A complete nicotinate degradation pathway in the microbial eukaryote *Aspergillus nidulans*

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Several strikingly different aerobic and anaerobic pathways of nicotinate breakdown are extant in bacteria. Here, through reverse genetics and analytical techniques we elucidated in *Aspergillus nidulans*, a complete eukaryotic nicotinate utilization pathway. The pathway extant in this fungus and other ascomycetes, is quite different from bacterial ones. All intermediate metabolites were identified. The cognate proteins, encoded by eleven genes (*hxn*) mapping in three clusters are co-regulated by a specific transcription factor. Several enzymatic steps have no prokaryotic equivalent and two metabolites, 3-hydroxypiperidine-2,6-dione and 5,6-dihydroxypiperidine-2-one, have not been identified previously in any organism, the latter being a novel chemical compound. Hydrolytic ring opening results in α-hydroxy glutaramate, a compound not detected in analogous prokaryotic pathways. Our earlier phylogenetic analysis of Hxn proteins together with this complete biochemical pathway illustrates convergent evolution of catabolic pathways between fungi and bacteria.
Nicotinic acid (niacin, vitamin B3), a precursor of NAD, can serve as a nitrogen and carbon source in bacteria. In prokaryotes nicotinic acid (NA) is first converted to 6-hydroxynicotinic acid (6-NA), a reaction catalyzed by MOCO (molybdenum cofactor)-containing nicotinate hydroxylase enzymes (reviewed in ref. 1), which evolved several times independently2-4. Four quite different pathways metabolizing 6-NA have been described in detail in bacteria5.

The only detailed study of nicotinate utilization in a eukaryotic microorganism was carried out by us in the ascomycete Aspergillus nidulans. A nicotinate hydroxylase was characterized, and mutants in a gene encoding this enzyme and a putative transcription factor necessary for its induction were described5-10. The genes encoding nicotinate hydroxylase (HxnS) and the HxnR transcription factor map in a six-gene co-regulated cluster (including also hxnZ, Y, P and T, cluster hxn1/VI)11. Recently, five additional hxn genes (hxnX, W, V, N, and M) were identified as members of the HxnR-regulon. In A. nidulans, these two gene clusters hxn2/VI and hxn3/I clusters11 (Fig. 1).

The nearest characterized homolog of HxnP is the high-affinity nicotinate transporter TNA1 of S. cerevisiae (27% identity), while there is no close characterized homolog of HxnZ. The most likely orthologue of TNA1 in A. nidulans (encoded by AN5650 and sharing 31% amino acid (AA) identity with TNA1) and also its apparent paralogue in the genome (AN11116) show higher similarity with TNA1 than HxnP. While expression of AN5650 is completely independent from HxnR and NA or 6-NA induction (Supplementary Fig. 1b), hxnP shows a pattern of regulation identical to that of hxnS and the other enzyme-encoding genes of the clusters12. This may signify a divergence in substrate specificity and/or redundancy of nicotinate transporters.

The growth tests indicate whether the tested metabolites are a nitrogen source for each strain, but also, whether in a given deletion strain the hitherto unidentified physiological inducer metabolite is synthesized or not (Fig. 3a). To this end we monitor the induction of hxnS. HxnS can catalyze the hydroxylation of hypoxanthine (Hx) to xanthine, which is further converted to uric acid by the XanA enzyme7,13-15, and differently from the canonical xanthine dehydrogenase (HxA), HxnS is resistant to allopurinol (Allp) inhibition10,13. Thus, if the physiological inducer metabolite is produced, a given strain would use Hx as a nitrogen source in the presence of Allp (Fig. 3a). This growth on Hx may be diminished or abolished if the accumulated pathway metabolite is toxic (Fig. 3a, b).

**Transporters.** Two genes, hxnP and hxnZ map in cluster 1/VI and encode putative transporters of the Major Facilitator Superfamily with 12-transmembrane domains (Supplementary Fig. 1a, c)11. The rationale for this pathway is detailed below.

**Nicotinamide utilization.** One equivalent of N can be obtained by deamination of one molecule NAA via the xanthine dehydrogenase (XanA) pathway, which is not as widespread as the Nicotinamide Nucleotide Transhydrogenase (NNT) pathway. This is likely the reason why these two pathways were diverged in eukaryotic and eubacterial lineages. Thus, the NAA deaminase, similar to Pnc1p in S. cerevisiae, may be encoded by the gene hxnR.

**Results and discussion**

Figure 2 shows the pathway of NA utilization in Aspergillus nidulans. The rationale for this pathway is detailed below.

