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Increased glycopeptide production after overexpression of shikimate pathway genes being part of the balhimycin biosynthetic gene cluster

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ABSTRACT

Amycolatopsis balhimycina produces the vancomycin-analogue balhimycin. The strain therefore serves as a model strain for glycopeptide antibiotic production. Previous characterisation of the balhimycin biosynthetic cluster had shown that the border sequences contained both, a putative 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (*dahp*), and a prephenate dehydrogenase (*pdh*) gene. In a metabolic engineering approach for increasing the precursor supply for balhimycin production, the *dahp* and *pdh* genes from the biosynthetic cluster were overexpressed both individually and together and the resulting strains were subjected to quantitative physiological characterisation. The constructed strains expressing an additional copy of the *dahp* gene and the strain carrying an extra copy of both *dahp* and *pdh* showed improved specific glycopeptide productivities by approximately a factor three, whereas the *pdh* overexpression strain showed a production profile similar to the wild type strain. In addition to the overexpression strains, corresponding deletion mutants, Δ*dahp* and Δ*pdh*, were constructed and characterised. Deletion of *dahp* resulted in significant reduction in balhimycin production whereas the Δ*pdh* strain had production levels similar to the parent strain. Based on these results the relation between primary and secondary metabolism with regards to Dahp and Pdh is discussed.

1. Introduction

Glycopeptides are a class of antibiotics produced by *actinomycetes*. They play an important role in treatment of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) strains with vancomycin and teicoplanin currently in clinical use. They have the status of being drugs of last resort. During the last 10 years Europe and North America have been facing increasing problems with infections caused by bacteria resistant to classical medical treatments (Shorr, 2007). This increased bacterial resistance towards traditional antibiotics has resulted in intensified search for new and more effective antibiotics.

The balhimycin producer *Amycolatopsis balhimycina* (Nadkarni et al., 1994) has been applied as model strain for research on glycopeptide synthesis. This strain belongs to the group of *actinomycetes* and unlike several related glycopeptide producers *A. balhimycina* is accessible to genetic modifications, which enables strain improvement through genetic engineering (Pelzer et al., 1999). In addition, balhimycin (Fig. 1) differs only in the glycosylation pattern from vancomycin and its properties are almost identical to those of vancomycin.

The 66-kb balhimycin biosynthetic gene cluster has been identified, sequenced, and characterized (Pelzer et al., 1999; Wohlleben et al., 2009). Based on the genetic characterizations of the balhimycin biosynthetic genes together with biochemical and chemical analyses, the biosynthetic pathway for balhimycin biosynthesis has been elucidated in detail. Interestingly, in the border region of the balhimycin biosynthetic gene cluster two genes *dahp* and *pdh* were identified, which showed high similarity to 3-deoxy-D-arabino-heptulosonate 7-phosphate (Dahp) synthase from *Amycolatopsis methanolica* and prephenate dehydrogenase from *Zymomonas mobilis*, respectively (Shawky et al., 2007).

Enzymatic activities of both Dahp and Pdh are key steps of the shikimate pathway (Fig. 1). Dahp synthase, the first enzyme of the shikimate pathway, condenses the pentose phosphate pathway intermediate D-erythrose 4-phosphate and the glycolytic pathway intermediate phosphoenol pyruvate to Dahp. The chorismate mutase catalyses the transformation of chorismate to prephenate.

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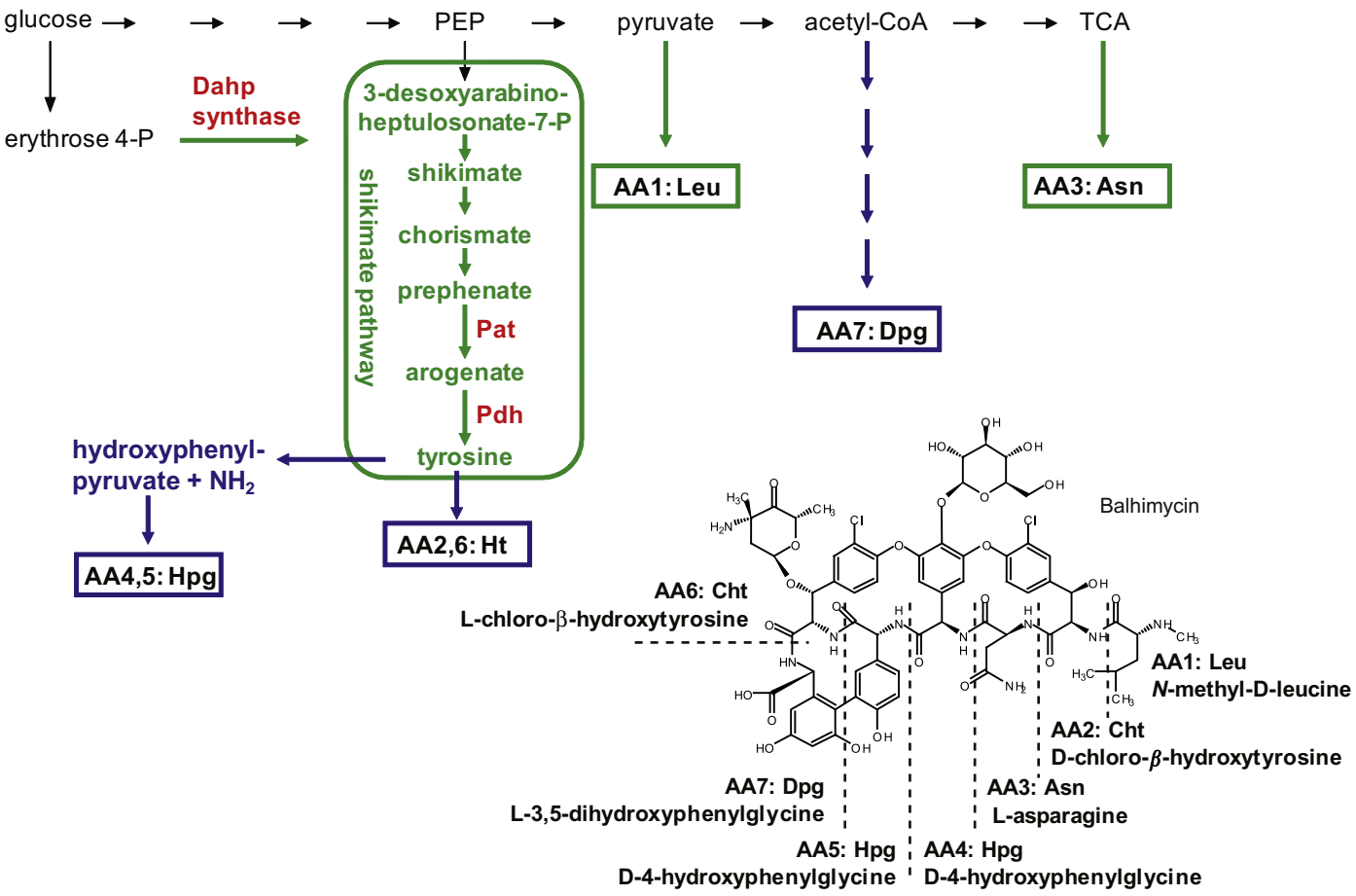

