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# Adaptive evolution of drug targets in producer and non-producer organisms

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## Synopsis

Mycophenolic acid (MPA) is an immunosuppressive drug produced by several fungi in *Penicillium* subgenus *Penicillium.* This toxic metabolite is an inhibitor of IMP dehydrogenase (IMPDH). The MPA biosynthetic cluster of *P. brevicompactum* contains a gene encoding a B-type IMPDH, IMPDH-B, which confers MPA-resistance. Surprisingly, all members of subgenus *Penicillium* contain genes encoding IMPDHs of both the A and B type, regardless of their ability to produce MPA. Duplication of the IMPDH gene occurred prior to and independent of the acquisition of the MPA biosynthetic cluster. Both *P. brevicompactum* IMPDHs are MPA-resistant while the IMPDHs from a nonproducer are MPA-sensitive. Resistance comes with a catalytic cost: while *P. brevicompactum* IMPDH-B is >1000-fold more resistant to MPA than a typical eukaryotic IMPDH, its value of $k_{cat}/K_m$ is 0.5% of "normal". Curiously, IMPDH-B of *Penicillium chrysogenum*, which does not produce MPA, is also a very poor enzyme. The MPA binding site is completely conserved among sensitive and resistant IMPDHs. Mutational analysis shows that the C-terminal segment is a major structural determinant of resistance. These observations suggest that the duplication of the IMPDH gene in *Pencillium* subgenus *Penicillium* was permissive for MPA production and that MPA production created a selective pressure on IMPDH evolution. Perhaps MPA production rescued IMPDH-B from deleterious genetic drift.

### Keywords

IMPDH; gene duplication; *Penicillium*; mycophenolic acid; neofunctionalization; drug resistance

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## Introduction

Filamentous fungi produce a vast arsenal of toxic natural products that require the presence of corresponding defense mechanisms to avoid self-intoxication. The importance of these defense mechanisms is demonstrated by the presence of resistance genes within biosynthetic gene clusters, yet how production and resistance co-evolved is poorly understood. Insights into the inhibition of enzymes involved in self-resistance provide an intriguing strategy for the development of antifungal agents. Further, the elucidation of the defense mechanisms is required for the design of heterologous cell factories producing bioactive compounds.

Self-resistance can involve expression of a target protein that is impervious to the toxic natural product, which suggests that resistance originates from a gene duplication event. The biosynthetic cluster of the immunosuppressive drug mycophenolic acid (MPA) offers an intriguing example of this phenomenon. MPA is a well-characterized inhibitor of IMP dehydrogenase (IMPDH) [1], and the *P. brevicompactum* MPA biosynthetic cluster contains a second type of IMPDH gene that confers MPA-resistance (IMPDH-B/*mpaF*; [2, 3]). Curiously, both MPA producers and non-producers in *Penicillium* subgenus *Penicillium* contain two IMPDH genes encoding IMPDH-A and IMPDH-B [3]. Phylogenetic analysis suggests that the gene duplication event occurred before or simultaneous with the radiation of *Penicillium* subgenus *Penicillium* [2, 3]. How this gene duplication event influenced the acquisition of MPA biosynthesis is not understood.

Here we investigate the relationship between MPA production, MPA resistance and the properties of IMPDH-A and IMPDH-B. While IMPDH-B from the producer organism *P. brevicompactum* is extraordinarily resistant to MPA, IMPDH-B from the nonproducer *P. chrysogenum* displays typical sensitivity. Both IMPDH-Bs are very poor enzymes, but *P. chrysogenum* IMPDH-B is almost nonfunctional. These observations suggest that acquisition of the MPA biosynthetic cluster may have rescued IMPDH-B from deleterious genetic drift.

## Experimental

### Fungal strains

*P. brevicompactum* IBT23078, *P. chrysogenum* IBT5857, *A. nidulans* IBT27263, and the 11 strains listed in Supplementary Table S1, were grown on Czapek yeast extract agar (CYA) at 25°C [4].

### MPA treatment of fungi

Spores from *P. brevicompactum* IBT23078, *P. chrysogenum* IBT5857 and *A. nidulans* IBT27263 were harvested and suspended in sterile water. 10 μl of serial 10-fold spore dilutions were spotted on CYA plates with or without 200 μg/ml MPA. All plates contained 0.8 % (v/v) methanol. Stock solution (25 mg/ml MPA in methanol) and MPA plates were made briefly before the spores were spotted on the plates in order to avoid MPA degradation. MPA was acquired from Sigma.

## RNA purification and cDNA synthesis

Spores from *P. brevicompactum* IBT23078 were harvested and used to inoculate 200 ml yeast extract sucrose (YES) medium in 300 ml shake flasks without baffles [4]. *P. brevicompactum* was grown at 25°C and 150 rpm shaking. After 48 hours the mycelium was harvested and RNA was purified using the Fungal RNA purification Miniprep Kit (E.Z.N.A) and cDNA was synthesized from the RNA using Finnzymes Phusion™ RT-PCR Kit according to the instructions of the two manufactures.

## Plasmid construction

Constructs for expression of His-tagged IMPDHs in *E. coli* were created by inserting the IMPDH CDSs into a pET28a that had been converted into a USER compatible vector (Figure S1). pET28U was created by PCR amplifying pET28 with the primers 527/528 followed by DpnI treatment to remove the PCR template (Figure S1). The *P. brevicompactum* IMPDH-B gene was amplified from cDNA from *P. brevicompactum.* Genes encoding *A. nidulans* IMPDH-A (*An*IMPDH-A), *P. brevicompactum* IMPDH-A (*Pb*IMPDH-A), *P. chrysogenum* IMPDH-A (*Pc*IMPDH-A) and *P. chrysogenum* IMPDH-B (*Pc*IMPDH-B) were obtained from gDNA. In all cases, the three exons of each gene were individually PCR amplified and purified and subsequently USER fused using a method previously described [5, 6]).

