Genetic and environmental factors determining heterogeneity in preservation stress resistance of Aspergillus niger conidia

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CHAPTER 2

Natural variation and the role of Zn_2Cys_6 transcription factors SdrA, WarA and WarB in sorbic acid resistance of *Aspergillus niger*

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Abstract

Weak acids, such as sorbic acid, are used as chemical food preservatives by industry. Fungi overcome this weak acid stress by inducing cellular responses mediated by transcription factors. In our research, a large-scale sorbic acid resistance screening was performed on 100 *A. niger* wild-type *sensu stricto* strains isolated from various sources to study strain variability in sorbic acid resistance. The minimal inhibitory concentration of undissociated (MIC_u) sorbic acid at pH = 4 in MEB of the *A. niger* strains varies between 4.0 mM and 7.0 mM, with the average out of 100 strains being 4.8 ± 0.8 mM, when scored after 28 days. MIC_u values were roughly 1 mM lower when tested in commercial ice tea instead of MEB. Genome sequencing of the most sorbic acid sensitive strain among the isolates, CBS 147320, was found to have a premature stop codon inside the sorbic acid response regulator encoding gene *sdrA*. Repairing this missense mutation to using CRISPR-Cas9 mediated genome editing increased the sorbic acid resistance, showing that the sorbic acid sensitive phenotype of this strain is caused by the loss of SdrA function. To identify additional transcription factors involved in weak acid resistance, a transcription factor knock-out library consisting of 240 *A. niger* deletion strains each lacking a single transcription factor was screened. The screen identified a novel transcription factor WarB, contributing to the resistance against a broad range of weak acids, including sorbic acid. The SdrA and WarA Zn_2Cys_6 transcription factors were previously shown to mediate sorbic acid resistance. The role of SdrA, WarA and WarB in weak acid resistance, including sorbic acid, was investigated by creating single, double and the triple knock-out strains. All three transcription factors were found to have an additive effect to the sorbic acid stress response, and the *ΔwarB* strain was found significantly more sensitive to benzoic acid compared to the *ΔsdrA* and *ΔwarA* strains.

Introduction

A significant portion of microbial food spoilage is caused by filamentous fungi, commonly referred to as moulds [1]. Several fungal species have the capacity to infect foods and beverages, and are able to proliferate in conditions with limited water availability, a lack of nitrogen or after heat treatment [2]. Fungal spoilage can affect the visual appearance, taste and other properties of food products [3]. Additionally, the production of mycotoxins by food spoiling fungi forms a direct risk for human health [4].

There are several ways in which the food industry preserves food and reduces microbial spoilage. Firstly, by the use of packaging thereby preventing microbes with access to food. Secondly, by inactivation of microorganisms in food by ionizing radiation and heat treatments such as pasteurization and sterilization [5]. Another tactic involves growth inhibition of microorganisms present on the foods; this includes storage at lowered temperatures, reducing the water activity of foods by drying products or reducing oxygen availability by vacuum packaging. One other growth inhibition technique relies on the addition of chemical substances which reduce microbial growth, such as the addition of weak acid preservatives. [2,5]

Weak acids are food preservatives that cause growth inhibition on a broad spectrum of microorganisms. Weak acids are both fungistatic and bacteriostatic [6]. Commonly used weak acids in the food industry include sorbic acid, benzoic acid [7,8], propionic acid [9], lactic acid, acetic acid [10] and citric acid [11]. Sorbic acid can be added in its acid form, recognized by the European food additive number E200, but is more commonly added as the salt components sodium sorbate (E201), potassium sorbate (E202) or calcium sorbate (E203). Sorbic acid is added to food products such as condiments, bread, fruit jams, juices and soft drinks [12]. The concentration of weak acids allowed in foods and beverages is tightly controlled by governmental organizations such as the Food and Drug Administration (FDA) and the European Food and Safety Authority (EFSA). The maximum concentration of sorbates strongly depends on the food product, for example, the EFSA states that a maximum of 300 mg/L (2.67 mM) sorbate is

allowed in flavored drinks (excluding dairy products), whereas a maximum of 500 mg/L (4.46 mM) sorbate is allowed in fruit and vegetable juices, and 2000 mg/L (17.84 mM) is allowed in processed cheeses [13]. The mode of action of these weak acids as preservatives is most commonly described in the “classical weak acid theory” [14].

