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Metabolic engineering of *Escherichia coli* for the biosynthesis of 2-pyrrolidone

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ABSTRACT

2-Pyrrolidone is a valuable bulk chemical with myriad applications as a solvent, polymer precursor and active pharmaceutical intermediate. A novel 2-pyrrolidone synthase, ORF27, from *Streptomyces aizunensis* was identified to catalyze the ring closing dehydration of γ-aminobutyrate. ORF27's tendency to aggregate was resolved by expression at low temperature and fusion to the maltose binding protein (MBP). Recombinant *Escherichia coli* was metabolically engineered for the production of 2-pyrrolidone from glutamate by expressing both the genes encoding GadB, a glutamate decarboxylase, and ORF27. Incorporation of a GadB mutant lacking H465 and T466, GadB_ΔHT, improved the efficiency of one-pot 2-pyrrolidone biosynthesis in vivo. When the recombinant *E. coli* strain expressing the *E. coli* GadB_ΔHT mutant and the ORF27-MBP fusion was cultured in ZYM-5052 medium containing 9 g/L of L-glutamate, 7.7 g/L of L-glutamate was converted to 1.1 g/L of 2-pyrrolidone within 31 h, achieving 25% molar yield from the consumed substrate.

1. Background

2-Pyrrolidone was identified by the US Department of Energy as an important C4 "Top Value-Added Chemical from Biomass" that can potentially be derived from glutamate (Werpy, 2004). 2-Pyrrolidone is currently used as precursor for the production of N-vinylpyrrolidone, a solvent for animal injection, a building block for active pharmaceutical ingredients, optical co-solvent for water-based ink formulation, process solvent for membrane filters and a copolymer for floor polish (BASF, 2015). 2-Pyrrolidone can also be used in ring-opening polymerization to produce nylon-4, a fiber material with better thermal stability and the highest hydrophilicity in the nylon family of materials (Park, 2013). With a variety of applications, 2-pyrrolidone continues to be a product of huge commercial interest.

Current industrial production of 2-pyrrolidone involves the dehydrogenation of 1,4-butanediol to form γ–butyrolactone, followed by reacting aqueous γ-butyrolactone with ammonia (Fig. 1A) (Harreus et al., 2011). By using low cost glutamate as starting material, as well as avoiding harsh reaction conditions, biological production of 2-pyrrolidone offers the potential for a cheaper and more environmentally friendly synthesis route. Therefore, we propose a two-step enzymatic process for 2-pyrrolidone biosynthesis from glutamate: (1) decarboxylation of glutamate to form γ-aminobutyrate (GABA), and (2) enzymatic ring closing of GABA into 2-pyrrolidone (Fig. 1B).

While the first enzymatic step is known (Ma, 2012; Park, 2013; Shi, 2013; Takahashi, 2012; Vo, 2012), the second step has not been demonstrated under any fermentation conditions (Stavila, 2013). We employed a targeted strategy to identify appropriate enzyme candidates for the GABA activation step by conducting retro-biosynthetic analysis of polyketides. Here we report the discovery in *Streptomyces aizunensis* of ORF27, an auxiliary enzyme in the linearmycin A biosynthetic cluster that performs the GABA activation step to form 2-pyrrolidone under mild fermentation conditions. Coupling this 2-pyrrolidone synthase with glutamate decarboxylase, which forms GABA from glutamate, we achieved the first demonstration of the full 2-pyrrolidone biosynthetic pathway in *Escherichia coli*.

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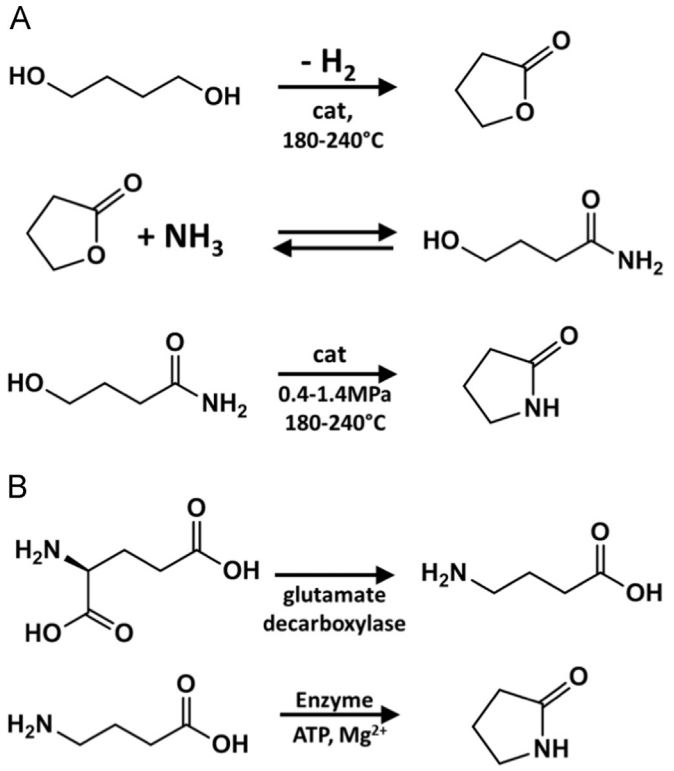

Fig. 1. Routes for production of 2-pyrrolidone. (A) BASF petrochemical route for 2-pyrrolidone production. (B) Microbial 2-pyrrolidone biosynthetic route.

