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The most heat-resistant conidia observed to date are formed by distinct strains of *Paecilomyces variotii*

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Summary

Fungi colonize habitats by means of spores. These cells are stress-resistant compared with growing fungal cells. Fungal conidia, asexual spores, formed by cosmopolitan fungal genera like *Penicillium*, *Aspergillus* and *Peacilomyces* are dispersed by air. They are present in places where food products are stored and as a result, they cause food spoilage. Here, we determined the heterogeneity of heat resistance of conidia between and within strains of *Paecilomyces variotii*, a spoiler of foods such as margarine, fruit juices, canned fruits and non-carbonized sodas. Out of 108 strains, 31 isolates showed a conidial survival >10% after a 10-min-heat treatment at 59°C. Three strains with different conidial heat resistance were selected for further phenotyping. Conidia of DTO 212-C5 and DTO 032-I3 showed 0.3% and 2.6% survival in the screening respectively, while survival of DTO 217-A2 conidia was >10%. The decimal reduction times of these strains at 60°C (D_{60} value) were 3.7 ± 0.08, 5.5 ± 0.35 and 22.9 ± 2.00 min respectively. Further in-depth analysis revealed that the three strains showed differences in morphology, spore size distributions, compatible solute compositions and growth under salt stress. Conidia of DTO 217-A2 are the most heat-resistant reported so far. The ecological consequences of this heterogeneity of resistance, including food spoilage, are discussed.

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Introduction

Fungi produce numerous spores that are distributed by water, air and other vectors (as insects, soil movements, etc.). Airborne spores are present in every cubic meter of air and cause fungal colonization in all environments. As reviewed recently, conidia vary in several parameters (Dijksterhuis, 2017). For instance, *Aspergillus niger* conidia of 5 days old showed higher trehalose content compared with spores of younger age (Teertstra *et al*., 2017). Conidia of *Aspergillus fumigatus* showed differences in pigmentation, compatible solute content and oxidative- and heat-resistance, dependent on the growth temperature of the conidia-forming colony (Hagiwara *et al*., 2017). In general, spore parameters are influenced by conditions during spore formation and maturation. However, there is a scarcity of information on the extent of heterogeneity of spore populations, even if they originate from one colony. Stress resistance of spores and germination properties are both important for colonization but different in nature. Colonies from individual spores that enter a new ecosystem often have longer lag times compared with a population of spores (Gougouli and Koutsoumanis, 2013; Dagnas *et al*., 2015). This is essentially caused by the phenomenon that the spore that germinates fastest determines the lag time of a population. Furthermore, germination of conidia is delayed markedly when conditions are suboptimal. In several studies, this is tested in the case of water activity and temperature (Dagnas *et al*., 2017; Nguyen Van Long *et al*., 2017; Stevenson *et al*., 2017).

Heterogeneity encountered in conidia is relevant in the area of food spoilage; the strongest spore will define if spoilage occurs. In nature, the strongest spore enlarges the limits of growth of a species. Fungal spoilage of processed foods and drinks causes food waste and economic losses (Gustavsson *et al*., 2011). In general, industry prevents fungal food spoilage in their products by using preservation hurdles like pasteurization, lowering water activity, lowering pH or decrease storage temperature. Consumer demands towards more natural and preservative free food put a pressure on shelf life of food (Leyva Salas *et al*., 2017). For instance, lowering heat treatments could lead to more beneficial organoleptic

characteristics of the product and better retainment of vitamins or other temperature-sensitive healthy components. On the other hand, this procedure would introduce survival of heat-resistant cells and more risk for the product to be spoiled. In addition, consumers demand healthier alternatives, including products containing lower salt concentrations and less use of antimicrobial compounds. These trends towards milder processing methods make processed food products more prone to microbial spoilage and put a tension on the microbial safety limits of processed foods and drinks.

Paecilomyces variotii is a common thermo-tolerant fungus that occurs worldwide and has been isolated from food products, soil samples, clinical samples and indoor environments (Houbraken *et al*., 2010). Recently, the genomes of *P. variotii* type strain CBS 101075 and CBS 144490 were sequenced (Urquhart *et al*., 2018). *P. variotii* produces conidia abundantly and to lesser extent chlamydospores. Besides these spore types, it is also known to produce highly heat-resistant ascospores in a heterothallic manner (Houbraken *et al*., 2008). Due to the resistant nature of these spores and its ability to grow at low oxygen concentrations, it is able to spoil pasteurized food products like fruit juices, canned fruits and non-carbonized sodas (Pitt and Hocking, 2009). Although *P. variotii* is a major spoilage fungus in a certain product niche, many aspects remain elusive in the resistance of its propagules.

Compatible solutes like trehalose, mannitol, glycerol, erythritol and arabitol are accumulated in fungal spores (Wyatt *et al*., 2013). Due to the kosmotropic nature of these compounds, they protect cells against various stressors (Cray *et al*., 2013). Kosmotropic solutes have typically a larger affinity to form hydrogen bonds with water than water molecules with themselves, resulting in a stabilizing effect on biomacromolecules like proteins (Washabaugh and Collins, 1986; Collins, 1997). It is thought that trehalose plays an important role in the heat resistance of fungal spores (Wyatt *et al*., 2013, 2015). A decrease in trehalose concentration in *Aspergillus nidulans* and *Aspergillus oryzae* conidia resulted in a higher sensitivity to heat stress (Fillinger *et al*., 2001; Sakamoto *et al*., 2008).

Based on sequences of marker genes, *P. variotii* showed a higher genetic variability than related members of the Eurotiales (Houbraken *et al*., 2008). In this research, we used the conidia of *P. variotii* as a model to explore the limits and variability of resistance to heat stress. We used 108 isolates from various substrates and locations to screen the conidia for heat resistance. Subsequently, we did an in-depth analysis of three strains of interest in their heat resistance, morphology and compatible solute composition. In addition, we explored the influence of salt stress on the growth rate. We used the results to reflect on the heterogeneity within and between the various *P. variotii* strains.

Results

Screening conidia for heat resistance

In total, 108 *P. variotii* isolates were used in this study. The strains were isolated from various substrates and locations, of which most originated from United States or The Netherlands (Table 1). Of the 108 strains, 65 were isolated from food products or industry environment, 27 originated from indoor and outdoor environments and 16 were medical isolates from human patients or hospital environment. All strains were identified by DNA sequencing of the β-tubulin locus (Samson *et al*., 2009). A maximum likelihood tree revealed that all *P. variotii* strains grouped together and were separated from *Paecilomyces brunneolus* the closest related species, with a bootstrap value of 100% (Fig. 1). More intraspecific clades were identified than described before (Houbraken *et al*., 2008). However, most of these clades showed bootstrap values below 70%. Within the *P. variotii* group, the overall mean distance was 0.013 ± 0.003 substitutions per site, indicating a low variation between the strains. Like recently described, the β-tubulin gene from the draft genome of the previously misidentified *Paecilomyces formosus* No5 as *P. variotii*, groups with the type strain of *P. formosus* in the tree (Oka *et al*., 2014; Urquhart *et al*., 2018).

