Weak Acid Sensitive A, a novel transcription factor required for regulation of weak-acid resistance and spore-spore heterogeneity in *Aspergillus niger*

Ivey A. Geoghegan[a], Malcolm Stratford[a], Mike Bromley[b], David B. Archer[a], and Simon V. Avery[a,#]

[a]School of Life Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

[b]Division of Infection, Immunity & Respiratory Medicine, School of Biological Sciences, University of Manchester, Manchester M13 9NT, UK

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[#]Address correspondence to: Simon.Avery@nottingham.ac.uk

ABSTRACT

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

IMPORTANCE

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

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

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 acids 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 4-hydroxybenzoate decarboxylase (OhbA1) and a phenylacrylic
80 acid decarboxylase (PadA1) (12-14). 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 (15). 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 (15). 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 (16). *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 (17). *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 (18). 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 (19). 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. (20) 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 (21). 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 (22-24). 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, 25, 26). 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 (27). 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 Sensitive A), that is required for resistance to weak acid preservatives in *A.*
120 *niger* and *A. fumigatus*. Furthermore, we identify and characterize genes that are putatively
121 regulated by WasA, 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.

126

127 **RESULTS**

128

129 **A screen for transcription-factor deletion strains sensitive to sorbic acid identifies**
130 ***wasA.*** To find genes associated with weak acid resistance, an *Aspergillus fumigatus*
131 transcription-factor deletant collection (28) was screened for sorbic acid sensitivity. This
132 resource comprised a library of 401 deletion strains of non-essential transcription factors. To
133 determine sensitivity of the deletion strains, radial growth was compared on agar medium
134 with and without sorbic acid (Fig. 1). This revealed two deletion strains which were highly
135 sensitive to sorbic acid compared with the wild-type strain (Δ*metR*, and Δ*AFUB_000960*) (Fig.
136 1 and Fig. 2A). A number of other strains exhibited moderate sensitivity to sorbic acid,
137 including Δ*creA* (Δ*AFUB_027530*), Δ*devA* (Δ*AFUB_030440*) (Fig. 1B and Fig. 2A), Δ*rfeD*
138 (*ΔAFUB_022280*), Δ*AFUB_020350* and Δ*AFUB_054360* (Fig. 1B).

139 MetR is a bZIP-type transcription factor mediating transcriptional regulation of genes
140 involved in sulphur uptake and utilization (29). Because sorbic acid is known to decrease
141 cellular uptake of some nutrients (6, 30), it was hypothesised that sulphur limitation could be
142 a cause of sorbic acid sensitivity in the Δ*metR* deletion strain. To test this, sorbic acid
143 sensitivity of the Δ*metR* strain was determined in medium supplemented with the sulphur-
144 containing amino acid methionine. This showed that the sorbic acid sensitivity of the Δ*metR*
145 strain was abolished in the presence of supplementary methionine (Fig. S1).

146 *AFUB_000960* encodes a Zn2Cys6-type transcription factor which contains a fungal
147 specific transcription factor domain (PF11951). To further investigate the role of
148 *AFUB_000960* in weak acid resistance, sensitivity of the deletion strain to a range of weak
149 acids was evaluated (Fig. 2B). The Δ*AFUB_000960* strain was sensitive to propionic, butanoic,

150 pentanoic, hexanoic, sorbic and benzoic acids. Because of these phenotypes, *AFUB_000960*
151 was named *Weak Acid Sensitive A* (*wasA)*.
152
153 **A WasA orthologue in *A. niger* is important for weak acid resistance.** *Aspergillus*
154 *fumigatus* is not commonly associated with food spoilage, and displays relatively high
155 sensitivity to weak acids such as sorbic acid. To determine whether orthologues of WasA are
156 present in other species of fungi, a BLAST-search using the *Afu*WasA protein sequence as a
157 query was conducted. In *A. niger,* this identified An08g08340, a protein with 46.2% identity
158 and 63% similarity to the *A. fumigatus* WasA protein. Orthologues of *AfuwasA* were also
159 found to be present in *Penicillium* and *Botrytis spp.*
160 *Aspergillus niger* is highly resistant to weak acids (especially sorbic and benzoic acids),
161 and can readily cause food and drink spoilage. To determine whether *wasA* is also required
162 for weak acid resistance in *A. niger*, *An08g08340* was deleted by a targeted gene replacement
163 approach, with successful deletion confirmed by PCR and Southern Blotting (Fig. S2).
164 Sensitivity of the Δ*An08g08340* deletion strain to different weak acids was evaluated (Fig. 3).
165 In contrast to the Δw*asA* deletant of *A. fumigatus*, the *A. niger* Δ*An08g08340* strain
166 demonstrated only slight sensitivity to sorbic acid, which was most apparent when individual
167 conidia of the Δ*wasA* strain were spread onto medium containing sorbic acid (Fig. S3A).
168 However, the deletant was highly sensitive to propionic, butanoic and benzoic acids as
169 evaluated by radial growth on agar (Fig. 3). Determination of Minimum Inhibitory
170 Concentrations (MIC) in broth also corroborated the radial growth data; the MIC in benzoic
171 acid was 4.5 ± 0.5 mM in the wild type (WT; n=3), compared with 3.2 ± 0.2 mM in Δ*wasA*
172 (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*wasA*
173 (n=3). Resistance was restored to WT levels when *An08g08340* was reintroduced into the

174 Δ*An08g08340* strain (Fig. S4). Thus, *An08g08340* has an important role in weak acid resistance
175 in *A. niger*, and was named as *wasA* (Weak Acid Sensitive A) also in this species.
176 Sorbic acid (and structurally related acids) are known to be detoxified by
177 decarboxylation in *A. niger*, but not in *A. fumigatus* (12, 13). The decarboxylation process
178 involves three linked genes: *ohbA1*, *padA1* and *sdrA* (13). OhbA1 is the key enzyme involved
179 in the decarboxylation, whereas *sdrA* is a transcription factor regulating expression of *OhbA*
180 and *padA1* synthesizes a cofactor for *OhbA*. It was hypothesized that mild sorbic acid
181 sensitivity of *A. niger* Δ*wasA* may be due to downregulation of *ohbA1*, *padA1* or *sdrA* genes
182 in the Δ*wasA* strain. To investigate the relationship between WasA and weak acid
183 decarboxylation, a ΔΔ*ohbA1/wasA* mutant was constructed. The ΔΔ*ohbA1/wasA* strain was
184 more sensitive to sorbic acid than the Δ*ohbA1* strain (Fig. S3B), suggesting an *OhbA1*-
185 independent role for *wasA* in resistance of *A. niger* to sorbic acid.
186
187 **Determination of WasA-regulated genes by transcriptomic analysis during weak acid**
188 **stress of *A. niger*.** The weak acid sensitivity of the Δ*wasA* mutant suggested that WasA
189 regulates genes which are important for weak acid resistance. Previously, genes upregulated
190 by sorbic acid exposure in *A. niger* were successfully identified by exposing conidia of the wild-
191 type (WT) to sorbic acid during germination (6). In order to identify which genes are
192 differentially regulated in *A. niger* Δ*wasA*, RNA-seq analysis was conducted with WT and
193 Δ*wasA* conidia germinated in the presence or absence of sorbic acid. Germination of WT
194 conidia for 1 h in the presence of sorbic acid resulted in 3,274 differentially expressed genes
195 (FDR adjusted p-value < 0.05) (1885 upregulated, 1467 downregulated) in comparison with
196 conidia germinated in the absence of sorbic acid. In Δ*wasA* conidia, 3,442 genes were
197 differentially expressed during germination in the presence of sorbic acid (1885 upregulated,

