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The interaction of *Saccharomyces paradoxus* with its natural competitors on oak bark

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Abstract

The natural history of the model yeast *Saccharomyces cerevisiae* is poorly understood and confounded by domestication. In nature, *S. cerevisiae* and its undomesticated relative *S. paradoxus* are usually found on the bark of oak trees, a habitat very different from wine or other human fermentations. It is unclear whether the oak trees are really the primary habitat for wild yeast, or whether this apparent association is due to biased sampling. We use culturing and high-throughput environmental sequencing to show that *S. paradoxus* is a very rare member of the oak bark microbial community. We find that *S. paradoxus* can grow well on sterile medium made from oak bark, but that its growth is strongly suppressed when the other members of the community are present. We purified a set of twelve common fungal and bacterial species from the oak bark community and tested how each affected the growth of *S. paradoxus* in direct competition on oak bark medium at summer and winter temperatures, identifying both positive and negative interactions. One *Pseudomonas* species produces a diffusible toxin that suppresses *S. paradoxus* as effectively as either the whole set of twelve species together or the complete community present in nonsterilized oak medium. Conversely, one of the twelve species, *Mucilaginibacter sp.*, had the opposite effect and promoted *S. paradoxus* growth at low temperatures. We conclude that, in its natural oak tree habitat, *S. paradoxus* is a rare species whose success depends on the much more abundant microbial species surrounding it.

Keywords: bacteria, competition, ecology, fungi, natural history, *Saccharomyces*

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Introduction

The ability of *Saccharomyces cerevisiae* to transform grape juice into wine has made it an integral component of human culture, and fermentation can be considered an ancient form of biotechnology (Walker 1998). In more recent times, *S. cerevisiae* has also become one of the best-studied laboratory model organisms. In grape juice or in sugar-rich laboratory media, *Saccharomyces* yeasts ferment anaerobically, even when oxygen is available for aerobic respiration. This trait, known as the 'Crabtree effect', has two potential benefits: it allows more rapid (but less energetically efficient) growth than aerobic respiration and it produces toxic ethanol which might inhibit natural competitors. Additionally, ethanol can later be consumed using aerobic respiration, and some of the energy wasted in fermentation can be recovered (Piskur *et al*. 2006; Goddard 2008). It has been proposed that the Crabtree effect evolved when fermentable fruit sugars became abundant following the radiation of angiosperms about 100 million years ago (Piskur *et al*. 2006). Initially, *S. cerevisiae* was thought to be a domesticated species, artificially selected by humans over the last 10 000 years to make alcoholic drinks and to raise bread (Vaughan-Martini & Martini 1995). However, recent phylogenetic analysis reveals the existence of a wild *S. cerevisiae* population in addition to clades associated with both grape wine and rice wine (Fay & Benavides 2005). Wild populations of

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S. cerevisiae have also been identified in primeval forests, far from human influence (Wang *et al*. 2012). Because of its use as a model organism, we know the basic biology of *S. cerevisiae* very well, but its natural history in the wild is almost entirely a mystery (Greig & Leu 2009).

Attempts to study *S. cerevisiae* in the wild are complicated by domestication. Whilst wild populations certainly exist, there is a risk that any individuals isolated from a natural source may have originated from human fermentations, or have recently interbred with domesticated strains. Researchers interested in the ecology and natural history of yeast therefore usually focus on the closest known relative of *S. cerevisiae*, *Saccharomyces paradoxus* (Replansky *et al*. 2008). The two species are phenotypically nearly indistinguishable 'sibling species' (Naumov 1987), sharing the same profiles of assimilation and fermentation of organic compounds (Vaughan-Martini & Martini 1998), suggesting that they evolved in similar environments, and indeed they can be found in sympatry in nature (Sniegowski *et al*. 2002; Sampaio & Gonçalves 2008). However, *S. paradoxus* is not domesticated and is not found in human alcoholic fermentations. *S. paradoxus* is therefore an ideal subject for natural studies, both for its own sake and for inferring the ecology and natural history of wild *S. cerevisiae*, which is expected to be similar (Replansky *et al*. 2008).

Oak trees are widely thought to be the principle natural habitat for *S. paradoxus, S. cerevisiae* and other *Saccharomyces* species (Sniegowski *et al*. 2002; Replansky *et al*. 2008; Sampaio & Gonçalves 2008). Given how well adapted *S. cerevisiae* is to growth at high density in sugar-rich fermenting grape juice, it is surprising that it lives on the relatively nutrient-poor surface of a tree, but nevertheless oaks are the main source of wild yeast for researchers. A recent survey of all *S. paradoxus* strains available from culture collections comprised 65 isolates from oak trees or from soil under oaks, 15 isolates from maple trees and none from other sources (Bozdag & Greig 2014). Despite this apparent association with oak, *Saccharomyces* is not detected in the majority of oak samples. Zhang *et al*. (2010) found *Saccharomyces* in only 24% of New Zealand oak samples. On oaks and surrounding soils in North America, 23% of samples contained *S. cerevisiae* or *S. paradoxus* (Sniegowski *et al*. 2002). *S. paradoxus* occurred in 0%, 10% and 28% of oak samples from southern England, depending on the tree and the sampling date (Koufopanou *et al*. 2006). Repeated sampling of 86 oak trees gave an overall success rate of 8%, but 70% of trees never yielded positive samples (Johnson *et al*. 2004). A survey of different Mediterranean oaks and closely related trees belonging to the *Fagaceae* family found *Saccharomyces* in over 70% of *Quercus pyrenaica* and *Quercus faginea* samples, but the same isolation methods were successful on 45% of *Quercus garryana* samples from Canada and on just 8% of *Quercus robur* samples from Canada and Germany (Sampaio & Gonçalves 2008). A more recent survey of Canadian trees found *S. paradoxus* in 12% of samples coming from trees belonging to the *Fagaceae* family (mostly oak species) and in 4% of maple samples (Charron *et al*. 2014). The low and variable rates of detection of *S. paradoxus* on oak, as well as the contrast between oak and the artificial winemaking environment in which *S. cerevisiae* thrives, raise the possibility that oak is not the main habitat for *S. paradoxus*. We have successfully isolated *S. paradoxus* from oak, larch, beech and spruce trees in the same forest (V. Kowallik & D. Greig, unpublished data). But we know of only one published study that uses a standardized, well-described sampling protocol that allows the abundance of yeast on oak bark to be compared directly to other potential habitats: Sampaio & Gonçalves (2008)found that oaks and other closely related species in the family *Fagaceae* were about three times more likely to yield *Saccharomyces* than other tree species. An unbiased, systematic survey of the abundance of wild yeast across a broader range of potential habitats would be very useful, but many wild yeast researchers want isolates for genetic analysis and so are not motivated to quantify yeast abundance in different habitats, or to sample new habitats in which yeast may not be discovered. Further, the way in which yeast is typically isolated from environmental samples may give little indication of its true abundance.