The expression constructs for chimeric and mutated IMPDHs were created by fusing the two parts of the IMPDHs using the USER fusion method to introduce the mutation in the primer tail [6]. Chimeric IMPDH of *Pb*IMPDH-B (N-term) and *Pb*IMPDH-A (C-term) was created by USER fusing two *P. brevicompactum* IMPDH-B and IMPDH-A fragments, which were PCR amplified with primer-pairs 529/667 and 668/540, respectively, into pET28U as described above. Similarly, chimeric IMPDH of *Pb*IMPDH-A (N-term) and *Pb*IMPDH-B (C-term) was created by amplifying *Pb*IMPDH-A and *Pb*IMPDH-B fragments with primer-pairs 539/669 and 670/530, respectively , followed by USER fusing into pET28U. Using gDNA from the proper source, the Y411F (Chinese hamster ovary IMPDH2 numbering) mutation was introduced into *A. nidulans* IMPDH-A and into *P. brevicompactum* IMPDH-A and B by amplifying and fusing two IMPDH fragments with primers 545/359 and 546/358; 539/455 and 540/454; and 529/361 and 530/360, respectively. For details on primers see Tables S2 and S3. The PfuX7 polymerase was used for PCR amplification in all cases [7].

## Cladistic analysis

Alignment of DNA coding regions and protein were performed with Clustal W implemented in the CLC DNA workbench 6 (CLC bio) using the following parameters: gap open cost = 6.0, gap extension cost = 1.0, and end gap cost = free. A cladogram was constructed with the same software using the neighbour-joining method and 1000 bootstrap replicates [PMID: 3447015]. The DNA sequence of IMPDH and *β*-tubulin from selected fungi were either generated by degenerate PCR (dPCR, Supplementary Table S1) or retrieved from NCBI. *A. nidulans β*-tubulin [GenBank:XM_653694], IMPDH-A DNA sequence [GenBank: BN001302 (ANIA_10476)], *Coccidioides immitis* β-tubulin [GenBank: XM_001243031], IMPDH-A DNA sequence [GenBank: XM_001245054], *P. bialowiezense* IBT21578 *β*-tubulin [GenBank:JF302653], IMPDH-A DNA sequence [GenBank:JF302658], IMPDH-B

DNA sequence [GenBank:JF302662], *P. brevicompactum* IBT23078 β-tubulin [GenBank:JF302653], IMPDH-A DNA sequence [GenBank:JF302657], *P. carneum* IBT3472 β-tubulin [GenBank:JF302650], IMPDH-A DNA sequence [GenBank:JF302656], IMPDH-B DNA sequence [GenBank:JF302660],)], *P. chrysogenum* IBT5857 β-tubulin [GenBank:XM_002559715], IMPDH-A DNA sequence [GenBank:XM_002562313], IMPDH-B DNA sequence[GenBank:XM_002559146], *P. paneum* IBT21729 β-tubulin [GenBank:JF302651], IMPDH-A DNA sequence [GenBank:JF302654], IMPDH-B DNA sequence [GenBank:JF302661], *P. roqueforti* IBT16406 β-tubulin [GenBank:JF302649], IMPDH-A DNA sequence [GenBank:JF302655], IMPDH-B DNA sequence [GenBank:JF302659]. The MPA gene cluster sequence from *P. brevicompactum* IBT23078, which contains the IMPDH-B gene sequence (*mpaF*) is available from GenBank under accession number [GenBank:HQ731031]. Full length protein sequences were retrieved from NCBI under the following accessions *Candida albicans*: [GenBank: EEQ46038], Chinese hamster IMPDH2 [GenBank:P12269], *E. coli* [GenBank:ACI80035], Human IMPDH type 2 [GenBank:NP_000875], *P. chrysogenum* IMPDH-A gene [GenBank: CAP94756], IMPDH-B [GenBank:CAP91832], Yeast IMD2 [GenBank:P38697], IMD3 [GenBank:P50095], IMD4 [GenBank:P50094].

## Degenerate PCR

Primers and PCR conditions for amplifying part of the genes encoding IMPDH-A, IMPDH-B and beta-tubulin sequence was as described in [3]. Genomic DNA from 11 fungi from the *Penicillium* subgenus *Penicillium* sub-clade (Table S1) were extracted using the FastDNA® SPIN for Soil Kit (MP Biomedicals, LLC). PCR primer pairs 236HC/246HC or 531/532 were used to amplify DNA from the gene encoding IMPDH-A. The primer pair 240 HC/241 HC was used to amplify DNA from the gene encoding IMPDH-B, and the primer pair 343/344 was used to amplify β-tubulin.

## Expression and purification of His-tagged proteins

Plasmids were expressed in a Δ*guaB* derivative of *E. coli* BL21(DE3) [8]. Cells were grown in LB medium at 30°C until $OD_{600}$ = 1.0 for *An*ImdA and 1.5 for all other enzymes, and induced overnight at 16°C with 0.5 mM IPTG for *An*ImdA and 0.1 mM IPTG for all other enzymes. Cells were harvested by centrifugation and resuspended in buffer containing 20 mM $Na_2HPO_4$, 500 mM NaCl, 5 mM β-mercaptoethanol, 5 mM imidazole and *Complete* protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Cell lysates were prepared by sonication followed by centrifugation. All enzymes were purified using a HisTrap affinity column (GE Healthcare, Piscataway, NJ) on an AKTA Purifier (Amersham Biosciences, GE Healthcare Piscataway, NJ) and dialyzed into buffer containing 50 mM Tris, pH 8.0, 100 mM KCl, 1 mM DTT and 10% glycerol. All enzymes were purified to >90% purity as determined by SDS-PAGE with the exception of *Pc*IMPDH-B, which was partially purified to ~45% purity due to poor expression and stability (Figure S2).

