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ORIGINAL ARTICLE

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The obligate alkalophilic soda-lake fungus *Sodiomyces alkalinus* has shifted to a protein diet

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Abstract

Sodiomyces alkalinus is one of the very few alkalophilic fungi, adapted to grow optimally at high pH. It is widely distributed at the plant-deprived edges of extremely alkaline lakes and locally abundant. We sequenced the genome of *S. alkalinus* and reconstructed evolution of catabolic enzymes, using a phylogenomic comparison. We found that the genome of *S. alkalinus* is larger, but its predicted proteome is smaller and heavily depleted of both plant-degrading enzymes and proteinases, when compared to its closest plant-pathogenic relatives. Interestingly, despite overall losses, *S. alkalinus* has retained many proteinases families and acquired bacterial cell wall-degrading enzymes, some of them via horizontal gene transfer from bacteria. This fungus has very potent proteolytic activity at high pH values, but slowly induced low activity of cellulases and hemicellulases. Our experimental and in silico data suggest that plant biomass, a common food source for most fungi, is not a preferred substrate for *S. alkalinus* in its natural environment. We conclude that the fungus has abandoned the ancestral plant-based diet and has become specialized in a more

protein-rich food, abundantly available in soda lakes in the form of prokaryotes and small crustaceans.

KEYWORDS
alkalophilic fungus, brine shrimps, enzymes, HGT, prokaryotes, *Sodiomyces alkalinus*

1 | INTRODUCTION

Soda (or alkaline) soils and lakes are the most alkaline natural habitats on Earth. With pH values typically ranging from 9 to 11, these environments are also often very salty—with high Na^+ concentrations. Soda soils are usually restricted to arid or semi-arid savanna inland areas interspersed with alkaline water basins called soda lakes, which fluctuate in size throughout the season due to evaporation and rain (Jones & Grant, 2000; Jones, Grant, Duckworth, & Owenson, 1998). A prominent reservoir of soda lakes and soils is located in the Western Siberia, Altai, Trans-Baikal areas (Russia), Mongolia and Africa, where Lakes Magadi and Natron have pH values approaching 12.

Extreme conditions of soda lakes and soils are not rendering them inhabitable as thought before, as an extensive diversity of microorganisms is adapted to thrive there (Grant & Sorokin, 2011; Zavarzin, 1993; Zavarzin, Zhilina, & Kevbrin, 1999). Fungi generally prefer acidic or neutral pH for optimal performance and were therefore not expected in saline soda lakes. However, soda lakes in Russia, Mongolia and Africa unexpectedly were found to harbour fungal species of different taxonomic lineages (Grum-Grzhimaylo, Debets, van Diepeningen, Georgieva, & Bilanenko, 2013; Grum-Grzhimaylo, Georgieva, Bondarenko, Debets, & Bilanenko, 2016; Grum-Grzhimaylo, Georgieva, Debets, & Bilanenko, 2013). Some of the recovered fungi not only tolerate high ambient pH, but even prefer that condition for optimal growth, a physiological category called alkalophiles (Horikoshi, 2011). The species that was identified in nearly every soda soil sample was *Sodiomyces alkalinus*—an obligate alkalophilic ascomycetous fungus, which is a member of the *Plectosphaerellaceae* family (Grum-Grzhimaylo, Debets et al., 2013). The high abundance of this fungus in such extreme environment is striking. First, the high pH makes growth of most fungi impossible. Second, the edges of soda lakes are deprived of plant biomass, which is the dominant food source of most fungi. We hypothesized that *S. alkalinus* has specific adaptations to deal with those challenges and has changed the preference towards nonplant diet in this plant-deprived environment.

To test this hypothesis, the genome sequence of *S. alkalinus* was determined and examined for footprints of adaptation to the extreme conditions of soda lakes. The set of encoded carbohydrate-active and proteolytic enzymes was analysed in combination with direct bioassays under various conditions, to shed light on the preferred growth substrates of *S. alkalinus*. Our results support the shift in preference of the fungus from plants to alternative protein-rich substrates, such as small crustaceans and prokaryotes. Results obtained here not only improve our understanding of how fungi evolved to thrive under extreme natural conditions of soda lakes, but also provide opportunities to exploit *S. alkalinus* for commercial purposes, as a source of alkaline-active compounds for industrial use.

2 | MATERIALS AND METHODS

2.1 | Strains, media and growth

The *Sodiomyces alkalinus* ex-type strain F11 (CBS 110278) was used throughout the study and routinely propagated on the alkaline agar (AA) medium at 28°C (Grum-Grzhimaylo, Debets et al., 2013). For pH-dependent growth experiments, we used appropriate inorganic buffers at final concentration 0.1 M to generate desired pH. We used *Aspergillus fumigatus* wild type (CBS 140053) and *Aspergillus oryzae* RIB40 as neutrophilic references. We tested various carbon sources and vitamin utilization on the minimal medium designed for *S. alkalinus*. For the enzyme assays, *S. alkalinus* was pregrown in liquid alkaline medium of the same content as AA, omitting agar. The collected pregrown mycelium was washed, filtered and inoculated in the liquid media, based on AA but with different pH values (6, 8 or 10) and substituting malt/yeast extracts for 1% carbon sources. For extended methods, see Supporting Information Appendix S1.

2.2 | SDS-PAGE and silver staining

We applied 20 μl of media extracts on 12% (w/v) SDS-PAGE topped with a 5% stacking gel using a MiniProtean II system (Bio-Rad), as described in (Laemmli, 1970). The gels were stained with silver nitrate to visualize the total proteins content (Blum, Beier, & Gross, 1987).

2.3 | Enzymatic assays

Culture filtrates after 48 hr of incubation were analysed for the selected enzyme activities involved in plant biomass degradation. All enzymatic activities were measured in biological duplicates. Each biological duplicate was measured in technical triplicates in 96-well plates at three pH values using 0.1 M buffers with pH 6, 8 and 10, and at 30°C. Activities were measured using either appropriate p-nitrophenyl (pNP) substrates (Sigma) or by the amount of reducing sugars released, using dinitrosalicylic acid (DNS) reagent (de Vries, Visser, & Graaff, 1999). One unit of enzymatic activity (U) was defined as the amount of enzymes that liberated 1 mmol of the corresponding product per minute of reaction, under assay conditions. The activities were expressed per volume of enzymatic

solution (U/ml). Total proteolytic activity was measured with Pierce Fluorescent Protease Assay Kit (Thermo Fisher Scientific, USA) using a fluorescein isothiocyanate (FITC)-labelled casein assay according to the manufacturer's instructions. We tested the assay with the standard kit TBS buffer of pH 7.2, but also ensured the assay works properly with other buffers the yield pH 6, 8 and 10. One unit (U) of protease activity was defined as the amount of protein that has an equivalent activity of 1 μg bovine pancreas trypsin (Thermo Fisher Scientific) at pH 8. The activities were expressed per volume of enzymatic solution (U/ml). For extended methods, see Supporting Information Appendix S1.

