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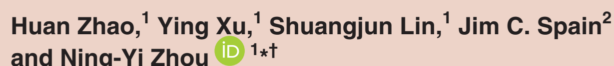
The molecular basis for the intramolecular migration (NIH shift) of the carboxyl group during *para*–hydroxybenzoate catabolism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Summary

The NIH shift is a chemical rearrangement in which a substituent on an aromatic ring undergoes an intramolecular migration, primarily during an enzymatic hydroxylation reaction. The molecular mechanism for the NIH shift of a carboxyl group has remained a mystery for 40 years. Here, we elucidate the molecular mechanism of the reaction in the conversion of *para*-hydroxybenzoate (PHB) to gentisate (GA, 2, 5-dihydroxybenzoate). Three genes (*phgABC*) from the PHB utilizer *Brevibacillus laterosporus* PHB-7a encode enzymes (*p*-hydroxybenzoyl-CoA ligase, *p*-hydroxybenzoyl-CoA hydroxylase and gentisyl-CoA thioesterase, respectively) catalyzing the conversion of PHB to GA via a route involving CoA thioester formation, hydroxylation concomitant with a 1, 2-shift of the acetyl CoA moiety and thioester hydrolysis. The shift of the carboxyl group was established rigorously by stable isotopic experiments with heterologously expressed *phgABC,* converting 2, 3, 5, 6-tetradeutero-PHB and [carboxyl-^{13}C]-PHB to 3, 4, 6-trideutero-GA and [carboxyl-^{13}C]-GA respectively. This is distinct from the NIH shifts of hydrogen and aceto substituents, where a single oxygenase catalyzes the reaction without the involvement of a thioester. The discovery of this three-step strategy for carboxyl group migration reveals a novel role of the CoA thioester in biochemistry and also illustrates the diversity and complexity of microbial catabolism in the carbon cycle.

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Introduction

Aromatic compounds rank behind only carbohydrates as the most abundant class of organic compounds (Kluczek-Turpeinen *et al*., 2003; Fuchs *et al*., 2011) and most of them can be utilized by microorganisms (Harwood and Parales, 1996; Gibson and Harwood, 2002; Fuchs *et al*., 2011). The classical oxygen-dependent catabolism is initially catalyzed by mono- or dioxygenases that hydroxylate the aromatic ring to form activated intermediates, such as catechol (1, 2-dihydroxybenzene), protocatechuate (3, 4-dihydroxybenzoate), gentisate (GA, 2, 5-dihydroxybenzoate), hydroquinone and substituted hydroquinones for ring cleavage by their corresponding dioxygenases (Ornston and Stanier, 1966; Harwood and Parales, 1996; Fuchs *et al*., 2011; Hayes *et al*., 2013; Liu *et al*., 2015). Biodegradation pathways for many lignin breakdown products including biphenyl, vanillic acid, *para*-hydroxybenzoate (PHB), converge at these central intermediates which illustrates their key role in the global carbon cycle (Kosa and Ragauskas, 2013; Sainsbury *et al*., 2013). The hydroxylation of the aromatic ring results in the loss of the original functional group that is directly replaced by a hydroxyl group. Hydroxylation of some aromatic compounds can also induce migration of the original functional group to the adjacent carbon (Guroff *et al*., 1966; 1967). This phenomenon of hydroxylation-induced migration (1, 2-shift) and retention in aromatic metabolism was named the NIH shift because it was initially observed at the National Institutes of Health (NIH) (Guroff *et al*., 1967). To date, the NIH shift has been found not only in bacteria (Guroff *et al*., 1966; Buswell and Clark, 1976; Crawford, 1976; Keenan and Chapman, 1978; Hartmann *et al*., 1999; Gabriel *et al*., 2012) and Archaea (Fairley *et al*., 2002), but also in eukarya, including *Homo sapiens* (Lindstedt and Odelhög, 1987; Hu *et al*., 2003). The substituents subject to NIH shifts include hydrogen (deuterium or tritium) (Hartmann *et al*., 1999; Hu *et al*., 2003), halogen (Mori *et al*., 2009), aceto substituent (Hareland

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et al., 1975; Rundgren, 1982; Moran *et al*., 2000; Moran, 2005; Shah *et al*., 2013), alkyl group (Gabriel *et al*., 2012) and carboxyl group (Buswell and Clark, 1976; Crawford, 1976; Keenan and Chapman, 1978; Fairley *et al*., 2002; Schoenian *et al*., 2012). The NIH shift of hydrogen is found to be involved in disorder of amino acid metabolism (phenylalanine and tryptophan) (Jequier *et al*., 1969; Waisbren and Levy, 1991) and in metabolism of therapeutic drugs (such as tamoxifen) (Hu *et al*., 2003). The NIH shift of the aceto substituent has been established to be involved in the modulation of blood tyrosine levels in human beings and animals and in the anabolic production of essential cofactors in plants (Moran, 2005). The NIH shifts of hydrogen and aceto substituents have been well studied and the molecular mechanisms are established (Eisensmith and Woo, 1991; Wang *et al*., 2002; Moran, 2005). The former is catalyzed by a single monooxygenase (Hartmann *et al*., 1999; Bassan *et al*., 2003), and the latter is catalyzed by a dioxygenase (Lindstedt and Odelhög, 1987; Moran, 2005), without involving additional reactions or enzymes.

para-Hydroxybenzoate (PHB) is a natural compound widely released into the environment mainly through lignin degradation (Kosa and Ragauskas, 2013) and has also been established to be involved in the full pathogenicity of *Xanthomonas campestris* (Wang *et al*., 2015). PHB can be efficiently utilized as a carbon source for growth by many microorganisms and the following three pathways are known for the aerobic utilization of PHB. In the first pathway, PHB 3-hydroxylase catalyzes conversion of PHB to protocatechuate in bacteria such as *Pseudomonas putida* WCS358 (Bertani *et al*., 2001) and *Corynebacterium glutamicum* RES167 (Huang *et al*., 2008). In the second pathway, PHB 1-hydroxylase (decarboxylating) catalyzes conversion of PHB to hydroquinone in *Candida parapsilosis* CBS604 (Eppink *et al*., 1997). Finally, PHB is utilized via the gentisate pathway in a few microorganisms (Buswell and Clark, 1976; Crawford, 1976; Keenan and Chapman, 1978; Fairley *et al*., 2002). In other systems, gentisate is derived from *meta*-hydroxybenzoate (MHB) via a reaction catalyzed by MHB 6-hydroxylase (Jones and Cooper, 1990; Xu *et al*., 2013; Montersino *et al*., 2017) and *ortho*-hydroxybenzoate (OHB) *via* a reaction catalyzed by OHB 5-hydroxylase (Zhou *et al*., 2001; 2002). In both of the latter pathways, the hydroxyl group is introduced *para* to the original hydroxyl group. In contrast, the direct hydroxylation of PHB to gentisate is impossible due to the presence of a carboxyl group in the *para* position to the original hydroxyl group (Fig. 1). Surprisingly, three *Bacillus* strains (Buswell and Clark, 1976; Crawford, 1976; Keenan and Chapman, 1978) and a haloarchaeal strain (Fairley *et al*., 2002) grow on PHB through the gentisate pathway and it was established that the introduction of a hydroxyl group at the *para* position induces the intramolecular migration of the carboxyl group. *Brevibacillus laterosporus* PHB-7a (ATCC29653) (formerly known as *Bacillus laterosporus* PHB-7a) was first reported to grow on PHB via a pathway involving gentisate and an initial NIH shift of the carboxyl group, but attempts to detect the enzyme(s) involved in the reaction were not successful (Crawford, 1976). For *Bacillus stearothermophilus* (Keenan and Chapman, 1978) and haloarchaeal strain D1 (Fairley *et al*., 2002), whole cell transformation of stable isotope-labeled PHB confirmed that the hydroxylation in the C-1 position induced the migration of the carboxyl group to yield GA. In the study of carboxyl group migration in PHB catabolism via GA by haloarchaeal strain D1 (Fairley *et al*., 2002), it was postulated that CoA thioester formation, migration and hydrolysis were involved in this unusual conversion. However, the genetic determinants and enzymes involved in the intramolecular migration of the carboxyl group remain unknown.

