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Dihydrophenylalanine: A Prephenate-Derived *Photorhabdus luminescens* Antibiotic and Intermediate in Dihydrostilbene Biosynthesis

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SUMMARY

2,5-Dihydrophenylalanine (H_2Phe) is a multipotent nonproteinogenic amino acid produced by various Actinobacteria and Gammaproteobacteria. Although the metabolite was discovered over 40 years ago, details of its biosynthesis have remained largely unknown. We show here that L-H_2Phe is a secreted metabolite in *Photorhabdus luminescens* cultures and a precursor of a recently described 2,5-dihydrostilbene. Bioinformatic analysis suggested a candidate gene cluster for the processing of prephenate to H_2Phe, and gene knockouts validated that three adjacent genes *plu3042–3044* were required for H_2Phe production. Biochemical experiments validated Plu3043 as a nonaromatizing prephenate decarboxylase generating an endocyclic dihydro-hydroxyphenylpyruvate. Plu3042 acted next to transaminate the Plu3043 product, precluding spontaneous exocyclic double-bond isomerization and yielding 2,5-dihydrotyrosine. The enzymatic products most plausibly on path to H_2Phe illustrate the versatile metabolic rerouting of prephenate from aromatic amino acid synthesis to antibiotic synthesis.

INTRODUCTION

2,5-Dihydrophenylalanine (H_2Phe, *(S)*-2-amino-3-(cyclohexa-1,4-dienyl) propanoic acid, Figure 1A) has long been known as an antimetabolite of Phe (Nass et al., 1971). Initially, H_2Phe was detected as an unintended by-product of the Birch reduction of antibiotics evolidine and bacitracin (Ressler and Kashelikar, 1966), though its synthesis was later intentionally optimized and its biological activity was investigated (Snow et al., 1968). The compound was subsequently identified as a natural product produced by several *Streptomyces* species (Scannell et al., 1970; Yamashita et al., 1970; Fickenscher et al., 1971), *Pseudomonas* sp. Strain I-30 (Onishi et al., 1982), and *Erwinia amylovora* (Feistner, 1988).

H_2Phe possesses a myriad of biological activities, most presumably arising from its ability to act as an antagonist of Phe. It has broad-spectrum antimicrobial activity (Genghof, 1970) and will inhibit *E. coli* growth unless external sources of Phe are provided in culture media (Fickenscher and Zähner, 1971). It is believed that this antibiotic action arises from false-feedback inhibition of the shikimate pathway because the planar structure (Shoulders et al., 1968) of H_2Phe allows it to serve as a Phe analog (Genghof, 1970). In fact, H_2Phe can be incorporated into proteins during translation in place of Phe (Pine, 1975). H_2Phe can also function as an antifungal via disruption of microtubule assembly (Kiso et al., 2004). Studies with mammalian cell lines have shown that in human promyelocytic leukemia cells H_2Phe induces cathepsin-dependent apoptosis (Kiso et al., 2001). In vivo studies on young rats show that addition of this compound to their diet induced anorexia and was cytotoxic (Snow et al., 1968). Plants are also negatively impacted by H_2Phe. The plant pathogen *E. amylovora*, which causes a devastating disease to apple and pear trees called Fire blight, produces H_2Phe as a virulence factor (Schwartz et al., 1991). This compound has been shown to decrease production of phytoalexins, indicating that the compound acted to decrease the plant's defensive responses to pathogens (Feistner, 1988; Schwartz et al., 1991).

Biosynthetic investigations into H_2Phe production have focused primarily on determination of the metabolic precursor to the compound. It has been shown that H_2Phe is not derived from reduction of Phe (Scannell et al., 1970) but is in fact produced from prephenate, an upstream primary metabolic precursor to Phe (Shimada et al., 1978). Furthermore, isotopic-labeling studies indicate that the enzymes that convert prephenate to H_2Phe are capable of distinguishing between the two enantiotopically related halves of the prephenate cyclohexadienyl ring (Figure 1A) (Shimada et al., 1978). Given that the conversion of prephenate to H_2Phe must at some point involve a reduction, the possibility that a reduced prephenate derivative (dihydroprephenate) would be used in the biosynthetic pathway was raised. However, feeding of the tritium-labeled dihydroprephenic acid to a H_2Phe-producing *Streptomyces* strain did not result in formation of radiolabeled H_2Phe, indicating that reduction proceeds not on the prephenate scaffold but on a downstream intermediate (Shimada et al., 1978).

Our recent work has established a new family of prephenate-utilizing enzymes that reroutes prephenate away from aromatic amino acid biosynthesis into secondary metabolism (Mahlstedt and Walsh, 2010; Mahlstedt et al., 2010). These prephenate

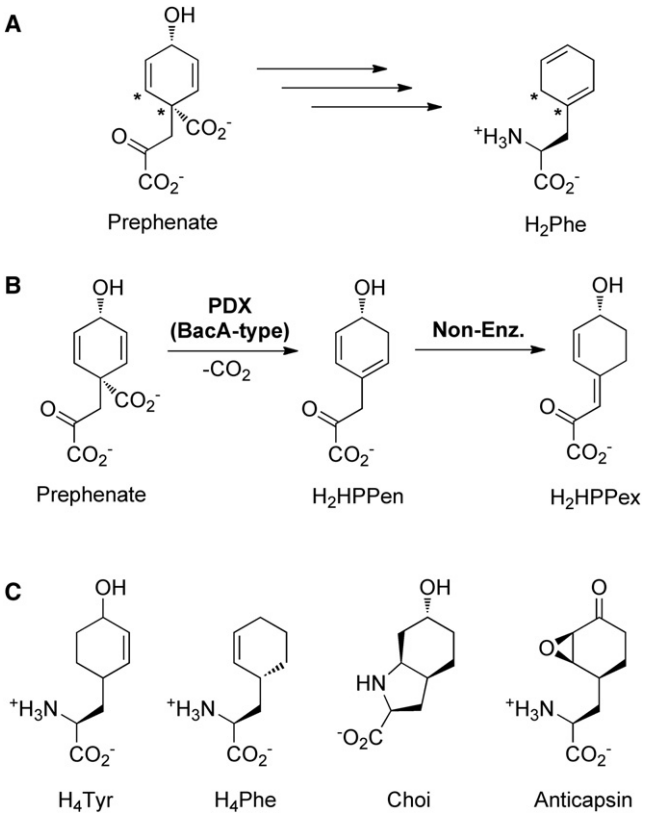


Figure 1. Prephenate-Derived Nonproteinogenic Amino Acids

(A) Prephenate is the metabolic precursor to H_2Phe. The biochemical transformations required to produce H_2Phe distinguish between the two halves of the starting material in production of the final amino acid product.

(B) PDX enzymes decarboxylate prephenate to produce H_2HPPen, which can nonenzymatically undergo isomerization to H_2HPPex. PDX catalyzed decarboxylative isomerization can yield the S or R configuration at the C7 position of H_2HPPen. Based on the isotopic-labeling studies in (A) and the double-bond positions in H_2HPPen, C7 is assumed to adopt the R configuration in H_2Phe biosynthesis.

(C) Selected examples of prephenate-derived nonproteinogenic amino acid products.

decarboxylase (PDX) enzymes decarboxylate but do not aromatize prephenate to produce an *en*docyclic *dihydro-h*ydroxy*ph*enyl*p*yruvate ("H_2HPPen," 3-(4-hydroxycyclohexa-1,5-dienyl)-2-oxopropanoic acid) (Figure 1B). This scaffold can then be transformed to a range of biologically relevant nonproteinogenic amino acids, such as tetrahydrotyrosine (H_4Tyr), tetrahydrophenylalanine (H_4Phe), choi, and anticapsin (Figure 1C) (Mahlstedt and Walsh, 2010; Mahlstedt et al., 2010). Given the similarity of H_2Phe to these amino acids and the knowledge that it is derived from prephenate, we suspected the involvement of this novel enzyme family. In this work, we identify L-H_2Phe as a secreted product of the insect-pathogenic bacterium, *Photorhabdus luminescens*, determine its intermediacy in the biosynthesis of the recently discovered 2,5-dihydrostilbene product (Kontnik et al., 2010), identify a gene cluster involved in H_2Phe biosynthesis, and begin a detailed in vitro biochemical investigation into the formation of this multipotent nonproteinogenic amino acid.