Inferring the abundance of *S. paradoxus* on oak, or another potential habitat, from the proportion of samples in which it has been detected is problematic. Many authors only need examples of wild yeast for population genetic analysis and so do not report standardized sampling procedures that would permit comparisons between studies. But the main problem is the method by which yeast is isolated from the samples. Typically, an environmental sample, such as a piece of oak bark, is incubated for a few days in a sugar-rich liquid medium, which is sometimes spiked with ethanol. These 'enrichment culture' conditions, which approximate the winemaking process, can favour the growth of Crabtree-positive *Saccharomyces* over other microbial species, allowing the yeast to outcompete them and to dominate the culture so that it can easily be purified. However, the successful isolation of *Saccharomyces* from an enrichment culture gives no indication as to the number of *Saccharomyces* cells in the original sample: the *Saccharomyces* that takes over an enrichment culture may, in principle, be derived from a single yeast cell in the original sample, or they might already be the dominant microbe in the sample. Worse, the failure of an

enrichment culture to yield *Saccharomyces* cannot be taken to mean that it was absent from the sample: it is possible that it was present but was outcompeted by other species. Variation in the proportion of enrichment cultures that successfully yield *Saccharomyces* may therefore reflect variation in the microbial community, rather than in *Saccharomyces* abundance.

Given these uncertainties, we decided to investigate the perceived association between yeast and oak, comparing traditional culturing methods, which suffer from well-known biases (Kell *et al.* 1998) including those discussed above, to culture-free high-throughput environmental sequencing, which suffers from different set of potential biases (e.g. Polz & Cavanaugh 1998; DeSantis *et al.* 2005; Feinstein *et al.* 2009; Delmont *et al.* 2011). As far as we know, this is the first description of this well-known wild yeast habitat using environmental metagenomics, although another recent study used the method to examine the domestic *S. cerevisiae* community in its vineyard habitat (Taylor *et al.* 2014). We first surveyed local oak trees for *S. paradoxus* using an enrichment culture isolation method. We then tested the sensitivity and repeatability of the enrichment assay by attempting to re-isolate known numbers of oak-isolated cells that we 'spiked' into samples that had previously yielded no yeast. We used these data to estimate the average density of *S. paradoxus* on the oak trees we sampled. Finding the density to be low, and given apparent disparity between the winery and the oak environment, we tested how well wild *S. paradoxus* could grow on medium containing only oak bark extract. We found that it grew well in sterile monoculture, but was strongly inhibited by the natural microbial community on the bark. We determined the composition of this community by high-throughput sequencing the microbial metagenome of oak bark from trees containing *S. paradoxus* by enrichment culture. We found no *Saccharomyces* sequences among the samples, confirming both the very low frequency of *S. paradoxus* within the community and the effectiveness of enrichment culturing method for detecting. To investigate the influence of more common microbial species on the success of *S. paradoxus*, we competed a suite of representative members of the microbial community directly with *S. paradoxus* in both solid and liquid oak bark extract medium at summer (26 °C) and winter (5.5 °C) temperatures, finding both positive and negative interactions.

Materials and methods

Isolation of S. paradoxus from oak

Sampling Plön oaks. We sampled a set of 22 oak trees in Plön, northern Germany on 26–28 October 2010 and on 13–14 January 2011 (see Table S1, Supporting information). We used two sampling methods: a sterilized increment hammer which removed ~4-mm-diameter plugs of bark, and sterile cotton buds moistened with sample solution (20% glycerol, 0.1% Tween 20) and rubbed against the bark in a ~4-mm-diameter spot. Samples were taken in groups of four in a 10 cm by 10 cm square; samples in groups of eight were taken directly above and below the vertices of this square. We resampled the same part of each tree on both occasions. Each bark sample (plugs, or the heads of the cotton buds) was vortexed and stored at −80 °C in 2 mL sample solution.

To make enrichment cultures, we first thawed and mixed each sample. A 100 μL of each sample was added to 900 μL of ME (malt extract medium: 5% malt extract, 0.4% lactic acid w/v) and to 900 μL YEPD (1% yeast extract, 2% peptone, 2% glucose). Enrichment cultures were incubated whilst shaking at room temperature for 2 days and tested for the presence of tetrad-forming yeast by spreading 5 μL onto sporulation agar (2% potassium acetate, 0.22% yeast extract, 0.05% glucose, 0.087% complete amino acid mix, 2.5% agar) and incubating for 2 days at room temperature before examining for tetrads using a microscope. Enrichment cultures containing tetrad-forming yeasts were purified by streaking to single colonies. Candidate colonies were tested to see whether they would mate with a haploid *S. paradoxus* tester strain [ho::KMX, lys5-], identifying them as members of the *Saccharomyces* sensu stricto group, and whether the resulting cross could produce viable meiotic spores, identifying them as *S. paradoxus*. This application of the biological species concept to yeast was originally developed by Naumov (1987) and successfully resolved the phenotypically indistinguishable *Saccharomyces* sensu stricto species (Naumov *et al.* 2000, 2010; for a current review of the *Saccharomyces* sensu stricto species see Boynton & Greig 2014).

Determining the sensitivity of ME enrichment culture assay on Plön oaks. We constructed oak bark enrichment cultures containing known numbers of *S. paradoxus* tetrads to test the sensitivity of our enrichment protocol. Tetrads are four haploid spores in an ascus and are built under starvation via meiosis by diploid cells. These spores are dormant and resistant, but germinate into metabolically active haploid gametes when returned to rich medium. As it is likely that we isolate *S. paradoxus* tetrads from oak bark, we decided to use tetrads in the sensitivity assay because other microbes present in the oak wash could outcompete *S. paradoxus* in the time the tetrads need to germinate.

We thawed and mixed together all the sample solutions from January 2011 that did not test positive for

S. paradoxus. We inoculated 120 900 μL ME cultures with 100 μL from this pool of previously negative sample solutions to create 'unspiked' cultures. We tested for the presence of *S. paradoxus* (see 1.1 above). Simultaneously, we tested 264 additional enrichment cultures that were initiated in the same way but which were also 'spiked' with *S. paradoxus*. To do this, we grew eleven strains of *S. paradoxus* (previously isolated from the oak tree: see 1.1 above) overnight in liquid YEPD. We serially diluted the cultures and plated onto YEPD agar plates to determine the density by colony counts. Simultaneously, we diluted to 10^{-6} and used 10 μL to inoculate each 'spiked' culture with the overnight culture, for 24 'spiked' cultures for each of the eleven *S. paradoxus* strains. This resulted in adding an average of 1.7 tetrads (range 1.3–2.8 cells) per spiked culture (see Table S1, Supporting information).

'Spiked' cultures that tested positive therefore contained *S. paradoxus* from either of two sources: from cells already present in the pool of previously negative sample solutions or from the diluted culture cells used to spike the samples. Using a Poisson distribution, we determined the probability that each enrichment culture was spiked with at least one cell. To determine the sensitivity of the assay, we used the following calculation:

a = observed proportion of 'spiked' enrichment cultures testing positive for *S. paradoxus*

b = probability of any enrichment culture testing positive if *S. paradoxus* cells present

c = Poisson-derived probability of a 'spiked' enrichment culture contains at least one cell of added *S. paradoxus*

d = probability that 'unspiked' enrichment culture contains *S. paradoxus*

*b*d is therefore the proportion of unspiked cultures that resulted in testing positive.*

Proportion observed positive culture = Probability of 'spiked' culture containing at least 1 cell * Probability of detecting if cells are present +Probability of 'spiked' cultures not containing at least 1 cell * Probability of detecting if cells are present * probability that 'unspiked' cultures contain cells.