2.4 | DNA and RNA extraction

Genomic DNA was extracted from mycelium of *S. alkalinus* grown on top of a cellophane membrane put onto AA medium. For genome sequencing, we obtained genomic DNA using the CTAB-based protocol from (Rogers & Bendich, 1988), with minor modifications. The quality and quantity of the DNA was verified on 0.6% agarose gels stained with EtBr, but also using a Nanodrop 2000 (Thermo Fisher Scientific) and Qubit Fluorometer dsDNA assays (Thermo Fisher Scientific). For transcriptome sequencing, total RNA was extracted using RNeasy Plant Mini Kit (Qiagen Inc., CA, USA) following the manufacturer's instructions. For PCRs, we used genomic DNA and RNA extracted with DNeasy and RNeasy Plant Mini Kits (Qiagen Inc.). For extended methods, see Supporting Information Appendix S1.

2.5 | Genome sequencing, assembly and annotation

The *S. alkalinus* genome and transcriptome were sequenced using Illumina, the former in combination with fragment and long mate pair (LMP) libraries. All libraries were quantified using KAPA Biosystem's next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. The quantified libraries were then prepared for sequencing on the Illumina HiSeq sequencing platform utilizing a TRUSEQ PAIRED-END CLUSTER KIT, version 3, and Illumina's cBot instrument to generate a clustered flowcell for sequencing. Sequencing of the flowcell was performed on the Illumina HiSeq2000 sequencer using a TRUSEQ SBS SEQUENCING KIT, version 3, following a 2 × 100 and 2 × 150 indexed run recipe for LMP and fragments/transcriptome, respectively.

Genomic reads from two libraries were filtered and assembled with AllPathsLG (Gnerre et al., 2011). RNA-seq data were de novo assembled into consensus sequences using Rnnotator (Martin et al., 2010). Fungal genome was annotated using the JGI Annotation pipeline and made available via JGI fungal genome portal MycoCosm (http://genome.jgi.doe.gov/Sodal1; Grigoriev et al., 2014), and DDBJ/ENA/GenBank database. For extended methods, see Supporting Information Appendix S1.

2.6 | Phylogeny

For the phylogenomic placement of *S. alkalinus*, we used a set of 1,347 single-copy orthologue genes among 32 ascomycetous fungi (Supporting Information Table S1), as identified with OrthoFinder (Emms & Kelly, 2015). Individual protein sequences were aligned and concatenated into a single matrix using MAFFT (Katoh & Standley, 2013). The ambiguously aligned areas and gaps were removed with Gblocks 0.91b (Castresana, 2000), leaving 320,204 characters (24.83% of total), of which 168,273 were informative. RAXML version 8. 2. 11 (Stamatakis, 2014) was used to infer an ML tree under the unpartitioned PROTGAMMAGTR model of amino acid substitution with 100 rapid bootstrapping replicates.

2.7 | CAZy annotation

Carbohydrate-Active enZyme (CAZy) modules were annotated as in Lombard, Ramulu, Drula, Coutinho, and Henrissat (2014) or obtained from Mycocosm portal as of May 2017 (http://genome.jgi.doe.gov/; Grigoriev et al., 2014). Extended CAZy data set is listed in Supporting Information Table S2.

2.8 | Proteinases

Putative proteinases were found by two successive searches against MEROPS databases (Rawlings, Waller, Barrett, & Bateman, 2014). At first, predicted proteomes of 32 fungi were used as queries for blastp against full MEROPS database (version 12) to remove false positives (Rawlings & Morton, 2008). Then, the output was used as queries for blastp against a smaller MEROPS Scan database of type domains. In both searches, *e*-value was set to 0.0001. Secretory peptidases were predicted with TargetP 1.1b under default settings (Emanuelsson, Brunak, von Heijne, & Nielsen, 2007).

2.9 | qRT-PCR

To track the expression profiles of the lysozyme GH25 (JGI ID 341929), DD-peptidase (JGI ID 350999) and racemase (JGI ID 322460) across various pH, we used RNA isolated from the mycelium grown in liquid (2 days old) at various pHs in biological triplicates. 1 μg of genomic RNA was converted into cDNA with iScript™ cDNA Synthesis Kit (Bio-Rad). The qPCR final mix volume was 8 μl and contained 4 μl 2x iQ SYBR Green SuperMix (Bio-Rad), 0.16 μl 10 μM of each primer (for the primer list, see Supporting Information Table S3), 0.68 μl MQ water and 3 μl of cDNA. Each reaction was run in technical triplicates, in a Bio-Rad CFX96 thermocycler. *act1* gene was used as a reference housekeeping gene, and annealing temperature for the qRT-PCRs was set to 61°C (primer efficiencies were verified in pilot runs). The technical triplicates were averaged and used for calculating C_t difference relative to the lowest pH point. The $\Delta\Delta C_t$ method was employed to calculate relative gene expression. qRT-PCR results were analysed by the BIO-RAD CFX MANAGER version 2.0 software. Baseline threshold line was set arbitrarily at exponential phase of PCR (500 relative fluorescence units), and results were normalized to the *act1* gene signal.

2.10 | DD-peptidase deletion

The strategy for the knockout was based on a double recombination event with the targeted incorporation of a hygromycin resistance cassette disrupting the gene of interest, as described by (Kars, McCalman, Wagemakers, & van Kan, 2005). In brief, two DNA fragments, flanking the ORF of the DD-peptidase, were amplified and fused by overlap PCR with the *hph* cassette, carrying a gene conferring resistance to hygromycin B (hygB, Formedium). The cassette was introduced into the protoplasts of *S. alkalinus* by PEG-mediated transformation as in (Kars et al., 2005) and (ten Have, Mulder, Visser, & van Kan, 1998) with a few adjustments. HygB-resistant colonies were subjected to a single-spore bottleneck to ensure a homokaryotic transformant. The putative knockouts were verified with PCR experiments, ensuring the deletion of the DD-peptidase. For extended methods, see Supporting Information Appendix S1 and Supporting Information Table S3.

2.11 | Horizontal gene transfer detection

The method described in Marcet-Houben and Gabaldón (2010) was used for the detection of putative horizontal gene transfer (HGT) cases. Shortly, if protein sequences are present in a few fungi as well as in a high number of bacterial genomes, this gene is assumed to have undergone HGT. To identify these cases, we blasted (blastp, *e*-values 10^{-5}, 10^{-10}, 10^{-15}, 10^{-20}, 10^{-30}, 10^{-50}) the predicted proteome of *S. alkalinus* against proteomes (248 fungal and 6,321 bacterial proteomes) obtained from the nonredundant database in NCBI as of April 2017. We tagged a given protein to be horizontally transferred if it fell in the quadrant limited by first 35-quantile for fungal occurrences and 100**SE* from loess fit in bacterial distribution. Substantial intersection of the proteins that we obtained after analyses ran at various *e*-values strengthens our results. We considered an analysis ran at the *e*-value of 10^{-20} in our results. For details, see Supporting Information Appendix S1.

