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Frequent, independent transfers of a catabolic gene from bacteria to contrasted filamentous eukaryotes

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Even genetically distant prokaryotes can exchange genes between them, and these horizontal gene transfer events play a central role in adaptation and evolution. While this was long thought to be restricted to prokaryotes, certain eukaryotes have acquired genes of bacterial origin. However, gene acquisitions in eukaryotes are thought to be much less important in magnitude than in prokaryotes. Here, we describe the complex evolutionary history of a bacterial catabolic gene that has been transferred repeatedly from different bacterial phyla to stramenopiles and fungi. Indeed, phylogenomic analysis pointed to multiple acquisitions of the gene in these filamentous eukaryotes—as many as 15 different events for 65 microeukaryotes. Furthermore, once transferred, this gene acquired introns and was found expressed in mRNA databases for most recipients. Our results show that effective inter-domain transfers and subsequent adaptation of a prokaryotic gene in eukaryotic cells can happen at an unprecedented magnitude.

1. Introduction

In nature, species need to constantly adapt to changing environments, and this can be achieved by modifying their genetic repertoire to acquire new functions. Indeed, gene duplications (followed by evolution of new functions) and other genomic rearrangements have shaped eukaryotic genomes [1]. However, genetic innovation can also result from the acquisition of exogenous genes by horizontal gene transfer (HGT). Prokaryotes adapt largely by HGT, and strains of a particular species can differ by large fractions of their genome [2–6].

Long thought to be a prokaryote specialty, HGT is now recognized as a mechanism of genetic innovation in eukaryotes as well [7–10]. Genome analysis of eukaryotes revealed that several genes had been horizontally transferred [11,12], with important implications for environmental adaptation [13–15]. Indubitably, HGT can enable acquisition of entirely novel functions, which is more drastic than the gradual evolutionary processes that rely on modification of pre-existing genes [16–18] and may enhance ecological opportunities. In this context, the prokaryotic gene pool can serve as a large reservoir of potential functions for eukaryotes [9]. Indeed, it appears that prokaryote-to-eukaryote inter-domain HGT events are more prevalent than eukaryote-to-eukaryote ones [19]. As described for the insect *Hypothenemus hampei*, where inter-domain HGT of a mannase-encoding gene from a Firmicute enabled the insect to parasitize coffee berries [7], the acquisition of a single gene can lead to enhanced competitiveness and ecological specialization. However, inter-domain HGT can involve more than one gene. Thus, previous studies reported that significant parts of genome, up to 10% of the gene repertoire of the multicellular rotifer *Adineta ricciae* [13], had been horizontally acquired. Although these acquisitions resulted from several transfers, the extent to which a given bacterial gene may undergo inter-domain transfers to eukaryotes remains unclear.

The bacterial gene *acd*S has been evidenced not only in taxonomically contrasted bacteria [20], notably in strains with plant growth-promotion activity, but also in a few fungi [21–23]. The AcdS enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (EC 4.1.99.4) can transform the plant's ethylene precursor ACC to α-ketobutyrate and ammonia. By degrading ACC in exudates, plant-interacting bacteria can indirectly lower ethylene level in roots, thus stimulating root growth and modulating plant stress resistance [24,25]. In addition to the *acdS* gene itself, AcdS catalytic activity was also found in fungi, namely the Ascomycota *Trichoderma asperellum* [22], *Cyberlindnera* (formerly *Hansenula*) *saturnus* [23], *Penicillium citrinum* [21] and *Magnaporthe oryzae* (M.B., C.P.-C., P.L., Y.M.-L. & D.M. 2013, unpublished data), raising the question of the evolutionary origin of this gene in eukaryotes.

In this study, we show that the bacterial gene *acdS* has been repeatedly transferred to a wide range of eukaryotic recipients (i.e. fungi and stramenopiles). Phylogenetic analysis pointed to multiple *acdS* acquisitions, from different bacterial phyla to different eukaryotes. Ancestral state character reconstruction confirmed past occurrence of multiple, independent transfers of *acdS* from each of these bacterial phyla to different types of eukaryotes. Moreover, transferred *acdS* genes were effectively transcribed and occasionally acquired introns in eukaryotic recipients. Altogether, these results show that prokaryote-to-eukaryote transfer of a single gene can happen at high frequency, with adaptation of the transferred gene to its new host cell machinery.