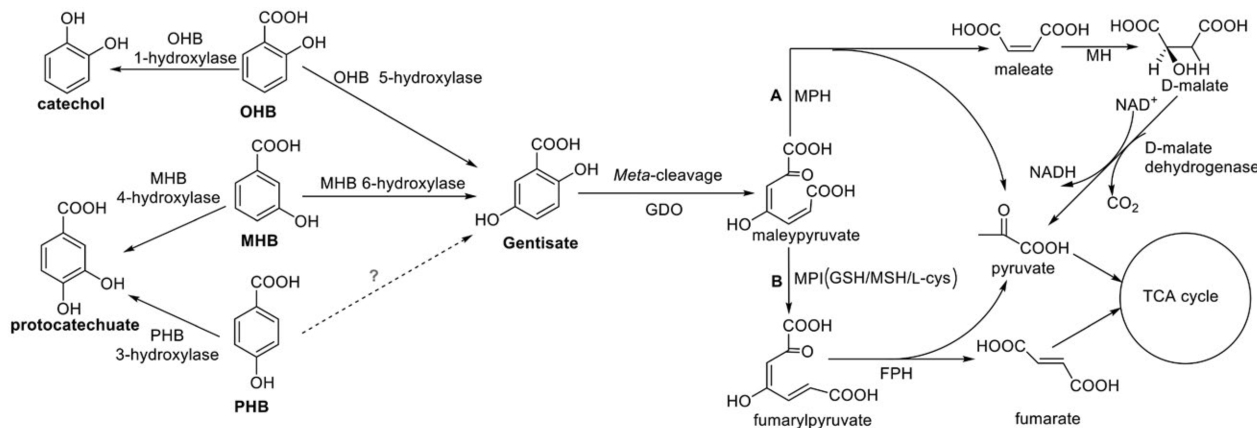


Fig. 1. Catabolic pathways of hydroxybenzoates. Left: Catabolic pathways of hydroxybenzoates to gentisate (peripheral (upper) pathways in the catabolism of mono-hydroxybenzoates); Right: Catabolic pathways of gentisate: A, direct hydrolysis; B, isomerization route. Compounds: OHB, *o*-hydroxybenzoate; MHB, *m*-hydroxybenzoate; PHB, *p*-hydroxybenzoate. Enzymes: GDO, gentisate 1, 2-dioxygenase; MPI, maleylpyruvate isomerase; FPH, fumarylpyruvate hydrolase; MPH, maleylpyruvate hydrolase. [Colour figure can be viewed at wileyonlinelibrary.com]

In this study, the molecular basis for the 1, 2-shift of the carboxyl group during the transformation of PHB to GA in strain PHB-7a was established. We discovered three inducible genes encoding enzymes that catalyze the conversion of PHB to GA via thioester formation, hydroxylation and hydrolysis. The genes and enzymes involved in the further catabolism of GA to TCA cycle intermediates were also determined. This completely characterized catabolic pathway of PHB to GA reveals the molecular basis for carboxyl group migration in the well-known NIH shift.

Results

Whole cell transformation of PHB to GA by strain PHB-7a

The degradation of PHB *via* GA in *Brevibacillus laterosporus* PHB-7a was previously established, but the molecular basis was unknown (Crawford, 1976). To determine whether the genes involved in the pathway were inducibly expressed, whole cell biotransformations of PHB by PHB-treated and -untreated cells of strain PHB-7a were conducted in the presence of 2, 2′-dipyridyl, a ring-cleavage dioxygenase inhibitor (Hopper and Chapman, 1971). PHB decreased substantially faster with PHB-treated cells than with untreated cells (Fig. S1), which established that the genes encoding enzymes involved in the conversion of PHB to GA were inducible. Stoichiometic conversion of PHB to GA confirmed previous observations about the initial step(s) in the catabolic pathway.

Identification of genes involved in gentisate catabolism by genome analysis

Genome sequencing established that the chromosome of strain PHB-7a was 5.44 Mb with a 38.2% GC content. A 0.08 Mb plasmid was also present in strain PHB-7a. Bioinformatics analysis revealed a cluster of four genes with high similarity to the functionally identified *bagXILK* cluster involved in the catabolism of MHB *via* GA in *Paenibacillus* sp. NyZ101 (Liu and Zhou, 2012). Functional identification showed that BagX in cell extracts and purified BagI, BagL and BagK from strain PHB-7a were MHB 6-hydroxylase (1.34 U mg^{-1} against MHB), gentisate 1, 2-dioxygenase (5.54 U mg^{-1} against gentisate), L-cysteine-dependent maleylpyruvate isomerase (1.98 U mg^{-1} against maleylpyruvate) and fumarylpyruvate hydrolase (2.98 U mg^{-1} against fumarylpyruvate), indicating that the gentisate formed from PHB conversion was catabolized to TCA cycle intermediates (Fig1).

The findings were also consistent with several previous reports of MHB 6-hydroxylase-encoding genes clustered with genes for GA degradation (Liu *et al*., 2011; Liu and Zhou, 2012). Indeed, PHB-7a was found to also grow on MHB as the sole source of carbon and energy.

Location of upregulated genes involved in the conversion of PHB to GA

Transcriptome analysis revealed that a total of 39 genes were upregulated in PHB-treated cells. Through

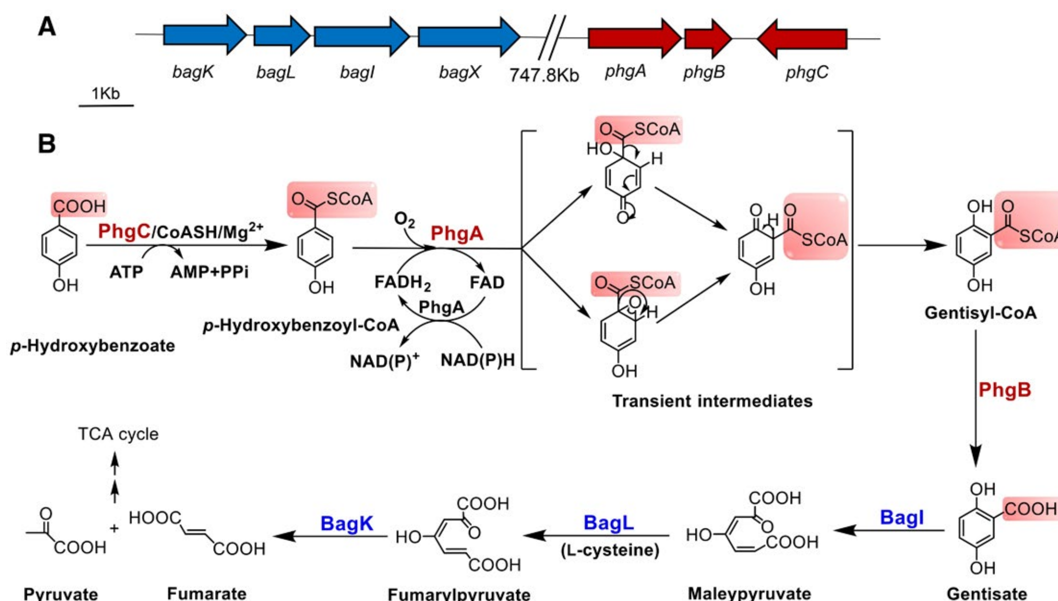


Fig. 2. Proposed catabolic pathway of PHB via GA involving carboxyl group migration in *Brevibacillus laterosporus* PHB-7a.
A. Catabolic gene cluster for PHB degradation. The arrows indicate the size and direction of transcription of each gene.
B. Catabolic reactions and intermediates of this pathway *in vivo*. PhgC: *p*-hydroxybenzoyl-CoA ligase; PhgA: *p*-hydroxybenzoyl-CoA 1-hydroxylase; PhgB: gentisyl-CoA thioesterase; BagI: gentisate 1, 2-dioxygenase; BagL: maleypyruvate isomerase; BagK: fumarylpyruvate hydrolase. [Colour figure can be viewed at wileyonlinelibrary.com]