$a = (c * b) + (1 - c) * (b * d)$

Therefore, the probability of detecting any *S. paradoxus* cell, if present, is given by:

$b = ([a - (b* d)] / c) + (b * d)$

We also used a maximum-likelihood model as a second way to estimate the probability of detecting any *S. paradoxus* cell, if present (see Table S1 and Appendix S1, Supporting information).

Sampling Nehmten oaks. We focused on four oak trees (*Quercus robur*) in an old mixed oak/beech/spruce/larch forest in Nehmten, northern Germany for all the following experiments on the oak microbial community. On 9 January 2013, we took 48 samples from each tree using wet cotton swabs but by putting the intact cotton swab directly in 1 mL ME. We streaked the ME samples onto sporulation medium and purified colonies. Because we intended to later use genetic and metagenomic sequencing methods to determine the microbial community (see 3.1 and 3.2, below) of these oak trees, individual *S. paradoxus* isolates were confirmed by Sanger sequencing the ITS region (see 3.1 below), rather than by mating to a tester strain as previously (1.1). There is good agreement between molecular and biological species definitions for *Saccharomyces* sensu stricto species (Naumov *et al.* 2000).

Growth of S. paradoxus on oak nutrients

We made oak bark infusion from the four local oak trees (above, section 1.3) as described by Belotte *et al.* (2003) for soil media preparation. Briefly, we placed 10 g samples of oak bark in sterile tea bags and incubated them in 150 mL sterile water for 24 h at room temperature. We sterilized oak bark infusion using one of three methods: autoclaving, filtration with a 0.22-μm filter and adding 0.4% G418 antibiotic (ENZO Life Sciences). To make oak bark infusion agar, we added 6% sterile agar for a final concentration of 1.5% agar.

For the growth study in sterile and unsterile conditions, we engineered strain Sp-Plön by inserting the KanMX4 cassette into both copies of its HO gene, thereby making it resistant to the G418 antibiotic (Goldstein & McCusker 1999). We grew Sp-Plön in ME overnight and determined the initial density through colony counts. A 30 μL of diluted culture (containing approximately 100 cells) was added to tubes containing 3 mL of the following 5 agar conditions: filter-sterilized oak bark infusion, heat-sterilized oak bark infusion, oak bark infusion with 0.4% G418, unsterilized oak bark infusion and YEPD. The inoculated tubes were incubated at room temperature for seven days. Growth on the surface of the agar in each tube was washed off with 1 mL H_2O, diluted and plated onto G418 agar plates (YEPD supplemented with 0.04% G418), which effectively suppress the growth of all microbes except the resistant strain Sp-Plön. We calculated the number of divisions Sp-Plön went through using the Malthusian parameter ln(final number of cells/initial number of cells)/ln(2) (Lenski *et al.* 1991).

Identification of oak micro-organisms

Identifying common oak micro-organisms by culturing. We serially diluted unsterile oak bark infusions from the four local oak trees (see 2, above), plating 100 μL of

10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions onto full-strength YEPD agar and 10% YEPD agar (0.1% yeast extract, 0.2% peptone, 0.2% glucose). Plates were incubated at room temperature and scored every second day for 10 days. We counted morphologically identical colonies and purified each morphotype by restreaking to new plates of the same nutrient concentration.

DNA was extracted from purified colonies using the MasterPure™ Yeast DNA Purification Kit (Epicentre) according to the manufacturer's instructions. Bacteria were identified by amplifying and Sanger sequencing the 16S region using Universal8f (AGAGTTTGA TCCTGGCTCAG) (Turner *et al.* 1999) and Universal1492r (ACGGCTACCTTGTTACGACTT) primers (Weisburg *et al.* 1991). Fungi were identified by amplifying and sequencing the ITS region using ITS1f (CTTGGTCATTTAGAGGAAGTAA) (Gardes & Bruns 1993) and ITS4 (TCCTCCGCTTATTGATATGC) (White *et al.* 1990). Taxonomy was determined with NCBI BLAST search using Geneious Pro v. 6.1.6. Altogether, we sequenced 48 colonies from YEPD and 36 from 10% YEPD.

High-throughput sequencing of the oak microbiome. We used 454 sequencing of the 16S and ITS regions of microbial communities from four oak trees to determine species composition without culturing biases. The sampling scheme illustrated in Fig. 2 was devised to allow us to compare the microbial community in oak infusion with the total microbial bark community, to examine microbial variation within and between trees and to compare the sampling methods (small whole bark pieces and infusions made out of bark). We made a 300 mL oak infusion from each tree as described in section 2 with 20 g of oak bark. Infusion pellets were created by centrifugation. We also sampled four individual whole pieces of oak bark of approximately 1 g (~1 cm^2 external surface) from Tree 1 and one piece from each of the other three trees. We ground bark pieces and infusion pellets using a bead-beating machine (Precellys® Peqlab). DNA was extracted from all samples using the Soil DNA Kit from Omega Bio-Tek according to the manufacturer's protocol. The resulting ten DNA pellets were resuspended in 20 μl TE buffer and sent to LGC Genomics (GmbH, Berlin, Germany) for amplification of the fungal ITS (ITS1, 5.8S and ITS2) sequences using ITS1f (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) primers (Gardes & Bruns 1993; White *et al.* 1990) and of the bacterial 16S rRNA (V1 to V5) sequences using GM3 (AGAGTTT GATCMTGGC) and 926R (CCGTCAATTCMTTTGAGT TT) primers (Muyzer *et al.* 1995, 1996). PCR conditions were 30 cycles, 30 s 95 °C, 30 s 50 °C and 60 s 72 °C, performed with the polymerase Kapa2G Enzym (Kapa Biosystems). The DNA from each sample was pooled and run on a 1/8 PicoTiterPlate on 454 GS FLX+ Titanium sequencer (Roche). A total of 127 694 ITS and 134 630 16S sequence reads were obtained.

16S sequences were analysed with the software package MOTHUR version 1.31.2 (Schloss *et al.* 2009). Raw reads were processed by trimming the primer sequences and any terminal sections with a mean quality score of 35 using a 50-bp sliding window. Resulting sequences <500 nucleotides were discarded. We removed homopolymers with greater than ten repeats using the trim.seqs command. Reads were randomly sequenced from both the forward and the reverse sites, so we used only the overlapping regions, reducing the average length of the sequences to ~330 bp. Sequences were then aligned to the comprehensive seed database from SILVA (Pruesse *et al.* 2007) downloaded on 29 April 2013. All potentially chimeric sequences were identified and removed with the mothur embedded de novo uchime function (Edgar *et al.* 2011). Sequence classification was performed using the mothur implementation of naive Bayesian classification based on the RDP Classifier (Wang *et al.* 2007) version 9, with a threshold bootstrap value of 70% for each taxonomic level. We removed all sequences classified as 'mitochondria', 'chloroplast', 'archaea', 'eukaryota' or 'unknown'. To allow fair comparisons of the microbial diversity to be made between the ten oak tree samples, we normalized the remaining sequences from every sample to 4000 sequences per sample. We created a distance matrix of aligned sequences and clustered them into operational taxonomic units (OTUs) at 97% sequence similarity using the average neighbour clustering algorithm. We analysed a total of 40 000 bacterial reads, from which 3604 OTUs were detected on 97% identity level.