We systematically deleted all hxn genes (hxnS and hxnR deletions were published previously10) in both hxnR+ (wildtype) and hxnRΔ (where the HxnR transcription factor is constitutively active) backgrounds. The resulting strains were tested for their ability to utilize the commercially available NA derivatives as N-sources or as inducers.

The rationale for this pathway is detailed below.

### Transcripts

**Fig. 1** Summary of organization and function of HxnR-regulon composed of three gene clusters in A. nidulans11. Arrows indicate specific hxn genes and relative gene orientation. Lines below the genes indicate gene clusters. Names of clusters are indicated below the lines. Above the arrows, reported roles of genes (hxnS and hxnRΔ) or roles deduced from domain functions (hxnM, T, P, Y, Z, W, V) are indicated. Black arrows indicate enzyme gene products, striped arrows indicate transporter gene products, and the white arrow denotes the pathway-specific transcription factor.
**Fig. 2 Nicotinate catabolic route in A. nidulans.** HxnP and HxnZ are transporters (represented by blue and green transmembrane domains, respectively) that transport the indicated compounds. HxnS hydroxylates nicotinic acid (NA) to 6-hydroxynicotinic acid (6-NA). HxnX is a 6-NA monooxygenase (Fig. 5a). Its closest known structural homolog is the 6-NA 3-monooxygenase, NicC (PDB 1KT244018 (Fig. 5a). The six additional AA residues, His47, His211 and Tyr215 residues of NicC from *P. putida* Supplementary Table 4). His232 and Tyr236 residues of HxnX operate in peroxisomes and convert 6-NA to 2,5-dihydroxypyridine (2,5-DP), which is subsequently hydroxylated by HxnV to 2,3,6-trihydroxypyridine (2,3,6-THP). HxnT is a yet-unknown alkene reductase (UEI) partially saturate the pyridine ring of 2,3,6-THP to (5,6R)-(−)-dihydroxypyridine-2-one (5,6-DHPip-2-O), which is then converted to 3-dihydroxypyridine-2,6-dione (3-HPip-2,6-DO) by HxnW, a NAD-dependent polyol dehydrogenase type enzyme. The ring of 3-HPip-2,6-DO is opened by the cyclic imidase HxnM between N-C2 resulting in (S)-(−)-α-hydroxyglutaramate (α-HGA) formation. The nitrogen is salvaged by HxnN amide hydrolase and results in α-hydroxyglutarate (α-HG) formation. This reaction can also be catalyzed by other amide hydrolases (UE2). NA can be formed endogenously by the hydrolytic cleavage of amide group of nicotinamide (NAA) by a non-HxnR regulated deamidase. Cellular components such as cell membrane, cytoplasm, and peroxisome are shown and indicated by pictograms. Reaction in the peroxisome pictogram indicates the spatial separation of the referred catabolic step in the peroxisome. The compound in square brackets denotes a predicted intermediate that was not detected by the UHPLC-HRMS method but deduced from the structure of the identified upstream and downstream metabolites. The structure of the compound in the dashed square brackets was deduced by the exact m/z value and MS/MS fragmentation pattern of the compound obtained by UHPLC-HRMS (Supplementary Table 1), the UHPLC-HRMS and NMR confirmed structures of the upstream and downstream metabolites (Supplementary Tables 1, 2) in line with the proposed ketoreductase activity of the HxnW. UE: unidentiﬁed enzyme. PI: physiological metabolite inducer of the pathway-related hxn genes; Compound names in red lettering denote metabolites, which have never been detected before neither in eukaryotic nor in prokaryotic organisms. Compound names in blue letter denote metabolites not detected in prokaryotic NA catabolic pathways. (Created with BioRender.com).

**Conversion of 6-NA to 2,5-DP occurs in the peroxisome by the 6-NA monooxygenase HxnX.** Previous work has shown that HxnS catalyzes the hydroxylation of NA to 6-NA (ref. 10 and references therein). Deletion of hxnS prevents the utilization of 6-NA but not 2,5-DP as a nitrogen source (Fig. 3a). Strains deleted for this gene are also defective in the induction of hxnS by 6-NA but not by 2,5-DP and an hxnS deletion blocks the 2,5-DP accumulation in hxnRΔ hxnVΔ mutant (Figs. 3a, 4a, b). HxnX is a monooxygenase (Fig. 5a). Its closest known structural homolog is the 6-NA 3-monooxygenase, NicC (PDB code: 5eow), from *Pseudomonas putida* KT244018 (Fig. 5a and Supplementary Table 4). His232 and Tyr236 residues of HxnX and their spatial orientation correspond to the 6-NA substrate-binding His211 and Tyr215 residues of NicC from *P. putida* KT244018 (Fig. 5a). The six additional AA residues, His47, Cys202, Met213, Val227, Thr228, and Gly229, which are involved in the formation of the active site18 are not conserved in HxnX (Gln59, Val223, Val234, Val247, Leu248, and Leu249, respectively) (Fig. 5a). Similarly to NicC-119, HxnX is proposed to require NADH, FAD, and O2 to replace the carboxyl group with a hydroxyl group on the 6-NA substrate that results in 2,5-DP formation.

