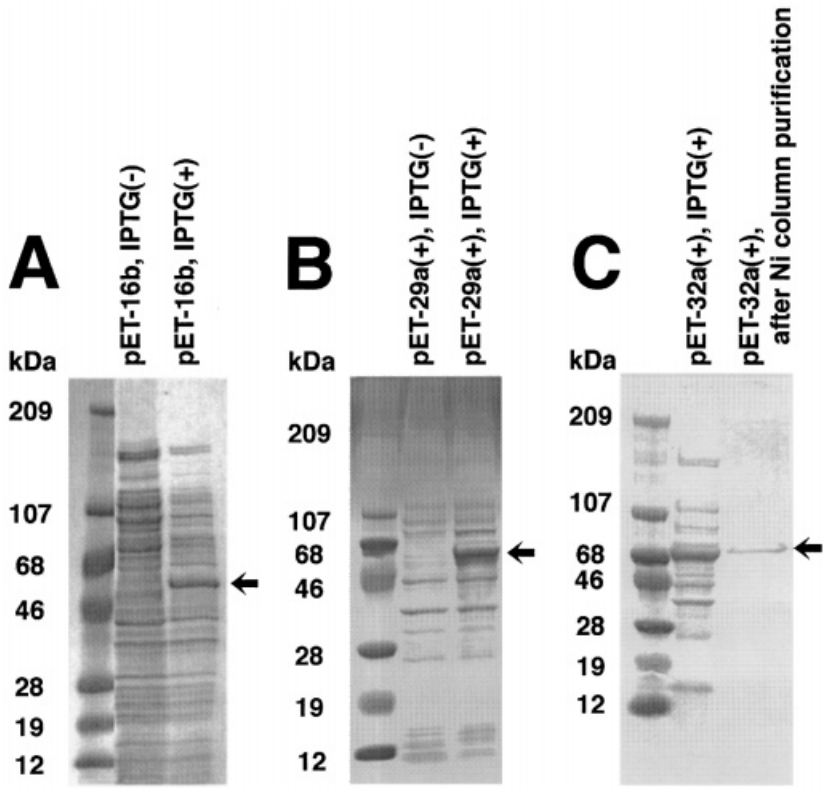

Figure 1. SDS-polyacrylamide gel electrophoresis of soluble fractions of *Escherichia coli* lysate expressing *Neurospora crassa* β-tubulin (A) by pET-16b; (B) by pET-29a(+); (C) by pET-32a(+). IPTG(−) means lysate of *E. coli* incubated without ITPG, and IPTG(+) lysate of *E. coli* incubated with IPTG. Arrows indicate bands of expressed proteins.

to the nickel resin and eluted with the buffer containing 1 M imadazole. The eluted fraction contained only one band of protein on the SDS-polyacrylamide gel electrophoresis (Fig 1-C). The yield of the expressed protein after this purification was about 10% of the total protein expressed in soluble fraction.

4 CONCLUSION

We succeeded in expressing a fungal β-tubulin, which is contained in a very small amount in fungal cells and difficult to purify, in a large amount using the pET expression vector systems in *E coli*. The protein expressed using pET-32a(+) was purified by nickel resin column chromatography. This will open the way to obtaining β-tubulin for three-dimensional structure analysis of the complex with fungicide by NMR. Codon 198 of the β-tubulin cCDN has been altered from GAG (Glu) to GGG (Gly) by a site-directed mutagenesis using PCR (unpublished data). This single base mutagenesis was reported to give a benzimidazole-resistant, diethofencarb-sensitive-type β-tubulin.[4] We have started cloning of the mutated β-tubulin gene into an expression vector for a study to compare the binding mode of benzimidazole fungicides with that of diethofencarb to β-tubulin.

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REFERENCES

1 Davidse LC, *Ann Rev Phytopathol* **24**:43–65 (1986).
2 Fujimura M, Kamakura T and Yamaguchi I, *Nihon Noyaku Gakkaishi* (*J Pestic Sci*) **17**:237–242 (1992).
3 Koenraadt H, Somerville SC and Jones AL, *Phytopathology* **82**:1348–1354 (1992).
4 Fujimura M, Kamakura T, Inoue H, Inoue S and Yamaguchi I, *Pestic Biochem Physiol* **44**:165–173 (1992).

