**CHEMICAL ENGINEERING TRANSACTIONS**, Volume 14, 2008
Edited by G. Marino, E. Bardone, A. Viglia
 ISBN 978-88-95608-02-0


# Interlocking of primary and secondary metabolism in antibiotic-producing actinomycetes

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Glycopeptides such as vancomycin are the drugs of last resort for the treatment of severe infections caused by antibiotic resistant gram-positive bacteria. As a model strain for analysing and manipulating glycopeptide biosynthesis we have chosen *Amycolatopsis balhimycina* which synthesizes the vancomycin-type glycopeptide balhimycin.
The 66-kb gene cluster encoding the biosynthesis of balhimycin was identified and sequenced. The biosynthetic pathway was elucidated and functions were assigned to all genes of the cluster.
Five out of seven amino acids of the heptapeptide backbone are – directly or indirectly – derived from the shikimate pathway: two molecules $\beta$-hydroxytyrosine ($\beta$-Ht) and hydroxyphenylglycine (Hpg) and one molecule dihydroxyphenylglycine (Dpg). In addition to the genes encoding the biosynthetic enzymes typical for secondary metabolism, the balhimycin gene cluster includes two genes (*pdh, dahp*) which encode key enzymes of the shikimate pathway. Since a second copy of each of these two genes is found in the genome outside of the balhimycin cluster, we assume that the "pathway-specific" enzymes are responsible for an optimized provision of tyrosine, a precursor of the non-proteinogenic amino acids $\beta$-Ht, Hpg and Dpg.
In the efficient excretion of balhimycin an ABC-transporter is involved whose inactivation led to an intracellular accumulation of balhimycin.
Bbr, a StrR-type regulator, is involved in the transcriptional control of the biosynthetic genes. Furthermore a two component regulator system (VanRS) was identified which may encode an overriding control system responding to the presence of glycopeptide.
In order to improve the yield of balhimycin, gene inactivation and overexpression studies were performed. The manipulation of the transcriptional regulation as well as targeted intervention in the primary metabolism should result in an increased balhimycin production.

## Primary and secondary metabolism

Primary metabolites are elementary puzzle pieces for all microorganisms: They are end products of catabolism, set up to form primary intermediates such as amino acids, nucleotides, vitamins, carbohydrates, and fatty acids. These biosynthetic intermediates are subsequently assembled into the complex and essential metabolites that give structure and biological function to the organism.

Secondary metabolites are produced only by small groups of organism and are not required for growth. They are structurally diverse, generally produced in mixtures with other members of the same chemical family, and formed at low specific growth rates.
Important classes of secondary metabolites are the antibiotics. The metabolic pathways of the primary metabolism often supply the precursors of the antibiotics. There is an early common part which then branches to the synthesis of a primary metabolite on the one hand and to a secondary metabolite on the other.
In some cases, primary end product feedback inhibits the common part of the pathway and thus impairs production of secondary metabolites and accumulation of the precursors. The amount of precursors and cofactors required for synthesis of antibiotics is usually sufficiently low to be easily accommodated by the central carbon metabolism of the cell. In cases that the precursors for antibiotic synthesis are cellular building blocks such as amino acids, carbon is drained from the anabolic routes rather than the central carbon metabolism. To obtain high yield strains it is not only important to increase expression of the biosynthesis genes, but also the activity of the anabolic pathway. The situation is generally more complex when there is a requirement for specific precursors that are synthesized by enzymes encoded by genes in the biosynthesis gene cluster (Gunnarsson et al., 2004).

## Glycopeptide biosynthesis

Synthesis of specific precursors plays an important role in the biosynthesis of glycopeptide antibiotics. Glycopeptides such as vancomycin are the drugs against antibiotic resistant gram-positive bacteria. The best analyzed strain for glycopeptide biosynthesis is *Amycolatopsis balhimycina*, the producer of the vancomycin-type glycopeptide balhimycin (Nadkarni et al., 1994) (Figure 1).