3 | RESULTS

3.1 | Growth in situ and in vitro

Sodiomyces alkalinus is an obligate alkalophilic fungus that was isolated from the edge of many soda lakes (Grum-Grzhimaylo, Debets et al., 2013). Figure 1a–e depicts the typical environment of a soda lake (Lake Tanatar-2, Altai area, Russia, during field trip of August

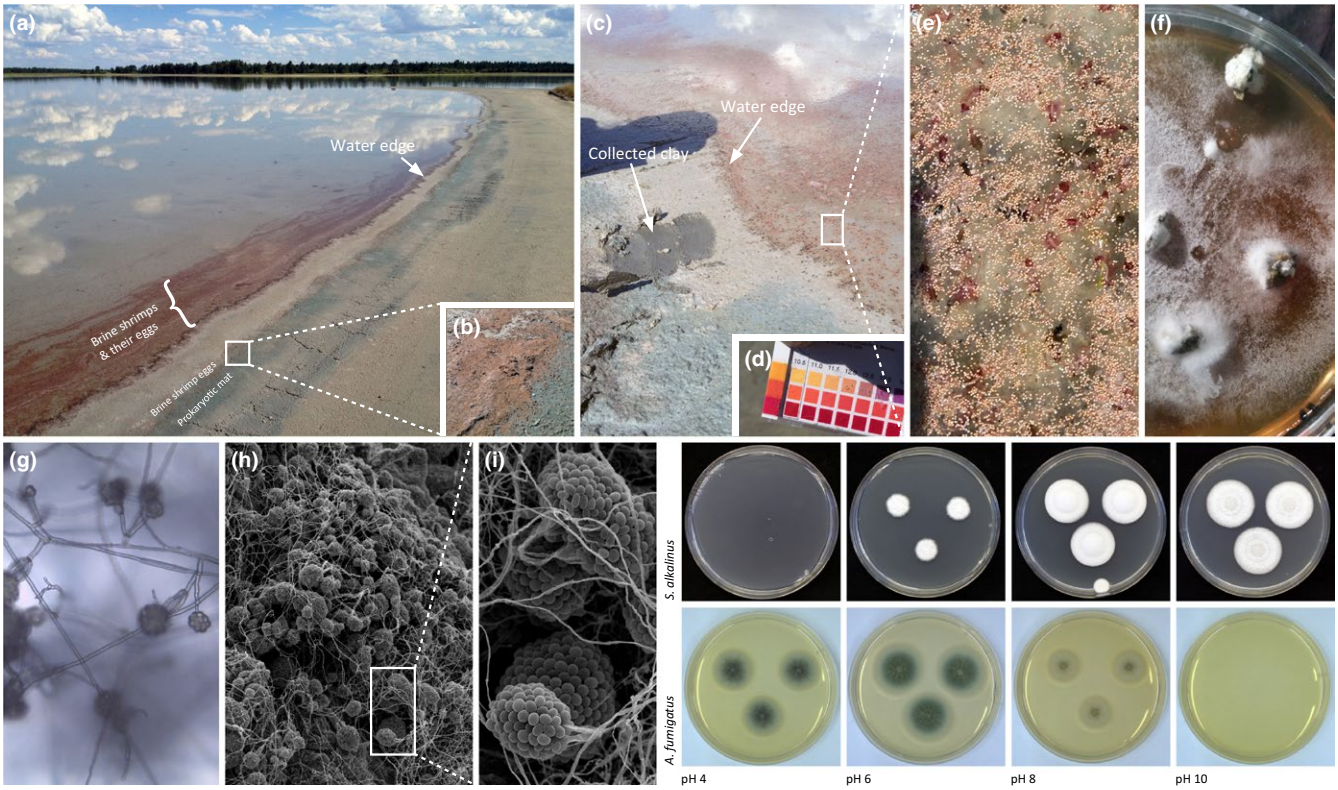

FIGURE 1 *Sodiomyces alkalinus* in situ and in vitro. (a) Soda lake—typical habitat for *S. alkalinus* (Lake Tanatar-2, Altai area, Russia). Note the absence of vegetation on the rim of the lake. (b) A close-up on thick crusts (a few cm) of brine shrimp eggs deposits and prokaryotic mats on the edge of the lake. (c) Water edge with a spot of collected clay. (d) Extremely alkaline pH (around 11.5) of the clay. (e) A close-up on brine shrimps (with eggs) floating in the water. (f) *Sodiomyces alkalinus* growing out of the collected clay clumps put on alkaline agar medium. (g–i) *Sodiomyces alkalinus* conidiophores with conidial heads grown on top of the clay clumps (g—light microscopy, h–i—scanning electron microscopy). Plates display growth of *S. alkalinus* at different pH, as compared to a neutrophilic reference fungus *Aspergillus fumigatus* (MYA medium based on 0.1 M buffers) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Genome and assembly statistics of *Sodiomyces alkalinus* with related species within the *Plectosphaerellaceae* family

Species	*Sodiomyces alkalinus*	*Acremonium alcalophilum*	*Verticillium dahliae*	*Verticillium alfalfae*	*Plectosphaerella cucumerina*
Strain	CBS 110278	JCM 7366	VdLs.17	VaMs.102	DS2psM2a2
Genome assembly size (Mb)	43.45	54.42	33.83	32.83	36.84
Average coverage	113.5×	8×	7×	4.08×	86.9×
No. of contigs	290	286	1,562	4,147	124
No. of scaffolds	29	13	52	26	26
Scaffold N50 (Mb)	3.52	7.2	1.27	2.32	4.03
Scaffold L50	4	3	9	6	4
No. of gaps	261	273	1,510	4,121	98
% of scaffold length in gaps	0.7	0.2	2.7	7.7	0.1
No. of predicted genes	9,411	9,521	10,535	10,221	14,254

2017) where *S. alkalinus* thrives. The clay clumps with pH around 11.5 that were collected at the plant-deprived (but prokaryote- and brine shrimp-rich) edge of the water basin and put on alkaline agar medium (pH c. 10) resulted in abundant growth of *S. alkalinus* on top of the clay, subsequently expanding onto the nutrient medium (Figure 1f–g). *Sodiomyces alkalinus* shows optimal growth on agar nutrient medium at pH values ranging from 8 to 10 and moderate Na^+ concentrations of 0.3–0.4 M (Supporting Information Figure S1). The obligate alkalophilic physiology of *S. alkalinus* drastically contrasts with the neutrophilic physiology of *Aspergillus fumigatus*, typical for most fungi (Figure 1). These in situ and in vitro growth data confirm that *S. alkalinus* is metabolically active in its natural environment of soda soils and lakes and are consistent with adaptation to the natural growth conditions with stable high pH values, but with variation in salt concentration, from very low to high (Grum-Grzhimaylo, Debets et al., 2013).

