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Metabolic engineering of the *E. coli* L-phenylalanine pathway for the production of D-phenylglycine (D-Phg)

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Abstract

D-phenylglycine (D-Phg) is an important side chain building block for semi-synthetic penicillins and cephalosporins such as ampicillin and cephalexin. To produce D-Phg ultimately from glucose, metabolic engineering was applied. Starting from phenylpyruvate, which is the direct precursor of L-phenylalanine, an artificial D-Phg biosynthesis pathway was created. This three-step route is composed of the enzymes hydroxymandelate synthase (HmaS), hydroxymandelate oxidase (Hmo), and the stereoinverting hydroxyphenylglycine aminotransferase (HpgAT). Together they catalyse the conversion of phenylpyruvate via mandelate and phenylglyoxylate to D-Phg. The corresponding genes were obtained from *Amycolatopsis orientalis*, *Streptomyces coelicolor*, and *Pseudomonas putida*. Combined expression of these activities in *E. coli* strains optimized for the production of L-phenylalanine resulted in the first completely fermentative production of D-Phg.

Keywords: Aromatic amino acids; D-Amino acids; Renewable; Sustainable; Fine chemicals

1. Introduction

The D-amino acids, D-phenylglycine (D-Phg) and D-4-hydroxyphenylglycine (D-HPG) are important precursors used for the preparation of semi-synthetic cephalosporins and penicillins. Currently, D-HPG is obtained in a two-step chemo-enzymatic synthesis based on hydantoinase technology (Liese et al., 2000), and D-Phg is made by classical or enzymatic resolution of a racemic mixture (reviewed in Wegman et al., 2001) derived from petrochemical feedstocks. As an alternative to current production methods, a complete fermentative route based on renewable and sustainable resources would be of commercial interest.

Several successful examples of metabolic engineering for microbial production of aromatic amino acids like L-phenylalanine, or L-tryptophan and derived compounds like indigo are known (LaDuca et al., 1999; Bongaerts et al., 2001). Broadening the aromatic amino acid pathway of *Escherichia coli* by metabolic pathway engineering to D-Phg would result in a microbial strain that allows the production of D-Phg in a single fermentation step.

Thus far, no direct biosynthetic pathway to free D-Phg has been identified in nature. Nevertheless several actinomycete peptide antibiotics like the streptogramin (Cocito, 1979) and vancomycin groups of antibiotics (Nagarajan, 1991) contain either Phg or hydroxylated derivatives thereof such as D-HPG and L-3,5-dihydroxyphenylglycine (L-DHPG), as building block.

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Recently the pathways for L-HPG and L-DHPG biosynthesis have been elucidated illustrating that the biosynthesis of each compound follows completely different routes. While the pathway to DHPG originates with malonyl-CoA and acetyl-CoA, and involves a type III polyketide synthase (Li et al., 2001b; Pfeifer et al., 2001), L-HPG is synthesized from tyrosine using a hydroxymandelate synthase (HmaS), a novel dioxygenase, as the pivotal enzyme (Choroba et al., 2000; Hubbard et al., 2000). In both cases the last biosynthetic step is a transamination catalysed by an L-hydroxyphenylglycine aminotransferase converting either 4-hydroxyphenylglyoxylate or 3,5-dihydroxyphenylglyoxylate to the corresponding L-amino acids L-HPG (Choroba et al., 2000) or L-DHPG (Pfeifer et al., 2001).

Orthologous genes encoding the enzymes involved in the biosynthesis of L-HPG have recently been identified in several actinomycete strains, e.g., *A. orientalis* (Choroba et al., 2000), *Streptomyces lavendulae* (Chiu et al., 2001), *Streptomyces coelicolor* (Hojati et al., 2002), and *Nocardia uniformis* (Gunsior et al., 2004), which, respectively, produce chloroeremomycin, complestatin, calcium dependent antibiotic, and nocardicin A.

These actinomycetes synthesize L-HPG from 4-hydroxyphenylpyruvate through the action of three enzymes, HmaS (decarboxylation and oxidation to L-4-hydroxymandelate), hydroxymandelate oxidase Hmo (oxidation to 4-hydroxyphenylglyoxlate) and L-4-hydroxyphenylglycine aminotransferase (transamination to L-HPG). The latter enzyme, L-4-hydroxyphenylglycine aminotransferase uses L-tyrosine as an amino-donor thereby releasing 4-hydroxyphenylpyruvate as the starting molecule again (Choroba et al., 2000; Hubbard et al., 2000).