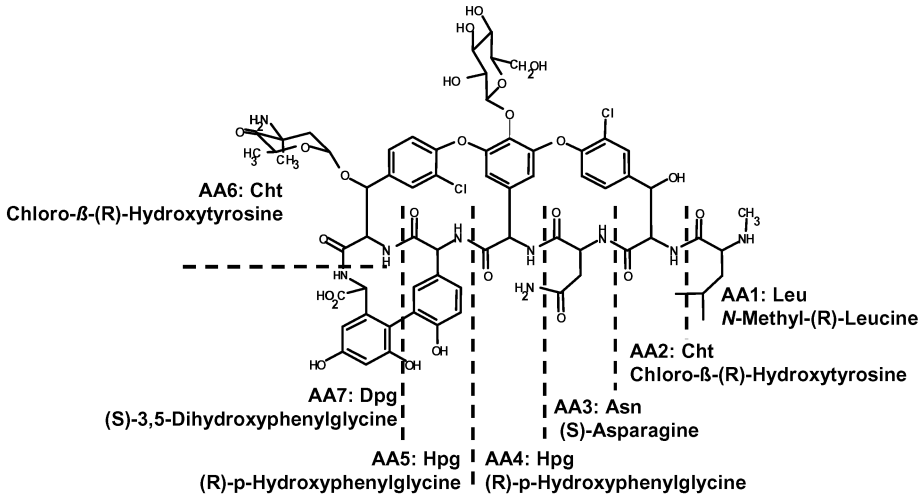


Figure 1: Structure of the glycopeptide balhimycin produced by *A. balhimycina*.

Using a “Reverse Genetics” approach the 66-kb gene cluster (Pelzer et al., 1999) encoding the biosynthesis of balhimycin has been identified. By a combination of genetics, biochemistry and analytical organic chemistry the biosynthetic pathway has been elucidated and the function of nearly all genes of the cluster has been proven.

The biosynthesis starts with the pathway-specific supply of the non-proteinogenic amino acids β-hydroxytyrosine (β-Ht), hydroxyphenylglycine (Hpg) and dihydroxyphenylglycine (Dpg) which form together with (N-methyl) leucine and asparagine the heptapeptide backbone of balhimycin. β-Ht and Hpg are derived from the shikimate branch of amino acid biosynthesis including unusual modification reactions (Hubbard et al., 2000; Puk et al., 2002, Puk et al. 2004).

Hpg is derived from the aromatic ring of tyrosine and the α-carbon of tyrosine is the carboxylic acid carbon of 4-Hpg. In the balhimycin biosynthetic gene cluster three *orfs* (*pgat*, *hmaS* and *hmo*) are located which show high similarity to the genes that participate in the biosynthesis of Hpg from prephenate in the chloroeremomycin producer *Amycolatopsis orientalis* (Hubbard et al. 2000). Therefore, an analogous biosynthetic pathway was postulated for the Hpg-biosynthesis in *A. balhimycina*.

The three genes which are required for the biosynthesis of β-Ht are also localized in the balhimycin biosynthetic gene cluster: the perhydrolase, Bhp, the nonribosomal peptide synthetase module, BpsD and the monooxygenase, OxyD. Inactivation of all these three genes resulted in null mutant strains of *A. balhimycina*. Supplementation of these mutant strains with β-Ht restored balhimycin production thus confirming the involvement of Bhp, BpsD and OxyD in β-Ht-biosynthesis. The proposed model suggested tyrosine being loaded onto BpsD with subsequent β-hydroxylation of BpsD-bound tyrosine catalysed by OxyD (Puk et al., 2004). The last step is the hydrolysis of β-Ht from the BpsD complex due to the action of the hydralase Bhp in order to deliver the free amino acid.

The nonproteinogenic amino acid Dpg is synthesized via a polyketide synthase mechanism (PKSIII) similar to that known from plant chalcon/stilben synthases (Pfeifer et al., 2001). Four genes forming an operon (*dpgABCD*) are required to provide Dpg. Inactivation of DpgA, the predicted polyketide synthase resulted in the loss of balhimycin production. Restoration was achieved by supplementation with 3,5-dihydroxyphenylacetic acid, which is both, a possible product of the PKS and a likely precursor of Dpg. Enzyme assays with the protein expressed in *Streptomyces lividans* showed that DpgA uses only malonyl-CoA as substrate (Pfeifer et al., 2001). The heterologous co-expression of all *dpg* genes in *S. lividans* led to the accumulation of 3,5-dihydroxyphenylglyoxylic acid. The final step in the pathway of Dpg is the transamination. Inactivation of the predicted transaminase gene (*pgat*) resulted in a null mutant strain and in accumulation of 3,5-dihydroxyphenylglyoxylic acid. Restoration of balhimycin production in this mutant was only possible by simultaneous supplementation with (S)-3,5-dihydroxyphenylglycine and (S)-4-hydroxaphenylglycine, indicating that the transaminase is essential for the formation of both amino acids (Pfeifer et al. 2001). Similar to Hpg, the formation of Dpg requires tyrosine as amino donor which thus interconnects Dpg biosynthesis with the shikimate pathway.

