1 **Weak Acid Resistance A (WarA), a novel transcription factor required for regulation of**
2 **weak-acid resistance and spore-spore heterogeneity in *Aspergillus niger***

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13 Running title: Weak acid resistance mechanisms in *A. niger* conidia

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16 Word count (main text, excl. legends, references and methods): 4241
17 Word count (Abstract): 234

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22 **ABSTRACT**

23 Propionic, sorbic and benzoic acids are organic weak acids that are widely used as food
24 preservatives, where they play a critical role in preventing microbial growth. In this study, we
25 uncovered new mechanisms of weak acid resistance in moulds. By screening a library of 401
26 transcription-factor deletion strains in *Aspergillus fumigatus* for sorbic acid hypersensitivity,
27 a previously uncharacterised transcription factor was identified, and named as WarA (Weak
28 Acid Resistance A). The orthologous gene in the spoilage mould *Aspergillus niger* was
29 identified and deleted. WarA was required for resistance to a range of weak acids, including
30 sorbic, propionic and benzoic acids. A transcriptomic analysis was performed to characterise
31 genes regulated by WarA during sorbic acid treatment in *A. niger*. Several genes were
32 significantly upregulated in the wild type compared with a Δ*warA* mutant, including genes
33 encoding putative weak acid detoxification enzymes and transporter proteins. Among these
34 was An14g03570, a putative ABC-type transporter which we found to be required for weak
35 acid resistance in *A. niger*. We also show that An14g03570 is a functional homologue of the
36 *Saccharomyces cerevisiae* protein Pdr12p, and therefore named as PdrA. Lastly, resistance to
37 sorbic acid was found to be highly heterogeneous within genetically-uniform populations of
38 ungerminated *A. niger* conidia, and we demonstrate that *pdrA* is a determinant of this
39 heteroresistance. This study has identified novel mechanisms of weak acid resistance in *A.*
40 *niger* which could help to inform and improve future food spoilage prevention strategies.

41

42 **IMPORTANCE**

43 Weak acids are widely used as food preservatives, as they are very effective at preventing
44 growth of most species of bacteria and fungi. However, some species of moulds can survive

45 and grow in the concentrations of weak acid employed in food and drink products, thereby
46 causing spoilage with resultant risks for food security and health. Current knowledge of weak
47 acid resistance mechanisms in these fungi is limited, especially in comparison to that in yeasts.
48 We characterised gene functions in the spoilage mould species *Aspergillus niger* which are
49 important for survival and growth in the presence of weak acid preservatives. Such
50 identification of weak acid resistance mechanisms in spoilage moulds will help to design new
51 strategies to reduce food spoilage in the future.
52

53 **INTRODUCTION**

54

55 Microbiological spoilage of food and drinks is a serious threat to food security and human
56 health. It is estimated that 25% of global food produced annually is lost due to contamination
57 and degradation by microorganisms (1). Such spoilage also directly imperils human health, for
58 example due to the production of toxins by the microorganism. Preventing microbial spoilage
59 is therefore a key to safeguarding food supply and safety. The use of chemical food
60 preservatives to inhibit growth of bacteria and fungi is a ubiquitous and generally effective
61 strategy in reducing spoilage (2). Some of the most commonly used preservatives are weak
62 organic acids such as propionic, sorbic and benzoic acids. These are usually included in food
63 and drink products in the form of calcium, potassium and sodium salts, respectively.

64 Weak acid preservatives are broad-spectrum antimicrobials that directly inhibit the
65 growth of yeasts, moulds and bacteria. Although their precise mechanism of action has not
66 yet been fully determined, it is known that weak acid preservatives cause a reduction of
67 cytoplasmic pH (3, 4), and inhibit nutrient uptake (5, 6). It is also known that weak acids tend
68 to be fungistatic rather than fungicidal, especially at the levels legally permitted in food and
69 drinks. In most cases, microbial growth is completely inhibited by the weak acid levels used
70 in food and drink products. However, certain species of yeasts and moulds demonstrate
71 elevated resistance to weak acids, and are therefore capable of causing food spoilage (7, 8).

72 Weak acid resistance can be attributed in part to the enzymatic degradation of certain
73 acids (e.g., benzoic, sorbic and cinnamic acids), which nullifies their antimicrobial effects.
74 Benzoate can be catabolized through a pathway involving an initial hydroxylation step. The
75 enzyme responsible (benzoate *para*-hydroxylase) has been found to be required for
76 resistance to benzoic acid in *Aspergillus niger* and *A. nidulans* (9, 10). Sorbic and cinnamic

77 acids (and certain other structurally related acids) are degraded by decarboxylation (11). In
78 moulds such as *A. niger*, a cluster of three genes is required for this process, encoding a
79 transcription factor (SdrA), a decarboxylase (CdcA; formerly OhbA1 or FdcA) and a
80 prenyltransferase (PadA) (12-15). Deletion of any of these genes reduces, but does not
81 eliminate, resistance to sorbic acid. Thus, additional and as-yet uncharacterized mechanisms
82 of weak acid resistance must operate in this mould species. Enzymatic decarboxylation of
83 weak acids also occurs in numerous yeast species (16). However, contrary to the case in
84 moulds, deletion of the phenylacrylic acid decarboxylase gene (*PAD1*) in the yeast *S. cerevisiae*
85 does not decrease weak acid resistance (16). Furthermore, certain spoilage yeasts do not
86 appear to decarboxylate weak acids at all, suggesting that alternative mechanisms of
87 resistance also operate in these species.

88 Mechanisms of weak acid resistance have been best characterized in *S. cerevisiae*. One
89 of the key genes required for resistance is *PDR12*, encoding an ATP-Binding Cassette (ABC)
90 transporter (17). *PDR12* is required for resistance to carboxylic acids with chain lengths
91 between 1 and 7, proposedly by mediating the efflux of weak acid anions from the cell in an
92 energy-dependent manner (18). *PDR12* is itself transcriptionally regulated by War1p, a
93 Zn2Cys6 zinc finger transcription factor that binds to weak acid response elements (WARE) in
94 the *PDR12* promoter (19). Another transcription factor, Haa1p, is also required for resistance
95 to weak acids in *S. cerevisiae*, by regulating the transcription of membrane multidrug
96 transporters (Tpo2p and Tpo3p) amongst other, less well characterized genes (20). High-
97 throughput mutant screens have helped to identify many other genes which influence weak
98 acid resistance in *S. cerevisiae*. For example, Mollapour et al. (21) reported 237 genes which
99 were required for wild-type resistance to sorbic acid, and a further 34 which resulted in
100 enhanced sorbic acid resistance when deleted. A similar study, also in *S. cerevisiae,* revealed

101 650 determinants of acetic acid resistance (22). Unfortunately, there is a distinct lack of
102 equivalent data in any other fungal species, including moulds. Considering the propensity of
103 mould fungi to cause food spoilage, understanding the genetic determinants of weak acid
104 resistance in these species is very important.

105 An additional and historically-overlooked determinant of antimicrobial resistance is
106 the phenotypic heterogeneity that exists within microbial cell populations. Phenotypic
107 heterogeneity is a phenomenon observed within isogenic cell populations, whereby individual
108 cells can display a markedly different phenotype despite being genetically identical. This has
109 been recognised as an important determinant of microbial cell survival in response to
110 antimicrobial agents and other environmental stressors (23-25). Phenotypic heterogeneity in
111 weak acid resistance (heteroresistance) has been found in cell populations of *S. cerevisiae* and
112 the spoilage yeast *Zygosaccharomyces bailli* (8, 26, 27). However, there has been no
113 investigation to date of whether weak acid resistant subpopulations exist in populations of
114 mould spores, although heterogeneity is known to arise in *A. niger* spore populations as a
115 consequence of asynchronous conidial maturation (28). The presence of weak acid resistant
116 spore subpopulations could have significant implications for spoilage control strategies and is
117 therefore worthy of investigation.

118 In this study, we report the identification and characterization of a novel transcription
119 factor (Weak Acid Resistance A), that is required for resistance to weak acid preservatives in
120 *A. niger* and *A. fumigatus*. Furthermore, we identify and characterize genes that are putatively
121 regulated by WarA, including a gene encoding a putative membrane transporter protein with
122 similarity to *S. cerevisiae* Pdr12p and which, we show, mediates weak acid resistance and
123 heteroresistance in *A. niger*. These data significantly enhance our understanding of weak acid
124 resistance in moulds, and highlight both similarities and differences in weak acid resistance

125 strategies between yeast and mould fungi. (This article was submitted to an online preprint

126 archive (29))

127 **RESULTS**

128

129 **A screen for transcription-factor deletion strains sensitive to sorbic acid identifies**
130 ***warA.*** To find genes associated with weak acid resistance, an *Aspergillus fumigatus*
131 transcription-factor deletant collection (30) was screened for sorbic acid sensitivity.
132 *Aspergillus fumigatus* is not commonly associated with food spoilage, and displays relatively
133 high sensitivity to weak acids such as sorbic acid (14). However, deletant collections are
134 available in *A. fumigatus*, unlike Aspergilli associated with food spoilage. It was reasoned that
135 transcription factors associated with weak acid resistance in *A. fumigatus* may be conserved
136 in related spoilage species such as *A. niger*. This resource comprised a library of 401 deletion
137 strains of non-essential transcription factors. To determine sensitivity of the deletion strains,
138 radial growth was compared on agar medium with and without sorbic acid (Fig. 1). This
139 revealed two deletion strains which were highly sensitive to sorbic acid compared with the
140 wild-type strain (Δ*metR*, and Δ*AFUB_000960*) (Fig. 1 and Fig. 2A). A number of other strains
141 exhibited moderate sensitivity to sorbic acid, including Δ*creA* (Δ*AFUB_027530*), Δ*devA*
142 (Δ*AFUB_030440*) (Fig. 1B and Fig. 2A), Δ*rfeD* (*ΔAFUB_022280*), Δ*AFUB_020350* and
143 Δ*AFUB_054360* (Fig. 1B).

144 MetR is a bZIP-type transcription factor mediating transcriptional regulation of genes
145 involved in sulphur uptake and utilization (31). Because sorbic acid is known to decrease
146 cellular uptake of some nutrients (6, 32), it was hypothesised that sulphur limitation could be
147 a cause of sorbic acid sensitivity in the Δ*metR* deletion strain. To test this, sorbic acid
148 sensitivity of the Δ*metR* strain was determined in medium supplemented with the sulphur-
149 containing amino acid methionine. This showed that the sorbic acid sensitivity of the Δ*metR*
150 strain was abolished in the presence of supplementary methionine (Fig. S1).

