

BIOBASED ANTIBIOTICS FROM BASIDIOS: A CASE STUDY ON THE IDENTIFICATION AND MANIPULATION OF A GENE CLUSTER INVOLVED IN PLEUROMUTILIN BIOSYNTHESIS FROM *CLITOPILUS PASSECKERIANUS*

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ABSTRACT

With bacteria becoming resistant to antibiotics, there is a growing need to find new sources of antibiotics. Our work has focussed on the organism *Clitopilus passeckerianus* which produces a natural antibiotic, pleuromutilin. Recently, a derivative of pleuromutilin, retapamulin (developed by GSK) was approved for use in humans. Clinical trials have demonstrated its efficacy against certain Gram-positive bacteria including MRSA. We have developed all the tools to manipulate this important organism, and will present results on transformation, gene manipulation and enhancement, as well as gene isolation and mapping. These tools have allowed us to isolate the pleuromutilin gene cluster. Using the molecular tools we have been able to identify all genes involved, their roles, and perhaps most importantly, the ability to manipulate to elevate levels of antibiotic production and deliberately alter products produced. These results demonstrate that we are able to manipulate and control the *Clitopilus* genome. This provides a molecular toolbox which makes it possible to identify and manipulate individual genes of this fungus, and leading to some major new drugs which are not compromised by antibiotic-resistant strains of bacteria. The results will open up major opportunities for other previously intractable systems and antibiotics in fungi.

Keywords: *Clitopilus passeckerianus*; antibiotic; pleuromutilin; diterpene; GGS

INTRODUCTION

The high incidence of antibiotic resistance in clinical isolates of bacteria is making the control of infection more and more difficult. This is illustrated by the frequency with which infections such as those caused by *Staphylococcus aureus* or *Clostridium difficile* are encountered in hospital situations where they can become life-threatening due to the limited range of control agents to which they are susceptible. This has resulted in increased interest in developing antibiotics that demonstrate new modes of action or overcome existing resistance mechanisms.

One such class of antibiotics is the mutilins, which although used in veterinary medicine for many years, had not been developed for human therapeutics. Retapamulin was approved for human use in 2007, and is a semisynthetic derivative of pleuromutilin (Fig. 1), an antibiotic first reported in 1951 from the homobasidiomycete *Pleurotus passeckerianus*, later reclassified as *Clitopilus passeckerianus* [1, 2]. It is a potent and highly selective antibiotic active against a

range of Gram positive bacteria. Pleuromutilin inhibits protein peptidyl transferase by binding to domain V of 23S rRNA on the 50S ribosomal subunit. Whilst other antibiotics also target this region (e.g. carbomycin), they interact with different target nucleotides so there is no cross resistance to currently used antibiotic classes. This has led to the development of many semi-synthetic analogues, such as valnemulin, tiamulin and retapamulin.

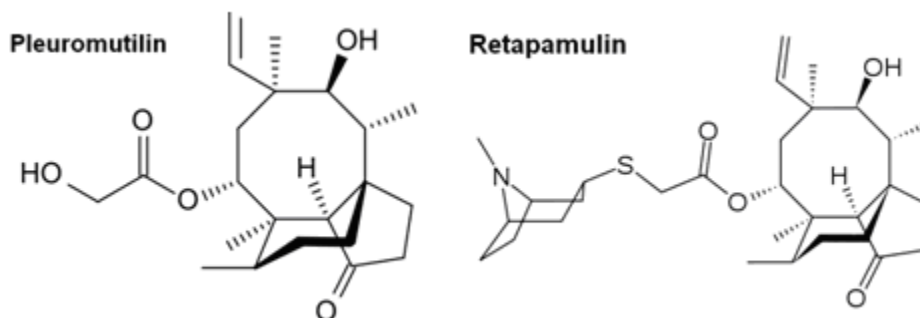


Figure 1: The structure of pleuromutilin and retapamulin.

Whilst retapamulin is only approved for use in creams and other methods of external application, there is ongoing research in a number of pharmaceutical companies seeking to develop derivatives which are suitable for oral administration. Pleuromutilin is a tricyclic diterpene which is commercially produced by fermentation of *C. passeckerianus*, however production is limited due to poor fermenter growth of the fungus, coupled with low titres of the antibiotic. If the present research into orally available derivatives is successful, there may be a substantial demand for the antibiotic, to the extent that production levels could become limiting, or add significantly to the production costs. To date, attempts to increase titre by conventional strain improvement have only met with limited success [3-5], likely due to the dikaryotic nature of the producing organism. It was therefore desirable to develop new methods to increase the titre of antibiotic production within the native host, or to isolate the genes responsible for its production to facilitate pleuromutilin production in a species more amenable to bulk fermentation.