Subsequently, the amino acids are linked by non-ribosomal peptide synthetases (Recktenwald et al., 2002). The aromatic side chains are interconnected by P450 monooxygenases (Süßmuth et al., 1999; Zerbe et al., 2002; Stegmann et al., 2006); a series of reactions which occur in a strict order (Bischoff et al., 2001a; 2001b) and lead to the first antibiotically active intermediate. The NADH/FAD-dependent halogenase BhaA catalyzes the chlorination of the two β-Hts (Puk et al. 2002). The substrates of the oxygenases as well as of the halogenase are not free biosynthetic precursors, but rather intermediates which are bound to the NRPS (Bischoff et al., 2005). The resulting cross-linked heptapeptide is then finally modified by methylation and glycosylation.

The final step in the secondary metabolite biosynthesis is the excretion of the products, often mediated by specific transporters. A gene (*tba*) encoding a putative ABC-transporter is part of the cluster. To prove its involvement in balhimycin excretion *tba* was inactivated by gene replacement which did not interfere with growth and did not affect balhimycin

resistance. However, in the supernatant of the *tba* mutant less balhimycin was detected compared to the wild type; and the intracellular balhimycin concentration was 10 times higher in the *tba* mutant than in the wild type.
The biosynthesis is regulated by a StrR-type regulator, which was shown to bind in front of different operons of the balhimycin gene cluster (Shawky et al. 2007). This probably ensures coordinated expression of the biosynthetic genes at the end of the exponential growth phase as shown by RT-PCR. However, it is not known, which signal controls the expression of the *bbr* gene.
In addition to the mentioned structural and regulatory genes the cluster also contains putative resistance genes with similarity to *vanY* (encoding a putative D,D carboxypeptidase) and *vanR* and *vanS* (putative two component regulatory system sensing cell wall antibiotics) (Shawky et al., 2007).

## Interconnection of primary and secondary metabolism in balhimycin biosynthesis

The supply of precursors or cofactors from primary metabolism might be a limiting factor for secondary metabolite production, in order to maximize product yields. Thus it is necessary to redirect primary metabolic fluxes in a manner that supports high secondary metabolite productivities (Thykaer and Nielsen, 2003). Current knowledge of the biochemistry of primary metabolism pathways in actinomycetes is limited but considered to be important for further rational improvement of strains overproducing aromatic amino acids and derived compounds (Hodgson, 2000). The non-proteinogenic amino acids $\beta$-Ht and Hpg of the heptapeptide backbone are directly derived from the aromatic amino acid tyrosine. In addition, tyrosine is the amino donor in the Dpg biosynthesis indicating a key role of the shikimate pathway in precursor supply. As a consequence, the supply of tyrosine may be rate controlling during high-yield glycopeptide production.
The aromatic amino acid L-tyrosine is derived from chorismate, the end product of the shikimate pathway. The key enzymes of the shikimate pathway are responsible for the following steps: DAHP synthase, the first enzyme of the shikimate pathway, condenses the pentose phosphate pathway intermediate D-erythrose 4-phosphate and the glycolytic pathway intermediate phosphoenolpyruvate to DAHP. The chorismate mutase (Cm) catalyses the transformation of chorismate to prephenate. L-tyrosine biosynthesis further proceeds by conversion of prephenate into 4-hydroxy-phenylpyruvate (via prephenate dehydrogenase, Pdh). 4-hydroxy-phenylpyruvate is subsequently transaminated (via aromatic amino acid aminotransferase) to yield L-tyrosine (Michal, 1999). Aromatic amino acid biosynthesis in bacteria is strictly regulated via feedback control mechanisms. Antibiotic biosynthesis may require specific metabolic adaptations, e.g. expression of isoenzymes that serve to avoid feedback regulation by aromatic amino acids.
The balhimycin biosynthetic gene cluster encodes two genes *pdh* and *dahp* which show high similarity to corresponding genes of the primary metabolism. Interestingly, *pdh* is also conserved within the glycopeptide biosynthesis gene clusters of chloroeremomycin (*cep*), dalbavancin (*dbv*), complestatin (*sta*) and teicoplanin (*tcp*), whereas *dahp* exists only in the *cep* cluster; a divergent Dahp synthase is encoded by the *tcp* cluster, but is counted among the unique functions (Donadio et al., 2005).
From the preliminary data of the *A. balhimycina* genome sequencing project it is known that a second copy of the *dahp* and *pdh* genes is located elsewhere in the genome. Therefore, for Dahp and Pdh in the balhimycin biosynthetic gene cluster “pathway specific” functions can be postulated. This assumption is further supported by regulatory studies where it has been demonstrated that these two genes are, like the structural biosynthetic genes, under the control of Bbr, the StrR like regulator of the