151 *AFUB_000960* encodes a Zn2Cys6-type transcription factor which contains a fungal
152 specific transcription factor domain (PF11951). To further investigate the role of
153 *AFUB_000960* in weak acid resistance, sensitivity of the deletion strain to a range of weak
154 acids was evaluated (Fig. 2B). The Δ*AFUB_000960* strain was sensitive to propionic, butanoic,
155 pentanoic, hexanoic, sorbic and benzoic acids, but not to acetic acid. Because of these
156 phenotypes, *AFUB_000960* was named *Weak Acid Resistance A* (*warA)*.
157

158 **A WarA orthologue in *A. niger* is important for weak acid resistance** To determine
159 whether orthologues of WarA are present in other species of fungi, a BLAST-search using the
160 *Afu*WarA protein sequence as a query was conducted. In *A. niger,* this identified An08g08340,
161 a protein with 46.2% identity and 63% similarity to the *A. fumigatus* WarA protein.
162 Orthologues of *AfuwarA* were also found to be present in *Penicillium* and *Botrytis spp.*
163 *Aspergillus niger* is highly resistant to weak acids (especially sorbic and benzoic acids),
164 and can readily cause food and drink spoilage. To determine whether *warA* is also required
165 for weak acid resistance in *A. niger*, *An08g08340* was deleted by a targeted gene replacement
166 approach, with successful deletion confirmed by PCR and Southern Blotting (Fig. S2).
167 Sensitivity of the Δ*An08g08340* deletion strain to different weak acids was evaluated (Fig. 3).
168 In contrast to the ΔwarA deletant of *A. fumigatus*, the *A. niger* Δ*An08g08340* strain
169 demonstrated only slight sensitivity to sorbic acid, which was most apparent when individual
170 conidia of the Δ*warA* strain were spread onto medium containing sorbic acid (Fig. S3A).
171 However, the deletant was highly sensitive to propionic, butanoic and benzoic acids as
172 evaluated by radial growth on agar (Fig. 3). Determination of Minimum Inhibitory
173 Concentrations (MIC) in broth also corroborated the radial growth data; the MIC in benzoic
174 acid was 4.5 ± 0.5 mM in the wild type (WT; n=3), compared with 3.2 ± 0.2 mM in Δ*warA*

175 (n=3). The MIC in butanoic acid was 8.6 ± 0 mM in the WT (n=2), and 6.9 ± 0.3 mM in Δ*warA*
176 (n=3). Resistance was restored to WT levels when *An08g08340* was reintroduced into the
177 Δ*An08g08340* strain (Fig. S4). Thus, *An08g08340* has an important role in weak acid resistance
178 in *A. niger*, and was named as *warA* (Weak Acid Resistance A) also in this species.
179 Sorbic acid (and structurally related acids) are known to be detoxified by
180 decarboxylation in *A. niger*, but not in *A. fumigatus* (14, 15). The decarboxylation process
181 involves three linked genes: *cdcA*, *padA* and *sdrA* (12, 14). CdcA is the key enzyme involved in
182 the decarboxylation, whereas SdrA is a transcription factor regulating expression of CdcA and
183 PadA synthesizes a cofactor for CdcA. It was hypothesized that mild sorbic acid sensitivity of
184 *A. niger* Δ*warA* may be due to downregulation of *cdcA*, *padA* or *sdrA* genes in the Δ*warA*
185 strain. To investigate the relationship between WarA and weak acid decarboxylation, a
186 ΔΔ*cdcA/warA* mutant was constructed. The ΔΔ*cdcA/warA* strain was more sensitive to sorbic
187 acid than the Δ*cdcA* strain (Fig. S3B), suggesting a *cdcA*-independent role for *warA* in
188 resistance of *A. niger* to sorbic acid.
189
190 **Determination of WarA-regulated genes by transcriptomic analysis during weak acid**
191 **stress of *A. niger*.** The weak acid sensitivity of the Δ*warA* mutant suggested that WarA
192 regulates genes which are important for weak acid resistance. Previously, genes upregulated
193 by sorbic acid exposure in *A. niger* were successfully identified by exposing conidia of the wild-
194 type (WT) to sorbic acid during germination (6). In order to identify which genes are
195 differentially regulated in *A. niger* Δ*warA*, RNA-seq analysis was conducted with WT and
196 Δ*warA* conidia germinated in the presence or absence of sorbic acid. Germination of WT
197 conidia for 1 h in the presence of 1 mM sorbic acid resulted in 3,274 differentially expressed
198 genes (FDR adjusted p-value < 0.05) (1885 upregulated, 1467 downregulated) in comparison

199 with conidia germinated in the absence of sorbic acid. In Δ*warA* conidia, 3,442 genes were
200 differentially expressed during germination in the presence of sorbic acid (1885 upregulated,
201 1,557 down regulated), in comparison with germination without sorbic acid. Importantly, a
202 number of genes were identified that were highly upregulated in the WT during sorbic acid
203 exposure, but not in Δ*warA* (Table 1 and Table S1). This included a gene encoding benzoate
204 *para*-hydroxylase (*bphA*), an enzyme known to be required for benzoate detoxification (9),
205 which had a Log Fold Change (Log_2FC) of 6.50 in the WT, compared with a Log_2FC of -0.51 in
206 Δ*warA.* A number of uncharacterized enzymes also required *warA* for normal upregulation by
207 sorbic acid, e.g., An12g09130 encoding a putative dienelactone hydrolase, and An12g02790
208 a putative isoflavone reductase (Log_2FC 5.95 in WT, Log_2FC 0.36 in Δ*warA*), as well as several
209 genes encoding putative transporter proteins. Of particular interest amongst these
210 transporters was An14g03570, an ABC-type transporter with 56% amino acid sequence
211 similarity to *S. cerevisiae* Pdr12p. Pdr12p has a crucial role in weak acid detoxification in *S.*
212 *cerevisiae* (17, 18). To support the RNA-seq data, five of the genes showing differential
213 expression between the WT and Δ*warA* strains were selected for qRT-PCR analysis (Fig. 4).
214 The qRT-PCR data supported the trends in gene expression seen in the RNA-seq dataset; all
215 the selected genes had a lower transcript abundance in Δ*warA*, compared with the WT during
216 sorbic acid treatment. In addition, transcript abundances of the selected genes were
217 compared by qRT-PCR during benzoic acid treatment. As expected, all the genes upregulated
218 by sorbic acid were also upregulated by benzoic acid, and had lower transcript abundances in
219 the Δ*warA* mutant, compared with the WT (Fig. 4).
220
221 **Characterization of *An02g09970* and *An14g03570.*** The transcriptomic analysis
222 identified genes that are downregulated in *A. niger* Δ*warA*, relative to the WT strain, during

223 weak acid stress. These genes may therefore have a role in weak acid resistance. To
224 investigate this, two genes of interest (*An02g09970* and *An14g03570*) were selected for
225 further characterization. *An02g09970* encodes a putative transmembrane transporter of the
226 Major Facilitator Superfamily (MFS), and was selected for further investigation due to its
227 extremely high transcript abundance during sorbic acid treatment, and large disparity in
228 transcript abundance between the WT and Δ*warA* strains (Log_2FC 8.56 in WT vs. Log_2FC 4.35
229 in Δ*warA*) (Table 1). The protein also shares significant sequence similarity with Tpo2 and
230 Tpo3, *S. cerevisiae* proteins involved in resistance to acetic, propionic and benzoic acids (20).
231 *An14g03570* encodes an ABC-type transporter with similarity to the weak acid detoxification
232 protein Pdr12p in *S. cerevisiae*, as stated above. Both genes were deleted in *A. niger* by a
233 targeted gene replacement approach, and mutant genotypes confirmed by PCR and Southern
234 blotting (Fig. S5). Sensitivity of the constructed deletion strains to weak acids was then
235 evaluated. The Δ*An02g09970* mutant did not exhibit altered sensitivity to any of the weak
236 acids tested (Fig. 5). However, the Δ*An14g03570* mutant was more sensitive to sorbic,
237 pentanoic and benzoic acids (Fig. 5 and Table 2), and resistance was restored to WT levels
238 when *An14g03570* was reintroduced into the Δ*An14g03570* strain (Fig. S6). Because of the
239 similarity in sequence and function between An14g03570 and Pdr12p, An14g03570 was
240 named PdrA.

241 **Complementation of *S. cerevisiae* Δ*pdr12* strain with PdrA (An14g03570).** The above
242 results showed that *pdrA* is required for resistance of *A. niger* to certain weak acids. Because
243 PdrA has significant protein sequence similarity with *S. cerevisiae* Pdr12p (36% sequence
244 identity, 56% similarity), it was hypothesized that these proteins could be functional
245 homologues. To test this hypothesis, functional complementation of the *S. cerevisiae* Δ*pdr12*
246 strain was attempted. The cDNA sequence of *A. niger pdrA* was cloned between the *S.*

247 *cerevisiae PDR12* promoter and terminator to allow for native regulation of *pdrA* in response
248 to weak acid stress in *S. cerevisiae*. The resulting plasmid (Fig. S7) was transformed into a *S.*
249 *cerevisiae* Δ*pdr12* deletion strain. Transformants were tested for sensitivity to a range of weak
250 acids. As hypothesized, *pdrA* could indeed functionally complement *PDR12*: sensitivity of the
251 Δ*pdr12* strain to weak acids was largely rescued in cells transformed to express *pdrA* (Fig. 6).
252 The resultant level of resistance was similar to that evident in Δ*pdr12* cells expressing *PDR12*
253 from the same vector backbone.
254
255 ***pdrA* is a determinant of heteroresistance to sorbic acid in *A. niger* conidia.**
256 Resistance to weak acid preservatives has been found to be heterogeneous between
257 individual cells of genetically-uniform cell populations of the yeasts *Zygosaccharomyces bailii*
258 and *S. cerevisiae* (8, 26, 27). We observed a similar phenomenon in populations of *A. niger*
259 conidia, whereby small subpopulations were capable of germinating and forming colonies at
260 high concentrations (>4 mM) of sorbic acid (Fig. 7A). Conidia harvested from colonies that
261 grew on these high concentrations of sorbic acid did not retain increased resistance upon
262 direct re-inoculation to sorbic acid-containing medium (data not shown), suggesting that
263 these were transient, non-heritable phenotypes, i.e., not due to genotypic variants within the
264 population. To test whether this heteroresistance had its origin in the ungerminated conidial
265 state, conidia were also pre-germinated for 6 hr, before spread plating onto sorbic acid-
266 containing medium. This showed that germinated conidia were much more susceptible to
267 sorbic acid (Fig. 7A). Moreover, resistance to sorbic acid among pre-germinated conidia was
268 much more homogeneous than when the resistance-assay commenced with ungerminated
269 conidia, as evidenced by the gradients of the dose inhibition curves: such dose response
270 curves reflect heterogeneity, with shallower curves indicating greater heterogeneity (23, 33).

