nagGH* genes encoding the oxygenase
476 component of salicylic acid 5-hydroxylase (S5H), thereby abolishing the ability of
477 strain JS3066 to transform salicylate via gentisate even if genes (*npaJIKL*) involved in
478 conversion of gentisate to intermediates for TCA cycle remained intact in strain JS3066.
479 To complete the pathway of 1NA degradation, strain JS3066 employs the *npaG* gene
480 encoding salicylic acid 1-hydroxylase (S1H) to transform salicylate into catechol prior
481 to ring fission. The evidence supports the hypothesis that the evolution of the 1NA

482 degradation pathway has occurred in a modular fashion, where assembly of catabolic
483 modules originating from different sources proceeded by transposition and subsequent
484 rearrangement. Ancestral strains similar to *Acinetobacter* YAA and *Ralstonia* U2 were
485 likely progenitors of JS3066, sharing common genetic traits and providing insights into
486 its evolutionary lineage and adaptation.

487 The glutamylation pathway plays a crucial role in the degradation of various amine
488 compounds (de Azevedo Wäsch et al., 2002; Krysenko et al., 2019; Kurihara et al.,
489 2005; Kurihara et al., 2008; Takeo et al., 2013). When the genes encoding the aniline
490 dioxygenase system were first discovered, one *orf* of unknown function was annotated
491 as “GS-like protein” (Rexer et al., 2006)(de Carvalho Fernandes et al., 2022).
492 Subsequently its function was established and it was renamed “N-glutamylanilide
493 synthase (EC6.3.1.18) (Takeo et al., 2013). Several other genes in various bacteria were
494 similarly initially annotated as GS-like proteins and subsequently renamed as their
495 functions were established (Krysenko et al., 2017). GoaS was considered a branch of
496 Glutamine Synthase I enzymes in the phylogeny described by deCarvalho(de Carvalho
497 Fernandes et al., 2022). However, combining the phylogenetic, function and structure
498 analysis in this research, the group seems sufficiently distinct from the glutamine
499 synthase enzymes that we propose designating them “glutamyl-organic amine
500 synthases” (GoaS).

501 Previous studies indicated that the GoaS protein lost the ability to catalyze the
502 formation of glutamine from ammonia (de Azevedo Wäsch et al., 2002; Ladner et al.,
503 2012; Rexer et al., 2006; Takeo et al., 2013). PA5508 catalyzes the glutamylation of

504 various aromatic amine substrates (e.g., spermidine, putrescine) (Ladner et al., 2012)
505 and is the sole enzyme in GoaS with a resolved structure. Despite structural
506 comparisons with GS, the catalytic mechanisms and binding properties of aromatic
507 amine compounds in GoaS have not been established. In this study, we investigated the
508 aromatic amine binding site in NpaA1 from both structural and biochemical
509 perspectives. Despite similarities in ATP, glutamate, and manganese ion binding sites
510 between NpaA1 and GSI, the amine binding pockets exhibit marked differences.
511 NpaA1 evolved a large and hydrophobic substrate binding pocket to accommodate the
512 catalysis of bulky organic amine compounds. To accommodate organic amines, both
513 the substrate binding pocket and tunnel of NpaA1 are rich in hydrophobic amino acids.
514 Besides hydrogen bonding interactions similar to GS proteins, hydrophobic interactions
515 play a crucial role in facilitating substrate binding in NpaA1. GoaS lost the conserved
516 catalytic residues found in GS during evolution, including the residues Glu357 involved
517 in the Glu flap and the catalytic Asp 50' in *StGS* (Figure 4—supplement figure 2) (Gill
518 & Eisenberg, 2001). The conserved catalytic residues in GS are replaced by
519 hydrophobic residues or loops in NpaA1. These differences could be significant reasons
520 why GoaS cannot catalyze ammonium glutamylation, indicating a fundamentally
521 different catalytic mechanism between NpaA1 and GSI.

522 Structure analysis suggests that amino acid differences in both the substrate
523 binding tunnel and the substrate binding pocket may be responsible for NpaA1's
524 broader substrate specificity compared to that of AtdA1. However, even with saturation
525 mutagenesis in the key amino acid V201 in the substrate binding pocket, we were

526 unable to identify determinants for expanding substrate specificity from the analysis of
527 this critical site. Differences in amino acids within the substrate binding tunnel may
528 also play a pivotal role. The collaborative action of multiple aromatic residues within
529 the substrate binding tunnel allows naphthylamine and its derivatives to enter smoothly.
530 To comprehend the mechanism by which different amino acids in NpaA1 influence
531 substrate selectivity, further experiments and computational studies are necessary. In
532 summary, the results of mutation analysis have revealed that several V201 mutants can
533 enhance the activity of NpaA1, indicating a high degree of plasticity in the large
534 substrate binding pocket of NpaA1, which is promising for future engineering
535 endeavors on NpaA1.

536 The discovery of *Pseudomonas* sp. strain JS3066 and its ability to degrade 1-
537 naphthylamine sheds light on the environmental fate of this toxic compound and
538 provides a potential bioremediation strategy. The biochemical and structural
539 characterization of the typical GoaS, NpaA1, for initial reaction of 1NA biodegradation
540 further expands our understanding of the glutamylation pathway and broadens the scope
541 of its application in the degradation of other aromatic amines.