3.2 | Genome statistics

The Illumina-sequenced genome of *S. alkalinus* resulted in a 43.45 Mb assembly with an average read-depth coverage of 113.5× in 290 contigs and 29 scaffolds. While the genome size is smaller than the 54.4 Mb genome assembly of the closely related *Acremonium alcalophilum*, both genomes encode a similar number of genes: 9,411 in *S. alkalinus* vs. 9,521 in *A. alcalophilum*, and represent a similar functional portrait (IPR domains, Supporting Information Table S4). The difference in genome size can be partly explained by higher repeat content in *A. alcalophilum* (49.8% vs. 33.2%). The analysis of the assembly and gene models using BUSCO (Simão, Waterhouse, Ioannidis, Kriventseva, & Zdobnov, 2015) suggests that we obtained the nearly complete genome of *S. alkalinus* (Supporting Information Table S5). The genome sequence, gene models and annotations are publicly available from the JGI genome portal Mycocosm (Grigoriev et al., 2014) at http://genome.jgi.doe.gov/Sodal1. Statistics pertinent to the genome assembly and comparisons with related species within the *Plectosphaerellaceae* are given in Table 1.

3.3 | Carbohydrate-active enzymes in silico and in vitro

Fungi decompose complex organic carbon compounds externally and use it as an energy source. The majority are soil saprobes that degrade dead organic matter using a versatile arsenal of extracellular enzymes including glycoside hydrolases, carbohydrate esterases, polysaccharide lyases and several auxiliary enzymes, which are classified in the CAZy database (Lombard et al., 2014) (http://www.cazy.org). Given the unique habitat of *S. alkalinus*, we compared its major enzyme sets with those of other fungal species. Figure 2 depicts the phylogenomic placement of *S. alkalinus* based on 1,347 single-copy orthologous genes, which confirms the affiliation of the fungus to the *Plectosphaerellaceae*. Each species on the tree is supplemented with its CAZyme profile obtained from genomic data. In addition to the numbers of enzymes in each CAZy class, we combined various CAZy families into groups, based on what substrate they are generally assumed to act upon (for extended data see Supporting Information Table S2). *S. alkalinus* and its sister alkalophilic species *A. alcalophilum* have CAZy patterns markedly different from their closest relatives, plant pathogens such as *Verticillium dahliae*, *V. alfalfae* and *Plectosphaerella cucumerina*, which are rich in CAZymes. The genome of *S. alkalinus* is larger than that of its closest plant-pathogenic relatives within the *Plectosphaerellaceae*, but encodes fewer proteins (Table 1). Notably, the genome of *S. alkalinus* encodes substantially fewer, about fifty per cent, CAZymes than its closest relatives. Reduction in lignin-, cellulose-, hemicellulose-, pectin-degrading and a complete loss of sucrose-degrading GH32 enzymes are evident. Loss of chitin-acting enzymes and modules (families AA11, GH18, CE4, CBM18) is less drastic (Supporting Information Table S2). In fact, retention of A11 family chitinases, but decrease

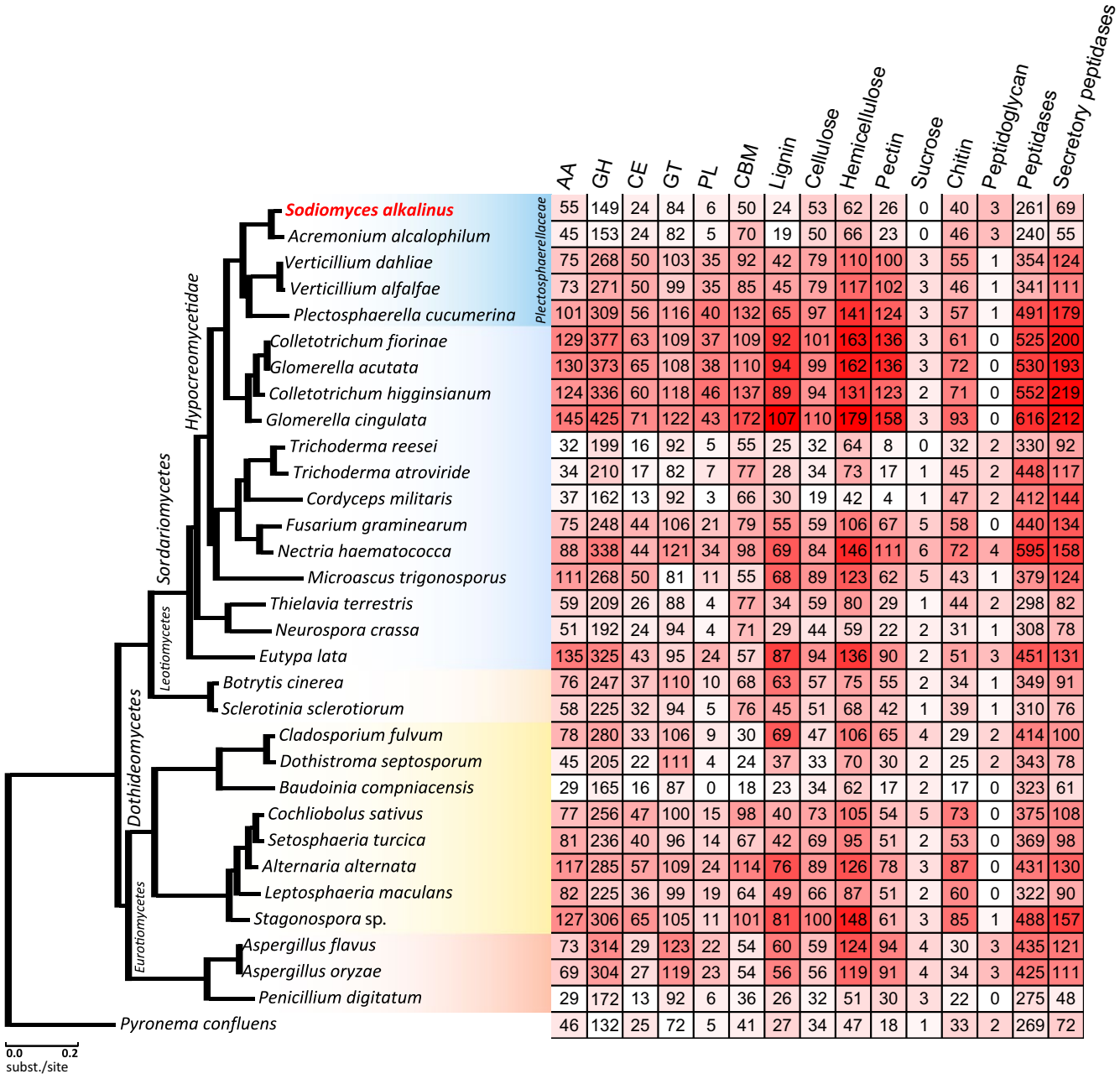

FIGURE 2 Phylogenomic placement of *Sodiomyces alkalinus* among ascomycetous fungi with their CAZyme profiles and peptidases encoded in the genomes. The maximum-likelihood phylogeny was reconstructed based on 1,347 single-copy orthologous proteins. All nodes received maximal support values. Colour intensity (from white to red) indicates fold difference from the average per column [Colour figure can be viewed at wileyonlinelibrary.com]

