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A thermophile *Hydrogenibacillus* sp. strain efficiently degrades environmental pollutants polycyclic aromatic hydrocarbons

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Summary

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants threatening ecosystems and human health. Here, we isolated and characterized a new strain, *Hydrogenibacillus* sp. N12, which is a thermophilic PAH-degrader. Strain N12 utilizes naphthalene as a sole carbon and energy source above 60°C and co-metabolizes many other PAHs as well. The metabolites were identified in the catabolism of naphthalene by gas chromatography–mass spectrometry (GC–MS) and stable isotopic analysis. Based on the identified metabolites, we proposed two possible metabolic pathways, one via salicylic acid and the other via phthalic acid. Whole-genome sequencing reveals that strain N12 possesses a small chromosome of 2.6 Mb. Combining genetic and transcriptional information, we reveal a new gene cluster for the naphthalene degradation. The genes, designated as *nar*AaAb that are predicted to encode the alpha and beta subunits of naphthalene dioxygenase, were subsequently subcloned into *Escherichia coli* and the enzyme activity was detected by whole-cell transformation. Capacity to degrade several other tricyclic-PAHs was also characterized, suggesting co-existence of other constitutively expressed enzyme systems in strain N12 in addition to the naphthalene degradation gene cluster. Our study provides insights into the potential of the thermophilic PAH-degrader in biotechnology and environmental management applications.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are one of the most common and persistent environmental pollutants. They are aromatic hydrocarbons consisting of two or more fused benzene rings, with low water-solubility and high melting-points (Haritash and Kaushik, 2009). Because of their toxicity, mutagenicity and carcinogenicity (Ugochukwu *et al*., 2018), PAHs are a focus of research worldwide. Decomposition of PAHs in natural conditions can be a time-consuming process. Although PAHs are susceptible to both chemical oxidation and photochemical alterations, the rigorous reactions that occur when these processes are conducted in the laboratory make it challenging for practical applications (Wild and Jones, 1995). However, microbes can catabolize PAHs with less input of time and energy. Therefore, bioremediation has become a highly promising way to deal with PAHs pollution.

PAH degraders, especially mesophilic bacteria, have been intensively investigated over the past few decades. Strains with diverse abilities to degrade different PAHs have been isolated. Biotransformation of PAHs with low-molecular weight, such as naphthalene, phenanthrene, and anthracene, has been widely explored in terms of molecular mechanism and genetic information (Denome *et al*., 1993; Menn *et al*., 1993; Iwabuchi and Harayama, 1998; van Herwijnen *et al*., 2003). Strains of *Sphingomonas* (Ye *et al*., 1996; Rentz *et al*., 2008), *Rhodococcus* (Walter *et al*., 1991), and *Mycobacterium* sp. (Heitkamp and Cerniglia, 1988; Rehmann *et al*., 1998) are well known for degrading even high-molecular weight PAHs. In general, knowledge of mesophilic degraders is desired for bioremediation in conventional environments. However, the degradation potential of extremophiles, particularly thermophiles, is gradually being recognized (Müller *et al*., 1998).

Thermophilic microorganisms are able to degrade xenobiotics including *n*-alkanes (Al-Maghrabi *et al*., 1999), chlorinated hydrocarbons (Michel *et al*., 1995), phenol (Mutzel *et al*., 1996), and PAHs (Zeinali *et al*., 2008b). Elevated temperature significantly influences the bioavailability and solubility of organic compounds, which is meaningful for hydrophobic substrates. According to the previous study (Viamajala *et al*., 2007), the equilibrium solubility concentrations and dissolution rates of PAHs are significantly increased at elevated temperatures, thereby improving bioavailability and degradation rate.

Previous research on thermophilic degraders mainly focused on the degradation of *n*-alkanes, while there is less research on refractory aromatic pollutions, such as PAHs. Nevertheless, some studies suggest differences in the biochemistry of PAH degradation between thermophilic and mesophilic bacteria. For example, naphthalene degradation by the thermophilic strain *Bacillus thermoleovorans* Hamburg 2 is different from that described in mesophilic bacteria (Annweiler *et al*., 2000). New metabolites were identified during the mineralization of phenanthrene by thermophilic *Nocardia otitidiscaviarum* strain TSH1 (Zeinali *et al*., 2008a). However, no further reports about their genetic or enzymatic information are available. In general, knowledge of thermophilic degraders is still lacking in terms of molecular mechanism or genetic information compared with mesophiles. Therefore, in-depth studies of the degradation mechanism of thermophilic bacteria are an important basis for technology development and engineering applications.

Here, we focused on the newly isolated *Hydrogenibacillus* sp. N12, a thermophilic strain that can utilize naphthalene as the sole carbon and energy source above 60°C. Strain N12 was characterized for its ability to metabolize a variety of PAHs and heterocyclic derivatives. Intermediates of naphthalene mineralization were analysed in detail by gas chromatography–mass spectrometry (GC–MS) and a stable isotopic test, and possible degradation pathways for phenanthrene (PHE), fluorine (FLN), and the heterocyclic derivatives dibenzothiophene (DBT), dibenzofuran (DBF), and carbazole (CA) were proposed. Combining the genetic and transcriptional information, we found a novel naphthalene gene cluster in strain N12 resembling the *nar* region, a gene cluster responsible for naphthalene degradation in *Rhodococcus* sp. strains. Due to limited studies of PAH degradation by thermophiles, our investigation provides useful information about a thermophilic PAH-degrader, which may contribute to assessing the fate of PAH contaminants in high-temperature habitats and allow researchers to tap the potential of thermophiles in environmental and biotechnological applications.

Results

Isolation and identification of thermophilic strain N12

A thermophilic strain that can use naphthalene as the sole carbon and energy source was isolated from compost. The phylogenetic tree based on the 16S rRNA gene showed that this strain had the highest similarity (99%) with *Hydrogenibacillus schlegelii* (Fig. S1), which was reclassified as a novel genus in 2013 (Kampfer *et al*., 2013). The pure culture of *H. schlegelii* was first reported in 1979 (Schenk and Aragno, 1979), and it was described as a thermophilic and chemolithotrophic bacteria able to oxidize hydrogen. According to Schenk and Aragno, isolation of *H. schlegelii* has a limited spectrum of possible carbon sources, mainly including some organic acids and amino acids. However, a strain of *Hydrogenibacillus* sp. that can use naphthalene as the sole carbon and energy source has not been reported. In addition to naphthalene, strain N12 can also catabolize PHE, FLN, and heterocyclic derivatives such as DBT, DBF, and CA.

The growth curve of strain N12 was measured at different temperatures and pH values when naphthalene or NaOAc served as the sole carbon source (Fig. 1A–C). The fastest growth rate appeared at 65°C with either NaOAc or naphthalene. The growth rate dropped sharply between 50°C and 45°C. When NaOAc served as the carbon source, the upper limit of growth temperature was nearly 10°C (80°C) higher than that when naphthalene was used (70°C). The optimal pH for growth was 7.0 (Fig. 1C).

Degradation of naphthalene

Growth on naphthalene was measured under the optimal conditions of temperature and pH (Fig. 1D). Naphthalene was degraded rapidly with bacterial growth, while the concentration remained almost unchanged in the medium without strain N12. As naphthalene was consumed, the growth of bacteria reached a plateau.

To clarify the metabolic pathway of naphthalene in strain N12, naphthalene metabolites were detected under optimal growth conditions, and samples were taken at 24 h intervals for 4 days. GC–MS analysis identified some metabolites well-known in naphthalene metabolism including salicylic acid (SA), and coumarin (Table 1). The results indicated that the classical route from naphthalene to SA may be present in strain N12. However, neither the ring fission product of dihydroxynaphthalene, 2-hydroxybenzal pyruvic acid, nor its further degradation product, 2-hydroxycinnamic acid, were detected. Only a trace amount of coumarin was identified, which was recognized as the cyclizing product of 2-hydroxycinnamic acid (Davies and Evans, 1964). On the contrary, the product 2-hydroxyphenylpropionic acid (BSTFA derivative) and its cyclizing compound hydrocoumarin seemed to be predominant in any parallel sample. These two compounds are structurally the hydrogenation products of 2-hydroxycinnamic acid and coumarin.