542 **Materials and Methods**

543 **Isolation and growth of 1NA degrading bacteria**

544 Isolates were obtained by selective enrichment under oxic conditions in nitrogen-
545 free minimal medium (BLK) (Bruhn et al., 1987) supplemented with 1-naphthylamine
546 (0.1 mM). Subsurface samples used to inoculate the enrichments were collected from
547 the capillary fringe at a former chemical manufacturing site in New Jersey, USA. When

548 the 1-naphthylamine disappeared from the enrichments as determined by high-
549 performance liquid chromatography (HPLC) the cultures were transferred to fresh
550 medium. After the process was repeated 3 times isolates were obtained by spreading on
551 agar plates containing BLK supplemented with 1-naphthylamine in the headspace and
552 individual colonies were tested for the ability to grow on 1-naphthylamine in liquid
553 medium.

554 **Bacterial strains, plasmids, primers, chemicals, media, and culture conditions**

555 The bacterial strains and plasmids used in this study are described in
556 Supplementary File 3, and the primers used are described in Supplementary File 4.
557 *Pseudomonas* sp. strain JS3066 was grown at 30°C in minimal medium (MM) with 0.3
558 mM 1NA as the sole carbon and nitrogen source. Aniline, 3,4-dichloroaniline, 2-
559 naphthylamine, 1,5-naphthalenediamine, 1,8-naphthalenediamine, salicylate, and
560 catechol were purchased from Aladdin Bio-Chem Technology Co., Ltd. (Shanghai,
561 China). 1-Naphthylamine and 1,2-dihydroxynaphthalene were supplied by Sigma-
562 Aldrich Co., Ltd. (Shanghai, China). 3-Chloroaniline was purchased from Damas-beta
563 Co., Ltd (Shanghai, China). 2,3-Naphthalenediamine was purchased from Meryer
564 Chemical Technology Co., Ltd. (Shanghai, China). 2,7-Naphthalenediamine was
565 purchased from Bide Pharmaceutical Technology Co., Ltd. (Shanghai, China). γ -
566 Glutamylated 1NA was supplied by J&K Scientific Chemical Co., Ltd. (Shanghai,
567 China). *Escherichia coli* strains were grown in lysogeny broth (LB) at 37°C, whereas
568 *P. putida* KT2440- $\Delta catA\Delta ggt$ was cultivated in LB at 30°C. Kanamycin (50 μ g/ml)
569 was added to the medium as needed.

570 **Genome sequencing of strain JS3066 and bioinformatics**

571 DNA of strain JS3066 was extracted with a Wizard Genomic Purification Kit
572 (Promega, USA). The genome sequencing and assembly was done by the BGI Medical
573 Examination Co., Ltd. (Wuhan, China) with the PacBio RSII platform. The complete
574 genome was annotated by Rapid Annotations using Subsystems Technology (RAST)
575 server (Overbeek et al., 2014). The sequence of the genomic DNA is available under
576 accession number (SUB13951314). BLASTp program was employed to deduce the
577 amino acid identities of potential 1NA degradative genes.

578 To analyze phylogenetic relationships, sequences were first aligned by Clustal X
579 version 2.1; subsequently, the phylogenetic tree was generated by the neighbor-joining
580 method using MEGA 11 (Tamura et al., 2021). The evolutionary distances between
581 branches were calculated using the Kimura two-parameter distance model, with
582 bootstrap analysis of 1,000 resamplings to evaluate the tree topology.

583 **General DNA techniques**

584 Routine isolation of genomic DNA, extraction of plasmids, restriction digestion,
585 transformations, PCR, and electrophoresis were carried out by following standard
586 procedures. The sequencing of PCR products and plasmids were performed by Tsingke
587 Biotech Co., Ltd. (Shanghai, China).

588 **Construction of recombinant plasmids and heterologous expression**

589 Genes from strain JS3066 were amplified using the corresponding primers
590 Supplementary File 4, the resultant amplified DNA fragments were cloned into digested
591 plasmids using a ClonExpress MultiS One Step Cloning kit (Vazyme Biotech Co., Ltd.,

592 Nanjing, China).

593 The vector pBBR1MCS-2 was employed for heterologous expression of suspected
594 functional genes in *P. putida* KT2440- Δ catA Δ ggt. A 5.1-kb DNA fragment containing
595 the entire set of *npaA1A2A3A4A5* genes was amplified and then fused with the
596 HindIII/XhoI-digested vector pBBR1MCS-2 to generate plasmid pNPA01. Afterwards,
597 pNPA01 derivatives lacking either *npaA1*, *npaA2*, or *npaA1* and *npaA2* (*npaA1A2*) were
598 constructed in the same way. Finally, the resulting recombinant plasmids harboring
599 different gene combinations (namely *npaA1A2A3A4A5*, *npaA1A3A4A5*, *npaA2A3A4A5*,
600 and *npaA3A4A5*) were introduced into *P. putida* KT2440- Δ catA Δ ggt by
601 electroporation to yield the recombinants KT/pNPA01, KT/pNPA01- Δ A1,
602 KT/pNPA01- Δ A2, and KT/pNPA01- Δ A12, respectively.