271 Thus, at least some factors determining heteroresistance to sorbic acid are specific to
272 ungerminated conidia, and are lost upon germination. Given the contributions of *warA* and
273 *pdrA* to sorbic acid resistance in *A. niger*, it was tested whether these genes could be
274 determinants of heteroresistance. Dose response curves of Δ*pdrA* and Δ*warA* conidia
275 demonstrated that *pdrA* makes a significant contribution to sorbic acid heteroresistance in *A.*
276 *niger* conidia, whereas *warA* does not (Fig. 7B, data for Δ*warA* not shown).
277

278 **DISCUSSION**

279

280 This study reports the discovery of novel factors determining weak acid resistance in moulds.
281 By screening >400 transcription factor deletion strains in *A. fumigatus*, we discovered a
282 previously uncharacterized transcription factor that is required for resistance to certain weak
283 organic acids. This transcription factor, here named *Weak Acid Resistance A* (*warA*) was also
284 found to be present in the food spoilage mould *A. niger*, where it also plays an important role
285 in weak acid resistance. However, WarA appears to mediate resistance to different weak acids
286 in *A. fumigatus* and *A. niger*. In *A. fumigatus*, the Δ*warA* strain is particularly sensitive to
287 linear-chain acids 3-6 carbons in length, whereas in *A. niger* the Δ*warA* strain is most sensitive
288 to propionic, butanoic and benzoic acids, but exhibited less sensitivity to 5 and 6 carbon acids.
289 Such differences in acid sensitivity may reflect differences in the WarA regulon between *A.*
290 *niger* and *A. fumigatus*, or the divergence in gene function within the WarA regulon.

291 We sought to gain insight to the WarA regulon in *A. niger* by conducting a comparative
292 transcriptomics experiment between germinating WT and Δ*warA* conidia treated with sorbic
293 acid. This approach identified several genes that appear to be regulated (either directly or
294 indirectly) by WarA. These include a number of putative enzymes and transporter proteins,
295 offering several candidates for future studies of weak acid resistance mechanisms in *A. niger*.
296 Amongst these candidates, we attempted to characterize two putative transporter-protein
297 genes. The first of these functions, An02g09970, is a transporter of the Major Facilitator
298 Superfamily, with sequence similarity to Tpo2p and Tpo3p in *S. cerevisiae*. Tpo2p and Tpo3p
299 are transporters of the DHA1 (Drug:H+ antiporter-1) family and are known to be required for
300 resistance to acetic, propionic and benzoic acids (20). However, deletion of *An02g09970* did
301 not sensitize *A. niger* to any of the acids tested, and so the role of this gene remains unknown.

302 It is possible that this transporter is responsible for detoxification of other xenobiotics not
303 tested here (if indeed it has a role in detoxification at all), or that *An02g09970* is functionally
304 redundant with other *A. niger* genes. We also attempted to characterize *pdrA*, encoding an
305 ABC-type transporter. Deletion of *pdrA* resulted in increased sensitivity to pentanoic,
306 hexanoic, sorbic and benzoic acids, substantiating a role for this protein in weak acid
307 resistance. Importantly, we were able to demonstrate that *pdrA* is a functional homologue of
308 *PDR12* in *S. cerevisiae*. Pdr12p is a key protein involved in weak acid resistance of *S. cerevisiae*
309 (17), where it is thought to efflux weak acid anions from the cytoplasm in an energy-
310 dependant manner (18). The identification of PdrA as a functional homologue of Pdr12p in a
311 mould species such as *A. niger* shows that a similar mechanism of weak acid detoxification by
312 active efflux may operate in yeasts and moulds. Interestingly, the Δ*pdr12* functional
313 complementation experiment demonstrated that *pdrA* confers resistance to a broader range
314 of weak acids than was suggested by the weak acid sensitivity of the Δ*pdrA* strain. For
315 example, *pdrA* complemented the propionic acid sensitivity of *S. cerevisiae* Δ*pdr12*, but the
316 *A. niger* Δ*pdrA* strain was not more sensitive to propionic acid than the WT. This may indicate
317 the presence of multiple, redundant mechanisms for resistance to certain weak acids in
318 *A.niger*, which may not operate in *S. cerevisiae*.

319 *pdrA,* as well as several other candidate WarA-regulated genes were all upregulated
320 in response to both sorbic and benzoic acids. This suggests a degree of overlap between
321 transcriptomic responses to different weak acids, as also found in *S. cerevisiae* (34). Thus,
322 although we characterized the WarA regulon by comparative transcriptomics in response only
323 to sorbic acid in the present study, it is likely that many of the differentially expressed genes
324 would be similarly regulated in response to other weak acids. There may be relevant
325 consensus sequences within WarA-regulated genes, although these are not apparent from

326 promoter sequence alignments we have carried out. In *S. cerevisiae*, a *cis*-acting weak acid
327 response element (WARE) was discovered in the promoter of *PDR12* which is required for
328 *PDR12* induction by the transcription factor War1p (19).

329 The regulation of WarA itself is also an outstanding question. Recent evidence in *S.*
330 *cerevisiae* suggests that weak acid anions bind directly to the transcription factors War1p and
331 Haa1p, thereby regulating their DNA-binding transcriptional activation (35). However, WarA
332 shares very little sequence homology with either War1p or Haa1p. In fact, a BLAST search of
333 the *S. cerevisiae* protein database with the WarA protein sequence yields no hits at all.
334 Nevertheless, a similar mechanism of transcription factor activation cannot be ruled out for
335 WarA, particularly as direct ligand binding has been established for a number of Zn2Cys6
336 family transcription factors (of which WarA is a member), including Pdr1p, Pdr3p, Leu3p and
337 Put3p (36-38).

338 During the course of this study, experiments with sorbic acid determined that
339 genetically-uniform populations of *A. niger* conidia demonstrate heteroresistance to this
340 weak acid. Phenotypic heterogeneity within microbial cell populations has been
341 demonstrated in a number of fungi in response to environmental stresses [reviewed in (23)],
342 however this is the first report of weak acid heteroresistance in fungal conidia. Interestingly,
343 heteroresistance was decreased within 6 h of conidial germination, suggesting that at least
344 some factors underlying this heterogeneity are limited to ungerminated conidia and are lost
345 upon germination. Resistance to sorbic acid was also markedly lower in germinated conidia,
346 which has also recently found to be the case for propionic acid (39). Heteroresistance to weak
347 acids in fungal conidia has significant implications for the food industry, because spoilage of
348 products may occur due to contamination with just a few conidia from a highly resistant
349 subpopulation. Thus, future spoilage control strategies may have to take into account the

350 presence of weak acid heteroresistance, perhaps by specifically targeting resistant
351 subpopulations.

352 Conidia of the Δ*pdrA* strain showed a significantly more homogeneous response to
353 sorbic acid. Heteroresistance typically arises from gene expression heterogeneity (or noise)
354 (40), so the present results suggest that *pdrA* could be expressed heterogeneously within
355 conidial populations; the conidia expressing more *pdrA* potentially able to withstand sorbic
356 acid stress. It is also noted that deletion of *pdrA* did not eliminate sorbic acid heteroresistance
357 in *A. niger*, so it likely that other genes also contribute.

358 In summary, this study markedly advances our understanding of weak acid resistance
359 mechanisms in *A. niger*. The identification of WarA as a key transcription factor involved in
360 weak acid resistance allowed us in turn to identify many more genes which may also be
361 important. Further work is required to determine how all these genes may contribute to weak
362 acid resistance. Moreover, we demonstrated here that a key weak acid resistance mechanism
363 operates in both *S. cerevisiae* and *A. niger*, in the form of the functionally homologous ABC-
364 transporters Pdr12p and PdrA respectively.

365

366 **MATERIALS AND METHODS**

367

368 **Strains and media.** The *Aspergillus fumigatus* transcription factor deletant library,
369 derived from wild-type strain MFIG001, was constructed by homologous recombination using
370 gene replacement cassettes and transformation methodologies as described (30, 41). Studies in
371 *Aspergillus niger* were performed in the *A.niger* N402 background (referred to as the *A. niger*
372 WT throughout) and an *A. niger ΔcdcA* deletion strain (14). *Aspergillus* strains were cultivated
373 on slopes of Potato Dextrose Agar (PDA) (Sigma) for 7 days at 28°C. Conidia were harvested
374 using 0.1% (v/v) Tween 80 and filtered through a 40 µm cell strainer (Fisher), before counting
375 on a haemocytometer. Studies in *S. cerevisiae* used the BY4743 background and isogenic
376 *Δpdr12* deletion strain, cultivated on YEPD agar (2% glucose, 2% bactopeptone (Oxoid), 1%
377 yeast extract (Oxoid), 1.5% agar) at 30°C. The *S. cerevisiae* strains were obtained from
378 EUROSCARF (Frankfurt). Growth assays with weak acids (below) were performed on YEPD
379 agar (pH 4).

380

381 **Deletant-library screening and growth assays.** The first round of *A. fumigatus*
382 transcription factor deletion library screening was performed in a 96-well array format.
383 Conidial suspensions of the *A. fumigatus* strains were initially supplied in 40% glycerol-0.01%
384 PBS solution at a concentration of 4 x 10^7 ml^{-1}. These were subsequently arrayed in 96-well
385 plates at a concentration of 4 x 10^5 conidia ml^{-1} in 0.01% Tween 20, and transferred using a
386 96-pin tool, to Nunc™ Omnitray™ single-well plates containing YEPD agar (pH 4), then
387 incubated at 28°C for 2 - 3 days. Radial growth was measured using ImageJ, and compared
388 between control medium, and medium containing sorbic acid. The second round of screening
389 was performed on 90 mm Petri dishes. Plates were inoculated with 10^5 conidia, and incubated

390 at 37°C for 3 days. Radial growth was compared between control medium (YEPD) and the
391 same medium containing sorbic acid.

392 Subsequent growth assays with weak acids on solid medium were performed in
393 *Aspergillus* spp. by inoculating YEPD agar (pH 4) supplemented with weak acids, with 5 µl of
394 conidial suspension, containing 10^5-10^2 conidia and subsequent incubation at 28°C for 2 – 3
395 days. Weak acid concentrations used for *A. fumigatus* were: 15 mM acetic acid , 4 mM
396 propionic acid, 1.5 mM butanoic acid, 0.75 mM pentanoic acid, 0.2 mM sorbic acid, 0.25 mM
397 hexanoic acid, 0.5 mM benzoic acid, 0.08 mM heptanoic acid, 0.05 mM octanoic acid. Weak
398 acid concentrations used for *A. niger*: 40 mM acetic acid, 10 mM propionic acid, 4 mM
399 butanoic acid, 2 mM pentanoic acid, 1.5 mM sorbic acid, 2 mM hexanoic acid, 2 mM benzoic
400 acid, 1 mM heptanoic acid, 0.75 mM octanoic acid.