RESULTS

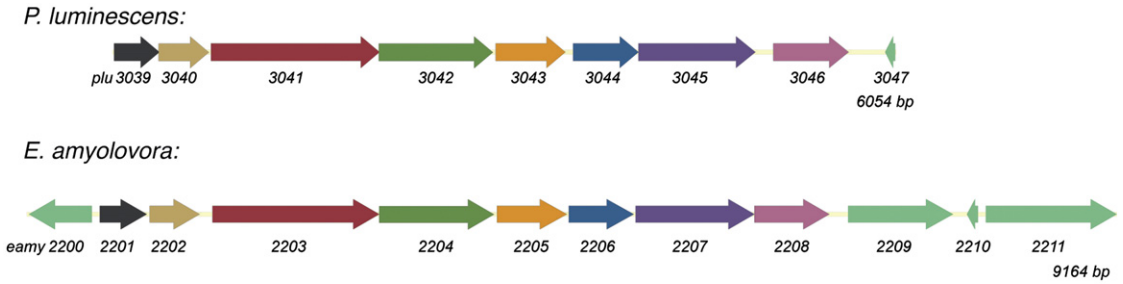
Identification of Candidate Gene Cluster

Putative PDXs were identified in the genomes of *E. amylovora* and *P. luminescens* encoded by *eamy2205* and *plu3043*, respectively. Intriguingly, inspection of the sequence surrounding these genes revealed the existence of an eight-gene set conserved in both organisms (Figure 2; see Table S1 available online). Based on the finding that this gene cluster encoded proteins that could be biosynthetic (*eamy2203* and *plu3041*, phenylacetate-CoA ligase; *eamy2204* and *plu3042*, PLP-dependent aminotransferase; *eamy2206* and *plu3044*, AhpD-family disulfide peroxidase; *eamy2207* and *plu3045*, ornithine cyclodeaminase), we suspected that this could be a natural product gene cluster. Additionally, a clustered amino acid efflux protein homolog (*eamy2208* and *plu3046*, transporter) would presumably provide the host with resistance to the encoded product by transporting the small molecule into the culture medium. A literature search revealed the existence of a prephenate-derived H_2Phe product produced by *E. amylovora* (Feistner, 1988). Although H_2Phe had not been identified as a product of *P. luminescens*, the recent discovery of a 2,5-dihydrostilbene product from the microorganism suggested that it could be produced as an intermediate in the construction of reduced stilbenes (Kontnik et al., 2010). With this knowledge we chose to determine whether H_2Phe could be isolated from *P. luminescens*, to examine this cluster's relevance in H_2Phe biosynthesis by constructing genetic knockouts, and to initiate in vitro biochemical characterization of the pathway.

Identification of L-H_2Phe in Wild-Type *P. luminescens* and Knockout Strains

To determine the presumed gene cluster's role in the biosynthesis of H_2Phe, scar-less deletions of *plu3042*, *plu3043*, *plu3044,* and *plu3045* were individually constructed in *P. luminescens* TT01 utilizing allelic exchange mutagenesis (Figure 3A). The resulting strains were cultivated in an L-proline rich medium to enhance production of secondary metabolites (Crawford et al., 2010). Using liquid chromatography-mass spectrometry (LC/MS), differential analysis of the secreted metabolites between wild-type cultures and mutant cultures was conducted to identify deleted products. Comparisons of ultraviolet and visible (UV/Vis) absorption traces showed minimal differences among the strains due to the complex metabolic backgrounds. However, comparisons of the MS profiles showed a dramatic difference in the production of a positive ion at 168 atomic mass units, consistent with protonated H_2Phe (Figure 3B). The gene products Plu3042, Plu3043, and Plu3044 were required for its synthesis because deletion of *plu3042*, *plu3043*, and *plu3044* completely abrogated production. The Δ*plu3045* strain continued production at levels comparable to wild-type under the conditions of the experiment.

We developed a method for the extraction and purification of the identified 168 positive ion from the lyophilized cleared culture medium. Due to the low ultraviolet absorption of the secreted product, it was tracked by LC/MS through each purification step. The purified product was subjected to detailed 1D- (^{1}H) and 2D-NMR analyses (gCOSY, HSQCAD, and gHMBC) (Table 1; Figures S1A–S1E) and high-resolution MS ($C_9H_{14}NO_2$;



Gene		Predicted Activity
plu3039	*eamy2201*	stress response α/β barrel
plu3040	eamy2202	no predictive homology
plu3041	*eamy2203*	phenylacetate-CoA ligase
plu3042	*eamy2204*	aminotransferase
plu3043	*eamy2205*	BacA prephenate decarboxylase
plu3044	*eamy2206*	AhpD ; carboxymuconolactone decarboxylase
plu3045	*eamy2207*	ornithine cyclodeaminase
plu3046	*eamy2208*	transporter

Figure 2. Gene Cluster in *P. luminescens* and *E. amylovora* Shown to Be Involved in H_2Phe Biosynthesis
See also Table S1 and Figure S3.

calculated 168.1025; observed, 168.1026; error 0.6 ppm). All data are in agreement with the conclusion that the identified ion produced by *P. luminescens* TT01 represents H_2Phe.

H_2Phe slowly undergoes spontaneous oxidative aromatization to Phe in the presence of air (Snow et al., 1968), and rotary evaporation causes aromatization (Feistner, 1988). To identify the absolute configuration of the *P. luminescens* product, isolated H_2Phe was subjected to several rounds of rotary evaporation from water, and the oxidation product was analyzed using the Marfey's method (Marfey, 1984; Fujii et al., 1997). The Marfey's derivatized product coeluted with L-Phe, but not D-Phe, indicating that the secreted metabolite is L-H_2Phe (Figure S1F).

L-H_2Phe Is an Intermediate in 2,5-Dihydrostilbene Biosynthesis

P. luminescens is involved in a multipartite symbiosis, in which the bacterium has formed an evolutionary alliance with *Heterorhabditis* nematodes to effectively kill a wide range of insect larvae in the soil (Waterfield et al., 2009). The bacterium also produces a broad range of antibiotics, in addition to H_2Phe identified here, to overcome the insect host and outcompete competitors in the decomposing insect carcass. During the *P. luminescens* lifecycle, the bacterium oscillates between its mutualistic nematode host and its pathogenic insect host. A LysR-type transcriptional repressor, HexA, was implicated in regulating the mutualist-pathogen transition in the related bacterium, *Photorhabdus temperata* (Joyce and Clarke, 2003). Bacteria belonging to the *Photorhabdus* genera are the only described bacterial producers of stilbenes (Richardson et al., 1988), which are common phenylpropanoid plant products (Chong et al., 2009), and deletion of the HexA regulator in *P. luminescens* and *P. temperata* led to an upregulation of the stilbene class (Kontnik et al., 2010). Interestingly, 2,5-dihydrostilbene ((*E*)-5-(2-(cyclohexa-1,4-dienyl)vinyl)-2-isopropylbenzene-1,3-diol, Figures 4A and 4B) is dramatically upregulated in the *P. luminescens hexA* mutant, which is barely detectable in wild-type cultures grown in rich lab media (Kontnik et al., 2010).

To determine if the secreted L-H_2Phe antibiotic also serves as an intermediate in 2,5-dihydrostilbene biosynthesis, a $^{13}C_6$-labeled H_2Phe derivative was synthesized via a Birch reduction of $^{13}C_6$-labeled L-Phe for isotopic-labeling studies (Figure 4A) (Skolaut and Rétey, 2001). Various concentrations of the synthetic $^{13}C_6$-L-H_2Phe were fed to the *P. luminescens hexA* mutant. As judged by LC/MS analysis of organic extracts, increasing concentrations of exogenous $^{13}C_6$-L-H_2Phe in the culture medium led to an increase in 2,5-dihydrostilbene production (Figures 4B and 4C) with a concomitant increase in $^{13}C_6$-isotopic enrichment (Figure 4D; Figure S2A). The isotopic-labeling studies indicate that whereas L-H_2Phe is a secreted metabolite, it can also serve as an intermediate in 2,5-dihydrostilbene biosynthesis. $^{13}C_6$-L-H_2Phe also incorporated to an extent into the major aromatic stilbene (Figure 4B, negative ion, *m/z* ($^{12}C_6$) = 253 and *m/z* ($^{13}C_6$) = 259), although oxidation of labeled H_2Phe, 2,5-dihydrostilbene, or any intermediate between could contribute to the isotopic enrichment.