In order to study the variation of heat resistance of conidia among the different strains, conidia of each strain were heat-inactivated in a water bath at 58 or 59°C during 10 min. Subsequently, conidia survival was determined by inoculating 10^3 conidia on malt extract agar (MEA). After 3 days incubation at 25°C, the number of colonies was counted. For 31 isolates, more than 10% of the conidia survived the 59°C heat treatment and showed more than 100 colonies on plates (Fig. S1). The remaining 77 strains showed a broad variety in the number of colonies, stretching from 1 to 94. After the 10 min-heat treatment at 58°C, 68 isolates showed more than 100 colonies after the treatment, while 40 strains showed between 13 and 95 colonies.

In-depth analysis of three strains

Three strains, DTO 032-I3, DTO 212-C5 and DTO 217-A2 were selected for an in-depth analysis in conidial heat resistance, morphology and compatible solute composition. Based on the screening, conidia from strain DTO 032-I3 were rather sensitive, showing 70 and 26 colonies after heat treatment at 58 and 59°C respectively. Conidia from strain DTO 212-C5 were one of the most heat sensitive, showing 58 and 3 colonies after heat

Table 1. Overview of the *P. variotii* isolates used in this study.

DTO No.	CBS No.	Substrate	Location	GenBank no. β-tubulin
DTO 013-C3		Indoor environment	The Netherlands	MN153213
DTO 021-B9		Spoiled sports drink	USA	MN153214
DTO 021-C3	CBS 145656	Spoiled sports drink	USA	MN153215
DTO 021-C4		Spoiled sports drink	USA	MN153216
DTO 021-C5		Spoiled sports drink	USA	MN153217
DTO 021-C9		Heat shocked sucrose	USA	MN153218
DTO 021-D3	CBS 145657	Heat shocked sucrose	USA	MN153219
DTO 027-B3	CBS 121577	Spoiled sports drink	USA	EU037084
DTO 027-B4	CBS 121578	Spoiled sports drink	USA	EU037083
DTO 027-B5	CBS 121579	Sucrose	USA	EU037082
DTO 027-B6	CBS 121580	Spoiled apple juice	The Netherlands	EU037081
DTO 027-B7	CBS 121581	Spoiled sweetened tea	USA	EU037080
DTO 027-B8	CBS 121582	Spoiled sweetened tea	USA	EU037079
DTO 027-B9	CBS 121583	Spoiled sports drink	USA	EU037078
DTO 032-I3	CBS 121585	High fructose corn syrup after heat shock	USA	EU037077
DTO 032-I4	CBS 121586	Spoiled bottle of bottle of sweetened tea	USA	EU037076
DTO 032-I5	CBS 121587	Spoiled sports drink	USA	EU037075
DTO 032-I6	CBS 121588	Heat shocked sucrose	USA	EU037074
DTO 034-C6	CBS 729.96	Beach sand	Spain	MN153220
DTO 034-C7	CBS 108945	Pectin	The Netherlands	MN153221
DTO 034-C8	CBS 110036	Cerebrospinal fluid of 60-year-old female with diabetes and cancer	Turkey	MN153222
DTO 038-B4	CBS 115809	Horse stable, air sample	Germany	MN153223
DTO 045-G8	CBS 145658	Drink	USA	MN153224
DTO 045-H1		Drink	USA	MN153225
DTO 045-H9		Pseudo-outbreak	UK	GU968685
DTO 048-I6		Pseudo-outbreak	UK	MN153226
DTO 049-D2		Unknown (patient, or hospital environment)	UK	MN153227
DTO 049-D3		Unknown (patient, or hospital environment)	UK	MN153228
DTO 063-D6		Human; mouthwash	The Netherlands	GU968692
DTO 063-D7		Human; faeces	The Netherlands	GU968693
DTO 063-D9		Human; mouthwash	The Netherlands	GU968695
DTO 063-E2		Unknown (patient, or hospital environment)	The Netherlands	GU968677
DTO 063-E4		Unknown (patient, or hospital environment)	The Netherlands	GU968679
DTO 063-E6		Unknown (patient, or hospital environment)	The Netherlands	GU968680
DTO 063-E7		Human; cerebral spinal fluid	The Netherlands	GU968681
DTO 063-E9		Human; mouthwash	The Netherlands	GU968682
DTO 063-F5		Human; abscess	The Netherlands	MN153229
DTO 065-A6		Indoor environment	Germany	MN153230
DTO 073-I6		Unknown (patient, or hospital environment)	The Netherlands	MN153231
DTO 073-I8		Unknown (patient, or hospital environment)	The Netherlands	MN153232
DTO 086-B2		Archive	The Netherlands	MN153233
DTO 086-F7		Filter flow cabinet Westerdijk institute	The Netherlands	MN153234
DTO 089-G8		Air in bedroom	The Netherlands	MN153235
DTO 089-H1		Air in bedroom	The Netherlands	MN153236
DTO 090-B8		Archive	The Netherlands	MN153237
DTO 122-F7		Bakery	Germany	MN153238
DTO 123-F7		Indoor environment	Germany	MN153239
DTO 164-E3	CBS 145659	Blueberry ingredients	The Netherlands	MN153240
DTO 166-F8		Ice pop, heat treated	The Netherlands	MN153241
DTO 166-F9		Ice pop, heat treated	The Netherlands	MN153242
DTO 166-G2		Pectin, heat treated	The Netherlands	MN153243
DTO 166-G3		Pectin, heat treated	The Netherlands	MN153244
DTO 166-G4	CBS 145660	Pectin, heat treated	The Netherlands	MN153245
DTO 166-G5		Pectin, heat treated	The Netherlands	MN153246
DTO 166-G6		Pectin, heat treated	The Netherlands	MN153247
DTO 166-G7		Pectin, heat treated	The Netherlands	MN153248
DTO 166-G8		Pectin, heat treated	The Netherlands	MN153249
DTO 169-A8		Indoor environment	USA	MN153250
DTO 169-B5		Indoor environment	USA	MN153251
DTO 169-B7		Indoor environment	USA	MN153252
DTO 169-B9		Indoor environment	USA	MN153253
DTO 169-C1		Indoor environment	USA	MN153254

(Continues)