198 1,557 down regulated), in comparison with germination without sorbic acid. Importantly, a
199 number of genes were identified that were highly upregulated in the WT during sorbic acid
200 exposure, but not in Δ*wasA* (Table 1 and Table S1). This included a gene encoding benzoate
201 *para*-hydroxylase (*bphA*), an enzyme known to be required for benzoate detoxification (9),
202 which had a Log Fold Change (Log_2FC) of 6.50 in the WT, compared with a Log_2FC of -0.51 in
203 Δ*wasA.* A number of uncharacterized enzymes also required *wasA* for normal upregulation
204 by sorbic acid, e.g., An12g09130 encoding a putative dienelactone hydrolase, and
205 An12g02790 a putative isoflavone reductase (Log_2FC 5.95 in WT, Log_2FC 0.36 in Δ*wasA*), as
206 well as several genes encoding putative transporter proteins. Of particular interest amongst
207 these transporters was An14g03570, an ABC-type transporter with 56% amino acid sequence
208 similarity to *S. cerevisiae* Pdr12p. Pdr12p has a crucial role in weak acid detoxification in *S.*
209 *cerevisiae* (16, 17). To support the RNA-seq data, five of the genes showing differential
210 expression between the WT and Δ*wasA* strains were selected for qRT-PCR analysis (Fig. 4).
211 The qRT-PCR data supported the trends in gene expression seen in the RNA-seq dataset; all
212 the selected genes had a lower transcript abundance in Δ*wasA*, compared with the WT during
213 sorbic acid treatment. In addition, transcript abundances of the selected genes were
214 compared by qRT-PCR during benzoic acid treatment. As expected, all the genes upregulated
215 by sorbic acid were also upregulated by benzoic acid, and had lower transcript abundances in
216 the Δ*wasA* mutant, compared with the WT (Fig. 4).
217
218 **Characterization of *An02g09970* and *An14g03570.*** The transcriptomic analysis
219 identified genes that are downregulated in *A. niger* Δ*wasA*, relative to the WT strain, during
220 weak acid stress. These genes may therefore have a role in weak acid resistance. To
221 investigate this, two genes of interest (*An02g09970* and *An14g03570*) were selected for

222 further characterization. *An02g09970* encodes a putative transmembrane transporter of the
223 Major Facilitator Superfamily (MFS), and was selected for further investigation due to its
224 extremely high transcript abundance during sorbic acid treatment, and large disparity in
225 transcript abundance between the WT and Δ*wasA* strains (Log_2FC 8.56 in WT vs. Log_2FC 4.35
226 in Δ*wasA*) (Table 1). The protein also shares significant sequence similarity with Tpo2 and
227 Tpo3, *S. cerevisiae* proteins involved in resistance to acetic, propionic and benzoic acids (19).
228 *An14g03570* encodes an ABC-type transporter with similarity to the weak acid detoxification
229 protein Pdr12p in *S. cerevisiae*, as stated above. Both genes were deleted in *A. niger* by a
230 targeted gene replacement approach, and mutant genotypes confirmed by PCR and Southern
231 blotting (Fig. S5). Sensitivity of the constructed deletion strains to weak acids was then
232 evaluated. The Δ*An02g09970* mutant did not exhibit altered sensitivity to any of the weak
233 acids tested (Fig. 5). However, the Δ*An14g03570* mutant was more sensitive to sorbic,
234 pentanoic and benzoic acids (Fig. 5 and Table 2), and resistance was restored to WT levels
235 when *An14g03570* was reintroduced into the Δ*An14g03570* strain (Fig. S6). 7Δ*An14g03570*
236 **Complementation of *S. cerevisiae* Δ*pdr12* strain with *An14g03570*.** The above results
237 showed that *An14g03570* is required for resistance of *A. niger* to certain weak acids. Because
238 An14g03570 has significant protein sequence similarity with *S. cerevisiae* Pdr12p (36%
239 sequence identity, 56% similarity), it was hypothesized that these proteins could be functional
240 homologues. To test this hypothesis, functional complementation of the *S. cerevisiae* Δ*pdr12*
241 strain was attempted. The cDNA sequence of *A. niger An14g03570* was cloned between the
242 *S. cerevisiae PDR12* promoter and terminator to allow for native regulation of *An14g03570* in
243 response to weak acid stress in *S. cerevisiae*. The resulting plasmid (Fig. S7) was transformed
244 into a *S. cerevisiae* Δ*pdr12* deletion strain. Transformants were tested for sensitivity to a range
245 of weak acids. As hypothesized, *An14g03570* could indeed functionally complement *PDR12*:

246 sensitivity of the Δ*pdr12* strain to weak acids was largely rescued in cells transformed to
247 express *An14g03570* (Fig. 6). The resultant level of resistance was similar to that evident in
248 Δ*pdr12* cells expressing *PDR12* from the same vector backbone.
249
250 ***An14g03570* is a determinant of heteroresistance to sorbic acid in *A. niger* conidia.**
251 Resistance to weak acid preservatives has been found to be heterogeneous between
252 individual cells of genetically-uniform cell populations of the yeasts *Zygosaccharomyces bailii*
253 and *S. cerevisiae* (8, 25, 26). We observed a similar phenomenon in populations of *A. niger*
254 conidia, whereby small subpopulations were capable of germinating and forming colonies at
255 high concentrations (>4 mM) of sorbic acid (Fig. 7A). Conidia harvested from colonies that
256 grew on these high concentrations of sorbic acid did not retain increased resistance upon
257 direct re-inoculation to sorbic acid-containing medium (data not shown), suggesting that
258 these were transient, non-heritable phenotypes, i.e., not due to genotypic variants within the
259 population. To test whether this heteroresistance had its origin in the ungerminated conidial
260 state, conidia were also pre-germinated for 6 hr, before spread plating onto sorbic acid-
261 containing medium. This showed that germinated conidia were much more susceptible to
262 sorbic acid (Fig. 7A). Moreover, resistance to sorbic acid among pre-germinated conidia was
263 much more homogeneous than when the resistance-assay commenced with ungerminated
264 conidia, as evidenced by the gradients of the dose inhibition curves: such dose response
265 curves reflect heterogeneity, with shallower curves indicating greater heterogeneity (22, 31).
266 Thus, at least some factors determining heteroresistance to sorbic acid are specific to
267 ungerminated conidia, and are lost upon germination. Given the contributions of *wasA* and
268 *An14g03570* to sorbic acid resistance in *A. niger*, it was tested whether these genes could be
269 determinants of heteroresistance. Dose response curves of Δ*An14g03570* and Δ*wasA* conidia

270 demonstrated that *An14g03570* makes a significant contribution to sorbic acid
271 heteroresistance in *A. niger* conidia, whereas *wasA* does not (Fig. 7B, data for Δ*wasA* not
272 shown).
273

274 **DISCUSSION**

275

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

287 We sought to gain insight to the WasA regulon in *A. niger* by conducting a comparative
288 transcriptomics experiment between germinating WT and Δ*wasA* conidia treated with sorbic
289 acid. This approach identified several genes that appear to be regulated (either directly or
290 indirectly) by WasA. These include a number of putative enzymes and transporter proteins,
291 offering several candidates for future studies of weak acid resistance mechanisms in *A. niger*.
292 Amongst these candidates, we attempted to characterize two putative transporter-protein
293 genes. The first of these functions, An02g09970, is a transporter of the Major Facilitator
294 Superfamily, with sequence similarity to Tpo2p and Tpo3p in *S. cerevisiae*. Tpo2p and Tpo3p
295 are transporters of the DHA1 (Drug:H+ antiporter-1) family and are known to be required for
296 resistance to acetic, propionic and benzoic acids (19). However, deletion of *An02g09970* did
297 not sensitize *A. niger* to any of the acids tested, and so the role of this gene remains unknown.

298 It is possible that this transporter is responsible for detoxification of other xenobiotics not
299 tested here (if indeed it has a role in detoxification at all), or that *An02g09970* is functionally
300 redundant with other *A. niger* genes. We also attempted to characterize *An14g03570*,
301 encoding an ABC-type transporter. Deletion of *An14g03570* resulted in increased sensitivity
302 to pentanoic, hexanoic, sorbic and benzoic acids, substantiating a role for this protein in weak
303 acid resistance. Importantly, we were able to demonstrate that *An14g03570* is a functional
304 homologue of *PDR12* in *S. cerevisiae*. Pdr12p is a key protein involved in weak acid resistance
305 of *S. cerevisiae* (16), where it is thought to efflux weak acid anions from the cytoplasm in an
306 energy-dependant manner (17). The identification of An14g03570 as a functional homologue
307 of Pdr12p in a mould species such as *A. niger* shows that a similar mechanism of weak acid
308 detoxification by active efflux may operate in yeasts and moulds. Interestingly, the Δ*pdr12*
309 functional complementation experiment demonstrated that *An14g03570* confers resistance
310 to a broader range of weak acids than was suggested by the weak acid sensitivity of the
311 ΔAn*14g03570* strain. For example, *An14g03570* complemented the propionic acid sensitivity
312 of *S. cerevisiae* Δ*pdr12*, but the *A. niger* ΔAn*14g03570* strain was not more sensitive to
313 propionic acid than the WT. This may indicate the presence of multiple, redundant
314 mechanisms for resistance to certain weak acids in *A.niger*, which may not operate in *S.*
315 *cerevisiae*.