in GH18 and CE4 members was observed in both alkalophiles (Supporting Information Table S2). Interestingly, we detected acquisition of lysozymes, which are enzymes involved in degradation of peptidoglycan, the major polysaccharide of bacterial cell walls.

To substantiate the in silico conclusions, a synthetic minimal medium (MM, pH 10) was developed for *S. alkalinus* and used to test its growth on different carbon sources (Figure 3). In the process of developing MM, deficiencies for two vitamins were detected in *S. alkalinus*—biotin and thiamine (Supporting Information Figure S2). Since vitamins are exclusively of biogenic origin, an inability to produce them indicates a dependence on other living organisms that synthesize them. Biotin and thiamine were therefore included in the MM for *S. alkalinus*. MM supplemented with 1% glucose supported the best growth of *S. alkalinus* and served as positive control for comparisons with other carbon sources. Xylan from maize resulted in abundant growth. The lack of growth on sucrose confirmed the inability of *S. alkalinus* to utilize sucrose as sole carbon source, as a result of the absence of GH32 family invertases in the genome. Weaker growth occurred on citrus pulp, a mixture of various carbohydrates enriched with pectins, but no growth on apple and citrus pectins confirms the

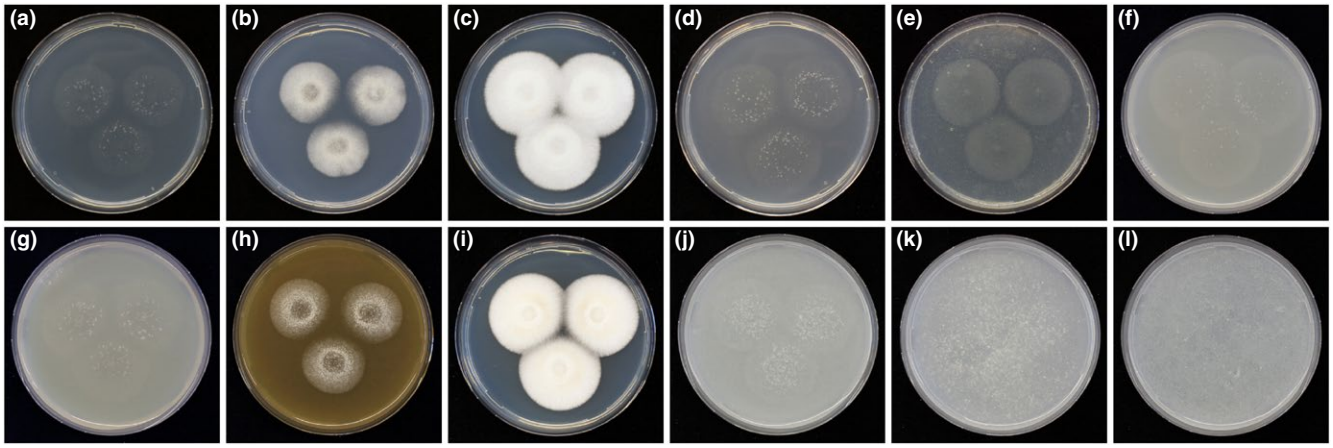

FIGURE 3 Growth of *Sodiomyces alkalinus* on various carbon sources in minimal medium at pH 10 (10-day-old). (a) No carbon source control. (b) 25 mM (=0.45%) D-glucose. (c) 1% D-glucose. (d) 1% sucrose. (e) 1% guar. (f) 1% apple pectin. (g) 1% citrus peel pectin. (h) 1% citrus pulp. (i) 1% xylan from maize. (j) 1% alpha-cellulose. (k) 1% fibrous cellulose. (l) 1% chitin [Colour figure can be viewed at wileyonlinelibrary.com]

poor pectinase arsenal encoded in the genome. Very faint colonies were visible on pure cellulose and chitin, one of the most recalcitrant polysaccharides in nature, suggesting that other carbon sources are required for optimal growth of *S. alkalinus*. The growth profile of *S. alkalinus* in many ways contrasts to both distantly related and closely related plant-degrading (or parasitic) fungi, such as *V. dahliae* and *A. oryzae*, which both grow very well on sucrose and pectins, typical plant-derived substrates (FUNG-GROWTH on-line resource http://www.fung-growth.org/DefaultInfo.aspx?Page=Home).

3.4 | *Sodiomyces alkalinus* produces weak but alkaline-active cellulases and hemicellulases

To obtain experimental evidence for the plant polysaccharides hydrolytic capabilities of *S. alkalinus*, enzyme assays were performed on the crude media extracts after 2 days of growth and compared to neutrophilic distantly related fungus *A. oryzae*. Extracts were obtained from three types of media at pH values of 8 and 10 (pH 6 for *A. oryzae*) that differ nutritionally—wheat bran (rich in arabinoxylans, cellulose, starch and proteins), sugar beet pulp (rich in pectins, cellulose and xyloglucan) and chitin. The enzyme bioassays were performed at pH 6, 8 and 10. The growth on chitin did not induce the secretion of any enzyme activity involved in the degradation of plant material.

Sodiomyces alkalinus grown at pH 10 displayed strong protein secretion after 1 day of incubation on wheat bran and sugar beet pulp, but not in chitin medium. In *A. oryzae*, the sets of proteins did not change over time, as the same banding pattern occurred after both days of growth although there was variation in the intensity of the bands (Supporting Information Figure S3). Especially evident for wheat bran medium, *S. alkalinus* produced different sets of proteins depending both on pH and incubation time, probably indicating a succession of the preferred carbon source utilization over time in the complex wheat bran substrate.