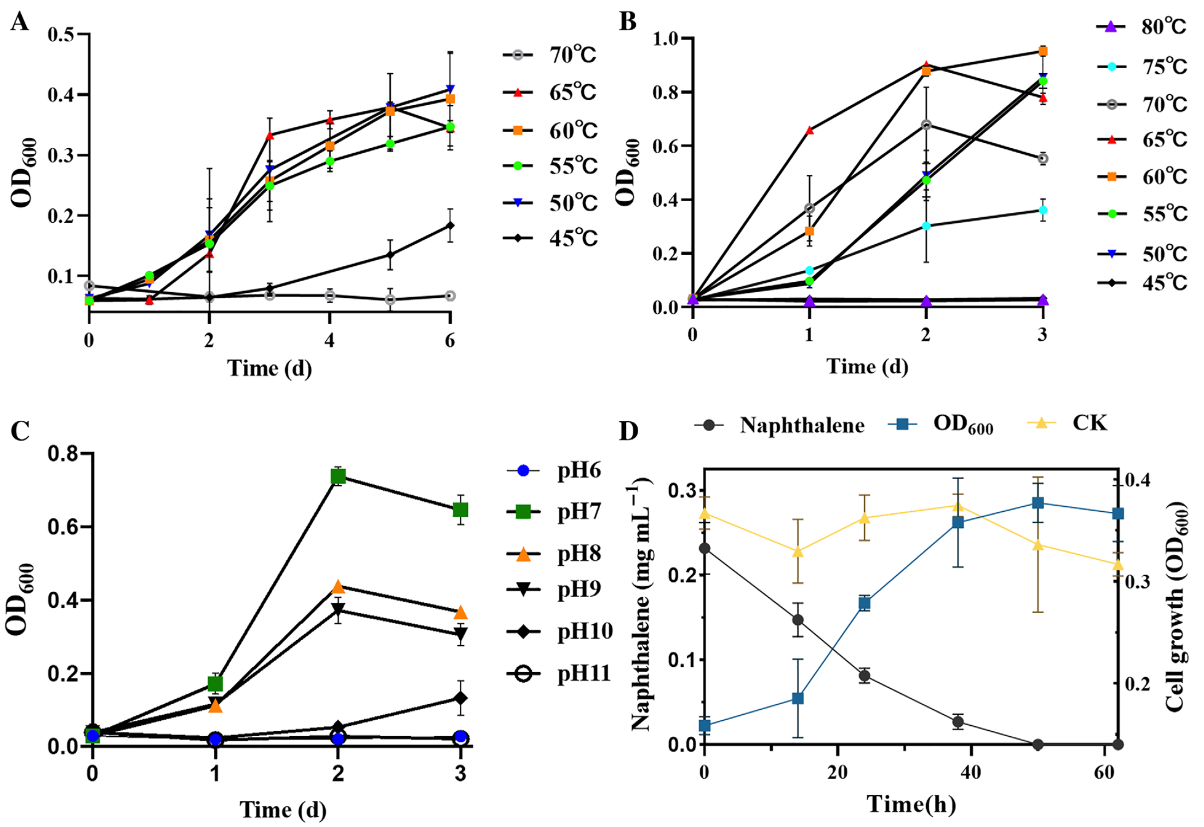

Fig 1. The growth of strain N12 at different temperatures and pH values. A. and B. The growth curve of strain N12 at different temperatures when naphthalene or NaOAc was used as the sole carbon source.C. Growth of strain N12 at various pH values with NaOAc as the sole carbon source.D. The growth and degradation curve of strain N12 on naphthalene for a short time scale under the optimal growth conditions. CK: Naphthalene in HY medium without strain N12.

Five metabolites rarely reported in mesophilic naphthalene degraders were detected by GC–MS, including 2-carboxycinnamic acid, phthalic acid, 3-hydroxybenzoic acid (BSTFA derivative), protocatechoic acid (BSTFA derivative), and 2,3-dihydroxybenzoic acid. These metabolites do not seem to be intermediate products belonging to the SA pathway (Fig. 2).

Deuterium-labelled naphthalene (naphthalene-d8) was used as supporting evidence for the detection of key metabolites. Intermediates during naphthalene-d8 mineralization were verified by GC–MS. When the mass spectrum of deuterium-labelled intermediates was compared to the unlabelled compound, masses of the fragment peaks were found to increase (larger fragments have a greater mass shift), while the retention time remained almost unchanged. As for metabolites produced in the mixed sample described in the methods, due to co-existence of labelled and unlabelled products, the relative abundance of isotope peaks changed, and the mass spectrum exhibited features of both the labelled and unlabelled products (Fig. S2A–F).

To determine the regulation mode of the initial stage of naphthalene degradation in strain N12, naphthalene dioxygenase activity was measured in whole cells. Results showed that the enzyme activity was only present in cells grown with naphthalene. As for NaOAc-grown cells, no enzyme activity was observed (Fig. S3). The findings indicated that degradation of naphthalene in strain N12 was an inducible process.

Degradation of phenanthrene, fluorene and heterocyclic derivatives

The ability to metabolize other PAHs and heterocyclic derivatives was also tested by resting cells of strain N12 (Fig. 3). However, unlike its ability to degrade naphthalene, which is an inducible process, it was found that both naphthalene-grown and NaOAc-grown cells could efficiently degrade PHE, FLN and other heterocyclic derivatives such as DBT, DBF and CA. Intermediates of co-metabolism of these substrates were separately analysed by GC–MS (Table 2). For PHE degradation, 2-hydroxy-1-naphthaldehyde,

Table 1. Metabolites of naphthalene degradation by GC–MS.

Metabolites	Retention time (min)	m/z of major ion peaks
2-Hydroxyphenylpropionic acid, BSTFA derivative	13.5	310(M+)(40),295 (59),192(78),177 (50),147(100),73(97)
Hydrocoumarin	10.48	148(M+)(86),120 (99),119(29),92 (32),91(100),78(62)
2-Carboxycinnamic acid	14.86	192(M+)(10),146 (100),105(51),77 (46),51(16),50(17)
Salicylic acid	9.47	138(M+)(37),120 (71),93(9),92(100),65 (14),64(25)
Coumarin	11.11	146(M+)(48),123 (28),118(100),90 (37),89(32),63(21)
Phthalic acid	9.69	148(M+)(10),104 (100),92(16),76 (63),74(24),71(17),50 (31)
1-Naphthol	14.49	144(M+)(50),116 (40),115(100),89 (11),65(14),63(15)
3-Hydroxybenzoic acid, BSTFA derivative	12.49	282(M+)(25),267 (100),223(71),193 (48),73(52)
Protocatechoic acid, BSTFA derivative	14.87	370(M+)(20),295 (17),193(41),147 (17),75(23),73(100)
2,3-Dihydroxybenzoic acid, BSTFA derivative	14.22	355(M+)(100),357 (17),356(32),193 (21),147(19),73(76)

3-(2-hydroxynaphthyl) propanoic acid, 1-naphthol and 2-naphthol were detected to accumulate in the system, with spectra that matched those provided by the database of National Institute of Standards and Technology (NIST) Library. The mass spectrum and retention time of 1-naphthol was further identified with an authentic compound. Further verification was carried out by resting cell transformation of 1-naphthol (Fig. S4A). As a result, 2-hydroxyphenylpropionic acid (BSTFA derivative) was found to be one of the downstream products (Fig. S4B). This may suggest that co-metabolism of PHE may converge with naphthalene metabolism, because 1-naphthol and 2-hydroxyphenylpropionic acid are metabolites shared by both. In addition, the detection of 9,10-phenan threnedione indicated another pathway for PHE degradation, which has oxidation on the 9th and 10th carbon atoms as the initial step.

Degradation of heterocyclic derivatives exhibited different colours (Fig. 3A), which usually indicates that some specific metabolites were generated. The coloured compound produced in DBT degradation suggested activation of the Kodama pathway (Gai *et al*., 2007). According to the results of GC–MS, four compounds were detected to be metabolites of DBT degradation, including benzo(b) thiophene-2-ol, benzo(b)thiophene-3-ol, dibenzothiophene-5-oxide and benzo(b)thiophene-2,3-dione.