316 *An14g03570,* as well as several other candidate WasA-regulated genes were all
317 upregulated in response to both sorbic and benzoic acids. This suggests a degree of overlap
318 between transcriptomic responses to different weak acids, as also found in *S. cerevisiae* (32).
319 Thus, although we characterized the WasA regulon by comparative transcriptomics in
320 response only to sorbic acid in the present study, it is likely that many of the differentially
321 expressed genes would be similarly regulated in response to other weak acids. There may be

322 relevant consensus sequences within WasA-regulated genes, although these are not apparent
323 from promoter sequence alignments we have carried out. In *S. cerevisiae*, a *cis*-acting weak
324 acid response element (WARE) was discovered in the promoter of *PDR12* which is required
325 for *PDR12* induction by the transcription factor War1p (18).

326 The regulation of WasA itself is also an outstanding question. Recent evidence in *S.*
327 *cerevisiae* suggests that weak acid anions bind directly to the transcription factors War1p and
328 Haa1p, thereby regulating their DNA-binding transcriptional activation (33). However, WasA
329 shares very little sequence homology with either War1p or Haa1p. In fact, a BLAST search of
330 the *S. cerevisiae* protein database with the WasA protein sequence yields no hits at all.
331 Nevertheless, a similar mechanism of transcription factor activation cannot be ruled out for
332 WasA, particularly as direct ligand binding has been established for a number of Zn2Cys6
333 family transcription factors (of which WasA is a member), including Pdr1p, Pdr3p, Leu3p and
334 Put3p (34-36).

335 During the course of this study, experiments with sorbic acid determined that
336 genetically-uniform populations of *A. niger* conidia demonstrate heteroresistance to this
337 weak acid. Phenotypic heterogeneity within microbial cell populations has been
338 demonstrated in a number of fungi in response to environmental stresses [reviewed in (22)],
339 however this is the first report of weak acid heteroresistance in fungal conidia. Interestingly,
340 heteroresistance was decreased within 6 h of conidial germination, suggesting that at least
341 some factors underlying this heterogeneity are limited to ungerminated conidia and are lost
342 upon germination. Resistance to sorbic acid was also markedly lower in germinated conidia,
343 which has also recently found to be the case for propionic acid (37). Heteroresistance to weak
344 acids in fungal conidia has significant implications for the food industry, because spoilage of
345 products may occur due to contamination with just a few conidia from a highly resistant

346 subpopulation. Thus, future spoilage control strategies may have to take into account the
347 presence of weak acid heteroresistance, perhaps by specifically targeting resistant
348 subpopulations.

349 Conidia of the Δ*An14g03570* strain showed a significantly more homogeneous
350 response to sorbic acid. Heteroresistance typically arises from gene expression heterogeneity
351 (or noise) (38), so the present results suggest that An14g03570 could be expressed
352 heterogeneously within conidial populations; the conidia expressing more *An14g03570*
353 potentially able to withstand sorbic acid stress. It is also noted that deletion of An14g03570
354 did not eliminate sorbic acid heteroresistance in *A. niger*, so it likely that other genes also
355 contribute.

356 In summary, this study markedly advances our understanding of weak acid resistance
357 mechanisms in *A. niger*. The identification of WasA as a key transcription factor involved in
358 weak acid resistance allowed us in turn to identify many more genes which may also be
359 important. Further work is required to determine how all these genes may contribute to weak
360 acid resistance. Moreover, we demonstrated here that a key weak acid resistance mechanism
361 operates in both *S. cerevisiae* and *A. niger*, in the form of the functionally homologous ABC-
362 transporters Pdr12p and An14g03570 respectively.

363

364 **MATERIALS AND METHODS**

365

366 **Strains and media.** The *Aspergillus fumigatus* transcription factor deletant library,
367 derived from wild-type strain MFIG001, was constructed by homologous recombination using
368 gene replacement cassettes and transformation methodologies as described (28, 39). Studies in
369 *Aspergillus niger* were performed in the *A.niger* N402 background (referred to as the *A. niger*
370 WT throughout) and an *A. niger ΔohbA1* deletion strain (13). *Aspergillus* strains were
371 cultivated on slopes of Potato Dextrose Agar (PDA) (Sigma) for 7 days at 28°C. Conidia were
372 harvested using 0.1% (v/v) Tween 80 and filtered through a 40 µm cell strainer (Fisher), before
373 counting on a haemocytometer. Studies in *S. cerevisiae* used the BY4743 background and
374 isogenic *Δpdr12* deletion strain, cultivated on YEPD agar (2% glucose, 2% bactopeptone
375 (Oxoid), 1% yeast extract (Oxoid), 1.5% agar) at 30°C. The *S. cerevisiae* strains were obtained
376 from EUROSCARF (Frankfurt). Growth assays with weak acids (below) were performed on
377 YEPD agar (pH 4).

378

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

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

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

399 Minimum inhibitory concentrations (MICs) of weak acids were determined by placing
400 10 ml of YEPD broth (pH 4) into 30 ml McCartney bottles, and inoculating with 10^4 conidia.
401 Bottles were incubated statically at 28°C for 28 days, and the concentration of acids required
402 to completely inhibit visible growth recorded.

403 Dose response curves were generated by harvesting conidia as stated above, diluting
404 to 500 spores ml^{-1} and spreading 200 µl of this onto YEPD agar (pH 4) containing sorbic acid.
405 Plates were incubated at 28°C for up to 28 days and colonies counted. For pre-germinated
406 conidia, conidia were first inoculated into 10 ml of YEPD/Tween 80 (6.66 ml YEPD/3.33 ml 0.1
407 % Tween 80) to a final concentration of 500 spores ml^{-1} and incubated statically at 28°C for 6
408 hr, before spread plating and incubation as above. For quantitative comparison of
409 heteroresistance, Hill slopes were fitted to plots (% Viability vs. log10[sorbic acid]) using Prism
410 software and arctangent values for the slopes calculated with Excel to estimate relative
411 heterogeneity (a shallower slope indicating higher heterogeneity) (31, 40).

412 **RNAseq and qRT-PCR.** For preparation of RNA, conidia of *A. niger* N402 were
413 inoculated into 1 litre of YEPD broth (pH 4) to a final concentration of 10^6 conidia ml^{-1} and
414 incubated at 28°C for 1 hr, with shaking at 150 rev. min^{-1}. For sorbic acid or benzoic acid
415 treatments, the medium was supplemented with 1 mM sorbic or 1 mM benzoic acid for the
416 1 h incubation. Conidia were harvested by filtration through a Corning vacuum filtration unit,
417 and immediately used for RNA extraction. RNA was extracted using a Norgen Biotek
418 Plant/Fungi Total RNA extraction kit, as per the manufacturer's instructions.

419 RNAseq analysis was performed by the University of Liverpool Centre for Genomic
420 Research. Three biological replicates were performed for each timepoint in each condition.
421 Between 463-1000ng of total RNA (depending on available material) was Poly A-treated using
422 the NEBNext® Poly(A) mRNA Magnetic Isolation Module, and subsequently purified using
423 Ampure RNA XP beads. Successful depletion of rRNA was confirmed using Qubit fluorometric
424 quantification (ThermoFisher) and Agilent 2100 Bioanalyzer. All of the depleted RNA was used
425 as input material for the NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina®.
426 Following 15 cycles of amplification the libraries were purified using Ampure XP beads. Each
427 library was quantified using Qubit and the size distribution assessed using the Bioanalyzer.
428 These final libraries were pooled in equimolar amounts using the Qubit and Bioanalyzer data.
429 The quantity was assessed using a Qubit® dsDNA HS Assay Kit, while the quality and average
430 fragment size was assessed using the High Sensitivity DNA Kit on the Agilent Bioanalyzer. The
431 RNA libraries were sequenced on an Illumina® HiSeq 4000 platform with version 1 chemistry
432 using sequencing by synthesis (SBS) technology to generate 2 x 150 bp paired-end reads.
433 Initial processing and quality assessment of the sequence data was performed as follows.
434 Briefly, base calling and de-multiplexing of indexed reads was performed by CASAVA version
435 1.8.2 (Illumina). The raw FASTQ files were trimmed to remove Illumina adapter sequences

436 using Cutadapt version 1.2.1 (41). The option "-O 3" was set, so the 3' end of any reads which
437 matched the adapter sequence over a stretch of at least 3 bp was trimmed off. The reads
438 were further trimmed to remove low quality bases, using Sickle version 1.200 with a minimum
439 window quality score of 20. After trimming, reads shorter than 20 bp were removed. Reads
440 were aligned to the *A.niger* CBS 588.13 genome sequence
441 (http://www.aspergillusgenome.org/download/sequence/A_niger_CBS_513_88/current/A_
442 niger_CBS_513_88_current_chromosomes.fasta.gz) using Tophat version 2.1.0 (42). The
443 expression of each gene was calculated from the alignment files using HTseq-count (43). The
444 raw count data were also converted into FPKM (Fragments per Kilobase per Million reads)
445 values. The count numbers per gene were used during the subsequent differential expression
446 analysis. All of the DGE (Differential Gene Expression) analyses were performed in R (version
447 3.3.3) environment using the DESeq2 package (44). Significantly differentially-expressed
448 genes were defined as those with FDR-adjusted P-value < 0.05.