401 Minimum inhibitory concentrations (MICs) of weak acids were determined by placing
402 10 ml of YEPD broth (pH 4) into 30 ml McCartney bottles, and inoculating with 10^4 conidia.
403 Bottles were incubated statically at 28°C for 28 days, and the concentration of acids required
404 to completely inhibit visible growth recorded. Concentrations of acids used were at 0.2 mM
405 increments for benzoic and sorbic acids, 0.3 mM increments for pentanoic and butanoic acids,
406 and 2 mM increments for propionic acid.

407 Dose response curves were generated by harvesting conidia as stated above, diluting
408 to 500 spores ml^{-1} and spreading 200 µl of this onto YEPD agar (pH 4) containing sorbic acid.
409 Plates were incubated at 28°C for up to 28 days and colonies counted. For pre-germinated
410 conidia, conidia were first inoculated into 10 ml of YEPD/Tween 80 (6.66 ml YEPD/3.33 ml 0.1
411 % Tween 80) to a final concentration of 500 spores ml^{-1} and incubated statically at 28°C for 6
412 hr, before spread plating and incubation as above. For quantitative comparison of
413 heteroresistance, Hill slopes were fitted to plots (% Viability vs. log10[sorbic acid]) using Prism

414 software and arctangent values for the slopes calculated with Excel to estimate relative
415 heterogeneity (a shallower slope indicating higher heterogeneity) (33, 42).

416 **RNAseq and qRT-PCR.** For preparation of RNA, conidia of *A. niger* N402 were
417 inoculated into 1 litre of YEPD broth (pH 4) to a final concentration of 10^6 conidia ml^{-1} and
418 incubated at 28°C for 1 hr, with shaking at 150 rev. min^{-1}. For sorbic acid or benzoic acid
419 treatments, the medium was supplemented with 1 mM sorbic or 1 mM benzoic acid for the
420 1 h incubation: these concentrations inhibit conidial germination over the course of the
421 experiment, but are not lethal (~25% of the MIC values for these acids). Conidia were
422 harvested by filtration through a Corning vacuum filtration unit, and immediately used for
423 RNA extraction. RNA was extracted using a Norgen Biotek Plant/Fungi Total RNA extraction
424 kit, as per the manufacturer's instructions.

425 RNAseq analysis was performed by the University of Liverpool Centre for Genomic
426 Research. Three biological replicates were performed for each timepoint in each condition.
427 Between 463-1000ng of total RNA (depending on available material) was Poly A-treated using
428 the NEBNext® Poly(A) mRNA Magnetic Isolation Module, and subsequently purified using
429 Ampure RNA XP beads. Successful depletion of rRNA was confirmed using Qubit fluorometric
430 quantification (ThermoFisher) and Agilent 2100 Bioanalyzer. All of the depleted RNA was used
431 as input material for the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina®.
432 Following 15 cycles of amplification the libraries were purified using Ampure XP beads. Each
433 library was quantified using Qubit and the size distribution assessed using the Bioanalyzer.
434 These final libraries were pooled in equimolar amounts using the Qubit and Bioanalyzer data.
435 The quantity was assessed using a Qubit® dsDNA HS Assay Kit, while the quality and average
436 fragment size was assessed using the High Sensitivity DNA Kit on the Agilent Bioanalyzer. The
437 RNA libraries were sequenced on an Illumina® HiSeq 4000 platform with version 1 chemistry

438 using sequencing by synthesis (SBS) technology to generate 2 x 150 bp paired-end reads.
439 Initial processing and quality assessment of the sequence data was performed as follows.
440 Briefly, base calling and de-multiplexing of indexed reads was performed by CASAVA version
441 1.8.2 (Illumina). The raw FASTQ files were trimmed to remove Illumina adapter sequences
442 using Cutadapt version 1.2.1 (43). The option "-O 3" was set, so the 3' end of any reads which
443 matched the adapter sequence over a stretch of at least 3 bp was trimmed off. The reads
444 were further trimmed to remove low quality bases, using Sickle version 1.200 with a minimum
445 window quality score of 20. After trimming, reads shorter than 20 bp were removed. Reads
446 were aligned to the *A.niger* CBS 588.13 genome sequence
447 (http://www.aspergillusgenome.org/download/sequence/A_niger_CBS_513_88/current/A_
448 niger_CBS_513_88_current_chromosomes.fasta.gz) using Tophat version 2.1.0 (44). The
449 expression of each gene was calculated from the alignment files using HTseq-count (45). The
450 raw count data were also converted into FPKM (Fragments per Kilobase per Million reads)
451 values. The count numbers per gene were used during the subsequent differential expression
452 analysis. All of the DGE (Differential Gene Expression) analyses were performed in R (version
453 3.3.3) environment using the DESeq2 package (46). Significantly differentially-expressed
454 genes were defined as those with FDR-adjusted P-value < 0.05.
455 For qRT-PCR analysis of gene expression, RNA was extracted as stated above. Genomic
456 DNA was removed by Turbo DNAse Free kit (Invitrogen). cDNA was synthesized using
457 Superscript IV reverse transcriptase (Invitrogen) and Oligo $d(T)_{20}$ primer (Invitrogen),
458 according to the manufacturer's instructions. Transcripts were amplified using SYBR green
459 Master Mix on an Applied Biosystems 7500 Real-Time PCR instrument and quantified against
460 a standard curve of *A. niger* gDNA. Primer pairs used are listed in Table S2.
461

462 **Gene deletion studies and complementation in *A. niger.*** Gene deletion studies were
463 performed in *A. niger* N402, the Open Reading Frames (ORFs) of the target genes being
464 replaced by a hygromycin resistance cassette (Fig. S2 and S6). Gene deletion cassettes were
465 constructed by Gap-Repair cloning in *S. cerevisiae* (47). Briefly, the hygromycin resistance
466 cassette, and approximately 1 kb upstream and downstream flanking regions of each target
467 gene were amplified from genomic DNA by PCR (Primers listed in Table S2). The hygromycin
468 resistance cassette had a 20-30 bp homology with the 1 kb flanking regions, and each flanking
469 region also had 20-30 bp homology with the multiple cloning site of the YEp351 plasmid. The
470 PCR products and *Hin*dIII-linearized YEp351 plasmid were transformed into *S. cerevisiae*
471 BY4743, and transformants selected by leucine prototrophy. Successful construction of the
472 gene deletion cassettes was confirmed by PCR. The resulting gene deletion cassettes were
473 amplified by PCR and purified using PCR purification columns (Machery-Nagel), to produce a
474 final linear gene deletion cassette. All PCR reactions were performed using Phusion High-
475 Fidelity DNA Polymerase (New England Biolabs). Production of protoplasts and their
476 transformation was performed using standard methods (48). Transformants were selected
477 using 200 μg ml^{-1} hygromycin (Roche) and confirmed by PCR and Southern blotting (Fig. S2
478 and S6), using standard methods (49).

479 For complementation of *A. niger* gene-deletion strains, the genes in question (*warA*
480 and *pdrA* (*An14g03570*)) were amplified by PCR (primers listed in Table S2) and cloned into
481 the *Sbf*I site of the pAN7.1*BAR* plasmid (Fig. S8), which contains the *BAR* gene as a selectable
482 marker (replacing the original hygromycin resistance cassette (50), and imparting resistance
483 to phosphinothricin). PCR amplification included ~1 kb upstream and ~300bp downstream of
484 the ORF. Transformation of the resulting plasmids was performed as described above, except
485 that transformants were selected using 5 mg ml^{-1} DL-phosphinothricin (Carbosynth) in YDA

486 agar (Yeast nitrogen base without amino acids, including 1.7 g l^{-1} ammonium sulfate, 10 g l^{1}
487 glucose, 2.25 g l^{-1} ammonium nitrate and 1 M sucrose; pH adjusted to 7.0 using Na_2HPO_4,
488 solidified with 1.2% (w/v) agar) (51). Transformants were subjected to an additional round of
489 selection by growth on YDA agar containing 5 mg ml^{-1} DL-phosphinothricin.
490
491 **Cloning and complementation in *S. cerevisiae.*** Complementation studies were
492 performed in the *S. cerevisiae* Δ*pdr12* strain. The complementation plasmid (Fig. S7) was
493 constructed by yeast gap-repair cloning (47). Briefly the *PDR12* promoter and terminator, and
494 PdrA ORF were amplified by PCR (primers listed in Table S2). Each amplified fragment included
495 a 20-30 bp region of homology either with the YEp351 plasmid or with a neighbouring
496 fragment. The PCR products and *Hin*dIII linearized YEp351 plasmid were transformed into *S.*
497 *cerevisiae Δpdr12*, and transformants selected by leucine prototrophy (47). Complementation
498 was also performed with the *PDR12* ORF, as a positive control for successful
499 complementation. The *S. cerevisiae Δpdr12* strain was also transformed with the empty
500 YEp351 plasmid, as a negative control.
501
502
503 **ACKNOWLEDGMENTS**
504 This work was supported by the Biotechnology and Biological Sciences Research Council
505 (Grant Number BB/N017129/1). This is an Industry Partnering Award in conjunction with
506 Lucozade Ribena Suntory and Mologic Ltd. This work was also supported by the Wellcome
507 trust (Grant Number 208396/Z/17/Z).
508