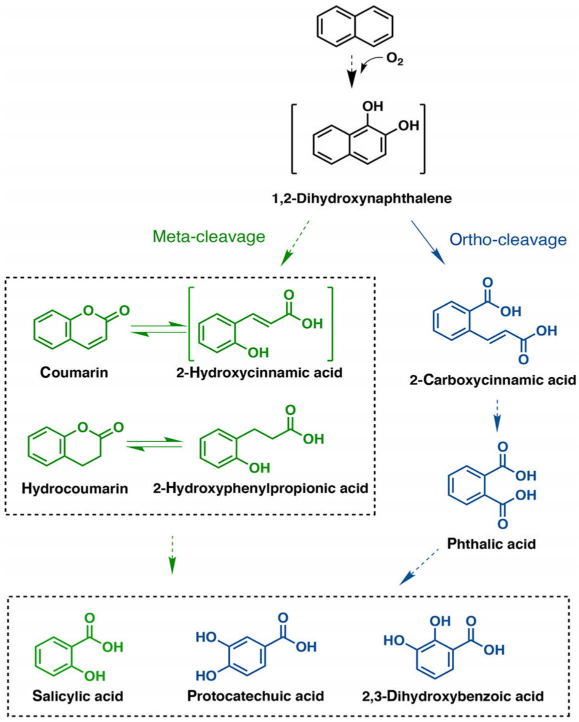

Fig 2. Proposed pathway for naphthalene degradation by thermophilic strain N12. Compounds in green are intermediate metabolites in the salicylic acid pathway, while compounds in blue are metabolites in the phthalic acid pathway. Solid arrows indicate one-step reactions. Dotted arrows indicate reactions with multiple steps. Two-way arrows represent compounds that may undergo mutual conversion.

DBF was rapidly degraded when incubated with strain N12 (Fig. 3B). Degradation of DBF produced metabolites with similar structures to intermediates of DBT, such as 3-benzofuranone and 2,3-benzofurandione. During degradation of CA, 2H-indol-2-one, 1H-Indole-2,3-dione and 1H-indole-3-carboxaldehyde were identified, indicating a similar oxidative pathway to DBF and DBT. In addition, 1-hydroxycarbazole was detected, which was assumed to be a spontaneous product of 1,2-dihydroxycarbazole in a previous report (Zhang *et al*., 2021).

Degradation of FLN seems to be suppressed in the early stage, and an accumulation of 9-fluorenone (identified with an authentic compound) was detected with the decrease of FLN, using both HPLC and GC–MS (Fig. S5A–D). Further degradation of 9-fluorenone by resting cells showed that the substrate can be slightly degraded, and the compound 2-hydrox yfluorenone was detected as its downstream product.

Genomic analysis of naphthalene degradation gene cluster

To further understand the mechanism of naphthalene degradation in strain N12, whole genome sequencing

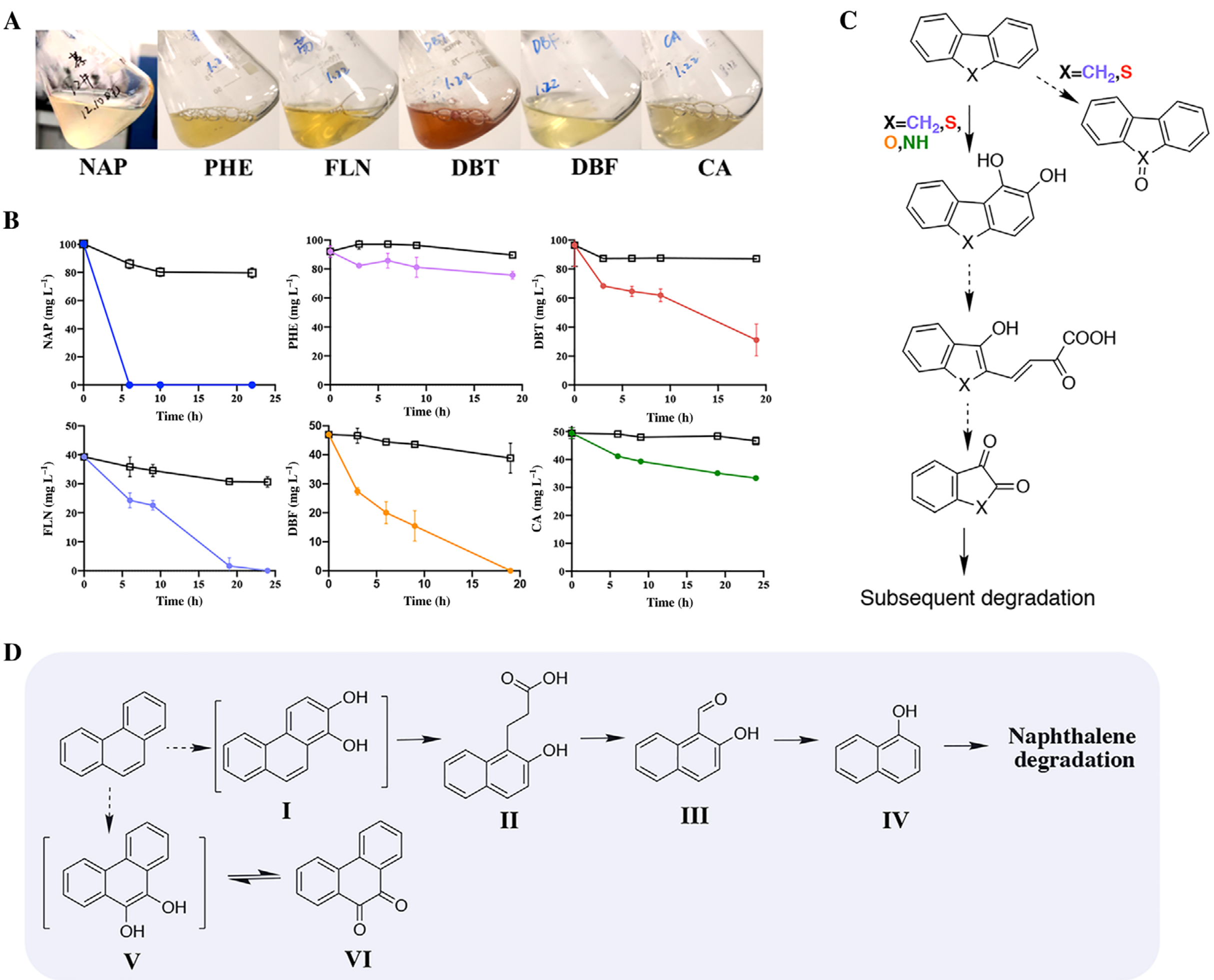

Fig 3. Metabolism of PAHs and heterocyclic derivatives by strain N12. A. Images of degradation phenomena of PAHs and heterocyclic derivatives by strain N12.B. Degradation curves of PAHs and heterocyclic derivatives by resting cell of strain N12. Concentrations of substrates are 100 mg L^{-1} NAP, 100 mg L^{-1} PHE, 100 mg L^{-1} DBT, 50 mg L^{-1} FLN, 50 mg L^{-1} DBF and 50 mg L^{-1} CA. The coloured dots represent the concentration of the corresponding substrate; white squires represent negative control without cells;C. The proposed pathway for catabolism of FLN and heterocyclic derivatives.D. The proposed pathway for metabolism of PHE; (I) 1,2-dihydroxyphenanthrene, (II) 3-(2-hydroxynaphthyl) propanoic acid, (III) 2-hydroxy-1-naphthaldehyde, (IV) 1-naphthol, (V) 9,10-dihydroxyphenanthrene and (VI) 9,10-phenanthrenedione. Dotted arrows represent multi-step reactions while solid arrows represent one-step reactions.

and analysis were performed. The size of the genome is 2.6 Mb, the GC content is 66%, and no plasmid was found (Fig. 4A). According to the gene annotation information of the NR database and homology alignment of reported gene sequences associated with naphthalene metabolism, Chr958 (shares 65% amino acid identity with previously reported NarAa from *Rhodococcus* sp.) and Chr959 (shares 53% amino acid identity with previously reported NarAb in *Rhodococcus* sp.) were predicted to be the alpha and beta subunits of the naphthalene dioxygenase (NDO) respectively (Kulakov *et al*., 2005). The protein sizes of the two putative genes (Chr958: 50.8 kDa, Chr959: 19.4 kDa) are almost the same as the reported subunits of NDO (Ensley and Gibson, 1983), and conserved sequence for the Rieske centre iron–sulfur-binding site was also found in the alpha subunit (Chr958) of the putative NDO. However, genes encoding ferredoxin and ferredoxin reductase essential for the function of NDO were not located in this region. In addition, we could not find genes sharing sequence identity to the reported reductase and ferredoxin.