HxnX includes canonical PTS-1 peroxisome targeting signal (SRL) at its C-terminal end (Supplementary Fig. 2). An N-terminal Gfp-HxnX fusion fully complements the growth phenotype of hxnXΔ and co-localizes with a peroxisomal marker (Fig. 5e). The PTS-1 signal is conserved among the HxnX proteins present in other *Pezizomycotina*19. No other hxn encoded enzyme carries a subcellular localization signal, which however does not exclude the possibility that the corresponding pathway-step(s) may occur in an organelle. While constructing double mutant strains, we were surprised that the hxnSΔ hxnTΔ double deletion strain utilizes 10 mM 6-NA more efficiently than the wild-type control or the single hxnSΔ or hxnTΔ deletion mutants (Fig. 3a). The ORFs of the two divergently transcribed genes were deleted in the double mutant, the intergenic region between the start codons was left intact, excluding any cis-acting regulatory effects on other genes of the cluster (see Methods section). The explanation of this phenotype may relate to the intracellular pool of NAD/NADH. NAD is the final electron acceptor of HxnS8, and the presumed electron
Fig. 3 Utilization, inducer, and inhibition tests of hxn mutants. a) Utilization of different nitrogen sources by mutants described in this article in an hxnR+ wild-type background (except for hxnΔ and hxnR7 controls). b) Utilization of different nitrogen sources by some hxn gene deletion mutants in an hxnR7 (constitutive) background. Above each column, we indicate the relevant mutation carried by each tested strain. Hx indicates 1 mM hypoxanthine as the sole nitrogen source. Hx, Allp, as above including 5.5 mM allopurinol, which fully inhibits HxA but not HxnS (therefore Hx utilization depends on the activation of HxnR-regulon-belonging HxnS (for details see10). NA and 6-NA indicate, respectively, nicotinic acid and 6-OH nicotinic acid added as the sodium salts (see Methods section). 2,5-DP and NAA indicate, 2,5-dihydroxypyridine and nicotinamide, respectively. Other relevant concentrations are indicated in the figure. Plates were incubated for 3 days at 37 °C except those marked by an asterisk (*), which were incubated for 4 days. The relevant hxn genes are symbolized by only the capital letter indicating the locus name. Strains used: parental control 1 (H2Z.120, parent of Sa, Ta, Ya), parental control 2 (TN02 A21, parent of Rα, Pa, Zα, Xα, Vα, Na) are wildtype for all hxn genes. Mutant strains: Sa (HZS.599), Rα (HZS.614), R7 (FGSC A872), Tα (HZS.222), Yα (HZS.223), Xα (HZS.502), Pa (HZS.221), Zα (HZS.226), Pα Zα (HZS.480), Sa Tα (HZS.892), Sa Yα (HZS.558), SaTα Yα (HZS.569), Vα (HZS.294), Vα (HZS.294), Wα (HZS.393), Mα (HZS.293), Nα (HZS.288), Vα R7 (HZS.309), Xα R7 (HZS.310), Mα R7 (HZS.517), Mα R7 (HZS.308), and Nα R7 (HZS.306). The complete genotypes are given in Supplementary Table 3.

Subsequent metabolism of 2,5-DP depends on the 2,5-DP monoxygenase, HxnV. N-source utilization tests showed that HxnV acts downstream of NA, 6-NA, and 2,5-DP (Fig. 3a). Induction tests (Hx Allp rows) are completely consistent with the above, in an hxnVΔ strain 2,5-DP does not act as an inducer. These results place the physiological inducer of the pathway downstream from 2,5-DP. In an hxnR7 background, where all other hxn genes are constitutively expressed10,11, an hxnVΔ strain accumulates 2,5-DP (Fig. 4a) in a medium supplemented with 10 mM 6-NA, indicating that 2,5-DP is its substrate. This strain also secretes a green pigment (detected both visually and by UHPLC-HRMS analysis), seen both in the solid medium around the colonies and in fermented broth (Fig. 4b, c). The green pigment was identified as the dimer form of 2,5-DP (Fig. 4d). A green pigment formation by a non-enzymatic transformation of 2,5-DP was reported in the P. putida NicX loss-of-function mutant, blocked in the catabolism of 2,5-DP20 and in a P. fluorescens strain grown on NA medium21. The formation of the pigment is almost completely blocked in an hxnR7 hxnXD hxnVΔ strain, consistent with the position of the HxnX protein in the pathway as the enzyme catalyzing the formation of 2,5-DP (see above) but also diminished in an hxnR7 hxnYΔ hxnVΔ strain. The fact that the deletion of hxnY diminishes the green pigment accumulation (Fig. 4c) may suggest, however, a role for HxnY in the detoxification of NA-catabolism-derived compounds.