449 For qRT-PCR analysis of gene expression, RNA was extracted as stated above. Genomic
450 DNA was removed by Turbo DNAse Free kit (Invitrogen). cDNA was synthesized using
451 Superscript IV reverse transcriptase (Invitrogen) and Oligo $d(T)_{20}$ primer (Invitrogen),
452 according to the manufacturer's instructions. Transcripts were amplified using SYBR green
453 Master Mix on an Applied Biosystems 7500 Real-Time PCR instrument and quantified against
454 a standard curve of *A. niger* gDNA. Primer pairs used are listed in Table S2.

455

456 **Gene deletion studies and complementation in *A. niger.*** Gene deletion studies were
457 performed in *A. niger* N402, the Open Reading Frames (ORFs) of the target genes being
458 replaced by a hygromycin resistance cassette (Fig. S2 and S6). Gene deletion cassettes were
459 constructed by Gap-Repair cloning in *S. cerevisiae* (45). Briefly, the hygromycin resistance

460 cassette, and approximately 1 kb upstream and downstream flanking regions of each target
461 gene were amplified from genomic DNA by PCR (Primers listed in Table S2). The hygromycin
462 resistance cassette had a 20-30 bp homology with the 1 kb flanking regions, and each flanking
463 region also had 20-30 bp homology with the multiple cloning site of the YEp351 plasmid. The
464 PCR products and *Hin*dIII-linearized YEp351 plasmid were transformed into *S. cerevisiae*
465 BY4743, and transformants selected by leucine prototrophy. Successful construction of the
466 gene deletion cassettes was confirmed by PCR. The resulting gene deletion cassettes were
467 amplified by PCR and purified using PCR purification columns (Machery-Nagel), to produce a
468 final linear gene deletion cassette. All PCR reactions were performed using Phusion High-
469 Fidelity DNA Polymerase (New England Biolabs). Production of protoplasts and their
470 transformation was performed using standard methods (46). Transformants were selected
471 using 200 µg ml^{-1} hygromycin (Roche) and confirmed by PCR and Southern blotting (Fig. S2
472 and S6), using standard methods (47).

473 For complementation of *A. niger* gene-deletion strains, the genes in question (*wasA*
474 and *An14g03570*) were amplified by PCR (primers listed in Table S2) and cloned into the *Sbf*I
475 site of the pAN7.1*BAR* plasmid (Fig. S8), which contains the *BAR* gene as a selectable marker
476 (replacing the original hygromycin resistance cassette (48), and imparting resistance to
477 phosphinothricin). PCR amplification included ~1 kb upstream and ~300bp downstream of
478 the ORF. Transformation of the resulting plasmids was performed as described above, except
479 that transformants were selected using 5 mg ml^{-1} DL-phosphinothricin (Carbosynth) in YDA
480 agar (Yeast nitrogen base without amino acids, including 1.7 g l^{-1} ammonium sulfate, 10 g l^{1}
481 glucose, 2.25 g l^{-1} ammonium nitrate and 1 M sucrose; pH adjusted to 7.0 using Na_2HPO_4,
482 solidified with 1.2% (w/v) agar) (49). Transformants were subjected to an additional round of
483 selection by growth on YDA agar containing 5 mg ml^{-1} DL-phosphinothricin.

484

485 **Cloning and complementation in *S. cerevisiae.*** Complementation studies were
486 performed in the *S. cerevisiae* Δ*pdr12* strain. The complementation plasmid (Fig. S7) was
487 constructed by yeast gap-repair cloning (45). Briefly the *PDR12* promoter and terminator, and
488 An14g03570 ORF were amplified by PCR (primers listed in Table S2). Each amplified fragment
489 included a 20-30 bp region of homology either with the YEp351 plasmid or with a
490 neighbouring fragment. The PCR products and *Hin*dIII linearized YEp351 plasmid were
491 transformed into *S. cerevisiae Δpdr12*, and transformants selected by leucine prototrophy
492 (45). Complementation was also performed with the *PDR12* ORF, as a positive control for
493 successful complementation. The *S. cerevisiae Δpdr12* strain was also transformed with the
494 empty YEp351 plasmid, as a negative control.

495

496

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502

503 **REFERENCES**

504 1. Bondi M, Messi P, Halami PM, Papadopoulou C, de Niederhausern S. 2014. Emerging microbial
505 concerns in food safety and new control measures. BioMed Res Int 2014:251512.
506 2. Kaczmarek M, Avery SV, Singleton I. 2019. Microbes associated with fresh produce: Sources,
507 types and methods to reduce spoilage and contamination. Adv Appl Microbiol 107:29-82.
508 3. Salmond CV, Kroll RG, Booth IR. 1984. The effect of food preservatives on pH homeostasis in
509 *Escherichia coli*. Microbiology 130:2845-2850.
510 4. Plumridge A, Hesse SJA, Watson AJ, Lowe KC, Stratford M, Archer DB. 2004. The weak acid
511 preservative sorbic acid inhibits conidial germination and mycelial growth of *Aspergillus niger*
512 through intracellular acidification. Appl Environ Microbiol 70:3506-3511.
513 5. Freese E, Sheu CW, Galliers E. 1973. Function of lipophilic acids as antimicrobial food additives.
514 Nature 241:321-325.
515 6. Novodvorska M, Stratford M, Blythe MJ, Wilson R, Beniston RG, Archer DB. 2016. Metabolic
516 activity in dormant conidia of *Aspergillus niger* and developmental changes during conidial
517 outgrowth. Fungal Genet Biol 94:23-31.
518 7. Warth AD. 1989. Relationships between the resistance of yeasts to acetic, propanoic and benzoic
519 acids and to methyl paraben and pH. Int J Food Microbiol 8:343-349.
520 8. Stratford M, Steels H, Nebe-von-Caron G, Novodvorska M, Hayer K, Archer DB. 2013. Extreme
521 resistance to weak-acid preservatives in the spoilage yeast *Zygosaccharomyces bailii*. Int J Food
522 Microbiol 166:126-134.
523 9. Boschloo JG, Moonen E, van Gorcom RFM, Hermes HFM, Bos CJ. 1991. Genetic analysis of
524 *Aspergillus niger* mutants defective in benzoate-4-hydroxylase function. Curr Genet 19:261-264.
525 10. Fraser JA, Davis MA, Hynes MJ. 2002. The genes *gmdA*, encoding an amidase, and *bzuA*, encoding
526 a cytochrome P450, are required for benzamide utilization in *Aspergillus nidulans*. Fungal Genet
527 Biol 35:135-146.
528 11. Stratford M, Plumridge A, Pleasants MW, Novodvorska M, Baker-Glenn CAG, Pattenden G, Archer
529 DB. 2012. Mapping the structural requirements of inducers and substrates for decarboxylation of
530 weak acid preservatives by the food spoilage mould *Aspergillus niger*. Int J Food Microbiol
531 157:375-383.
532 12. Plumridge A, Stratford M, Lowe KC, Archer DB. 2008. The weak-acid preservative sorbic acid is
533 decarboxylated and detoxified by a phenylacrylic acid decarboxylase, PadA1, in the spoilage mold
534 *Aspergillus niger*. Appl Environ Microbiol 74:550-552.
535 13. Plumridge A, Melin P, Stratford M, Novodvorska M, Shunburne L, Dyer PS, Roubos JA, Menke H,
536 Stark J, Stam H, Archer DB. 2010. The decarboxylation of the weak-acid preservative, sorbic acid,
537 is encoded by linked genes in *Aspergillus spp*. Fungal Genet Biol 47:683-692.
538 14. Payne KAP, White MD, Fisher K, Khara B, Bailey SS, Parker D, Rattray NJW, Trivedi DK, Goodacre
539 R, Beveridge R, Barran P, Rigby SEJ, Scrutton NS, Hay S, Leys D. 2015. New cofactor supports α, β-
540 unsaturated acid decarboxylation via 1, 3-dipolar cycloaddition. Nature 522:497-501.
541 15. Stratford M, Plumridge A, Archer DB. 2007. Decarboxylation of sorbic acid by spoilage yeasts is
542 associated with the *PAD1* gene. Appl Environ Microbiol 73:6534-6542.
543 16. Piper P, Mahé Y, Thompson S, Pandjaitan R, Holyoak C, Egner R, Mühlbauer M, Coote P, Kuchler
544 K. 1998. The Pdr12 ABC transporter is required for the development of weak organic acid
545 resistance in yeast. EMBO J 17:4257-4265.
546 17. Holyoak CD, Bracey D, Piper PW, Kuchler K, Coote PJ. 1999. The *Saccharomyces cerevisiae* weak-
547 acid-inducible ABC transporter Pdr12 transports fluorescein and preservative anions from the
548 cytosol by an energy-dependent mechanism. J Bacteriol 181:4644-4652.
549 18. Kren A, Mamnun YM, Bauer BE, Schuller C, Wolfger H, Hatzixanthis K, Mollapour M, Gregori C,
550 Piper P, Kuchler K. 2003. War1p, a novel transcription factor controlling weak acid stress response
551 in yeast. Mol Cell Biol 23:1775-1785.

