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Microbial conversion of glucose to a novel chemical building block, 2-pyrone-4,6-dicarboxylic acid

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ABSTRACT

2-Pyrone-4,6-dicarboxylic acid (PDC) is a catabolic intermediate in *Sphingobium* sp. SYK-6 (previously characterized as *Sphingomonas paucimobilis* SYK-6), which is a degrader of lignin-derived aromatic compounds. Recently, PDC has been also characterized as a novel starting material for several potentially useful synthetic polymers. In a previous study, we constructed a biosynthetic system in which PDC was generated efficiently from a chemically synthesized compound, protocatechuate. In order to develop an alternative system for production of PDC, we tried to generate it from glucose, which is a low-cost sugar that can be obtained from abundant cellulosic wastes and biomass crops. We designed a metabolic bypass to PDC from the shikimate pathway in recombinant *Escherichia coli* cells. PDC accumulated in the medium of recombinant *E. coli* cells that had been transformed with genes isolated from *Emericella niger*, *E. coli*, *Pseudomonas putida*, and *Sphingobium* sp. SYK-6. The yield of PDC depended on the combination of genes that we introduced into the cells and on the specific of host strain. Under optimal conditions, the yield and titer of PDC were, respectively, 17.3% and 0.35 mg/l when the concentration of glucose was 2 g/l and the culture volume was 50 ml. Our results open up the possibility of novel utilization of biomass as the source of a useful chemical building block.

1. Introduction

The dicarboxylic acid 2-pyrone-4,6-dicarboxylic acid (PDC) is a central metabolic intermediate in the degradation of low-molecular-mass model lignin compounds in *Sphingobium* sp. SYK-6 (previously characterized as *Shingomonas paucimobilis* SYK-6). In this bacterium, various monomeric and dimeric lignin-model compounds are degraded via PDC and can be exploited as the only sources of carbon via the activities of various catabolic enzymes (Masai et al., 2007).

PDC has a polar pseudo-aromatic ring system and two carboxylic acid moieties. The molecular shape of PDC resembles that of isophthalic acid, which can be used as a monomer for the synthesis of various polyesters and polyamides (Shigehara et al., 2001a, 2001b). In recent studies, we succeeded in producing novel polymers from PDC by direct dehydration polycondensation with diols such as 1,2-ethanediol, 1,3-propanediol, and bis(2-hydroxyethyl) terephthalate (Michinobu et al., 2008a). These polymers have unique characteristics and are potential components of functional films and adhesives. In addition, a polymer derived from PDC with improved characteristics can be also synthesized by a Cu-catalyzed azide-alkyne click reaction (Michinobu et al., 2008b).

Since, to our knowledge, no protocol for the chemical synthesis of PDC has been developed, in a previous study we investigated the production of PDC in microbial systems from phenolic compounds, such as chemically synthesized protocatechuate (PCA). Efficient production of PDC from PCA was achieved with recombinant *Pseudomonas putida*, in which genes for protocatechuate 4,5-dioxygenase (ligA and ligB) and 4-carboxy-2-hydroxymuconate-6-semialdehyde (CHMS) dehydrogenase (ligC) were overexpressed (Otsuka et al., 2006). After growth of recombinant *P. putida* in liquid medium with glucose as the sole carbon source, we added PCA gradually to the culture medium, and the PCA was converted to PDC with a yield of over 90%. The resultant PDC was successfully purified from the medium by our original protocol (Michinobu et al., 2007).

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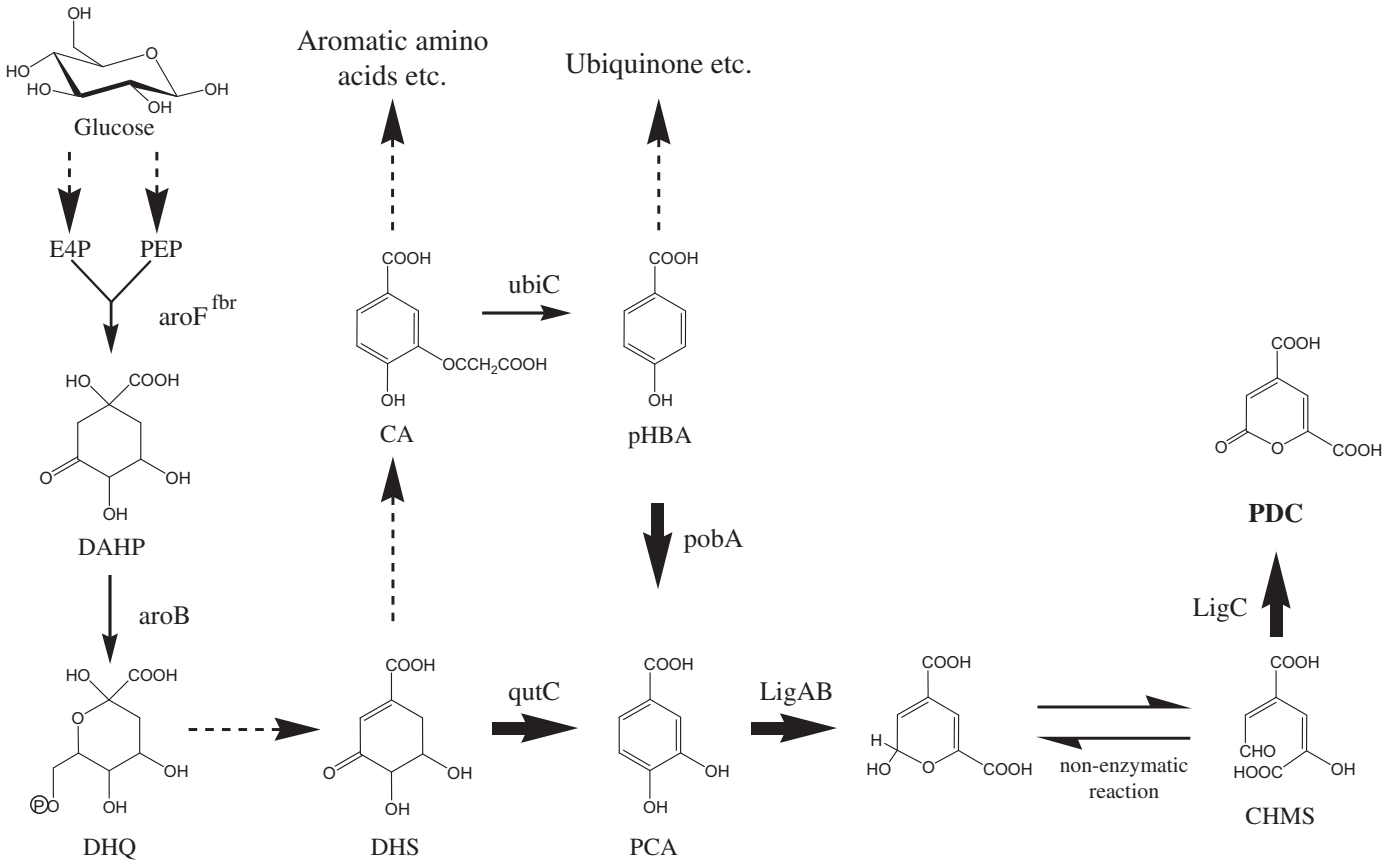