HxnV includes a phenol 2-monoxygenase domain (PRK08294) and shows remarkable structural similarity to 3-hydroxybenzoate hydroxylase (MBHB), from Comamonas testosteroni (PDB code: 2dkh) (Fig. 5b) as well as to phenol 2-monoxygenase (PHOX) from Trichosporon cutaneum (PDB code: 1pnt) (Fig. 5b, Supplementary Fig. 4 and Supplementary Table 4). The phenol ring interacting residues of MBHB (Asp75, Leu258, Ile260, and Tyr271) together with their spatial orientation are fully conserved in HxnV, and by the analogy between HxnV and its known structural homologs, HxnV may hydroxylate the 6-carbon of 2,5-DP resulting in 2,3,6-trihydroxypyridine (2,3,6-THP) formation (Fig. 5b). Thus, it is not unreasonable and in agreement with the data shown above that 2,5-DP is the substrate of HxnV, and by the analogy between HxnV and its known structural homologs, HxnV may hydroxylate the 6-carbon of 2,5-DP resulting in 2,3,6-trihydroxypyridine (2,3,6-THP) formation (Fig. 2). This metabolite was not detected in the metabolome of any of the mutants, however, the structurally identified upstream and downstream metabolites (2,5-DP and (5S,6R)-(−)-dihydroxyxypiperidine-2-one (see below), respectively) suggest that 2,3,6-THP is almost certainly the product of HxnV (Figs. 2, 4a).

The 2,3,6-THP alkeno reductase HxnT catalyzes the reduction of the pyridine ring. Accumulation of a saturated derivative of 2,3,6-THP, (5S,6R)-(−)-dihydroxyxypiperidine-2-one (5,6-DHPip-2-O) was exclusively observed in the metabolome of an hxnR7 hxnWΔ mutant (Fig. 4a and see Supplementary Table 2 for NMR results). This compound has not been detected previously in either eukaryotes or prokaryotes, and has not been synthesized chemically. 5,6-DHPip-2-O is an altogether novel compound. The accumulation pattern identifies 5,6-DHPip-2-O as the substrate of HxnW but also implies that an upstream alkeno reductase enzyme (HxnT, see Fig. 2 and below) acts on the...
hitherto undetected product of HxN. Logically the latter has to be 2,3,6-THP. The putative alkene reductase, which supposedly converges 2,3,6-THP to the 5,6-DHPip-2-O, is HxNT (a member of the “old yellow enzymes” group). Comparison of the structural model of HxNT with its closest known structural homolog, old yellow enzyme 1 (OYE1) of Saccharomyces pastorianus (PDB code: 1oya) showed that the para-hydroxybenzaldehyde binding residues of SpOYE1 (His191, Asn194, Tyr375) are remarkably conserved in HxNT (His183, Asn186, and Tyr372), and that the FMN binding residues are almost completely conserved in HxNT.23 (Fig. 5c, Supplementary Fig. 5, and Supplementary Table 4 for further details). An hxnTΔ strain shows a leaky growth phenotype, most noticeably on 2,5-DP and NA (Fig. 3a). The utilization of Hx in the inducer-test media is reduced but still clearly visible. Both results imply that while HxNT is responsible for the metabolism of the putative 2,3,6-THP metabolite to 5,6-DHPip-2-O, an additional unidentified enzyme must be catalyzing the same step. The deletion of hxnW identifies 5,6-DHPip-2-O as the physiological inducer of the pathway (NA and 6-NA serve as inducer precursors in the Hx Allp test in hxnWΔ, but not in hxnXΔ and to a reduced extent in hxnTΔ). 2,5-DP serves as an inducer precursor in hxnXΔ but not in hxnVΔ and to a reduced extent in hxnTΔ, which is in line with a redundant functioning additional enzyme. While induction of a whole pathway by a
metabolite such as the product of the first metabolic step has been described long ago (e.g., refs 24–27 and most recent28 with references therein), the pathway described in this article reports the unprecedented occurrence of concerted induction by an almost terminal metabolite in a degradative, catabolic pathway (as opposed to repression by end products in biosynthetic pathways). This result implies that non-induced levels of upstream enzymes are sufficient to result in intracellular concentrations of 5,6-DHPip-2-O resulting in the formation of 3-hydroxypiperidine-2,6-dione (3-HPip-2,6-DO) (Figs. 2 and 4a).