Phylogenetic analysis of Chr958 (NarAa) and Chr959 (NarAb) with other related proteins showed that they clustered with groups of subunits of aromatic-ring-hydroxylating dioxygenases. The large and small subunits exhibited moderate identity with their homologues in *Candidatus Carbobacillus altaicus* (Kadnikov *et al*., 2018) and

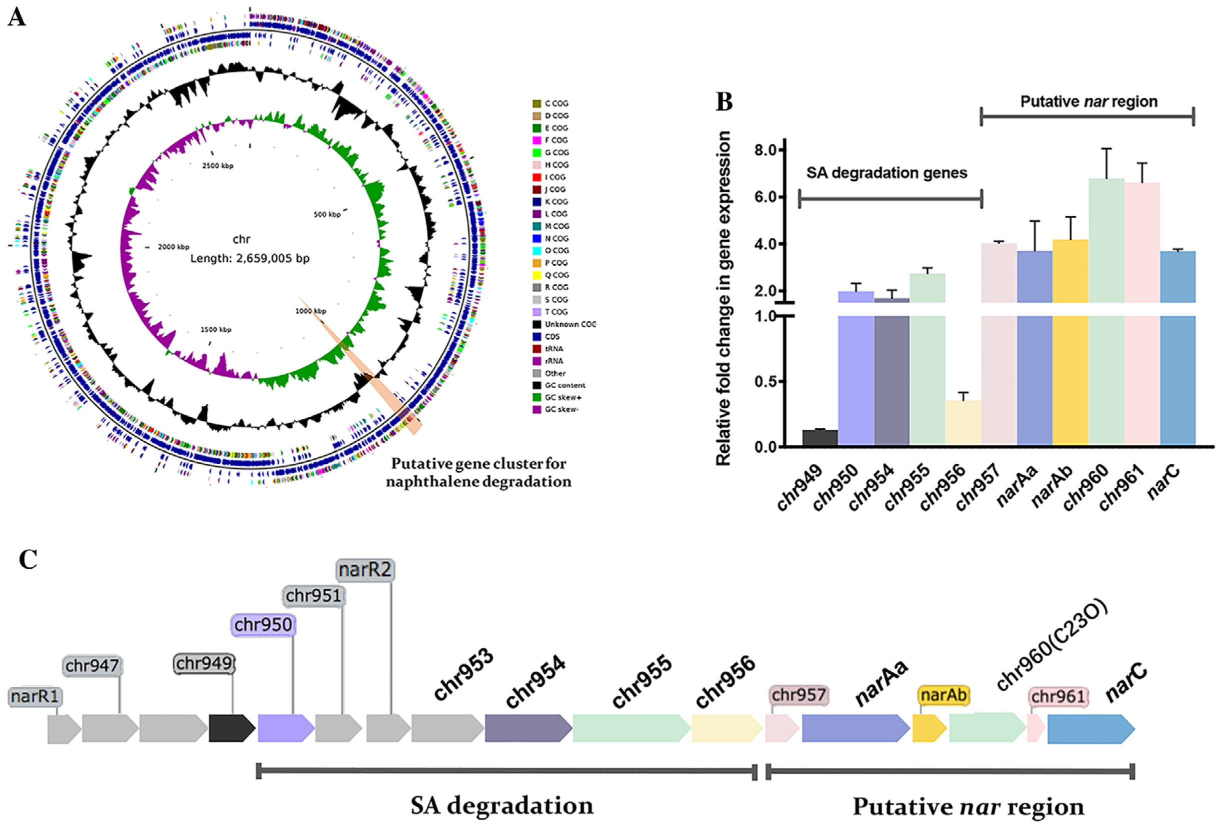

Fig 4. Transcription analysis of the putative gene cluster for naphthalene degradation. A. Genome map. From inside to outside: circle 1, scale; circle 2, G + C skew; circle 3, G + C content; circles 4 and 7, clusters of orthologous groups; circles 5 and 6, locations of coding sequence, tRNA, and rRNA genes.B. RT-qPCR analysis of the putative gene region for naphthalene degradation. Different bars indicate different genes.C. Physical and genetic map of the naphthalene degradation gene region. The same gene in B) and C) is indicated by the same colour shading. Genes in light grey indicate genes that have not been analysed by RT-qPCR; C23O: Catechol 2,3-dioxygenase.

Rhodococcus species, and had lower identity (< 40%) to homologues in *Pseudomonas* sp. strains (Fig. 5).

Surrounding regions were also analysed to find other genes belonging to the degradation pathway (Table S3). Two ORFs adjacent to *nar*AaAb were found to be related to naphthalene degradation: the amino acid of *chr*960 shares homology with catechol 2,3-dioxygenase. *chr*962 was predicted to encode an aldolase sharing 43% sequence identity with the trans-2′-carboxybenzalpyruvate hydratase-aldolase from *Nocardioides* sp. strain KP7 (Iwabuchi and Harayama, 1997) and 64% identity with the NarC (aldolase) from *Rhodococcus* P400.

In addition, a gene cluster for salicylate degradation was found upstream of *nar*AaAb, suggesting one of the pathways from naphthalene to salicylic acid. Two putative transcription regulators belonging to the GntR family were found upstream of the NDO components. The putative transcription regulators showed similarity (33.5% amino acid identity) with putative naphthalene degradation regulator NarR in *Rhodococcus* sp. P200, which is also a regulator of the GntR family (Kulakov *et al*., 2005).

RT-qPCR of the predicted naphthalene gene cluster

Each subunit of the predicted NDO shows a modest (< 65% sequence identity) sequence identity to previously reported sequences, so further verification was performed by the RT-qPCR analysis of the predicted gene region for naphthalene degradation (Fig. 4B,C).

We found that the putative gene region (chr958–chr962) responsible for initial naphthalene degradation was up-regulated in the naphthalene-grown group. In addition, catechol 2,3-dioxygenase (Chr960), which catalyses meta-cleavage of catechol to 2-hydroxymuconic-semialdehyde was up-regulated by (5.7-fold) when compared with the control group. Up-regulation in clusters indicate that these genes may be located in the same operon, which is induced by naphthalene or its downstream metabolites.

Functional analysis of initial enzyme NarAaAb in the naphthalene degradation

In order to further confirm the function of NarAaAb, the genes *nar*AaAb were heterologously expressed in