Gene disruption and biochemical characterisation of 3-isopropylmalate dehydrogenase from *Stagonospora nodorum*

R Neil Cooley,* Tracy P Monk, Sheila B McLoughlin, Stephen G Foster and Jane E Dancer
AgrEVO UK Ltd, Chesterford Park, Saffron Walden, Essex CB10 1XL, UK

Abstract: This summary reports work with auxotrophs of the pathogenic fungus *Stagonospora nodorum*, the causal agent of leaf spot and glume blotch in wheat and barley, with a view to investigating the biochemical basis of pathogenicity. Biosynthetic enzymes may serve as targets for novel fungicides and genetic target validation can identify new candidates for a biochemical approach to fungicide discovery.

* Correspondence to: R Neil Cooley, AgroEvo UK Ltd, Chesterford Park, Saffron Walden, Essex CB10 IXL, UK.
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Keywords: pathogenicity; biosynthetic enzymes; amino acid biosynthesis; novel fungicide targets; fungicides

The molecular basis of pathogenicity in fungal plant pathogens has received considerable interest in recent years. A major aim of this research is the molecular dissection of the pathogenesis process via the identification and characterization of pathogenicity determinants.[1] This basic research satisfies the strategic goals of the agrochemical industry through the identification of potential targets for use in fungicide discovery. With the increasing contribution made by high-throughput screening and rational design strategies to the agrochemical research process, the demand for novel biochemical targets is high.[2] The search for new targets extends beyond pathogenicity determinants to include any function whose disruption through the intervention of a chemical leads to a loss of pathogenesis. Thus, housekeeping functions, which may be common to both saphrophytic and pathogenic growth, are also potential agrochemical targets. The link between loss of function and loss of pathogenicity is essentially the principle behind target validation.[3]

The contribution of basic biosynthetic pathways, such as those responsible for amino acid and nucleotide synthesis, to pathogenicity is difficult to predict. It could be envisaged that the fungus does not require these enzymes for growth *in planta* since it has access to such nutrients from its host. There are, however, a number of examples where disruption of these pathways has an effect on pathogenicity. Mutants of *Cladosporium fulvum* Cke and *Sclerotinia sclerotiorum* De Bary deficient in pyrimidine biosynthesis have been reported as non-pathogenic to their hosts.[4,5] Similarly, arginine auxotrophs of the apple scab fungus, *Venturia inaequalis* (Cooke) Wint were unable to infect apple leaves unless supplied with this amino acid, whilst a histidine auxotroph of the rice blast fungus, *Magnaporthe grisea* (Hebert) Barr showed reduced pathogenicity to its host.[6,7] There is also evidence from chemical intervention that amino acid biosynthesis may be required for pathogenicity, in that methionine biosynthesis has been implicated in the mode of action of the anilinopyrimidine and anilide fungicides.[8,9] In herbicide discovery, amino acid and nucleotide biosynthesis have been identified as the target for several highly active compounds, some of which have achieved considerable commercial success and, as a result, enzymes from these pathways have been the focus of recent herbicide design programmes. It was therefore of interest to determine whether amino acid biosynthesis offered potential targets for use in fungicide discovery.

The approach adopted was to investigate auxotrophs of the phytopathogenic fungus *Stagonospora nodorum* (Berk.) Cast & EG Germans. *S nodorum* is a pathogen of wheat and barley which causes the commercially significant leaf spot and glume blotch diseases.[10] It is easily grown in culture and can be readily transformed, so it is amenable to biochemical and molecular approaches.[11] As an Ascomycete it is representative of the major agriculturally important fungal pathogens. *S nodorum* is therefore an appropriate organism in which to evaluate the potential of novel fungicide targets.[12]

A classical mutagenesis approach was used to isolate a mutant of *S nodorum* which was auxotrophic for the amino acid leucine. When inoculated onto wheat leaves this mutant was found to be non-pathogenic. To define the site of the lesion within the leucine biosynthesis pathway a combination of auxonography, using intermediates from the pathway, and biochemical analysis was used. These approaches suggested that the mutant was deficient in the penultimate enzyme in the pathway, 3-isopropylmalate dehydrogenase (IPMDH, EC 1.1.1.85) (Fig 1). The site of the lesion was confirmed genetically by complementation of the auxotroph back to leucine prototrophy by transformation with the IPMDH gene of *Neurospora crassa* Shear & Dodge (leu1).[13]

A cDNA clone of the *S nodorum* IPMDH gene (*leu*A) was isolated by complementation of an *E coli* *leu*B6 mutant with a library generated from RNA extracted from mycelium grown on minimal medium and constructed in the unidirectional cloning vector Unizap XR (Stratagene). The sequence of the insert was determined and found to encode a 365 amino acid polypeptide that showed considerable homology with other fungal IPMDH polypeptides. An overexpression vector was constructed by inserting a PCR generated *leu*A open reading frame into pJLA503 in order to supply enzyme for biochemical characterisation.