2. Results

(a) *acdS* prevalence in eukaryotes

We found as many as 65 *acdS* homologues in eukaryotes—four in stramenopiles (all oomycetes) and 61 in fungi—after analysis of 149 sequenced genomes based on the AcdS protein sequence of the proteobacterium *Pseudomonas fluorescens* F113. Significant sequence identity (at least 38% amino acid identity) was evidenced between bacterial and eukaryotic AcdS proteins. Moreover, conservation of nucleotide sequences between bacterial and eukaryotic *acdS* genes was also high (at least 40% identity). It reached as much as 65% between the actinobacterium *Streptomyces violaceusniger* Tu4113 and the ascomycotan *M. oryzae* 70–15, and 78% between the proteobacterium *Acidovorax radicis* N35 and the stramenopile *Phytophthora infestans* T30-4. Such gene sequence conservation across two different domains of life suggests both a common evolutionary origin and genetic transfer(s) between them.

(b) Distribution of *acdS* homologues in eukaryotes

Phylogenetic analysis of AcdS protein homologues retrieved by BLAST showed that eukaryotic AcdS sequences were distributed in three distinct clades (figure 1; electronic supplementary material, figures S1–S3). The first clade is rooted by actinobacterial AcdS sequences and corresponds exclusively to fungi (Ascomycota and a few Basidiomycota). The second clade is rooted by gammaproteobacterial AcdS sequences and only includes Ascomycota. The third clade is rooted by betaproteobacterial AcdS sequences, and includes a stramenopile subclade (*Phytophthora* species) and a fungal subclade. This topology was retrieved both with maximum-likelihood and Bayesian reconstructions [26] (see the electronic supplementary material, figure S1). Thus, our results strongly support a bacterial origin for *acdS* eukaryotic homologues, and also indicate that Actinobacteria, Betaproteobacteria and Gammaproteobacteria served as distinct *acdS* donors for eukaryotes. Fungal recipients belonged to several taxonomical classes, and different possibilities may account for the uneven distribution of *acdS* homologues in oomycetes and fungi. The first hypothesis is an ancestral acquisition of the gene followed by multiple losses in a broad range of eukaryotic lineages. The second hypothesis entails multiple HGT events, perhaps even between different types of eukaryotes.

(c) Estimated *acdS* gains and losses along fungal evolutionary history

To assess the extent of *acdS* transfer and loss events, we reconstructed the ancestral states of *acdS* presence/absence along fungal phylogenetic history, using 150 sequenced fungi. For actinobacterial donors, ancestral state reconstruction showed four supported acquisitions in Basidiomycota, which concerned *Gymnopus luxurians*, *Schizophyllum commune*, *Gloeophyllum trabeum* (Agaricomycetes class) and a *Cryptococcus* ancestor (Tremellomycetes class) (figure 2). Similarly, four acquisitions were identified for Ascomycota: a recent one in *Oidiodendron maius* (Leotiomycetes class), as well as three more ancient ones in the Eurotiomycetes class ancestor, in the Sordariomycetes class ancestor and in a Mycosphaerellaceae subclade (Dothideomycetes class). Ancestral state reconstruction for the *acdS* clade of betaproteobacterial origin pointed to two recent *acdS* acquisitions in the Basidiomycota *Fomitopsis pinicola* SS1 and *Punctularia strigosozonata* (Agaricomycetes class), and another acquisition by an unidentified ancestor of the Dothideomycetes *Hysterium pulicare*, *Rhytidisteron rufulum* and *Botryosphaeria dothidea*. Complete reconstructions for these two clades can be found in the electronic supplementary material, figures S4 and S5.

For gammaproteobacterial *acdS* donors, ancestral state reconstruction did not provide a clear scenario using maximum-likelihood reconstruction (see the electronic supplementary material, figure S6), but Bayesian reconstruction strongly pointed to independent acquisitions in every $acdS^{+}$ fungus (see the electronic supplementary material, table S1). Yet these two reconstructions strongly support the absence of *acdS* in the most ancestral nodes for the Saccharomycetes, refuting the hypothesis of a single, ancestral acquisition of the gene.

For each putative ancestral recipient, no case of subsequent *acdS* gene loss was detected in the descent, regardless of whether the gene originated from Actinobacteria, Betaproteobacteria or Gammaproteobacteria. Thus, our results point to recurrent HGT events of *acdS* towards oomycetes and fungi.