Given that the biosynthesis of phenylpropanoids, such as the stilbenes, is initiated by a Phe ammonia lyase that converts Phe to cinnamate (Vogt, 2010), one would expect the corresponding reduced 2,5-dihydrocinnamate ((*E*)-3-(cyclohexa-1,4-dienyl) acrylic acid) to also be involved in 2,5-dihydrostilbene biosynthesis. As in our H_2Phe LC/MS analysis above (Figure 3), a positive ion peak (*m/z* = 151) consistent with protonated 2,5-dihydrocinnamic acid was also detected as a secreted product in the cleared culture medium only in wild-type and in the *plu3045* mutant strain cultures (Figure S2B). Low production of the intermediate precluded detailed structural characterization. Complete abrogation of the ion product in the three mutants, *plu3042–plu3044*, provides genetic support for the production of

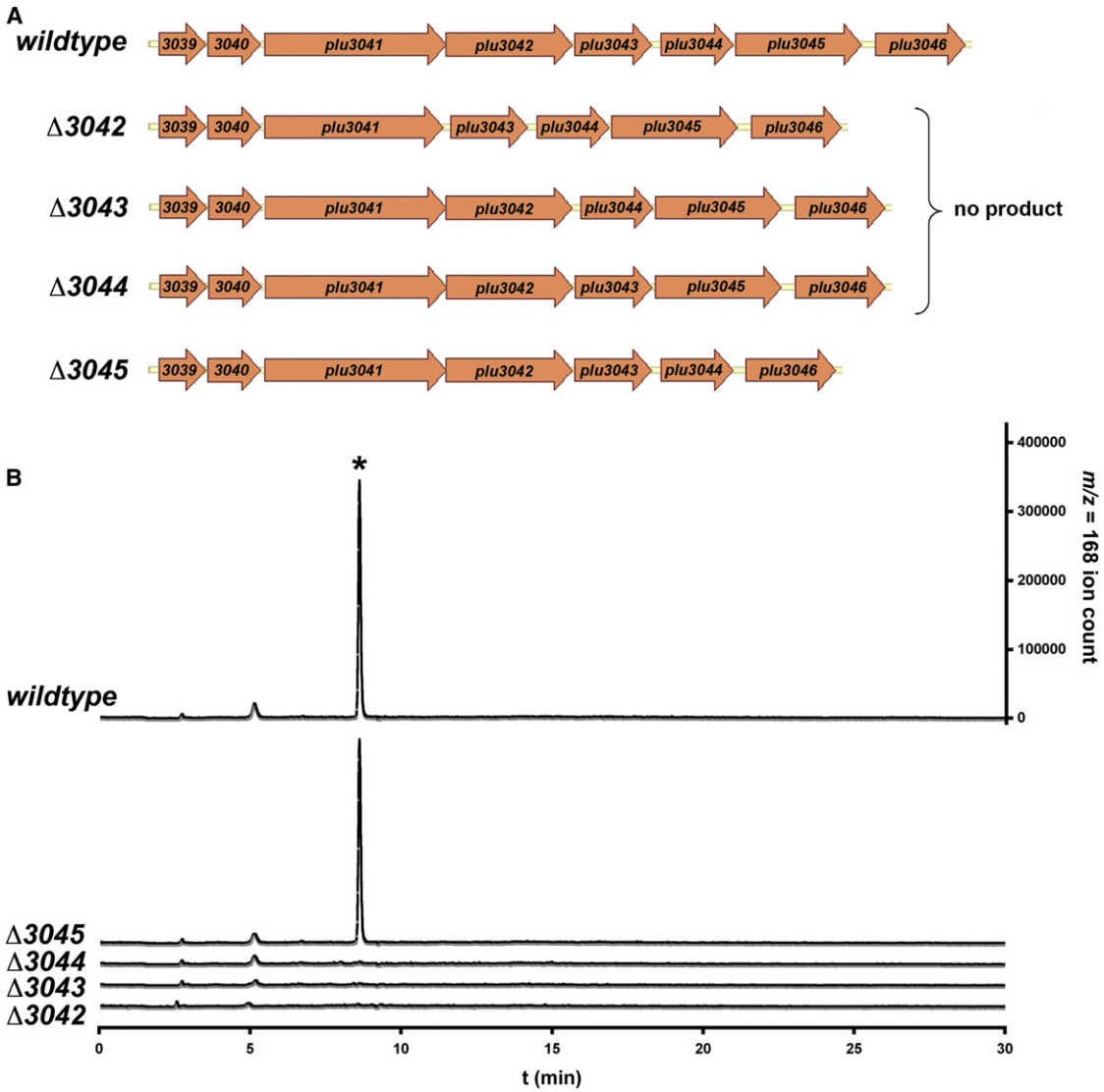


Figure 3. Genetic Connection of Candidate Biosynthetic Gene Cluster to H_2Phe

(A) Scar-less deletion mutants constructed in *P. luminescens* using allelic-exchange mutagenesis.

(B) Genes *plu3042*, *plu3043*, and *plu3044* were required for the synthesis of H_2Phe. Deletion of *plu3045* showed no effect. The LC/MS ion current window (167.7–168.7) for protonated H_2Phe (*m/z* = 168 ion) is shown for wild-type and each of the mutants.

See also Table S3.

2,5-dihydrocinnamate as a downstream intermediate in 2,5-dihydrostilbene biosynthesis. Aromatization of the cyclohexadienyl side chain of the secreted H_2Phe, 2,5-dihydrocinnamate, or 2,5-dihydrostilbene would likely occur when subjected to the insect's oxidative defenses (Kontnik et al., 2010). Strikingly, the aromatic stilbene derivative has been shown to serve as an antimicrobial (Hu and Webster, 2000), insect immunosuppressant (Eleftherianos et al., 2007), and nematode development signal (Joyce et al., 2008).

Expression of Plu3042, Plu3043, and Plu3044 in *E. coli* and Protein Purification

To begin investigating the biochemical transformations from prephenate to L-H_2Phe, the biosynthetically required genes *plu3042*, *plu3043*, and *plu3044* were amplified by PCR from *P. luminescens* TT01 genomic DNA. The PCR products were cloned into a pET-28b expression vector that was then transformed into the *E. coli* BL21(DE3) expression strain. The expressed proteins each harbor a N-terminal His_6 tag, allowing purification of the protein by Ni-NTA affinity chromatography to near homogeneity (SDS-PAGE analysis, Figure S3A). Plu3042 was acquired at 19.5 mg/l yield, Plu3043 at 19.5 mg/l, and Plu3044 at 25 mg/l. A minor truncation product of Plu3042 was observed by SDS-PAGE analysis. Both the major and minor protein products were identified to be Plu3042 by proteolytic digestion (trypsin) and LC/MALDI-MS/TOF-TOF analysis (Figure S3B).

Table 1. ^{1}H and ^{13}C Data for L-H_2Phe in D_2O

	^{13}C ppm	^{1}H ppm, mult. (Hz)	gCOSY	gHMBC
1	177.6			
2	52.9	3.68, dd (4.7, 9.3)	3a/3b	1, 3, 4
3a	39.8	2.37, dd (14.5, 9.2)	2, 3b, 9 w	1, 2, 4, 5, 9
3b		2.56, dd (14.3, 4.5)	2, 3a, 9 w	1, 2, 4, 5, 9
4	129.8			
5	27.6	2.6–2.75 (2.63 m)[a]	6	4, 8, 6/7/9 o
6	124.61	5.79 m	5	4, 5, 8, 7/9 o
7	124.61	5.79 m	8	5, 8, 6/9 o
8	26.3	2.7–2.75 (2.73 m)[a]	7, 9	4, 5, 6/7/9 o
9	124.58	5.66 br s	3a/3b w, 8	3, 5/8 w, 6/7 o

The ^{1}H signal was referenced to solvent, and the ^{13}C signal was referenced by VNMRJ software (Varian). br, broad; w, weak; o, overlapped; mult, multiplicity.