## Enzyme concentration determination

Enzyme concentration was determined by the BioRad assay according to manufacturer's instructions using IgG as a standard. The BioRad assay overestimates IMPDH concentration

by a factor of 2.6 [9]. Concentration of active enzyme was determined by MPA titration using an equation for tight binding inhibition (eq 1).

$$v_i/v_0 = \left(1 - \left((\mathrm{E} + [\mathrm{I}] + IC_{50}) - \left(\left((\mathrm{E} + [\mathrm{I}] + IC_{50})^2\right) - 4 \cdot \mathrm{E} \cdot [\mathrm{I}]\right)^{0.5}\right) / (2 \cdot \mathrm{E})\right) \quad (1)$$

### Enzyme assays

Standard IMPDH assay buffer consists of 50 mM Tris, pH 8.0, 100 mM KCl, 1 mM DTT, and varying concentrations of IMP and $NAD^+$ (concentrations used for each enzyme is listed in Table S4. Enzyme activity was measured by monitoring the production of NADH by changes in absorbance increase at 340 nm on a Cary Bio-100 UV-vis spectrophotometer at 25°C ($\varepsilon_{340}$ = 6.2 $mM^{-1}$ $cm^{-1}$). MPA inhibition assays were performed at saturating IMP and half-saturating $NAD^+$ concentrations to avoid complications arising from $NAD^+$ substrate inhibition. IMP-independent reduction of $NAD^+$ was observed in the partially purified preparation of *Pc*IMPDH-B at ~25% of the rate in the presence of IMP. The rate of this background reaction was subtracted from rate with IMP for each MPA concentration. Initial velocity data obtained by fitting to either the Michaelis-Menten equation (eq 2) or an uncompetitive substrate inhibition equation (eq 3) using SigmaPlot (Systat Software, Inc.).

$$v = V_m [\mathrm{S}] / (K_M + [\mathrm{S}]) \quad (2)$$

$$v = V_m / (1 + (K_M / [\mathrm{S}]) + ([\mathrm{S}] / K_{ii})) \quad (3)$$

### Bacterial complementation assays

Cultures were grown overnight in LB medium with 25 μg/ml kanamycin. 5 μl of 1:20 serial dilutions were plated on M9 minimal media containing 0.5% casamino acids, 100 μg/ml L-tryptophan, 0.1% thiamin, 25 μg/ml kanamycin, and 66 μM IPTG. Guanosine supplemented plates contain 50 μg/ml guanosine. MPA plates contain 100 μM MPA. Plates were incubated overnight at 37°C and then grown at room temperature.

### Sequences generated in this study

See Table S1 for GenBank accession numbers.

## Results

### The presence of IMPDH-B is not linked to MPA biosynthesis

The first gene encoding IMPDH-B was identified in *P. brevicompactum* where it is part of the MPA biosynthetic cluster and confers MPA resistance (*P. brevicompactum* IMPDH-B is also known as *mpaF*) [2]. Of the full genome sequences currently available for filamentous fungi, only *P. chrysogenum* (the sole representative of *Penicillium* subgenus *Penicillium*) contains an IMPDH-B gene. We previously analyzed 4 other fungi in *Penicillium* subgenus *Penicillium* for the presence of IMPDH genes, and all contain both IMPDH-A and IMPDH-B [3]. We have now expanded this search to include 11 additional *Penicillium* strains of the

*Penicillium* subgenus. Degenerate PCR analysis revealed that all 11 strains contain both genes encoding IMPDH-A and IMPDH-B (Supplementary Table S1 and Figure S3), suggesting that the presence of IMPDH-B is widespread in species of *Penicillium* subgenus *Penicillium*, even though many of these strains do not produce MPA [10]).

It is possible that these MPA non-producer strains may contain latent or cryptic MPA biosynthetic genes. A previously described example is that although *P. chrysogenum* does not produce viridicatumtoxin, griseofulvin and tryptoquialanine, it does contain sequence regions with high identity to these biosynthetic clusters, which suggests that these gene functions were lost during evolution [11, 12]. Likewise, *P. chrysogenum* does not produce MPA. However, in this case BLAST searches failed to identify any genes with significant sequence identity to the MPA biosynthesis gene cluster in *P. chrysogenum*. These searches did identify an IMPDH-B pseudogene in *P. chrysogenum.* This 260 bp gene fragment has highest identity to IMPDH-B from *P. chrysogenum* (77%) and 64% identity to IMPDH-B from *P. brevicompactum*.

We analyzed the regions surrounding the MPA biosynthetic cluster in *P. brevicompactum* and used BLAST searches to identify similar genes in *P. chrysogenum*. In this way, adjacent genes that are highly similar and syntenic to genes found in *P. chrysogenum* were identified (Figure 1). Interestingly, the genes flanking one side of the cluster correspond to a locus on contig Pc0022, while the other side of the cluster corresponds to a locus located at another contig, Pc0021. Regions further away from the MPA cluster in *P. brevicompactum* contain additional homologs to *P. chrysogenum* genes, but these genes are located in many different contigs (Table S5). *P. chrysogenum* contains small sequence regions with high identity to the flanking region of the MPA cluster. This identity is in the promoter regions and most likely represents conserved regulatory regions. Despite a detailed search, we found no sequence similarity to the regions around the *Pc*IMPDH-B and the pseudo IMPDH-B (Pc22g07570) (data not shown). Together these findings suggest that the MPA cluster has never been present in *P. chrysogenum* and that a high degree of genome shuffling has occurred since the divergence of *P. brevicompactum* and *P. chrysogenum*.

### *Pb*IMPDH-B is MPA-resistant, but *Pc*IMPDH-B is MPA-sensitive

The *Pb*IMPDH-B gene confers MPA resistance on *A. nidulans*, suggesting that MPA resistance might be a common feature of all IMPDH-B proteins [3]. Consistent with this hypothesis, *P. chrysogenum* is less sensitive to MPA than *A. nidulans*, though significantly more sensitive than *P. brevicompactum* (Figure 2A). These observations suggest that *P. chrysogenum* IMPDH-B may be semi-resistant to MPA.