(d) Functionality and selection patterns of eukaryotic *acdS*

Functionality of eukaryotic *acdS* is indicated by the conservation of the catalytic function. Indeed, the key residues K^{51}, Y^{269}, Y^{295} and E^{296} needed for ACC deaminase catalytic activity in the yeast *C. saturnus* [23] were conserved in all eukaryote sequences (both in *Phytophthora* and fungi), as were the amino acids adjacent to these residues (figure 3). This highlights the conservation of the bacterial *acdS* catalytic function across distant eukaryotic lineages that experienced independent *acdS* acquisitions. In addition, direct evidence for *acdS* transcription in eukaryotes was also obtained, as

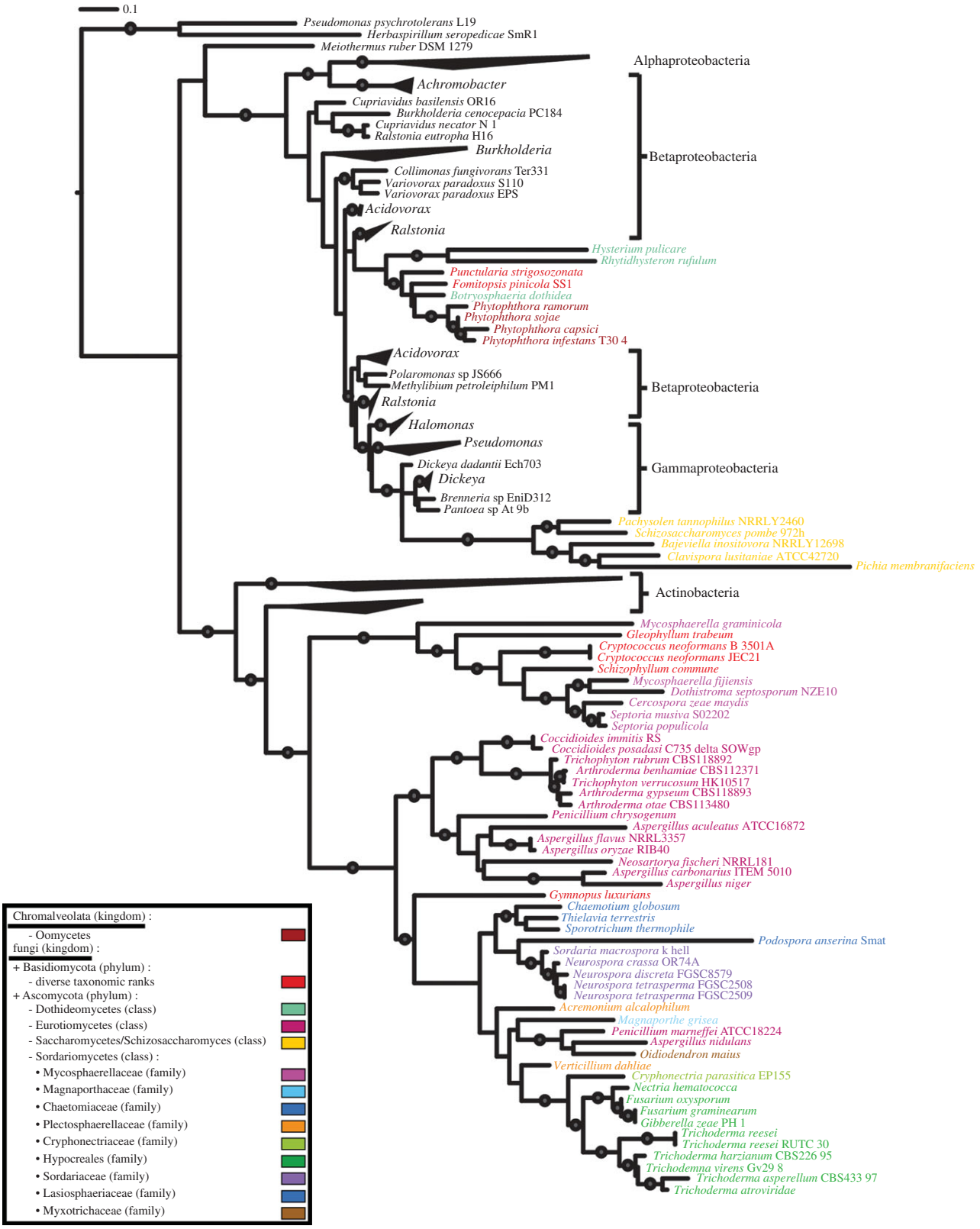

Figure 1. Maximum-likelihood phylogenetic tree of *acdS* protein sequences. The tree was rooted using D-cysteine desulfhydrase sequences as outgroup (electronic supplementary material, figure S3). Supported nodes are indicated with grey circles (bootstrap > 70).

mining transcript databases allowed the identification of all or part of *acdS* mRNA in almost all stramenopiles and fungi studied (see the electronic supplementary material, figure S7). This indicated that differences in promoter regions between bacteria and eukaryotes were not a barrier for successful genetic transfers.