603 For overexpression of the *npaA1* gene, it was amplified from genomic DNA of
604 strain JS3066 by PCR and then cloned into pET-29a to obtain the expression construct
605 pET-*npaA1* which was transformed into *E. coli* BL21 (DE3). The expression and
606 purification of NpaA1, an N-terminal Strep II-tagged fusion protein, were performed
607 according to procedures described previously (Ji et al., 2019). The eluted proteins were
608 further fractionated by gel filtration on a Superdex 200 Increase 10/300 GL column
609 (Cytiva) with the buffer containing 50 mM Tris-HCl and 200 mM NaCl. The purity of
610 Strep II-tagged NpaA1 was analyzed by 12.5% SDS-PAGE. Protein concentration was
611 determined by using the Bradford method. The expression and purification of NpaG
612 and AtdA1, also N-terminal Strep II-tagged fusion proteins, were performed in the same
613 way as for NpaA1. The primers used for constructing mutant vectors are listed in

614 Supplementary File 5, and the mutants of NpaA1 were overexpressed and purified by
615 the same methods as above.

616 **SEC-Multi angle light scattering (SEC-MALS)**

617 SEC-MALS was used to determine the molecular weight of NpaA1. Purified
618 NpaA1, separated by gel filtration, was diluted to a final concentration of 2 mg/ml and
619 dissolved in a 50 mM Tris buffer for sample loading.

620 **Crystallization and data collection**

621 Purified NpaA1 protein was concentrated to 12 mg/ml in the buffer containing 30
622 mM Tris-HCl (pH 8.0) and 120 mM NaCl. Crystals were obtained at 20 °C in 1-2 weeks
623 by sitting-drop vapor diffusion. Apo-NpaA1 was obtained in the buffer containing 0.1
624 M magnesium chloride hexahydrate, 0.1 M sodium acetate trihydrate, 0.1 M Bis-Tris
625 6.5 and 15 % v/v PEG smear broad (the ratio of protein to reservoir solution was 1:2).
626 To obtain crystals of NpaA1–ADP–MetSox-P complex, ATP and MetSox were added
627 to NpaA1 to a final concentration of 2 mM, and the protein solution was mixed 1:2 with
628 the buffer containing 0.1 M magnesium chloride hexahydrate, 0.1 M rubidium chloride,
629 0.1 M PIPES 7.0 and 20 % v/v PEG smear low. The NpaA1–ADP was obtained under
630 the same conditions as the NpaA1–ADP–MetSox-P complex above.

631 All the X-ray diffraction data were collected on BL19U1 beamline at the Shanghai
632 Synchrotron Radiation Facility. The initial data were processed by the HKL3000
633 program.

634 **Structure determination and refinement**

635 The crystal structure of apo-NpaA1 was determined by molecular replacement

636 using the model of NpaA1 built by AlphaFold2 (Jumper et al., 2021). The structure of
637 apo-NpaA1 was used as the model for the other structures. The refinements of these
638 structures were performed using Coot (Emsley et al., 2010) and Phenix (Liebschner et
639 al., 2019).

640 **Similarity searches and sequence comparison**

641 Amino acids of NpaA1 were used to search for similar sequences in the Swissprot
642 database. Sequences with similarity above 27% were selected for multiple sequence
643 alignment. Multiple sequence alignment and phylogenetic tree construction were
644 performed using MEGA 11(Tamura et al., 2021).

645 **Biotransformation of 1NA in cell suspensions of *P. putida* KT2440- Δ catA Δ ggt** 646 **harboring various recombinant plasmids**

647 Recombinants KT/pNPA01, KT/pNPA01- Δ A1, KT/pNPA01- Δ A2, and
648 KT/pNPA01- Δ A12 were individually grown in 250-ml Erlenmeyer flasks with 100 ml
649 of LB medium containing kanamycin (50 μ g/ml) at 30°C and 180 rpm, harvested by
650 centrifugation (4°C, 6,000 rpm, 5 min), washed twice with Tris-HCl buffer (50 mM,
651 pH 8.0) and finally resuspended in the same buffer. The optical density at 600 nm
652 (OD₆₀₀) of cell suspensions was adjusted to approximately 8.0. The substrate 1NA (final
653 concentration of 0.4 mM) was added to the suspension, and degradation experiments
654 were performed at 30°C and 170 rpm on a rotary shaker. Samples were collected at
655 regular intervals, and the change in concentrations of 1NA was analyzed by HPLC.

656 **Enzyme assays**

657 Activities of NpaA1 and AtdA1 against different substrates were analyzed

658 spectrophotometrically with a Lambda 25 spectrophotometer (PerkinElmer/Cetus,
659 Norwalk, CT) by following the disappearance of tested substrates at individual defined
660 wavelengths. The reaction system (0.5-ml volume) contained 1.5 mM ATP, 1.0 mM *L*-
661 glutamate, 2.0 mM MgCl₂, and 0.28 mg of NpaA1 in 50 mM Tris-HCl buffer (pH 8.0).
662 The assay was initiated by adding different substrates. The molar extinction coefficients
663 of various potential substrates were obtained by measuring their absorbance values in
664 reaction buffer at each characteristic wavelength. As for NpaA1 and AtdA1, one unit of
665 enzyme activity (U) is defined as the amount of enzyme required for the consumption
666 of 1 μmol of substrate in 1 min at 30°C. Specific activities for NpaA1 and AtdA1 are
667 expressed as units per gram of protein. The activity of NpaG against salicylate was
668 measured by monitoring NADH oxidation at 340 nm and the molar extinction
669 coefficient for NADH was taken as 6,220 M⁻¹·cm⁻¹. The reaction system (0.5-ml
670 volume) contained 200 μM NADH, 40 μM FAD, 80 μM salicylate, and 0.38 μg of
671 NpaG in 50 mM potassium phosphate buffer (pH 7.2). For NpaG, one unit of enzyme
672 (U) is defined as the amount of enzyme required for the consumption of 1 μmol of
673 NADH in 1 min at 30°C. Specific activity for NpaG against salicylate is expressed as
674 units per milligram of protein.