Table 1. Continued

DTO No.	CBS No.	Substrate	Location	GenBank no. β-tubulin
DTO 169-C2		Indoor environment	USA	MN153255
DTO 169-C4		Indoor environment	USA	MN153256
DTO 169-C6	CBS 145661	Indoor environment	USA	MN153257
DTO 169-C7		Indoor environment	USA	MN153258
DTO 169-D1		Indoor environment	USA	MN153259
DTO 169-D2		Indoor environment	USA	MN153260
DTO 169-D4		Indoor environment	USA	MN153261
DTO 169-D7		Indoor environment	USA	MN153262
DTO 169-D9		Indoor environment	USA	MN153263
DTO 169-E4		Indoor environment	USA	MN153264
DTO 169-E5	CBS 145662	Indoor environment	USA	MN153265
DTO 169-E6		Indoor environment	USA	MN153266
DTO 176-F5		Seal of Tetra package	Germany	MN153267
DTO 195-A7		Margarine	Belgium	MN153268
DTO 195-F2	CBS 145663	Margarine	Belgium	MN153269
DTO 207-G8	CBS 145664	Fruit, ingredient	The Netherlands	MN153270
DTO 212-C5	CBS 145665	Vanilla	The Netherlands	MN153271
DTO 215-H8		Ice pop, heat treated	The Netherlands	MN153272
DTO 215-H9		Ice pop, heat treated	The Netherlands	MN153273
DTO 215-I1		Ice pop, heat treated	The Netherlands	MN153274
DTO 217-A2	CBS 145666	Ice pop, heat treated	The Netherlands	MN153275
DTO 217-A4		Pectin	The Netherlands	MN153276
DTO 217-A6		Pectin	The Netherlands	MN153277
DTO 217-A7		Pectin	The Netherlands	MN153278
DTO 235-B7		Apple mixture	The Netherlands	MN153279
DTO 271-C1		Ice tea	South Africa	MN153280
DTO 271-C2		Ice tea	South Africa	MN153281
DTO 271-C5		Ice tea	South Africa	MN153282
DTO 271-C7		Ice tea	South Africa	MN153283
DTO 271-D1		Industry environment	Unknown	MN153284
DTO 271-D2		Industry environment	Unknown	MN153285
DTO 271-D3	CBS 145667	Industry environment	Guatemala	MN153286
DTO 271-G3	CBS 145668	Ice tea	South Africa	MN153287
DTO 271-G4		Ice tea	South Africa	MN153288
DTO 271-H4		Ice tea	South Africa	MN153289
DTO 271-H5		Ice tea	South Africa	MN153290
DTO 271-H6		Ice tea	South Africa	MN153291
DTO 271-I4		Ice tea	South Africa	MN153292
DTO 280-E3	CBS 109072	Pectin	The Netherlands	EU037071
DTO 280-E4	CBS 109073	Pectin	The Netherlands	EU037070
DTO 280-E5	CBS 101075^T	Heat processed fruit beverage	Japan	EU037069
DTO 282-E4		Food product isolate	Unknown	MN153293
DTO 282-E5	CBS 145669	Margarine	Italy	MN153294
DTO 282-E9		Recycled paper packaging	Brazil	MN153295
DTO 282-F1		Recycled paper packaging	Brazil	MN153296
DTO 282-F9	CBS 145670	Wall covering, industry environment	UK	MN153297

^{T}Type strain.

treatment at 58 and 59°C respectively. Conidia from strain DTO 217-A2 showed high heat resistance as judged by the outgrowth of more than 100 colonies after both heat treatments (Fig. 2A). To quantify the heat resistance of the conidia of the three strains, the D_{60} values of each strain were determined. Conidia were treated in a water bath at 60°C and at various time points, the number of colony-forming unit (cfu) was determined. DTO 032-I3, DTO 212-C5 and DTO 217-A2 showed a D_{60} value of 5.5 $\pm$ 0.35, 3.7 $\pm$ 0.08 and 22.9 $\pm$ 2.00 min respectively (Fig. 2B). In the case of strain DTO 032-I3 and DTO 212-C5, a log 6 reduction was achieved in 33 and 20 min respectively. Strain DTO 217-A2 showed less than log 3 inactivation after 60 min of heat treatment. The inactivation kinetics of strain DTO 032-I3, DTO 212-C5 and DTO 217-A2 revealed a linear character and showed an R^2 = 0.996, 0.998 and 0.989 respectively. These results indicate that the D_{60} value of conidia among the three strains varies 6.2 times.

The morphology of colonies and conidiophores (spore-forming structures) of the three strains was studied in detail. Stereomicroscopy and Cryo-Electron Microscopy

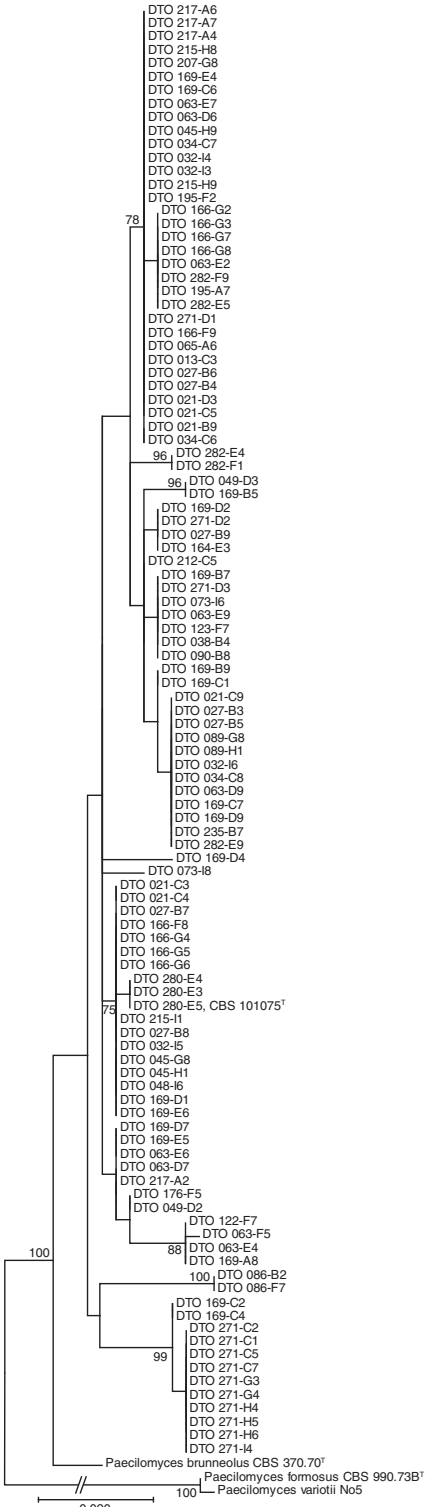

Fig. 1. Maximum likelihood tree of the partial *β-tubulin* gene of all the *P. variotii* strains used in this study. The type strain of *P. brunneolus* and *P. formosus* were used as outgroup. At the nodes, bootstrap values >70% are shown.

of 3-day-old colonies showed variation in appearance. Heat-resistant strain DTO 217-A2 showed colonies that formed spores from conidiophores that were present at the level near the agar surface, designated as velvety (Fig. 3A and B). DTO 032-I3 produced more aerial structures, mostly elongated conidiophores, compared with DTO 217-A2 (Fig. 3B and E). On the other hand, heat-sensitive strain DTO 212-C5 showed extensive formation of aerial hyphae, which contained the conidiophores (Fig. 3C and F). According to *Penicillium* morphology, the appearance of DTO 212-C5 could be designated as lanose (Samson *et al*., 2010). These results showed that the colony morphology of *P. variotii* strains could vary in branching patterns and formation of aerial structures.