19. Fernandes AR, Mira NP, Vargas RC, Canelhas I, Sá-Correia I. 2005. *Saccharomyces cerevisiae* adaptation to weak acids involves the transcription factor Haa1p and Haa1p-regulated genes. Biochem Biophys Res Commun 337:95-103.
20. Mollapour M, Fong D, Balakrishnan K, Harris N, Thompson S, Schüller C, Kuchler K, Piper PW. 2004. Screening the yeast deletant mutant collection for hypersensitivity and hyper-resistance to sorbate, a weak organic acid food preservative. Yeast 21:927-946.
21. Mira NP, Palma M, Guerreiro JF, Sá-Correia I. 2010. Genome-wide identification of *Saccharomyces cerevisiae* genes required for tolerance to acetic acid. Microb Cell Fact 9:79.
22. Hewitt SK, Foster DS, Dyer PS, Avery SV. 2016. Phenotypic heterogeneity in fungi: importance and methodology. Fungal Biol Rev 30:176-184.
23. Band VI, Weiss DS. 2019. Heteroresistance: A cause of unexplained antibiotic treatment failure? PLoS Pathog 15:e1007726.
24. Andersson DI, Nicoloff H, Hjort K. 2019. Mechanisms and clinical relevance of bacterial heteroresistance. Nature Rev Microbiol 17:1.
25. Stratford M, Steels H, Nebe-von-Caron G, Avery SV, Novodvorska M, Archer DB. 2014. Population heterogeneity and dynamics in starter culture and lag phase adaptation of the spoilage yeast *Zygosaccharomyces bailii* to weak acid preservatives. Int J Food Microbiol 181:40-47.
26. Fernández-Niño M, Marquina M, Swinnen S, Rodríguez-Porrata B, Nevoigt E, Ariño J. 2015. The cytosolic pH of individual *Saccharomyces cerevisiae* cells is a key factor in acetic acid tolerance. Appl Environ Microbiol 81:7813-7821.
27. Teertstra WR, Tegelaar M, Dijksterhuis J, Golovina EA, Ohm RA, Wösten HAB. 2017. Maturation of conidia on conidiophores of *Aspergillus niger*. Fungal Genet Biol 98:61-70.
28. Takanori F, van Rhijn, N., Fraczek, M., Gsaller, F., Davies, E., Carr, P., Gago, S., Fortune-Grant, R., Rahman, S., Mabey Glisenan, J., Houlder, E., Kowalski, C.H., Raj, S., Paul, S., Parker, J.E., Kelly, S., Cramer, R.A., Latge, J.-P., Cook, P., Moye-Rowley, S., Bignell, E., Bowyer, P., Bromley, M.J. The Negative Cofactor 2 complex is a master regulator of drug resistance in *Aspergillus fumigatus*. Nature Comm In revision.
29. Natorff R, Sieńko M, Brzywczy J, Paszewski A. 2003. The *Aspergillus nidulans metR* gene encodes a bZIP protein which activates transcription of sulphur metabolism genes. Mol Microbiol 49:1081-1094.
30. Melin P, Stratford M, Plumridge A, Archer DB. 2008. Auxotrophy for uridine increases the sensitivity of *Aspergillus niger* to weak-acid preservatives. Microbiology 154:1251-1257.
31. Holland SL, Reader T, Dyer PS, Avery SV. 2014. Phenotypic heterogeneity is a selected trait in natural yeast populations subject to environmental stress. Environ Microbiol 16:1729-1740.
32. Legras JL, Erny C, Le Jeune C, Lollier M, Adolphe Y, Demuyter C, Delobel P, Blondin B, Karst F. 2010. Activation of two different resistance mechanisms in *Saccharomyces cerevisiae* upon exposure to octanoic and decanoic acids. Appl Environ Microbiol 76:7526-7535.
33. Kim MS, Cho KH, Park KH, Jang J, Hahn JS. 2018. Activation of Haa1 and War1 transcription factors by differential binding of weak acid anions in *Saccharomyces cerevisiae*. Nucleic Acids Res doi:10.1093/nar/gky1188.
34. Thakur JK, Arthanari H, Yang F, Pan S-J, Fan X, Breger J, Frueh DP, Gulshan K, Li DK, Mylonakis E. 2008. A nuclear receptor-like pathway regulating multidrug resistance in fungi. Nature 452:604-609.
35. Sze J-Y, Woontner M, Jaehning JA, Kohlhaw GB. 1992. *In vitro* transcriptional activation by a metabolic intermediate: activation by Leu3 depends on alpha-isopropylmalate. Science 258:1143-1145.
36. Sellick CA, Reece RJ. 2003. Modulation of transcription factor function by an amino acid: activation of Put3p by proline. EMBO J 22:5147-5153.
37. Dijksterhuis J, Meijer M, van Doorn T, Houbraken J, Bruinenberg P. 2019. The preservative propionic acid differentially affects survival of conidia and germ tubes of feed spoilage fungi. Int J Food Microbiol 306:108258.

603 38. Avery SV. 2006. Microbial cell individuality and the underlying sources of heterogeneity. Nature
604 Rev Microbiol 4:577-587.
605 39. Zhao C, Fraczek, M. G., Dineen, L., Lebedinec, R., Macheleidt, J., Delneri, D., Bowyer, P., Brakhage,
606 A.A., Bromley, M. 2019. High-throughput gene replacement in *Aspergillus fumigatus*. Curr Protoc
607 Microbiol 54:e88.
608 40. Stratford M, Steels H, Novodvorska M, Archer DB, Avery SV. 2019. Extreme osmotolerance and
609 halotolerance in food-relevant yeasts and the role of glycerol-dependent cell individuality. Front
610 Microbiol 9:3238.
611 41. Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads.
612 EMBnet J 17:10-12.
613 42. Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357-
614 359.
615 43. Anders S, Pyl PT, Huber W. 2015. HTSeq—a Python framework to work with high-throughput
616 sequencing data. Bioinformatics 31:166-169.
617 44. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-
618 seq data with DESeq2. Genome Biol 15:550.
619 45. Oldenburg KR, Vo KT, Michaelis S, Paddon C. 1997. Recombination-mediated PCR-directed
620 plasmid construction in vivo in yeast. Nucleic Acids Res 25:451-452.
621 46. Ballance DJ, Turner G. 1985. Development of a high-frequency transforming vector for *Aspergillus*
622 *nidulans*. Gene 36:321-331.
623 47. Green MR, Sambrook J. 2012. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor
624 Laboratory Press.
625 48. Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH, van den Hondel CAMJJ. 1987. Transformation
626 of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. Gene 56:117-
627 124.
628 49. Ahuja M, Punekar NS. 2008. Phosphinothricin resistance in *Aspergillus niger* and its utility as a
629 selectable transformation marker. Fungal Genet Biol 45:1103-1110.