675 **Analytical methods**

676 To isolate and identify the metabolites, the biotransformation sample was acidified
677 to pH 2 with concentrated HCl and then extracted with an equal volume of ethyl acetate
678 which was subsequently removed by evaporation. Bis (trimethylsilyl)
679 trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) (volume ratio: 99/1)

680 was used as the derivatization reagent so that active hydrogen atom(s) of the 1NA
681 metabolites were replaced by a trimethylsilyl (TMS) group ($\text{Si}(\text{CH}_3)_3$, m/z 73). The
682 pellet was dissolved in BSTFA-TMCS and then incubated at 60°C for 30 min prior to
683 gas chromatography-mass spectrometry (GC-MS) analysis. GC-MS analyses were
684 performed on a TRACE 1310 gas chromatograph (Thermo Fisher Scientific, MA, USA)
685 using a capillary column HP-5MS (0.25 mm \times 30 m, Agilent Technologies, CA, USA).
686 The column temperature gradient was 0-5 min, 60°C; 5-27 min, 60-280°C (10°C min⁻¹);
687 27-32 min, 280°C. The detector was a TSQ8000 Triple Quadrupole MS (Thermo Fisher
688 Scientific, USA). The following conditions were applied for mass analysis: ionization
689 mode, EI⁺; ionizing electron energy, 70 eV; source temperature, 250°C and mass range
690 m/z 0-500. Mass spectra of individual total ion peaks were identified by comparison
691 with authentic standards and the *Wiley.275L* mass spectra data base.

692 HPLC analysis of substrates and their oxidation products was performed on a
693 Waters e2695 Separation Module equipped with a C18 reversed-phase column (5 μm ,
694 4.6 \times 250 mm). The mobile phase consisted of water containing 0.1% (vol/vol) acetic
695 acid (A) and methanol (B) at a flow rate of 1.0 ml min⁻¹. A gradient elution program
696 was as follows: 0-15 min 20%-80% B; 15-25 min 80% B; 25-25.1 min 80%-20% B;
697 25.1-40 min 20% B. The column temperature was 30°C and the injection volume was
698 20 μl . Qualitative analysis of the oxidation product from the reaction of 1NA
699 degradation catalyzed by NpaA1 was executed with ultra-performance liquid
700 chromatography-quadrupole-time of flight-mass spectrometry (UPLC-QTOF MS),
701 with the electrospray ionization (ESI) source in positive ion mode.

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707

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711

712 **Author contributions**

713 Shu-Ting Zhang, Investigation, Validation, Methodology, Software, Writing –
714 original draft, Writing – review and editing; Shi-Kai Deng, Investigation, Validation,
715 Methodology, Writing – original draft; Tao Li, Conceptualization, Methodology,
716 Writing – review and editing; Megan E. Maloney, Investigation, Validation,
717 Methodology; De-Feng Li, Validation, Methodology, Software; Jim C. Spain, Funding
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719 Zhou, Funding acquisition, Investigation, Conceptualization, Writing – review and
720 editing.

721 **Data Availability**

722 The complete genome of *Pseudomonas* sp. strain JS3066 is available in the NCBI

723 database under BioProject identifier (ID) PRJNA1035437 or BioSample accession
724 SUB13951314. The structures of apo-NpaA1, NpaA1-AMPPNP, NpaA1-ADP-
725 MetSox-P, have been deposited in PDB (www.rcsb.org/) under accession codes 8X6Z,
726 8WWU, 8WWV, respectively. These PDB entries will be hold for publication. All data
727 are contained within the manuscript and the supporting information.

728

729

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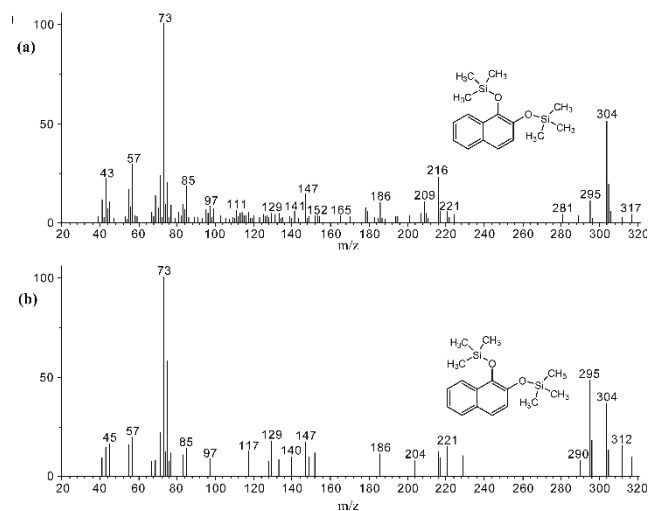
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**Discovery of the 1-naphthylamine biodegradation pathway reveals an
enzyme that catalyzes 1-naphthylamine glutamylation**