The 5,6-DHPip-2-O ketoreductase HxnW converts the 6-hydroxy group to a 6-oxo group. 5,6-DHPip-2-O, the product of HxnT, is the substrate of HxnW. HxnW is a short chain dehydrogenase/reductase and has a structurally conserved NAD_B-Rossmann fold domain30 with a TG(X)_2GXG (14-21 AAs) motif that is characteristic of the fungal ketoreductases (Supplementary Fig. 7). Comparison of HxnW to its closest known structural homologs, NAD(H)-dependent polyol dehydrogenase Gox2181 from Gluconobacter oxidans (PDB code: 3awd) and carveol dehydrogenase CDH from Mycobacterium avium (PDB code: 3uve)31,32 showed the striking conformity of the active site residues in Gox2181 and CDH to that of HxnW, characteristic of the fungal-type ketoreductases. Quality assessments of the Hxn models and superpositions with their closest known structural homologs are summarized in Supplementary Table 4. Subcellular localization of the Gfp-HxnX fusion protein. Gfp-HxnX is co-expressed with DsRed-SKL (peroxisome targeted red fluorescent protein54,55) in strain HZS.579. Fluorescent microscopy was carried out by using Zeiss 09 and 15 filter sets for DsRed and Gfp, respectively. Conidia were germinated for 6.5 h at 37 °C on the surface coverslips submerged in MM prior to microscopy. Scale bar represents 10 μm.
of 3-HPip-2,6-DO was deduced by the (i) exact m/z value; (ii) MS/MS fragmentation pattern of the compound obtained by UHPLC-HRMS analysis compared to the in silico fragmentation using Competitive Fragmentation Modeling-ID (CFM-ID 4.0) (Supplementary Table 1); and (iii) the UHPLC-HRMS and NMR confirmed structures of the upstream and downstream metabolites (Supplementary Tables 1, 2) in line with the proposed ketoreductase activity of the HxnW enzyme (see paragraph above). HxnM shares 74.3% identity (with 100% query coverage) with a Candida boidinii enzyme (OWB68015) belonging to the EC 3.5.99 enzyme class (GOterm: 0016810, hydrolase activity on non-peptide C-N bonds) and 64.2% identity (with 95.4% query coverage) with AAY98498, a cyclic imide hydrodase homolog, from P. putida (Supplementary Table 9). HxnM shows striking structural similarity with its closest known structural homolog, the so-called "peptidoglycan deacetylase" HpPgdA from Helicobacter pylori (PDB code: 3buq) (its substrate specificity being unknown) that is related to cyclic imidases (Supplementary Fig. 9 and Supplementary Table 4). The closest characterized phylogenetic relative of HpPgdA is an allantoicase (Pseudoomonas aeruginosa), whose natural substrate is a small cyclic imide (Supplementary Table 4). We propose that HxnM opens the ring of 3-HPip-2,6-DO between a C2 carbon and nitrogen, generating (S)-(+) α-hydroxylglutarate (α-HGA), a compound which was detected exclusively in the metabolome of an hxnR7 strain (Figs. 2, 4a and Supplementary Table 2). A ring-opening is a necessary step to generate NH4+, which can serve as a nitrogen source. In A. nidulans, uniquely among studied NA-catabolizing organisms, the generation of a piperidine ring from a pyridine ring precedes the hydrolysis of a C-N bond. In the different pathways described in bacteria, the ring-opening may take place by an oxidative process in an aromatic ring (such as in P. putida) or in a hydrolytic process on saturated or partially saturated rings (Eubacteriumarkeri and Azorhizobium caulinodans) (Fig. 6).