To further investigate this hypothesis, IMPDHs from *P. brevicompactum*, *P. chrysogenum* and *A. nidulans* were heterologously expressed as N-terminal $His_6$-tagged proteins in an *E. coli* Δ*guaB* strain that lacks endogenous IMPDH; these enzymes are denoted *Pb*IMPDH-A, *Pb*IMPDH-B, *Pc*IMPDH-A, *Pc*IMPDH-B and *An*ImdA (Figure 2B). All strains grow on minimal media in the presence of guanosine (Figure 2B). Bacteria expressing *An*ImdA, *Pb*IMPDH-A, *Pb*IMPDH-B and *Pc*IMPDH-A grow on minimal media in the absence of guanosine, indicating that active IMPDHs were expressed in all four cases. Only bacteria

expressing *Pb*IMPDH-B were resistant to MPA (Figure 2B). *Pc*IMPDH-B failed to grow on minimal media in the absence of guanosine, suggesting that this protein was not able to complement the *guaB* deletion. This observation suggests that *Pc*IMPDH-B may be nonfunctional.

All five fungal IMPDHs were isolated to further investigate the molecular basis of MPA resistance. With the exception of *Pc*IMPDH-B, all enzymes were purified to >90% purity in one step using a nickel affinity column (Figure S2). *Pc*IMPDH-B was unstable and could only be purified to ~45% homogeneity. Western blot analysis using anti-human IMPDH2 polyclonal antibodies showed that *Pc*IMPDH-B had undergone proteolysis. This degradation could not be prevented by the inclusion of protease inhibitor cocktails during the purification. We performed MPA titrations to determine the concentration of active enzyme in all five protein samples. MPA traps an intermediate in the IMPDH reaction, so that only active enzyme will bind MPA [13]. All of the proteins were ~100% active with the exception of *Pc*IMPDH-B, which was 48% active as expected. The oligomeric states of *An*ImdA, *Pb*IMPDH-A and *Pb*IMPDH-B were examined by gel filtration chromatography. All three enzymes eluted at a molecular weight of approximately 220 kDa, consistent with being tetramers (Figure S3).

*An*ImdA, *Pc*IMPDH-A and *Pc*IMPDH-B are all sensitive to MPA, with values of $IC_{50}$ ranging from 26-60 nM (Figure 3 and Table 1). Similar MPA inhibition has been reported for the mammalian and *C. albicans* enzymes [13, 14]. These observations indicate that MPA resistance is not a general property of IMPDH-Bs.

MPA is a poor inhibitor of both *Pb*IMPDH-A and *Pb*IMPDH-B (Figure 3 and Table 1). *Pb*IMPDH-A is 10-fold more resistant than IMPDHs from the other filamentous fungi while *Pb*IMPDH-B is a remarkable ~$10^3$-fold more resistant. Thus the ability of *P. brevicompactum* to proliferate while producing MPA can be explained by the intrinsic resistance of its target enzymes. These observations suggest that MPA production creates a selective pressure on IMPDH evolution. Curiously, similar $IC_{50}$ values have been reported for Imd2 and Imd3 from *Saccharomyces cerevisiae* ($IC_{50}$ = 70 μM and 500 nM for Imd2 and Imd3, respectively;[15]). These enzymes are the only other examples of naturally occurring MPA-resistant IMPDHs from eukaryotes. Cladistic analysis indicates that the *S. cerevisiae* IMPDHs arose in a distinct gene duplication event (Figure 4). Therefore *P. brevicompactum* IMPDH-B is unlikely to share a common ancestry with *S. cerevisiae* Imd2.

## IMPDH-Bs are very poor IMPDHs

The reactions of the fungal IMPDHs were characterized to determine how MPA resistance affects enzymatic function. The values of $k_{cat}$ are similar for *An*ImdA, *Pc*IMPDH-A, *Pb*IMPDH-A and *Pb*IMPDH-B and generally typical for eukaryotic IMPDHs (Table 1, [13]). Typical values of $K_M$ ($NAD^+$) and $K_M$ (IMP) are also observed for *An*ImdA and *Pc*IMPDH-A; the values of $K_M$ for *Pb*IMPDH-A are high, but not unprecedented. In contrast, the value of $K_M$(IMP) = 1.4 mM for *Pb*IMPDH-B is ~100-fold higher than the highest value reported for any other IMPDH. Like *Pb*IMPDH-B, *Pc*IMPDH-B exhibits an abnormally high value of $K_M$ (IMP). The value of $k_{cat}$ of *Pc*IMPDH-B is more than a factor of 100 less than those of the other fungal IMPDHs. The corresponding values of $k_{cat}/K_M$ are

the lowest ever observed for an IMPDH by factors of $10^3$ (IMP) and 200 ($NAD^+$) (see [13] for compilation of values). Therefore both IMPDH-Bs are inferior enzymes.

### Identification of a major determinant of MPA resistance

MPA inhibits IMPDHs by an unusual mechanism [16]. The IMPDH reaction proceeds via the covalent intermediate E-XMP*, which forms when the active site cysteine residue attacks the C2 position of IMP, transferring a hydride to $NAD^+$ [13]. The resulting NADH dissociates, allowing MPA to bind to E-XMP*, preventing hydrolysis to form XMP. The crystal structure of the E-XMP*•MPA complex has been solved for Chinese hamster IMPDH type 2 [17], revealing that MPA stacks against the purine ring of E-XMP* in a similar manner to the nicotinamide of $NAD^+$. Surprisingly, all of the residues within 4 Å of MPA are completely conserved in the fungal IMPDHs (Figure 5). Likewise, the IMP binding site is also highly conserved. Only residue 411 is variable (Chinese hamster ovary IMPDH2 numbering); the IMPDH-A enzymes contain a tyrosine residue at this site, while IMPDH-B enzymes have phenylalanine residues (Figure 5). Substitution of Tyr411 with Phe did not increase the MPA resistance of *Pb*IMPDH-A, nor did the Phe411 to Tyr substitution decrease the MPA resistance of *Pb*IMPDH-B (Table 2). Therefore the structural determinants of MPA resistance must reside outside the active site.