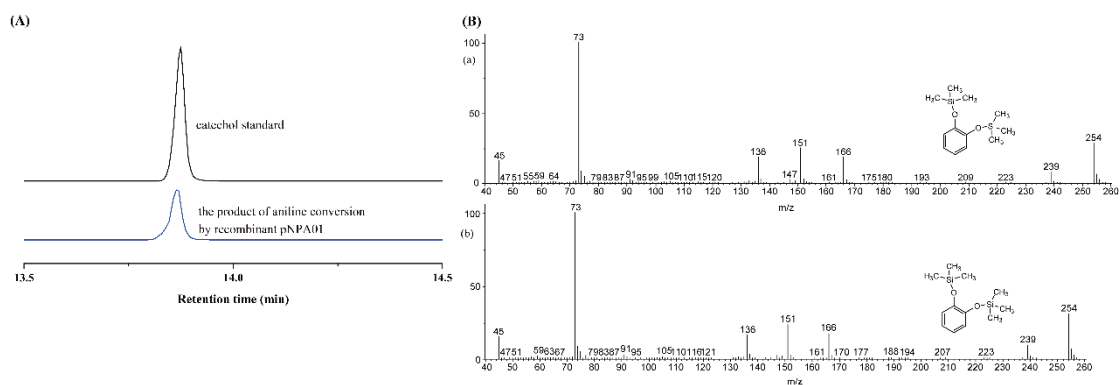
Zhang *et al.*

1 SUPPLEMENTARY FIGURES



2

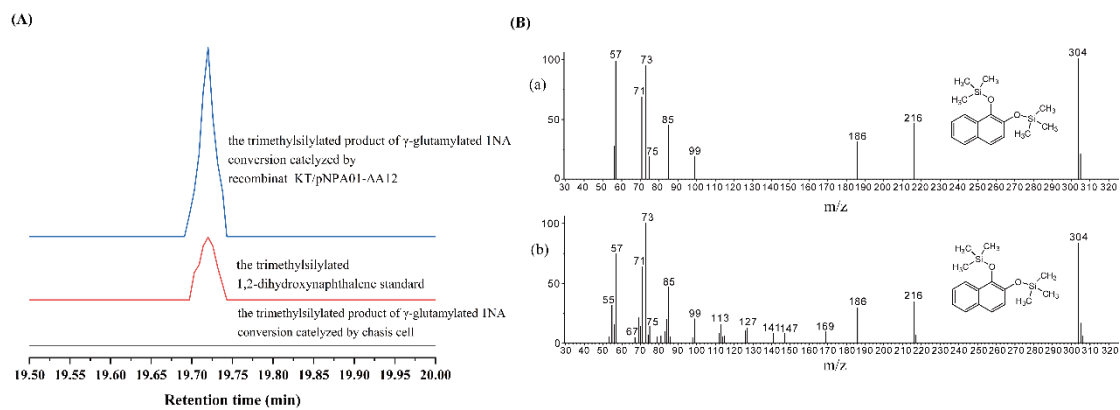
3 Figure 1—figure supplement 1. GC-MS analysis of the intermediate accumulated during 1NA
4 transformation by the cell suspension of *P. putida* KT2440- Δ catA Δ ggt harboring pNPA01. The
5 mass spectra of theoretical trimethylsilylated 1,2-dihydroxynaphthalene (a) and authentic
6 trimethylsilylated 1,2-dihydroxynaphthalene (b) are shown.



7

8

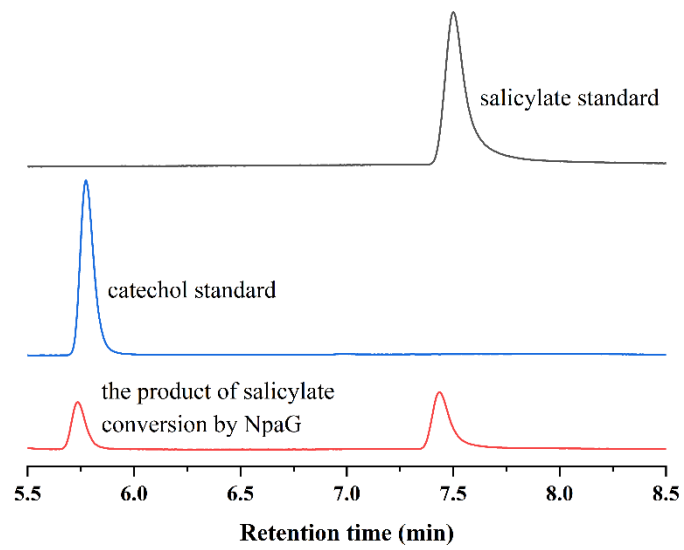
9 Figure 1—figure supplement 2. Conversion of aniline by strain *P. putida* KT2440-
10 $\Delta catA\Delta ggt$ harboring plasmid pNPA01 (recombinant KT/pNPA01). (A) The ion current
11 chromatograms at $m/z\ 254.00\pm 0.70$ extracted from the total ion current chromatograms
12 of standard catechol and the product of aniline conversion by recombinant KT/pNPA01.
13 (B) GC-MS analysis of the intermediate captured during aniline degradation by the cell
14 suspension of KT/pNPA01. The mass spectra of proposed trimethylsilylated catechol
15 (A) and authentic trimethylsilylated catechol (B) are shown.



16

17 Figure 1—figure supplement 3. Conversion of γ -glutamylated 1NA by recombinant
18 strain KT/pNPA01- $\Delta A12$. (A) The ion current chromatograms at m/z 304.00 ± 0.70
19 extracted from the total ion current chromatograms of standard 1,2-
20 dihydroxynaphthalene and the product of γ -glutamylated 1NA conversion by
21 recombinant KT/pNPA01- $\Delta A12$ are presented. (B) GC-MS analysis of the intermediate
22 captured during γ -glutamylated 1NA transformation by the cell suspension of
23 KT/pNPA01- $\Delta A12$. The mass spectra of theoretical 1,2-dihydroxynaphthalene (a) and
24 authentic 1,2-dihydroxynaphthalene (b) are shown.