ITS sequence reads were also quality processed using mothur. Reads were trimmed to a minimum length of 350 bp with the same parameters as described above. Chimeric sequences were detected and removed, and the sequence classification performed as described for the 16S sequences. We removed all sequences classified as 'chloroplast', 'archaea', 'bacteria' or 'unknown'. Each sample was normalized by subsampling to 4000 sequences per sample. For taxonomic classification, we used the same parameters as described for the 16S sequences and the 'dynamic' mothur release from 08.12.2013 of the UNITE database containing 40 679 representative sequences (RepS) and 2 441 reference sequences (RefS). All reference sequence sets represent a nonredundant version of all fungal rDNA ITS sequences in the current UNITE+INSD (International Nucleotide Sequence Databases) release of circa 350 000 sequences clustered approximately at the species level. All OTUs composed of two or more sequences are

referred to as species hypotheses (SHs) (Kõljalg *et al.* 2013). The 'dynamic' representative sequence file contains varying threshold values. Additionally, we used the taxonomy file associated with each individual sequence of the UNITE database with minor reformatting. We did not align our ITS sequences to the database as our ITS sequences are too variable in length for a global alignment. We used instead the 'pairwise.seqs' command in mothur for the OTU-based analysis with ignoring the penalization of the sequence ends. We analysed a total of 40 000 fungal reads, from which 2881 OTUs were detected on 97% identity level.

We calculated Shannon's diversity indices and community similarities to investigate whether samples differ in microbial diversity according to sampling method and/or tree location. All statistical tests were performed with the statistical software R Version 0.98.977 (R Core Team 2012) based on OTUs at 97% similarity. We investigated whether sampling effort accounted for all OTUs present using intersample rarefaction curves produced using mothur with 1000 randomizations (Fig. S1A,B, Supporting information). Within-sample diversity (alpha diversity) was calculated using Shannon's index (Shannon & Weaver 1949) using the VEGAN package in R (Oksanen *et al.* 2013) (Fig. S2A,B, Supporting information). We verified that our data were normally distributed before testing for significant differences in alpha diversity using *t*-tests.

To determine whether the samples are more similar according to sampling method or source tree, we first calculated the Bray–Curtis dissimilarity, a statistic used to quantify the community compositional differences between different sites. The Bray–Curtis dissimilarity is ranged between 0 and 1, where 0 indicates the sites have the same composition (share all the species) and 1 indicates that the sites are completely different and do not share any species. To visualize and interpret these differences, we used the ordination technique nonmetric multidimensional scaling (NMDS), which uses rank orders based on the Bray–Curtis dissimilarity, to collapse the multidimensional dissimilarity matrix into two arbitrary dimensions, so that it can be plotted. Bray–Curtis dissimilarities were calculated using mothur, and NMDS plots were produced using VEGAN package in R. We further tested for significant differences ('location effect') between method, tree and interaction between method and tree using analysis of dissimilarity (ADONIS), implemented in VEGAN. ADONIS is a multidimensional analysis of variance on the Bray–Curtis dissimilarity matrix.

Selection of oak microbes for competition experiments. We selected the twelve most common bacteria and fungi (six isolates each) isolated from the culturing assay (3.1, above) to represent a simplified and experimentally tractable oak community for further competition experiments with *S. paradoxus*. We wanted to determine the abundance of these representative, culturable organisms within the oak bacterial and fungal microbiomes. This is not possible on the taxonomic 454 output because of the well-known problem of classifying a given sequence to the species level, especially for the bacterial 16S rRNA sequences (Fox *et al.* 1992), but also for many fungal ITS sequences (Schoch *et al.* 2012). So we determined the frequency of the specific ITS sequences of each our cultured species within the oak metagenome by building 'minor databases' containing our high-quality forward and reverse Sanger sequences and then searching these databases for the 454 16S or ITS sequences of the cultured species. We used the mothur implementation of naive Bayesian classification based on the RDP Classifier (Wang *et al.* 2007), with a threshold bootstrap value of 90% for the taxonomic level. We also searched the database of ITS reads for the presence of *Saccharomyces* sequences.

Competition experiments between S. paradoxus and representative oak micro-organisms

To test how different species within the oak bark microbiome affect the growth of *S. paradoxus*, we competed it directly against the twelve representative microbial species (see 3.3, above) on both liquid and solid oak infusion medium (see 2, above) at summer (26 °C) and winter (5.5 °C) temperatures.

The *S. paradoxus* strain we used, Sp-Nehmten, was isolated from one of the four focal trees (Tree 2) used for the initial oak infusion growth experiments (see 2, above) and for the analysis of the oak microbiome (see 3, above). We engineered this strain to be resistant to the antibiotic G418 by replacing both its homologous copies of the *HO* gene with the *KanMX4* cassette (Goldstein & McCusker 1999). For liquid experiments at 26 °C, all twelve representative microbes as well as strain Sp-Nehmten were separately grown in 2 mL filter-sterilized oak infusion at 26 °C for seven days with shaking. All cultures were diluted 10^{-2} and 15 μL of the Sp-Nehmten culture was then transferred into 12 new tubes each containing 2 mL filter-sterilized oak infusion, followed by 15 μL of one of each of the twelve microbes. Three further tubes were prepared as controls: one was inoculated with 15 μL of Sp-Nehmten alone, one was inoculated with 15 μL of Sp-Nehmten into unsterilized oak bark medium, and one was inoculated with 15 μL of Sp-Nehmten as well as 15 μL of a mix of all the twelve microbial species. Tubes were mixed, and a sample was serially diluted and plated onto G418 agar plates to yield single colonies, which

were counted to determine the initial number of *S. paradoxus* cells. Tubes were incubated at 26 °C for 4 days with shaking, and then a second sample was taken from each tube and plated on G418 as before to determine the change in density of Sp-Nehmten. For the experiment in liquid at 5.5 °C, the incubations were conducted with shaking for 20 days. For the experiments on solid, tubes containing 2 mL oak infusion combined with 0.5 mL 6% agar were used without shaking, washed and plated (see 2, above). All experiments were independently replicated three times.

Halo assays

We performed halo assays (inhibition assays) to visualize how patches of each of the twelve representative microbes affected the local growth of a lawn of Sp-Nehmten. We grew all representative oak microorganisms individually to saturation in oak infusion as described in 4, above. A 200 μL of an overnight culture of Sp-Nehmten was pelleted by microcentrifugation, resuspended in 1.9 mL of 0.75% soft agar at 45 °C and plated onto a petri dish of YEPD agar. Attempts to visualize haloes on solid oak medium did not work, presumably because the low nutrient level in oak medium does not support large enough colonies, so instead we also compared haloes on 10% YEPD agar, which presumably lies somewhere between laboratory YEPD medium and natural oak bark in its nutrient richness. We then placed 5 μL of each of the twelve cultures at equally spaced intervals around the plate and incubated at 26 °C and 5.5 °C. We examined the plate daily for evidence of growth interference on the Sp-Nehmten lawn.