In order to construct a biosynthetic pathway to the free D-amino acids D-Phg or D-HPG, part of the L-HPG pathway needs to be combined with a D- rather than an L-4-hydroxyphenylglycine aminotransferase. The presence of such a D-4-hydroxyphenylglycine and D-phenylglycine aminotransferase has previously been described for *Pseudomonas putida* LW-4 (Van den Tweel et al., 1986, 1988) and *Pseudomonas stutzeri* (Wiyakrutta and Meevootisom, 1997).

We investigated whether the combination of the first two steps of the L-HPG actinomycete biosynthetic pathway and a D-4-hydroxyphenylyglycine aminotransferase results in a new metabolic pathway for D-Phg starting from the intermediate phenylpyruvate (Fig. 1), the direct precursor of L-phenylalanine. The introduction of this 'artificial' pathway into an L-phenylalanine producing *E. coli* strain resulted in fermentative production of D-Phg. To our knowledge, this is the first report of multi-step engineering to produce free D-Phg from renewable resources such as glucose.

2. Material and methods

2.1. Bacterial strains and culture media

Amycolatopsis orientalis NRRL 18098 (US patent 5,843,437) was obtained from the ARS (Agricultural Research Service) Patent Culture Collection, Peoria, Illinois, USA. *A. orientalis* was cultivated in $10\,g \cdot L^{-1}$ glucose, $5\,g \cdot L^{-1}$ yeast extract, $20\,g \cdot L^{-1}$ starch, $1\,g \cdot L^{-1}$ casamino acids, pH 7.5 with NaOH at 28 °C.

Streptomyces coelicolor A3(2) M145, kindly obtained from Professor M.J. Bibb of the John Innes Institute, Norwich (UK), was cultivated at 28 °C in YE-ME medium containing $3\,g \cdot L^{-1}$ yeast extract, $5\,g \cdot L^{-1}$ peptone, $3\,g \cdot L^{-1}$ malt extract, $10\,g \cdot L^{-1}$ glucose, $340\,g \cdot L^{-1}$ sucrose. $10\,g \cdot L^{-1}$ glycine and 5 mM $MgCl_2$ were added after sterilization.

Pseudomonas putida LW-4 (NCIMB 12565) was obtained from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, UK. *P. putida* was grown at 30 °C in LB medium.

Escherichia coli strains and plasmids used in this study are listed in Tables 1 and 2. DH5α, and TOP10 were used as hosts for plasmid construction. During strain construction cultures were grown at 30, 33, 37, or 42 °C in LB broth. Antibiotics were used when needed at the following concentrations: kanamycin (25 or $50\,mg \cdot L^{-1}$), chloramphenicol ($25\,mg \cdot L^{-1}$), ampicillin ($100\,mg \cdot L^{-1}$).

To test in vivo D-Phg production, the recombinant *E. coli* strains were cultivated in different mineral media of the following compositions:

Mineral salt medium A: Na citrate $\cdot 3H_2O$ ($1.0\,g \cdot L^{-1}$), $MgSO_4 \cdot 7H_2O$ ($0.3\,g \cdot L^{-1}$), KH_2PO_4 ($3.0\,g \cdot L^{-1}$), K_2HPO_4 ($12.0\,g \cdot L^{-1}$), NaCl ($0.1\,g \cdot L^{-1}$), $(NH_4)_2SO_4$

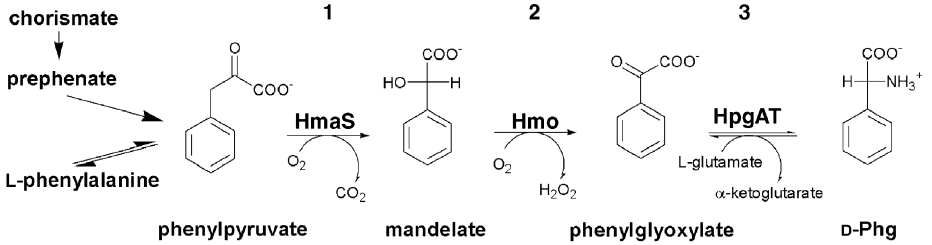

Fig. 1. Biosynthesis of D-phenylglycine (D-Phg). 1. Hydroxymandelate synthase (HmaS) from *A. orientalis* ($hmaS_{Ao}$) or *S. coelicolor* ($hmaS_{Sc}$); 2. Hydroxymandelate oxidase (Hmo) from *A. orientalis* (hmo_{Ao}) or *S. coelicolor* (hmo_{Sc}); 3. D-(4-Hydroxy)phenylglycine aminotransferase (HpgAT) of *Pseudomonas putida* (*hpgAT*).