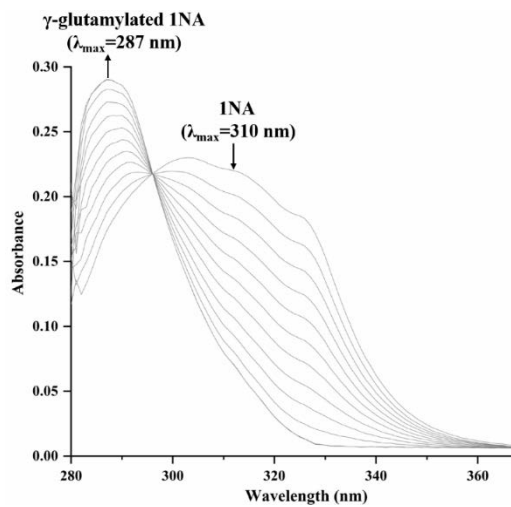
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26

27 Figure 1—figure supplement 4. HPLC chromatographs of salicylate standard, catechol
28 standard, and the reaction product of salicylate conversion catalyzed by the recombinant
29 protein NpaG.

30

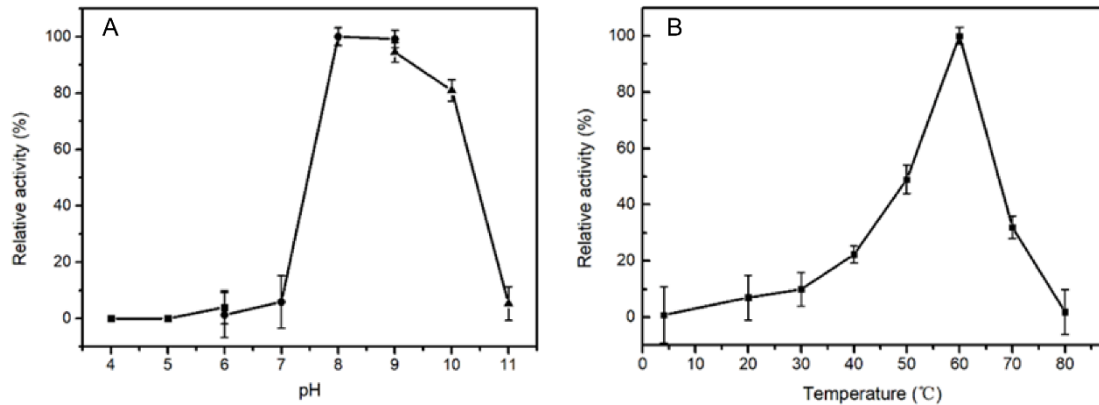


31

32 Figure 2—figure supplement 1. Spectral changes during the transformation of 1NA by
33 purified N-terminal Strep II-tagged NpaA1. The maximum characteristic absorption
34 peaks of 1NA and γ -glutamylated 1NA are 310 nm and 287 nm. The arrows indicate
35 the directions of spectral changes.

36 Figure 2-Source data 1. Raw data for the spectral changes shown in Figure 2—figure
37 supplement 1.

38



39

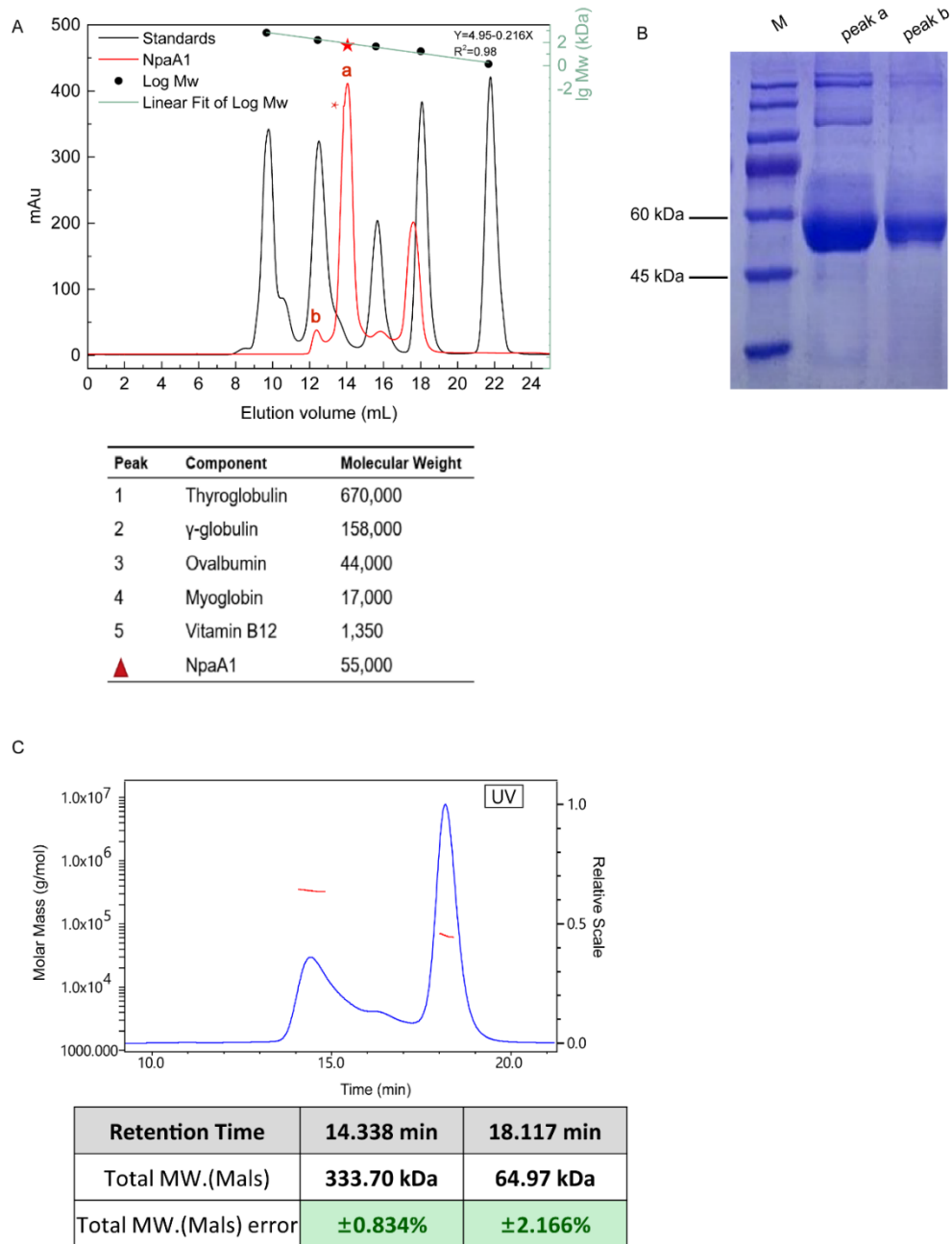
40 Figure 3—figure supplement 1. The optimal pH (A) and temperature (B) of wild type
41 NpaA1 activity. The optimal pH was determined at 25°C using Bis -Tris buffer for pH
42 4-6, Tris buffer for pH 6-9, and glycine-NaOH buffer for 9-11. The activity of NpaA1
43 was measured in a range from 5-80°C at pH 8.0 (50 mM Tris-HCl buffer). Error bars
44 represent the standard deviation of three biological replicates.