509 **REFERENCES**

510 1. Bondi M, Messi P, Halami PM, Papadopoulou C, de Niederhausern S. 2014. Emerging
511 microbial concerns in food safety and new control measures. BioMed Res Int
512 2014:251512.
513 2. Kaczmarek M, Avery SV, Singleton I. 2019. Microbes associated with fresh produce:
514 Sources, types and methods to reduce spoilage and contamination. Adv Appl Microbiol
515 107:29-82.
516 3. Salmond CV, Kroll RG, Booth IR. 1984. The effect of food preservatives on pH homeostasis
517 in *Escherichia coli*. Microbiology 130:2845-2850.
518 4. Plumridge A, Hesse SJA, Watson AJ, Lowe KC, Stratford M, Archer DB. 2004. The weak
519 acid preservative sorbic acid inhibits conidial germination and mycelial growth of
520 *Aspergillus niger* through intracellular acidification. Appl Environ Microbiol 70:3506-
521 3511.
522 5. Freese E, Sheu CW, Galliers E. 1973. Function of lipophilic acids as antimicrobial food
523 additives. Nature 241:321-325.
524 6. Novodvorska M, Stratford M, Blythe MJ, Wilson R, Beniston RG, Archer DB. 2016.
525 Metabolic activity in dormant conidia of *Aspergillus niger* and developmental changes
526 during conidial outgrowth. Fungal Genet Biol 94:23-31.
527 7. Warth AD. 1989. Relationships between the resistance of yeasts to acetic, propanoic and
528 benzoic acids and to methyl paraben and pH. Int J Food Microbiol 8:343-349.
529 8. Stratford M, Steels H, Nebe-von-Caron G, Novodvorska M, Hayer K, Archer DB. 2013.
530 Extreme resistance to weak-acid preservatives in the spoilage yeast *Zygosaccharomyces*
531 *bailii*. Int J Food Microbiol 166:126-134.
532 9. Boschloo JG, Moonen E, van Gorcom RFM, Hermes HFM, Bos CJ. 1991. Genetic analysis
533 of *Aspergillus niger* mutants defective in benzoate-4-hydroxylase function. Curr Genet
534 19:261-264.
535 10. Fraser JA, Davis MA, Hynes MJ. 2002. The genes *gmdA*, encoding an amidase, and *bzuA*,
536 encoding a cytochrome P450, are required for benzamide utilization in *Aspergillus*
537 *nidulans*. Fungal Genet Biol 35:135-146.
538 11. Stratford M, Plumridge A, Pleasants MW, Novodvorska M, Baker-Glenn CAG, Pattenden
539 G, Archer DB. 2012. Mapping the structural requirements of inducers and substrates for
540 decarboxylation of weak acid preservatives by the food spoilage mould *Aspergillus niger*.
541 Int J Food Microbiol 157:375-383.
542 12. Lubbers RJM, Dilokpimol A, Navarro J, Peng M, Wang M, Lipzen A, Ng V, Grigoriev IV,
543 Visser J, Hilden KS, de Vries RP. 2019. Cinnamic acid and sorbic acid conversion are
544 mediated by the same transcriptional regulator in *Aspergillus niger*. Front Bioeng
545 Biotechnol 7:249.
546 13. Payne KAP, White MD, Fisher K, Khara B, Bailey SS, Parker D, Rattray NJW, Trivedi DK,
547 Goodacre R, Beveridge R, Barran P, Rigby SEJ, Scrutton NS, Hay S, Leys D. 2015. New
548 cofactor supports α, β-unsaturated acid decarboxylation via 1, 3-dipolar cycloaddition.
549 Nature 522:497-501.
550 14. Plumridge A, Melin P, Stratford M, Novodvorska M, Shunburne L, Dyer PS, Roubos JA,
551 Menke H, Stark J, Stam H, Archer DB. 2010. The decarboxylation of the weak-acid
552 preservative, sorbic acid, is encoded by linked genes in *Aspergillus spp*. Fungal Genet Biol
553 47:683-692.

554 15. Plumridge A, Stratford M, Lowe KC, Archer DB. 2008. The weak-acid preservative sorbic
555 acid is decarboxylated and detoxified by a phenylacrylic acid decarboxylase, PadA1, in the
556 spoilage mold *Aspergillus niger*. Appl Environ Microbiol 74:550-552.
557 16. Stratford M, Plumridge A, Archer DB. 2007. Decarboxylation of sorbic acid by spoilage
558 yeasts is associated with the *PAD1* gene. Appl Environ Microbiol 73:6534-6542.
559 17. Piper P, Mahé Y, Thompson S, Pandjaitan R, Holyoak C, Egner R, Mühlbauer M, Coote P,
560 Kuchler K. 1998. The Pdr12 ABC transporter is required for the development of weak
561 organic acid resistance in yeast. EMBO J 17:4257-4265.
562 18. Holyoak CD, Bracey D, Piper PW, Kuchler K, Coote PJ. 1999. The *Saccharomyces cerevisiae*
563 weak-acid-inducible ABC transporter Pdr12 transports fluorescein and preservative
564 anions from the cytosol by an energy-dependent mechanism. J Bacteriol 181:4644-4652.
565 19. Kren A, Mamnun YM, Bauer BE, Schuller C, Wolfger H, Hatzixanthis K, Mollapour M,
566 Gregori C, Piper P, Kuchler K. 2003. War1p, a novel transcription factor controlling weak
567 acid stress response in yeast. Mol Cell Biol 23:1775-1785.
568 20. Fernandes AR, Mira NP, Vargas RC, Canelhas I, Sá-Correia I. 2005. *Saccharomyces*
569 *cerevisiae* adaptation to weak acids involves the transcription factor Haa1p and Haa1p-
570 regulated genes. Biochem Biophys Res Commun 337:95-103.
571 21. Mollapour M, Fong D, Balakrishnan K, Harris N, Thompson S, Schüller C, Kuchler K, Piper
572 PW. 2004. Screening the yeast deletant mutant collection for hypersensitivity and hyper-
573 resistance to sorbate, a weak organic acid food preservative. Yeast 21:927-946.
574 22. Mira NP, Palma M, Guerreiro JF, Sá-Correia I. 2010. Genome-wide identification of
575 *Saccharomyces cerevisiae* genes required for tolerance to acetic acid. Microb Cell Fact
576 9:79.
577 23. Hewitt SK, Foster DS, Dyer PS, Avery SV. 2016. Phenotypic heterogeneity in fungi:
578 importance and methodology. Fungal Biol Rev 30:176-184.
579 24. Band VI, Weiss DS. 2019. Heteroresistance: A cause of unexplained antibiotic treatment
580 failure? PLoS Pathog 15:e1007726.
581 25. Andersson DI, Nicoloff H, Hjort K. 2019. Mechanisms and clinical relevance of bacterial
582 heteroresistance. Nature Rev Microbiol 17:1.
583 26. Stratford M, Steels H, Nebe-von-Caron G, Avery SV, Novodvorska M, Archer DB. 2014.
584 Population heterogeneity and dynamics in starter culture and lag phase adaptation of the
585 spoilage yeast *Zygosaccharomyces bailii* to weak acid preservatives. Int J Food Microbiol
586 181:40-47.
587 27. Fernández-Niño M, Marquina M, Swinnen S, Rodríguez-Porrata B, Nevoigt E, Ariño J.
588 2015. The cytosolic pH of individual *Saccharomyces cerevisiae* cells is a key factor in acetic
589 acid tolerance. Appl Environ Microbiol 81:7813-7821.
590 28. Teertstra WR, Tegelaar M, Dijksterhuis J, Golovina EA, Ohm RA, Wösten HAB. 2017.
591 Maturation of conidia on conidiophores of *Aspergillus niger*. Fungal Genet Biol 98:61-70.
592 29. Geoghegan IA, Stratford M, Bromley M, Archer DB, Avery SV. 2019. Weak Acid Resistance
593 A (WarA), a novel transcription factor required for regulation of weak-acid resistance and
594 spore-spore heterogeneity in *Aspergillus niger*. BioRxiv https://doi.org/10.1101/788141.
595 30. Takanori F, van Rhijn, N., Fraczek, M., Gsaller, F., Davies, E., Carr, P., Gago, S., Fortune-
596 Grant, R., Rahman, S., Mabey Glisenan, J., Houlder, E., Kowalski, C.H., Raj, S., Paul, S.,
597 Parker, J.E., Kelly, S., Cramer, R.A., Latge, J.-P., Cook, P., Moye-Rowley, S., Bignell, E.,
598 Bowyer, P., Bromley, M.J. The Negative Cofactor 2 complex is a master regulator of drug
599 resistance in *Aspergillus fumigatus*. Nature Comm In revision.

600 31. Natorff R, Sieńko M, Brzywczy J, Paszewski A. 2003. The *Aspergillus nidulans metR* gene
601 encodes a bZIP protein which activates transcription of sulphur metabolism genes. Mol
602 Microbiol 49:1081-1094.
603 32. Melin P, Stratford M, Plumridge A, Archer DB. 2008. Auxotrophy for uridine increases the
604 sensitivity of *Aspergillus niger* to weak-acid preservatives. Microbiology 154:1251-1257.
605 33. Holland SL, Reader T, Dyer PS, Avery SV. 2014. Phenotypic heterogeneity is a selected
606 trait in natural yeast populations subject to environmental stress. Environ Microbiol
607 16:1729-1740.
608 34. Legras JL, Erny C, Le Jeune C, Lollier M, Adolphe Y, Demuyter C, Delobel P, Blondin B, Karst
609 F. 2010. Activation of two different resistance mechanisms in *Saccharomyces cerevisiae*
610 upon exposure to octanoic and decanoic acids. Appl Environ Microbiol 76:7526-7535.
611 35. Kim MS, Cho KH, Park KH, Jang J, Hahn JS. 2018. Activation of Haa1 and War1 transcription
612 factors by differential binding of weak acid anions in *Saccharomyces cerevisiae*. Nucleic
613 Acids Res doi:10.1093/nar/gky1188.
614 36. Thakur JK, Arthanari H, Yang F, Pan S-J, Fan X, Breger J, Frueh DP, Gulshan K, Li DK,
615 Mylonakis E. 2008. A nuclear receptor-like pathway regulating multidrug resistance in
616 fungi. Nature 452:604-609.
617 37. Sze J-Y, Woontner M, Jaehning JA, Kohlhaw GB. 1992. *In vitro* transcriptional activation
618 by a metabolic intermediate: activation by Leu3 depends on alpha-isopropylmalate.
619 Science 258:1143-1145.
620 38. Sellick CA, Reece RJ. 2003. Modulation of transcription factor function by an amino acid:
621 activation of Put3p by proline. EMBO J 22:5147-5153.
622 39. Dijksterhuis J, Meijer M, van Doorn T, Houbraken J, Bruinenberg P. 2019. The preservative
623 propionic acid differentially affects survival of conidia and germ tubes of feed spoilage
624 fungi. Int J Food Microbiol 306:108258.
625 40. Avery SV. 2006. Microbial cell individuality and the underlying sources of heterogeneity.
626 Nature Rev Microbiol 4:577-587.
627 41. Zhao C, Fraczek, M. G., Dineen, L., Lebedinec, R., Macheleidt, J., Delneri, D., Bowyer, P.,
628 Brakhage, A.A., Bromley, M. 2019. High-throughput gene replacement in *Aspergillus*
629 *fumigatus*. Curr Protoc Microbiol 54:e88.
630 42. Stratford M, Steels H, Novodvorska M, Archer DB, Avery SV. 2019. Extreme
631 osmotolerance and halotolerance in food-relevant yeasts and the role of glycerol-
632 dependent cell individuality. Front Microbiol 9:3238.
633 43. Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing
634 reads. EMBnet J 17:10-12.
635 44. Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods
636 9:357-359.
637 45. Anders S, Pyl PT, Huber W. 2015. HTSeq—a Python framework to work with high-
638 throughput sequencing data. Bioinformatics 31:166-169.
639 46. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion
640 for RNA-seq data with DESeq2. Genome Biol 15:550.
641 47. Oldenburg KR, Vo KT, Michaelis S, Paddon C. 1997. Recombination-mediated PCR-
642 directed plasmid construction in vivo in yeast. Nucleic Acids Res 25:451-452.
643 48. Ballance DJ, Turner G. 1985. Development of a high-frequency transforming vector for
644 *Aspergillus nidulans*. Gene 36:321-331.
645 49. Green MR, Sambrook J. 2012. Molecular Cloning: a Laboratory Manual. Cold Spring
646 Harbor Laboratory Press.