bioinformatics analysis, seven of the genes in two clusters including aforementioned four genes *bagXILK* were annotated to encode enzymes for aromatic catabolism and thus considered as candidates for involvement in PHB catabolism via the GA pathway (Fig. 2A).The other three *orfs* were designated *phg* genes (*para*-hydroxybenzoate to gentisate). *phgABC* are clustered although *phgC* is divergently transcribed compared to *phgAB* (Fig. 2A). The products from *phgABC* were analyzed using the software of the conserved domain database (CDD) of NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). PhgA is an FAD-dependent hydroxylase which belongs to FAD_binding_3 superfamily (pfam01494) and it is similar to salicylate (or OHB) 1-hydroxylase (EC 1.14.13.1) from *Pseudomonas stutzeri* AN10 (Bosch *et al*., 1999) but with a low identity (23%). Protein classification showed that PhgB is an acyl-CoA thioesterase which is 48% identical to gentisyl-CoA thioesterase from *Bacillus halodurans* C-125 (Zhuang *et al*., 2004). PhgB contains three conserved domains: FadM (COG0824), 4HBT (cd00586) and 4HBT_2 (pfam13279), and belongs to the hot_dog superfamily (cd03440) (Heath and Rock, 1996; Dillon and Bateman, 2004). PhgC, a phenylacetate-CoA family protein, is similar to phenylacetate-CoA ligase from *Pseudomonas putida* U (Martínez-Blanco *et al*., 1990) with a low identity (29%). The CDD software predicted an AMP binding site, a CoA binding site and an AMP-binding domain in PhgC. Based on the assumption that PhgB was likely a gentisyl-CoA thioesterase responsible for hydrolyzing gentisyl-CoA to GA, we hypothesized the involvement of a CoA thioester in the conversion of PHB to GA. Finally, a hydroxylase would be essential for addition of a hydroxyl group to the aromatic ring during this conversion. Thus, the products from *phgABC* were deduced to be able to transform PHB to GA via a route involving CoA thioester formation, hydroxylation and thioester hydrolysis. Based on the above preliminary evidence, a PHB catabolic pathway was proposed (Fig. 2B) and subsequently confirmed (see below).

E. coli BL21 (DE3) [pETDuet-phgABC] converted PHB to GA

Heterologous expression experiments were conducted to establish rigorously the ability of PhgABC to transform PHB to GA. Because *phgC* is transcribed divergently from *phgAB*, pETDuet™-1 containing two multiple cloning sites was used for coexpression of *phgABC* or any two of the three genes. The resulting plasmids pETDuet-*phgABC*, pETDuet-*phgAB*, pETDuet-*phgAC*, pETDuet-*phgBC* were used to transform *E. coli* BL21 (DE3) and the resulting strains were tested for the ability to transform PHB to gentisate by whole cell biotransformation. IPTG-induced *E. coli* BL21 (DE3) [pETDuet-*phgABC*] converted PHB to GA (20.04 μmol h^{-1} g^{-1} cell dry weight)

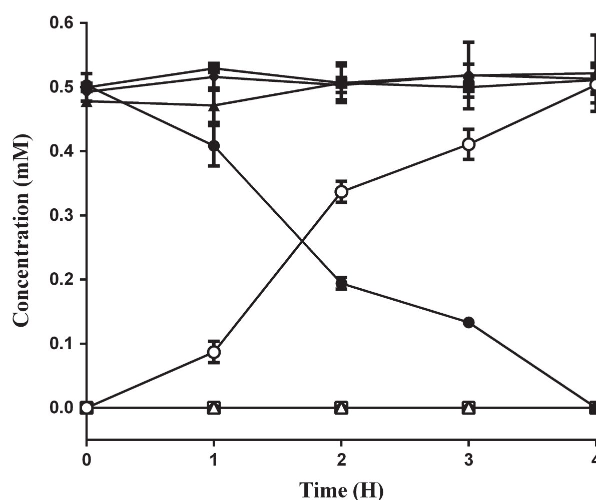


Fig. 3. PHB consumption and GA accumulation in whole cell transformations by *E. coli* containing different constructs. (●) PHB of pETDuet-*phgABC*, (○) GA of pETDuet-*phgABC*, (▲) PHB of pETDuet-*phgAB*, (△) GA of pETDuet-*phgAB*, (◆) PHB of pETDuet-*phgAC*, (◇) GA of pETDuet-*phgAC*, (■) PHB of pETDuet-*phgBC*, (□) GA of pETDuet-*phgBC*. Note that the lines of GA accumulation by *E. coli* containing any two of three genes are overlapped due to no accumulation of GA. All points represent the mean values of triplicate trials with error bars denoting the standard deviations.

as determined by HPLC analysis (Fig. 3). In contrast, neither PHB consumption nor GA accumulation was detected in IPTG-induced *E. coli* BL21 (DE3) containing any two of the three genes. The identity of produced GA was confirmed by LC-MS analysis by comparison with the mass spectra of authentic GA (Fig. S2). These results clearly demonstrated that PhgABC indeed are capable of converting PHB to GA and each of the three expressed enzymes is indispensable.

PhgC is p-hydroxybenzoyl-CoA ligase

phgC was cloned into pET28a (+) and transformed into *E. coli* BL21 (DE3) for overexpressing PhgC with an N-terminal His-tag. Finally, purified H_6-PhgC with a specific activity of 0.074 U mg^{-1} against PHB was obtained. During purification procedures, 10% glycerol for H_6-PhgC stability was indispensable. It was also helpful to protect H_6-PhgC from precipitating by addition of 0.2 mM DTT during desalinating. H_6-PhgC was determined to be 45 KDa by SDS-PAGE, consistent with its deduced molecular mass. Purified H_6-PhgC was fairly stable in 10% glycerol at −80°C; no obvious loss of enzyme activity was observed after one week.

Spectrophotometric assays provided evidence for the formation of *p*-hydroxybenzoyl-CoA (λ_{max}, 330 nm) at pH 9.2 (Fig. 4A). Because the molar extinction coefficient of *p*-hydroxybenzoyl-CoA is pH dependent (Webster *et al*., 1974), the optimum pH for enzyme catalysis was

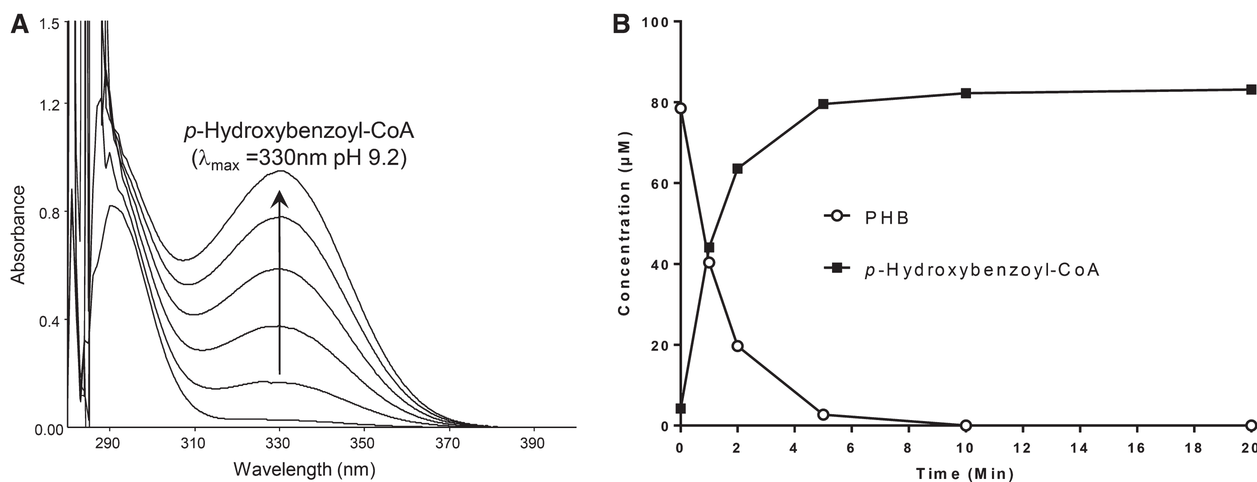


Fig. 4. Spectrophotometric analysis of purified H_6-PhgC (*p*-hydroxybenzoyl-CoA ligase) activity (A) and time course of PHB transformation by purified H_6-PhgC (B). Absorbance increase of *p*-hydroxybenzoyl-CoA (λ_{max}, 330 nm) at pH 9.2 is shown. PHB and *p*-hydroxybenzoyl-CoA were quantified by HPLC.