The α-HGA amide hydrolase HxnN is involved in nitrogen salvage from NA. HxnN is a putative amide hydrolase, its closest structural homolog is the fatty acid amide hydrolase 1 (FAAH1) from Rattus norvegicus (Supplementary Table 4). Deletion of hxnN diminishes but does not abolish the utilization of NA, 6-NA and 2,5-DO as sole nitrogen sources. While hxnN encodes the last enzyme of the hxn regulon, the growth tests demonstrate that (a) yet-unidentified hydrolase(s) contribute(s) to the deamidation of α-HGA (Fig. 3a). Several genes encoding putative paralogues of HxnN are extant in the genome of A. nidulans with identities to HxnN up to 39%. Superposition of the structural model of HxnN with its closest known structural homolog, FAAH1 (PDB code: 2vya), shows that the catalytic triad residues from FAAH1 involved in the hydrolysis of the amide bond, "the oxoanion hole" forming residues and the Ser residue that interacts with the catalytic triad residues are fully conserved in HxnN (Supplementary Fig. 10 and Supplementary Table 4). None of the prokaryotic amide hydrolases operating in the bacterial NA catabolic routes (ω-amidases) show considerable similarity to HxnN. Amide hydrolysis of α-HGA generates α-hydroxylglutarate (α-HG) (Figs. 2, 4a), which has not been detected as an intermediate in any of the elucidated prokaryotic NA catabolic routes.

Toxicity of intermediate catabolic compounds. In an hxnR7 background, all hxn genes are constitutively transcribed. We can thus investigate the accumulation of NA metabolites bypassing the physiological induction of the pathway. The accumulated 2,5-DP in hxnVA is a strong inhibitor of growth, while 2,6-DHPip-2-

O in hxnWA mildly, and 6-NA, 3-HPip-2,6-DO, and α-HGA in hxnXΔ, hxnMΔ, and hxnNΔ, respectively, slightly inhibit growth (Fig. 3b). Growth inhibition by pathway metabolites was also detected when acetamide was the main N-source.

HxnY is an α-ketoglutarate-dependent dioxygenase. Among enzymes of this class, its closest structural homolog is the thymine-7-hydroxylase (T7H) of Neurospora crassa (PDB code: 5c3q), which catalyzes the sequential conversion of the methyl group of thymine to a carboxyl group. The conservation of the α-ketoglutarate and Fe2+/Fe3+ binding residues and those involved in π-π stacking and hydrophobic interactions with the pyrimidine ring of T7H are consistent with the putative activity of HxnY on a pyridine derivative related to the pathway (Supplementary Fig. 8 and Supplementary Table 4). On the basis of the hxnYΔ-related phenotypes, we could not propose a function for HxnY that directly relates to nicotinate catabolism. The deletion of hxnY diminishes both the utilization of 6-NA (Fig. 3a) and the accumulation of 2,5-DO derived green pigment (Fig. 4c). The fact that the hxnXΔ phenotype is not leaky (Fig. 3a), makes unlikely that HxnY contributes significantly to the NA-derived nitrogen pool by conversion of NA/6-NA to 2,5-DO. The fact that the deletion of hxnY diminishes the green pigment formation, however, may suggest a role in the detoxification of NA-catabolism-derived compounds.

Conclusions

The eukaryotic NA catabolic pathway described above shows clear differences from previously described prokaryotic pathways in steps that precede (compounds 5,6-DHPip-2-O and 3-HPip-2,6-DO) and follow (compounds α-HGA and α-HG) ring-opening. Conversion of NA to 2,3,6-THP through 2,5-DO was not detected in prokaryotes, albeit each of the 2,3,6-THP and 2,5-DO intermediates appear in several pathways (2,3,6-THP appears in Bacillus sp. and Arthrobacter nicotinovorans; 2,5-DO appears in P. putida) (Fig. 6). 2,5-DO is formed from 6-NA in Pseudomonas sp., which is not hydroxylated further but the pyridine ring is cleaved between C5-C6 (Fig. 3a). Deletion of hxnY diminishes the green pigment formation, however, may suggest a role in the detoxification of NA-catabolism-derived compounds.

In aerobic prokaryotic pathways the ring-opening occurs either between C-C of 2,5-DO (by extradiol dioxygenase) or 2,3,6-THP (in Pseudomonas sp. and Bacillus sp., respectively) or between C-N of 2,3,6-THP (in Rhodococcus sp. and Arthrobacter sp. by polyketide cyclase) generating N-formyl maleamic acid or α-ketoglutaramate (Fig. 6). In the following steps in prokaryotes, the amide is hydrolyzed by an ω-amidase not related to the HxnN amidase. The anaerobic pathway described in E.arkeri and Azorhizobium caulinodans involves the partial saturation of the pyridine ring of 6-NA that results in 1,4,5,6-tetrahydro-6-oxonicotinic acid (THON), followed by hydrolytic ring-opening of THON between C-N and the simultaneous deamination (by a bifunctional enamiadase in E.arkeri) resulting in (S)-2-formylglutarate formation (Fig. 6). While no redundantly functioning enzymes are involved in the
prokaryotic routes, two steps of the fungal catabolism involve alternative enzymes (two unidentified enzymes, one functioning redundantly with HxnT, the other with HxnN) (Fig. 2). Catabolic steps downstream to 2,3,6-THP differ from those in prokaryotes and lead to the newly identified intermediate metabolites 5,6-DHpip-2-O and 3-HPip-2,6-DO (Fig. 6). The identification of these new metabolites may be of industrial or agricultural importance. The complete description of this eukaryotic pathway further illustrates convergent evolution, both at the level of individual enzymes and at the level of a whole pathway.