Fig. 1. Proposed microbial pathway for the synthesis of PDC from glucose. The dashed arrows correspond to metabolic steps in wild-type *E. coli*. The arrows correspond to accelerated metabolic steps that result from overexpression of genes from *E. coli* (*aroF*^{fbr}, *aroB*, and *ubiC*). The bold arrows correspond to the metabolic steps introduced by genes from other microorganisms (*qutC*, *pobA*, *ligA*, *ligB*, and *ligC*). See text for abbreviations.

Although synthesis from phenolic petrochemicals, such as PCA, is an attractive strategy for production of PDC, a fermentative approach based on renewable and sustainable resources would be more environmental friendly and of commercial interest. Thus, in an attempt to develop alternative methods for production of PDC, we tried to produce it from glucose by exploiting microbial activities in the shikimate pathway. The shikimate pathway is of pivotal importance in the production of numerous aromatic compounds in plants, bacteria, and fungi. Many aromatic metabolites and their derivatives, such as aromatic amino acids, ubiquinone and folate, are synthesized via this pathway. The pathway starts with the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) to yield D-arabinoheptulosonate 7-phosphate (DAHP) and then chorismate (CA), which is a central intermediate in the synthesis of various downstream metabolites of interest. Fermentative approaches to the production of metabolites and their derivatives via the shikimate pathway have been reviewed (Frost and Draths, 1995; Sprenger, 2007).

Although PDC is a metabolite in *Sphingobium* sp. SYK-6, this bacterium lacks the ability to utilize most of the simple sugars that we can recover from various types of renewable resource. Thus, in the present study, we designed methods for the production of PDC from glucose by recombinant strains of *Escherichia coli*. Since wild-type *E. coli* does not produce PCA, which is a key intermediate in the production of PDC, introduction of a metabolic pathway for the biosynthesis of PCA in *E. coli* was one of the most important aspects of this study. We designed two putative routes to PCA using the shikimate pathway (Fig. 1). In the first case, PCA was synthesized from CA by two enzymatic reactions that were catalyzed by chorismate pyruvate-lyase (ubiC) and then by 4-hydroxybenzoate hydroxylase (pobA), and the resultant PCA was converted to PDC by ligA, ligB, and ligC functions. To exploit a more direct and shorter pathway, in the second case, we generated PCA directly from 3-dehydroshikimate (DHS) via the action of dehydroshikimate dehydratase (qutC) and then PCA was converted to PDC as above. In addition to these reactions, we enhanced the central shikimate pathway to CA in recombinant *E. coli* by overexpression of the genes for 3-dehydroquinate synthase (aroB) and for feedback-resistant (fbr) 3-deoxy-7-phosphoheptulonate synthase (aroF^{fbr}; Frost and Draths, 1995; Bongaerts et al., 2001). We used a mutant strain of *E. coli*, which lacked either *pheA* or *tyrA*, as host to avoid conversion of CA to phenylalanine or tyrosine, respectively. Simultaneous co-expression of the various genes resulted in the biosynthesis of PDC from glucose in recombinant *E. coli* cells.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli BL21(DE3), XL-1 blue and JM109 were purchased from TAKARA BIO Inc. (Otsu, Japan). *E. coli* Δ*pheA* (JD23488) and Δ*tyrA* (JD23490) mutants and their parent strain, KP7600, were provided by the National BioResource Project for *E. coli* at the National Institute of Genetics (Mishima, Japan). *E. coli* KP7600 can grow without addition of any aromatic amino acids to the medium, while the two mutants require phenylananine and tyrosine, respectively. The λDE3-lysogenized Δ*pheA* and Δ*tyrA*