In another strain (CBS 121579), a large variation in the size of conidia could be observed. Interestingly, a row of conidia retained the same size, but large variation was found between different chains of conidia (Fig. S2). Light microscopy pictures confirmed that colonies of strains DTO 032-I3, DTO 212-C5 and DTO 217-A2 produced conidia of various sizes (Fig. 4B). In addition, these pictures suggested that differences in size might occur between the strains. To study the spore-size distribution more into detail, spore size of the three strains was measured by flow cytometry (fluorescence-activated cell sorters [FACS]) and Coulter Counter (CC). We tested if we could describe the distributions by one component ($k = 1$, normal distribution) or two components ($k = 2$, bimodal distribution) by bootstrapping the data. In all cases, the three strains showed $P(k > 1) < 0.001$ for the FACS data, as well for CC data. This suggested that the spore-size distributions were heterogeneously distributed and that it is valid to describe spore size by means of two components. To this end, we computed a two-component mixture model using an expectation–maximization (EM) algorithm for each distribution (Fig. 4B and C). In this model, component 1 represents a subpopulation of small spores and component 2 represents a subpopulation of large spores. The mean spore size (diameter in μm) of the total population and the two subpopulations, as well as the standard deviation (σ) and the weight (λ) of the two subpopulations are summarized in Table 2. The histograms of the spore-size distributions measured by FACS and CC showed large similarity. Least variation in spore size was found for conidia produced by strain DTO 032-I3, while most variation was found for conidia produced by strain DTO 217-A2. For DTO 032-I3, the CC data showed that the weight of the subpopulation containing the small spores ($\lambda_1 = 0.61$) is larger than the subpopulation of large spores ($\lambda_2 = 0.39$). In contrast, DTO 217-A2 showed a larger weight for the subpopulation of large spores ($\lambda_1 = 0.39$, $\lambda_2 = 0.61$). The weight of the subpopulations of conidia produced by DTO 212-C5 was almost equal ($\lambda_1 = 0.48$, $\lambda_2 = 0.52$). DTO 217-A2 produced the largest mean conidia size with a diameter of 3.51 μm, while DTO 032-I3 and DTO 212-C5 showed a mean conidia size of 2.90 and 3.04 μm respectively. It is noteworthy that the

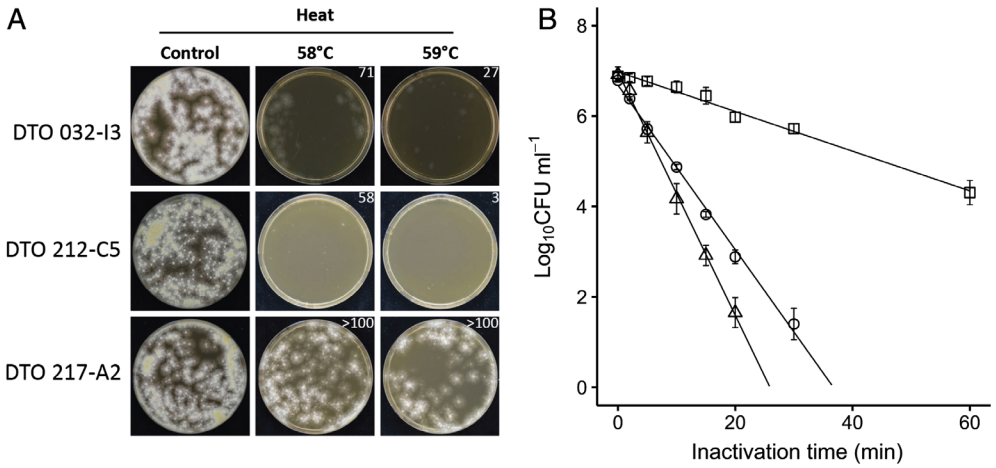

Fig. 2. Strain variation in heat resistance of dormant *P. variotii* conidia. The screening results of three strains used for the in-depth analysis (A). The numbers in the pictures of the plates indicate the amount of colonies. After heat treatments at 58 and 59°C for 10 min, DTO 217-A2 showed least reduction in number of colonies and growth. The most sensitive conidia were formed by DTO 212-C5. Inactivation curves of conidia at 60°C of DTO 217-A2 (□), DTO 212-C5 (Δ) and DTO 032-I3 (○) (B). Mean values of three biological replicates ± SD are shown. [Color figure can be viewed at wileyonlinelibrary.com]

subpopulation of small spores of DTO 217-A2 showed a larger mean spore size than the total population of the other two strains. Altogether, these results showed that heterogeneity in conidia size is not only found between strains, but also within strains.

To see whether there is difference in compatible solute composition between the strains, we extracted them from the conidia and quantified them using HPLC. Since we found differences in spore size between the strains, we corrected the quantified compounds with the mean spore volumes measured by CC. This allowed us to estimate the intracellular concentration of trehalose, glycerol, mannitol and arabitol in mole per liter (Fig. 5). In all strains, conidia contained predominantly trehalose. The heat-resistant conidia of DTO 217-A2 contained a concentration of trehalose that was 40% higher compared with the other two strains. This might explain the differences in heat resistance. On the other hand, DTO 032-I3 conidia contained significantly more mannitol, while DTO 212-C5 conidia contained significantly more arabitol. In all strains, minor amounts of glycerol were found, but without significant differences. Every strain tested showed a different compatible solute composition. This might suggest that lower trehalose concentrations are substituted by other compatible solutes as mannitol or arabitol.

The differences in compatible solute composition could point towards adaptation to different stress conditions. For this reason, we assessed if we could find a difference between strains in germination and early growth rates in NaCl-enriched medium. Germination of conidia and early growth rates in complete medium (CM), supplemented with various concentrations of NaCl, were followed for 48 h using the oCelloScope. Photos were taken every 30 min. After 24 h, pictures of germinated conidia at 1 mol l^{-1} NaCl showed differences between strains (Fig. 6A–C). DTO 032-I3 and DTO 212-C5 both showed more growth than DTO 217-A2. In addition, we used the background-corrected absorption (BCA) algorithm provided by the oCelloScope software to model early growth rates. This algorithm measures object pixel intensities that increase during germination and early growth. The maximum growth rate (μ) was calculated by fitting a Gompertz curve to the BCA values. For all three strains, a lower μ was found when the medium was enriched with NaCl (Fig. 6D). Lowest μ was found at the highest NaCl concentrations. In all samples in medium with additional NaCl, μ was higher for DTO 032-I3 and DTO 212-C5 compared with DTO 217-A2. No difference was found in the control samples in medium without additional NaCl. This might imply that DTO 032-I3 and DTO 212-C5 are better adapted to salt stress and are able to germinate faster under salt stress conditions, compared with heat-resistant DTO 217-A2.

Discussion

In this study, we explored strain variability in conidial heat resistance of the important food spoilage fungus *P. variotii*. We screened conidia of 108 isolates, which gave us a good overview of the variation in sensitivity to heat stress. We selected three strains and characterized the heat resistance, morphology, spore size distribution, compatible solute compositions and early growth under salt stress conditions. In all aspects, we found variation between the three selected strains. To our knowledge, *P. variotii* DTO 217-A2 conidia showed with 22.9 min the highest *D* value for this cell type at 60°C ever measured. Compared to DTO 032-I3 and DTO 212-C5, DTO 217-A2

