Social wasps are a *Saccharomyces* mating nest

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The reproductive ecology of *Saccharomyces cerevisiae* is still largely unknown. Recent evidence of interspecific hybridization, high levels of strain heterozygosity, and prion transmission suggest that outbreeding occurs frequently in yeasts. Nevertheless, the place where yeasts mate and recombine in the wild has not been identified. We found that the intestine of social wasps hosts highly outbred *S. cerevisiae* strains as well as a rare *S. cerevisiae*×*S. paradoxus* hybrid. We show that the intestine of *Polistes dominula* social wasps favors the mating of *S. cerevisiae* strains among themselves and with *S. paradoxus* cells by providing a succession of environmental conditions prompting cell sporulation and spores germination. In addition, we prove that heterospecific mating is the only option for European *S. paradoxus* strains to survive in the gut. Taken together, these findings unveil the best hidden secret of yeast ecology, introducing the insect gut as an environmental alcove in which crosses occur, maintaining and generating the diversity of the ascomycetes.

yeasts | *Saccharomyces cerevisiae* | *Saccharomyces paradoxus* | hybrids | social wasps

Since the birth of agriculture, the budding yeast *Saccharomyces cerevisiae* has flourished in human-made fermented products (1). However, insects such as social wasps have been recently shown to host *S. cerevisiae* in their intestine and spread them in the wild (2). For a long time it was agreed that the mating of *S. cerevisiae* spores mostly occurs between spores belonging to the same ascus (self-fertilization/inbreeding) and that outbreeding (mating of spores belonging to different asci) is a very uncommon event (3). However, several recent findings have called this hypothesis into question. Evidence of interspecific hybridization (4–6), a high level of strain heterozygosity (7, 8), and prion transmission (9) have suggested that outbreeding could occur more frequently than previously estimated (9).

We calculated the outbreeding rate from the heterozygosity level at polymorphic sites in three genes selected as able to reproduce the topology generated with the genomes of *S. cerevisiae* (10). Calculation of the outbreeding rate was carried out only on diploid strains for which the sequences of the three genes were available ($n = 34$; *SI Appendix*, Table S1), and was based on a modified model, accounting for the possibility of diploid individuals to derive either from intra-ascus mating or from outcrossing (11). Isolates from wasp gut were more likely to have originated from outbreeding compared with strains isolated from other sources (Fig. 1*A*). There are two possible reasons that could have led to this situation: either wasps prefer to feed on mated yeasts or the insect intestine makes yeast mating more likely.

If wasps prefer to feed on mated yeasts, a possibility suggested by the evidence that fruit flies are differentially attracted by *S. cerevisiae* strains (12), we should have inferred almost the same outbreeding rate for strains isolated from wasp intestines and grapes, although this was not the case (Fig. 1*A*). The second possibility is supported by previous findings reporting dissolution of yeast ascospores in the *Drosophila melanogaster* intestine (13). Nevertheless, this finding by itself does not demonstrate a role of the insects in favoring outbreeding: spore dissolution is necessary but not sufficient for outbreeding to occur. Indeed, dissolved spores should germinate into viable cells and mate with cells from other strains, rather than self diploidizing (14).

Potentially, fruit flies could deliver dissolved spores onto ripe fruits, where the yeasts could meet other strains already present (15), but in this case we should have found the highest outbreeding rate for strains isolated from grape skin, rather than for those isolated from wasp gut. To justify the high outbreeding rate calculated for wasp isolates, several capital conditions should have been satisfied, either before wasp uptake or during the passage through the insect intestine: the simultaneous presence of different strains in the same environment and environmental changes allowing yeast cell sporulation, spore germination, and mating outside the ascus. The alimentary canal of adult insects, furnishing chemical–physical characteristics varying from the foregut to the hindgut (i.e., pH and oxygen availability) (16) and hosting for prolonged periods yeast cells collected from the wide range of environments where the wasps feed (2), is a good candidate for satisfying these conditions and allowing yeast mating. We tested the hypothesis of *S. cerevisiae* (Sce) crossing within the insect intestine by feeding *Polistes dominula* wasps five different Sce strains, each one recognizable by auxotrophic profile and genetic markers (*SI Appendix*, Tables S2 and S3). To prevent external introduction of yeast strains or exchange between insects, the wasps were kept individually separated in natural hibernation conditions. The Sce populations present within the intestine were investigated after a hibernation period lasting at least 2 mo (60 d) and compared with the population observable by