We have recently explored the taxonomy of this group, showing that the various species reported to produce pleuromutilin may in fact be the same on the basis of ITS sequence, with pleuromutilin being made by only a small clade within the Entolomataceae [6, 7]. On the basis of transformation systems established for *Coprinopsis cinerea* and *Agaricus bisporus* [8-10], we then set up methods for transformation of *C. passeckerianus*, using both *Agrobacterium* and protoplast-based techniques [11]. As is typical in many such basidiomycetes [12], efficient expression of heterologous genes has been shown to need the presence of a 5' intron. We have previously used GFP as a target for establishing silencing in other fungi [13-15] and applied this to *C. passeckerianus* showing that gene silencing could be used to suppress activity of a GFP transgene [11]. We therefore have a set of tools which would allow investigation into the molecular basis of pleuromutilin in this fungus.

In other fungi, it is common for all the genes needed for production of a secondary metabolite to be clustered together within the genome, meaning that if you can identify one key gene, the remaining genes ought to be adjacent. Diterpene pathways have been characterized in several ascomycete fungi, and in most cases they contain a gene encoding a pathway-specific

geranylgeranyldiphosphate synthase. We therefore set out to use this as the basis for isolation of the pleuromutilin biosynthetic pathway. We also sought to further characterize *C. passeckerianus* since improved understanding of its biology and genetics may serve to underpin future development of antibiotic production in this species.

MATERIALS AND METHODS

Strains and growth conditions. *C. passeckerianus* DSMZ1602 was obtained from the Deutsche Sammlung von Microorganism and Zellkulturen and was cultivated on Malt Extract Agar (MEA) at 25⁰C.

Pleuromutilin assays. For plate-based bioassays, the fungus was cultured on plates containing 20ml of Tryptic Soy Agar for four days and then overlaid with 5ml agar containing spores of *Bacillus subtilis* ATCC 6633, as detailed in [6]. Colony diameters and clearing zones were measured after an additional 48h incubation at 30⁰C. Cultures for HPLC analysis were prepared as in [6]. Briefly, a starter culture of CS01 medium was inoculated with five 4mm plugs from an agar plate and incubated until uniform mycelial slurry was obtained. This was used to inoculate 100 ml of proprietary high production medium and cultured for six days at 25⁰C with shaking at 220rpm. Aliquots were then homogenised and mixed with three volumes of acetonitrile before centrifugation to remove matriculate matter. This was then analysed by HPLC as described previously [6].

Classical genetics methods. Fruiting bodies of *C. passeckerianus* were obtained after 28 days incubation of MEA plates at 20⁰C under a white light illumination regime of 16h day, 8h night. Fruiting bodies were excised and placed in a Petri-dish to allow spores to be shed. Spores were then harvested in water and diluted appropriately before plating onto MEA to obtain discrete colonies. These were observed by microscopy after 24h and those colonies thought to originate from single spores were identified and sub-cultured. For attempted mating, agar plugs of different lines were placed 5mm apart on MEA plates and incubated for up to 21 days, with frequent observation to note any morphological changes upon mycelial contact. Small-scale DNA extractions and PCR analysis of monokaryons was based on [6] using 30b allele-specific PCR primers designed on derived sequences [16] and with annealing conditions optimised for each primer combination.

Gene isolation and analysis. Genomic DNA was extracted from *C. passeckerianus* as previously described [11] and a lambda genomic library prepared in λ Gem11 (Promega) by partial digested of gDNA with *Sau3A*, partial fill-in of the ends and ligation into pre-prepared vector following manufacturers recommended methods. The ligation mixture was packaged into phage particles and aliquots were transfected into *E.coli*. Approximately 25000 phages were screened in each round of analysis as outlined in [17]. Positive plaques were subcultured and then insert-containing plasmids excised for subsequent analysis.

Degenerate primers were used to amplify fragments of target genes from *C. passeckerianus* by PCR. The products were gel purified (Promega Wizard) and cloned into pCR2.1topo (Invitrogen). Plasmids were extracted (Qiagen miniprepII) and 10 μ g used for sequencing with universal primers (Agowa).

For Northern blotting, RNA was isolated using the method of [17]. Gels were electrophoresed, blotted and probed according to [17] using appropriate $\alpha^{32}\text{PdCTP}$ -labelled PCR fragments as probes.