A genomic *leu*A clone was isolated by colony hybridisation of an *S nodorum* cosmid library.[14] The gene was localised on a 7.5 kb *Pst*I fragment and sequencing the appropriate region of the subclone has revealed that the *leu*A gene contains five introns all with 5′ and 3′ splice sites similar to consensus for fungal introns. A disruption vector was made by excising an internal portion of the *leu*A gene and replacing this with the hygromycin B resistance cassette from pAN7-1.[15] This disruption vector was linearised and transformed into a wild-type strain of *S nodorum*. Integration of the vector by homologous double-crossover events results in the replacement of the wild-type *leu*A gene by a disrupted form containing the hygromycin B resistance cassette. Amongst the transformants recovered, a number of gene replacement strains were identified. These strains were leucine auxotrophs and when tested were found to be non-pathogenic. The *leu*A replacement strains are specifically mutated at the IPMDH locus, indicating that disruption of this gene alone is sufficient to result in the loss of pathogenicity.

IPMDH catalyses the irreversible oxidation of β-isopropylmalate to α-ketoisocaproate with the con-

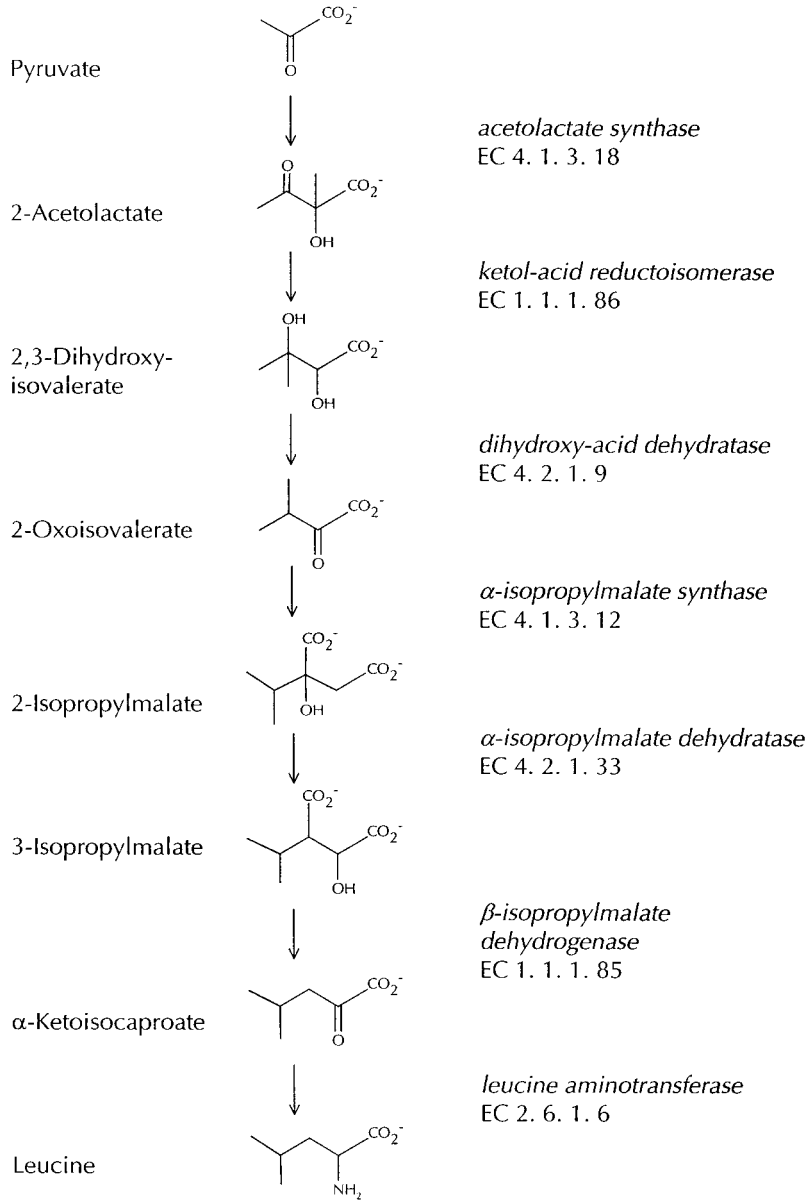

Figure 1. Biosynthesis pathway to leucine.