Significance

Despite the widespread interest on *Saccharomyces cerevisiae*, its wild lifestyle is far from being completely understood, with one of the most resounding examples being its sexual attitude. We show that the intestine of social wasps favors the mating of *Saccharomyces* strains by providing a succession of environmental conditions prompting sporulation and germination. We also demonstrate that the insect intestine favors hybridization of *S. cerevisiae* and *Saccharomyces paradoxus*. Although *S. paradoxus* survives in wild environments and rarely mates with *S. cerevisiae*, we discover that two European *S. paradoxus* strains cannot survive the wasp's intestinal environment but can be rescued through interspecific hybridization with *S. cerevisiae*. These findings are introducing insects as environmental alcoves in which yeast cells can meet and mate.

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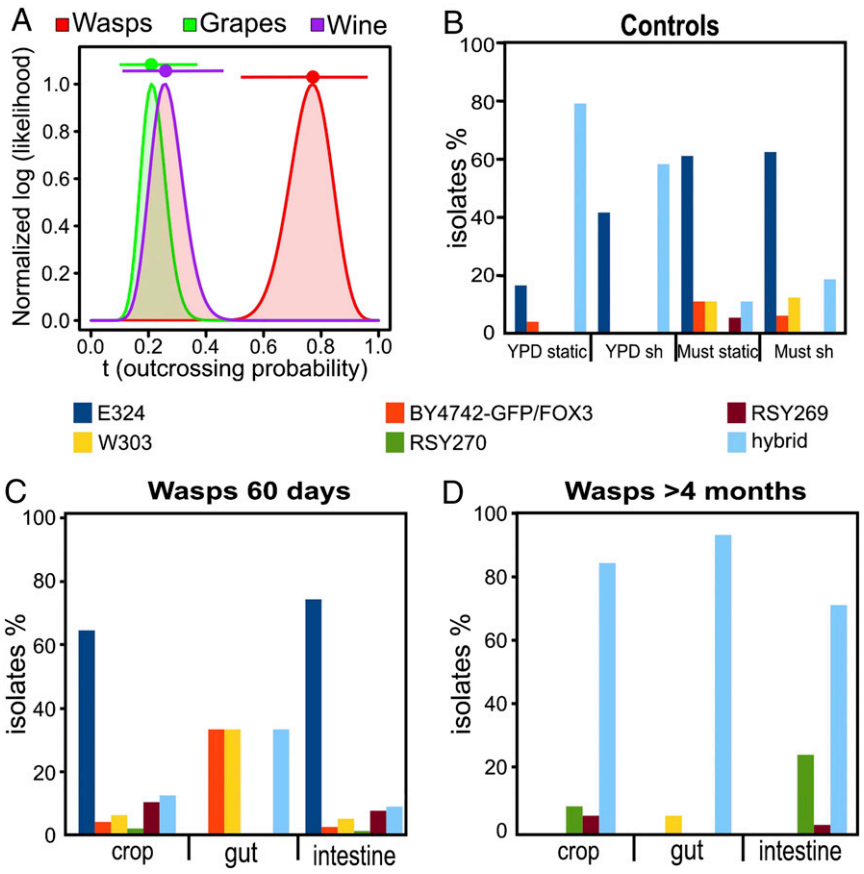

Fig. 1. *Saccharomyces cerevisiae* inbreeding rate and mating. (*A*) Inbreeding rate inferred with a R script based on a modified model, accounting for the possibility of diploid individuals to derive either from intra-ascus mating or from random outcrossing (11). The analysis was carried out on the heterozygous sites in three genes whose SNPs sequences are able to reconstruct the topology of the tree generated from whole-genome sequences (10). Only diploid strains for which the sequences of the three genes were available were included in the analysis. (*B*) Identification of strains isolated from control treatments: YPD and sterile must inoculated with BY4742 GFP/FOX3, W303, E324, RSY269, RSY270. Next, 10^8 cells of a mixture of these strains (added in equal amounts) were kept in agitated or static conditions for more than 4 mo (126 d). Percentages indicate the fraction of each strain represented within the respective specimen. (*C*) Identification of strains isolated from *Polistes* spp intestine ($n = 8$) inoculated with 10^8 cells, in total, of the mix of yeasts used in the control experiments (added in equal amounts). Wasps were hibernated for 2 mo (60 d). Percentages indicate the fraction of each strain represented within the respective specimen. (*D*) Identification of strains isolated from *Polistes* spp intestine ($n = 24$), inoculated with the mix of yeasts used in the other feeding experiments. Wasps were hibernated for 4 mo (119–126 d). Percentages indicate the fraction of each strain represented within the respective specimen. Crop and gut localization in the wasp abdomen are shown in Fig. 2*A*; "intestine" refers to the whole intestinal tract.