The classic weak acid theory explains that weak acids, when present in low pH, can cause the acidification of cells. In a liquid solution, a weak acid can be present in its undissociated form, in which the weak acid is present in its full molecule formation, or in its dissociated form in which the molecule has dissociated into the charged anion (WA^-) and proton (H^+). In solutions where the pH is equal to the pKa of the weak acid, the amount of undissociated acid (WAH) is equal to the amount dissociated acid (WA^- and H^+). When the pH decreases, the proportion of undissociated acid (WAH) increases. Only weak acids in their undissociated form (WAH) are able to diffuse through the plasma membrane into the cytoplasm [15,16]. Therefore, when the pH < pKa, the weak acid molecules diffuse through the plasma membrane, and because of the near neutral pH of the cytoplasm, the undissociated acids (WAH) are forced to dissociate into charged ions (WA^- and H^+) [17,18]. The charged ions (WA^- and H^+) are not able to diffuse back through the plasma membrane and accumulate in the cytoplasm, resulting in acidification of the cytoplasm [19]. In this classic theory, weak acids are thought to be most effective in solutions with a low pH.

However, recent studies show that not all weak acids inhibit growth equally, and some weak acids do not even cause a lowered internal pH [20]. Therefore, alternative mode of actions have been proposed in literature, describing the function of specific weak acids in food preservation. For example, a study in *Saccharomyces cerevisiae* suggested that sorbic acid accumulates in mitochondrial membranes [20]. This study proposed that sorbic acid inhibits the O_2 uptake by inducing ROS and thereby negatively influencing the respiration of yeast. Similarly, in *Aspergillus niger* sorbic acid acts as a membrane active compound which inhibits glucose and O_2 uptake, thereby inhibiting conidial germination [21]. These examples show that weak acid preservatives can have inhibiting effects on cells besides acidification of the cytoplasm as described in the clas-

sical weak acid theory.

Several food spoiling fungi have been reported with resistance to these weak acid preservatives, the most well-known species being spoilage yeasts. The spoilage yeast *Zygosaccharomyces bailii* has been reported to survive up to 9.45 mM of sorbic acid and 11 mM of benzoic acid [3]. However, besides yeast species, mostly Ascomycetes are found as food contaminants [1]. Commonly found food spoiling Ascomycetes include for example *A. niger*, *Paecilomyces variotii* and *Penicillium roqueforti* [2]. Therefore, preservative resistance of Ascomycetes have been investigated before, especially in relation to specific food products such as bread [22–25]. Investigation on the stress responses and molecular mechanisms behind weak acid resistance in filamentous fungi has been limited, however the role of two transcription factors has been revealed in *A. niger*.

Transcription factors play an important role in acquiring weak acid resistance by food spoiling fungi. *A. niger* has the ability to decarboxylate the weak acids sorbic acid and cinnamic acid, mediated by the enzymes phenyl transferase PadA, and cinnamic acid decarboxylase CdcA [26,27]. These enzymes provide resistance towards sorbic acid and cinnamic acid, and is most effective during conidial germination and outgrowth [6]. CdcA and PadA are regulated by the Zn_2Cys_6-finger transcription factor sorbic acid decarboxylase regulator SdrA [27]. These genes are also present in other *Aspergilli* which are able to grow on sorbic acid and cinnamic acid [27]. The deletion of *cdcA*, *padA*, or *sdrA* results in increased sensitivity towards sorbic acid and cinnamic acid, but did not completely eliminate it [27]. This suggests the presence of a separate yet uncharacterized set of genes that also add to the sorbic acid resistance in *A. niger*.

The transcription factor ‘weak acid resistance A’ (WarA) has been recently described in *A. niger*, and is required for resistance against a range of weak acid preservatives [28]. The knock-out strain (*ΔwarA*) showed sensitivity to propionic, butanoic, pentanoic, hexanoic, sorbic and benzoic acids. WarA is described as having a CdcA-independent role in the sorbic acid resistance of *A. niger*, since the double knock-out strain

lacking WarA and CdcA shows increased sensitivity to sorbic acid when compared to either of the single knock-out strains. Geoghegan and colleagues propose that WarA is possibly required for weak acid resistance by regulating the expression of PdrA, an ATP binding cassette (ABC) type transporter, which the authors show is a homologue to Pdr12p, an ABC-type transporter known to pump out weak-acid anions in *S. cerevisiae* [29]. In *S. cerevisiae* Pdr12p is regulated by War1p, a Zn_2Cys_6-finger transcription factor, which binds to weak acid response element, WARE, in the Pdr12p promotor [30]. It should be noted that the *S. cerevisiae* War1p and *A. niger* WarA protein are both Zn_2Cys_6 transcription factors, but they do not shown significant sequence similarity apart from the DNA binding domain [28].