comitant reduction of the cofactor NAD^+ to NADH (Fig 2). This enzyme has been isolated previously and characterised from a number of different plants and micro-organisms including the fungi *Saccharomyces cerevisiae* Meyer ex Hansen and *Candida maltosa*.[16,17] It is not present in mammals. The enzyme from *S nodorum* was found to have very similar properties to those reported for IPMDH from other sources. The pH optimum is between 8 and 9 and activity is dependent on a divalent cation with Mn^{2+} proving most effective. The Km_{app} values for isopropylmalate and NAD^+ are 13 and 111 μM respectively. IPMDH is an attractive target for rational design approaches. The crystal structure of the enzyme from the extreme thermopile, *Thermus thermophilus*, has been solved to 2.2 Å resolution and, more recently, a 2.5 Å structure has been obtained with substrate and cofactor bound.[18] The mechanism of the reaction catalysed has been elucidated and potent inhibitors which mimic the transition state have already been described[19] These are reported to have moderate herbicidal activity. In developing fungicides active at this target the potential for phytotoxicity is an obvious issue; however, there is plenty of precedent for selectivity through uptake and metabolism. The availability of an abundant source of the fungal enzyme provides opportunities for the discovery of novel inhibitors through high-throughput screening approaches as well as biochemical design.

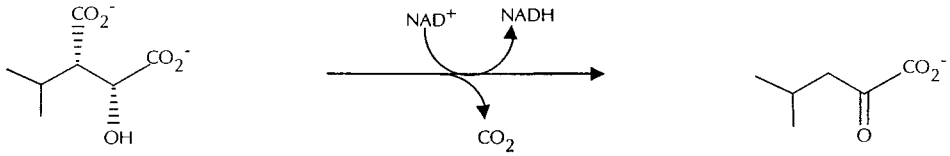

Figure 2. Reaction catalysed by IPMDH.

The loss of pathogenicity associated with an inability to biosynthesise leucine has validated IPMDH as a potential fungicide target. This provides further evidence that biosynthetic enzymes may serve as targets for novel fungicides and has demonstrated that genetic target validation can identify new candidates for biochemical approaches to fungicide discovery.