maintaining the same initial set of strains in two control conditions. Controls were carried out in the standard conditions used to allow Sce to grow and mate (yeast peptone-dextrose; YPD) and in sterilized wine must, reproducing the environment in which several different Sce strains can live simultaneously (17). Strains were inspected by dissecting the wasps and immediately plating intestine contents on solid YPD after serial dilutions to obtain an average of 10 yeast colonies per plate, to prevent eventual mating occurring outside the wasp intestine. After 2 mo of wasp hibernation, the percentage of outbred Sce strains within the insect gut was comparable to that in must fermentation (12.50%, 33.33%, and 10.85% of crop, gut, and entire intestine Sce isolates, respectively; 11.11% must static, 18.75% must shaking; Fig. 1*C*), and 30.93% of wasps bore Sce hybrids (*SI Appendix*, Fig. S1). Surprisingly, after a hibernation period of more than 4 mo (119–126 d), the amount of outbred Sce strains occurring within the insect intestine reached the level obtained in the most favorable conditions (90.00%, 88.89%, and 87.30% of crop, gut, and intestine Sce isolates, respectively; 79.17% and 58.33% of YPD static and YPD shaking isolates respectively; Fig. 1*D*). *S. cerevisiae* can outbreed within the insect intestine, and this seems to be the best currently known condition for this event to occur (*SI Appendix*, Fig. S2 and Table S4).

The pool of Sce yeasts we fed the wasps encompassed *IME1* (inducer of meiosis) deletion mutants, unable to sporulate (E324) (18), and *UME6* (unscheduled meiotic gene expression) deletion mutants, whose spores are unable to germinate (RSY270) (19). No hybrids resulting from the mating of either E324 or RSY270 were expected to be found, as these two steps in the cell cycle are essential for diploid yeasts to mate, although ScexSce hybrids having the E324 genome were isolated both from the crop and the gut (*SI Appendix*, Fig. S3*A*), especially after a long period of wasp hibernation (*SI Appendix*, Fig. S3*B*). Sporulation of *IME1* deletion strains can only occur if cells adopt an atypical sporulation pathway in alkalized environments (20). The insect intestine showed a gradual pH increase from the anterior toward the posterior end (16, 20), thus providing a favorable environment for mating, even for *IME1* deletion and low-sporulating Sce strains. The latter probably sporulate in the alkaline trait of the intestine (the foregut, as indicated by the presence of E324 hybrids) and then germinate (as indicated by the lack of RSY270 hybrids). This suggests that spores formed in the wasps crop are liberated from their ascus while passing to the hindgut, and then germinate in an environment where the probability of the neighboring spore of being self is equal or less statistically frequent than nonself. To complete all these stages, it is necessary that

yeast cells inhabit the insect intestine for a sufficiently long period (*SI Appendix*, Fig. S1*C*), as it occurs while social wasps and other insects are hibernating.