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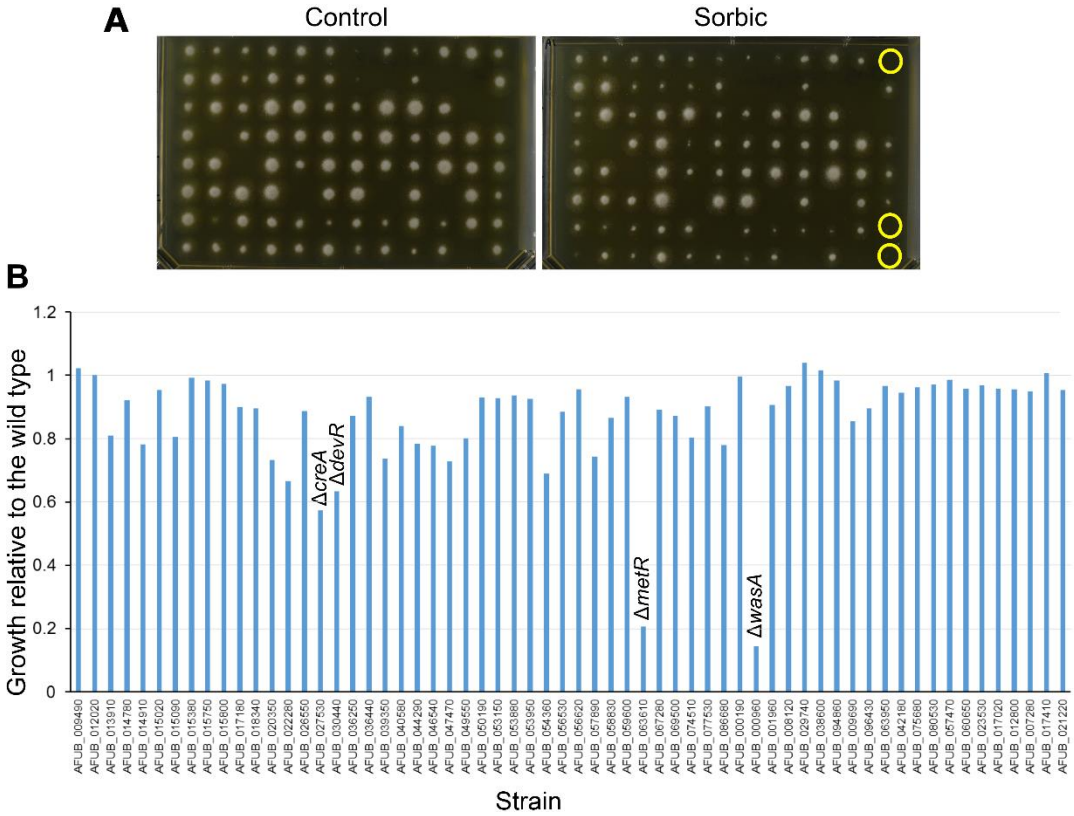

FIG 1 Screening of *A. fumigatus* deletion library. (A) Example of *A. fumigatus* deletant library screen. Conidial suspensions of the different deletants were arrayed in 96-well plates, and transferred to growth medium using a 96-pin tool. Examples of putatively sorbic acid sensitive strains are circled 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*wasA*" refers to Δ*AFUB_000960.*

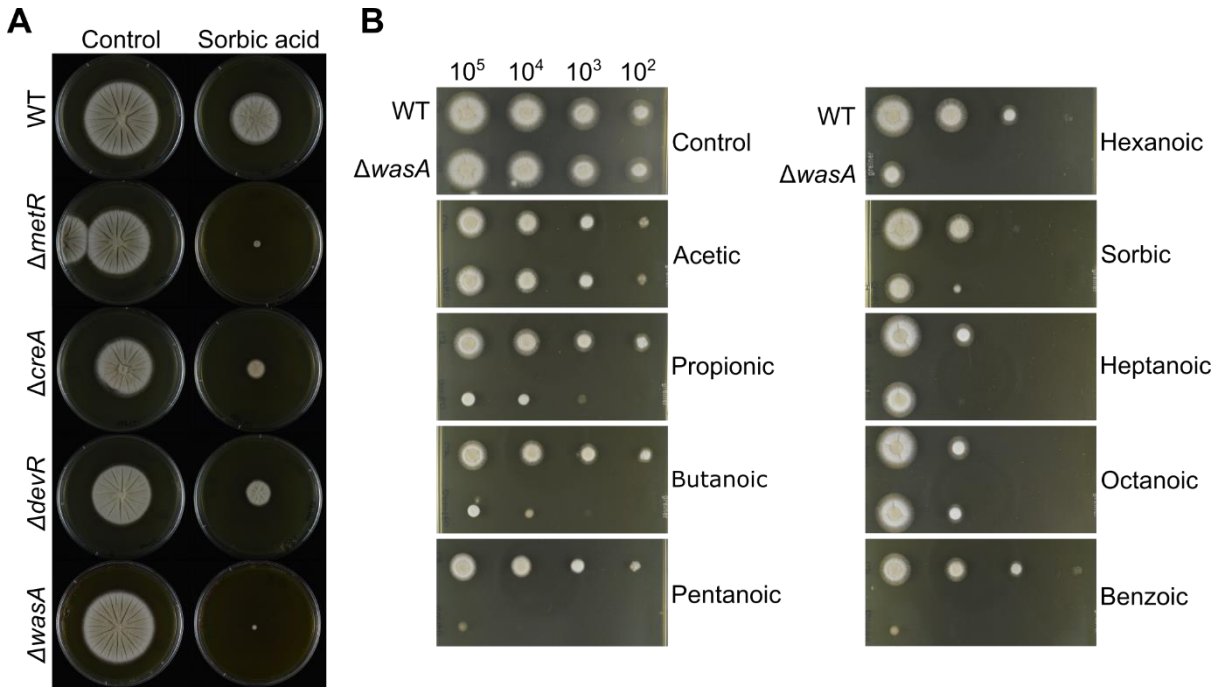

FIG 2 Growth of *A. fumigatus* transcription factor deletion strains on medium containing weak acids. (A) Radial growth of *A. fumigatus* transcription factor deletion strains on agar containing 0.5 mM sorbic acid. Images were captured after 3 d growth at 37°C. (B) Radial growth of *A. fumigatus* Δ*wasA* 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.

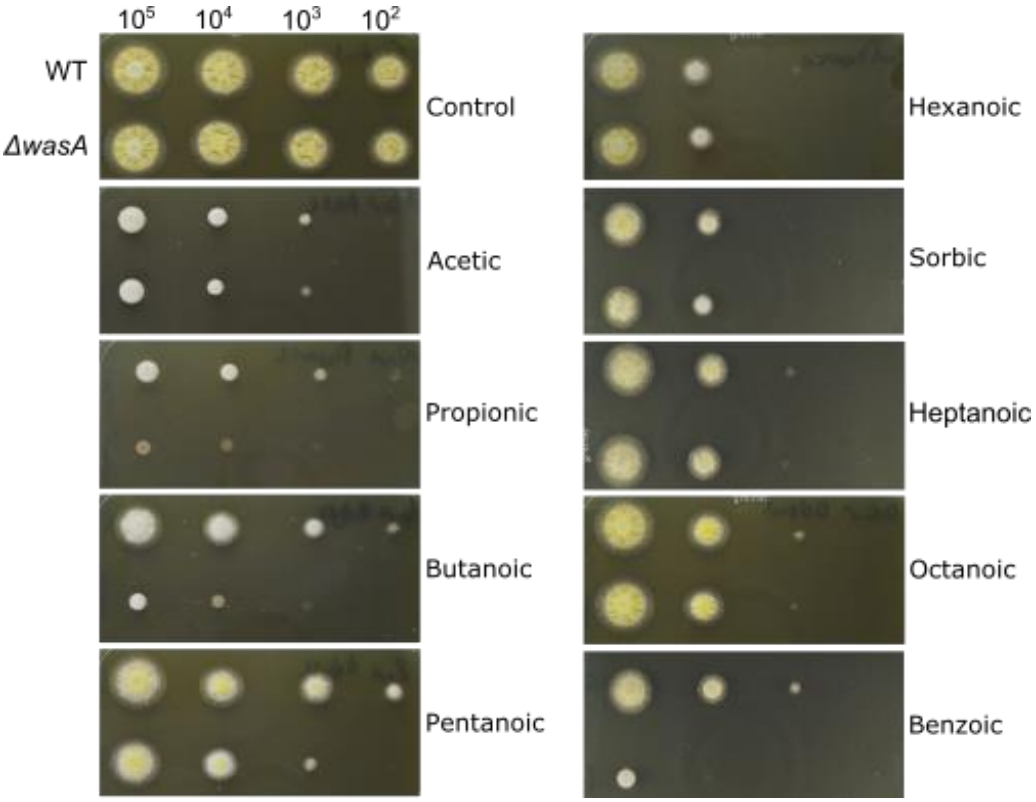

FIG 3 Radial growth of *A. niger* Δ*wasA* growing on different weak acids. Plates were inoculated with a 10-fold dilution series of conidial suspensions; approximate numbers of conidia are indicated above the pictures. Images were captured after 2 d growth at 28°C, and are representative of 2-3 independent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

TABLE 1 Transcriptomics data for selected genes upregulated in the WT during sorbic acid treatment and differentially expressed in WT versus Δ*wasA*.[a]