647 50. Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH, van den Hondel CAMJJ. 1987.
648 Transformation of *Aspergillus* based on the hygromycin B resistance marker from
649 *Escherichia coli*. Gene 56:117-124.
650 51. Ahuja M, Punekar NS. 2008. Phosphinothricin resistance in *Aspergillus niger* and its utility
651 as a selectable transformation marker. Fungal Genet Biol 45:1103-1110.

652

653 **TABLE 1** Transcriptomics data for selected genes upregulated in the WT during sorbic acid treatment and differentially expressed in WT versus

654 Δ*warA*.[a]

Gene_ID	Log_2FC^b.WT _Sorbic vs WT control	Log_2FC.Δ*warA* _Sorbic vs Δ*warA* control	WT sorbic RPKM[c]	Δ*warA* sorbic RPKM	Function
An14g03570	9.35	7.1	611.3	146	ABC-type transporter with similarity to S.cerevisiae Pdr12p
An12g09130	9.31	5.98	6055.3	224.7	Possible dienelactone hydrolase function
An02g09970	8.56	4.35	1532.7	7.3	Ortholog(s) have role in drug response, hexose transport, pathogenesis
An13g02460	8.24	3.08	283	0.3	Protein similar to nonribosomal peptide synthases (NRPS-like)
An06g02170	7.98	1.51	80.3	3	Ortholog(s) have S-adenosylMet-dependent methyltransferase activity
An13g03170	7.25	5.73	1433.3	465.3	Unknown
An12g09120	7.14	2.87	312.3	31.7	Unknown
An09g03500	6.5	-0.51	226.3	4	Putative benzoate-para-hydroxylase; 3-hydroxybenzoate 4- hydroxylase
An12g02790	5.95	0.36	135.3	2.7	Isoflavone reductase - phenylcoumaran benzylic ether reductases type
An08g07850	5.81	3.77	11809.3	3248.7	Unknown
An01g05850	5.59	1.5	75.7	3	Thioesterase domain protein
An08g01560	5.22	-1.47	802.7	5.7	Ortholog(s) have role in meiotic cell cycle, regulation of TORC1 signaling
An04g05240	5.02	2.84	833	259.7	Unknown
An11g04385	4.9	2.02	74	5.7	Possible ubiquitin hydrolase
An13g02450	4.21	0	492.7	0	Six hairpin glycosidase
An08g01980	3.31	1.71	309	74.7	Unknown
An13g02290	3.06	-0.95	73.3	1.7	Possible 3-dehydroshikimate dehydratase
An09g05760	2.74	-0.47	397.7	40	Ortholog(s) have actomyosin contractile ring, intermediate layer localization
An18g03550	2.45	-2.43	1506.7	22.3	Similar to yeast Arr3 arsenate transporter
An08g05750	2.26	0.76	1095	298	Unknown
An16g00700	0.96	-3.07	345	20.7	Has domain(s) with predicted 2Fe,2S cluster binding, oxidoreductase activity

655 [a]See supplementary Table 1 for full list of genes. [b]Log_2 Fold Change. [c]Reads per Kilobase per Million.

656

657 **TABLE 2** MIC values (in mM) for *A. niger* WT and the Δ*An14g03570* (Δ*pdrA*) deletion strain.[a]

Acid	**MIC (mM)**	
	WT	**Δ*pdrA***
Benzoic	4.67 ± 0.12	3.40 ± 0.20
Pentanoic	3.70 ± 0.35	2.60 ± 0.30
Sorbic	5.00 ± 0.35	3.80 ± 0.35

658 [a]Values are averages of 3 biological replicates ± StdDev.

659

660

LEGENDS TO FIGURES

FIG 1 Screening of *A. fumigatus* deletion library. (A) Example of *A. fumigatus* deletant library screen. Conidial suspensions of the different deletants were arrayed in 96-well plates, and transferred to growth medium using a 96-pin tool. Examples of putatively sorbic acid sensitive strains are circled in yellow. (B) Sensitivity of *A. fumigatus* transcription factor deletion strains to sorbic acid. 62 strains were identified from the initial screen in (A) as putatively sorbic acid hypersensitive, and subjected to a second round of screening as outlined in Materials and Methods. Sensitivity to sorbic acid relative to the WT strain is shown (a value of 1 indicates identical sensitivity of the deletion strain to the WT, according to radial growth). "Δ*warA"* refers to Δ*AFUB_000960.*

FIG 2 Growth of *A. fumigatus* transcription factor deletion strains on medium containing weak acids. (A) Radial growth of *A. fumigatus* transcription factor deletion strains on agar containing 0.5 mM sorbic acid. Images were captured after 3 d growth at 37°C. (B) Radial growth of *A. fumigatus* Δ*warA* and wild-type on agar containing weak acids. Plates were inoculated with a 10-fold dilution series of conidial suspensions; approximate numbers of conidia are indicated above the pictures. Images were captured after 2 d growth at 28°C, and are representative of 2-3 independent experiments. Concentrations of acids used are given in the Materials and Methods.

FIG 3 Radial growth of *A. niger* Δ*warA* growing on different weak acids. Plates were inoculated with a 10-fold dilution series of conidial suspensions; approximate numbers of conidia are indicated above the pictures. Images were captured after 2 d growth at 28°C, and are

685 representative of 2-3 independent experiments. Concentrations of acids used are given in the

686 Materials and Methods.

687 **FIG 4** qRT-PCR of genes differentially regulated in WT and Δ*warA* strains of *A. niger.* Transcript

688 abundances in WT (black bars) and Δ*warA* (grey bars) conidia germinated in control media, or

689 in the presence of 1 mM sorbic acid or 1 mM benzoic acid. Error bars are standard deviation

690 of 3 technical replicates. WT and Δ*warA* transcript abundances were compared by Student's

691 t-test (*p < 0.05, **p <0.01, *** p<0.001).

692

693 **FIG 5** Radial growth of Δ*An02g09970* and Δ*An14g03570 (ΔpdrA)* deletion strains growing on

694 weak acids. Plates were inoculated with a 10-fold dilution series of conidial suspensions.

695

696 **FIG 6** Growth of *S. cerevisiae* complemented strains on weak acids. Ten-fold dilution series

697 of *S. cerevisiae* strains (isogenic with the BY4743 wild type) were inoculated onto medium

698 containing weak acids. The Δ*pdr12* strain was transformed with either empty plasmid

699 (+YEp351), YEp351 plasmid containing *PDR12* (+*PDR12)* or YEp351 plasmid containing the

700 *pdrA* ORF and *PDR12* promoter and terminator (+*pdrA*). Two independent transformants of

701 the +*pdrA* strain are shown.

702

703 **FIG 7** Sorbic acid dose response curves for *A. niger* conidia. (A) Dose response curves of

704 germinated (blue lines) and ungerminated (black/grey lines) WT conidia, and comparison of

705 slope values. Dose response curve slope values were compared with 2-way Welch's T-test (p=

706 0.0404) n = 2-3. (B) Dose response curves of WT (black/grey lines) and Δ*pdrA* (pink/red lines)

707 conidia and comparison of dose response curve slope values, compared with 2-way Welch's

708 T-test (p = 0.0468) n = 3. Two representative independent experiments are shown in the dose

709 response curve.

710

711 **LEGENDS TO SUPPLEMENTARY FIGURES**

712

713 **FIG S1** Radial growth of the *A. fumigatus* Δ*metR* strain on medium containing 0.5 mM sorbic

714 acid, with or without 0.5 mM methionine.

715

716 **FIG S2** PCR and Southern Blot confirmation of *warA* deletion. (A) Targeted gene deletion

717 strategy. The *warA* ORF was replaced with a hygromycin resistance cassette. (B) PCR

718 confirmation of *warA* deletion. Primer pair 1 was used to confirm deletion of *warA* ORF –

719 deletion strains are negative, WT is positive. Primer pair 2 was used to confirm integration of

720 *HygR* at the *warA* locus. (C) Southern blotting of *warA* and *cdcA/warA* deletion strains. gDNA

721 of strains was digested with *Hin*dIII restriction enzyme. Membranes were hybridised with a

722 probe consisting of Digoxigenin-UTP labelled *HygR*. Single bands confirm single integration of

723 deletion cassette into the *A. niger* genome. Transformants Δ*warA_12* and ΔΔ*cdcA/warA_12*

724 were used for experiments.

725

726 **FIG S3** (A) Growth of the Δ*warA* strain conidia on medium containing sorbic acid. Plates were

727 inoculated with ~100 conidia and incubated for 2 d (control medium) or 3 d (medium

728 containing 1 mM sorbic acid). (B) Radial growth of ΔΔ*cdcA/warA* strain on medium containing

729 1 mM sorbic acid. Plates were inoculated with a 10-fold dilution series of conidial suspensions;

730 approximate numbers of conidia are indicated at the top. Images were captured after 2 d

731 growth at 28°C, and are representative of 2-3 independent experiments.

732 **FIG S4** Radial growth of complemented Δ*warA* strains on medium containing 2 mM benzoic
733 acid. Plates were inoculated with a 10-fold dilution series of conidial suspensions.
734 Approximate numbers of conidia are indicated above the pictures. Images were captured
735 after 2 d growth at 28°C. Two independent complemented lines are shown.
736
737 **FIG S5** PCR and Southern Blotting of *An02g09970* and *An14g03570* (*pdrA*) deletion strains.
738 (A) Targeted gene deletion strategy. The *An02g09970* and *An14g03570* ORFs were replaced
739 with a hygromycin resistance cassette. (B) PCR confirmation of *An02g09970* deletion. Primer
740 pair 1 was used to confirm deletion of the *An02g09970* ORF – deletion strains are negative,
741 WT is positive. Primer pairs 2 and 3 were used to confirm integration of *HygR* at the
742 *An02g09970* locus. (C) PCR confirmation of *An14g03570* deletion. Primer pair 1 was used to
743 confirm deletion of the *An14g03570* ORF – deletion strains are negative, WT is positive.
744 Primer pairs 2 and 3 were used to confirm integration of *HygR* at the *An14g03570* locus. (D)
745 Southern blotting of ΔA*n02g09970* and ΔA*n14g03570* deletion strains. gDNA of strains was
746 digested with the restriction enzymes *Xba*I (for ΔA*n02g09970*) or *Eco*RV (for ΔA*n14g03570*).
747 Membranes were hybridised with a probe consisting of Digoxigenin-UDP labelled *HygR*. Single
748 bands confirm single integration of deletion cassette into the *A. niger* genome (multiple
749 integrations apparent for Δ*An02g09970_39*). Transformants ΔA*n14g03570_4* and
750 Δ*An02g09970_29* were used for experiments.
751
752 **FIG S6** Radial growth of complemented Δ*pdrA* strains on medium containing 2mM benzoic
753 acid. Plates were inoculated with a 10-fold dilution series of conidial suspensions. Two
754 independent complemented lines are shown. A Δ*pdrA* strain containing the empty
755 pAN7.1*BAR* plasmid is also shown.