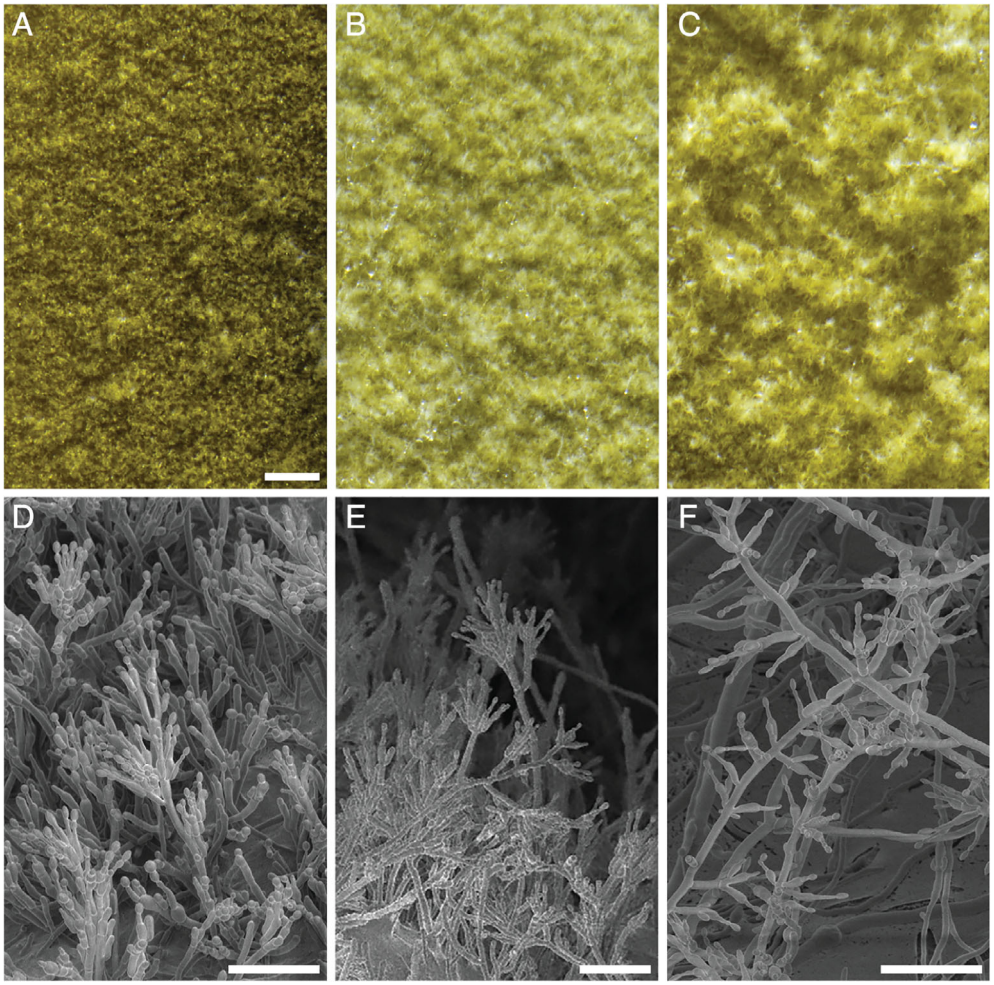

Fig. 3. Colony morphology of DTO 217-A2 (A, D), DTO 032-I3 (B, E) and DTO 212-C5 (C, F). The stereomicroscopy pictures show a detailed top view of a 3-day-old colony (A–C). DTO 217-A2 produced short aerial hyphae with conidiophores and showed a velvety appearance. On the other hand, DTO 212-C5 produced aerial structures with conidiophores to a large extend and has a lanose appearance. DTO 032-I3 showed a morphology between the other two strains, it also produced larger aerial structures, but not as extensively as DTO 212-C5. CryoSEM pictures confirmed these findings (D–F). Scale bars indicate 500 μm (A–C) or 50 μm (D–F). [Color figure can be viewed at wileyonlinelibrary.com]

conidia were more heat resistant, produced less aerial structures when cultured and contained larger average conidia in size having a higher trehalose concentration. On the other hand, DTO 217-A2 showed less growth and slower germination under salt stress conditions.

The D_{60} values of *P. variotii* conidia were in the same range as *Saccharomyces cerevisiae* ascospores, which showed D_{60} values between 4.6 and 22.5 min (Put and Jong, 1982; Milani *et al*., 2015). In general, conidial heat resistance is evaluated at temperatures lower than 60°C, because conidia of most species do not survive heat treatments at this temperature for several minutes (Wyatt *et al*., 2013). However, we showed that inactivation at 60°C revealed large differences between the tested *P. variotii* strains. In a study with xerophilic fungi, the most heat-resistant conidia of *Aspergillus ruber* showed 3% survival after a 10 min heat treatment at 60°C (Pitt and Christian, 1970). Conidia of *A. niger* showed a D_{60} value of 2.2 min, about 10 times shorter than *P. variotii* DTO 217-A2 (Baggerman, 1984). Although no *D* values were determined, comparable heat resistance was found for *A. fumigatus* conidia (Hagiwara *et al*., 2017). After a 15-min heat treatment at 60°C, approximately 7% of the conidia survived when cultures were grown at 25°C. This would roughly correspond to a D_{60} value of 13 min. Interestingly, increasing the culture incubation temperature to 37 and 45°C dramatically increased the heat resistance of *A. fumigatus* conidia and hardly showed any reduction after identical heat treatments. These findings suggest that the limits of *P. variotii* conidial heat resistance could possibly be stretched further by growing cultures at higher temperatures.

The heat resistance of the *P. variotii* ascospores is much higher than observed for conidia. Ascospores of closely related *Paecilomyces fulvus* and *Paecilomyces niveus* (both species have a byssochlamys-morph) survive heat