Gene_ID	Log_2FC[b].WT _Sorbic vs WT control	Log_2FC.Δ*wasA* _Sorbic vs Δ*wasA* control	WT sorbic RPKM[c]	Δ*wasA* 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

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

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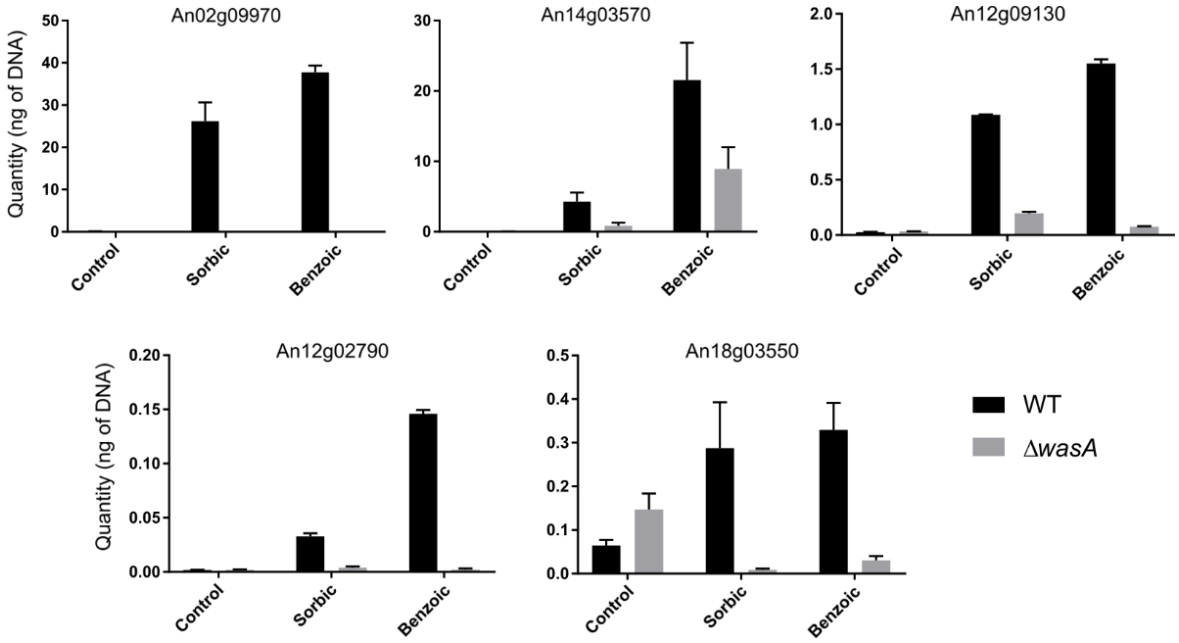

664

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

666 abundances in WT (black bars) and Δ*wasA* (grey bars) conidia germinated in control media,

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

668 of 3 technical replicates.

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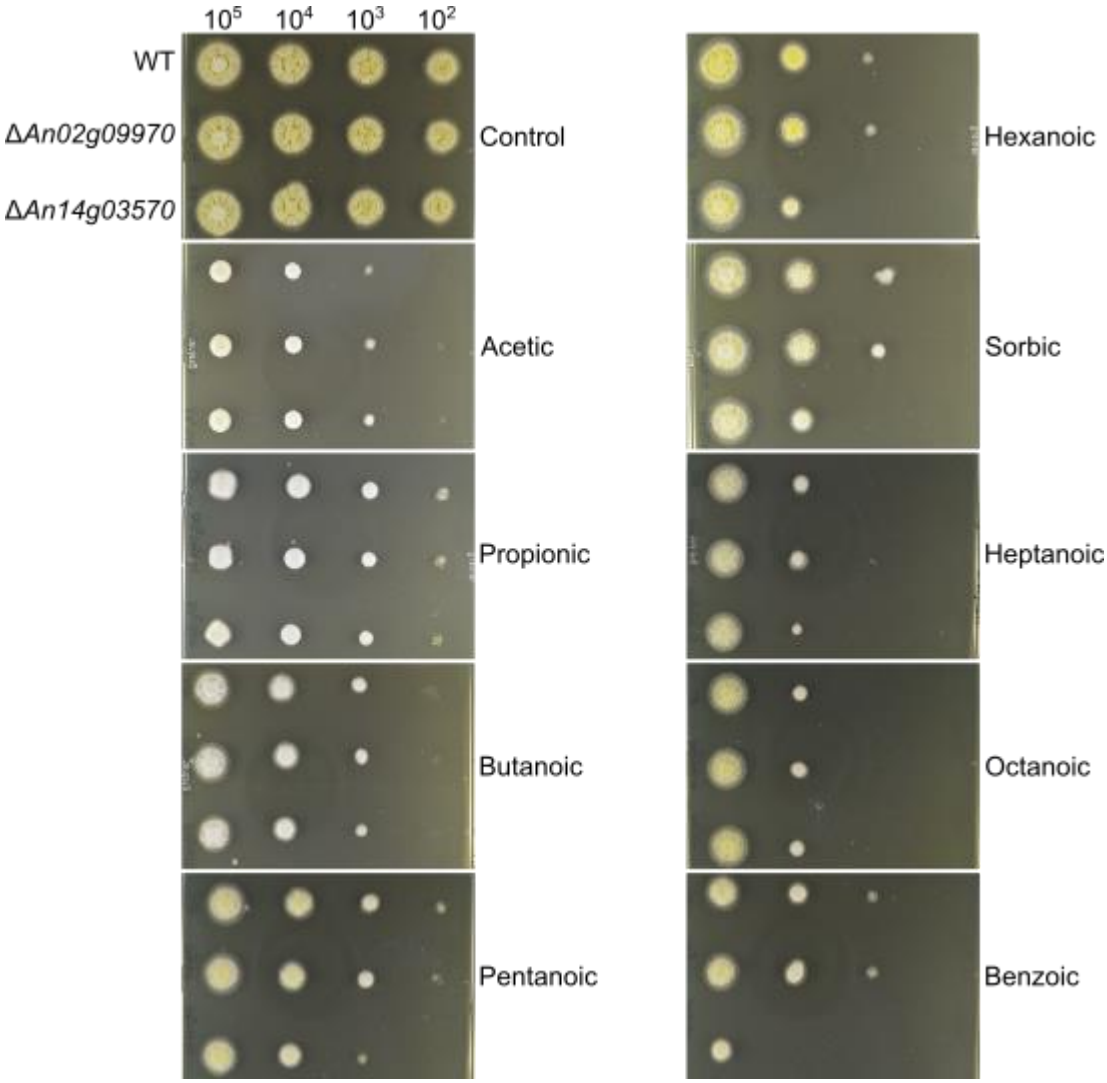

671

672 **FIG 5** Radial growth of Δ*An02g09970* and Δ*An14g03570* deletion strains growing on weak

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

674

675

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

Acid	MIC (mM)	
	WT	**Δ*An14g03570***
Benzoic	4.67 ± 0.12	3.4 ± 0.20
Pentanoic	3.70 ± 0.35	2.6 ± 0.30
Sorbic	5.00 ± 0.35	3.8 ± 0.35

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

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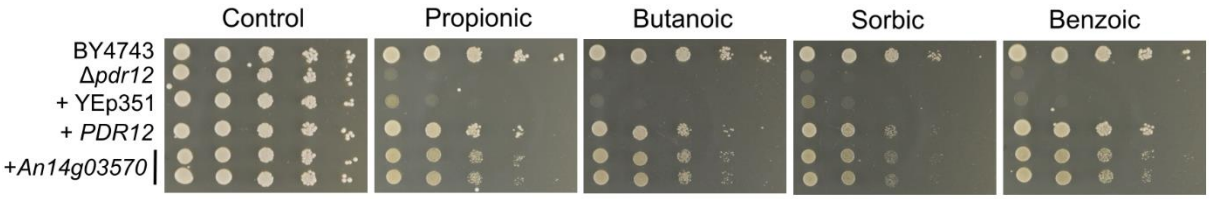

681

682 **FIG 6** Growth of *S. cerevisiae* complemented strains on weak acids. Ten-fold dilution series
683 of *S. cerevisiae* strains (isogenic with the BY4743 wild type) were inoculated onto medium
684 containing weak acids. The Δ*pdr12* strain was transformed with either empty plasmid
685 (+YEp351), YEp351 plasmid containing *PDR12* (+*PDR12)* or YEp351 plasmid containing
686 *An14g03570* ORF and *PDR12* promoter and terminator (+*An14g03570*). Two independent
687 transformants of the +*An14g03570* strain are shown.