Among the strains isolated from the intestine of social wasps caught in nature ($n = 16$), two different interspecific hybrids were found (*S. cerevisiae*×*S. uvarum*, Sce×Suva; and *S. cerevisiae*×*S. paradoxus*, Sce×Spa; *SI Appendix*, Table S1). Sce×Suva often generates during must fermentation (21), and it could have been picked up by the wasps when they were feeding around wineries or on rotting fruit. Although Sce and Spa strains are often isolated simultaneously from the same source (*SI Appendix*, Fig. S4), they rarely mate in natural settings, and the described hybrids have mostly been generated in vitro (21–24). The isolation of such an uncommon interspecific hybrid reinforces the evidence that social wasp gut conditions can favor yeast mating. To verify this, *P. dominula* wasps were fed an equal amount of five different strains of Sce and two Spa strains (a haploid and a diploid strain), all identifiable by specific genetic markers. More than one third of wasps fed with yeasts bore cells intraspecific hybrids and Sce×Spa hybrids (34.39% and 36.73%, respectively; *SI Appendix*, Fig. S5). After 2 mo of insect hibernation, Sce×Spa hybrids were found within the insect intestine (14.29% of the intestine content), but they increased considerably after longer hibernation (mean of 28.40% and 68.39% after 119 and 126 d of hibernation, respectively; *SI Appendix*, Fig. S6). After 2 mo of wasp hibernation, both Sce×Sce and Sce×Spa cells were preferentially isolated from the posterior part of the intestine (gut, Fig. 2*A*), thus confirming the previous results on the ability of the E324 strain to sporulate in alkaline environments. Sce pure strains (such as those fed to the wasps) disappeared after the longest period of wasp hibernation, suggesting outbred and hybrid cells are fitter than the studied pure Sce strains during long periods within the wasp intestine (Fig. 2*A*). Although these results could be enhanced by the use of auxotrophic strains, as complementation of strain auxotrophy through mating could represent a fitness advantage, the findings still support the high outbreeding rate calculated for wasp isolates.

The two Spa strains, completely prototrophic, had already disappeared from the insect intestine at the first point (57 d hibernation) (Fig. 2*B*). Only the haploid Spa strain was found, and only as a Sce×Spa hybrid. These results show that *S. paradoxus* can survive within the insect intestine with a higher success as an interspecies hybrid than as "pure species" and is supported by the fact that it has never been found in insect guts. The same number of inter- and intraspecific hybrids were isolated, thus nullifying, at least in wasp gut, the supposed preference of *S. cerevisiae* for intraspecific hybridization (25). The strain unable to sporulate, RSY270, was never isolated as a hybrid from the insect intestines. This strain and the corresponding wild-type (RSY269) were recovered even after the longer hibernation period (*SI Appendix*, Fig. S5), suggesting a high tolerance of these strains to the intestinal environment. Nevertheless, although the wild-type RSY269 strain was found as hybrid (both inter- and intraspecies), the deletion strain was not isolated as