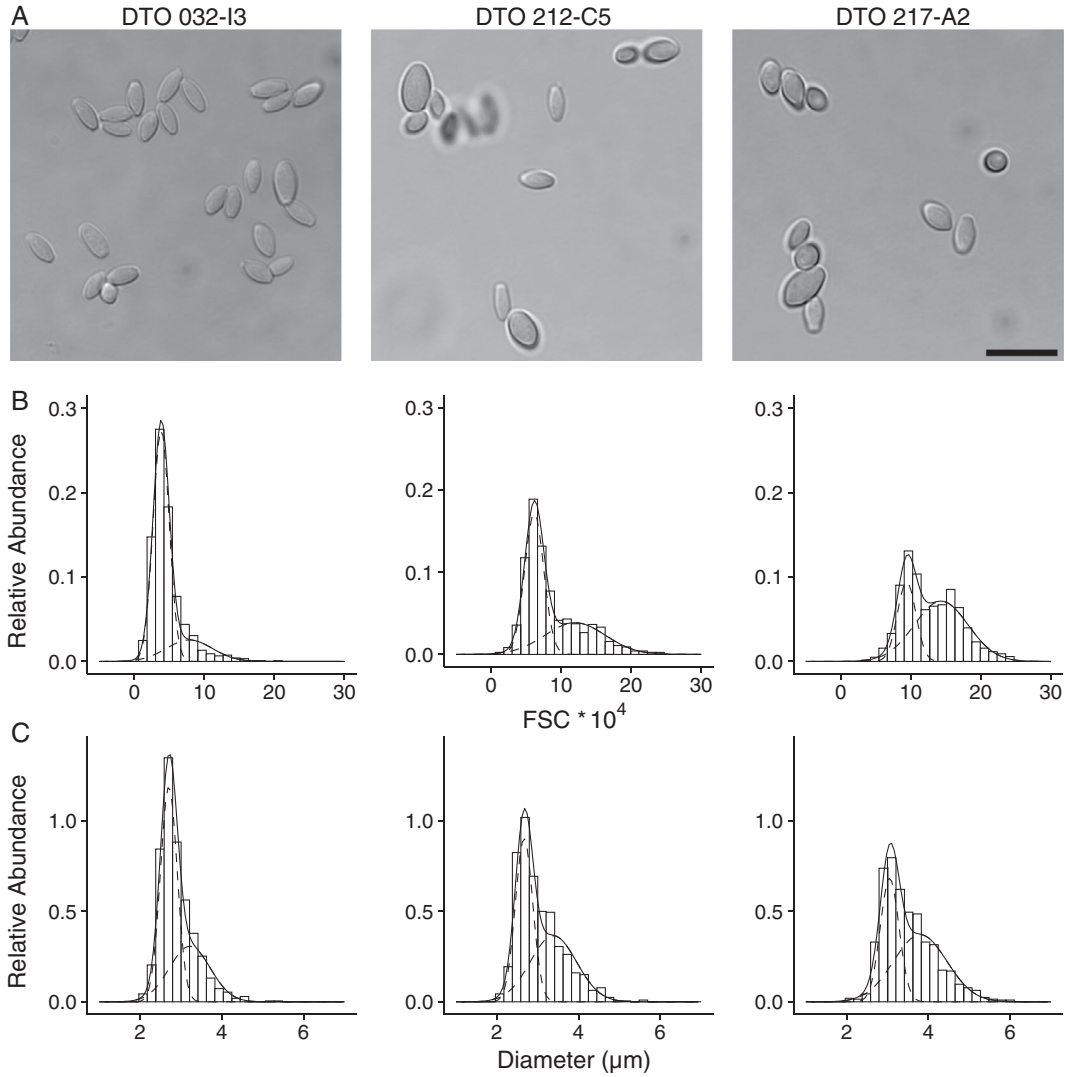

Fig. 4. Conidia size distributions of DTO 032-I3, DTO 212-C5 and DTO 217-A2. Light microscopical pictures of conidia of various sizes produced by a single colony (A). Histograms show spore-size distributions as measured by flow cytometry (B) and coulter counter (C). Dashed lines indicate two computed subpopulations of small and large spores. The black line indicates the sum of the two subpopulations. The total surface area of each histogram equals 1. Scale bar indicates 10 µm.

treatments of 85°C for several minutes (Beuchat and Rice, 1979; Dijksterhuis, 2019). The ascospores of *P. variotii* are much more heat resistant, surviving 85°C for more than an hour, although *D* values were not accurately measured (Houbraken *et al*., 2006). Ascospores are produced after mating of two compatible strains, and formed within the mycelium and as such not distributed easily compared with airborne conidia. It has to be expected that ascospores can spoil food that is extensively (heat) processed, although being present at very low numbers (dos Santos *et al*. 2018).

Table 2. Conidia size heterogeneity of colonies grown on MEA as measured by coulter counter (CC).

Strain	$Size_{total}$	$Size_1$	$Size_2$	σ_1	σ_2	λ_1	λ_2
DTO 032-I3	2.90 ± 0.01	2.70 ± 0.01	3.21 ± 0.11	0.21 ± 0.01	0.50 ± 0.04	0.61 ± 0.07	0.39 ± 0.07
DTO 212-C5	3.04 ± 0.02	2.67 ± 0.06	3.38 ± 0.01	0.21 ± 0.03	0.57 ± 0.01	0.48 ± 0.05	0.52 ± 0.05
DTO 217-A2	3.51 ± 0.02	3.05 ± 0.01	3.80 ± 0.07	0.23 ± 0.01	0.65 ± 0.06	0.39 ± 0.05	0.61 ± 0.05

Spore sizes are expressed by diameter (µm) of the spherical equivalent (Heywood diameter) for the CC data. The mean spore size is shown for the total population ($size_{total}$) and two subpopulations ($size_1$ and $size_2$) as used in the modelling. The standard deviation of the size distributions and the relative weight of each subpopulation are expressed by σ and λ respectively. All values are shown ± the bootstrapped SE.

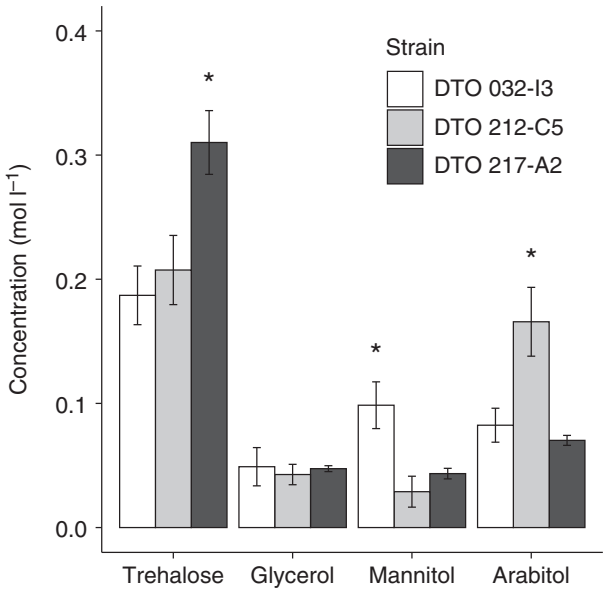

Fig. 5. Compatible solute composition of three strains in mole per litre. Mean values of three biological replicates are shown ± SD. Asterisk indicates statistic significant differences of one strain compared with the other two strains (Tukey HSD $P < 0.01$).

On the other hand, ubiquitous airborne conidia could be expected to contaminate mildly heat-treated processed food products. This is a subject that will need attention in future research, as well as the question if heat-resistant conidia-forming strains also form more resistant ascospores.

Over the past decades, various HPLC methods and enzymatic assays have been developed to quantify the internal compatible solute compositions (Hallsworth and Magan, 1994b; D'Enfert and Fontaine, 1997; Sakamoto *et al*., 2009; Al-Bader *et al*., 2010; Novodvorska *et al*., 2013; van Leeuwen *et al*., 2013; Hagiwara *et al*., 2014; Nguyen Van Long *et al*., 2017). These methods usually do not consider spore size and give values in pg spore^{-1}, or μmol (g dry weight)$^{-1}$. By obtaining accurate data about the cell volumes by CC, we were allowed for the first time to estimate the internal concentration of compatible solutes in fungal spores. *P. variotii* conidia showed a predominant concentration of trehalose, similar to *A. fumigatus* conidia (Hagiwara *et al*., 2017). Despite the larger conidia size of heat-resistant strain DTO 217-A2 compared with DTO 032-I3 and DTO 212-C5, the conidia of this strain contained a significant higher concentration of trehalose.

Interestingly, HPLC analysis showed that DTO 212-C5 conidia contained higher arabitol concentrations than DTO 032-I3 and DTO 217-A2. Arabitol is described to accumulate in fungal spores and vegetative cells under intermediate osmotic stress conditions (Hallsworth and Magan, 1994a; Ruijter *et al*., 2004; Rangel *et al*., 2015). Like glycerol, it has a lower molecular weight and higher solubility than trehalose and mannitol. Therefore, this compatible solute can accumulate at higher concentrations to protect cells better to osmotic stress conditions (de Lima Alves *et al*., 2015). For this reason, we tested if we could find differences in germination and early growth under salt stress conditions with NaCl between the three strains. DTO 217-A2 showed to have a lower maximum growth rate under salt stress conditions compared with the other two strains. Surprisingly, DTO 032-I3 was equally adapted to salt stress conditions as DTO 212-C5 while it showed a comparable arabitol concentration as