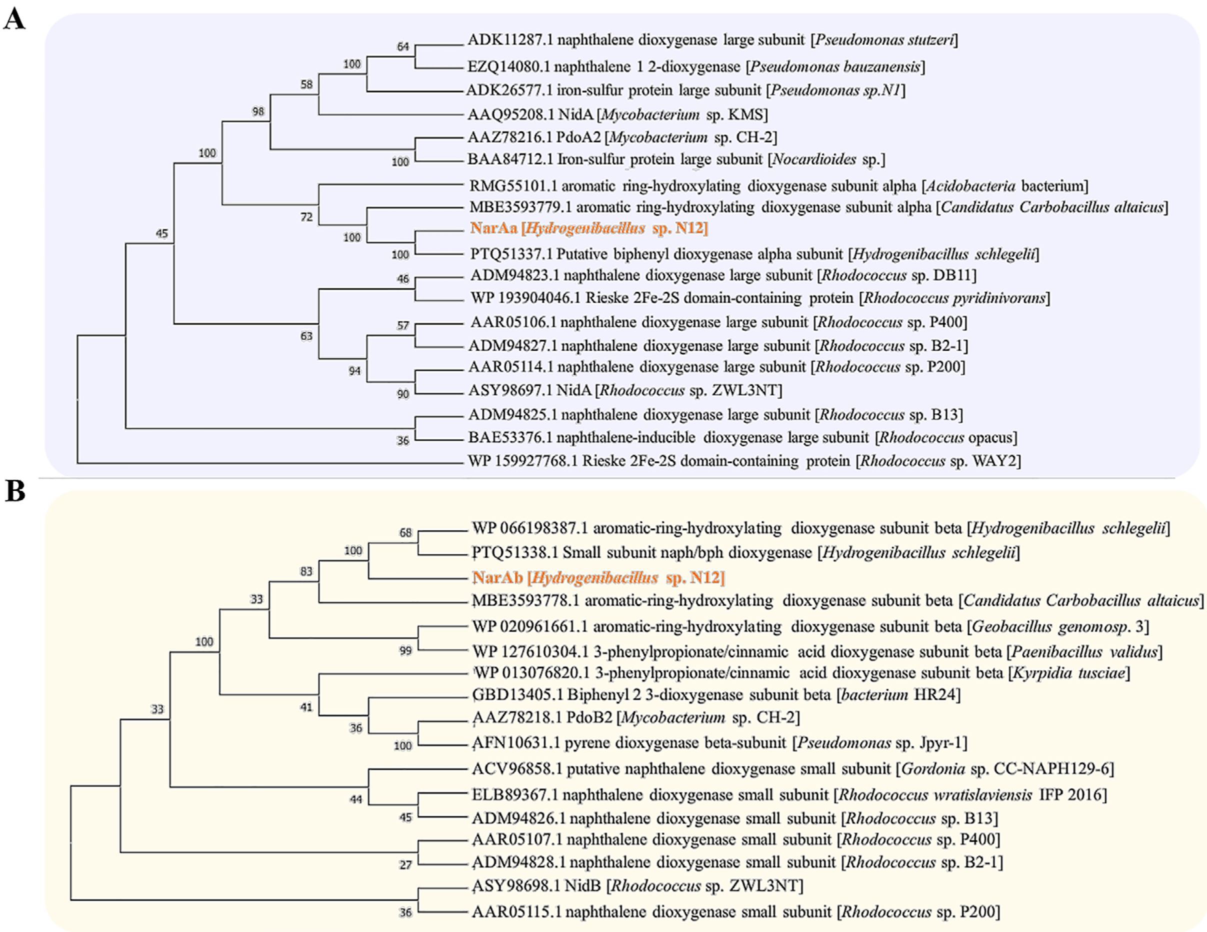

Fig 5. Neighbour-joining phylogenetic tree of amino acid sequences of NarAa (A) and NarAb (B) of strain N12 with published sequences. Bootstrap values (1000 replications) are shown as percentages at nodes.

E. coli BL21(DE3). Nearly, 50- and 22-kDa polypeptides were identified by SDS-PAGE (Fig. 6A). Most of the large subunit was expressed in the pellet, but soluble large subunit could be detected by western blot. The enzyme activity was observed in the recombinant *E. coli* harbouring plasmid pET-*nar*AaAb by whole-cell transformation of naphthalene. The enzyme activity was strengthened when it was co-expressed with additional electron transfer components PhdCD and PhtAcAd from *Nocardioides* sp. strain KP7 and *Mycobacterium vanbaalenii* PYR-1 respectively (Fig. 6B,C). The intermediates in naphthalene degradation were analysed by GC–MS. The trimethylsilylated naphthalene dihydrodiol was detected in the sample after 24-h incubation (Fig. 6E, F). Accumulation of naphthalene dihydrodiols suggests that NarAaAb exhibits naphthalene dioxygenase activity. 1-Naphthol was also detected as trimethylsilylated derivatives, which was considered as the dehydration product of *cis*-naphthalene dihydrodiols according to the previous report (Fig. 6G) (Kim *et al*., 2006). Substrate specificities of NarAaAb for PHE, FLN, DBT and DBF were determined, indicating that these substrates can also be degraded by NarAaAb with different degradation rates (Fig. 6D).

Discussion

In the past few decades, there have been many studies on the degradation of PAHs by bacteria. However, mining and utilization of strain resources in extreme habitats are rare. Extremophiles usually possess unique physiological characteristics, such as being thermophilic, acidophilic, alkaliphilic and halophilic (Margesin and Schinner, 2001). However, the degradation mechanisms of these microorganisms are not well understood. As more research has focused on biotransformation in extreme habitats, the abilities of these specialized degraders have been gradually uncovered, among which the thermophiles are the

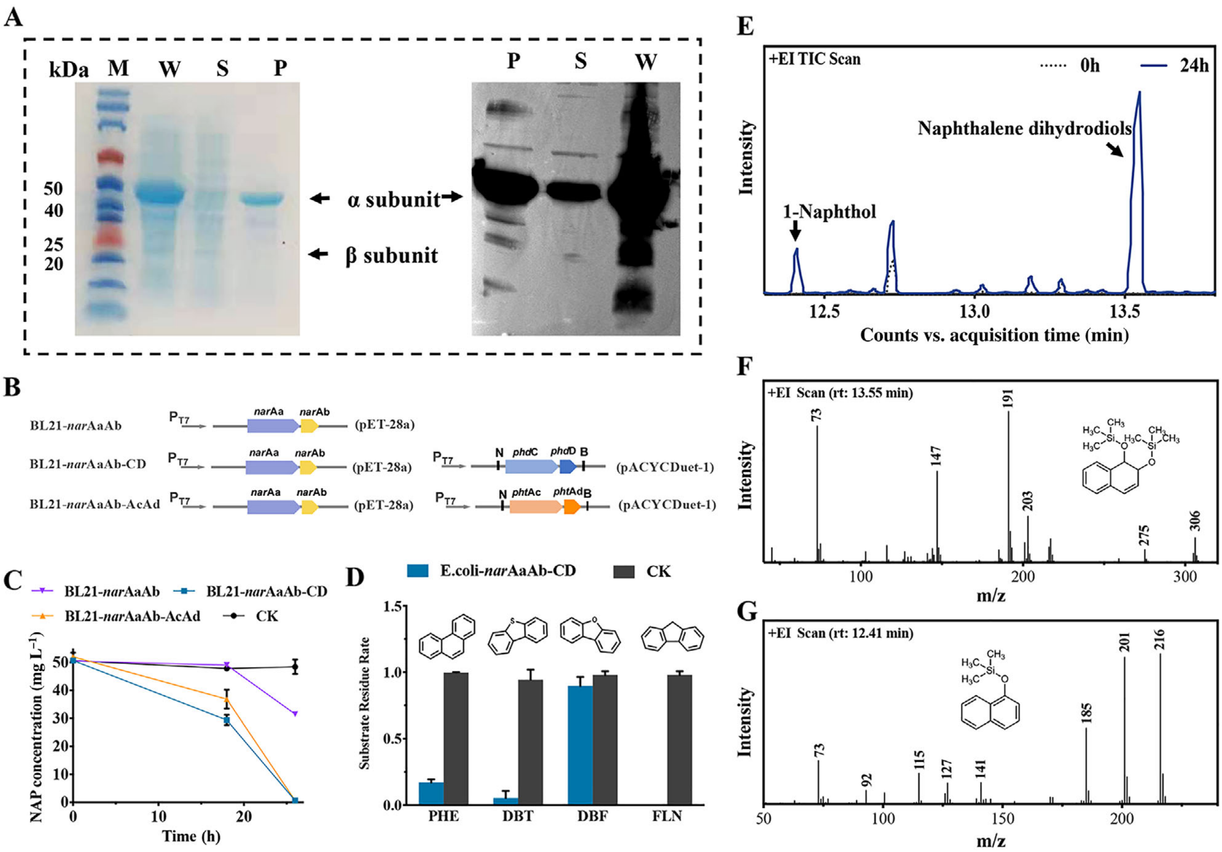

Fig 6. Functional analysis of NarAaAb in *Hydrogenibacillus* sp. N12. A. SDS-PAGE analysis of NarAaAb (left) and western blot analysis (right) of the alpha subunit (NarAa) expressed in *E. coli* BL21(DE3). Lane M, molecular size marker; lane W, whole-cell lysate of BL21(DE3)-pET-*nar*AaAb-pACYCDuet-*phd*CD; lane S, supernatant of the whole-cell lysate；lane P, pellet of the whole-cell lysate.B. Structure of the constructed plasmid. N, NcoI; B, BamHI.C. Detection of enzyme activity of NarAaAb in *E. coli* BL21(DE3). CK, sample without cells; BL21-*nar*AaAb represents strain BL21-pET-*nar*AaAb; BL21-*nar*AaAb-AcAd represents strain BL21(DE3)-pET-*nar*AaAb-pACYCDuet-*pht*AcAd; BL21-*nar*AaAb-CD represents strain BL21(DE3)-pET-*nar*AaAb-pACYCDuet-*phd*CD.D. Substrate specificity and degradation rate of NarAaAb for other PAHs and heterocyclic derivatives. PHE, phenanthrene；DBF, dibenzothiophene; DBF, dibenzofuran; FLN, fluorene.E. GC–MS spectra of the product formed from the whole-cell transformation of naphthalene. The black dotted line represents sample at the beginning of the reaction, the dark blue line represents sample after 24 h reaction.F. Mass spectra of the trimethylsilylated naphthalene dihydrodiols.G. Mass spectra of the trimethylsilylated 1-naphthol.