756

757 **FIG S7** Plasmid map of YEp351 containing *An14g03570* (*pdrA*)

758

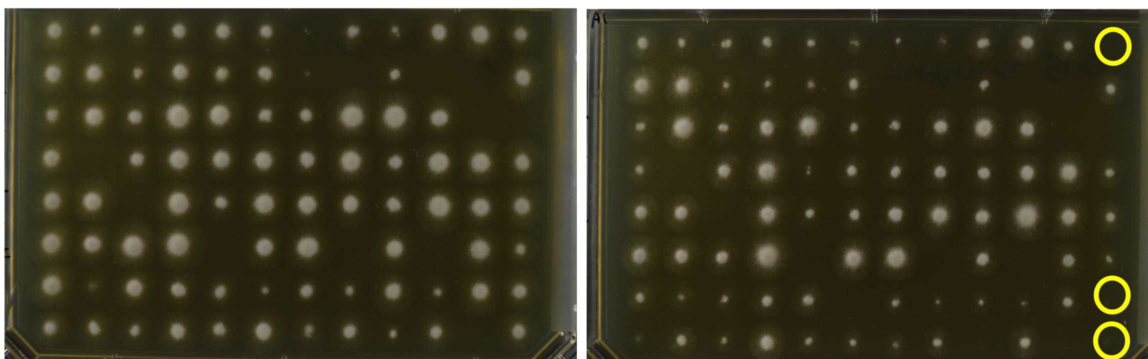
759 **FIG S8** Plasmid map of pAN7.1*BAR*.

760

761 **TABLE S1** RPKM and Log_2FC values for *A. niger* genes (Excel file).

762

763 **TABLE S2** List of primers used in this study (Excel file).


A
Control
Sorbic

B
Growth relative to the wild type
1.2
1
0.8
0.6
0.4
0.2
0
ΔcreA
ΔdevR
ΔmetR
ΔwarA
AFUB_009490
AFUB_012020
AFUB_013910
AFUB_014780
AFUB_014910
AFUB_015020
AFUB_015090
AFUB_015380
AFUB_015750
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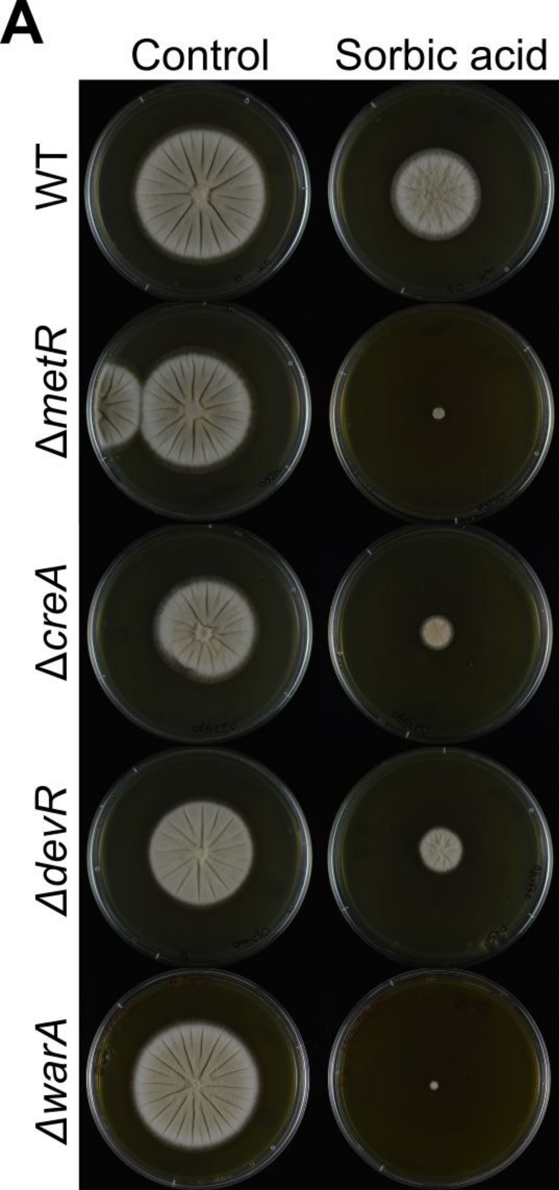
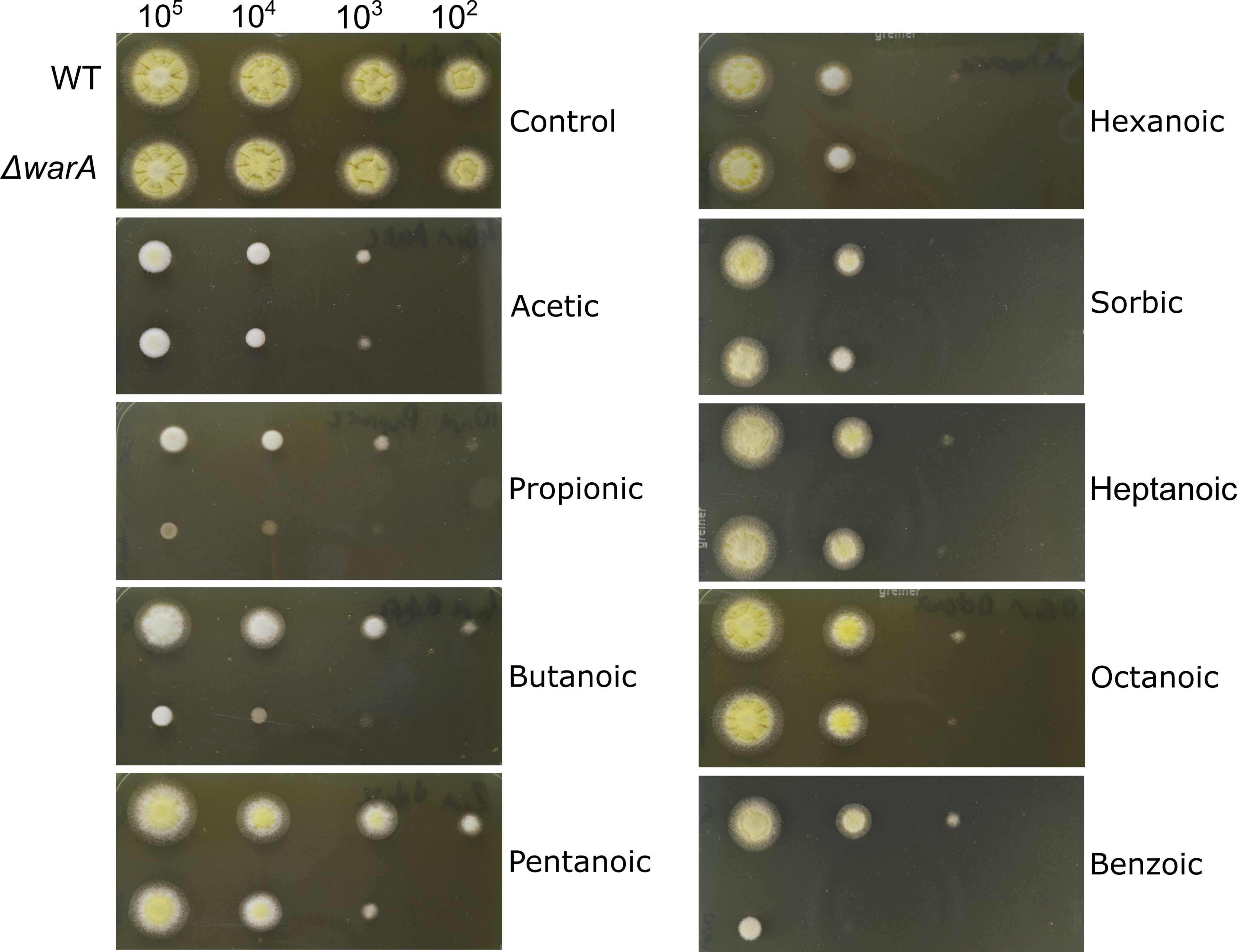
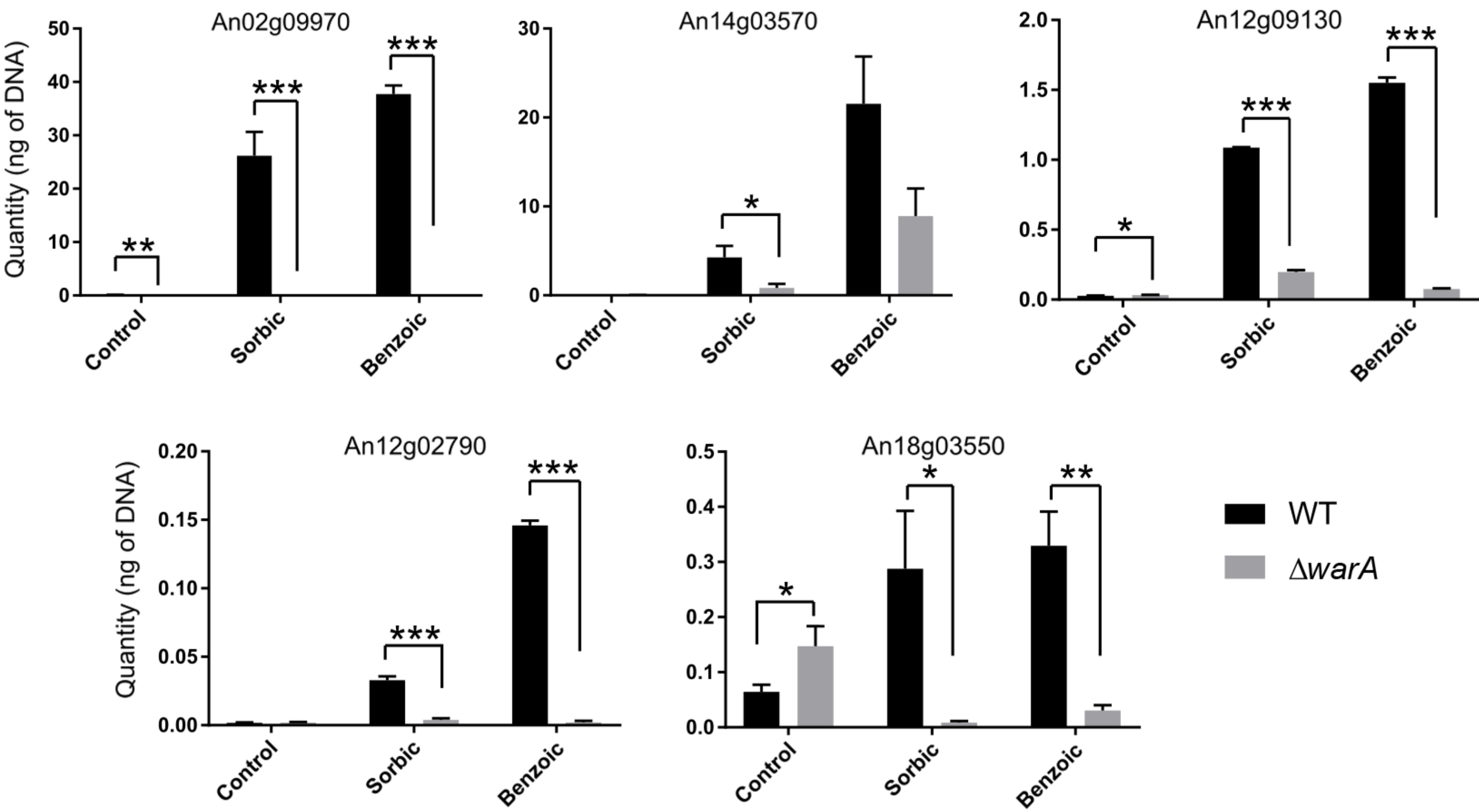
AFUB_036440
AFUB_039350
AFUB_040580
AFUB_044290
AFUB_046540
AFUB_047470
AFUB_049550
AFUB_050190
AFUB_053150
AFUB_053880
AFUB_053950
AFUB_054360
AFUB_056530
AFUB_056620
AFUB_057890
AFUB_058830
AFUB_059600
AFUB_063610
AFUB_067280
AFUB_069500
AFUB_074510
AFUB_077530
AFUB_086680
AFUB_000190
AFUB_000960
AFUB_001960
AFUB_008120
AFUB_029740
AFUB_038600
AFUB_094860
AFUB_009690
AFUB_096430
AFUB_063950
AFUB_042180
AFUB_075680
AFUB_080530
AFUB_057470
AFUB_060650
AFUB_023530
AFUB_017020
AFUB_012800
AFUB_007280
AFUB_017410
AFUB_021220
Strain