Fungal Transformation. Plasmids for fungal transformation were prepared by yeast-based recombination, where individual fragments (either restriction fragments or PCR products) were designed to include 30bp of homology between each piece. These were co-transformed into *S. cerevisiae* Y10000 along with appropriate linearised pYES-based vectors [9, 10] and uracil-independent transformants were selected. Plasmids were extracted from yeast colonies using Zymoprep II (Zymogen) and rescued into *E. coli* prior to confirmation by PCR, restriction digestion or sequencing.

Protoplasts of *C. passeckerianus* were prepared from CS01A starter cultures and transformed as described in [11], with hygromycin-resistant transformants being selected on PDA supplemented with 0.6M sucrose, 100 $\mu\text{g/ml}$ hygromycin. These were subjected to three rounds of subculture on PDA with 50 $\mu\text{g/ml}$ hygromycin before analysis of pleuromutilin titre.

RESULTS AND DISCUSSION

Isolation of the pleuromutilin pathway. Alignments of the available GGS enzymes identified conserved regions which were suitable for the design of degenerate oligonucleotide primers for a PCR-based gene isolation approach. PCR using genomic DNA from *C. passeckerianus* as the template, yielded a mixed PCR product. This was cloned and sequencing of numerous clones revealed the presence of four different families of products. One of these showed an expression profile on Northern analysis that was closely correlated with the production of pleuromutilin. The other two did not show increased levels during pleuromutilin production and so were assumed to have housekeeping roles or to be involved in other secondary metabolism pathways.

The pleuromutilin-associated GGS region was used to probe a genomic lambda library resulting in the isolation of several clones. Probes derived from these were then used to walk into the library in each direction and eventually a region spanning 32kb was isolated and fully sequenced. Bioinformatic analysis of this region highlighted the presence of not just the GGS gene, but twelve additional genes (Fig. 2).



Figure 2: The pleuromutilin locus. ORF 5 is the pleuromutilin associated GGS

In addition to isolation of the suspected pleuromutilin gene cluster other target genes were amplified by degenerate PCR and genomic clones identified and sequence, including α -actin (AAGTACCCCATCGAGCACGG and AAGATGACTCAAATCATGTTCGAGAC), β -tubulin (CGATTCCCTGGTCAACTCAACTC and GTGAACTCCATCTCGTCCAT) to use as controls in expression studies and sources of constitutive strong promoters, and the farnesyl diphosphate

synthase (FDS) which may be involved in substrate supply for the pathway. In all cases, two alleles were isolated for each gene.

Confirmation of involvement in pleuromutilin biosynthesis. Genes 1 and 11-13 were unlikely to be involved in pleuromutilin biosynthesis on the basis of similarity to other known proteins, but genes 2-10 were all similar to those involved in secondary metabolite biosynthesis in other species, many of them with functions predicted to be needed for pleuromutilin biosynthesis. Northern analysis was performed using each of these genes to probe expression levels during induction or repression of pleuromutilin biosynthesis. The northern for the pleuromutilin-associated GGS is shown in Figure 3 and was typical of the induced expression during production. This showed that genes 2-8 were coordinately expressed, with increased titres during pleuromutilin biosynthesis, and so are all likely to be part of the pleuromutilin pathway.

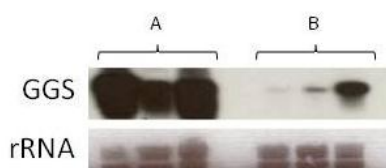


Figure 3: Northern analysis using the candidate pleuromutilin-specific GGS product to probe mRNA from growth conditions with induction (A) and repression (B) of pleuromutilin production at days 3, 4&5, showing induction of expression during pleuromutilin production.

Whilst the gene cluster contained all the genes believed necessary for pleuromutilin biosynthesis, coordinated expression alone was not sufficient proof that this was the correct gene cluster. Therefore gene silencing was used to knock down transcript levels to determine whether this resulted in reduced pleuromutilin titre. Central regions of each gene were amplified using primers designed to allow yeast-based recombination into a suitable vector to express each in an antisense orientation. The vector pYes hph 004cbx [9] was digested with *Xho*I and *Bam*HI to remove the cbx open reading frame and the antisense GGS or other target genes integrated in its place. When transformed into *C. passeckerianus*, approximately 20% of the GGS antisense transformants showed reduced pleuromutilin production as indicated by smaller clearing zones on plate-based bioassay (Fig. 4). Similar results were obtained for some of the other genes believed to be involved in biosynthesis. Where plate-based bioassay indicated reduced titres, this was also observed by HPLC analysis. The reduction in pleuromutilin yields upon this specific gene silencing confirmed that this cluster was indeed responsible for pleuromutilin production.