most promising for dealing with both industrial and environmental problems. Organic pollutants with low water solubility, such as PAHs, have been increasingly recognized as needing to be degraded.

Many researchers study composting as a bioremediation process. The critical stage of the composting process is the thermophilic phase (Fogarty and Tuovinen, 1991), during which biotransformation of thermophiles is crucial for removing pollution or solid waste. Some studies have also reported the application of thermophilic bacteria in the treatment of high-temperature wastewater. For example, a study previously reported removal of phenol in wastewater under thermophilic conditions, showing that over 99% of phenol was effectively degraded in an up-flow anaerobic sludge blanket (UASB) reactor at 55°C (Fang *et al*., 2006). Therefore, due to their adaptability to high-temperature, thermophiles have great potential to cope with the decomposition of pollutants in thermal environments, even the in-situ remediation of extreme habitats. However, the lack of relevant research indicates unknown aspects of these specialized degraders. In this study, we described the metabolism of PAHs and heterocyclic derivatives by thermophilic *Hydrogenibacillus* sp. strain N12. To our knowledge, this is the first report of PAH degradation by this genus.

Basic physiological tests on strain N12 showed that it is an obligate thermophile with the fastest growth rate near 65°C. N12 prefers neutral conditions and even weakly acidic environments will produce strong growth inhibition (Fig. 1C). As mentioned before, strains of *Hydrogenibacillus* sp. can use a limited spectrum of carbon sources. In this study, some common carbon sources (e.g., sucrose, glucose, and glycerol) were tested for growth, but all failed. Strains of this genus possess a relatively small genome (2.6 Mb in this study) compared to most other bacteria (Maker *et al*., 2017), which may possibly explain their narrow spectrum of carbon sources.

In our study, strain N12 grew on naphthalene as the sole source of carbon and energy. During the mineralization of naphthalene, we identified several major intermediates (Table 1). It was noted that some metabolites barely reported in naphthalene degraders of *Pseudomonas* sp. or other known mesophilic bacteria were produced, such as 2-carboxycinnamic acid and phthalic acid. The 2-carboxycinnamic acid was first reported as a metabolite during naphthalene degradation by the thermophilic *Bacillus thermoleovorans* Hamburg 2 (Annweiler *et al*., 2000). It was later reported in other thermophilic naphthalene degraders (Bubinas *et al*., 2008), as a ring fission product of dihydroxynaphthalene. According to previous reports, phthalic acid was supposed to be the further transformation product of 2-carboxycinnamic acid. Therefore, the appearance of 2-carboxycinnamic acid and phthalic acid revealed a new branch in the pathway, which is totally separate from the SA route.

Pseudomonas sp. strains are usually considered a model for studying naphthalene degradation (Brunet-Galmes *et al*., 2012; Tay *et al*., 2014). The naphthalene degradation pathway in *Pseudomonas* sp. is usually separated in two operons: the upper pathway involved in the conversion of naphthalene to salicylate, and the lower pathway involved in the conversion from salicylate to pyruvate and acetyl coenzyme A (Yen and Gunsalus, 1982). We also found a SA pathway in strain N12 and compared it with that in *Pseudomonas* sp. strains. In the upper pathway of *Pseudomonas*, 2-hydroxycinnamic acid and coumarin are the main products, while in strain N12, only a trace amount of coumarin was detected and no 2-hydroxycinnamic acid was observed. Instead, we found the accumulation of 2-hydroxyphenylpropionic acid and hydro coumarin in the system, which are two predominant products in this pathway. However, the two metabolites were also identified in other thermophilic naphthalene-degraders, suggesting that metabolites forming under thermophilic conditions are quite different from those under mesophilic conditions (Annweiler *et al*., 2000). Whether these new compounds are formed because of enzymatic activity or only products of a spontaneous reaction of 2-hydroxycinnamic acid and coumarin at thermophilic conditions still need further investigation.

We provide a possible schematic diagram for naphthalene utilization in strain N12 as follows: Naphthalene was first transformed to 1,2-dihydroxynaphthalene, from where the pathway branches. The meta-cleavage pathway produces 2-hydroxyphenylpropionic acid and subsequently salicylic acid, while the ortho-cleavage pathway leads to 2-carboxycinnamic acid and phthalic acid (Fig. 2). The similarity of the naphthalene degradation pathway in thermophiles, which is quite different from that in *Pseudomonas* sp. or other mesophilic strains, possibly revealed a different evolutionary branch of enzyme systems for naphthalene degradation (Table S2).

PAH-degrading bacteria usually degrade more than one aromatic substrate, which is generally due to the diversity of the substrate profiles of the relevant degrading enzymes (Moody *et al*., 2001; Garrido-Sanz *et al*., 2020). Sometimes, one gene cluster is responsible for degrading multiple substrates. *Pseudomonas putida* B6-2 was reported to degrade a variety of PAHs and dioxins by biphenyl-grown cells, and the biphenyl degradation gene cluster *bph*ABC is the only cluster responsible for the upper metabolic pathway of these substrates (Wang *et al*., 2021). Therefore, we tested the ability of strain N12 to degrade other tricyclic PAHs and heterocyclic derivatives. We found that strain N12 can degrade PHE, FLN, DBT, DBF, and CA by co-metabolism (Fig. 3). But it is worth noting that inducing for enzyme activities was not necessary: cells fed with NaOAc were able to degrade these substrates as well, suggesting constitutive expression of related enzymes. The results indicated that the NDO, whose activity was strictly regulated by naphthalene, is not the only one responsible for the initial conversion of these co-metabolized substrates. Therefore, we propose that strain N12 possesses other oxygenase enzymes for degrading aromatic compounds.

Mineralization of heterocyclic derivatives in strain N12 was found mainly through the lateral dioxygenation pathway (Fig. 3C). Degradation of these compounds produced metabolites that were similar in structure, indicating that the same cluster of genes was functioning. SA was detected in the DBF samples (Table 2), which indicates that DBF can be completely degraded by co-metabolism, rather than being blocked at a certain step to accumulate toxic intermediates. Although strain N12 can degrade FLN rapidly, we detected high-abundance accumulation of 9-fluorenone, which indicates that the further conversion of 9-fluorenone is a rate-limiting step in this degradation pathway.

We also screened the genes encoding NDO or other enzymes responsible for naphthalene mineralization. Many studies have shown that due to horizontal transfer of genes between different species, gene clusters with high-sequence homology can be found in different genera of PAH-degrading bacteria (DeBruyn *et al*., 2012; Yuan *et al*., 2017). However, sequences with high similarity were not found in strain N12 by sequence homology comparison. This suggested a new naphthalene degradation gene cluster in *Hydrogenibacillus* sp. N12,

Table 2. Intermediates of PHE, FLN and heterocyclic derivatives catabolism by GC–MS.