determined by measuring the formation of *p*-hydroxybenzoyl-CoA *via* HPLC analysis. Tricine-NaOH buffer gave higher activities than Tris-HCl or PB buffer at the same pH values and the optimum activity was obtained at pH 9.2. Addition of Mg^{2+} and Mn^{2+} (5 mM) increased the enzyme activity by 88 or 96 % respectively. Co^{2+}, Ca^{2+}, Zn^{2+} inhibited the activity, and Cu^{2+} led to complete inactivation. Under the optimum condition, the K_m value of H_6-PhgC for PHB was 40.1 ± 4.9 μM, and the corresponding K_{cat} value was 82.3 ± 3.3 min^{-1}. H_6-PhgC was not active against benzoate, OHB, MHB, GA, protocatechuate, 2, 4-dihydroxybenzoate, phenylacetate and 4-hydroxyphenylacetate, which indicated an extremely narrow specificity.

HPLC analysis during assay of H_6-PhgC activity under optimum condition showed that PHB (82.7 μM) was completely transformed to *p*-hydroxybenzoyl-CoA (83.2 μM) (Fig. 4B). LC-LTQ MS/MS comparison with chemically synthesized *p*-hydroxybenzoyl-CoA confirmed that the product was *p*-hydroxybenzoyl-CoA (Fig. S3). The results established rigorously that H_6-PhgC is a *p*-hydroxybenzoyl-CoA ligase catalyzing the conversion of PHB to *p*-hydroxybenzoyl-CoA, an initial and critical activating step to form a thioester in the enzymatic conversion of PHB to GA.

PhgA is a p-hydroxybenzoyl-CoA hydroxylase

phgA was cloned into pET28a (+) and transformed into *E. coli* BL21 (DE3) for overexpression. H_6-PhgA purified by renaturation of inclusion body protein formed during the expression yielded a low concentration (0.217 mg ml^{-1}) and a low specific activity. The molecular mass of the purified protein was 45 kDa determined by SDS-PAGE. In the presence of H_6-PhgA, UV spectrophotometric analysis revealed a decrease in *p*-hydroxybenzoyl-CoA (λ_{max}, 285 nm, pH 7.6) and NADPH (λ_{max}, 340 nm), concomitant with the formation of gentisyl-CoA ($\lambda_{shoulder}$, 380 nm) (Zhuang *et al*., 2004). The isobestic point at 360 nm indicated a direct conversion of *p*-hydroxybenzoyl-CoA to gentisyl-CoA (Fig. 5). When PHB was used as substrate, no spectrophotometric changes occurred. This indicated that PhgA acted upon *p*-hydroxybenzoyl-CoA rather than PHB. Due to the unavailability of gentisyl-CoA and our failure to synthesize it following a published protocol (Zhuang *et al*., 2004), the enzymatically formed product was further identified as gentisyl-CoA by LC-LTQ MS (Fig. 6). The MS spectrum containing a single peak at m/z 902.21 under negative ion mode corresponded to the predicted mass of gentisyl-CoA (903.13). The peak at m/z 902.21 was not detected in the reaction mixture without purified H_6-PhgA. The results established that PhgA is a *p*-hydroxybenzoyl-CoA 1-hydroxylase that catalyzes the transformation of *p*-hydroxybenzoyl-CoA to gentisyl-CoA, clearly indicating that the substituent subject to NIH shift is the acyl-CoA but not the carboxyl group itself.

PhgB was similarly purified as a His-tagged protein as above and determined to be 16 kDa by SDS-PAGE. As stated above, it shares 48% amino acid identity to the functionally identified BH1999, a gentisyl-CoA thioesterase converting gentisyl-CoA to gentisate in *Bacillus halodurans* C-125 (Zhuang *et al*., 2004). Furthermore, when purified H_6-PhgB was added to the reaction mixture containing gentisyl-CoA formed from *p*-hydroxybenzoyl-CoA hydroxylation by H_6-PhgA, gentisate was detected by LC-MS (Fig. S4). Taken with its essential role in the conversion of PHB to GA by heterologously expressed *phgABC* described above, these results established that PhgB is a gentisyl-CoA thioesterase catalyzing the hydrolysis of gentisyl-CoA to gentisate.

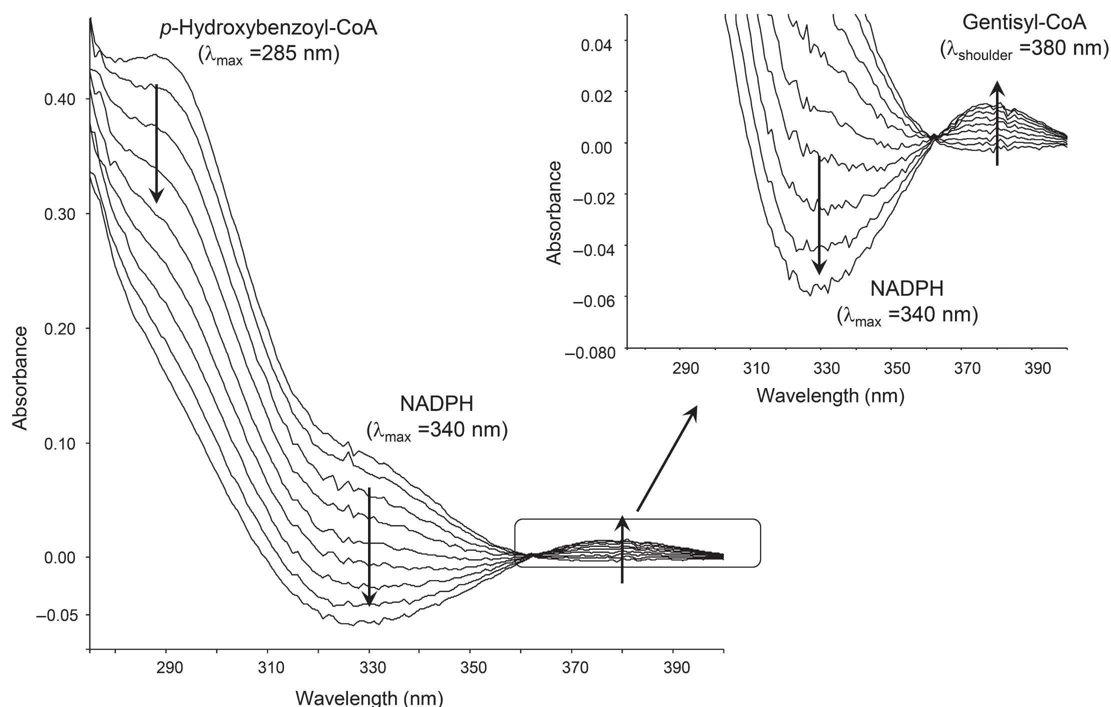


Fig. 5. Spectral changes associated with the transformation of *p*-hydrxoybenzoyl-CoA by H_6-PhgA. Sample and reference cuvettes contained 0.2 mM NADPH, 25 μM FAD, 40 μM Zn^{2+}, and 33 μg of purified PhgA in 500 μl of 50 mM phosphate buffer (pH 7.6). Reactions were started by addition of *p*-hydroxybenzoyl-CoA to a final concentration of 36 μM to the sample cuvettes, and spectra were recorded every 1 min.