Fig. 1. Schematic overview of the shikimate pathway together with the biosynthesis of balhimycin. Green: reactions or substrates of the primary metabolism. Blue: reactions or substrates of the secondary metabolism. Abbreviations: AA, amino acid; Hpg, 4-hydroxyphenylglycine; Dpg, dihydroxyphenylglycine; Ht, hydroxytyrosine; Asn, asparagine; Leu, leucine; PEP, phosphoenol pyruvate; TCA, tricarboxylic acid; Dahp, 3-deoxy-D-arabino-heptulosonate 7-phosphate (Dahp) synthase; Pat, prephenate aminotransferase; Pdh, Prephenate dehydrogenase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Prephenate is further modified by the action of an aminotransferase and a dehydrogenase to yield L-tyrosine. The dehydrogenases comprise a family of homologues (TyrA homologues) that have different specificities of the cyclohexadienyl substrate: one homologues ($TyrA_a$) is specific for L-arogenate, one ($TyrA_p$) is specific for prephenate, and finally one is able to use both, L-arogenate and prephenate. The arogenate dehydrogenase $TyrA_a$ converts arogenate to tyrosine. Precondition for this reaction is the transamination of prephenate to obtain L-arogenate catalysed by the prephenate aminotransferase. Alternatively, prephenate dehydrogenase ($TyrA_p$) converts prephenate to L-hydroxyphenylpyruvate which is then transaminated to tyrosine. A broad-specificity cyclohexadienyl dehydrogenase is competent to catalyse either the $TyrA_a$ or the $TyrA_p$ reaction (Song et al., 2005).

As indicated in Fig. 1, amino acid 2, 4, 5, 6, 7 of balhimycin are – directly or indirectly – derived from the shikimate pathway. Biosynthesis of both β-hydroxytyrosine (β-Ht) at amino acid positions 2 and 6 and hydroxyphenylglycine (Hpg) at amino acid positions 4 and 5 require the aromatic amino acid tyrosine or hydroxyphenylpyruvate as precursor. Furthermore, tyrosine is also the amino donor in Hpg and dihydroxyphenylglycine (Dpg) biosynthesis. As a consequence, their supply may be the limiting factor for high-yield glycopeptide production.

Metabolic engineering has proven to be a rational alternative to classical strain improvement for optimisation of antibiotic production as well as for the development of heterologous antibiotics production in well characterized organisms (Siewers et al., 2009). Recently, attempts were made to create a cell factory for amphotericins production (antifungal antibiotics) in *Streptomyces nodosus* (Nic Lochlainn and Caffrey., 2009). A common approach for improving product formation is to increase precursor supply and this approach has been successfully applied in several *actinomycetes*. Overexpression of acetyl-CoA carboxylase has been shown to result in increased actinorhodin production in *Streptomyces coelicolor* (Ryu et al., 2006). Thus, through genetic engineering, carbon flux from acetyl-CoA was directed towards malonyl-CoA and since these two metabolites are important precursors in actinorhodin production, a positive effect on production was observed. A similar observation was made in connection with heterologous 6-MSA production in the yeast *Saccharomyces cerevisiae* where the overexpression of Acc1p resulted in substantially improved production (Wattanachaisaereekul et al., 2008). In *Saccharopolyspora erythraea*, a 50% increase in erythromycin production was observed when higher availability of methylmalonyl-CoA was generated by overexpression of the entire methylmalonyl-CoA mutase cluster (Reeves et al., 2007).

In the present study, we investigated whether the *dahp* and *pdh* genes in the biosynthetic cluster of *A. balhimycina* are responsible for sufficient precursor supply for balhimycin biosynthesis. This was achieved by constructing several overexpression and mutant