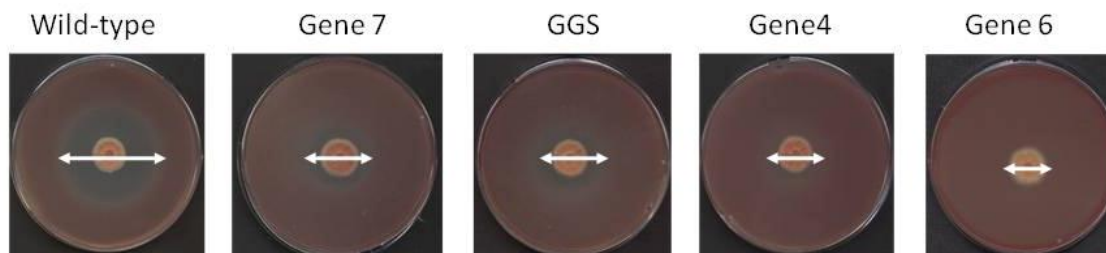


Figure 4: plate-based bioassays showing the reduced titre of pleuromutilin (indicated by arrows) for selected silenced lines.

Reduced titres were not seen for all of the genes analysed, however silencing was not expected to cause a reduction in titre for all the genes, given that some of the later pathway intermediates still have antimicrobial activity. Therefore even if the later stages of the pathway were completely blocked, the accumulating intermediates should still give good clearing zones when assessed by plate-based assay.

Classical genetics on *C. passeckerianus*. The wild-type isolate DSMZ1602 is the strain on which commercial production has been based. During *in vitro* culture we were able to produce fruiting bodies (Fig. 5) which yielded viable basidiospores, indicating that this isolate is a fertile dikaryon as was suspected from the presence of two allelic forms of each gene that had been sequenced. 100 individual lines have been raised from these basidiospores. These strains show variation in growth rates and morphology as would be expected from sexual progeny.



Figure 5: A fruiting body of *Clitopilus passeckerianus*. This is a mature basidiocarp (spore producing), approximately four weeks after plate inoculation. The colouration below the basidiocarp is the brown-pigmented spores.

Allele-specific PCR was performed on 24 progeny to investigate their karyotype. In 20/24 cases these have been confirmed to be monokaryons as determined by the presence of single allelic types for loci including tubulin, actin, FDS as well as various locations within the pleuromutilin cluster, with just four lines giving both patterns suggesting possible dikaryons. Amongst the 20 presumed monokaryon progeny, no recombination events were detected between markers separated by ~11kb within the pleuromutilin gene cluster, indicating that recombination frequencies are low in this region. This low level of recombination means that natural mating and selection may not be a realistic means of strain improvement using alleles within the gene cluster, however it may be a means to alter the genetic background to modify factors such as substrate supply or regulatory processes.

We found that monokaryon lines were able to produce pleuromutilin, often with titres similar to, or only slightly less than the dikaryotic parental strain. These monokaryotic strains were amenable to transformation and to gene silencing. Given that these contain just one nuclear type, such isolates might prove to be more appropriate hosts for genetic modification aimed at strain improvement.

Over-expression of GGS. Whilst gene-silencing showed it was possible to reduce antibiotic titre, thus confirming the involvement of particular genes in pleuromutilin biosynthesis, the overall aim of this research was to produce a strain with an increase in antibiotic yield. The first committed step in pleuromutilin biosynthesis is the pathway-specific GGS, so we set out to over-express this gene in an attempt to increase the rate of entry of precursors into the pathway and hence increase final titre. The GGS cDNA was fused with a 5' intron and placed under the control of the *gpdII* promoter of *A. bisporus*. This promoter plus 5' intron system has previously been shown to give high-level expression in various basidiomycetes [11, 12]. The plasmid was transformed into protoplasts of *C. passeckerianus*, and the resulting transformants screened for antibiotic production. Some of the transformants were found to have increased clearing zones on plate-based bioassay. These were then investigated in more detail by fermentation and HPLC. One such transformant, GGS 16 was shown to have a 116% increase in titre. This is a dramatic increase in yield, given that GGS is already expressed at comparatively high levels during production as assessed by Northern analysis. This highlights the potential for further increases in titre by manipulation of the other genes in this pathway. It may be that increasing the transcripts of some or all of the pathway could lead to even greater increases in titre, particularly if the growth conditions and media were then optimized for such improved strains.

CONCLUSIONS

By means of PCR with degenerate primers we were able to isolate a GGS-like gene that had an expression pattern corresponding to pleuromutilin production and so was likely to be involved in its biosynthesis. This enabled the cloning, sequencing and characterization of a cluster of genes responsible for the production of the diterpene antibiotic pleuromutilin from *Clitopilus passeckerianus*. Co-localisation of these genes shows that the phenomenon of gene clusters for secondary metabolite pathways does occur in basidiomycetes and could be valuable in isolation such pathways from other fungi.