[a] Signals in multiplet were distinguishable in gCOSY and HSQCAD experiments. The ^{1}H and ^{13}C chemical shift values of the isolated metabolite were similar to the previously reported synthetic H_2Phe trifluoroacetic acid salt (Skolaut and Rétey, 2001), except the pH-sensitive C1 carbon and the α- and β-proton signals were slightly shifted. See also Figure S1.

Plu3043 Produces H_2HPP

Plu3043 is a predicted homolog to the BacA, SalX, and AerD enzymes, which were previously shown to convert prephenate to the nonaromatized H_2HPPen (Figure 1B) (Mahlstedt and Walsh, 2010; Mahlstedt et al., 2010). The endocyclic alkene of H_2HPPen spontaneously isomerizes to the exocyclic alkene of H_2HPPex (3-(4-hydroxycyclohex-2-enylidene)-2-oxopropanoic acid). To test whether Plu3043 catalyzes an identical reaction as its characterized homologs BacA, AerD, and SalX, we turned to NMR. The ^{1}H-NMR spectrum of the Plu3043-catalyzed reaction product was collected and was identical to that of the previously characterized H_2HPPex (Table S2 and Figure S4) (Mahlstedt and Walsh, 2010). This verifies that Plu3043 does indeed catalyze the reaction characteristic of the PDX enzyme family.

Plu3043 Transaminates H_2HPPen to H_2Tyr

PDXs convert prephenate to two possible scaffolds that can be transformed to unique nonproteinogenic amino acids. The first is the direct product of the reaction, H_2HPPen (Figure 1B). However, this product is unstable and nonenzymatically isomerizes to the compound H_2HPPex. Our past work has indicated that H_2HPPex is on the pathway to the amino acids anticapsin (producer, *Bacillus subtilis*), choi (*Planktothrix agardhii*), H_4Tyr (*Bacillus subtilis*), and H_4Phe (*Salinispora tropica*; Figure 1C). In the case of anticapsin and choi biosynthesis, the biosynthetic gene clusters encode an enzyme (BacB and AerE, respectively) that can accelerate the H_2HPPen to H_2HPPex isomerization. However, with H_4Phe biosynthesis we have not identified an isomerase that can perform a similar reaction, and so at the present, we must work with the assumption that this conversion occurs nonenzymatically in vivo. In considering the biosynthesis of H_2Phe, we propose a novel use of the H_2HPPen scaffold that contrasts with the previously studied pathways. Ultimately, in forming H_2Phe, the ring olefins need to be brought out of conjugation. We suspected that this could be accomplished more readily on the less stable, less extensively conjugated H_2HPPen as opposed to the fully conjugated H_2HPPex. We hypothesized that the nonenzymatic isomerization could be prevented by transamination of the α-keto acid H_2HPPen to the amino acid L-H_2Tyr ((*S*)-2-amino-3-((*R*)-4-hydroxycyclohexa-1,5-dienyl) propanoic acid). This reaction replaces the pyruvyl ketone with a primary amine and removes the driving force for isomerization because the C = O π electrons would no longer be present. Given the homology to aminotransferases, we tested the enzyme Plu3042 for activity with the H_2HPPen product.

This hypothesis was first assessed by UV/Vis spectroscopy. The H_2HPPex product has a characteristic chromophore at 295 nm. Reactions containing prephenate and Plu3043 would ultimately display this 295 nm chromophore if left long enough for the nonenzymatic H_2HPPen to H_2HPPex transformation to occur. However, reactions containing Plu3042 and an accepted amino acid cosubstrate in addition to Plu3043 and prephenate would produce the less colorful H_2Tyr, and H_2HPPex would not be formed. To determine the best amino acid cosubstrate for Plu3042, all 20 proteinogenic amino acids were tested in reactions, and the products were analyzed by scanning from 220 to 600 nm (Figure 5B; Table S4). It was found that inclusion of L-Gln best precluded formation of the 295 nm chromophore, supporting our hypothesis and implicating L-Gln as the optimal amino donor. Other amino acids, such as L-Leu, displayed some activity with Plu3042, though to a lesser extent than L-Gln. This is not unexpected given general substrate promiscuity of transaminases (Taylor et al., 1998).

Further structural confirmation of the Plu3043-Plu3042 product came from NMR experiments. These reactions contained both the cosubstrate L-Gln and coproduct 2-oxo-glutaramate, which complicated analysis of spectra. To mask signal from these compounds, a poly-deuterated Gln (2,3,3,4,4-D5) was utilized in the enzyme reactions. ^{1}H, gCOSY, gHSQC, and gHMBC spectra were collected on the enzymatic product, which confirmed its assignment as H_2Tyr (Table 2 and Figures 5A and 5C; S5A–S5C). Three olefinic protons are observed in the ^{1}H spectrum: H_5 at δ_H 6.07, H_6 at δ_H 6.02, and H_9 at δ_H 5.78. Coupling between H_5 and H_6 is observed, as well as between H_6 and the H_7 proton α to the alcohol at δ_H 4.26. The downfield chemical shift of H_7 indicates retention of the C_7-OH group. 2D gCOSY reveals coupling of H_7 to a proton peak at δ_H 2.48, which is in turn coupled to the olefinic proton H_9. Integration of the ^{1}H-NMR peak at 2.48 indicates two protons, H_{8a} and H_{8b}, and the gHSQC phase information verifies that an even number of protons are bound to C_8 at δ_C 31.52. This extensive spin system confirms assignment of the cyclohexadienyl side chain. The α proton H_2 is present at δ_H 3.77, and is coupled to the H_{3a} and H_{3b} protons at δ_H 2.56 and 2.70. By gHMBC we observe a spin system between each of these protons and C_1 at δ_C 174.83, completing the assignment.

DISCUSSION

The compound H_2Phe has been long known both as a synthetically reduced form of Phe (Snow et al., 1968) and as a natural product (Scannell et al., 1970; Yamashita et al., 1970; Fickenscher et al., 1971; Onishi et al., 1982; Feistner, 1988), though little has been known about its biosynthesis until this work.