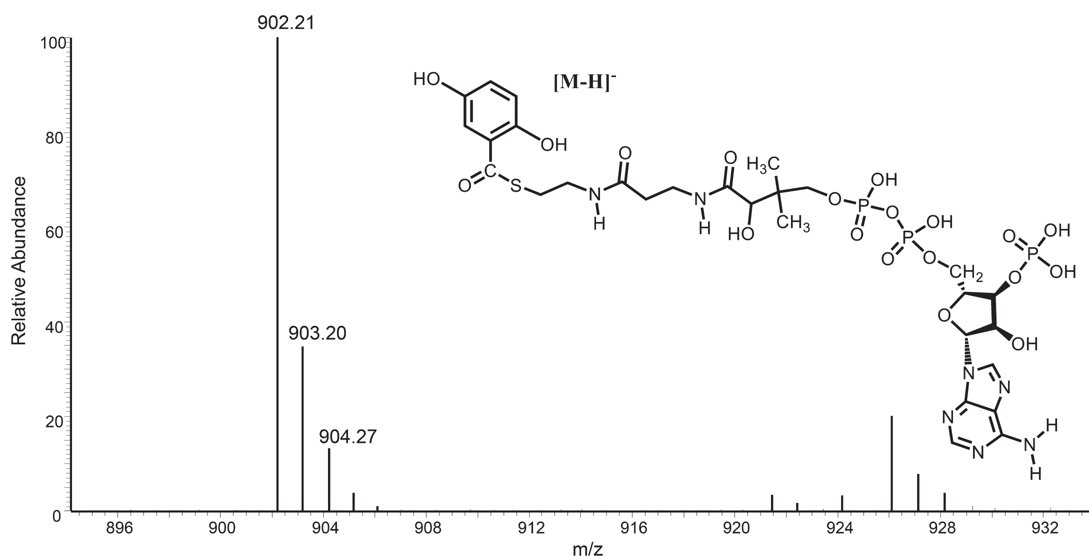


Fig. 6. LC-LTQ MS analysis of gentisyl-CoA produced from *p*-hydroxybenzoyl-CoA by purified PhgA.

Carboxyl group migration

Previously, whole cell transformations with stable isotope-labeled PHB in two wild-type strains have confirmed the involvement of carboxyl group migration in transformation of PHB to GA (Keenan and Chapman, 1978; Fairley *et al.*, 2002). Here, the migration was confirmed by whole cell transformations with *E. coli* cells containing pETDuet-*phgABC*. Stable isotope-labeled 2, 3, 5, 6-tetradeutero-PHB was used as the substrate to determine the migration of the carboxyl group (Fig. 7A) and [carboxyl-^{13}C]-PHB was used to determine the retention of the original carboxyl group (Fig. 7B). The product from 2, 3, 5,

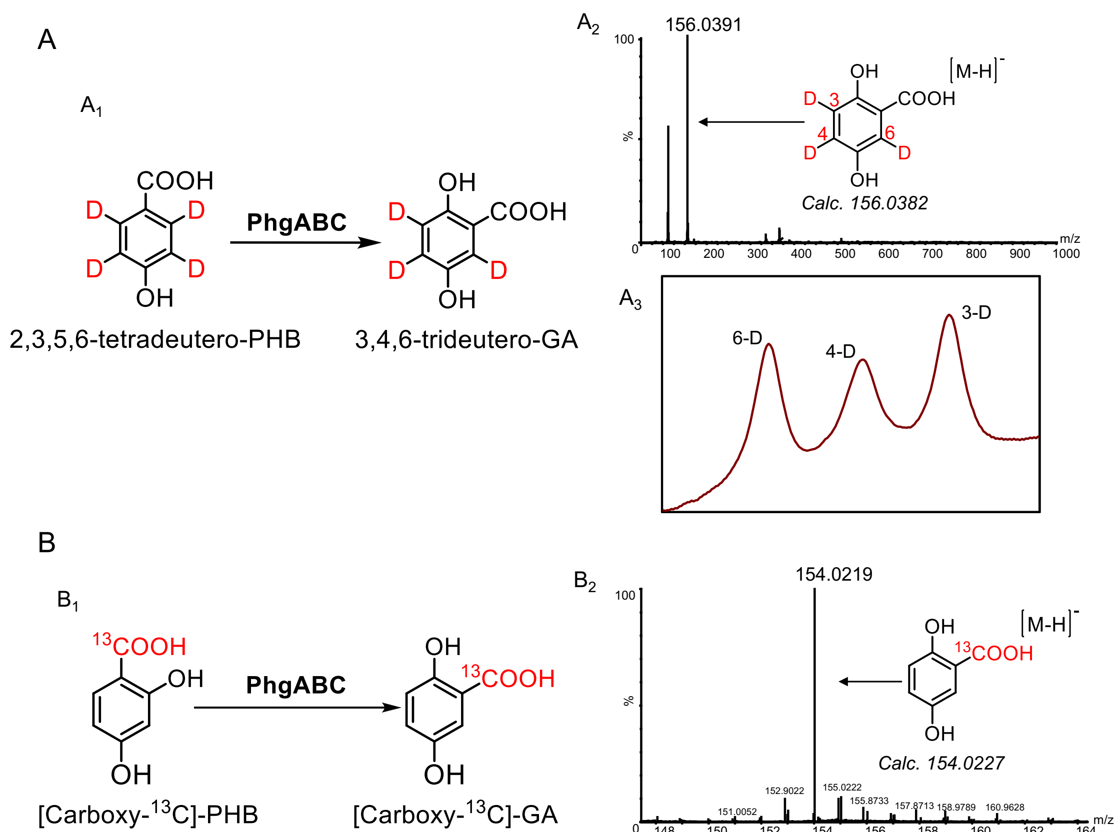


Fig. 7. Intramolecular migration of carboxyl group determined by whole cells biotransformation of isotopically labeled PHB by *E. coli* containing *phgABC.*
A. The deuterium at the *ortho* position was replaced by the carboxyl group. A_1. Biotransformation of 2, 3, 5, 6-tetradeutero-PHB to 3, 4, 6-trideutero-GA; A_2. LC-MS spectrum of 3, 4, 6-trideutero-GA; A_3. HNMR analysis of 3, 4, 6-trideutero-GA.
B. The retention of original carboxyl group. B_1. Biotransformation of [carboxyl-^{13}C]-PHB to [carboxyl-^{13}C]-GA; B_2. LC-MS spectrum of [carboxyl-^{13}C]-GA. [Colour figure can be viewed at wileyonlinelibrary.com]

6-tetradeutero-PHB was confirmed to be 3, 4, 6-trideutero-GA by LC-MS (Fig. 7A) and HNMR (Fig. 7A), which supports the involvement of the migration of a carboxyl group, but not the hydroxyl group. When [carboxyl-^{13}C]-PHB was used as substrate, identification of the product by LC-MS (Fig. 7B) established that the original carboxyl group was retained. Taken together, the above results established clearly that carboxyl group migration during the conversion of PHB to GA was catalyzed by PhgABC.