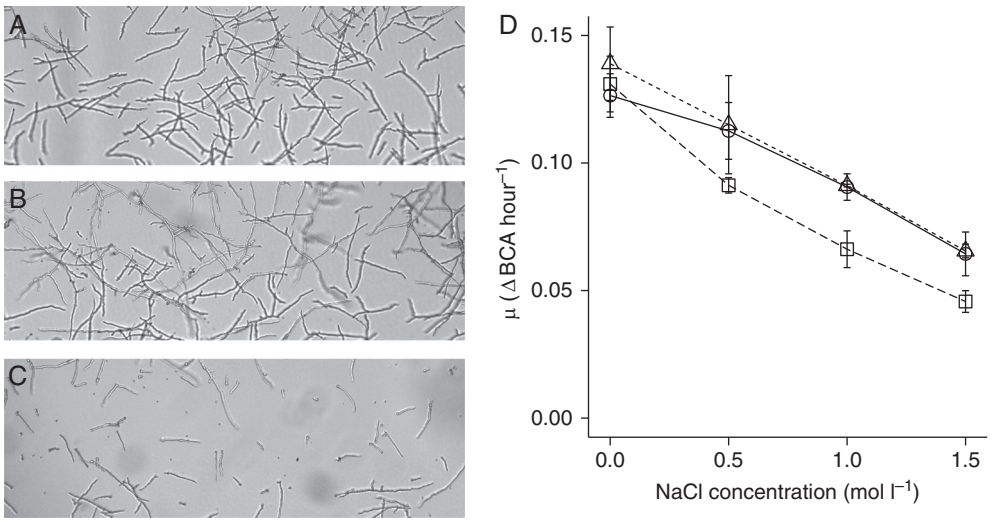

Fig. 6. Growth of three *P. variotii* strains on NaCl supplemented media. Germination and growth were studied in a 96 wells plate in an oCelloScope for 48 h. Pictures were taken of each well every 30 min. After 24 h, DTO 032-I3 (A) and DTO 212-C5 (B) showed more growth than DTO 217-A2 (C) at 1.0 mol l^{-1} NaCl. (D) The maximum growth rate (μ) of DTO 032-I3 (○, solid line), DTO 212-C5 (Δ, dotted line) and DTO 217-A2 (□, dashed line) was calculated by fitting a Gompertz curve to the background corrected absorption (BCA) at various NaCl concentrations. The mean of biological triplicates ± SD are shown.

DTO 217-A2. Although more mannitol was found in DTO 032-I3 conidia, mannitol can only be involved in osmoregulation to a limited extent due to its low solubility (de Lima Alves *et al*., 2015). The ability to grow under NaCl stress is determined by the accumulation of compatible solutes in germinating spores and mycelium and not of dormant conidia. Measuring the mycelial compatible solutes would provide information in how these strains adapt to NaCl stress. Nevertheless, with this experiment we have shown that the three strains react differently to NaCl stress. These results imply that the three isolates tested are adapted to different stress environments.

With the phylogenetic analysis of β-tubulin of the isolates used in this study, we have shown that all belong to *P. variotii* according to the current taxonomy (Samson *et al*., 2009). Among the 108 strains, sequence variation was found, resulting in formation of various clades. However, the bootstrap values <70% of most clades could indicate that this variation is statistically irrelevant to the phylogeny (Hillis and Bull, 1993). Furthermore, low bootstrap values indicate that recombination events may occur among the *P*. *variotii* isolates. We did not find indications that the results of screening for conidial heat resistance correlated with the position in the β-tubulin tree (data not shown). The strains that showed more than 100 colonies after the 10-min heat treatment of conidia at 59°C were positioned in various clades in the tree. In addition, we could not find a link between the location and substrate of isolation and conidial heat resistance. Altogether, these results suggest that the variation found among the strains could be the same at every location and environment.

Notably, we found variation between the three strains in the morphological characteristics of their colonies. Largest differences were found between DTO 217-A2, which produced conidia on short aerial hyphae close to the medium, and DTO 212-C5, which produced its conidiophores on aerial mycelium. Aerial mycelum is physiologically different and this may cause variation in conidia physiology (Bleichrodt *et al*., 2013). Presumably, conidia formed on aerial structures are developed differently than conidia developed close to substrate hyphae. In addition, the spore-size distributions were different in three strains with respect to range, bimodal distribution and average size. Surprisingly, the presence of larger spores differed among the strains; the resistant strain produced relatively more conidia of large size than the two sensitive strains. These data showed that heterogeneity occurs between strains, but also within strains. This heterogeneity in size distribution was confirmed with two different counting techniques, namely flow cytometry and CC. A bimodal spore-size distribution has been observed earlier in the case of *Gloeosporium orbiculare* (Chapela, 1991). This, however, is certainly not common for other species. *Penicillium glabrum*, *Penicillium roqueforti* and *A*. *niger* showed narrow and symmetrical distributions (unpublished results).

Altogether, heterogeneity in the conidia can be found within and between *P*. *variotii* strains in all aspects discussed in this article. Physiological heterogeneity may be beneficial for *P*. *variotii* as a species and could enhance its ability to adapt to different environments (Hewitt *et al*., 2016). This might be one of the reasons why *P*. *variotii* can be isolated from a broad range of environments. With the recently sequenced genome and the development of molecular tools, *P*. *variotii* conidia could potentially be a useful model to study fungal spore resistance and heterogeneity on a genetic and molecular level (Urquhart *et al*., 2018).

Experimental procedures

Strain selection, sequencing and phylogenetic analysis

Strains were selected from the working collection of the Applied and Industrial Mycology (DTO) group at the Westerdijk Fungal Biodiversity Institute. All strains were stored in 30% glycerol at −80°C. All isolates were identified by sequencing the β-tubulin gene (*benA*) as described before (Houbraken *et al*., 2008). In short, DNA from 3 to 4-day-old cultures grown on MEA (Oxoid, Hampshire, UK) was isolated using DNeasy UltraClean Microbial kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. *BenA* was amplified using the primers Bt2a and Bt2b (Houbraken *et al*., 2008), followed by a sequencing PCR using ABI Prism BigDye Terminator v.3.0 ready reaction cycle sequencing kit (Applied Biosystems, Foster City, CA). The samples were sequenced by a 3730XL DNA Analyser (Applied Biosystems) and analysed with SeqMan (DNAstar, Madison, WI). Sequences were analysed using MEGA7 and aligned using ClustalW (Kumar *et al*., 2016). The maximum likelihood tree was computed with the Tamura 3-parameter model including gamma distribution and 1000 bootstrap replications.

Culture conditions and harvesting conidia

Conidia were harvested from 7-day-old colonies grown on MEA at 25°C (Samson *et al*., 2010). The conidia were rubbed into 10 ml ice-cold ACES buffer [10 mM *N*-(2-acetamido)-2-aminoethanesulfonic acid, 0.02% Tween 80, pH 6.8] using a T-spatula. After homogenizing, the conidia were filtered through sterile glass wool in a 30 ml syringe. Fifty millilitres Falcon tubes were filled up to 30 ml with ice-cold ACES buffer and centrifuged for 5 min at 1260*g* at 4°C. The pellet was washed with 40 ml ice-cold ACES buffer, followed by centrifugation. Finally, the conidia were resuspended in 10 ml ice-cold ACES buffer.

The conidia were counted using a haemocytometer (Bürker-Türk, VWR, Amsterdam, The Netherlands) or the Beckman CC Multisizer 3 (Beckman Coulter, Fichtenhain, Germany). Conidia were stored on ice until further treatment.