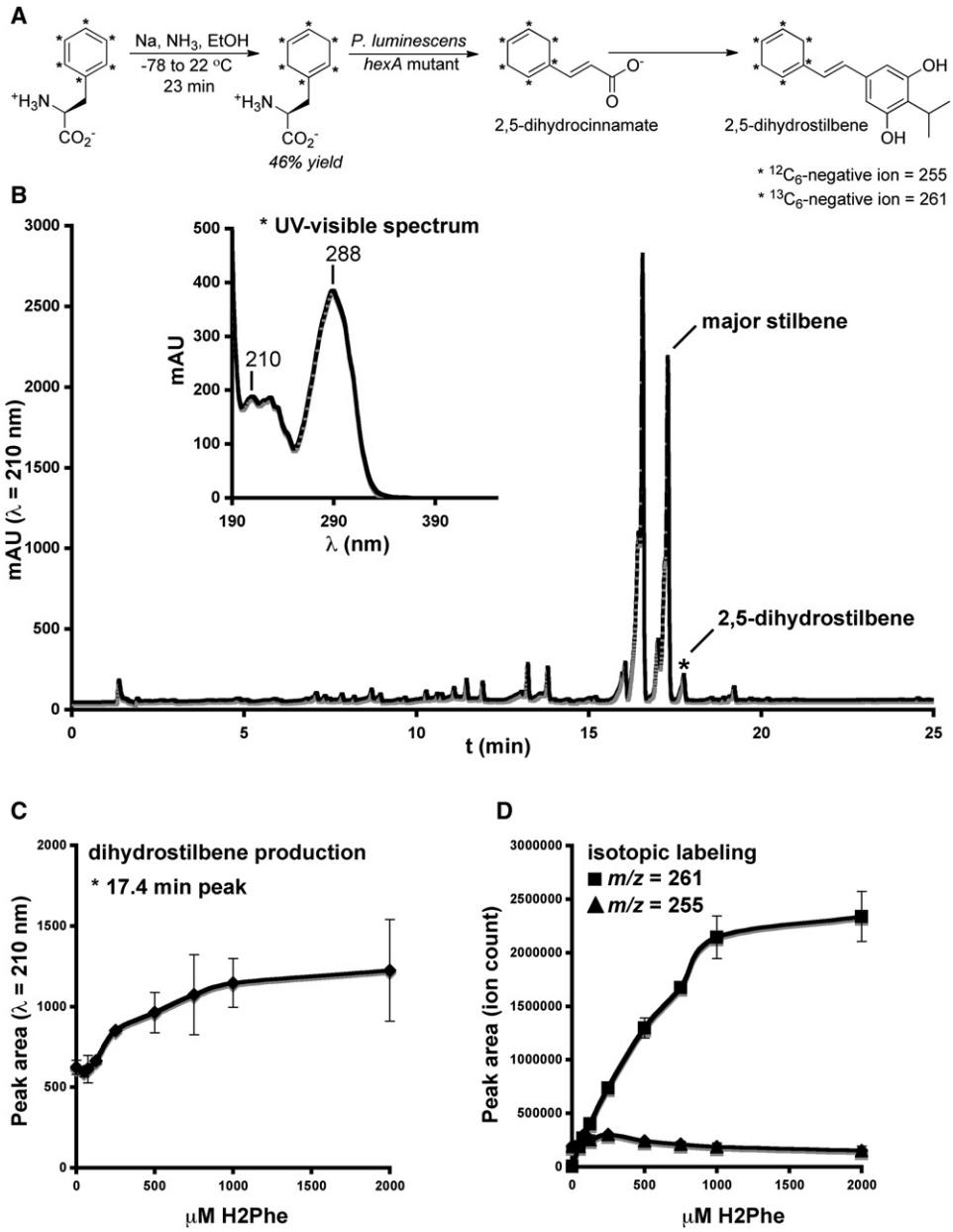


Figure 4. Isotopic-Labeling Studies on 2,5-Dihydrostilbene Biosynthesis

(A) Birch reduction of $^{13}C_6$-L-Phe to $^{13}C_6$-L-H_2Phe, which was used to label 2,5-dihydrostilbene in the *P. luminescens hexA* mutant.

(B) Representative ultraviolet absorption trace from the LC/MS data set. The asterisk (*) marks the 2,5-dihydrostilbene peak in the organic extract analysis. The major aromatic stilbene is also indicated, which primarily utilizes Phe as a metabolic precursor, but oxidative aromatization of the 1,4-cyclohexadienyl ring in 2,5-dihydrostilbene also leads to the aromatic stilbene.

(C) Increasing quantities of exogenous $^{13}C_6$-L-H_2Phe led to increased 2,5-dihydrostilbene production in the *hexA* mutant, as judged by integrating the LC peak (λ = 210 nm; error bars represent SD).

(D) Isotopic enrichment of 2,5-dihydrostilbene. The MS ion current peaks were integrated for the ^{12}C- (ion current window, 254.7–255.7) and $^{13}C_6$-labeled (260.7–261.7) products (error bars represent SD).

See also Figure S2.

Given evidence that H_2Phe is derived from prephenate (Shimada et al., 1978), we hypothesized the involvement of a member of the newly characterized PDX class of enzymes in catalyzing the first transformation in the H_2Phe biosynthetic pathway. A BLAST search revealed a gene encoding a likely PDX in the known H_2Phe producer *E. amylovora*, as well as in *P. luminescens*, an organism not known to produce H_2Phe. Moreover, the PDX candidates were identified in an eight-gene cluster in both bacterial genomes. This eight-gene cluster is also found in *Pseudomonas syringae* pv *syringae* FF5. Although

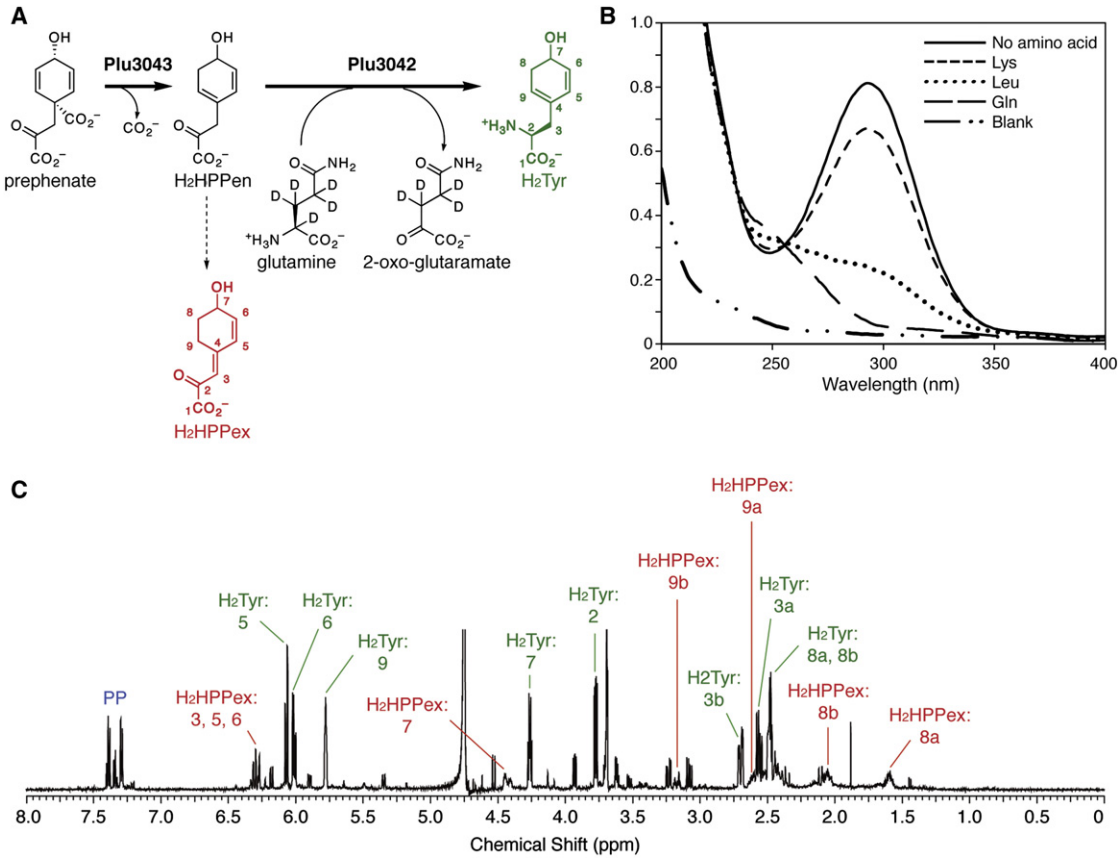


Figure 5. Transamination of H_2HPPen Averts Spontaneous Exocyclic Double-Bond Isomerization to Yield L-H_2Tyr Most Plausibly on Path to L-H_2Phe

(A) Dual-enzyme reaction to produce H_2Tyr. See also Figure S4 and Table S2.

(B) UV/Vis test of Plu3042 activity. Prephenate, Plu3042, Plu3043, and a variety of amino acids were incubated overnight at room temperature. Plu3043 decarboxylates prephenate to H_2HPPen. When the added amino acid serves as a cosubstrate for the PLP-dependent transamination of H_2HPPen, nonenzymatic isomerization to H_2HPPex is prevented, precluding observance of the H_2HPPex peak at 295 nm (as in the case with Gln, and to a lesser extent with Leu and Ile). See also Table S4.

(C) ^{1}H-NMR of H_2Tyr from reaction in (A). H_2HPPex contaminant peaks are labeled in red; H_2Tyr peaks in green.

this organism has not yet been shown to produce H_2Phe, another *Pseudomonas* has been shown to produce the compound (Onishi et al., 1982). In this work, we have established H_2Phe as a product of *P. luminescens* and used genetic deletions of genes *plu3042*, *plu3043*, *plu3044*, and *plu3045* to verify that this cluster is involved in production of H_2Phe.