Discussion

The molecular mechanism for NIH shift of the carboxyl group elucidated in this study is different from previously studied NIH shifts for hydrogen and aceto substituents. The NIH shift of hydrogen can be observed in the conversion of phenylalanine to tyrosine catalyzed by a tetrahydrobiopterin-dependent hydroxylase (Bassan *et al*., 2003), or hydroxylation of tamoxifen mediated by P450 enzymes (Hu *et al*., 2003), or dearomatization of 2-aminobenzoyl-CoA catalyzed by a flavoprotein 2-aminobenzoyl-CoA monooxygenase/reductase (Hartmann *et al*., 1999). The other well-studied example is the aceto substituent 1, 2-shift during the conversion of *p*-hydroxyphenylpyruvate to homogentisate involving decarboxylation, aromatic hydroxylation and aceto group migration in a single catalytic cycle catalyzed by an Fe(II)-dependent, non-heme iron dioxygenase (Moran, 2005). Nevertheless, both the migrations of hydrogen and aceto substituent mentioned above are catalyzed by a single oxygenase, without the formation of thioesters. In contrast, the migration of the carboxyl group in this study is considerably more complex, involving three independent catalytic steps including CoA thioester formation as the initial reaction, hydroxylation-induced acyl-CoA moiety migration and hydrolysis of CoA thioester (Fig. 2B). The results reported here solidly confirmed the prescient predictions from the earlier work in haloarchaeal strain D1 (Fairley *et al*., 2002).

In most cases, PHB is aerobically catabolized by microorganisms through hydroxylation without carboxyl group migration to yield a dihydroxylated product, such as protocatechuate (Fig. 1) and hydroquinone (Suemori *et al*.,

1995; Bertani *et al.*, 2001; Huang *et al.*, 2008). Under anaerobic conditions, PHB is converted to *p*-hydroxybenzoyl-CoA by a *p*-hydroxybenzoyl-CoA ligase, followed by an oxygen-sensitive dehydroxylation to form benzoyl-CoA which undergoes further reductive metabolism (Geissler *et al.*, 1988; Merkel *et al.*, 1989; Gibson *et al.*, 1997; Harwood *et al.*, 1998). Here the aerobic degradation of PHB in strain PHB-7a incorporates the aforementioned key features of both anaerobic and aerobic catabolism in that it is also initiated by *p*-hydroxybenzoyl-CoA ligase to form *p*-hydroxybenzoyl-CoA but the ligase is phylogenetically distinct. The subsequent two steps involve aromatic ring hydroxylation with the help of oxygen and hydrolysis of thioester to form gentisate for oxygen-dependent aromatic ring cleavage. The *p*-hydroxybenzoyl-CoA ligases in both anaerobic and aerobic pathways are insensitive to oxygen (Gibson *et al.*, 1994); therefore, *p*-hydroxybenzoyl-CoA is a common intermediate and a branch point for aerobic and anaerobic pathways. Evidence that the evolution of oxidative catabolic pathways retain the vestiges of anaerobic processes (activated by CoA addition) is also present in the catabolism of other aromatic acids, including benzoate (Fairley *et al.*, 2006; Carlström *et al.*, 2015), phenylacetate (Teufel *et al.*, 2010), OHB (Ishiyama *et al.*, 2004) and MHB (Altenschmidt *et al.*, 1993). The involvement of CoA thioesters in this aerobic-hybrid catabolic pathway was suspected to be a strategy for microorganisms to adapt to low or fluctuating oxygen conditions (Fuchs *et al.*, 2011; Carlström *et al.*, 2015). In addition, the intracellular conversion of free aromatic compounds to membrane impermeable CoA thioesters is thought to be important for microorganisms to uptake and retain the carbon source (Gibson *et al.*, 1994; Fuchs *et al.*, 2011), particularly at low substrate concentrations.

Because PHB was not a substrate for *p*-hydroxybenzoyl-CoA hydroxylase (PhgA), the strain PHB-7a has to utilize PHB through the CoA addition pathway, despite the process being somewhat futile at first glance. In addition, in *p*-hydroxybenzoate, electrons of the carboxyl oxygen anion are more likely to delocalize to the carbonyl, leading to decreasing the effect of the π bond and the stability of the transition state. In contrast, the thioester, as an electron-withdrawing substituent, has high migratory aptitudes in related processes of enzymatic or nonenzymatic rearrangement (Dagli *et al.*, 1975; Marx and Merken, 2004) and migrates substantially faster than the carboxyl group (Marx and Merken, 2004).

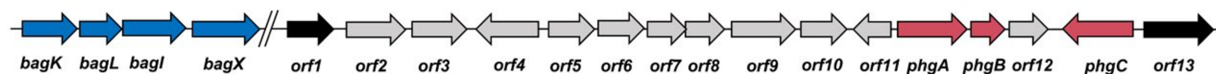
A similar 1, 2-shift of a carboxyl group activated by CoA addition also occurs in the biosynthesis of the 3-aminosalicylate moiety of antimycins in *Streptomyces ambofaciens* (Schoenian *et al.*, 2012) and *Streptomyces albus* J1074 (Sandy *et al.*, 2012). A putative multicomponent monooxygenase was suggested to catalyze the formation of 3-aminosalicyloyl-CoA from anthraniloyl-CoA via a reaction involving carboxyl group migration, but its activity was not demonstrated in *vitro* (Sandy *et al.*, 2012; Schoenian *et al.*, 2012). An anthraniloyl-CoA ligase (AntF) encoding gene was located near the monooxygenase encoding genes (*antHIJKL*) in strain J1074 (Sandy *et al.*, 2012). It was suggested that anthranilate was activated by AntF to form anthraniloyl-CoA which was then hydroxylated by the monooxygenase (Sandy *et al.*, 2012). The above findings indicate that CoA activation of substrate and carboxyl-CoA group migration can occur in microbial anabolism as well as catabolism. In the domain of bacteria, NIH shift of the carboxyl group in PHB degradation was so far found only in *Bacillus* (Buswell and Clark, 1976; Keenan and Chapman, 1978) and related species such as *Brevibacillus laterosporus* PHB-7a. No genomic information is currently available for other strains reported to utilize PHB *via* GA. To determine whether the *phg* gene cluster is also distributed in other microbial genomes, we conducted bioinformatics analysis of all sequenced microbial genomes. The results revealed that three *Bacillus* strains, including *Bacillus* sp soil768D1, *Bacillus butanolivorans* AFS003229 and *Bacillus butanolivorans* DSM18926, not only possess gene clusters with the same organization as the *phg* gene cluster of strain PHB-7a, but the genes also share high DNA and protein sequence identity (99–100%). The close relatedness suggests a recent common origin for the conversion of PHB to GA. Moreover, these three *Bacillus* strains also contain gene clusters highly similar to *bagXILK*, the gentisate degradation gene cluster of strain PHB-7a (Fig. 8), but do not have PHB 3-hydroxylase-encoding genes for the protocatechuate pathway. The above bioinformatics evidence indicates that these three *Bacillus* strains are also likely able to utilize PHB via GA with the same mechanism found in strain PHB-7a.

Experimental procedures

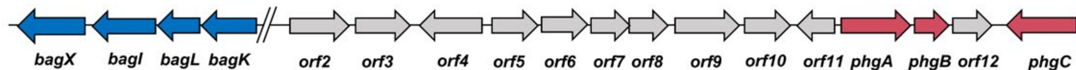
Bacterial strains, plasmids, growth conditions and chemicals

All bacterial strains and plasmids used in this study are listed in Table 1 and all primers are listed in Table 2. *Brevibacillus laterosporus* PHB-7a (ATCC29653) was purchased from the Bioresource Collection and Research Center (https://www.bcrc.firdi.org.tw/). All *E. coli* strains were cultivated at 37°C in lysogeny broth (LB) medium supplemented with kanamycin (50 μg ml^{-1}), ampicillin (100 μg ml^{-1}), as required. *Brevibacillus laterosporus* PHB-7a was grown aerobically in LB medium or M9 minimal medium (Sambrook, 2001) containing *p*-hydroxybenzoate (2 mM) or glucose (2 mM) at 30°C.