45 Figure 3-Source data 1: Raw data for the NpaA1 activity shown in Figure 3—figure
46 supplement 1.

47

SEQUENCE NAME	E-VALUE	MOTIF DIAGRAM
P0A9C7	3.5e-184	12-[11]-34-[7]-14-[9]-10-[8]-31-[14]-3-[6]-5-[1]-4-[4]-14-[2]-9-[5]-7-[3]-17-[12]-34
P12425	9.9e-175	15-[11]-33-[7]-15-[9]-9-[8]-5-[14]-2-[6]-5-[1]-4-[4]-15-[2]-9-[5]-6-[3]-19-[12]-32
P9WN38	1e-158	15-[11]-34-[7]-14-[9]-10-[8]-34-[14]-3-[6]-5-[1]-4-[4]-15-[2]-9-[5]-8-[3]-17-[12]-35
P0A1P6	2.5e-186	12-[11]-34-[7]-14-[9]-10-[8]-31-[14]-3-[6]-5-[1]-4-[4]-14-[2]-9-[5]-7-[3]-17-[12]-34
3NG0	3.6e-181	14-[11]-34-[7]-14-[9]-10-[8]-31-[14]-3-[6]-5-[1]-4-[4]-14-[2]-9-[5]-8-[3]-17-[12]-35
4LNF	9.6e-175	14-[11]-33-[7]-15-[9]-9-[8]-5-[14]-2-[6]-5-[1]-4-[4]-15-[2]-9-[5]-6-[3]-19-[12]-32
DcaQ	1.4e-145	37-[11]-[15]-23-[7]-12-[9]-8-[8]-63-[6]-5-[1]-5-[4]-16-[2]-9-[5]-7-[3]-10-[12]-43
TdnQ	2.8e-143	29-[11]-[15]-23-[7]-12-[9]-8-[8]-63-[6]-5-[1]-5-[4]-16-[2]-9-[5]-7-[3]-10-[12]-42
AJR26810	2.4e-139	37-[11]-[15]-22-[7]-12-[9]-8-[8]-63-[6]-5-[1]-5-[4]-16-[2]-9-[5]-7-[3]-10-[12]-42
ATDA1	7.8e-140	31-[11]-[15]-26-[7]-12-[9]-8-[8]-63-[6]-5-[1]-6-[4]-16-[2]-9-[5]-7-[3]-10-[12]-6-[15]-9
NpaA1	2.1e-144	31-[11]-[15]-22-[7]-12-[9]-8-[8]-63-[6]-5-[1]-5-[4]-16-[2]-9-[5]-7-[3]-10-[12]-6-[15]-9
CAC81335	6e-95	18-[11]-47-[7]-12-[9]-8-[8]-13-[10]-[6]-5-[1]-4-[4]-16-[2]-9-[5]-6-[3]-14-[12]-32
GlnA4	3.9e-91	29-[11]-47-[7]-12-[9]-8-[8]-54-[6]-5-[1]-3-[4]-13-[2]-9-[5]-5-[3]-11-[12]-32
P78061	6.4e-73	34-[11]-78-[9]-8-[8]-57-[6]-6-[1]-4-[4]-14-[2]-9-[5]-6-[3]-55-[8]-2
GlnA3	1.1e-79	27-[11]-[15]-14-[7]-12-[9]-9-[8]-4-[14]-2-[6]-5-[1]-4-[4]-15-[2]-9-[5]-10-[3]-4-[9]-[12]-3
4HPP	2.4e-86	4-[11]-[15]-13-[7]-17-[9]-8-[8]-40-[6]-5-[1]-4-[4]-16-[2]-9-[5]-13-[3]-19-[12]-32
P20478	4.6e-29	131-[8]-53-[6]-5-[1]-5-[4]-53-[5]-37
P15104	6e-37	70-[7]-13-[9]-6-[8]-28-[5]-10-[6]-5-[1]-5-[4]-53-[5]-8-[3]-4
Q09179	1.4e-36	105-[9]-6-[8]-53-[6]-5-[1]-5-[4]-49-[5]-36
AAA34644	2.4e-38	77-[9]-6-[8]-53-[6]-5-[1]-5-[4]-49-[5]-8-[3]-8
AAA62803	8.6e-32	99-[8]-52-[6]-5-[1]-5-[4]-47-[5]-8-[3]-16
Q8RNI4	6.8e-99	116-[13]-40-[8]-62-[6]-5-[1]-4-[4]-37-[15]-55-[3]-226
WP_036880422	6.5e-99	56-[4]-49-[13]-40-[8]-62-[6]-5-[1]-4-[4]-37-[15]-55-[3]-226
WP_008765057	1.3e-99	116-[13]-40-[8]-62-[6]-5-[1]-4-[4]-37-[15]-55-[3]-226
P15623	4.1e-97	116-[13]-40-[8]-62-[6]-5-[1]-4-[4]-37-[15]-55-[3]-142-<1>-55
48 WP_010933078	1.3e-72	98-[13]-45-[8]-62-[6]-5-[1]-4-[4]-37-[15]-209-[5]-[2]-56