The lack of *acdS* deletion and the high AcdS sequence conservation suggest that this gene confers a selective advantage to microeukaryotes. In addition, comparing relative fixation rates of synonymous (silent) and non-synonymous (amino acid altering) mutations showed strong negative selection (dN/dS ratio < 1) in most species (see the electronic supplementary material, figure S8), meaning that functional mutational modifications were selected against. Despite this purifying selection, positive selection (dN/dS ratio > 1) was also found in ancestral branches, notably in the Ascomycota *Aspergillus* (Eurotiomycetes class), *Trichoderma* and *Fusarium* (Sordariomycetes class), and in the Dothideomycetes class.

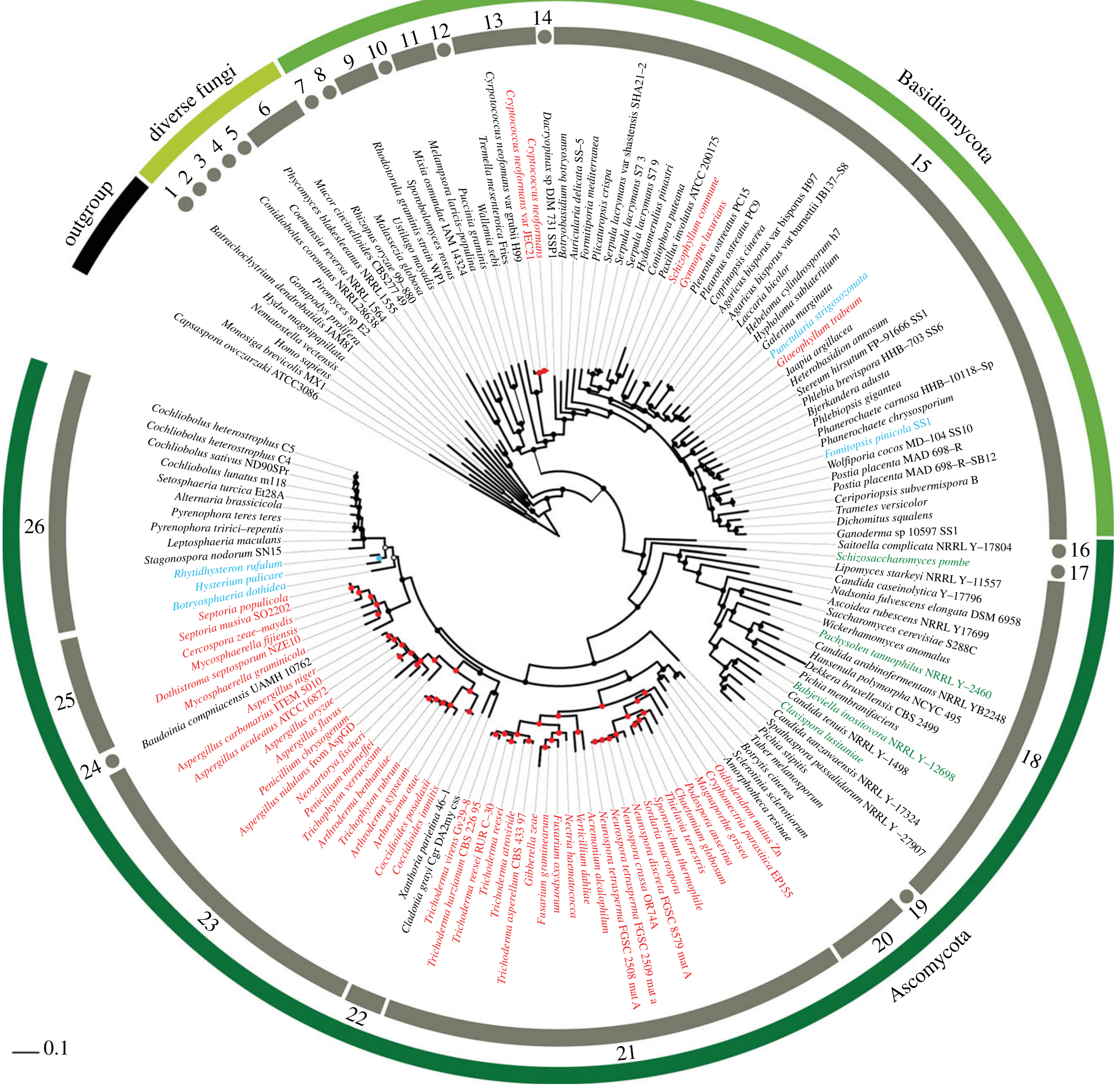

Figure 2. Ancestral state character reconstruction of *acdS* gene gains/losses. Coloured nodes and species names indicate gene presence while black means absence. Actinobacterial, betaproteobacterial and gammaproteobacterial *acdS* origins are shown in, respectively, red, blue and green for these names and nodes. Uncertainty in reconstruction is indicated by a white node. Fungal classes: (1) Chytridiomycetes; (2) Monoblepharidomycetes; (3) Neocallimastigomycetes; (4) Entomophthoromycotina; (5) Kickxellomycotina; (6) Mucoromycotina; (7) Exobasidiomycetes; (8) Ustilaginomycetes; (9) Microbotryomycetes; (10) Mixiomycetes; (11) Pucciniomycetes; (12) Wallemiomycetes; (13) Tremellomycetes; (14) Dacrymycetes; (15) Agaricomycetes; (16) Taphrinomycotina; (17) Schizosaccharomycetes; (18) Saccharomycetes; (19) Pezizomycetes; (20) Leotiomycetes; (21) Sordariomycetes; (22) Lecanoromycetes; (23) Eurotiomycetes; (24) Teratosphaeriaceae; (25) Mycosphaerellaceae; (26) Dothideomycetes.

This diversifying selection, which means that functional AcdS modifications could be selected, is more likely to reflect sequence adaptation to gene biology in eukaryotes rather than a true change in protein function. This hypothesis is strengthened by the conservation of key residues implicated in the catalytic function of the protein, as well as demonstration of AcdS enzymatic activity in fungi tested [21–23].

(e) Intron acquisitions in eukaryotic *acdS* sequences