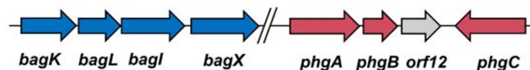
Brevibacillus laterosporous PHB-7a



Bacillus butanolivorans AFS003229



Bacillus sp soil768D1



Bacillus butanolivorans DSM18926

Fig. 8. Comparison of genetic organization of PHB catabolic clusters from different strains. The *bag* cluster encoding for catabolism of MHB and gentisate; The *phg* cluster encoding enzymes for the conversion of PHB to gentisate; Unidentified *orfs*; IS110 family transposases. The *phg* gene cluster in strain PHB-7a shares 99–100% gene and protein sequence identities with the other three *Bacillus* strains. *orf 2* to *phgC* in strain PHB-7a share 99–100% gene and protein sequence identities with their counterparts in strain AFS003229. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strain		
Brevibacillus laterosporus PHB-7a	Wild type	Crawford, 1976
E. coli DH5α	*supE44ΔlacU169 (φ80dlacZΔM15) hsdR17 recA1 endA1 hsdR17 thi-1 gyrA96 relA1*	Novagen
E. coli BL21(DE3)	*F⁻ ompT hsdSB (Rb⁻mB⁻) gal (λc I 857 ind1 Sam7 nin5 lacUV5 T7gene1) dcm (DE3)*	Novagen
Plasmid		
pET-28a (+)	Kan^r, overexpression vector	Novagen
pETDuet™-1	Amp^r, co-expression vector	Novagen
pET-28a-*phgA*	*PhgA* cloned into pET-28a	This study
pET-28a-*phgB*	*PhgB* cloned into pET-28a	This study
pET-28a-*phgC*	*PhgC* cloned into pET-28a	This study
pETDuet-(*phgABC*)	*phgC* and *phgAB* cloned into pETDuet™-1	This study
pETDuet-(*phgAC*)	*phgC* and *phgA* cloned into pETDuet™-1	This study
pETDuet-(*phgBC*)	*phgC* and *phgB* cloned into pETDuet™-1	This study
pETDuet-(*phgAB*)	*phgA* and *phgB* cloned into pETDuet™-1	This study

p-Hydroxybenzoate and gentisate were purchased from Sigma (St. Louis, MO). NAD(P)H, CoASH and ATP were obtained from Aladdin Reagent Co. Ltd. (Shanghai, China). Compounds 2, 3, 5, 6-Trideutero-PHB and [carboxyl-^{13}C]-PHB were purchased from Shanghai Research Institute of Chemical Industry and Reer Technology, Ltd respectively. *p*-Hydroxybenzoyl-CoA was synthesized as reported previously (Merkel *et al*., 1989).

Whole cell biotransformations

Brevibacillus laterosporus PHB-7a was grown in 200 ml of M9 minimal medium (Sambrook, 2001) supplemented with glucose (2 mM) at 30°C. The culture was divided into two 250-ml Erlenmeyer flasks until the A_{600} was approximately 0.6. PHB was added into one flask to a final concentration of 0.5 mM. The control flask received no PHB. Cells were harvested by centrifugation (6000 × *g*, 5 min, 4°C) after being cultivated for another 3 h. The harvested cells were washed twice with PB buffer (50 mM, pH 7.4), and then suspended in the same buffer to a final optical density of 10.0 at 600 nm. After incubation for 20 min in the presence of 2 mM 2, 2'-dipyridyl (Hopper and Chapman, 1971), both PHB-treated cells and control cells were supplemented with PHB to a final concentration of 2 mM and incubated at 30°C in a shaker (200 rpm). Samples were removed at appropriate intervals. All the samples were centrifuged (19,000 × *g*, 1 min) and filtered with a 0.22-μm membrane filter before being analyzed by HPLC.

Table 2. Primers used in this study

Primer	Sequence (5-3)	Purpose
phgC-F	TTCGGATCCATGGTAGCAAGTCAACAGCAACC	Amplification of *phgC* gene for expression
phgC-R	GATGTCGACTTAGAAGGCGTCTCTTTTGTCCA	
phgA-F	GTGGGATCCATGCAAACGAATCAAACGGATC	Amplification of *phgA* gene for expression
phgA-R	TAAGTCGACTTAGTATTTTGTTGTCAATTCCGGC	
phgB-F	TAGGGATCCATGGGTGCAGTTACTTATGATTTTC	Amplification of *phgB* gene for expression
phgB-R	CAAGTCGACTTATGCGCCTATTTCCATTTTCG	
bagX-F	ATTGAATTCGGGAATTGAATAATGAACACAG	Amplification of *bagX* gene for expression
bagX-R	TTTGCGGCCGCTGAAGCAAATGTAGGTTAT	
bagI-F	AAAGAATTCATGAAAGTAGGGAGTAATGTGGC	Amplification of *bagI* gene for expression
bagI-R	TAAGCGGCCGCACTATCAGGTTGGAAGCTCC	
bagL-F	AAAGAATTCATGGCTTATCAGTCAATCGGTAC	Amplification of *bagL* gene for expression
bagL-R	TCTGCGGCCGCAGCCACATTACTCCCTACTTTC	
bagK-F	ATAGAATTCGCCTTGTTTCAGACAAAGATAGG	Amplification of *bagK* gene for expression
bagK-R	AAAGCGGCCGCTCGATGTACCGATTGACTGAT	
DuET(*phgC*)-F	TCATCACCACAGCCAGGATCCGATGGTAGCAAGTCAACAGCAACC	Amplification of *phgC* gene for expression with pETDuet™-1
DuET(*phgC*)-R	GCCGAGCTCGAATTCGGATCCTTAGAAGGCGTCTCTTTTGTCCA	
DuET(*phgAB*)-F	GCGATCGCTGACGTCGGTACCATGCAAACGAATCAAACGGA	Amplification of *phgAB* gene for expression with pETDuet™-1 or coexpression with pETDuet-(*phgC*)
DuET(*phgAB*)-R	TTTACCAGACTCGAGGGTACCTTATGCGCCTATTTCCATTTTCG	
DuET(*phgA*)-F	GCGATCGCTGACGTCGGTACCATGCAAACGAATCAAACGGA	Amplification of *phgA* gene for coexpression with pETDuet-(*phgC*)
DuET(phgA)-R	GCCGAGCTCGAATTCGGATCCTTAGTATTTTGTTGTCAATTCCGGC	
DuET(*phgB*)-F	TCATCACCACAGCCAGGATCCGATGGGTGCAGTTACTTATGATTTTC	Amplification of *phgB* gene for coexpression with pETDuet-(*phgC*)
DuET(*phgB*)-R	TTTACCAGACTCGAGGGTACCTTATGCGCCTATTTCCATTTTCG	

Genome and transcriptome sequencing

The complete genome sequence of strain PHB-7a was carried out using the PacBio RSII platform (giving 163.15× coverage) by Wuhan Institute of Biotechnology. The complete genome has been deposited in Genbank (accession number: CP030926). For transcriptome sequencing, strain PHB-7a was cultured and harvested as described above. Extraction of total RNA was performed with a Spin Column Bacterial Total RNA Purification Kit (Sangon Biotech, Shanghai, China). RNA sequencing was accomplished using the Illumina Hiseq 4000× platform (giving 400× coverage) by OEbiotech Company (Shanghai, China). The RNA seq data have been deposited in SRA (accession number: SRP153765).

Construction of pETDuet-phgABC and whole cell transformation of PHB

All the genes were cloned from strain PHB-7a genomic DNA by PCR amplification with T100^{TM} Thermal Cycler (Bio-Rad). To construct the coexpression vector of pETDuet-*phgABC*, *phgC* alone was cloned and ligated to MCS1 of pETDuet™–1 to yield pETDuet-*phgC,* according to the protocol of pEASY-Uni Seamless Cloning and Assembly Kit (Transgen, China). Both *phgA* and *phgB* were then amplified together and ligated to MCS2 of pETDuet-*phgC* to yield pETDuet-*phgABC* with the same protocol. The recombinant plasmids pETDuet-*phgAB*, pETDuet-*phgAC* and pETDuet-*phgBC* were constructed similarly. All constructs were transformed into *E. coli* BL21 (DE3).