49 Figure 3—figure supplement 2. Motif order and spacing for GS-like proteins.



50

51 Figure 4—supplement figure 1. Aggregate state analysis of NpaA1. (A) Gel filtration

52 of NpaA1. (B) SDS-PAGE showed that peak a and peak b were both NpaA1. (C)

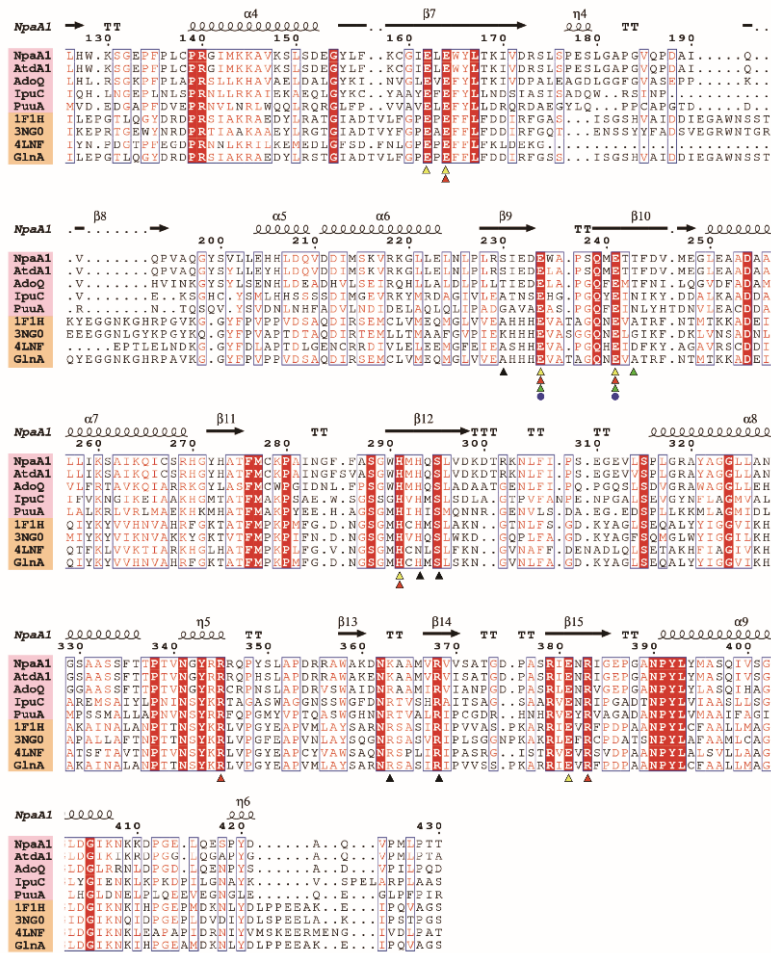
53 Multi-angle light scattering (MALS) analysis of NpaA1. The molecular weight and

54 error of each peak are counted in the table.

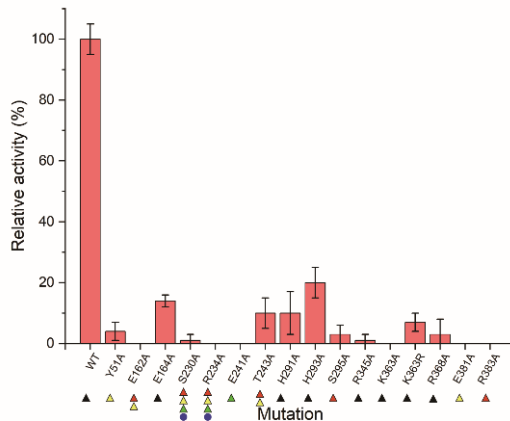
55 Figure 4-Source data 1: Raw data for Gle filtration shown in Figure 4—supplement

56 figure 1.

A



B



57

58 Figure 4—figure supplement 2. Conserved residues and mutant analysis of ligand
59 binding sites (A) Sequence alignment of GS-like proteins (pink background) with GS
60 proteins (yellow background). Residues involved in binding ATP, glutamate, Mg²⁺, and
61 ammonium are marked with black triangles, red triangles, yellow triangles, and green
62 triangles, respectively. (B) Mutational analysis of important residues participating in
63 binding substrate and catalysis. Error bars represent the standard deviation of three
64 biological replicates.

65

66 Figure 4—Source data 1: Raw data for enzyme activities shown in Figure 4—figure
supplement 2.