Using isotopic-labeling studies, we showed that H_2Phe is incorporated into the reduced stilbene 2,5-dihydrostilbene. The biosynthesis of the aromatic stilbene derived from Phe was previously described (Joyce et al., 2008). In part, a Phe ammonia lyase converts Phe to cinnamate, which accumulates in the culture medium (Paik et al., 2005; Chalabaev et al., 2008). During expression of the stilbene pathway, cinnamate is utilized as a precursor of the downstream type II polyketide synthase proteins involved in bacterial stilbene biosynthesis. Phe ammonia lyases are known to accept H_2Phe to produce the corresponding 2,5-dihydrocinnamate (Hanson et al., 1979). As expected, we observed a low-abundance ion product in the cleared spent culture medium consistent with 2,5-dihydrocinnamate. It is likely that H_2Phe is converted to 2,5-dihydrostilbene via promiscuity in the stilbene biosynthetic machinery.

In this study, in vitro biochemical investigations have found that formation of H_2Phe likely arises from the H_2HPPen product of PDX Plu3043. This pathway contrasts with the prephenate-derived secondary metabolic pathways previously investigated, which use the stable H_2HPPex in formation of nonproteinogenic amino acids (Mahlstedt and Walsh, 2010; Mahlstedt et al., 2010). The PLP-dependent transaminase Plu3042 then converts H_2HPPen to the amino acid L-H_2Tyr, a compound that no longer isomerizes to an exocyclic diene-containing counterpart (Figure 5).

Although we have shown that prephenate can be converted to H_2Tyr, the enzymatic reactions that would be required to convert this to H_2Phe remain to be elucidated. This transformation would require a two-electron reduction and a dehydration. Through our genetic work we have identified an additional gene product, Plu3044, that is critical to H_2Phe production. Intriguingly, this protein displays only weak homology to other characterized enzymes including AhpD (Table S1), a disulfide

Table 2. ^{1}H and ^{13}C Data for H_2Tyr in D_2O

	^{13}C[a] ppm	^{1}H ppm, mult (Hz)	gCOSY	gHMBC
1	174.83			
2	53.83	3.77, ddd (8.2, 4.7, 1)	3a, 3b	1, 3, 9, 4/5/6[c]
3a	36.57	2.7, dd (14.7, 4.7)	2, 3b	1, 2, 9, 4/5/6[c]
3b		2.56, dd (14.7, 8.2)	2, 3a	1, 2, 9, 4/5/6[c]
4	127.5[b]			
5	127.6	6.07, dd (9.7, 1)	6, 9	3, 7, 9, 4/5/6[c]
6	127.38	6.02, dd (9.7, 5)	5, 7	7, 8, 4/5/6[c]
7	61.87	4.26, dt (7, 4.8)	6, 8a/b	8, 9, 4/5/6[c]
8a	31.52	2.48, m	7, 9	2, 7, 9, 4/5/6[c]
8b		2.48, m	7, 9	2, 7, 9, 4/5/6[c]
9	125.17	5.78, br t (4)	5, 8a/b	3, 7, 8

See also Figure S5. mult, multiplicity.
[a] Carbon shifts assigned by HSQC and HMBC.
[b] An exact ^{13}C chemical shift for C_4 could not be assigned, though HMBC analysis suggests that it is in the range of 127–128 ppm.
[c] Carbons 4/5/6 are indistinguishable by HMBC due to similar chemical shifts.

peroxidase (Bryk et al., 2002; Nunn et al., 2002; Koshkin et al., 2003) and MA3736, a disulfide reductase that contains an iron-sulfur cluster (Lessner and Ferry, 2007). Each of these enzymes contains a crucial CXXC motif. However, Plu3044 only contains one of these critical Cys residues. In fact, there is only one additional Cys in the entire 180 amino acid Plu3044 sequence, located significantly C terminal to the first. This makes it unlikely that this protein binds a [FeS] cluster and suggests that it may have a function distinct from its disulfide reductase homologs.

We have purified this protein to near homogeneity through heterologous expression in *E. coli* at high yield of soluble protein (25 mg/l), suggesting that the protein is properly folded. The purified protein displays no absorbance characteristic of an iron-sulfur cluster or any other readily identifiable cofactor. Unfortunately, to date, we have not detected any conversion of H_2Tyr to H_2Phe in the presence of Plu3044 or a codon-optimized *E. amylovora* homolog Eamy2206, despite testing with a selection of protein constructs and reaction conditions (detailed in Supplemental Experimental Procedures). It is not yet clear how H_2Tyr is converted by net replacement of the C_4-OH by -H on the way to H_2Phe. Completion of the pathway will require future work, likely investigating the other genes in the eight-gene cluster for unexpected biosynthetic activity.

SIGNIFICANCE

Because microbial genome sequence information is generated at an exponential rate, we continue to observe a growing biosynthetic potential for the construction of structurally diverse small molecules, some of which would be expected to regulate complex symbiotic interactions or have therapeutic applications. Most of these biosynthetic pathways observed in microbial genome sequences are "orphaned," and they either encode completely new molecular scaffolds yet to be discovered or previously discovered molecules in which we know very little about their biosynthesis (Davies, 2011; Winter et al., 2011). The connection of the previously orphaned eight-gene cluster located in the genomes of the plant pathogen *E. amylovora* and the insect pathogen *P. luminescens* to the synthesis of H_2Phe illustrates how primary metabolites such as prephenate can be routed in diverse ways to antibiotic synthesis. In this case, the known broad activities of L-H_2Phe against other microbes, plants, and animals would contribute to a successful pathogenesis. More broadly, the endocyclic or exocyclic H_2HPP intermediates can be selectively processed to produce a range of products, such as H_2Phe, H_4Phe, H_2Tyr, H_4Tyr, choi, and anticapsin. Given the structural diversity arising from PDX (prephenate decarboxylase) homologs and their involvement in antibiotic synthesis, PDX sequences could be utilized as a powerful genome-mining tool for the identification of novel nonproteinogenic amino acids or elaborated natural products. Indeed, in *P. luminescens*, H_2Phe is further advanced to a dihydrostilbene derivative. Aromatization of the cyclohexadienyl side chain when subjected to the insect's oxidative defenses would lead to a product known to serve as an antibiotic and a developmental modulator of its animal hosts.

EXPERIMENTAL PROCEDURES

Materials

E. coli TOP10 and BL21(DE3) competent cells were purchased from Invitrogen. Plasmid pET-28b was purchased from Novagen. DNA oligomers were ordered from Integrated DNA Technologies. PfuTurbo DNA polymerase was acquired from Stratagene. Restriction enzymes, Phusion DNA polymerase, and *Taq* DNA Ligase were purchased from New England BioLabs. T5 exonuclease was acquired from Epicentre Biotechnologies. Plasmid and oligonucleotide purification was performed with kits from QIAGEN. The Molecular Biology Core Facilities at the Dana-Farber Cancer Institute (Boston) performed DNA sequencing. Nickel-nitrilotriacetic acid agarose (Ni-NTA) resin was purchased from QIAGEN. Protein concentration was determined using a Coomassie Plus Kit from Pierce with BSA as a standard. Bio-Rad Mini-Protean TGX gels were used for SDS-PAGE. Proteolytic digestion and protein identification using an ABI4800 MALDI/TOF-TOF were performed at the Dana-Farber/Harvard Cancer Center Cancer Proteomics Core facility.

Chemicals were purchased from Sigma-Aldrich, except (2,3,3,4,4-D5)-L-glutamine, which was obtained from Cambridge Isotope Laboratories. Enzyme reactions were quenched by filtration using 0.5 ml Millipore Amicon Ultra 10 kDa MWCO centrifugal filter devices. NMR solvents were obtained from Cambridge Isotope Laboratories. The 100 × 2.1 mm 5 μm Hypercarb column used for analytical HPLC was acquired from Thermo Scientific.

Instrumentation

UV/Vis spectroscopic analysis was performed using a Varian Cary 50 Bio UV-Visible Spectrophotometer. ^{1}H- and 2D-NMR spectra were recorded on a Varian Inova 600 MHz spectrometer at the Harvard Medical School East Quad facility. High-resolution LC/MS data were collected on an Agilent 6520 Accurate-Mass Q-TOF Mass Spectrometer fitted with an electrospray ionization (ESI) source. A 50 × 2.1 mm 5 μm Hypercarb column from Thermo Scientific was used for high-resolution-LC/MS. Low-resolution LC/MS data were collected on an Agilent 6130 single quadrupole with an ESI source. Analytical reverse-phase HPLC was performed on a Beckman Coulter System Gold instrument (126 solvent module, 168 detector) or on an Agilent 1100 system.