Substrate	Metabolites	m/z of major ion peaks
DBT	Benzo(b)thiophene-2-ol	121(100),150(66),122 (62),78(24),57(24)
	Benzo(b)thiophene-3-ol	121(100),150(97),122 (29),78(20),76(15)
	Dibenzothiophene 5-oxide	171(100),200(92),172 (61),184(33),139(22)
	Benzo(b)thiophene-2,3-dione	136(100),108(47),69 (24),191(18),82(11)
CA	2-Hydroxycarbazole	183(100),154(34),182 (15),184(15),127(14)
	1H-Indole-2,3-dione	119(100),92(76),147(46) 81(32),57(29)
	1-H-Indole-3-carboxaldehyde	144(100),145(87),178 (62),89(35),116(32)
	2H-Indol-2-one	104(100),133(99),78 (49),105(43),77(22)
DBF	3(2H)-Benzofuranone	134(100),105(82),76 (67),77(35),50(15)
	2-Coumaranone	78(100),134(54),106 (32),51(21),77(15)
	2,3-Benzofurandione	92(100),120(88),63 (38),64(34),121(31)
	Salicylic acid	92(100),120(79),138 (49),57(30),64(27)
PHE	2-Hydroxy-1-naphthaldehyde	172(100),115(73),171 (63),144(46),116(15)
	3-(2-Hydroxynaphthyl) propanoic acid	198(100),128(86),170 (61),169(54),156(33)
	1-Naphthol	144(100),115(95),116 (45),69(34),103(34)
	2-Naphthol	144(100),115(61),103 (18),69(17),85(17)
	2-Hydroxyphenylpropionic acid, BSTFA derivative	147(100),73(55),295 (20),148(15),129(9)
	9,10-Phenanthrenedione	180(100),152(83),181 (48),210(46),151(37)
FLN	9-Fluorenone	180(100),152(40),151 (23),181(16),150(14)
	2-Hydroxyfluorenone, BSTFA derivative	253(100),268(76),73 (49),75(30),71(26)

compared to previous reports. Instead, two groups of genes with relatively lower similarity (*chr*958 and *chr*959, *chr*966 and *chr*967) were found and proposed to encode the large and small subunits of NDO. Amino acid sequences of these genes share nearly 50% sequence similarity with the NDO components, NarAa and NarAb, in *Rhodococcus* sp. (Chr958 and Chr966 share 65% and 51% amino acid sequence identity with NarAa, respectively, Chr959 and Chr967 share 53% and 45% amino acid sequence identity with NarAb respectively).

Based on both genomic and RT-qPCR analysis, the region *chr*958–*chr*962, which contains the *nar*AaAb genes, is notably induced by naphthalene. Therefore, it was proposed to be related to the upper pathway of naphthalene degradation. In contrast, the transcriptional levels of *chr*966 and *chr*967 were downregulated with the addition of naphthalene (Fig. S6). The terminal dioxygenase subunit genes (*nar*AaAb) were cloned, expressed and biochemically analysed in *E. coli*, indicating that they are functional enzymes for naphthalene degradation. Most often, ferredoxin and reductase are indispensable for the NDO function (Haigler and Gibson, 1990). It is hypothesized that NarAaAb might borrow the electron transfer chain from some other systems in *E. coli*; this phenomenon has once been reported in the functional analysis of NidAB dioxygenase subunits (Khan *et al*., 2001). We found that the enzyme activity was strengthened by extra ETC components PhdCD and PhtAcAd. They are type V electron transfer chains, which consist of a [3Fe–4S]-type ferredoxin and a glutathione reductase (GR)-type reductase. Preliminary substrate specificity test showed that the enzyme could efficiently degrade PHE, DBT and FLN as well. Therefore, we preliminarily suggest NarAaAb from *Hydrogenibacillus* sp. N12 as a novel nonheme iron aromatic ring-hydroxylating oxygenase.

Because the predicted *nar* region in strain N12 shares moderate similarity with their equivalence in *Rhodococcus* species, it was compared with gene clusters from some naphthalene-degrading *Rhodococcus* sp. strains (Fig. S8 and Table S4). It was found that unlike the *nah* gene cluster in *Pseudomonas*, the electron transport chain could not be found nearby the dioxygenase subunits in the *nar* region, which was speculated to be scattered in other positions on the genome.

In addition, part of the gene cluster (*paa*KFGHJ) for metabolism of phenylacetic acid was found on the genome. It was reported that *paa*KFGHJ is responsible for conversion of phenylacetic acid to succinyl-CoA and acetyl-CoA (Teufel *et al*., 2010). In our study, it was significantly up-regulated in the naphthalene groups compared with the control in the transcriptional data (Fig. S7). Therefore, we speculates that it may be involved in the metabolism of downstream product of naphthalene to centre carbon metabolism.

In this study, we characterized thermophilic *Hydrogenibacillus* strain N12 for its ability to metabolize a variety of PAHs and heterocyclic derivatives. We analysed the naphthalene mineralization pathway of strain N12 in detail by the isotope labelling method, from which we found differences between thermophilic and mesophilic bacteria in the pathway of naphthalene degradation. Study of co-metabolic substrates, including PHE, FLN, DBT, DBF and CA, revealed the presence of an abundant and efficient oxidase system in strain N12. Based on genomic, transcriptional and enzymatic analyses, we suggest the *nar* region in strain N12 as a gene cluster responsible for initial oxidation of naphthalene. Moreover, as a heat-resistant and efficient PAH decomposer, *Hydrogenibacillus* strain N12 is a potential candidate for the *in situ* remediation of high-temperature habitats or some thermal biological treatment processes, such as composting or high-temperature organic wastewater. In conclusion, knowledge of biodegradation by thermophilic strains is still limited, and while our research provides improved comprehension of this special group of microorganisms, their full potential in bioremediation still needs to be exploited.

Experimental procedures

Chemicals and culture media

Naphthalene (NAP, 99% purity), phenanthrene (PHE, 95% purity), dibenzothiophene (DBT, 99% purity) and dibenzofuran (DBF, 98% purity) were purchased from J&K Scientific (Beijing). Naphthalene-d8 (99 atom% D, ≥98%) and fluorene (FLN, 99% purity) were purchased from Aladdin (Shanghai Aladdin Biochemical Technology Co., Ltd.). Carbazole (CA, 99% purity) was purchased from Macklin (Shanghai Macklin Biochemical Co., Ltd.). Mineral salt medium (MSM) (Lu *et al*., 2019) was used in the enrichment culture. HY medium (adjusted from a basic material medium described previously (Schenk and Aragno, 1979) was used for cultivation and degradation. HY medium is composed of (litre $^{-1}$): 4.5 g $Na_2HPO_4{\cdot}2H_2O$, 1.5 g KH_2PO_4, 1 g NH_4Cl, 0.1 g $MgSO_4$, 0.01 g $CaCl_2$, 0.005 g ferric ammonium citrate, and 0.5 ml trace element solution (0.3 g $FeCl_2{\cdot}4H_2O$, 0.02 g $MnCl_2{\cdot}4H_2O$, 0.0124 g H_3BO_3, 0.0034 g $CuCl_2{\cdot}2H_2O$, 0.038 g $CoCl_2{\cdot}6H_2O$, 0.014 g $ZnCl_2$, and 0.04 g $Na_2MoO_4{\cdot}2H_2O$ per 1 L of distilled water).

Strain isolation and identification

Hydrogenibacillus sp. strain N12 was isolated from the compost of municipal sludge (Jinzhou, Liaoning, China). The enrichment procedure was performed by incubating the samples in MSM with naphthalene at 60°C and then repeating the incubation several times. Single colonies were obtained on MSM plates with 5 g L^{-1} sodium acetate (NaOAc) in naphthalene.

The 16S rRNA gene was amplified using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCA-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′), and the sequence was analysed by alignment with the GenBank program BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Genome sequencing and analysis

Whole genome sequencing (WGS) was performed on the Illumina MiSeq and Pacbio Sequel platforms. GeneMarkS (http://topaz.gatech.edu/GeneMark/) was used to predict the entire gene sequence. For functional annotations of protein-coding genes, sequence alignment was performed by diamond (http://github.com/bbuchfink/diamond). The databases used for sequence alignment were NR (ftp://ftp.ncbi.nih.gov/blast/db/) and Swiss-Prot (http://www.uniprot.org/), the critical value of sequence alignment was $1e^{-6}$, and one of the best Hits was chosen.