Methods

Strains and growth conditions. The A. nidulans strains used in this study are listed in Supplementary Table 3. Standard genetic markers are described in http://www.fgsc.net/Aspergillus/gene_list/. Minimal media (MMs) with glucose as the sole carbon source and different sole nitrogen sources were used45,46. The media were supplemented with vitamins (http://www.fgsc.net) according to the requirements of each auxotrophic strain. Nitrogen sources, inducers, repressors, and inhibitors were used at the following concentrations: 10 mM NA or 10 mM 6-NA (1:100 dilution from 1 M NA or 6-NA dissolved in 1 M sodium hydroxide), 10 mM 2,5-DP added as a powder, 10 mM NAA added as a powder, 10 mM 5,6-DHpip-2-O added as a powder, 1 mM Hx added as a powder, 10 mM acetamide as sole N-sources; NA sodium salt, 6-NA sodium salt, 2,5-DP, NAA, 5,6-DHpip-2-O in 1 mM or 100 µM final concentration as inducers; 5.5 µM Allp as an inhibitor of purine hydroxylase I (HxA) enzyme activity. Strains were grown at 37 °C for the indicated times.

For metabolite extraction, the mycelia of hxnRc7 strains with different hxn gene deletions(s) were grown for 16 h on MM with 10 mM acetamide as the sole N-source at 37 °C with 150 rpm agitation, which was followed by shifting the mycelia to MM with 10 mM 6-NA as substrate without additional utilizable N-source and incubated for further 24 h.

Gene deletions. Deletion of hxnT/R/Y/Z/P/X/W/V/M/N genes were constructed as described previously47. The gene targeting substitution cassette was constructed by double-joint PCR48, where the riboB+, pabaA+, or pyroA+ genes were used as transformation markers. Construction of double and triple deletion mutants or changing the hxnR+ genetic background of mutants to hxnRc7 was carried out by standard genetic crosses or transformation followed by checking via PCR and Southern blots. DNA was prepared from A. nidulans as described by ref.49. Hybond-N membranes (Amersham/GE Healthcare) were used for Southern blots50. Southern hybridizations were done by DIG DNA Labeling and Detection Kit (Roche) according to the manufacturer’s instructions. Transformations of A.

Fig. 6 A comparison of the nicotinate catabolic pathway of the ascomycete Aspergillus nidulans with known prokaryotic pathways. The catabolism of nicotine by A. nicotinovorans involves the opening and release of the pyrrolidine ring, leading to 2,6-DP, which is further catabolized through 2,3,6-THP, an intermediate of pathways in Bacillus sp. as well as in A. nidulans. The nicotine pathway upstream to 2,6-DP (indicated by linked arrows) is not relevant to the present work. Red-colored text indicates completely novel metabolites, while blue-colored text indicates metabolites that have never been identified in prokaryotic NA catabolic pathways. While the eukaryotic NA catabolic pathway has only been studied experimentally in A. nidulans, genes encoding the whole or part of the pathway are present in many ascomycete fungi11. The site of ring-opening (either between two carbons or carbon and nitrogen) is indicated by red wavy arrows. (o): ring-opening is oxidative; (h): ring-opening is hydrolytic.
**Construction and microscopy of Gfp-HxnX (N-terminal fusion) expressing strains.** Construction of the gfp-hxnX expressing strain is described in detail in Supplementary Methods 3. Briefly, a bipartite cassette of the gfp-hxnX fusion was constructed by double-joint PCR (DJ-PCR) and cloned into the PAN-HZS-1 vector yielding the gfp-hxnX expression vector PAN-HZS-13, which was used to transform Aspergillus nidulans strain (HZS534), which carries a permissiveness marker (expresses DsRed-SKL) (Supplementary Methods 3). Transformants carrying the gfp-hxnX transgene from one to ten copies were isolated. Gfp-HxnX localization was studied in HZS579 that carried the transgene in seven copies. Coindiosides of HZS579 was germinated for 6.5 h on the surface of coverslips submerged in MM at 37 °C. Young hyphae were examined by fluorescence microscopy using Zeiss 09 and 15 filter sets for DsRed and GFP, respectively.