Cellulolytic and hemicellulolytic activities were detected in both *S. alkalinus* and *A. oryzae*, and these activities remained at pH 8 and 10 in *S. alkalinus* (Supporting Information Figures S4 and S5). By contrast, none of the *A. oryzae* enzymes that break down cellulose or hemicellulose were active at pH 10, consistent with its inability to grow at high pH values (Supporting Information Figures S4 and S5). In absolute terms, *A. oryzae* had a higher activity of cellulases and hemicellulases, which suggests either low intrinsic capacity of *S. alkalinus* to degrade cellulose and hemicellulose or their activities are induced at later stages of incubation. Further experiments that follow cellulolytic activities for an extended period are needed to clarify this. The enzyme assays suggest weak (and/or slowly induced) cellulase and hemicellulase activities in *S. alkalinus*, which, however, are capable of acting at high pH.

3.5 | Proteinases in the genome of *Sodiomyces alkalinus*

Along with CAZymes, proteinases can play a major role in nutrition of fungi (Yike, 2011). To address the evolution of proteolytic enzymes in *S. alkalinus*, we searched for the genes that encode putative proteinases and compared them with other fungi. Similarly to CAZymes, we found that the genome of *S. alkalinus* (and sister *A. alcalophilum*) has lost about 40%–55% of its total and secreted proteinases suits when compared with its closest relatives, plant pathogenic *Verticillium* and *Plectosphaerella* species (Figure 2). Most dramatic losses were observed for the serine-type peptidases (S-clan, Rawlings et al., 2014) such as subtilisins (S08), S09 family peptidases and prolyl aminopeptidases of the family S33 (Supporting Information Table S6). Despite overall losses, many peptidases from the C-, M- and T-clans (cysteine, metallopeptidases and threonine peptidases) seem to be retained in *S. alkalinus*, specifically aminopeptidases of the M-clan, such as M01, M18, M24 and secreted peptidases of the M28 family (Supporting Information Table S6). Global losses, coupled with retention of certain groups of proteinases, suggest *S. alkalinus* may have specialized towards a narrow diversity of proteins the fungus encounters in its natural setting of soda lakes.

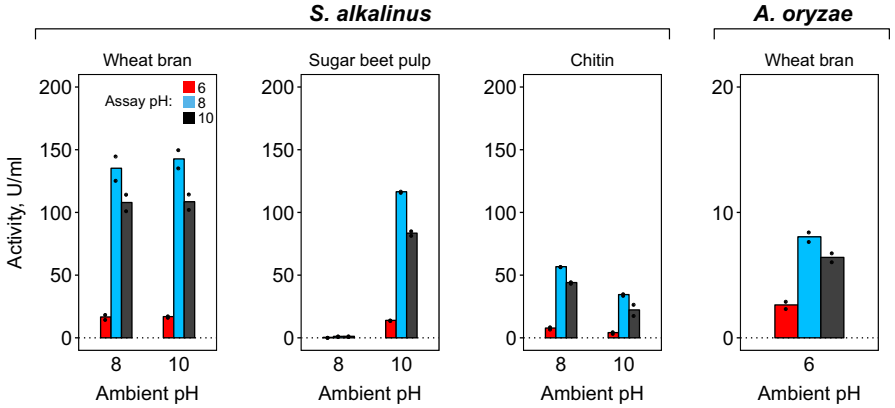

FIGURE 4 Total proteolytic activity in *Sodiomyces alkalinus* and *Aspergillus oryzae* grown on different carbon sources at various pH after 2 days. Bars indicate mean values from two biological replicates (individual replicate values are indicated by dots) [Colour figure can be viewed at wileyonlinelibrary.com]

3.6 | *Sodiomyces alkalinus* produces strong alkaline proteases

To directly assess proteolytic capabilities of *S. alkalinus*, we measured activity of proteases in crude media extracts obtained after growing *S. alkalinus* on various substrates at different pH. The crude media samples of *S. alkalinus* displayed very potent protease activities. The highest induction was observed on wheat bran at pH 8 and 10 (Figure 4). Less protease activity was detected on sugar beet pulp at pH 10, whereas at pH 8 protease activities were not induced on this substrate. Interestingly, the chitin medium also induced proteolytic activity. Total proteolytic activity had its optimum at pH 8 and retained about 80% of its activity at pH 10. *A. oryzae* also produced proteases that were active at high pH, but the activity was 17 times lower than in *S. alkalinus*. The very active and quickly induced peptidases coupled with global losses of peptidase-encoding genes and retention of certain families of peptidases are consistent with the hypothesis of specialization of *S. alkalinus* towards narrow diversity of protein substrates found at soda lakes. Our data support the view that protein-rich small crustaceans (or their eggs), and/or prokaryotes, present in bulk at soda lakes (Figure 1a–c,e), can be the primary food source for *S. alkalinus*. This hypothesis was corroborated by good growth of the fungus on an array of proteinaceous substrates as sole carbon source in the minimal medium, including crude brine shrimp eggs (Supporting Information Figures S6 and S7). The bacterial cell extracts did not yield abundant growth probably due to low substrate concentration, but the difference with no carbon source control could still be seen, indicating *S. alkalinus* can extract energy from bacterial substrates as well.

3.7 | Horizontally transferred prokaryotic genes into *Sodiomyces alkalinus*

Horizontal gene transfer (HGT; also known as lateral gene transfer) is defined as a relocation and stable integration of genetic material between the species' genomes (Doolittle, 1999). It has been shown that fungi are capable of acquiring genes from both prokaryotic and eukaryotic donors (Ros & Hurst, 2009; Slot & Rokas, 2011). Given the extremophilic lifestyle of *S. alkalinus* and the abundance of prokaryotes from where this fungus was isolated, we searched for evidence of HGT into the genome of *S. alkalinus*. The distribution of protein occurrences at various *e*-values among the sampled bacterial (n = 6,321) and fungal (n = 248) predicted proteomes is displayed in Supporting Information Figure S8 with the nine potential HGT cases ($e < 10^{-20}$). All detected HGTs involve structural genes, encoding enzymes that probably have a narrow catalytic mode. None of the acquired genes are unique to *S. alkalinus*, as they were found in other fungi as well, often displaying a mosaic distribution, which suggests multiple gains and losses. The acquired genes are involved in several processes: protein and amino acid metabolism, bacterial cell metabolism and some others. The list is enriched for genes (4 out of 9 genes) that encode proteins involved in amino acid and protein metabolism providing an extra line of evidence that these substrates are relevant to *S. alkalinus*.