Results

Isolation of S. paradoxus from oak

Plön oaks. Overall, 14 of 352 primary samples from 22 Plön oak trees yielded wild *S. paradoxus* yeast (Table S1, Supporting information). We found the increment hammer and swab methods each yielded seven isolates. The positive samples came from only 9 of the 22 trees, but we did not find significant differences among the trees in their probability of yielding isolates ($P = 0.0522$, Fisher's exact test on a 22×2 contingency table); however, given the low rate of positive samples, we had limited statistical power to detect tree-to-tree variation. Given how close the *P*-value was to being critical, it seems that a future survey with more samples per tree might reveal tree-to-tree variation in *S. paradoxus* abundance. YEPD yielded 10 positive samples compared to 4 positive samples in ME, but this difference was not significant ($P = 0.175$, Fisher's exact test on a 2×2 contingency table), again as expected given the low power of the test. We decided to use ME for the remainder of this study, as this is one of the standard media for *Saccharomyces* isolation enrichment culture (Naumov *et al.* 1992; Johnson *et al.* 2004) and better prevented growth of filamentous fungi.

Sensitivity of malt extract enrichment culture assay. The 120 'unspiked' enrichment cultures inoculated with 100 μL from a pool of oak bark washes that had not previously yielded *S. paradoxus* yielded 14 further isolates of *S. paradoxus* (11.7%, see Table S1, Supporting information). In contrast, *S. paradoxus* was re-isolated from 243 (92%) of the 264 'spiked' enrichment cultures. We calculated the sensitivity of the assay (e.g. the probability of detecting a spiked cell when present, averaged across the eleven strains used for spiking, see Methods 1.2), as approximately 1, that is we expect all tetrads present to be detected. Using the Poisson distribution, the average estimated sensitivity was 1.1 (range 0.88–1.26) and using the maximum-likelihood model the estimated average sensitivity was 1.39 (range 0.60–2.30). Probabilities higher than 1 can best be explained by error associated with estimating the proportion of cultures with at least 1 spiked yeast tetrad. Also, probabilities higher than 1 could have resulted if tetrads in the 'spiked' cultures formed colonies on solid medium at a lower frequency than germination and growth in the liquid enrichment cultures used in the sensitivity assays.

Nehmten oaks. We focused on four oak trees in Nehmten for the remaining experiments. We took 48 bark samples from each of the four trees. *S. paradoxus* was isolated from three of the samples from Tree 2, two samples from Tree 3 and none of the samples from trees 1 and 4. We found no significant variation among the four Nehmten oak trees in the proportion of positive samples ($P = 0.177$, Fisher's exact test on a 4×2 contingency table).

Growth of S. paradoxus on oak nutrients

Figure 1 shows how the natural microbiota in oak bark affected the growth of Sp-Plön, a wild *S. paradoxus* genetically modified to be resistant to the antibiotic G418. The average number of Sp-Plön cell doublings on nonsterilized oak bark infusion is 3.2, but this increases to 10.9 cell doublings when G418 is added, or when the medium is autoclaved (11.9 cell doublings) or filtered (13 cell doublings). These three sterilization treatments significantly improved growth compared to unsterile medium ($P = 0.0024$, pairwise Wilcoxon rank sum test with Bonferroni correction). Filtered medium supported significantly better growth than either autoclaved

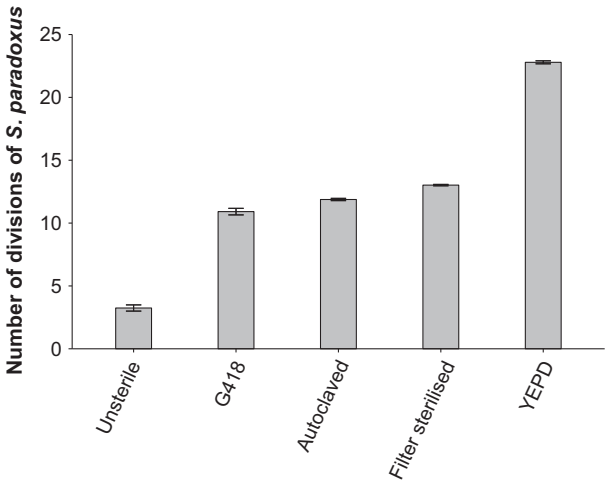

Fig. 1 The effects of sterilization on growth success of *Saccharomyces paradoxus*. Number of divisions of *S. paradoxus* on untreated, G418-amended, autoclaved and filtered solid oak infusion medium and in addition on solid high-sugar laboratory medium at room temperature after seven days. The plotted means are from nine measurements. Error bars indicate the standard errors of the means.

($P = 0.0024$) or G418-treated medium ($P = 0.0024$). Therefore, we used filtration for all further competition assays.

Identification of oak micro-organisms

Culturing. To determine a set of culturable microbial species to represent the oak microbiota, we plated unsterile oak infusion from the four local trees onto YEPD and 10% YEPD agar, purified the most common colony morphs by restreaking and genotyped them by sequencing their 16S rRNA (for bacteria) or ITS rRNA (for fungi) genes. We found the bacterial clones all contained one of 14 different 16S sequences, and the fungi all contained one of six different ITS sequences. Given the difficulty of classifying many microbes down to the species level using only 16S (Fox *et al*. 1992) or ITS (Schoch *et al*. 2012) sequences, we simply named each according to its genus (Fig. S1, Supporting information). We decided to use the six most frequently detected bacterial species (named as *Burkholderia*, *Sphingomonas*, *Massilia*, *Mucilaginibacter, Pseudomonas I and Pseudomonas II*) and all six fungal species (named as *Umbelopsis*, *Rhodotorula*, *Cryptococcus, Penicillium I, Penicillium II and Penicillium III*). Consistent with the low abundance of *S. paradoxus* determined from our enrichment isolation data, we did not find any *Saccharomyces* colonies during this screening.

High-throughput sequencing. We expected that culturing the oak bark microbiota would be highly biased, as culture methods typically underestimate the size and diversity of microbial populations (Kell *et al*. 1998). We therefore used 454 sequencing of the unsterile oak bark infusion, and of whole oak pieces, from the four focal trees to better determine the microbial community.

Figure 2 shows how the trees were sampled and how the different samples varied in microbial composition. NMDS for both bacterial 16S (Fig. 2B; stress = 0.215; $R^2 = 0.785$) and fungal ITS (Fig. 2C; stress = 0.23; $R^2 = 0.817$) sequences showed that our samples clustered according to the method we used to sample the microbial community, rather than according to which tree the samples were taken from. Two-factor ADONIS supported this interpretation. For 16S sequences, we found a significant effect of the sampling method ($R^2 = 0.29$, DF = 1, P-value =0.001), but not of tree ($R^2 = 0.11$, DF = 1, P-value = 0.283) or interaction between method and tree ($R^2 = 0.06$, DF = 1, P-value = 0.724). Likewise for fungal ITS sequences, we found a significant effect of the sampling method ($R^2 = 0.31$, DF = 1, P-value = 0.009), but not of tree ($R^2 = 0.07$, DF = 1, P-value = 0.665) or interaction between method and tree ($R^2 = 0.05$, DF = 1, P-value = 0.938). Rarefaction curves for species richness showed that our sampling did not approach saturation (Fig. S2, Supporting information). Wilcoxon rank sum test showed that average fungal (ITS) alpha diversity was significantly higher in infusions than in whole bark samples ($W = 24$, $P = 0.0095$), but no significant difference was found for the 16S alpha diversity ($W = 13$, $P = 0.914$) (Fig. S3A,B, Supporting information).