**Metabolite analysis.** For metabolite extraction, 1 ml of methanol/water (8/2) was added to both 25 mg of freeze-dried mycelium and 2 ml freeze-dried fermentation broth then cultivated for 1 min and vortexed at 500 W for 3 × 5 min in ice to prevent the samples for 30 s. After centrifugation (20,000×g, 10 min, 4 °C) the supernatants were subjected to UHPLC-HRMS analysis. UHPLC-HRMS measurements were performed using a DionexUltimate 3000 UHPLC system (Thermo Scientific) coupled to a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific) operating with a heated electrospray interface (HESI). Metabolites were separated on an Acquity UPLC BEH Amide (2.1 × 100 mm, 1.7 μm) column (Waters, Hungary) thermostatted at 40 °C. Acetonitrile (A) and water (B) both supplemented with 0.1% formic acid served as mobile phases. A gradient elution program was applied as follows: 0–5 min: 97% A, 0.3–5 min: 97% A, 0.4–10 min: 98% A–40% B, 10–13 min: 40%–97% A, 13.5–27.5 min: 97% A. The flow rate was kept at 0.3 ml/min, and the injection volume was 3 μl.

All samples were analyzed in both positive and negative ionization mode using the following ion source settings: the temperature of the probe heater and ion transfer capillary, spray voltage, sheath gas flow rate, auxiliary gas flow rate, and S-lens RF level were set to 300 °C, 350 °C, 3.5 kV, 40 arbitrary unit, 10 arbitrary unit, and 50 arbitrary unit, respectively. For data acquisition full-scan/data dependent MS/MS method (Full MS/ddMS2) was applied, where the full scan MS spectra were acquired at a resolution of 70,000 from m/z 50 to 500 with a maximum injection time of 100 ms. For every full scan, five ddMS2-scans were carried out with a resolution of 17,5000, and a minimum automatic gain control target of 1.00 × 105. The isolation window was 0.4 m/z. Instrument control and data collection were carried out using Trace Finder 4.0 (Thermo Scientific) software. The raw data files were processed by Compound Discoverer 2.1 software for chromatographic alignment, compound detection, and accurate mass determination.

All NMR experiments were accomplished on a Bruker Ushields 500 Plus spectrometer, solvent residual signals (methanol, DMSO) adopted as internal standards. Optical rotations were measured with a Jasco P 2000 Polarimeter.

**Purification of 5,6-DHPip-2-O and α-HGA.** About 4 and 14 g of freeze-dried mycelia of 5,6-DHPip-2-O and α-HGA accumulating strains were extracted in 160 and 560 ml of methanol, respectively. The extracts were then evaporated to dryness and were purified with dry sample loading injection on a CombiFlash EZPrep flash chromatography (Teledyne Isco, USA) using 0.063–0.2 mm spherical silica (Molar Chemicals, Hungary) as solid phase. The metabolite detected at m/z 132.0656 was separated with ethyl acetate/methanol, 4/1 (V/V) supplemented with 5% aqueous ammonia as a mobile phase resulting in 5 mg material. For the metabolite detected at m/z 146.0461, the separation using ethyl acetate/methanol, 7/3 (V/V) supplemented with 5% aqueous ammonia was followed by an additional purification step, where a mixture of methanol/water (95/5, V/V) as mobile phase was applied to achieve 5 mg purified material. At each step of the purification, the purities of the metabolites were determined via the UHPLC-HRMS method described above. To assign the stereochemistry of the isolated α-HGA, (S)-- and (R)--α-HGA were prepared commercially available (S)-- and (R)--5-oxo-2-tetrahydrofurancarboxylic acid (Merck Co.) following literature and measuring optical rotations. The observed optical rotation of isolated α-HGA ([α]D +7.8°, c = 0.077, MeOH) clearly proved the presence of (S)--α-HGA ([α]D +1.2°, c = 2.5, H2O; measured of the compound prepared from (S)--5-oxo-2-tetrahydrofurancarboxylic acid: [α]D +6.3°, c = 0.076, MeOH). Since the NOESY measurements and coupling constants of H-5 and H-6 in 5,6-DHPip-2-O clearly show trans relative configuration and because the configuration of H-5 is the same as in α-HGA, the stereochemistry of 5,6-DHPip-2-O was determined as S6R6. Optical rotation of S5R6-5,6-DHPip-2-O was [α]D +5.16°, c = 0.133, MeOH.
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