Screening and quantification of heat resistance

To screen conidia of different strains for heat resistance, dilutions of 2×10^4 conidia ml^{-1} were used for heat inactivation. A quantity of 150 μl of this suspension was placed in a 1.5 ml Eppendorf tube and transferred into a water bath at 58 or 59°C for 10 min. Of the heat-treated sample, 50 μl was used to inoculate approximately 10^3 conidia on the surface of 9 cm MEA plates. All plates were inoculated in duplicate and incubated for 3 days at 25°C. After incubation, pictures were made of the plates and colonies were counted.

To quantify conidial heat resistance, we determined the D_{60} values, which expresses the time it takes to have 1 log reduction in cfu at 60°C. For the determination of *D* values, 19 ml ACES buffer was pre-heated in 100 ml Erlenmeyer flasks in a water bath (120 rpm, 60°C). At $t = 0$, a volume of 1 ml of a 2×10^8 conidia ml^{-1} suspension was added to pre-heated buffer to reach a final concentration of 10^7 conidia ml^{-1}. After 2, 5, 10, 15, 20, 30 and 60 min incubation, 1 ml was removed from the flasks and immediately cooled on ice. These samples and the untreated spore suspensions were further diluted to 10^3 conidia ml^{-1}. Of each dilution, 100 μl was inoculated on the surface of a MEA plate, resulting in inoculation of 10^6, 10^5, 10^4, 10^3 and 10^2 conidia per plate. After 3 days incubation at 25°C, colonies were counted and pictures of the plates were made. The inactivation curves were described by a linear regression ($R^2 > 0.98$). *D* values were calculated by $\frac{-1}{\text{slope}}$.

Microscopy

Pictures of conidia were taken with a Zeiss AX10 microscope with 1000× magnification. Colonies surfaces of 3-day-old plates of *P. variotii* DTO 032-I3, DTO 212-C5 and DTO 217-A2 were studied by means of stereomicroscopy with a Nikon SMZ25. Both microscopes were equipped with a Nikon DS-Ri2 camera.

For cryo-electron scanning microscopy (cryoSEM) small rectangular $5 \times mm^2$ blocks of agar at the rim of colonies were carefully excised with a scalpel and transferred into a copper cup for rapid freezing in nitrogen slush. The agar blocks were glued with frozen tissue medium (KP-Cryoblock; Klinipath, Duiven, The Netherlands) into the copper cup, which was placed on the transfer rod. The sample was coated 3 min (3 times 1 min) using a gold target. Electron microscopy was done with a JEOL 5600LV scanning electron microscope (JEOL, Tokyo, Japan) equipped with an Oxford CT1500 Cryostation. Electron micrographs were taken at an acceleration voltage of 2.5–5 kV.

Spore-size distributions and measuring spore size

After harvesting the conidia, the spore suspensions were diluted 2×10^3 times in ISOTON II diluent (Beckman Coulter, Fichtenhain, Germany). A volume of 100 μl was used to determine spore size concentration and size distributions by a Beckman Coulter Counter Multisizer 3 equipped with a 70-μm aperture tube. Approximately 10^3 data points were extracted and used for further analysis. Size of particles is expressed by the equivalent spherical diameter in micrometer. Conidia with a diameter below 2 and above 8 μm were excluded from the data set. Particle volumes were calculated by $V = \frac{4}{3}\pi\left(\frac{\text{Diameter}}{2}\right)^3$. A concentration of 2×10^7 conidia ml^{-1} was used to measure the spore-size distribution by a FACS machine (FACSVerse™; Becton Dickinson, Franklin Lakes, NJ). The forward scatter values of the particles were used as size parameter. A thousand data points were extracted from the data set and used for further analysis.

Spore-size distributions were analysed by the R package mixtools (Benaglia *et al*., 2009). With the boot.comp function, we evaluated if we could describe the distributions by a two-component mixture model by a parametric bootstrap. The data were bootstrapped 10^3 times. Subsequently, we computed the mixture model using EM algorithm by the normalmixEM function. Next, a parametric bootstrap with 10^3 bootstrap samples was performed for standard error approximation of the parameters in the mixture model using the boot.se function.

Compatible solute concentrations

To extract the compatible solutes, 10^8 conidia were centrifuged 1 min at 21 000*g* at 4°C in an Eppendorf centrifuge. The supernatant was discarded and the pellet was flash-frozen in liquid nitrogen. After adding two stainless steel beads (diameter 3.2 mm), the tubes were transferred into pre-cooled adapters at −80°C and the pellet was homogenized for 6 min at 30 Hz in a Qiagen Tissuelizer. Subsequently, 1 ml of water was added to the broken conidia and samples were placed in a water bath at 95°C for 30 min. Samples were centrifuged at 20 817 g for 30 min at 4°C and the supernatant was filtered through an Acrodisc 0.2 μm nylon syringe filter (Pall Life Science, Mijdrecht, The Netherlands). Samples were stored at −20°C, prior to HPLC analysis.

The HPLC equipment consisted of a Waters 515 HPLC pump (0.6 ml min^{-1}) with control module, a Waters 717 plus Autosampler and a Waters IR 2414 Refractive

Index detector. To achieve a better separation between glycerol and mannitol, two Waters Sugar-Pak I columns were placed in line and kept at 70°C in a WAT380040 column heater module. A sample volume of 20 µl was injected in the mobile phase consisting of 0.1 mmol l^{-1} Ca EDTA in ultrapure water and samples were followed for 30 min. A mixture of trehalose, glucose, glycerol, erythritol, mannitol and arabitol (0.002% – 0.10% w/v) was used as reference. All calibration curves showed an R2 > 0.999 with a limit of detection <0.0005% (w/v) for each compatible solute. Empower software (Waters, Etten-Leur, The Netherlands) was used for peak integration and quantification. The mean volume of the conidia measured by the CC was used to calculate the concentration in mole per liter.

Germination under salt stress

To study germination under salt stress conditions, 10^4 conidia were inoculated into a 96 wells plate in 100 µl CM (de Vries *et al*., 2004) supplemented with an additional 0, 0.5, 1.0, 1.5 mol l^{-1} NaCl. Germination was followed in an oCelloScope (Philips BioCell A/S, Denmark) for 48 h at 25°C (Fredborg *et al*., 2013). To allow the conidia to sediment, measurements started after 1 h of incubation (Aunsbjerg *et al*., 2015). Pictures were made each 30 min with a 4× lens magnification factor and analysed with the UniExplorer software (version 8.1.0.7424, BioSense). The BCA normalized algorithm was used to quantify the optical growth at each data point. BCA normalized was further analysed using the R package grofit (Kahm *et al*., 2010). Maximum growth rate μ (ΔBCA hour^{-1}) was calculated by fitting a Gompertz curve to the BCA normalized data.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1 The results of screening 108 *P. variotii* strains for conidia heat resistance. The bars show number of colonies counted after heat treatment of the conidia at 58 °C (blue bars) and 59 °C (red bars). Average values of two plates ± SD are shown.

Figure S2 Conidia of various sizes produced by one colony of *P. variotii* CBS 121579. Conidia were formed in chains and are produced by the phialides. Two chains of conidia depicted by the arrows clearly contained conidia of different sizes. Scale bar indicates 10 μm.