Unlike in prokaryotes, eukaryotic genes typically display a combination of introns and exons, and indeed we found one or several spliceosomal introns (up to eight in the Basidiomycota *G. trabeum*) in eukaryotic *acdS* sequences (figure 4). Thus, *acdS* acquisition by eukaryotes was followed by intron formation(s) in around half the identified transfers (31 of 65 eukaryotes). Most Sordariomycetes (corresponding to nine distinct taxonomic families) presented a conserved region of intron insertion, located 186–195 nucleotides from the *acdS* start codon. Introns were also found in this region in species belonging to distant taxonomic classes, such as the Lecanoromycete *O. maius* Zn and the Eurotiomycete *Penicillium marneffei* ATCC18224. However, the intron sequences themselves showed no conservation, except in closely related species (data not shown). Taken together, the data point to lineage-specific intronization, which might have gone on par with *acdS* evolution and domestication within these lineages.

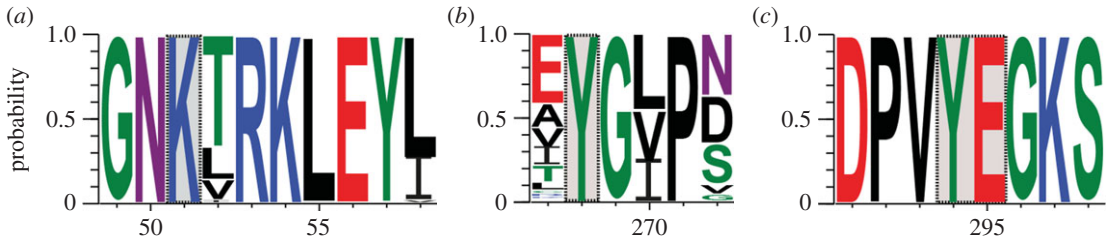

Figure 3. Conservation of key amino acids in *acdS*. Conserved amino acids are shown in (*a*) the Lys[51] region, (*b*) the Tyr[268] region and (*c*) the Tyr[294]-Glu[295] region, which are important for catalytic activity in bacterial and eukaryotic AcdS sequences [23]. Positions indicated refer to those in the protein sequence of the Ascomycota model *C. saturnus*, for which key residues (boxed) were identified.

3. Discussion

HGT is a key feature of bacterial evolution [27,28], but recent studies have also reported HGT events from prokaryotes to eukaryotes [3,7–10,12]. A significant portion of the genome of certain eukaryotic species was acquired horizontally [13–15], yet it was not clear at which order of magnitude such transfers could take place for a given gene. This work demonstrates that the bacterial gene *acdS* is extensively present in filamentous eukaryotes, based on the recovery of this gene in as many as 44% of the 149 sequenced genomes available. Different processes can explain the uneven distribution of genes in a given lineage, such as convergent evolution [29,30], lineage-specific gene loss [31,32] or HGT [8,33,34]. In our case, convergent evolution is unlikely as *acdS* nucleotide sequence identity of bacterial and eukaryotic homologues (40–78%) is particularly high, indicating a similar evolutionary origin.