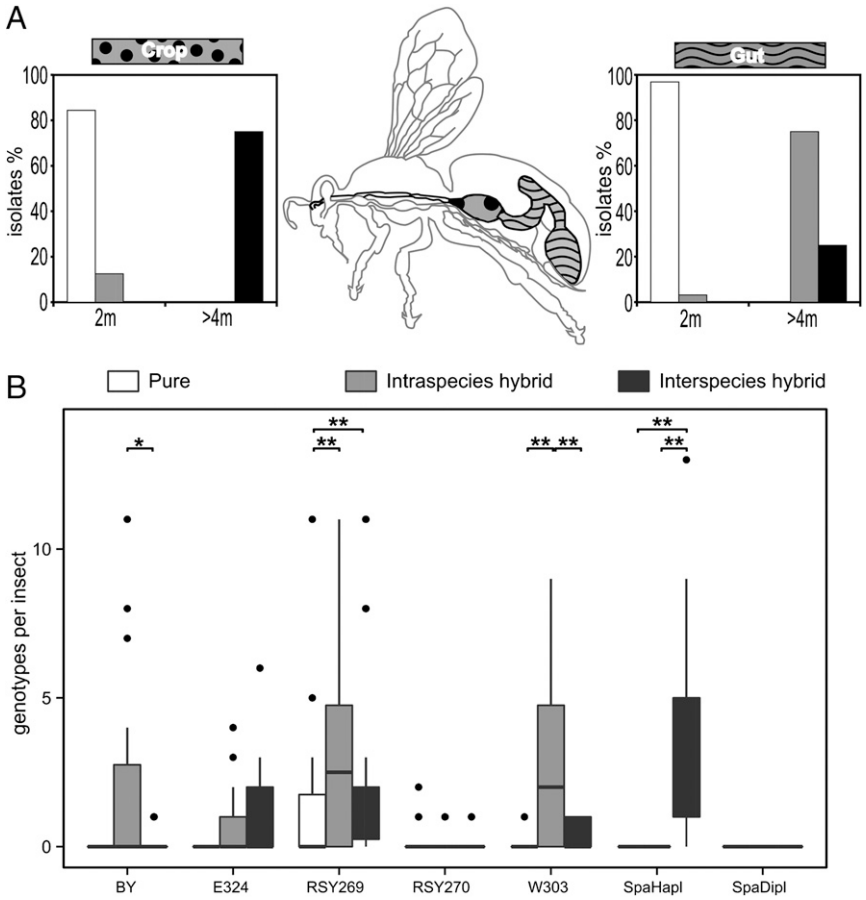

Fig. 2. *Saccharomyces cerevisiae* and *S. paradoxus* mating within the wasp intestine. (*A*) Distribution of strains bearing the E324 genotype within wasp intestine parts. 2m, 2 mo wasp hibernation; >4m: more than 4 mo hibernation. (*B*) Composition of the strains isolated from wasp intestine after more than 4 mo (119–126 d) controlled hibernation. Values represent the number of strains per insect intestine having the relative genetic background (the genetic background was identified using auxotrophic and genetic markers). $*P < 0.05$, $**P < 0.01$ Wilcoxon test. Outliers are cases laying outside 1.5 times the interquartile range above the upper quartile and below the lower quartile.

hybrid (*SI Appendix*, Fig. S7). This could indicate that the strains face several sporulation-germination cycles while residing in the insect intestines. As inoculated diploid cells were able to hybridize (i.e., E324 and W303), they should have sporulated and then germinated before mating. Both RSY strains may be able to form hybrids, but the isolation of hybrids of RSY269 only indicates that after the mating, another sporulation event occurred within the insect intestines. Heterozygous *UME6* deletion strains, generated by the mating of RSY269 with one of the other strains inoculated to the wasps, could also have sporulated, but we were not able to isolate them because of their reduced germination ability (19).

We can conclude that the social wasp intestine provides *S. cerevisiae* with an environment favoring outbreeding and likely represents a highly selective environment for its "wild cousin" *S. paradoxus*. The Spa strains used were selected to represent the European *S. paradoxus* population (22). We are aware that the use of two *S. paradoxus* strains from the European clade of the *paradoxus* tree (22) could potentially lead to genotype-specific observations, favored by peculiar adaptations to a given environment (26). However, the common "mittel-European" geographic origin of the yeast strains and the wasps used in this study is coherent with the aim to mimic the ecological settings under investigation. Even if the evidence that the tested *S. paradoxus* were able to survive in the insect intestine only as hybrids with *S. cerevisiae* could be biased by the genotype of the selected strains, the lack of reports on the isolation of pure Spa strains from this environment in natural settings could indicate a general lower fitness of the pure strains compared with the interspecific hybrids. Further studies with strains and insects from other geographical areas will be needed to generalize our findings. However, the reported ability of the yeast strains studied here to sporulate, germinate, and mate, giving rise to intra- and interspecific hybrids, represents a milestone in our knowledge regarding the natural history of *Saccharomyces*, elucidating how their evolution depends on interaction with insects, favoring the formation of new genetic combinations. Ultimately, the high rate of outbreeding in insect intestine can provide a key to explaining the growing evidence for the existence of a high level of diversity and mosaicism in yeast populations (27). This diversity could be instrumental to surviving and thriving both in the wasps' intestine and in the novel environmental microenvironments where insect-borne *Saccharomyces* cells will land, thanks to the hybrid fitness phenomenon (28, 29).

Finally, the direct link between social insects and the yeast species biodiversity is relevant to human industry, as the genetic diversity generated in the wasp's gut could favor adaptation to the ever-changing fermentative environment, as demonstrated by the evidence that several of the most successful industrial strains are interspecific hybrids (30). Thus, preserving the treasure potentially hidden in the gut of vineyard wasps could be relevant from both the ecological and biotechnological standpoints.