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REFERENCES

1 Oliver R and Osbourne A, Molecular dissection of fungal phytophathogenicity. *Microbiology* **141**:1–9 (1995).
2 Dancer J, Schulz A, Peine T and Wright K, The value of biochemistry in pesticide discovery research. *Pesticide Outlook* June 1998, 36–41.
3 Foster SG and Höfgen R, Towards a rational design of pesticides, in *Opportunities for molecular biology in crop protection*, ed by Beadle DJ, Bishop DHL, Copping LG, Dixon GK and Hollomon DW, BCPC, Farnham, UK. pp 75–81 (1993).
4 Marmeise R, van den Ackerveken, GFJM, Goosen T, de Wit PJGM and van den Broek HWJ, Disruption of the avirulence gene *avr*9 in two races of the tomato pathogen *Cladosporium fulvum* causes virulence on tomato genotypes with the complementary resistance gene *Cf9*. *Mol Plant-Microbe Interact* **6**:412–417 (1993).
5 Miller RV, Ford EJ, Zidack NJ and Sands DC, A pyrimidine auxotroph of *Sclerotinia sclerotiorum* for use in biological weed control. *J Gen Microbiol* **135**: 2085–2091 (1989).
6 Boone DM, Stauffer JF, Stahmann MA and Keitt GW, *Venturia inaequalis* (Cke) Wint VII. Induction of mutants for studies on genetics, nutrition and pathogenicity. *Am J Bot* **43**:199–204 (1956).
7 Sweigard JA, Carroll AM, Farrall L, Chumley FG and Valent B, *Magnaporthe grisea* pathogenicity genes obtained through insertional mutagenesis. *Mol Plant-Microbe Interact* **11**:404–412 (1998).
8 Fritz R, Lanen C, Colas V and Leroux P, Inhibition of methionine biosynthesis in *Botrytis cinerea* by the anilinopyrimidine fungicide pyrimethanil. *Pestic Sci* **49**:40–46 (1997).
9 Baldwin BC, Brownwell KH, Rees SB, Wiggens TE, Furter R, Masner P and Zobrist P, The mode of action of the anilide fungicide SC-0858. 8th *IUPAC Internat Congr Pestic Chem, Washington, USA*, poster 423 (1994).
10 King JE, Cook RJ and Melville SC, A review of *Septoria* diseases of wheat and barley. *Ann Appl Biol* **103**:345–373 (1983).
11 Cooley RN, Shaw RK, Franklin FCH and Caten CE, Transformation of the phytopathogenic fungus *Septoria nodorum* to hygromycin B resistance. *Curr Genet* **13**:383–389 (1988).
12 Dancer J, Daniels A, Cooley N and Foster S, *Septoria tritici* and *Stagonospora nodorum* as model pathogens for fungicide discovery *Proc 15th Long Ashton International Symposium: Understanding pathosystems: A focus on* Septoria (in press).
13 Jarai G, Yagmai B, Fu Y-H and Marzluf GA, Regulation of branched chain amino acid biosynthesis in *Neurospora crassa:* Cloning and characterization of the *leu*-1 and *ilv*-3 genes. *Mol Gen Genet* **224**:383–388 (1990).
14 Cooley RN, van Gorcom RFM, van den Hondel CAMJJ and Caten CE, Isolation of a benomyl-resistant allele of the β-tubulin gene from *Septoria nodorum* and its use as a dominant selectable marker. *J Gen Microbiol* **137**:2085–2091 (1990).
15 Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH and van den Hondel CAMJJ, Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. *Gene* **56**:117–124 (1987).
16 Hsu Y-P and Kohlaw GB, Leucine biosynthesis in *Saccharomyces cerevisiae:* purification and characterization of β-isopropylmalate dehydrogenase. *J Biol Chem* **255**:7255–7260 (1980).
17 Bode R and Birnbaum D, Some properties of the leucine-biosynthesizng enzymes from *Candida maltosa*. *J Basic Microbiol* **31**:21–26 (1991).
18 Hurley JH and Dean AM, Structure of 3-isopropylmalate dehydrogenase in complex with NAD^+: ligand-induced loop closing and mechanism for cofactor specificity. *Structure* **2**:1007–1016 (1994).
19 Wittenbach VA, Rayner DR and Schloss JV, Pressure points in the biosynthetic pathway for branched-chain amino acids. *Current Topics in Plant Physiol* **7**: 69–88 (1992).

Involvement of the alternative oxidase in cellular energy production in the wheat 'take all' fungus, *Gaeumannomyces graminis* var *tritici*

Tim Joseph-Horne,[1]* Paul M Wood,[2] Claudia Heppner[3] and Derek W. Hollomon[1]

[1] *IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol, BS41 9AF, UK*

[2] *University of Bristol, Department of Biochemistry, School of Medical Sciences, University Walk, Bristol, BS8 1TD, UK*

[3] *Monsanto Technical Centre, Rue Laid Burnait, B-1348 Louvain-la-Neuve, Belgium*

Abstract: This summary describes a functional role for the alternative respiratory pathway of the wheat 'take-all' fungus, *Gaeumannomyces graminis* var *tritici* (Ggt), which utilises the alternative oxidase as its terminal oxidase. Unlike other plant and fungal alternative oxidases, the alternative oxidase of Ggt is both constitutively expressed and active. We have demonstrated that for cellular respiration, and therefore for ATP synthesis, to occur both the cytochrome and alternative pathways must remain active.

Keywords: *Gaeumannomyces graminis*; alternative respiration; ATP synthesis; electron transfer

Cellular energy production occurs in a tightly regulated manner with the complete oxidative metabolism of sugars producing carbon dioxide, water and ATP. Whilst this process normally yields between 32 and 38 ATP molecules per glucose molecule, only four of these ATPs are produced during glycolysis and within the TCA cycle. The remainder are formed by the mitochondrial ATP synthase, in a

* Correspondence to: Tim Joseph-Horne, IACR-Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF, UK
E-mail: TIM.JOSEPH-HORNE@bbsrc.ac.uk
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