Inspection of the structure suggested that the C-terminal segment (residues 498-527) was another likely candidate for a structural determinant of MPA resistance (Figure 5). This region includes part of the site that binds a monovalent cation activator, as well as segments that interact with the active site residues. Deletion of this segment inactivates the enzyme [18]. Swapping the 30 residue C-terminal segments revealed that this region is responsible for ~7 of the 60-fold difference in MPA resistance between *Pb*IMPDH-A and *Pb*IMPDH-B, see Table 2. Additional structural determinants of MPA resistance remain to be identified.

## Discussion

Phylogenetic analysis indicates that the IMPDH gene was duplicated prior to the radiation of *Penicillium* subgenus *Penicillium* (Figure 4 and Figure S4). No remnants of the MPA biosynthetic cluster are present in *P. chrysogenum*, which suggests that the ability to produce MPA arrived after duplication of the IMPDH genes. Consistent with this hypothesis, both IMPDHs from the MPA-producer *P. brevicompactum* are more resistant to MPA than the IMPDHs from *A. nidulans* and *P. chrysogenum*. Thus, both *P. brevicompactum* IMPDH-A and IMPDH-B have evolved in response to the presence of MPA. *Pb*IMPDH-B is 1000x more resistant than typical eukaryotic IMPDHs. The structural basis of these functional differences is not readily discerned. Whereas prokaryotic IMPDHs contain substitutions in their active sites that can account for MPA-resistance, no such substitutions are present in the *P. brevicompactum* IMPDHs. The substitution of Ser or Thr for Ala249 is associated with MPA resistance in *C. albicans* and *S. cerevisiae* [14, 15]. This substitution is found in *Pb*IMPDH-A, but not in the more resistant *Pb*IMPDH-B. Substitutions at positions 277 and 462 have also resulted in modest MPA resistance [19], and substitutions at 351 are proposed to cause resistance [20], but again no correlation is observed in the fungal IMPDHs. We identified the C-terminal segment as a new structural

determinant of MPA sensitivity, but this segment accounts for only 7x of the 60x difference in resistance between *Pb*IMPDH-B and *Pb*IMPDH-B. Importantly, the extraordinary resistance of *Pb*IMPDH-B comes with a catalytic cost as this enzyme has lost much of the catalytic power of a typical IMPDH, with values of $K_m$(IMP) that are ~100x greater and $k_{cat}/K_m$(IMP) that are $10^2$-$10^3$x lower than "normal". This observation suggests that *Pb*IMPDH-B would be a very poor catalyst at normal cellular IMP concentrations of 20-50 μM [21]. However, IMP concentrations increase 10-35 fold in the presence of MPA [1, 22, 23]. Thus *P. brevicompactum* may contain millimolar concentrations of IMP during MPA biosynthesis, so that the turnover of *Pb*IMPDH-B would be comparable to a "normal" fungal IMPDH.

Curiously, MPA production does not follow the phylogenetic relationship established by analysis of the IMPDH and beta-tubulin genes (Figure 4). *P. chrysogenum* does not contain MPA biosynthetic genes although MPA-producers are found on the neighboring branches of the phylogenetic tree. As noted above, *P. chrysogenum* is more resistant to MPA than *A. nidulans* (Figure 2A). This resistance could be the result of a gene dosage effect as MPA resistance is associated with amplification of IMPDH genes in several organisms, consistent with this hypothesis [20, 24-27]. We propose that the presence of two IMPDH genes in *Pencillium* subgenus *Penicillium* was permissive for MPA production. The failure to observe MPA production in other *Pencillium* species may reflect inappropriate culture conditions rather than absence of the biosynthetic cluster. If so, it is possible that MPA biosynthesis has been obtained in several independent events. It will be interesting to map the evolution of MPA biosynthesis and resistance as more genomic sequences of filamentous fungi become available.

Gene duplication is generally believed to be a driving force in evolution, allowing one copy to diverge constraint-free while the other copy maintains essential functions. The "escape from adaptive conflict" subfunctionalization model is particularly attractive for the evolution of new enzyme activities [28, 29]. In this model, duplication relieves the constraint of maintaining the original function and allows the emergence of enzymes with new catalytic properties. The blemish in this appealing hypothesis has long been recognized: most mutations are deleterious, so that the extra copy is quickly lost or converted into a pseudogene. Though several elegant and rigorous investigations of the *in vitro* evolution of new enzyme function demonstrate the feasibility of subfunctionalization (reviewed in [29, 30]), genetic drift is widely recognized as the dominant mechanism in the wider evolution field. Several observations suggest that the emergence of MPA-resistant *Pb*IMPDH-B could be an example of neofunctionalization by a classic Ohno type mechanism - a duplication followed by (largely deleterious) drift until a new environment, MPA production, provides a selection.

*Pc*IMPDH-B displays the effects of deleterious genetic drift: this protein is labile to proteolysis, and the value of $k_{cat}$ ~1% of that of typical eukaryotic IMPDH. Microarray data indicate that *P. chrysogenum* expresses both IMPDH-A and IMPDH-B under most conditions, so at present there is no evidence for specialized functions for these genes [31, 32]. We cannot rule out that *Pc*IMPDH-B plays another role within the cell, perhaps by interacting with another protein, or even as a heterotetramer with *Pc*IMPDH-A.