E. coli BL21 (DE3) containing *phgABC* or any two of the three genes were cultured in LB medium supplemented with ampicillin (100 μg ml^{-1}) at 37°C. When the A_{600} reached approximately 0.6 at 600 nm, isopropyl 1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.2 mM and the cells were incubated at 16°C. After 12 h cells were harvested, washed twice with PB buffer (50 mM, pH 7.4) and suspended to a final 15.0 of A_{600}. PHB was added to a final concentration of 0.5 mM for biotransformation in the presence of 5 mM glucose and the products were identified by HPLC and LC-MS. Specific activity of transformation is expressed as the production of 1 μmol of product per hour per milligram of dry weight of cells.

Cloning, overexpression and purification of PhgA, PhgB, PhgC

phgA, phgB, phgC genes were amplified with primers listed in Table 2 and inserted into pET-28a (+) to yield pET-28a-*phgA*, pET-28a-*phgB*, pET-28a-*phgC* with the pEASY-Uni Seamless Cloning and Assembly Kit. *E. coli* BL21 (DE3) carrying pET-28a-*phgA or* pET-28a-*phgB*

or pET-28a-*phgC* was cultivated with kanamycin (50 μg ml^{-1}) and induced as described above. For purification of PhgB and PhgC, harvested cells were washed twice with PB buffer (50 mM, pH 7.8) and then suspended in binding buffer (50 mM PB buffer, 135 mM NaCl, 10% glycerol, 5mM β-mercaptoethanol, 20 mM imidazole, pH 7.8) before being disrupted with a high pressure homogenizer (AH-1500; ATS Engineering Limited, Vancouver, Canada). After the cell lysates were centrifuged at 19,000 × *g* for 45 min at 4°C, and filtered through a 0.45-μm membrane filter, the cell supernatant was used for protein purification. The His-tagged proteins were purified using a 5-ml HisTrap HP column (GE Healthcare) and ÄKTA start system (GE Healthcare) according to manufacturer's instructions. A total of 30 ml of binding buffer was used to equilibrate the 5-ml HisTrap HP column before sample injection. The proteins released by elution buffer (50 mM PB, 135 mM NaCl, 10% glycerol, 5 mM β-mercaptoethanol, 250 mM imidazole, pH 7.8) were collected and then desalted with HiTrap™ 5 ml Desalting (GE Healthcare). The desalting buffer was 50 mM PB, 135 mM NaCl, 10% glycerol, 0.2 mM DTT, pH 7.8. The purified protein was determined by SDS-PAGE and stored at -80°C.

PhgA formed inclusion bodies in *E. coli.* Renaturation of the inclusion bodies was accomplished as follows. After being washed twice with washing buffer (50 mM pH 8.0 Tris-HCl, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM DTT), the inclusion bodies were dissolved in binding buffer (50 mM pH 8.0 Tris-HCl, 150 mM NaCl, 8 M Urea, 20 mM imidazole, 2 mM DTT). A Ni-IDA column was equilibrated by binding buffer and used for purifying the dissolved protein from inclusion bodies. The proteins released by elution buffer (50 mM pH8.0 Tris-HCl, 150 mM NaCl, 8 M Urea, 2 mM DTT) containing 100 mM and 500 mM imidazole were collected. The eluted proteins were in turn dialyzed against buffer 1 (10 mM pH 7.4 PBS buffer, 4 mM GSH, 0.4 mM GSSG, 2 mM EDTA, 0.4 M L-arginine, 1 M urea, 2 mM DTT) and buffer 2 (10 mM pH 7.4 PBS buffer, 20% glycerol, 2 mM DTT) in dialysis bags for 8 hours and filtered with a 0.22-μm membrane filter. Finally, the soluble proteins were stored at -80°C for further study.

Enzyme assays

p-Hydroxybenzoyl-CoA ligase was assayed as reported previously (Gibson *et al*., 1994). Optimal reaction conditions and kinetic parameters were assayed by HPLC. To determine the optimal buffer and pH for *p*-hydroxybenzoyl-CoA ligase activity, PB buffer (pH 7.2, pH 7.6, pH 7.8, and pH 8.0), Tris-HCl buffer (pH 7.2, pH 7.6, pH 8.0, pH 8.3, pH 8.6, and pH 8.9) or Tricine-NaOH buffer (pH 7.6, pH 8.0, pH 8.4, pH 8.8, pH 9.0, pH 9.2, and pH 9.6) were used in the reaction mixture described above. To determine the optimal ions for enzyme activity of PhgC, the H_6-PhgC was incubated with six types of ions (Mg^{2+}, Mn^{2+}, Co^{2+} ,Ca^{2+}, Zn^{2+}, Cu^{2+}) at 5 mM. All of the reactions mentioned above were started by addition of PHB, then the reactions were stopped after 1.0 min by adding 14 μl of 10% formic acid. The samples were vortexed and centrifuged for 15 min at 19,000 × *g* before being analyzed by HPLC.

p-Hydroxybenzoyl-CoA hydroxylase activity was assayed in 1 ml of 50 mM phosphate buffer (pH 7.6) containing NADPH (0.2 mM), FAD (25 μM) and H_6-PhgA (20 μg). The assay was initiated by addition of *p*-hydroxybenzoyl-CoA (50 μM). The activity was assayed by measuring the decrease in absorbance at 285 nm for *p*-hydroxybenzoyl-CoA and 340 nm for NADPH. The formation of gentisyl-CoA was measured at 380 nm (molar extinction coefficient 17,800 M^{-1} cm^{-1}) (Zhuang *et al*., 2004). The enzyme activities of MHB 6-hydroxylase, gentisate 1, 2-dioxygenase, maleylpyruvate isomerase and fumarylpyruvate hydrolase were assayed using the methods reported previously (Zhou *et al*., 2001; Liu *et al*., 2011; Liu and Zhou, 2012). One unit of enzyme activity was defined as the amount required for the production of 1 μmol of product per min at 30°C. Specific activities are expressed as units per milligram of protein.

Analytical techniques

Identification of aromatic compounds by HPLC was performed on a Waters e2695 Separation Module with a 2998 PDA Detector. A reverse-phase LP-C18 column (5 μm, 4.6 × 250 mm) was used to separate PHB and GA. The mobile phase containing 80% 50 mM ammonium acetate (pH 4.2) and 20% methanol was pumped isocratically at a flow rate of 1 ml min^{-1} at 30°C for 10 min. The detection wavelengths for PHB and GA were 254 and 320 nm respectively (Fairley *et al*., 2002). A reverse-phase XB-Phenyl column was used to separate PHB and *p*-hydroxybenzoyl CoA. The mobile phase was acetonitrile as solvent A and 50 mM ammonium acetate (pH 5.0) as solvent B with a linear gradient from 10 to 25% A over 7 min followed by a linear gradient of 25 to 40% A within 1 min and an isocratic hold for 2 min. The detection wavelength was 254 nm. LTQ-MS/MS analysis of CoA thioester was performed using a linear ion trap mass spectrometer (LTQ XL, Thermo Fisher Scientific) equipped with an electrospray ionization (ESI) source in negative ion mode, mass range: m/z 50–1500. The HPLC-MS (Surveyor, Thermo Fisher Scientific, San Jose, CA, USA) system consisted of a diode array detector and a HILIC Amide column (2.1 × 150 mm, 3 μm; Wekcg, China). NMR analyses for purified *p*-hydroxybenzoyl CoA, 3, 5, 6-trideutero-GA and [carboxyl-^{13}C]-PHB

were conducted with a Bruker AVANCE III600 MHz NMR spectrometer (Germany). The samples were suspended in 0.2 ml of D_2O.

Acknowledgments

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.