Even though the gene loss hypothesis cannot be fully rejected, data clearly point to multiple and independent HGT events for *acdS*. In particular, eukaryotic *acdS* homologues do not form an independent clade outside bacterial *acdS* homologues (as expected if genes were vertically transmitted) but are rather interspersed within bacterial sequences. Thus, *acdS* phylogeny identified three major incongruences with the species tree (electronic supplementary material, figure S2), indicating HGT from three bacterial phyla to many eukaryotic lineages. Moreover, incongruences were also found inside eukaryotic clades, suggesting eukaryote-to-eukaryote transfers. Such events can take place [31,34], but here poor node supports failed to strengthen the hypothesis.

For two of the three major incongruences—namely those involving (i) branching of various fungi and stramenopiles (two distant eukaryotic lineages [35,36]) inside Betaproteobacteria and (ii) Basidiomycota and Ascomycota in relation to Actinobacteria—this could entail a unique acquisition of *acdS* by a eukaryotic ancestor followed by extensive gene loss during evolution and speciation of the different eukaryotic lineages. Considering vertical transfer to explain the patterns of *acdS* presence would imply an ancestral acquisition in an ancestor common to various eukaryotes. However, this hypothesis is rather unlikely considering the high conservation of *acdS* sequences in the eukaryotic lineages in each case. An alternative possibility is a series of multiple, distinct *acdS* transfers to different microeukaryotic clades. The two ancestral character reconstruction methods that were used clearly pointed to the latter possibility, which is very well supported statistically when considering *acdS* distribution in relation to fungal and stramenopile evolutionary histories.

The third incongruence involves Gammaproteobacteria and diverse Saccharomycetes/Schizosaccharomycetes. In this case, the two methods of ancestral character reconstructions gave conflicting results, thus inferences should be taken with caution. Maximum-likelihood reconstruction pointed to a unique, ancestral acquisition of *acdS* followed by subsequent vertical transmission and differential gene losses. However, 10 of 16 nodes of the Saccharomycetes/Schizosaccharomycetes clade were not statistically supported, limiting conclusions on the current analysis. By contrast, Bayesian reconstruction inferred the presence of the gene in a last eukaryotic common ancestor, but this was unlikely (and thus might reflect analysis bias) because only a few Saccharomycetes/Schizosaccharomycetes fungi possessed *acdS*. Nevertheless, a robust Bayesian reconstruction was obtained when constraining the model to infer the absence of *acdS* at the root of the tree. In this case, the analysis favours multiple and independent transfers with strong statistical support at each node of the Saccharomycetes/Schizosaccharomycetes clade.

In the 65 *acdS*$^+$ microeukaryotes, we estimated that *acdS* acquisition entailed at least 15 different HGT events based on ancestral character reconstructions (figure 2). Such a magnitude for genetic transfer of a bacterial gene across a vast range of filamentous eukaryotes has never been described before [37,38] and challenges our understanding of eukaryote evolution, as most current models in evolutionary biology assume gene duplication as a key process of biochemical innovation [39]. In contrast to duplication, which gives rise to slow genetic innovation, gene acquisition might play a distinct role in enabling rapid phenotypic or ecological adaptation. Nevertheless, the actual mechanism(s) by which *acdS* was acquired by filamentous eukaryotes remain(s) unknown. We did not find any remnant of a mobile genetic element in the vicinity of *acdS* insertion sites. Furthermore, other putative bacterial genes in the vicinity of *acdS* were not found in eukaryotic genomes based on sequence identity search, except one encoding a putative monooxygenase downstream *acdS* in Eurotiomycetes, but that is also largely present in *acdS*-negative fungi (data not shown). Thus, it seems that *acdS* could have been transferred alone. Phagotrophy, the consumption of a whole cell by another one, is seen as a driving force in bacteria-to-unicellular eukaryote HGT [40], but cannot explain HGT in fungi [41].