Phylogenetic analysis

Homology analysis was performed using BLAST to find related genes and proteins. DNA or protein sequences were aligned with CLUSTALW program (Chenna *et al*., 2003). The software MEGAX (https://www.megasoftware.net) was used to construct a neighbour-joining phylogenetic tree by comparing homologues of the sequences.

Degradation conditions of naphthalene

To optimize the cultivation and degradation conditions of strain N12, different temperatures and pH values were used. The optimization experiment was carried out under the conditions of 5% (v/v) inoculum size, 600 mg L^{-1} naphthalene or 5 g L^{-1} sodium acetate as the sole source of carbon, without shaking. To measure the optimum temperature for growth, the strain was cultured at different temperatures (37°C, 45°C, 50°C, 60°C, 65°C, 70°C, 75°C, and 80°C). The growth rate at different pH values (6.0, 7.0, 8.0, 9.0, and 10.0) was measured at the optimum temperature with 5 g L^{-1} sodium acetate as the sole source of carbon. The pH of the medium was modified with either NaOH or HCl.

Degradation of PAHs and heterocyclic derivatives by whole-cell transformation

To measure the ability of strain N12 to degrade aromatic compounds, cells grown on naphthalene or NaOAc were collected at the late exponential phase by centrifugation, washed twice and resuspended with 0.9% NaCl solution (OD_{600} 3.0–4.0). The cell suspensions were incubated at 65°C for 2–3 h without carbon sources to prepare resting cells. Then substrates were added into cell suspensions at appropriate concentrations and in all cases, the temperature for whole-cell transformation was 65°C. The samples used for HPLC analysis were extracted with equal volumes of ethyl acetate. The analytical method for HPLC detection was 80% methanol–water, 0.8 ml min^{-1} flow rate, at a detection wavelength of 254 nm. All the tests above were conducted in triplicate.

Identification of intermediate metabolites

To detect intermediate metabolites, the supernatant of the whole-cell transformation medium was collected and acidified to pH 2.0 with HCl. The acidified sample was extracted twice with an equal volume of ethyl acetate, then treated with anhydrous sodium sulfate. The remaining extract was concentrated using a rotary evaporator. Derivatization of hydroxyl and carboxyl groups was performed with *N*, *O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Both derivatized and underivatized samples were analysed by GC–MS (Agilent & GC-7890B; MS-5977B;) with an HP-5MS column (30 m × 0.25 mm, 0.25 μm), using a previously described method (Krivibok *et al*., 2003).

Stable isotope labelling experiment

The isotope labelling experiment was performed to verify naphthalene degradation metabolites. Three groups of cultures were prepared for analysis: the naphthalene group, the naphthalene-d8 group and the group cultured with naphthalene and naphthalene-d8 mixture. The supernatant was collected for GC–MS detection.

RT-qPCR analysis

Strain N12 cultured with naphthalene or NaOAc (control group) was collected at the mid-exponential phase. Total RNA of strain N12 was extracted with the RNAprep pure cell/bacteria kit (TianGen, China). After removing residual genomic DNA by treatment with DNase I, reverse transcription was conducted with Hifair II 1st Strand cDNA Synthesis Kit (Yeasen) to obtain cDNA. RT-qPCR was performed using Hieff qPCR Green Master Mix (no Rox) (Yeasen) with the quantitative PCR apparatus (qTOWER3G). The program for RT-qPCR was as follows: 95°C for 5 min, followed by 40 cycles of denaturation (95°C, 20 s), annealing (58°C, 20 s) and elongation (72°C, 20 s). The melting curve analysis was as follows: 95°C for 1 min, 55°C for 65 s and finally held at 95°C. The fold change in gene expression was calculated using the equation $\text{ratio} = \frac{(E_{target})^{\Delta Ct_{target}\,(\text{control}-\text{sample})}}{(E_{ref})^{\Delta Ct_{ref}\,(\text{control}-\text{sample})}}$, $E = 10^{\frac{-1}{k}}$; the coefficient k for each pair of primers is the slope of the curve obtained by fitting the template concentration as the independent variable and Ct value as the dependent variable. The 16S rRNA gene was used as the reference gene. Primers for each gene are listed in Table S1.

Table 3. Strains and plasmids used in this study.

Strains or plasmid	Description[a]	Source
Strains		
Hydrogenibacillus sp. N12	Thermophilic PAH-degrading strain	This study
Escherichia coli DH5α	F^- *recA1 endA1 thi-1 hsdR17 supE44 relA1 deoR(lacZYA-argF)* U169 80d*lacZM*15	TransGen
Escherichia coli BL21(DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm*(DE3)	TransGen
Plasmids		
pET-28a	$Kana^r$; expression vector	
pACYCDuet-1	Cm^r; expression vector	
pET-*nar*AaAb	pET-28a containing *nar*Aa and *nar*Ab	This study
pACYCDuet-*phd*CD	pACYCDuet-1 containing *phd*CD	This study
pACYCDuet-*pht*AcAd	pACYCDuet-1 containing *pht*AcAd	This study

[a] $Kana^r$, kanamycin resistance; Cm^r, chloromycetin resistance.

Construction of enzyme expression system

A DNA fragment containing *nar*AaAb was amplified from the genomic DNA of *Hydrogenibacillus* sp. strain N12 with primers 958-F (5′-CGCGGCAGCCATATGATGACGATT TATGCTCAGTC-3′) and 959-R (5′-ACGGAGCTCGAATT CTCAAAACAGAATCGCCAGAT-3′). The PCR amplicon was cloned into pET-28a to generate the recombinant plasmid pET-*nar*AaAb. Two different electron transport chains (ETC), *phd*CD (from *Nocardioides* sp. strain KP7) (Kim *et al*., 2006) and *pht*AcAd (from *Mycobacterium vanbaalenii* PYR-1) (Stingley *et al*., 2004), were synthesized and the codons were optimized with *E. coli* as the host. The coding sequences of the ETC components were cloned into pACYCDuet-1 between restriction enzyme recognition sites NcoI and BamHI, resulting in plasmids pACYCDuet-*phd*CD and pACYCDuet-*pht*AcAd. The recombinant plasmids (listed in Table 3) were transformed in *E. coli* BL21(DE3) for protein expression.

SDS-PAGE and western-blot analysis of products of narAaAb

The recombinant *E. coli* strain harbouring plasmid pET-*nar*AaAb was cultivated in 1000 ml of LB medium at 37°C. When the OD_{600} reached a value of 0.6, the culture was induced by adding IPTG (isopropyl-β-D-thiogalactopyranoside) at a final concentration of 0.1 mM and further incubated at 16°C for 20 h. The culture was harvested by centrifugation and resuspended in the PBS buffer. Cells were broken by a French press. The suspension was centrifuged (10,000 rpm, 35 min) to remove cell debris. The supernatant was used for SDS-PAGE and western blot analysis. SDS-PAGE was performed with electrophoresis buffer containing 1.0 g L^{-1} SDS, 18.8 g L^{-1} glycine, and 3.0 g L^{-1} Tris. For western blot analyses, gels were then transferred to a nitrocellulose membrane using a Trans-blot Turbo Transfer System

(Bio-Rad). The alpha subunit was determined using an anti-6x His tag antibody (Abcam, China). Protein bands were detected with ECL western blotting detection reagents (Share-Bio, Shanghai).

Biotransformation assay of NarAaAb

The enzyme activity of NarAaAb was measured using the whole-cell biotransformation. A final concentration of 0.1 mM IPTG was added to the cell culture when the optical density at 600 nm reached 0.6, and the cells were further cultivated at 16°C for 20 h. The culture was harvested by centrifugation, washed twice with PBS buffer and finally resuspended in the same solution. The suspension was incubated at 30°C for 3 h to prepare resting cells. PAHs were added to a final concentration of 50 mg L^{-1}. The whole-cell transformation assay was conducted at 30°C. Residual substrate concentration was detected by HPLC. The analysis and identification of metabolites by GC–MS was performed as described previously.

Accession number(s)

Hydrogenibacillus sp. strain N12 has been deposited in the China Center for Type Culture Collection (CCTCC) under accession number M20211078. The whole-genome sequence of *Hydrogenibacillus* sp. strain N12 was deposited into the NCBI database under the accession number CP080953.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supporting Information