3.8 | Bacterial DD-peptidase, racemase and lysozyme in *Sodiomyces alkalinus*

The putative bacterial cell wall-degrading enzymes, such as DD-peptidase and amino acid racemase, are acquired by HGT and characteristic to both alkalophilic fungal species, *S. alkalinus* and *A. alcalophilum*, and are not present in the sister group. To address whether these enzymes may have functional significance in the natural habitat of *S. alkalinus*, the expression of these genes on rich medium at different pHs was quantified. Additionally, we included peptidoglycanase (lysozyme) of the family GH25 into this analysis. All three genes showed strong upregulation (fivefold to 15-fold) at pH 10, compared to acidic pH and the lysozyme GH25 showed an extra peak of upregulation at neutral pH (Supporting Information Figure S9). A DD-peptidase knockout mutant, constructed by homologous recombination-mediated replacement by an antibiotic resistance cassette, did not produce obvious phenotypic alterations and showed no defects on MM at pH 10 when grown on glucose. On the proteinaceous carbon sources, the mutant showed healthier looking edges of the colonies, a phenomenon which we cannot explain at the moment (Supporting Information Figures S6 and S7). Nonetheless, the results suggest that the DD-peptidase is not essential for the performance at high pH on synthetic media supplemented with these substrates. However, when grown with bacterial cell extract as a sole carbon source, the DD-peptidase knockout

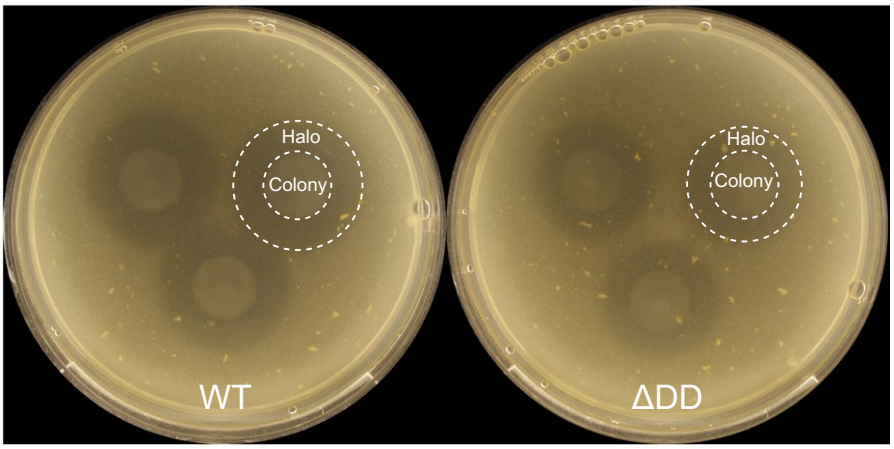

FIGURE 5 *Sodiomyces alkalinus* wild type (strain F11) and the DD-peptidase knockout mutant grown (MM, pH 10, 5-day-old, 28°C, dark) on 0.3% (wet weight) *Escherichia coli* cell extract as a sole carbon source. Note smaller halos around the growing colonies of the mutant [Colour figure can be viewed at wileyonlinelibrary.com]

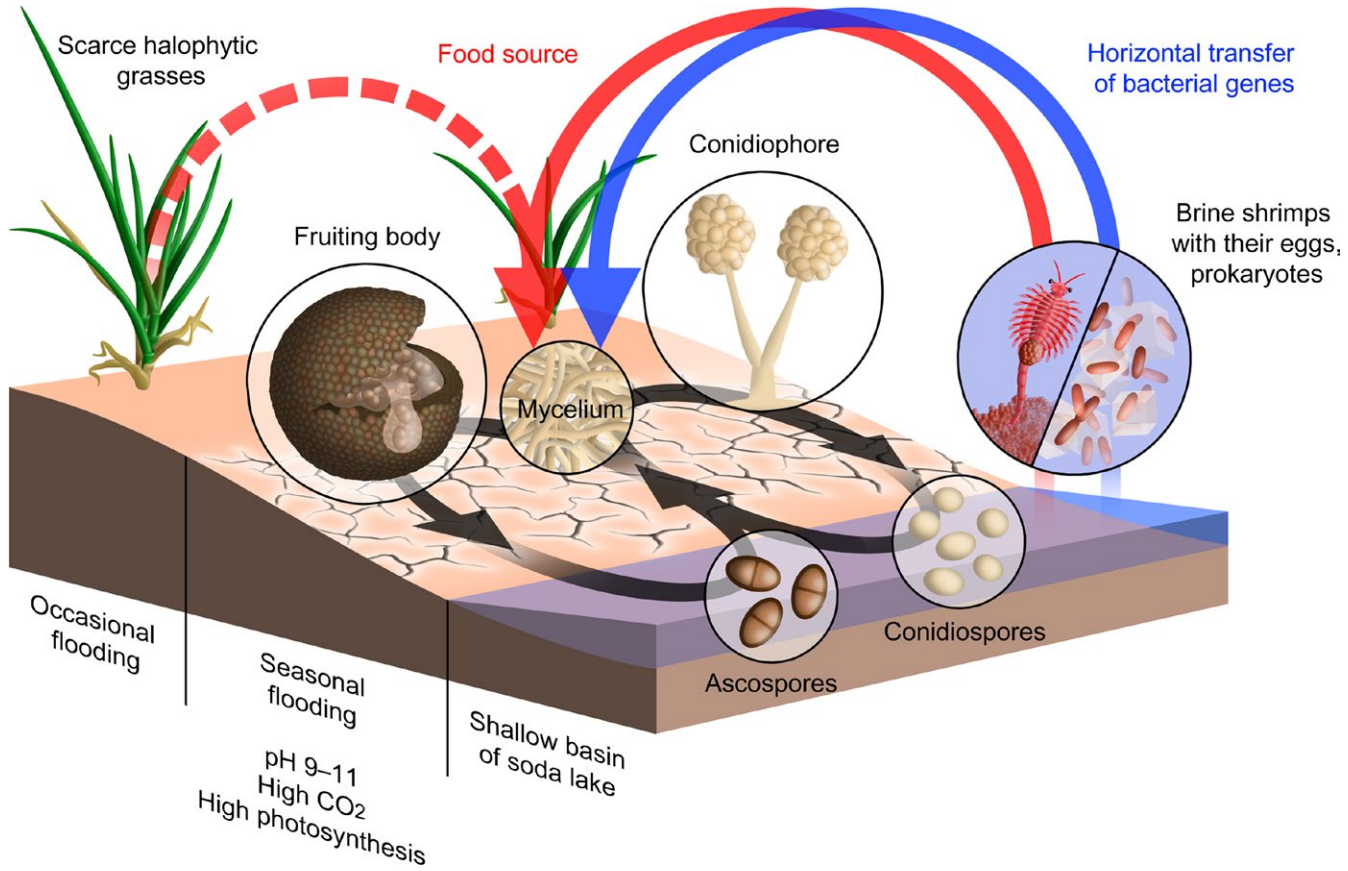

FIGURE 6 Ecology of *Sodiomyces alkalinus* thriving at soda lakes [Colour figure can be viewed at wileyonlinelibrary.com]

mutant produced considerably smaller halos (clearing zones) around the growing colonies compared to the wild type (Figure 5). This indicates that the DD-peptidase in *S. alkalinus* is acting externally and is involved in the degradation of bacterial substrates.