Further analysis was conducted by pooling the sequences from the four oak infusion samples. Figure S4 in the supplemental material represents the bacterial (A) and fungal (B) composition of the oak infusion microbiota. We could identify sequences of all 12 of the representative bacteria and fungi selected in the culturing experiments (above) and used in the competition experiments (below). The frequencies of these sequences in the 454 data set of the total microbial communities are shown in Fig. S4 (Supporting information). No sequences corresponding to *Saccharomyces* were found in any sample.

Competition experiments

The ecological interactions between the tested species and *S. paradoxus* strongly depended on temperature. Effects range from killing (*Pseudomonas* I at 5.5 °C), through complete suppression of growth (*Pseudomonas* I at 26 °C), through neutrality (*Pencillium* II at 26 °C), to promotion of growth (*Mucilaginibacter* at 5.5 °C) (Fig. 3). On solid oak bark infusion medium, most species had the same effect on *S. paradoxus* as they did in liquid,

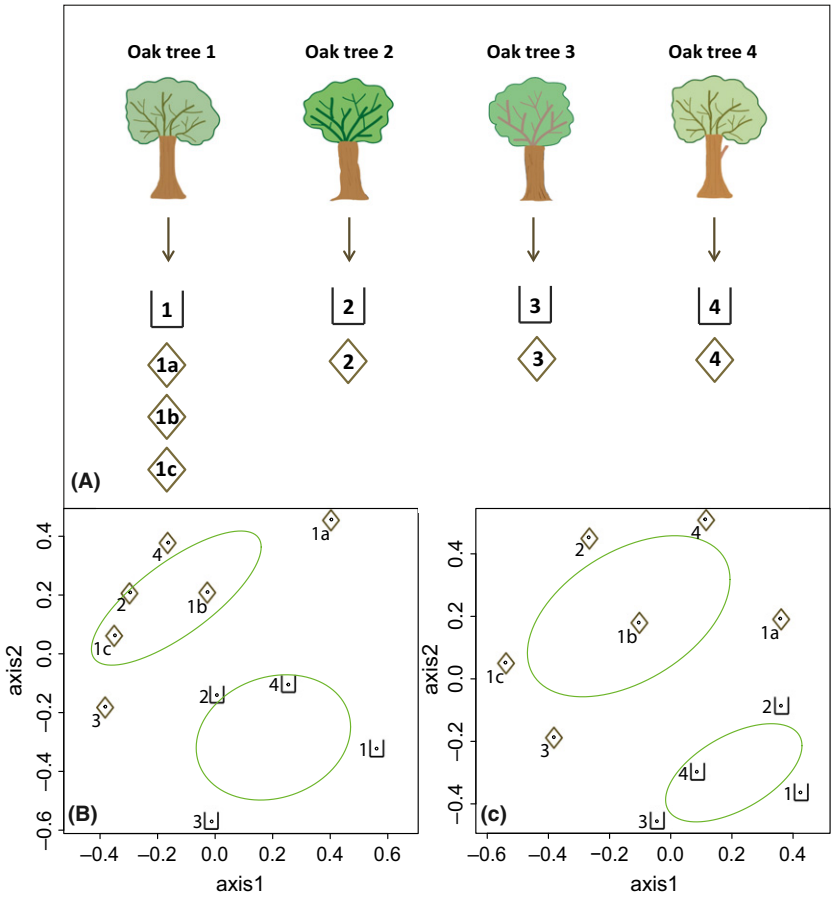

Fig. 2 Sampling scheme (A) From each of four oaks, one liquid infusion was prepared. In addition, three oak pieces were taken from one tree and one piece from each of the other oaks. DNA was extracted from all these samples, and the microbial community was determined by sequencing. Community differences at the 97% sequence OTU identity level were evaluated using NMDS of Bray–Curtis dissimilarities. This produced two-dimensional approximations of the distances between the points for bacterial 16S (B) and fungal ITS (C) sequences. Each point symbolizes a single oak sample's community, and the two symbols indicate the methods used (infusion and piece). The ellipses represent the standard deviation around the centroids of the respective isolation method in NMDS distance.

but overall the effects were less extreme (Fig. S5, Supporting information). An exception is *Pseudomonas* II, which inhibits the yeast much more strongly on solid than in liquid medium (Fig. 3, Fig. S5, Supporting information). Overall, there was a greater variance in effects among the different species at 5.5 °C than at 26 °C, and many organisms showed almost no effect on *S. paradoxus* growth at 26 °C.

Halo assays

To visualize effects that the twelve different microbes had on *S. paradoxus* growth, we performed halo assays on YEPD at 26 °C and 5.5 °C (Fig. 4). The patch containing *Pseudomonas I* produced a large halo in the surrounding lawn of *S. paradoxus* at 5.5 °C, and a smaller one at 26 °C, consistent with its effects on *S. paradoxus* growth in liquid oak infusion medium (Fig. 3). Consistent with its behaviour on solid oak infusion medium (Fig. S5, Supporting information), *Pseudomonas* II also produced a visible halo at 5.5 °C. No other clear halo effects could be seen. On 10% YEPD medium, the halos are hard to see at 5.5 °C because *S. paradoxus* is not growing much, but the halos are bigger compared to YEPD and all competitors form visible colonies; similarly, the halo around *Pseudomonas I* at 5.5 °C is larger than at 26 °C (Fig. S6A,B, Supporting information).

Discussion

Saccharomyces paradoxus is present on oak bark at very low density

Our results confirm that enrichment culturing is a very sensitive method for detecting *S. paradoxus* on oak tree bark. Even though most of our experimental samples were spiked with an average of 1.7 cells of wild *S. paradoxus*, 92% of them scored positive – actually more than predicted, based on the Poisson distribution of spiked cells combined with the background rate of isolation of new strains. We can therefore confirm that the method is so sensitive that a sample testing negative is unlikely to contain *S. paradoxus*. Given this, we can estimate the average density of *S. paradoxus* cells on oak bark. We took 352 4-mm-diameter samples from the 22 Plön oak trees and used 5% of each to inoculate ME cultures, yielding 4 *S. paradoxus* isolates. Thus, the 221 mm^2 of oak bark we tested probably contained only 4 cells,

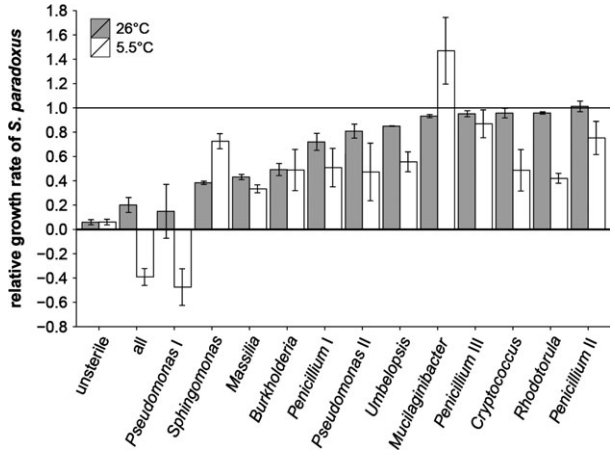

Fig. 3 Growth of *Saccharomyces paradoxus* relative to its growth in the absence of competition in liquid oak infusion medium. We used the initial and final G418 colony counts for each tube to calculate the average number of divisions Sp-Nehmten went through. We standardized each measure by dividing it by the number of divisions Sp-Nehmten went through when growing alone in the sterile control treatment of the same replicate block, to give a measure of the growth of Sp-Nehmten in each treatment relative to how well it can grow alone (the ratio of number of cell divisions; values >1 indicate that growth was promoted, values <1 indicate that growth was suppressed, values <0 indicate a net decline in cell numbers). The bars indicate the effect of the complete community ('unsterile'), the complete set of 12 representative microbes ('all') and the microbes tested individually. Error bars indicate the standard deviation across 3 replicates for each treatment.