The fact that most *acdS*$^+$ microeukaryotes live in the vicinity of plants is of primary interest, because sharing a same ecological habitat may facilitate physical interaction between HGT protagonists [42,43] and is likely to promote *acdS* transfer. In addition, plants are the main natural source of ACC, which may represent a significant source of carbon (α-ketoglutarate) and nitrogen (ammonia) for AcdS$^+$ plant-associated eukaryotes [44]. In return, the latter act on plants by decreasing ethylene levels. In non-pathogenic fungi like *T. asperellum* T203, this

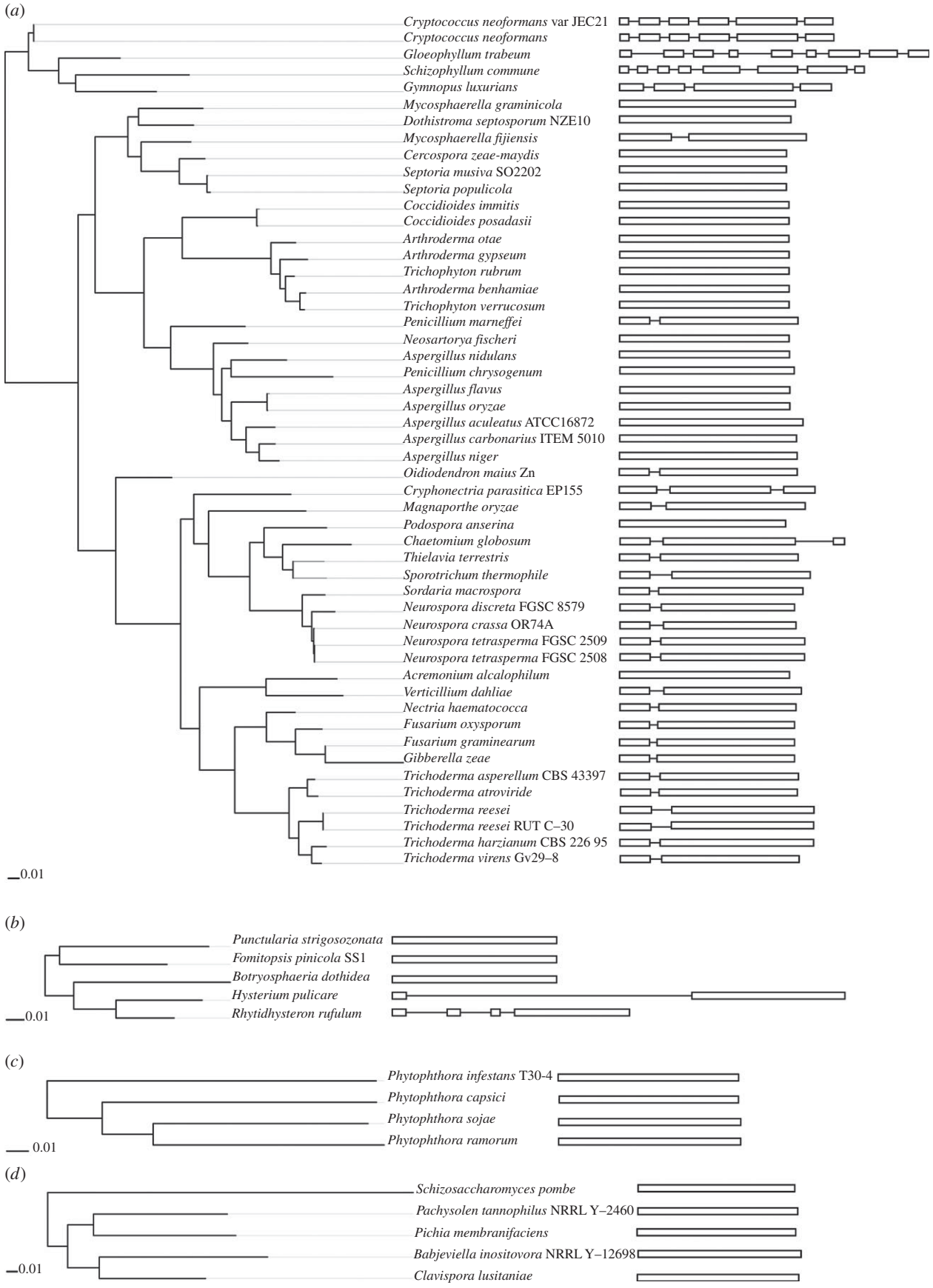

Figure 4. Genetic structure of eukaryotic *acdS* in relation to the position in the *acdS* phylogenetic gene tree. Coding sequences are represented by white rectangles and introns by black lines. (*a*) Fungal *acdS* of actinobacterial origin, (*b*) fungal *acdS* of betaproteobacterial origin, (*c*) *Phytophthora acdS* of betaproteobacterial origin and (*d*) fungal *acdS* of gammaproteobacterial origin.

promotes root elongation of canola and cucumber, favouring plants' nutrient uptake and in turn their growth and yields [22,45]. In phytopathogenic oomycetes and fungi, however, the main effect of ACC degradation might be to facilitate plant infection, because ethylene signalling acts synergistically with jasmonate to induce plant defence responses [46,47].