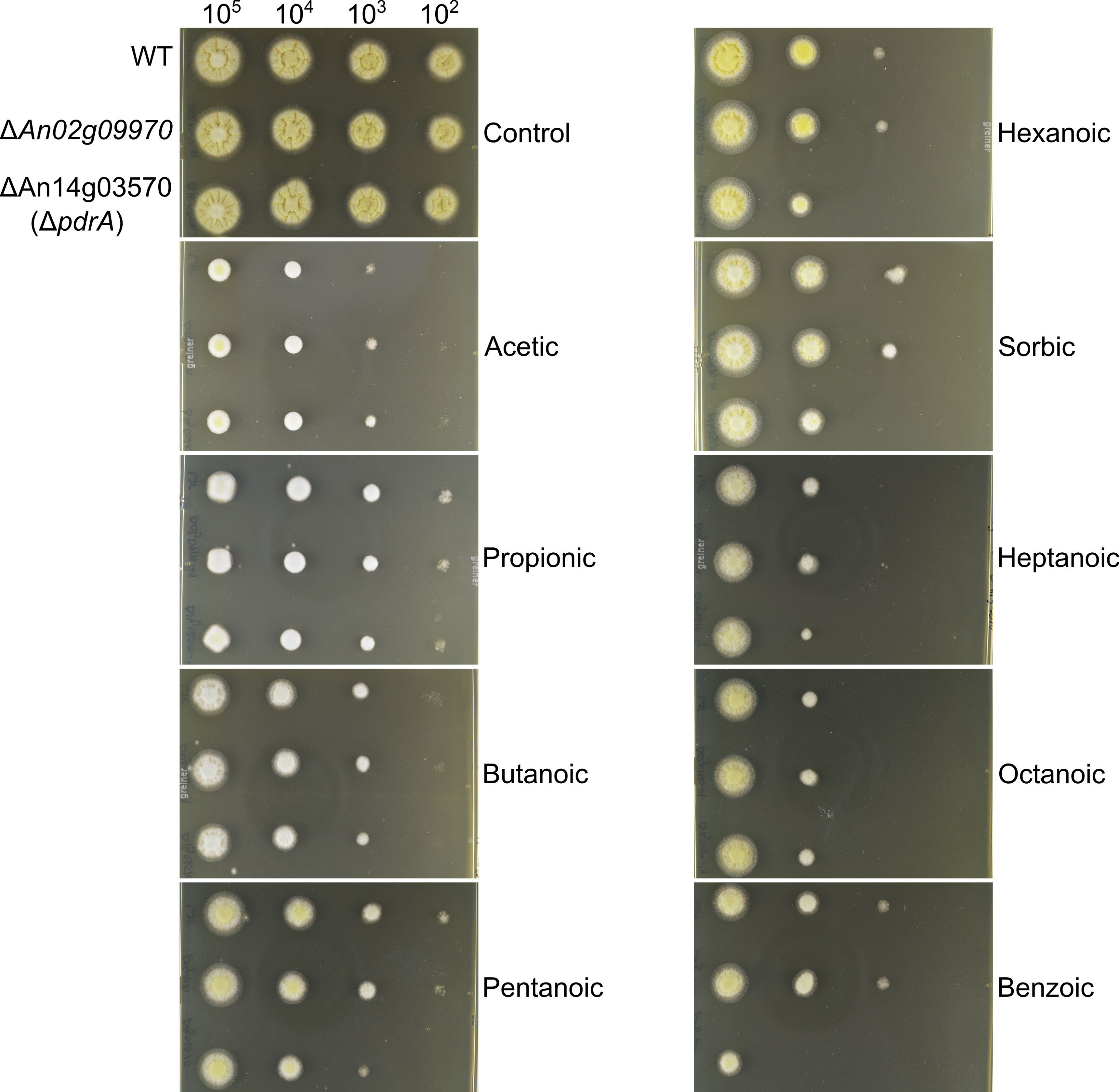

A
Control
Sorbic acid
WT
ΔmetR
ΔcreA
ΔdevR
ΔwarA
B
10^5
10^4
10^3
10^2
WT
ΔwarA
Control
Acetic
Propionic
Butanoic
Pentanoic
WT
ΔwarA
Hexanoic
Sorbic
Heptanoic
Octanoic
Benzoic



10^5
10^4
10^3
10^2
WT
ΔwarA
Control
Acetic
Propionic
Butanoic
Pentanoic
Hexanoic
Sorbic
Heptanoic
Octanoic
Benzoic



An02g09970
An14g03570
An12g09130
An12g02790
An18g03550
Quantity (ng of DNA)
Control
Sorbic
Benzoic
WT
ΔwarA



10^5
10^4
10^3
10^2
WT
Δ*An02g09970*
ΔAn14g03570
(Δ*pdrA*)
Control
Acetic
Propionic
Butanoic
Pentanoic
Hexanoic
Sorbic
Heptanoic
Octanoic
Benzoic

Control Propionic Butanoic Sorbic Benzoic

BY4743

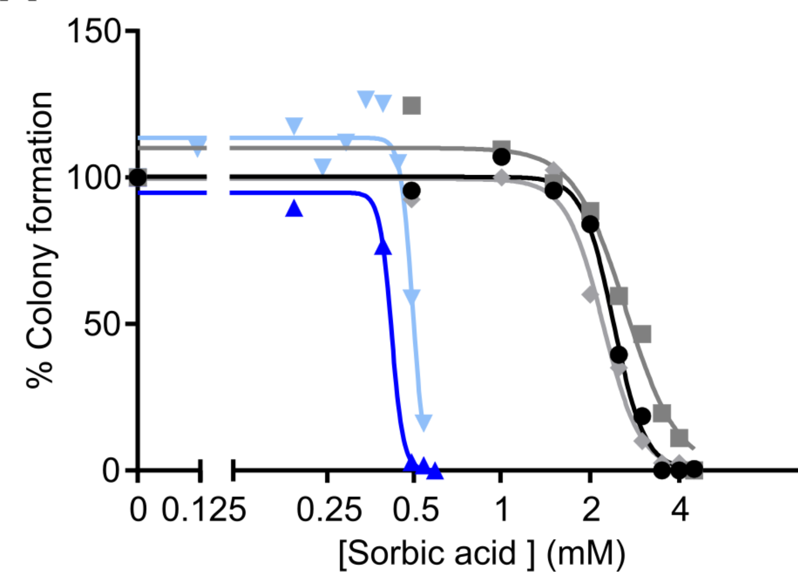
Δ*pdr12*

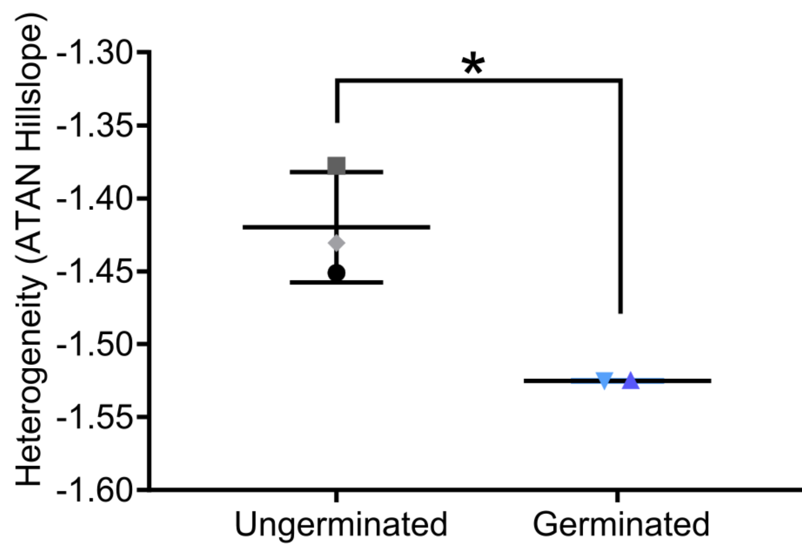
+ YEp351

+ *PDR12*

+ *pdrA*

A





B