Materials and Methods

Determination of the Inbreeding of *S. cerevisiae* Strains. The 34 *S. cerevisiae* isolates analyzed in this study in terms of inbreeding rate are listed in *SI Appendix*, Table S1. This set of strains encompasses strains isolated from natural (wasp intestine, grapes), industrial (wine fermentation), and human-related (human feces) environments. Population genetics analysis was carried out on the polymorphic sites of three genes whose sequences are able to reproduce the structure of the tree constructed on the whole-genome sequences of *S. cerevisiae* strains, *EXO5*, *URN1*, and *IRC8* (10). Only sequences of (at least) diploid *S. cerevisiae* strains were included in the study, downloaded from SGD (Saccharomyces Genome Database) and from NCBI (National Centre for Biotechnology Information) (last access, May 2015). SNPs were identified by multiple alignment, and a unique sequence was generated by concatenating them, as previously described (10). Heterozygous sites were maintained as ambiguous bases. The outbreeding rate was estimated for *S. cerevisiae* strains through an R-script calculating the Johnson's outbreeding rate (11). Results were compared with inbreeding levels calculated with the fstat() function of the adegenet R package (31) and with FSTAT (32) and RMES (33). The model proposed by Johnson and coworkers is considered to be the most suitable for calculating the inbreeding rate of microorganisms such as *S. cerevisiae* and *S. paradoxus*, as it takes into account the possibility of diploid individuals deriving either from intra-ascus mating or from random outcrossing. We thus referred the text to the results of this calculation, even considering that the inbreeding levels calculated with FSTAT, RMES, and the fstat() function of the adegenet R package gave similar results. Differences between strains, grouped according to the isolation source (wasp intestines, grapes, wines), were evaluated by applying the Wilcoxon test [wilcox.test() function of the stats R package (34)].

In Vivo Experiments: Insect Feeding and Dissection, Yeast Isolation, and Identification. Wasps were inoculated with two different types of strain mixes. The first, encompassing only *S. cerevisiae* strains, was made up of equal amounts of W303, BY4742 FOX3/GFP, E324 (17), RSY269, and RSY270 (18) strains (*SI Appendix*, Table S2). The second mix of strains encompassed both *S. cerevisiae* (the same as the first mix) and *S. paradoxus* strains CBS432 and Q95.3 (23). Before inoculation, the strains were grown separately overnight in rich medium [YPD: 1% (wt/vol) yeast extract, 2% (wt/vol) peptone, 2% (wt/vol) dextrose]. After the overnight preculture, strains were counted through a Bürker chamber and were diluted in sterile water to obtain a 6× (for the mix encompassing *S. cerevisiae* strains only) or 8× (for the mix encompassing *S. cerevisiae* and *S. paradoxus* strains) cell suspension. The mixes were prepared just before wasp feeding to avoid eventual mating of strains before inoculation. Each yeast mix, having a final cell concentration of 10^{10} cells/mL, contained the same amount of each strain (1.5×10^9 cells/mL and 1.25×10^9 cells/mL for the mix made up only of *S. cerevisiae* and for the mix made up of *S. cerevisiae* and *S. paradoxus*, respectively). To make the yeast solution more appealing to wasps, the mixes were supplemented with small amounts of sugar (sucrose, <0.1% wt/vol). Each wasp was fed individually: the wasp was collected with tweezers and 10 μL cell mix (containing 10^8 yeast cells) was injected directly into its mouth with a pipette, both to avoid cell dispersion and to ensure the ingestion of all of the cells.

Wasp Treatment and Dissection. Healthy and parasite-infected wasps were caught before hibernation and were inoculated with 10^8 yeast cells suspended in water, with the addition of sugar. Control wasps were fed with sugared water. Treated wasps were kept separate from each other in 6-well plates at seasonal temperatures. Wasp survival was scored twice a week. After 2 or 4 hibernation months (for *S. cerevisiae* only or for *S. paradoxus* and *S. cerevisiae* mixes, respectively), the surviving wasps were washed twice with water to avoid external contamination and then dissected in sterile conditions in sterile Petri dishes, using sterile clamps under a stereomicroscope. When possible, crop and gut were analyzed separately. Crop and gut contents were suspended in 100 μL sterile water and plated at low density (yielding a maximum of 10 colonies per plate) on rich solid medium (YPD, 2% Agar) supplemented with penicillin and streptomycin to prevent bacterial growth (35). Plates were kept at 27 °C for at least 2 d, and then colonies were further isolated. *Saccharomyces* sensu stricto strains were identified by means of ITS1-4 RFLP (4); *S. cerevisiae* and *S. paradoxus* strains were identified by means of *MET2* RFLP (36). Isolated *S. cerevisiae* strains were identified by means of their auxotrophies, by spotting 5 μL water cell suspension on YNB [0.67% (wt/vol) YNB with ammonium sulfate, without amino acids, 2% (wt/vol) dextrose, 2% (wt/vol) agar] lacking specific amino acids or bases (*SI Appendix*, Table S2) and confirmed by means of strain-specific DNA amplification (*SI Appendix*, Table S3).

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