Nevertheless, our data suggest that the enzymatic properties of both IMPDH-Bs have declined over the course of evolution. In the case of *P. brevicompactum*, gaining the ability to produce MPA may have rescued IMPDH-B from nonfunctionalization.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations used

| | |
|---|---|
| **IMPDH** | inosine-5'-monophosphate dehydrogenase |
| **IMP** | inosine 5'-monophosphate |
| **$NAD^+$** | nicotinamide adenine dinucleotide |
| **MPA** | mycophenolic acid |
| ***An*ImdA** | IMPDH from *Aspergillus nidulans* |
| ***Pb*IMPDH-A** | IMPDH-A from *Penicillium brevicompactum* |
| ***Pb*IMPDH-B** | IMPDH-B from *P. brevicompactum* |
| ***Pc*IMPDH-A** | IMPDH-A from *Penicillium chrysogenum* |
| ***Pc*IMPDH-B** | IMPDH-B from *P. chrysogenum* |

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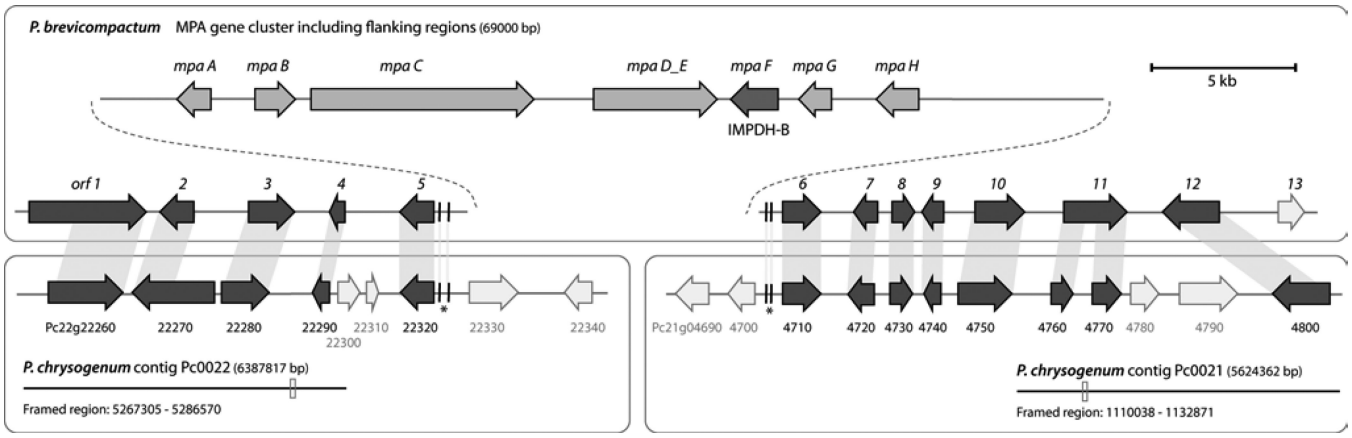



**Figure 1. Flanking regions of the MPA-biosynthesis gene cluster**

*P. brevicompactum* and the corresponding syntenic loci on *P. chrysogenum* contig Pc0022 and Pc0021 are shown. Regions marked with (*) indicate short conserved sequences (length < 53 bp, identity > 86%).

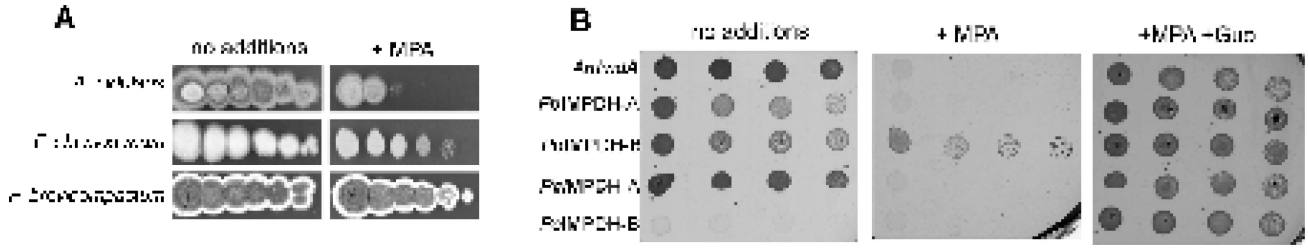



**Figure 2. MPA resistance in fungi and bacteria expressing fungal IMPDHs**

A. Spot assay to determine sensitivity towards MPA. A ten-fold dilution series of spores from the *A. nidulans*, *P. chrysogenum* and *P. brevicompactum* were grown on CYA plates containing no additives or 6.2 μM MPA. The spot with the highest number of spores in each row contains ~$10^6$ spores. B. *E. coli* Δ*guaB* was cultured on minimal media supplemented with 100 μM MPA, no additions or 50 μg/ml guanosine and 100 μM MPA.

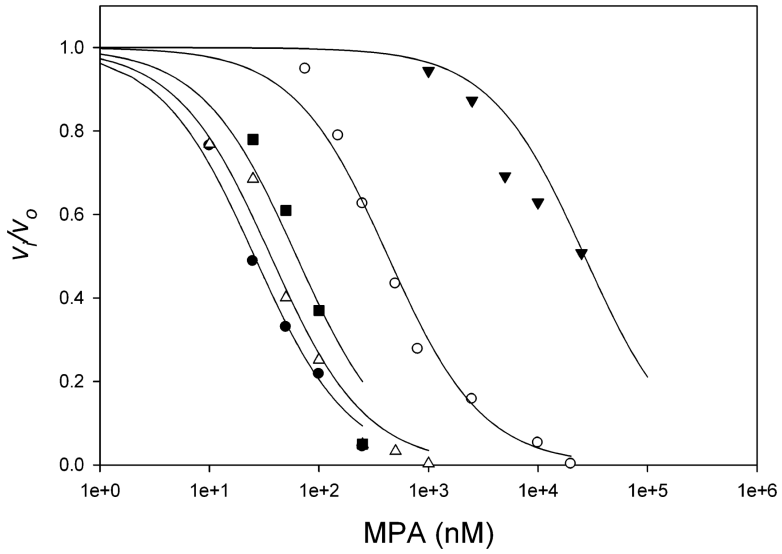



**Figure 3. MPA inhibition of fungal IMPDHs**

*An*ImdA, closed circles, *Pb*IMPDH-A, open circles, *Pb*IMPDH-B, closed triangles, *Pc*IMPDH-A, open triangles, *Pc*IMPDH-B, closed squares.