The wild type fungus is a fertile dikaryon and enabled the isolation of monokaryotic sexual progeny, which displayed a range of phenotypes including variable pleuromutilin production, highlighting that conventional mating might feasibly be a method to increase production, but recombination was rather limited and there may not be sufficient natural variants available to make such a route productive. Gene silencing has proven to be very effective in confirming involvement of several of the genes in pleuromutilin biosynthesis, whilst over-expression has been used to create strains with greatly increased titre. Should pleuromutilin derivatives be developed as oral antibiotics, techniques such as these will be invaluable for making production commercially viable. This highlights that basidiomycete fungi are amenable to strain improvement by means of genetic modification and opens the way for future development of this and other pathways using such methods. The development of the necessary tools for manipulating this species should be appropriate to other basidiomycetes.

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REFERENCES

- [1] Kavanagh F. et al. (1951). Antibiotic substances from Basidiomycetes. VIII. *Pleurotus multilus* (Fr.) Sacc. and *Pleurotus passeckerianus* Pilat. *Proc. Natl. Acad. Sci. USA* 37: 570–574.
- [2] Kavanagh F. et al. (1952). Antibiotic substances from Basidiomycetes. IX: *Drosophila subatrata* (Batsch ex Fr.) Quel. *Proc. Natl. Acad. Sci. USA* 38: 555–560.
- [3] Stewart KR. (1986). A method for generating protoplasts from *Clitopilus pinsitus*. *J. Antibiot.* 39: 1486–1487.
- [4] Papa IA. et al. (2006). Increasing pleuromutilin activity of *Clitopilus passeckerianus* by chemical mutagenesis and improvement of production medium. *Philipp. Agric. Sci.* 89: 20–33.
- [5] Knauseder F. & Brandl E. (1976). Pleuromutilins: fermentation, structure and biosynthesis. *J. Antibiot.* 29: 125–131.
- [6] Hartley AJ. et al. (2009). Investigating Pleuromutilin Producing *Clitopilus* Species and related Basidiomycetes. *FEMS Microbiol. Lett.* 297: 24-30.
- [7] Moreno G. et al. (2007). Molecular phylogenetic studies show *Omphalina giovanellae* represents a new section of *Clitopilus* (Agaricomycetes). *Mycol. Res.* 111: 1399–1405.
- [8] Burns AC. et al. (2006). Evaluation of agrobacterium-mediated transformation of *Agaricus bisporus* using a range of promoters linked to hygromycin resistance. *Mol. Biotechnol.* 32:129-138.
- [9] Kilaru S. et al. (2009). Investigating dominant selection markers for *Coprinopsis cinerea*: a carboxin resistance system and re-evaluation of hygromycin & phleomycin resistance vectors. *Curr. Genet.* 55: 543-550.
- [10] Collins CM. et al. (2010). Improvement of the *Coprinopsis cinerea* molecular toolkit using new construct design and additional marker genes. *J. Microbiol. Meth.* 82:156-162.
- [11] Kilaru S. et al. (2009). Establishing molecular tools for genetic manipulation of the pleuromutilin producing fungus *Clitopilus passeckerianus*. *Appl. Env. Microbiol.* 75: 7196-7204.
- [12] Burns C. et al. (2005) Efficient GFP expression in the mushrooms *Agaricus bisporus* and *Coprinus cinereus* requires introns. *Fung. Genet. Biol.* 42: 191-199.
- [13] Heneghan MN. et al. (2007). A Comparison of Methods for Successful Triggering of Gene Silencing in *Coprinus cinereus*. *Mol. Biotechnol.* 35: 283-296.
- [14] Costa AMS. et al. (2008). Oligonucleotide sequences forming short self-complimentary hairpins can expedite the down-regulation of *Coprinopsis cinerea* genes. *J. Microbiol. Meth.* 75: 205-208.
- [15] Costa AMS. et al. (2009). Quantifiable down-regulation of endogenous genes in *Agaricus bisporus* mediated by expression of RNA hairpins. *J. Microbiol. Biotechnol.* 19: 271-276.
- [16] Bailey AM. et al. (2011) Methods of Increasing Yields of Pleuromutilins. *Patent number WO/2011/051820*.
- [17] Sambrook J. and Russell D.W. *Molecular Cloning: a laboratory manual*. 3rd Ed. Cold Spring Harbour Laboratory Press (2001).