suggesting that the density of *S. paradoxus* on the Plön trees is 1.81 cells per square centimetre of oak bark. Although *S. paradoxus* is very rare on oak compared to other microbial species, this low absolute density is nevertheless sufficient to support a substantial local population size. Whittaker & Woodwell (1967) estimate that there is at least 1 m^2 of tree bark above each square metre of forest floor, implying that the overall density of cells is at least $2*10^{10}$ cells per square km of oak forest. The trees in our study were sampled in winter, and it is reasonable to expect that abundance of *S. paradoxus* changes seasonally, perhaps because nutrient availability on the bark changes, or because yeast migrate between oak bark and another habitat, or because yeast are consumed as a food source by insects. Our ability to wash *S. paradoxus* off oak bark into suspension also suggests that rain would carry the yeast from the tree to the leaf litter below. Any changes in seasonal abundance would provide valuable insights into the natural history of *S. paradoxus*. Our data did not show significant tree-to-tree variation in the probability of isolating *S. paradoxus*, either the 22 Plön oaks or the four Nehmten oaks, but the low proportion of positive samples offers us little statistical power to detect such variation.

Some important caveats should be considered. Our population density estimate assumes that the cells were efficiently washed off the bark into suspension. If they adhere to the bark, then the true density could be much higher and placing bark pieces directly into the enrichment medium would yield more isolates. Further, the eleven strains we used for spiking the samples to determine the enrichment culture sensitivity had previously been isolated from the same trees using ME and thus may have genotypes that are amenable to these enrichment conditions. It is possible that more *S. paradoxus* cells were present on the bark samples, but were not selected by the enrichment culture method, either because their genotypes do not give them high fitness in the malt extract enrichment conditions or because they are in the form of spores that do not germinate rapidly enough in the conditions. Our estimate of the *S. paradoxus* population density therefore applies only to nonadhering cells of the type that can be isolated in malt extract medium. Nevertheless, it is noteworthy that the density of such *S. paradoxus* cells on oak bark is this low, especially if oak bark is indeed the primary natural ecological niche of *S. paradoxus*.

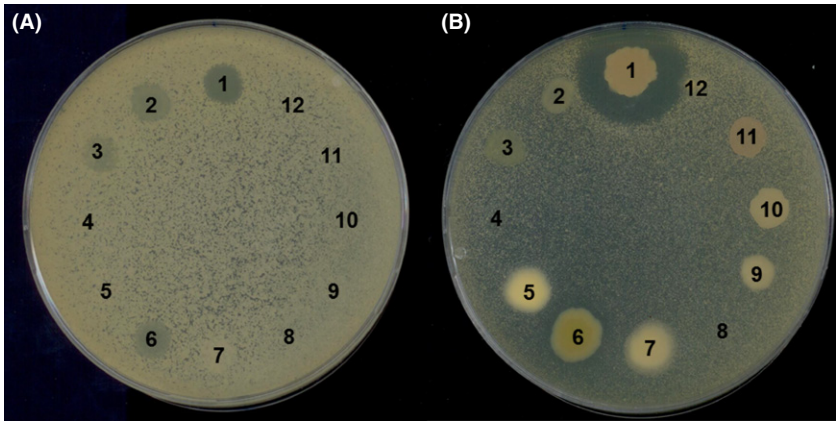

Fig. 4 Halo assays on YEPD at 26 °C (left) and 5.5 °C (right). The numbers indicate the different competitors. 1 = *Pseudomonas I*; 2 = *Sphingomonas*; 3 = *Massilia*; 4 = *Burkholderia*; 5 = *Penicillium I*; 6 = *Pseudomonas* II; 7 = *Umbelopsis*; 8 = *Mucilaginibacter*; 9 = *Penicillium III*; 10 = *Cryptococcus*; 11 = *Rhodotorula*; 12 = *Penicillium II*.

The oak bark community

Both culturing and environmental sequencing are known to produce biased estimates of microbial community size and diversity. Microbes vary greatly in how well they grow in culture; indeed the majority of microbes in typical environmental samples cannot be cultured at all (Amann *et al.* 1995; Kell *et al.* 1998). Our enrichment cultures are a good demonstration of this phenomenon: the offspring of a single, spiked *S. paradoxus* strain dominates all the other microbial species initially present in environmental samples. Next generation sequencing provides some information independent of our ability to culture organisms, but there also exist some biases that can skew relative abundance information, such as DNA extraction bias (DeSantis *et al.* 2005; Feinstein *et al.* 2009; Delmont *et al.* 2011) and PCR bias (Polz & Cavanaugh 1998). Even DNA from dead organisms can be included in sequencing-based estimates of community compositions. Different operon copy numbers of ribosomal rRNA encoding genes lead to a biased abundance picture of organisms; for example, the 16S rRNA copies range from 1 to 15 depending on the bacterial species (Lee *et al.* 2009). Amplification bias according to the sequencing primers is recognized for bacteria (Engelbrektson *et al.* 2010; Schloss *et al.* 2011) as well as fungi. Some primers (e.g. ITS1-F, ITS1 and ITS5) show a bias towards amplification of basidiomycetes, whereas ITS2, ITS3 and ITS4, are biased towards ascomycetes (Bellemain *et al.* 2010). In our study, we tried to control some of these biases using different isolation methods from whole oak bark pieces and from oak bark infusion, and by sequencing in both directions.

Direct plating and culturing of oak bark infusion allowed us to identify 12 abundant microbial species with distinct colony morphologies to use for culture-based experiments (Fig. S1, Supporting information). A total of 454 sequencing confirmed that DNA from all these species except for *Cryptococcus* was present in the oak infusion metagenome (Fig. S4, Supporting information). Sequences from the 12 species were more common in DNA extracted from oak bark infusion than in DNA extracted from whole oak bark pieces, as expected, given that the species were cultured from infusion. Thus, sampling method affects the apparent community composition and indeed, we found significant differences in the sequence compositions between the whole oak bark pieces and the oak bark infusion, but not between one tree and another (Figs 2 and S3, Supporting information).