The ACC deamination case is unusual in that the function can be ecologically important for microeukaryotes (and thus

was selected), easy to perform with existing cell machinery (requiring only B6 vitamin), novel in comparison with pre-existing eukaryotic capacities (and thus was maintained as such) and involves a highly conserved gene (also suggesting that *acdS* transfers were recent). Consistent with this, we found that *acdS* was acquired by different types of filamentous eukaryotes (from both stramenopile and fungi kingdoms), which obtained the gene from contrasted bacterial phyla (both Actinobacteria and Proteobacteria). Furthermore, this took place with a conservation of the original AcdS catalytic function, a rapid domestication process (based on intronization dynamics), and effective expression of *acdS* in most oomycetes and fungi studied (based on mRNA database analyses). Thus, this study provides an estimate for the higher transfer rate that could be expected for this type of inter-domain HGT event.

In summary, this study shows that HGT between prokaryotes and eukaryotes can happen in high magnitude, along with the conservation of the original catabolic function and a successful adaptation of the transferred gene to very distantly related recipients.

4. Material and methods

(a) Homologous sequence retrieval and re-annotation

The AcdS protein sequence (YP_005208895) of *P. fluorescens* F113 was queried with BLASTp [48] against the NCBI RefSeq database [49] to retrieve prokaryotic homologues, with an *E*-value threshold of 1×10^{-20} to filter results. To retrieve eukaryotic homologues, the same sequence was simultaneously queried with BLASTp against the JGI Mycocosm [50] and NCBI RefSeq databases, with same 1×10^{-20} *E*-value threshold. ACC deaminase sequences were further selected among the retrieved homologues based on functional domain identification, using RPS-BLAST [48].

(b) Phylogenetic analysis

Protein sequences were aligned with Clustal Omega [51]. Sequences were manually filtered to discard gaps and aligned regions of low quality. For *acdS* sequences, the phylogenetic trees were inferred with PhyML [52] with the GTR model, 1000 bootstraps, SPR topology search [53] and the estimation of the proportion of invariant sites. Paralogues were identified by a phylogenetic approach and removed from the analysis.

(c) Fungal phylogenetic tree reconstruction

The fungal species tree was inferred from 43 concatenated protein markers [54]. For the latter, the markers were obtained for 145 sequenced fungi using HMMER3. When multiple homologues of a given marker were retrieved for a given fungal species, redundancy was resolved using a tree-based approach, and markers showing major incongruences were discarded [55]. A few sequences aligning poorly were also discarded in the final alignment to limit the possibility of false homologues, which does not compromise tree topology if sufficient data are provided [56]. Sequences were aligned as explained above (alignment of 15 813 positions) and rooted using an outgroup composed of *Homo sapiens*, *Capsaspora owczarzaki* ATCC3086, *Monosiga brevicolis* MX1, *Nematostella vectensis* and *Hydra magnipapillata*. The phylogenetic tree was obtained as described above, except that NNI topology search [57] was used to infer the topology and 100 bootstraps were done.

(d) Ancestral state reconstruction

Maximum-likelihood and Bayesian reconstructions were used to mitigate potential methodological biases of each approach [58,59]. The maximum-likelihood method allows quick reconstruction of ancestral state with a good sensitivity [59], whereas Bayesian methods of reconstruction have the advantage of taking into account tree uncertainty by reconstructing ancestral states over a set of phylogenetic trees [60]. Analyses were done with the previously inferred fungal tree and matrices of presence/absence of fungal *acdS* homologues, using Mesquite (http://mesquiteproject.org/mesquite/mesquite.html) with maximum likelihood Asymmk2 model of rate variations [61] and the reversible jump Markov chain Monte Carlo method of BayesTraits [60]. Statistical confidence was assessed with a likelihood ratio test and the Bayes factor ratio, respectively.

(e) Conservation of key amino acids in eukaryotic *acdS* homologues

Unfiltered alignments were used to verify the presence of amino acids required for ACC deaminase activity. Structural analysis along with site-directed mutational studies of the AcdS protein in *C. saturnus* identified four important amino acids (K^{51}, Y^{269}, Y^{295} and E^{296} [23]). Multiple alignments were numbered according to *C. saturnus* sequence and represented using WebLogo (http://weblogo.berkeley.edu).

(f) Intron annotation

Introns were re-annotated in nucleotide sequences using Wise v. 2.1.20 (http://www.ebi.ac.uk/Tools/psa/genewise). A hidden Markov model (HMM) profile was generated using HMMER3 and served as query to align against nucleotide sequences. Wise software was set to consider GT/AG splicing sites only.

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