Preparation of Knockout Constructs

Deletion constructs for the generation of scar-less mutants were individually prepared for genes *plu3042*, *plu3043*, *plu3044*, and *plu3045* in *P. luminescens* TT01. The gDNA from *P. luminescens* TT01 was isolated as previously described (Syn and Swarup, 2000), and the direct upstream region

of each gene was amplified by PCR using primer pairs Plu304(2, 3, 4, or 5)-A5 and Plu304(2, 3, 4, or 5)-A3 (Table S3). The direct downstream region of each gene was amplified using primer pairs Plu304(2, 3, 4, or 5)-B5 and Plu304(2, 3, 4, or 5)-B3. These upstream and downstream PCR products for each gene served as templates in a final overlap-extension PCR reaction, using primer pairs Plu304(2, 3, 4, or 5)-A5 and Plu304(2, 3, 4, or 5)-B3. The resulting fused exchange sequences cleanly excised the coding sequences of interest. The exchange sequence PCR product was digested with SacI and inserted into the suicide plasmid pDS132 (Philippe et al., 2004), which contains a chloramphenicol-resistance marker, generating pDΔ3042, pDΔ3043, pDΔ3044, and pDΔ3045. Cloning was carried out in the *E. coli* DH5α λ-pir strain (WM3618 λ-pir) to propagate the suicide plasmid. The deletion constructs were verified by restriction analysis and sequencing.

Construction of Scar-less Deletion Mutants

Scar-less deletion mutants of genes *plu3042*, *plu3043*, *plu3044*, and *plu3045* were constructed using similar methods as previously described (Crawford et al., 2010). The deletion constructs, pDΔ3042, pDΔ3043, pDΔ3044, and pDΔ3045, were transformed by heat shock into the diaminopimelic acid auxotroph donor *E. coli* strain WM6026 λ-pir (Blodgett et al., 2007), and transformants were selected on Luria Bertani (LB) agar plates containing 25 μg/ml chloramphenicol. *P. luminescens* TT01 wild-type was grown on LB plates without selection. The donor *E. coli* strains and the recipient *P. luminescens* TT01 were then cultivated at 37°C and 30°C, respectively, in suspension with or without appropriate drug selection. The LB suspension cultures were timed so that they would simultaneously reach OD_{600} = 0.5–0.6. The mid-exponential phase cultures were mixed at a 2:8 and 1:1 (donor:recipient) ratio (1 ml final), diluted to 5 ml with fresh LB medium, and filtered through a 0.2 μM sterile filter. The filter was removed, placed on an LB agar plate supplemented with 0.3 mM diaminopimelic acid, and grown overnight at 30°C. The mating mixture was resuspended in fresh LB and plated on LB agar plates supplemented with 25 μg/ml chloramphenicol but lacking supplemental diaminopimelic acid. Single *P. luminescens* colonies growing on the chloramphenicol selection plates were carefully picked and restreaked on LB sucrose (5%) plates lacking drug for SacB counter-selection. Successful scarless deletion mutants from the allelic-exchange process were screened by colony PCR, using primer pairs Plu3042-5genome and Plu3045-3genome (Table S3). Deletion mutants were restreaked three times on LB sucrose (5%) plates, and the single colonies were validated by colony PCR a second time to ensure no wild-type contamination. The final PCR products were cloned into pCR2.1 TOPO (Invitrogen) and end sequence verified.

Metabolite Analysis of Mutants

P. luminescens TT01 Δ3042, Δ3043, Δ3044, Δ3045, and the wild-type parent strains were individually streaked on LB agar plates and grown for 2 days at 30°C. Single colonies were inoculated into 5 ml of LB and grown overnight at 30°C and 250 rpm. The overnight culture (500 μl) was used to inoculate 50 ml of M9 minimal medium, except the glucose carbon source was replaced with 0.4% casamino acids and 0.576% L-proline to enhance metabolite production (Crawford et al., 2010). The culture was incubated for 48 hr at 30°C and 250 rpm. At 48 hr, the cells were removed by centrifugation and then the cleared medium was filtered through a 0.2 μm nylon membrane. The cell-free aqueous solutions containing the secreted small molecules were analyzed directly by HPLC/MS (Agilent 6130) in positive and negative ion modes. Separation was carried out over a Hypercarb HPLC column (100 × 4.6 mm, 5 μM particle size, Thermo Scientific) with an acetonitrile:water gradient containing 0.1% formic acid at 0.7 ml/min: 0–2 min, 5% MeCN; 2–25 min, 5%–100% MeCN (Figure 3B; Figure S2B).

Isolation of H_2Phe

The M+H = 168 ion eluting at 8.6 min in the wild-type and Δ3045 *P. luminescens* cultures was isolated for NMR characterization. *P. luminescens* (600 ml) was grown as described in the "Metabolite Analysis of Mutants" section. The filtered spent medium was lyophilized, and the powder was extracted with 200 ml of methanol and filtered to remove salts and highly polar metabolites. The methanol extract was dried, redissolved in water, and separated over a 2 g C18 SepPak column (Waters) using a methanol gradient (0%, 20%, 40%, and 100% methanol). The compound of interest eluted in both the 0% and 20% methanol washes, but the 20% fraction was considerably more pure and enriched primarily in Phe and H_2Phe. A semi-preparative Luna phenyl-hexyl HPLC column (250 × 10 mm, 5 μM particle size; Phenomenex) was needed to separate and isolate H_2Phe from the undesired Phe using a 5% acetonitrile isocratic method (23.4 min retention time, isolation 1) or a 5% methanol isocratic method (40.5 min retention time, isolation 2) at 2 ml/min.

Structural Characterization of H_2Phe

The high-resolution mass of the M+H ion for the purified product was consistent with the molecular formula of protonated H_2Phe ($C_9H_{14}NO_2$; calculated 168.1025; observed, 168.1026; error 0.6 ppm). NMR characterizations (^{1}H, gCOSY, HSQCAD, and gHMBC) were performed with a Varian INOVA 600 MHz NMR using deuterated water and a symmetrical NMR microtube susceptibility matched to the solvent (Shigemi, Inc.).

Determination of Absolute Configuration of L-H_2Phe

The absolute configuration was determined by Marfey's analysis (Marfey, 1984; Fujii et al., 1997). A total of 50 μl (10 mg/ml) of Nα(2,4-dinitro-5-fluoro-phenyl)-L-alaninamide in acetone was added to 100 μl of L-Phe (1 mg/ml), DL-Phe (1 mg/ml), or isolated H_2Phe (subjected to ten rounds of rotary evaporation from water to aromatize a portion of the product) in 1 N $NaHCO_3$. The mixture was reacted for 15 min at 55°C in a water bath. The reaction was quenched with 50 μl of 2 N HCl. The products were dissolved by adding 150 μl of acetonitrile and further diluted with 150 μl of water (500 μl total). The products (15 μl) were analyzed by LC/MS (Agilent 6130) using a Kinetex C18 HPLC column (100 × 4.60 mm, 100 Å, 2.6 μm particle size; Phenomenex) with an acetonitrile:water gradient containing 0.1% formic acid at 0.5 ml/min: 0–50 min, 20%–70% MeCN; 50–51 min, 70%–100% MeCN; 51–56 min, 100% MeCN (Figure S1F).