Although we analysed a total of 40 000 fungal reads, we did not find a single *Saccharomyces* sequence. This supports our enrichment culture results which show that *S. paradoxus* is rare on oak tree bark and thus comprises only a tiny fraction of the total microbial community. Our results are comparable with the first study of the metagenome of vineyard grapes, which finds *Saccharomyces* sequences at a frequency of only one in 20 000 reads (Taylor *et al.* 2014). Considering how reliable winemaking is, it is remarkable to find that *Saccharomyces* species are so rare in both of their habitats, the natural oak tree habitat and even in the domestic winery habitat. Further, because we sequenced ground whole bark pieces as well as cells washed off into an infusion, we can be confident that we have not underestimated the abundance of *S. paradoxus* by missing cells that adhere to or are trapped within the oak bark matrix. The DNA we sequenced came from a total of 80 grams of bark processed by infusion and ~6 g processed by grinding whole bark. Our above estimates of 2–4 *S. paradoxus* cells per cm^2 of bark surface would suggest that the total sequenced sample should contain about 174 cells of *S. paradoxus*. However, the rarefaction curve (Fig. S2, Supporting information) clearly shows that we did not capture all the microbial sequence diversity within the sample. Greater sampling depth would be necessary to detect *S. paradoxus* reliably by environmental sequencing to determine its frequency within the total oak bark community. It is clear though that *S. paradoxus* is sparse on oak bark and a very rare member of the oak bark microbial community, at least in winter at this location in northern Germany.

Yeast growth on oak bark

The low density of *S. paradoxus* on oak bark prompts the question: can yeast actually grow there? Our results show that oak bark can indeed provide nutrients to support substantial growth of wild *S. paradoxus* (Fig. 1), strengthening the case that oak bark may be the natural niche for this species. Other authors have previously grown *S. cerevisiae* in sterilized oak infusion. Bell (2010) measured the effect on growth of systematic gene knockouts, and Giraldo-Perez & Goddard (2013) determined how a homing endonuclease affected growth rate and carrying capacity. As both authors measured growth using optical density, it is unclear how many cell divisions the medium supported, compared to normal laboratory media. However, oak infusion is clearly much poorer in nutrients than the standard laboratory medium YEPD. Glucose, sucrose and fructose are undetectably low in the bark of the oak *Quercus robur* (Sampaio & Gonçalves 2008), and *Saccharomyces* yeasts are not able to utilize cellulose directly as a carbon source (Van Rensburg *et al.* 1998). It is possible that *S. paradoxus* depends on the release of sugars from cellulose digestion by other members of oak bark communities,

and our observation that a *Mucilaginibacter* species promotes the growth of *S. paradoxus* is consistent with this. *Mucilaginibacter* is a member in the family *Sphingobacteriaceae,* and different members of this genus have the ability to hydrolyse organic matter such as xylan, pectin and laminarin (Pankratov *et al*. 2007; Madhaiyan *et al*. 2010; Han *et al*. 2012). *Mucilaginibacter* are also known to produce large amounts of extracellular polysaccharides containing the sugars glucose, galactose, mannose and rhamnose and may thus provide a carbon source for *Saccharomyces* (Urai *et al*. 2008). Whilst the *Mucilaginibacter* species can promote the growth of *S. paradoxus* on oak, most other species we tested (Fig. 3), as well as the community as a whole (Fig. 1), strongly inhibit its growth. One of the representative species we tested, a Pseudomonad, not only inhibited the growth of *S. paradoxus* but actively killed it by producing a toxin (Figs 3 and 4). Species of *Pseudomonas* are known to produce a wide range of different antifungal metabolites such as phenazines and pyrrolnitrin (Leisinger & Margraff 1979). Thus, even within the small number of species we tested, we discovered several forms of ecological interactions with yeast. We know that diversity within communities both promotes and depends upon a wide range of ecological interactions (Boddy & Wimpenny 1992). Some microbes may produce inhibitory peptides, proteins or glycoprotein, such as killer toxins, and enzymes that can lyse the cell walls of other species (Fleet 2003) or making new sugars available through digesting polysaccarides (Deak 2006). We found that the strength of both positive and negative ecological interactions between yeast and members of its community also depend on a simple abiotic factor, temperature, raising the possibility that seasonal changes as well as climatic conditions in different geographic regions could have significant impact on the abundance, range and life history of *S. paradoxus*.

Conclusions

Oak trees are widely considered to be the primary natural habitat for *S. paradoxus*, but it is possible that the special association between yeast and oak is actually an artefact due to various potential sampling biases. Here, we show that *S. paradoxus* is not only scarce on oak bark, but that it is also only a very rare member of the oak bark microbial community. Whilst we find that it can grow on nutrients present in oak bark, its growth is strongly suppressed by much more abundant microbial species. We do not know how the abundance of *S. paradoxus* on oak differs from place to place or from season to season, but it appears that the lifestyle of *S. paradoxus* in this habitat is very different from that of *S. cerevisiae* growing in fermenting wine must. Whilst *S. cerevisiae* rapidly consumes abundant grape sugars, growing to massive density until it dominates the community in a near laboratory-like monoculture, *S. paradoxus* in its oak habitat must eke out a living as a rare scavenger, its fate subject to numerous superior species. Given the physiological similarity between the two 'sibling' species (Vaughan-Martini & Martini 1998), it is probable that *S. cerevisiae* has an evolutionary history similar to that of *S. paradoxus*: indeed, both species can be found on the same oak trees (Sniegowski *et al*. 2002; Sampaio & Gonçalves 2008). It is therefore reasonable to suppose that the results we present here for *S. paradoxus* also apply to wild *S. cerevisiae* populations inhabiting oak.

Whilst our growth assays using the natural yeast community are necessarily highly simplified, they have helped us to identify positive, negative and neutral interactions that can be modulated easily by temperature. We wish to encourage more ecologists to study yeast, and we hope that yeast will become a useful model system for experimental ecology. Although quantifying the natural habitat of *S. paradoxus* is a daunting task, there are great potential benefits, both to yeast geneticists who would wish to better understand the environment in which their species evolved and to ecologists who might seek a tractable and genetically well-characterized model system.

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V.K. and D.G. conceived the study. E.M. did the sampling on oaks in Plön, engineering of Sp-Plön strain and determined the sensitivity of malt extract enrichment culture assay. V.K. did sampling in Nehmten, engineering of Sp-Nehmten strain, culturing, 454 sequence analysis, experiments and statistics. D. G. and V.K. wrote the manuscript. E.M. edited the manuscript.

Data accessibility

The sequences determined in this study have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under Accession no. SRP052992.

Mothur files; Taxonomic output and OTU tables for ITS and 16S sequences: Dryad doi:10.5061/dryad.c8n18.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 The relative abundance of bacteria [b] and fungi [f] colonies on YEPD and 10% YEPD cultured from oak infusions of four trees.

Fig. S2 (A): Rarefaction analysis of OTUs based on 97% identity between bacterial community diversity in oak infusion (blue) and on oak pieces (red).

Fig. S2 (B): Rarefaction analysis of OTUs based on 97% identity between fungal community diversity in oak infusion (blue) and on oak pieces (red).

Fig. S3 (A, B): Alpha diversity based on OTUs at 97% similarity, with the Shannon index for 16S (A) and ITS (B) sequences.

Fig. S4 (A, B): The taxonomic abundance on the genus level of fungi and bacteria in the four oak infusions.

Fig. S5 Growth of *S. paradoxus* relative to its growth in the absence of competition on solid oak infusion medium.

Fig. S6 (A, B): halo assay on 10% YEPD at 26 °C (left) and 5.5 °C (right).

Table S1 Raw sampling and sensitivity data of enrichment culture assay including Poisson distribution and maximum likelihood model results

Appendix S1 Maximum likelihood model description