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Mutasynthesis in Streptomyces for production of novel calcium-dependent antibiotics

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The calcium-dependent antibiotic (CDA) is a lipopeptide synthesised non-ribosomally and produced by *Streptomyces coelicolor* A3(2). CDA contains several non-proteinogenic amino acid residues. Hydroxyphenylglycine (4-HPG) is one of the unusual amino acids in the structure of the CDA and vancomycin groups of antibiotics. For the members of the vancomycin group of antibiotics, the 4-HPG residue plays crucial roles in the structure and function of the final glycopeptide antibiotic.To reveal the putative biosynthetic pathway of this amino acid in CDA, a standard "double crossover replacement strategy" was used to delete 4-hydroxymandelic acid synthase (4-HMAS, encoded by *hpd*) from *different strains of S. coelicolor*, using the delivery plasmid pZMH3. An in frame-deletion removed the *hpd* gene from the 4-HPG operon and generated null mutations in *S. coelicolor* MT1110 and 2377 (*S. coelicolor* MT1110Δ*hpd* and *S. coelicolor* 2377Δ*hpd*). The deleted regions in both strains were confirmed initially using PCR and finally by Southern analysis. There was no CDA production in the disrupted strains. Plates containing a gradient of hydroxymandelic acid were used to restore CDA production in both *S. coelicolor* MT1110Δ*hpd*. Exogenous supply of 4- hydroxyl phenylglyoxylate and 4-hydroxyphenylglycine re-established CDA production by the *hpd* mutant. Feeding analogs of these precursors to the mutant resulted in the directed biosynthesis of **novel lipopeptides** with modified arylglycine residues (mutasynthesis). The high level of calcium dependent antibiotic activity was detected in these novel antibiotics.

Keywords: mutasynthesis ; novel antibiotic ; Streptomyces ; calcium-dependent antibiotic ; vancomycin; non-robosomally synthesized ;gene deletion.

Introduction:

The calcium-dependent antibiotic (CDA) is a lipopeptide synthesised non-ribosomally and produced by *Streptomyces coelicolor* A3(2). Hydroxyphenylglycine (4-HPG) is one of the unusual amino acids in the structure of the CDA and vancomycin groups of antibiotics, including complestatin (Hubbard *et al*., 2000& Chiu *et al*., 2001). Vancomycin is the last line of defence in the treatment of infections caused by multi-drug-resistant bacteria, and the emergence of vancomycin-resistant enterococci (VRE) is feared, given the possibility of resistance transfer to β-lactam-insensitive staphylococci and streptococci (Chadwick and Wooster, 2000). To be able to make new antibiotics, however, each step in the biosynthetic pathway has first to be elucidated (Pfeifer *et al*., 2001). To establish a biosynthetic pathway for this non-proteinogenic amino acid, a putative biosynthetic pathway was postulated for it in CDA biosynthesis, using the completed sequences from the Sanger Centre and the data from the vancomycin group antibiotics (J. Micklefield; personal communication, Choroba *et al*., 2000). Here, the deletion of the *hpd* gene from *Streptomyces coelicolor* MT1110 is described. The objective was to assess the role of this gene in 4-HPG biosynthesis and thereafter in CDA production. Then the involvement of the *hpd* disrupted strains with the anticipated product of this enzyme was investigated using MT1110Δ*hpd*. In another attempt, the HMAS protein was over-expressed in *E. coli* for biochemical analysis and HPLC-based enzyme assay.

Materials and methods:

CsCl-EtBr gradient preparation of the pZMH3construct was demethylated (*using E. coli* ET12567: dam$^-$ *dcm*$^-$, *hsdM*$^-$) and denatured. It then was used to transform fresh protoplasts from *S. coelicolor* MT1110. Single crossover recombinants were first selected for using

hygromycin, spores from the recombinant colonies were then plated on R5 (without antibiotic selection), for isolating the disrupted strain from its parental strain, (where replica plating was used). Twenty-four "double crossover" recombinants were initially screened using PCR-based assay. To further confirm the deletion in MT1110Δ*hpd*, Southern hybridisation analysis was carried out using the α-^{32}P dCTP labelled 'hpd-AB' fragment as a probe and chromosomal DNA cut with *Pst*I. CDA bioassays were carried out using spores of wild type MT1110 and the disrupted strains.