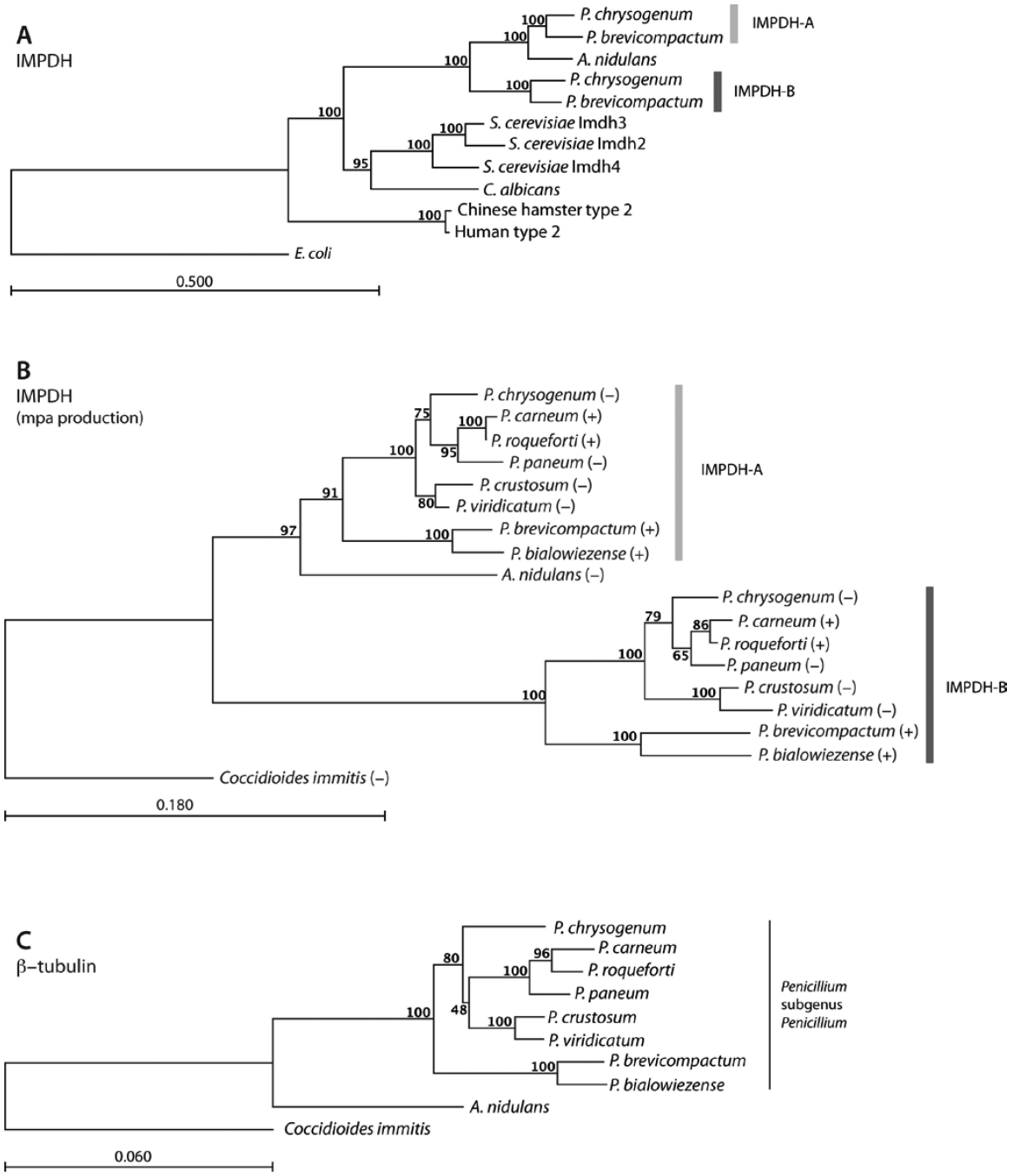



**Figure 4. IMPDH phylogeny**

A) Rooted cladogram based on full length amino acid sequences. B) and C) Rooted cladograms based on B) IMPDH cDNA sequences (372 – 387 bp) and C) β-tubulin cDNA sequences (950 bp) from eight species from *Penicillium* subgenus *Penicillium* and two filamentous fungi outside. *P.*: *Penicillium* and *A.*: *Aspergillus*. Bootstrap values (expressed as percentage of 1000 replications) are shown at the branch points, and the scale represents changes per unit. In analysis B, MPA production is indicated by "+" or "−". The sub-clades with *Penicillium* subgenus *Penicillium* genes are marked by light gray (IMPDH-A) and dark

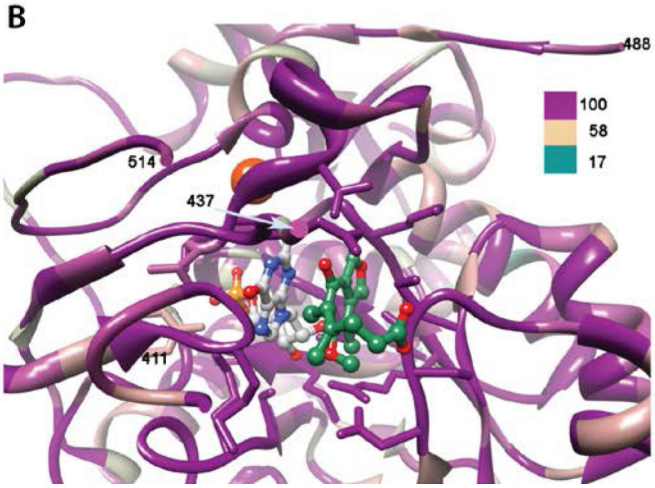
gray (IMPDH-B). *E. coli* has been used as outgroup in analysis A, and *Coccidioides immitis* as outgroup in analyses B and C.