balhimycin gene cluster. Analysis on possible operon structures within the balhimycin biosynthesis gene cluster reveals a co-transcription of several genes (Pfeifer et al., 2001; Puk et al., 2004, Shawky et al., 2005). For example it can be suggested that *bbr* and *pdh* comprise an operon as well as the genes expand from *dvaA* to *dahp*. Since gel retardation assays revealed that Bbr binds specifically to the upstream regions of the regulator gene *bbr*, and *dvaA,* involved in the dehydrovancosamine synthesis, *pdh* and *dahp* are Bbr regulated genes. Further, in RT-PCR experiments the expression of *bbr* was analyzed in correlation to growth and antibiotic production together with the expression pattern of some biosynthetic genes. The expression of *bbr* occurs simultaneously with that of the studied biosynthetic genes starting at least 8h before the onset of antibiotic production confirming the results of the gel retardation experiments. These results indicate that components of the primary metabolism are important for a higher antibiotic production (Figure 2). Since Pdh is the first enzyme of the tyrosine branch efficient flux towards hydroxyphenylpyruvate is an essential precondition. Whilst much attention has been paid to the pathways and regulation of the balhimycin biosynthesis, less is known of the pathways and the regulation of primary metabolism. With the imminent completion of the total genome sequence of *A. balhimycina*, understanding of the primary metabolism pathways and their interconnection to the secondary metabolism will be gained. This knowledge will be used for rational improvement of aromatic amino acids and balhimycin production in *A. balhimycina*.

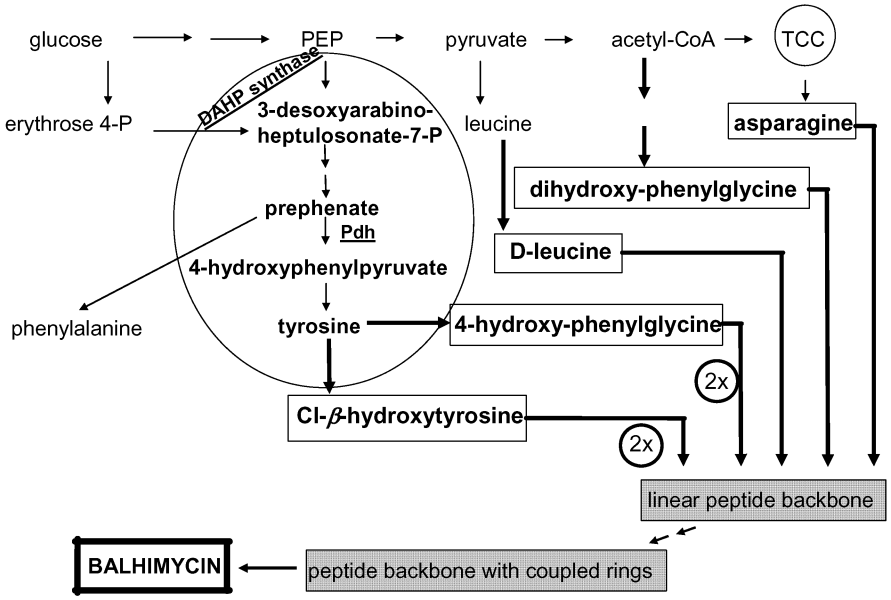


Figure 2: Interconnection of primary and secondary metabolism in balhimycin biosynthesis.

Highlighted by the circle is the tyrosine biosynthesis. Underlined are the enzymes DAHP synthase, 3-desoxyarabino-heptulosonate-7-phosphate synthase, and pdh, prephenate dehydrogenase. The thick arrows indicate the connection to balhimycin biosynthesis. Proteinogenic (leucine and asparagines) and non proteinogenic amino acids (dihydroxy-phenylglycine, 4-hydroxy-phenylglycine and Cl-β-hydroxytyrosine) are shown in white boxes , balhimycin intermediates in grey boxes. P, phosphate; PEP, phosphoenyl pyruvate; TCC, Tri-carbon cycle;

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