2. Materials and methods

2.1. Enzyme scouting by retro-biosynthetic analysis of PKS natural products

It was hypothesized that an enzyme employing a GABA activation mechanism on GABA's acid group followed by spontaneous irreversible intramolecular cyclization could be utilized as a 2-pyrrolidone synthase. However, there has been no documentation of AMP-activating enzymes using GABA as a substrate. Type I polyketide synthases (PKSs) are mega-synthases whose enzymatic domain organization predictably correlate with their natural product's chemical structure – usually referred to as the colinearity rule (Du, 2001, 2010; Dutta, 2014; Khosla, 2009; Li, 2009; Menzella, 2005; Tran, 2010; Weissman, 2008; Wong, 2010; Yadav, 2009). PKSs are responsible for biosynthesis of the characteristic polyketide aglycone backbone, and auxiliary enzymes are involved in starter unit activation and/or post aglycone modification (Kalaitzis, 2009; Li, 2005; Llewellyn, 2007; Moorea, 2002; Ogasawara, 2004; Shinohara, 2011; Simunovic, 2006). The close positioning of PKS genes with auxiliary enzymes in the same gene cluster makes it relatively easy to identify the substrates upon which these auxiliary enzymes act. PKS databases were explored to identify polyketides with positively charged, amine-containing starter units and auxiliary enzymes clustered near the PKS (see Table S1 for lists of PKS databases). Desertomycin A/B, Eco-0501, linearmycin A were selected for further retrobiosynthetic analysis (Fig. S1A). From the arrangement of the polyketide synthase genes, 4-aminobutyrate-like starter unit was predicted to be loaded onto the ACP0 domain (Fig. S1B). Since polyketide starter unit loading requires the substrate to be activated by an ATP dependent mechanism, the linearmycin A biosynthetic gene cluster was further analyzed for auxiliary enzymes with this function. Two ORFs in the gene cluster, ORF27 and ORF36, were predicted to be AMP-dependent synthetases (Fig. S1B). The hypothesized candidates were reverse transcribed according to the optimized *E. coli* codon usage using DNA 2.0's algorithm and synthesized.

2.2. Bacterial strains and chemicals

E. coli strain DH10B [F− mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ− rpsL (Strr) nupG] was used for all molecular biology manipulations. DH10B or BL21 Star (DE3) [F− ompT hsdSB (rB− mB−) gal dcm rne131 (DE3)] were used as hosts for production of 2-pyrrolidone. All the strains and plasmids utilized in this study are listed in Table 1. For high-density shake flask cultures, Studier's autoinduction ZYM-5052 medium was prepared according to the published protocol (Studier, 2005). Chloramphenicol (25 μg/ml), kanamycin (20 μg/ml) and ampicillin (100 μg/ml) were added where desired to provide selective pressure for plasmid maintenance. During 2-pyrrolidone production, the following antibiotic concentration was used: chloramphenicol (6.25 μg/ml), kanamycin (5 μg/ml) and ampicillin (25 μg/ml). 2-Pyrrolidone, glutamic acid, GABA, and ATP were purchased from Sigma-Aldrich (St. Louis, Missouri).

2.3. Plasmid construction

Genes encoding ORF27 (GenBank: AAX98201.1) and ORF36 (GenBank: AAX98210.1) were recoded using *E. coli* codon usage with biobrick overhangs 5′-gaattcaaaAGATCTAGGAGGCAT-3′ on the 5′ end and 5′-TAAGGATCCAAACTCGAG-3′ on the 3′ end. DNA 2.0 (Menlo Park, CA) cloned the genes into plasmid vectors creating pDNA2.0-ORF27 and pDNA2.0-ORF36, respectively. The genes encoding wild-type GadB and the variant GadB_ΔHT, were amplified from *E. coli* MG1655 genomic DNA using the primers specified. GadB_ΔHT lacks histidine 465 and threonine 466 of *E. coli* GadB. The construction of each plasmid is described in Table 2 and Table S2.

2.4. 2-Pyrrolidone, GABA and glutamate product assays

2.4.1. Liquid chromatography method for 2-pyrrolidone, GABA and glutamate separation

Liquid chromatography (LC) separation of 2-pyrrolidone was conducted at 55 °C with an Inertsil ODS-3 reverse-phase C18 column (250 mm length, 2.1 mm internal diameter, 3 μM particle size; GL Sciences) using a 1100 series high-performance LC system (Agilent Technologies). The mobile phase was composed of 0.1% formic acid in H_2O (solvent A) and 0.1% formic acid in MeOH (solvent B). Butyrolactam was separated with the following gradient: 40–60% B for 4.5 min, 60–100% B for 0.5 min, 100–40% B for 0.5 min, held at 10% B for 8.5 min. A flow rate of 0.18 mL/min was used throughout.

2.4.2. Mass spectrometry analysis of 2-pyrrolidone, GABA and glutamate

The LC system was coupled to an Agilent Technologies LC-MSD SL electrospray ionization mass spectrometer (ESI MS). Nitrogen gas was used as both the nebulizing and drying gas to facilitate the production of gas-phase ions. The drying and nebulizing gases were set to 10 L/min and 20 psig, respectively, and a drying gas temperature of 300 °C was used throughout. ESI was conducted in the positive-ion mode with a capillary voltage of 4 kV. Mass measurements were carried out in the selected ion monitoring (SIM) mode (2-pyrrolidone, m/z 86; GABA, m/z 104; glutamate, m/z 148) for the detection of $[M+H]+$ ions. Data acquisition and processing were performed using ChemStation (Agilent technologies).