A

| | Residues within 4 Å of XMP* | | | | | | | | | | | | | | | | | | | MPA interactions | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 68 | 70 | 303 | 328 | 329 | 330+ | 331 | 364 | 365 | 366 | 387 | 388 | **411** | 413+ | 414 | 415 | 416 | 417 | 429+ | 430+ | 441 | 274 | 276 | 303 | 322 | 324 | 326 | 333 | 336 | 441 |
| CHO 2 | S | M | N | G | S | I | C | D | G | G | G | S | Y | G | M | G | S | L | R | Y | Q | D | S | N | R | G | G | T | V | Q |
| *Pb*IMPDH-A | S | M | N | G | S | A | C | D | G | G | G | G | Y | G | M | G | S | I | R | Y | Q | D | S | N | R | G | G | T | V | Q |
| *Pb*IMPDH-B | S | M | N | G | S | A | C | D | G | G | G | S | F | G | M | G | S | I | R | Y | Q | D | S | N | R | G | G | T | V | Q |
| *Pc*IMPDH-A | S | M | N | G | S | A | C | D | G | G | G | G | Y | G | M | G | S | I | R | Y | Q | D | S | N | R | G | G | T | V | Q |
| *Pc*IMPDH-B | S | M | N | G | S | A | C | D | G | G | G | S | F | G | M | G | S | I | R | Y | Q | D | S | N | R | G | G | T | V | Q |
| *An* ImdA | S | M | N | G | S | A | C | D | G | G | G | G | Y | G | M | G | S | I | R | Y | Q | D | S | N | R | G | G | T | V | Q |

(+)Not interacting in open conformation

B




C

```
                487                                                 515 (stop)
CHO 2           ELKFEKRTSSAQVEGGVHSLHSYEKRLF--
Pb IMPDH-A      TVRFEMRSASAMTEGNVHGLHSYDKKLYA-
Pb IMPDH-B      QVRFEMRSASAQTEGNVHGLHSHEKKLYSS
Pc IMPDH-A      TVRFEMRSASAMTEGNVHGLHSYDKKLYS-
Pc IMPDH-B      TVRFEMRSASAQTEGNVHGLHTHEKKLYS-
An ImdA         TVRFEVRSASAMAEGNVHGLHSYDKKLYS-
```

**Figure 5. The MPA binding site is conserved among drug sensitive and drug resistant eukaryotic IMPDHs**

A) An alignment of IMPDH-A and IMPDH-B from *P. brevicompactum* 23078 and *P. chrysogenum*, IMPDH-A from *A. nidulans* and IMPDH2 from Chinese hamster was mapped onto the x-ray crystal structure of the MPA complex of Chinese hamster IMPDH (PDB accession 1JR1 [17]. 100% conserved, dark magenta; 58% conserved, tan; 17% conserved, dark cyan. MPA, green ball and stick; IMP intermediate, gray ball and stick; $K^+$, gold; residues within 4 Å of MPA and IMP are shown in stick.

B) Amino acid polymorphism in IMPDH of *A. nidulans* (*An*), *P. brevicompactum* (*Pb*) and *P. chrysogenum* (*Pc*). Shown are residues within 4 Å of XMP* and MPA.

C) Alignment of the C-terminal region. The region aligned in the figure represents the fragments that were swapped in the chimerics of *Pb*IMPDH-A and *Pb*IMPDH-B. Underlined: residues that are polymorphic.

## Table 1
### Characterization of IMPDHs from filamentous fungi

Data analyzed as described in Materials and Methods. n.a., not applicable. *Pb*IMPDH-B and *Pc*IMPDH-B did not show $NAD^+$ substrate inhibition up to 5 mM. The concentrations of IMP and $NAD^+$ used for $IC_{50}$ determination for each enzyme is included in the Supporting information.

| | $IC_{50}$ (MPA) (μM) | $k_{cat}$ ($s^{-1}$) | $K_M$ (IMP) (μM) | $k_{cat}/K_M$ (IMP) ($M^{-1}s^{-1}$) | $K_M$ ($NAD^+$) (μM) | $k_{cat}/K_M$ (NAD) ($M^{-1}s^{-1}$) | $K_{ii}$ ($NAD^+$) (mM) |
|---|---|---|---|---|---|---|---|
| *An*ImdA | 0.026 ± 0.002 | 0.74 ± 0.06 | 10 ± 1 | 74,000 ± 8,000 | 170 ± 70 | 4,400 ± 100 | 1.5 ± 0.6 |
| *Pb*IMPDH-A | 0.43 ± 0.03 | 0.70 ± 0.08 | 130 ± 30 | 5,400 ± 1,200 | 340 ± 90 | 2,100 ± 900 | 4.7 ± 1.1 |
| *Pb*IMPDH-B | 27 ± 9 | 0.41 ± 0.02 | 1,400 ± 200 | 300 ± 80 | 790 ± 140 | 500 ± 100 | n.a. |
| *Pc*IMPDH-A | 0.036 ± 0.004 | 0.84 ± 0.03 | 40 ± 6 | 21,000 ± 2,000 | 290 ± 60 | 2900 ± 300 | 2.4 ± 0.5 |
| *Pc*IMPDH-B | 0.06 ± 0.01 | 0.0075 ± 0.001 | 600 ± 300 | 12 ± 3 | 640 ± 130 | 11 ± 6 | n.a. |

## Table 2
### MPA inhibition of fungal IMPDH variants

Residue Phe411 is substituted with Tyr in IMPDH-B and residue Tyr411 is substituted with Phe in IMPDH-A. nd, no data.

| | $IC_{50}$ (μM) | | | |
|---|---|---|---|---|
| | **WT** | **411*** | **C-terminal swap** | **C-terminal + 411*** |
| *Pb*IMPDH-A | 0.43 ± 0.03 | 0.16 ± 0.02 | 3 ± 0.2 | 4.1 ± 0.3 |
| *Pb*IMPDH-B | 27 ± 9 | 25 ± 2 | 4.1 ± 0.3 | 3.1 ± 0.3 |
| *An*ImdA | 0.026 ± 0.002 | 0.026 ± 0.002 | nd | nd |