4 | DISCUSSION

Data obtained here from genome analyses, enzyme assays and growth experiments, in addition to our previous studies, enabled us to portray our current understanding of the ecology and life cycle of the alkalophilic ascomycetous fungus *Sodiomyces alkalinus* (Figure 6). The fungus grows at plant-deprived edges of soda lakes and produces slime-covered heads of asexual spores that facilitate their dispersal by water currents during occasional flooding of the lake rim. The sexual fruiting bodies have a similar adaptation by which they crack open forcibly, releasing a mass of sexual spores embedded in a slimy matrix. Occasional scarce plant material can potentially serve as carbon source, but the primary food appears to be protein-rich substrates, such as prokaryotes and brine shrimps with their numerous eggs.

Our genome analysis supports the hypothesis of a dietary preference of the obligate alkalophilic fungus *S. alkalinus* towards nonplant substrates. This hypothesis fits with the absence of plant material, but abundance of brine shrimps and prokaryotes at the edges of soda lakes, the typical habitat of this fungus. Given the relatively short evolutionary distance to related species, plant pathogens such as *Verticillium* and *Plectosphaerella* which have a large number of CAZymes (Klosterman et al., 2011; O'Connell et al., 2012), we conclude that the transition to saprotrophy and concurrent heavy losses of CAZymes in the *S. alkalinus* genome have occurred rapidly upon the colonization of the soda soil niche. Good in vitro growth on xylan from maize as sole carbon source suggests that *S. alkalinus* still can utilize plant material when it is present. Partial retention of cellulolytic and hemicellulolytic activity at pH 10, which was not observed in the neutrophilic fungus *Aspergillus oryzae* nevertheless suggests that alkaline-active plant-degrading enzymes in *S. alkalinus* still provide a selective benefit.

Strong and fast induction of alkaline-active proteases indicates that protein-rich sources are the potential diet of *S. alkalinus*. Brine shrimps with eggs and prokaryotes match this criterion, especially given their high prevalence at soda lakes (Figure 1a–c,e; Grant & Sorokin, 2011; Schneider, Herzig, Koinig, & Sommaruga, 2012). Although alkaline proteases were detected in *A. oryzae*, their relevance remains questionable since the fungus cannot sustain growth at high pH. Conversely, the production of alkaline proteases in *S. alkalinus* seems to be in line with its natural environment. Retention of some aminopeptidase families in the genome of *S. alkalinus* may also be linked to the ecology of the fungus, as these enzymes tend to have their optimum at neutral or alkaline pH (Pel et al., 2007). We did not detect expansions of chitinases in the genome of *S. alkalinus*, but the present arsenal may be sufficient to degrade the exoskeleton of brine shrimps and reach their proteinaceous interior. Gains of lysozymes suggest an enhanced capability for the degradation of bacteria. As bacterial cells on average contain about 50% of proteins by dry weight (Neidhardt, 1963), *S. alkalinus* may have adapted to bacteria as a source of food. The constant physical proximity to brine shrimps with eggs and vitamin-producing prokaryotes at soda soils may also explain vitamin deficiencies that we detected in *S. alkalinus*, since complementation via diet is possible. Very potent proteases of *S. alkalinus* can also indicate a strong need for nitrogen in natural environments of soda lakes, as this element becomes limiting at high pH condition due to losses in the form of ammonia gas (NH_3). Evolution of very active proteases in *S. alkalinus* can be additionally governed by strong competition with prokaryotes for the easily accessible nitrogen-rich protein substrates.

To follow the notion that bacteria could have influenced the evolution of *S. alkalinus* in soda soils, we searched for genes that may have been horizontally acquired from bacteria. The documented cases of HGT into fungi suggest that some ecologically specialized organisms are rich in horizontally transferred genes (e.g., Schönknecht et al., 2013). The exact mechanisms of HGT are poorly understood, but it is clear that physically intimate and continuous association of microorganisms greatly enhance the chances for gene transfer (Keeling & Palmer, 2008). However, the magnitude of HGT into *S. alkalinus* from bacteria was similar to that estimated for other fungi (Marcet-Houben & Gabaldón, 2010), indicating no elevated rate of foreign gene acquisition, as one might expect given the extreme environmental niche of this fungus. Despite average HGT rate, some of the transferred genes seem significant as they encode enzymes that can be beneficial under soda-lake conditions. For instance, the acquired DD-peptidase and amino acid racemase most probably act on bacterial cell wall compounds facilitating their decomposition. In the current study, we were able to confirm the bacteria-degrading function at least for the DD-peptidase. Interestingly, this enzyme was also found in the genome of a social amoeba *Dictyostelium discoideum*, also hypothesized to have entered by HGT, and proposed to facilitate degradation of bacterial cell walls inside the amoeba cells (Eichinger et al., 2005). This is a curious example of how unrelated organisms, a fungus and a slime mould, acquired similar enzymes through HGT to meet similar needs.

The genome of *S. alkalinus* harbours a gene cluster that encodes the core enzymes required for the biosynthesis of beta-lactam antibiotics (e.g., penicillins, cephalosporins; van den Berg et al., 2008). This three-gene cluster (*pcbC*, *pcbAB*, *penDE*) is also present in the sister alkalophilic species *Acremonium alcalophilum*, but not in other closely related fungi. It remains to be demonstrated whether *S. alkalinus* is capable of producing antibiotics in vivo and if such production would provide any benefit to *S. alkalinus* in its natural habitat, as beta-lactams rapidly degrade at high pH (Deshpande, Baheti, & Chatterjee, 2004). We are not excluding the possibility that *S. alkalinus* can produce structurally different antibiotics, which retain stability and activity at high pH, as well as other alkaline metabolites that can be of commercial interest.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

The research was designed by A.A.G-G. and A.J.M.D. Isolation of the fungus and microscopy analyses were preformed by E.N.B. The research was performed by A.A.G-G. and D.L.F. The HGT search was performed by J. v d H. The phylogenetic analysis was performed by C.A.V-J. The genome was sequenced, assembled and annotated by B.M., I-G.C., A.L., C.G.D. and I.V.G. CAZy screen was performed by B.H. Transformation of the fungus was guided by J.A.L. v K. The paper with contributions from D.K.A., A.T., B.H., R. d V., J. A.L. v K., I.V.G. and A.J.M.D. was written by A.A.G-G.

DATA ACCESSIBILITY

DNA sequences: Mycocosm: http://genome.jgi.doe.gov/Sodal1; Genbank: This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession NITX00000000. The version described in this paper is version NITX01000000. Phylogenetic analysis: TreeBASE #22172.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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