Results and Discussion:

The putative *hpd* operon for the biosynthesis of the 4-HPG contains three genes *hpd glo* (encodes a putative glycolate oxidase) and *amt*. *hpd* occupies the first position in the operon. Two flanking regions were chosen to make a suitable cassette for this deletion, hpd-A and hpd-B. The cosmid 6E4 was subjected to digestion with *Pst*I to obtain the 2.38 kb hpd-B fragment. For the initial cloning, the pBluescript vector was used. In pZHB the hpd-B is located in the desired orientation as illustrated by restriction digestion with *Kpn*I and and *Bgl*II. To obtain the hpd-A (flanking the other side of the *hpd* gene) cosmid E63 was used as a template for PCR amplification of the desired region. PCR primers were designed to allow the creation of a *Bgl*II and *Xba*I sites in the hpd-A fragment The 2.1 kb amplified hpd-A fragment was digested with *Bgl*II and *Xba*I, gel purified and cloned into pZHB, creating pZHAB(Figure 1). This construct subsequently was lifted out from pBs and **ligated into a suitable *Streptomyces* non-replicative vector (pMAH), resulting pZMH3.** The Southern Analysis results confirmed the existence of the *hpd* deletion in the MT1110 chromosome (Figure 2). The pattern expected was a 2.5 kb band in *S. coelicolor* MT1110 and 2377 wild type and 4.8 kb in successful deletants, due to the loss of one *PstI* site inside of the *hpd* gene. A 0.7 kb band also is seen in both wild type and disrupted strains, since it is a *Pst*I fragment inside the hpd-B. The CDA bioassay results showed no CDA production in *S. coelicolor* MT1110Δ*hpd*. Exogenous supply of 4- hydroxyl phenylglyoxylate and 4- hydroxyphenylglycine re-established CDA production by the *hpd* mutant. Feeding analogs of these precursors to the mutant resulted in the directed biosynthesis of **novel lipopeptides** with modified arylglycine residues (mutasynthesis). The high level of calcium dependent antibiotic activity was detected in these novel antibiotics.

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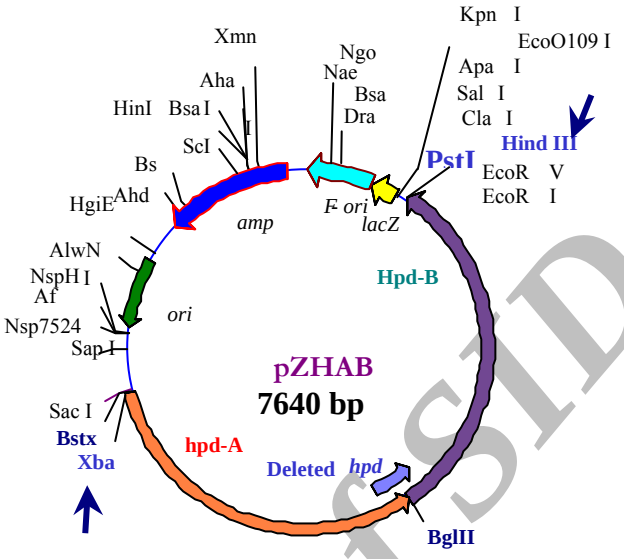

Figure 1: Recombinant plasmid, pZHAB, was constructed during several different steps. The hpd-A fragment in pZHAB was sequenced entirely to check for occurrence of any mistake(s) during PCR amplification. Eight primers were used to sequence the entire hpd-A fragment and the sequence was found to contain no mistakes (data not shown).

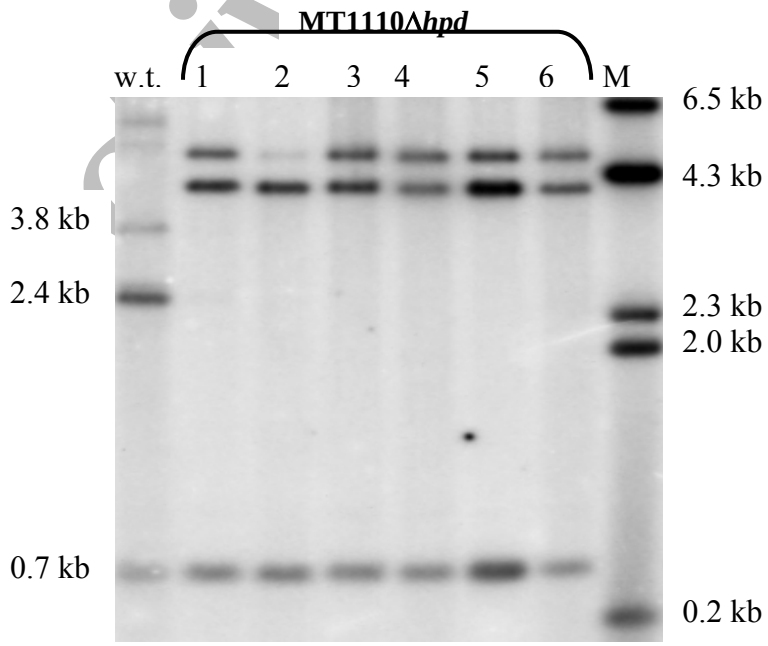

Figure 2: Southern hybridisation to confirm deletion of *hpd* gene in *S. coelicolor* MT1110 creating MT1110Δ*hpd*. The 4.6 kb AB fragment from *Hin*dIII-*Xba*I digested pZHAB was labelled with α-^{32}P dCTP in a reaction using hexanucleotides as a random primer and used as probe. M is for α-^{32}P dCTP labelled λ*Hin*dIII size markers.