Synthesis of $^{13}C_6$-Labeled L-H_2Phe

The procedure was adapted from a known protocol (Skolaut and Rétey, 2001). Under an inert atmosphere of dry N_2, a flame-dried 25 ml two-neck round-bottom flask, fitted with a Dewar condenser and equipped with a magnetic stir bar, was charged with $^{13}C_6$-labeled L-Phe (144 mg, 0.842 mmol). The Dewar condenser was chilled to −78°C (dry ice/acetone), and the flask was also allowed to cool to −78°C (dry ice/acetone). Ammonia (~10 ml) was then condensed into the round-bottom flask via the Dewar condenser; the homogeneous solution was allowed to stir. Ethanol (1 ml) was added. Finely cut pieces of sodium (excess) were then added through the second neck of the flask (otherwise sealed with a glass stopper) until a blue color persisted. After 8 min, the reaction mixture solidified, and so the cooling bath was removed (mixture melted shortly thereafter). After an additional 15 min, the mixture was exposed to the atmosphere and allowed to warm to room temperature, permitting the ammonia to evaporate; the ethanol was removed in vacuo. The remaining white solids were dissolved in minimal water, and the solution was adjusted to pH 7 by the dropwise addition of 6 M aqueous HCl (exothermic). Purification by preparative HPLC (19 × 250 mm SunFire C18 OBD 5 μM column, 15 ml/min, 100% H_2O [0.1% TFA] for 3 min, then linear gradient to 95:5 MeCN:H_2O [0.1% TFA] over 17 min) afforded the $^{13}C_6$-labeled 2,5-dihydrophenylalanine (67.5 mg, 0.390 mmol, 46.3% yield) as a colorless solid. ^{1}H-NMR (600 MHz, D_2O): δ 5.90–5.48 (3H, m), 3.99 (1H, app ddd, J = 8.4, 3.6, 3.6 Hz), 2.80–2.59 (3H, m), 2.59–2.40 (3H, m); ^{13}C-NMR (100 MHz, D_2O) δ 171.9, 128.9–123.7 (4C, m), 53.6, 37.8 (d, J_{C-C} = 41.0 Hz), 27.7–25.6 (2C, m); High Res LCMS $[M+H]^+$ calc: 174.1226, found: 174.1222.

Isotopic Labeling of 2,5-Dihydrostilbene

The *P. luminescens hexA* insertional inactivant (Kontnik et al., 2010) was streaked on LB agar plates supplemented with 25 μg/ml chloramphenicol and grown for 3 days at 30°C. The colonies were used to inoculate liquid LB (5 ml) with 25 μg/ml chloramphenicol. The suspension culture was grown overnight at 30°C and 250 rpm. A total of 100 μl of overnight seed culture was used to inoculate 5 ml of M9 minimal medium lacking drug selection, in which the M9 glucose carbon source was substituted with 0.4% casamino acids and 0.576% L-proline. The M9 suspension cultures were grown at 30°C and 250 rpm until OD_{600} = 0.5. Synthetic $^{13}C_6$-L-H_2Phe (350 mM stock in water) was added to the cultures at a final concentration of 0, 0.05, 0.075, 0.125, 0.25, 0.5, 0.75, 1, and 2 mM in triplicate (27 cultures total). The cultures

were incubated at 30°C and 250 rpm until a final time of 48 hr. At 48 hr, whole cultures were individually extracted with 5 ml of ethyl acetate, and the liquid phases were nicely separated by centrifugation. The top 4 ml of ethyl acetate was transferred to a separate glass vial, dried, and resuspended in 500 μl methanol for LC/MS analysis. The products (25 μl) were analyzed by LC/MS (Agilent 6130) using a Kinetex C18 HPLC column (100 × 4.60 mm, 100 Å, 2.6 μm particle size; Phenomenex) with an acetonitrile:water gradient at 0.7 ml/min: 0–2 min, 10% MeCN; 2–25 min, 10%–100% MeCN; 25–27 min, 100% MeCN (Figure 4A).

Cloning, Expression, and Purification of Plu3042, Plu3043, and Plu3044

Genes encoding Plu3042, Plu3043, and Plu3044 were cloned from *P. luminescens* genomic DNA using the following primers:

plu3042, 5′-AGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGACAAACAATATCGTTAGCCCAGTTAAT-3′ and 5′-ACCCATTTGCTGTCCACCAGTCATGCTAGCCTAATAAACCGGATTTAACCAATGTGAGAA-3′;
plu3043, 5′-AGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGAATGTGTTTTTTTTAGTTTCTACAAA-3′ and 5′-ACCCATTTGCTGTCCACCAGTCATGCTAGCTTATGCGGCTGTAAATACTGACCAAAGCAT-3′;
plu3044, 5′-AGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGTCTTTACTTACGCTACACACCATTACT-3′ and 5′-ACCCATTTGCTGTCCACCAGTCATGCTAGCTTAAAGGTCGTCCAGTTTGAATTTATCATC-3′.

After PCR purification, the genes were ligated into plasmid pET-28b nicked at the Nde1 restriction site using the one-step isothermal DNA assembly protocol previously developed (Gibson et al., 2009). Plasmids were transformed into chemically competent *E. coli* TOP10, then purified and sequenced to verify correct gene insertion. Purified plasmid encoding each gene with a N-terminal His_6 tag was transformed into chemically competent BL21(DE3) cells for protein expression.

Cultures of expression cells in LB Broth supplemented with 50 μg/ml kanamycin were grown at 37°C until the OD_{600} reached 0.4. The temperature was then dropped to 15°C. Once cooled, the cells were induced with 120 μM IPTG and grown overnight. Cells were harvested by centrifugation at 5,000 × g for 8 min, resuspended in 50 mM potassium phosphate (pH 8), 150 mM sodium chloride (lysis buffer), then lysed via three passes at 5,000–10,000 psi in an Avestin EmulsiFlex-C5 high-pressure homogenizer. Cell lysate was clarified by centrifugation at 35,000 × g for 35 min at 4°C. The supernatant was then incubated with 1.5 ml Ni-NTA resin, while rocking, at 4°C for 2 hr. The resin was washed once with lysis buffer, and once with lysis buffer supplemented with 5 μM imidazole. A stepwise gradient of lysis buffer supplemented with 20, 40, 60, and 200 mM imidazole was used to elute protein from the column. SDS-PAGE was used to identify fractions containing the desired protein, which were then combined and dialyzed in lysis buffer to remove imidazole. Protein was flash frozen and stored at −80°C.

Structural Elucidation of Plu3043 Reaction Product

Plu3043 is a predicted PDX with homology to enzymes BacA, AerD, and SalX that we have previously characterized (Mahlstedt and Walsh, 2010; Mahlstedt et al., 2010). To verify that Plu3043 produces the same H_2HPP product, NMR spectroscopy was used, and the resulting ^{1}H-NMR spectrum compared to the previously reported ^{1}H spectra for H_2HPPex. Reactions were set up using previously reported methodology (Mahlstedt et al., 2010). The reaction product was dissolved in D_2O before NMR analysis.

Determination of Amino Acid Cosubstrate for Plu3042

As a predicted PLP-dependent aminotransferase, the enzyme Plu3042 requires an amino acid cosubstrate for activity. To identify the best amino acid cosubstrate, an UV/Vis spectroscopic assay was utilized. A total of 1 mM prephenate was incubated with 2 mM each of 3 of the 20 standard proteinogenic amino acids (Trp and Tyr were tested individually), 20 μM PLP, 10 μM Plu3042 and 10 μM Plu3043 in 50 mM potassium phosphate (pH 8) (50 μl total volume) at room temperature overnight. Controls with no amino acid added or no Plu3043 added were set up at the same time under identical conditions. Following incubation, 20 μl of each reaction mixture was diluted with 200 μl water and scanned from 220 to 600 nm. When no amino acid was included, the reaction mixture displayed absorbance at 295 nm, characteristic of H_2HPPex. The same is true when amino acids that are not capable of serving as cosubstrates for Plu3042 were included. However, when a correct amino acid was included, the samples did not absorb at 295 nm. The sample yielding the least absorbance at 295 nm included Gln, Ile, and Leu. To identify which of these three amino acids was the optimal cosubstrate, reactions were set up and analyzed as described above; however, they only contained 2 mM of one amino acid (Gln, Ile, or Leu). Reactions with no amino acid and with Lys were used as controls.

Structural Elucidation of Plu3042 Product

NMR structural characterization of the product of the Plu3043-Plu3042 reaction was desired. To produce enough sample for analysis, a 500 μl reaction including 20 mM prephenate, 40 mM (2,3,3,4,4-D5)-L-glutamine, 30 μM PLP, 20 μM Plu3042, and 20 μM Plu3043 in 50 mM potassium phosphate (pH 8) was incubated overnight at room temperature. The reaction was quenched by filtration to remove the enzyme and lyophilized. The solid-reaction product was dissolved in 700 μl D_2O, and ^{1}H, gCOSY, gHSQC, and gHMBC spectra were collected.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four tables, five figures, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2011.07.009.

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