688

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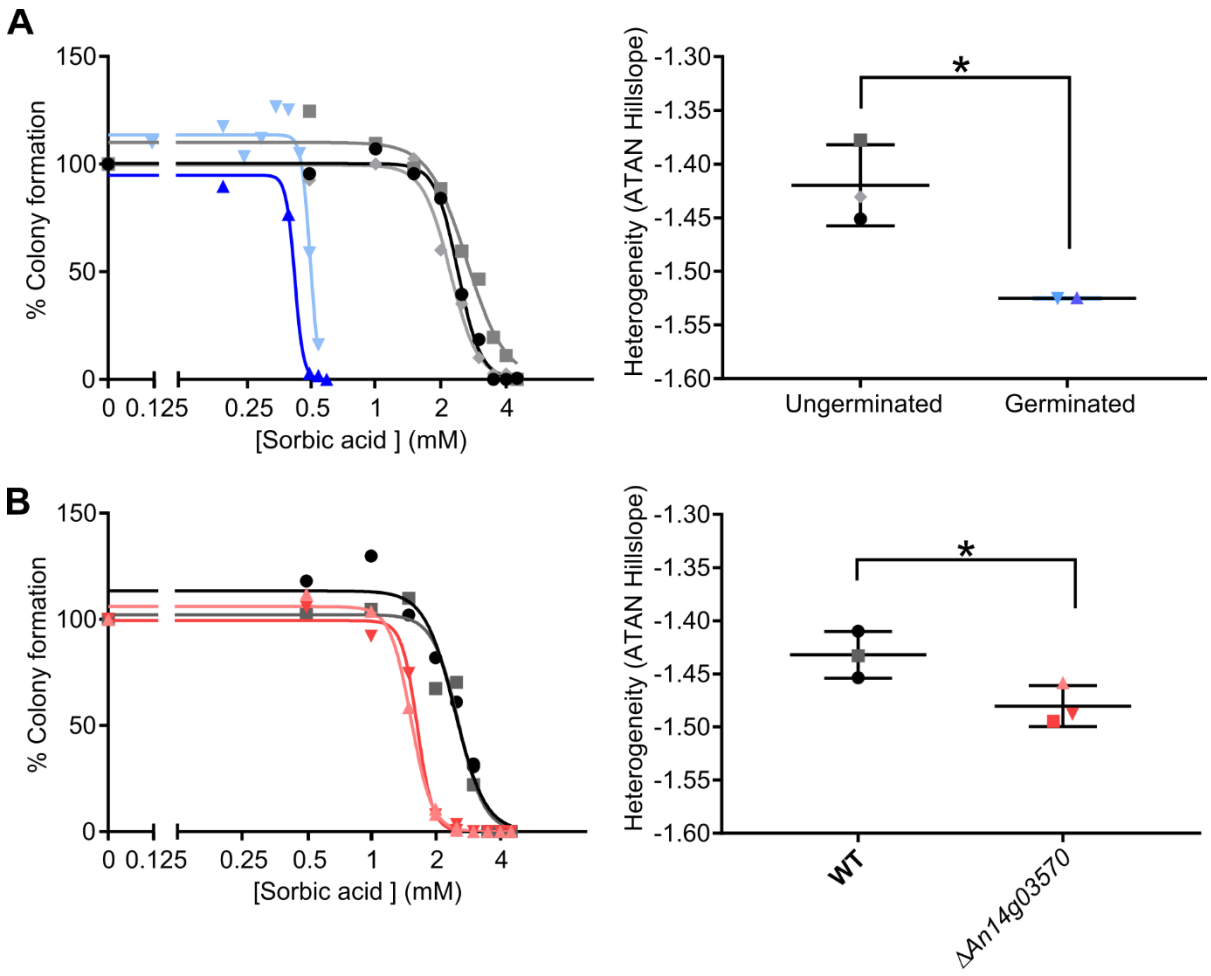

690

691 **FIG 7** Sorbic acid dose response curves for *A. niger* conidia. (A) Dose response curves of
692 germinated (blue lines) and ungerminated (black/grey lines) WT conidia, and comparison of
693 slope values. Dose response curve slope values were compared with 2-way Welch's T-test (p=
694 0.0404) n = 2-3. (B) Dose response curves of WT (black/grey lines) and Δ*An14g03570* (pink/red
695 lines) conidia and comparison of dose response curve slope values, compared with 2-way
696 Welch's T-test (p = 0.0468) n = 3. Two representative independent experiments are shown in
697 the dose response curve.

698

699

700 **LEGENDS TO SUPPLEMENTARY FIGURES (data provided in separate attachments)**

701

702 **FIG S1** Radial growth of the *A. fumigatus* Δ*metR* strain on medium containing 0.5 mM sorbic
703 acid, with or without 0.5 mM methionine.

704

705 **FIG S2** PCR and Southern Blot confirmation of *wasA* deletion. (A) Targeted gene deletion
706 strategy. The *wasA* ORF was replaced with a hygromycin resistance cassette. (B) PCR
707 confirmation of *wasA* deletion. Primer pair 1 was used to confirm deletion of *wasA* ORF –
708 deletion strains are negative, WT is positive. Primer pair 2 was used to confirm integration of
709 *HygR* at the *wasA* locus. (C) Southern blotting of *wasA* and *ohbA1/wasA* deletion strains.
710 gDNA of strains was digested with *Hin*dIII restriction enzyme. Membranes were hybridised
711 with a probe consisting of Digoxigenin-UTP labelled *HygR*. Single bands confirm single
712 integration of deletion cassette into the *A. niger* genome. Transformants Δ*wasA_12* and
713 ΔΔ*ohbA1/wasA_12* were used for experiments.

714

715 **FIG S3** (A) Growth of the Δ*wasA* strain conidia on medium containing sorbic acid. Plates were
716 inoculated with ~100 conidia and incubated for 2 d (control medium) or 3 d (medium
717 containing 1 mM sorbic acid). (B) Radial growth of ΔΔ*ohbA1/wasA* strain on medium
718 containing 1 mM sorbic acid. Plates were inoculated with a 10-fold dilution series of conidial
719 suspensions; approximate numbers of conidia are indicated at the top. Images were captured
720 after 2 d growth at 28°C, and are representative of 2-3 independent experiments.

721

722 **FIG S4** Radial growth of complemented Δ*wasA* strains on medium containing 2 mM benzoic
723 acid. Plates were inoculated with a 10-fold dilution series of conidial suspensions.
724 Approximate numbers of conidia are indicated above the pictures. Images were captured
725 after 2 d growth at 28°C. Two independent complemented lines are shown.
726
727 **FIG S5** PCR and Southern Blotting of *An02g09970* and *An14g03570* deletion strains. (A)
728 Targeted gene deletion strategy. The *An02g09970* and *An14g03570* ORFs were replaced with
729 a hygromycin resistance cassette. (B) PCR confirmation of *An02g09970* deletion. Primer pair
730 1 was used to confirm deletion of the *An02g09970* ORF – deletion strains are negative, WT is
731 positive. Primer pairs 2 and 3 were used to confirm integration of *HygR* at the *An02g09970*
732 locus. (C) PCR confirmation of *An14g03570* deletion. Primer pair 1 was used to confirm
733 deletion of the *An14g03570* ORF – deletion strains are negative, WT is positive. Primer pairs
734 2 and 3 were used to confirm integration of *HygR* at the *An14g03570* locus. (D) Southern
735 blotting of ΔA*n02g09970* and ΔA*n14g03570* deletion strains. gDNA of strains was digested
736 with the restriction enzymes *Xba*I (for ΔA*n02g09970*) or *Eco*RV (for ΔA*n14g03570*).
737 Membranes were hybridised with a probe consisting of Digoxigenin-UDP labelled *HygR*. Single
738 bands confirm single integration of deletion cassette into the *A. niger* genome (multiple
739 integrations apparent for Δ*An02g09970_39*). Transformants ΔA*n14g03570_4* and
740 Δ*An02g09970_29* were used for experiments.
741

742 **FIG S6** Radial growth of complemented Δ*An14g03570* strains on medium containing 2mM
743 benzoic acid. Plates were inoculated with a 10-fold dilution series of conidial suspensions.
744 Two independent complemented lines are shown. A Δ*An14g03570* strain containing the
745 empty pAN7.1*BAR* plasmid is also shown.
746
747 **FIG S7** Plasmid map of YEp351 containing An14g03570
748
749 **FIG S8** Plasmid map of pAN7.1*BAR*.
750
751 **TABLE S1** RPKM and Log_2FC values for *A. niger* genes (Excel file).
752
753 **TABLE S2** List of primers used in this study (Excel file).