In our research, a large-scale sorbic acid resistance screening was performed on 100 *A. niger* wild-type strains to study strain variability in sorbic acid resistance. Additionally, the screening of 240 transcription factor knock-out strains revealed the importance of multiple transcription factors in the weak acid stress response, including WarB. We show that WarB is important for sorbic, benzoic, cinnamic, propionic and acetic acid stress resistance, and that the *warB* deletion has an additive effect on the sorbic acid resistance when combined with the *sdrA* and *warA* deletions.

Results

Natural variation of sorbic acid resistance among 100 *A. niger* sensu stricto strains

All 100 wild-type *A. niger sensu stricto* strains were obtained from the CBS collection, Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands. These strains originate from all over the word and were isolated from diverse sources (Table 2.S1). In order to investigate food spoiling capacity of these strains, a 96-wells plate assay was performed testing sorbic acid resistance of *A. niger* strains in two types of liquid media; malt extract broth (MEB) (Figure 2.1) and commercial ice tea (Lipton Peach Ice Tea) (Figure 2.2). The 100 *A. niger* strains were subjected to 0 – 9 mM of undissociated sorbic acid and scored for growth after seven and 28 days.

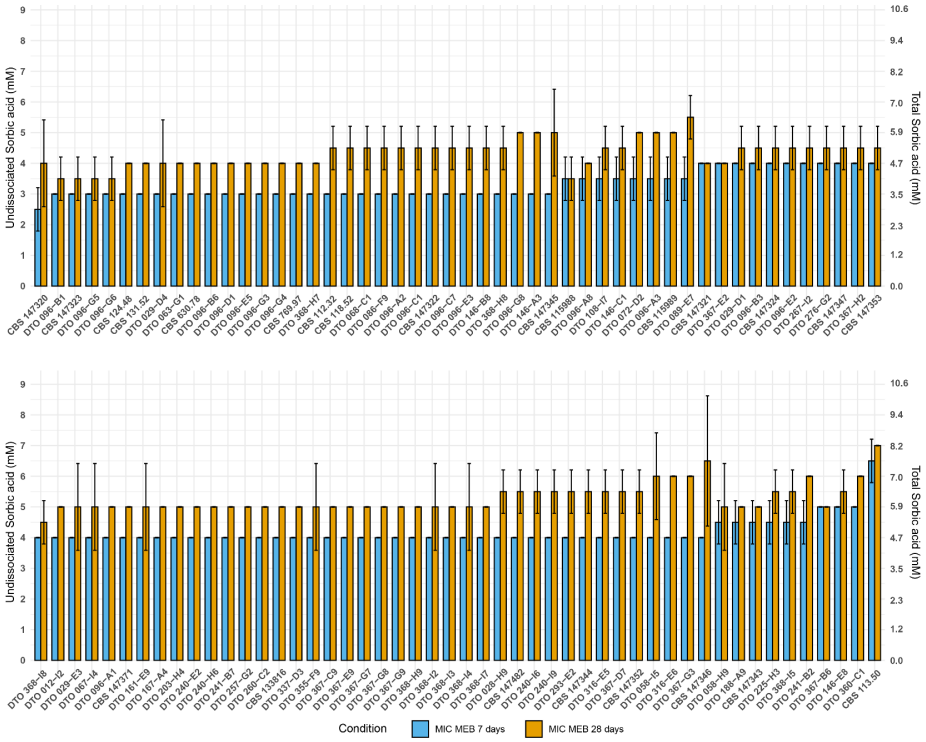

Figure 2.1. MIC assay showing strain diversity of 100 *A. niger* strains grown in MEB. Mean MIC values for each strain was determined after 7 days (blue), and 28 days (orange) of growth at 25 °C, from biological duplicates. The error bar indicates the standard deviation between the duplicates. The primary Y-axis indicates the undissociated sorbic acid concentration, whereas the secondary Y-axis indicates the total sorbic acid concentration.

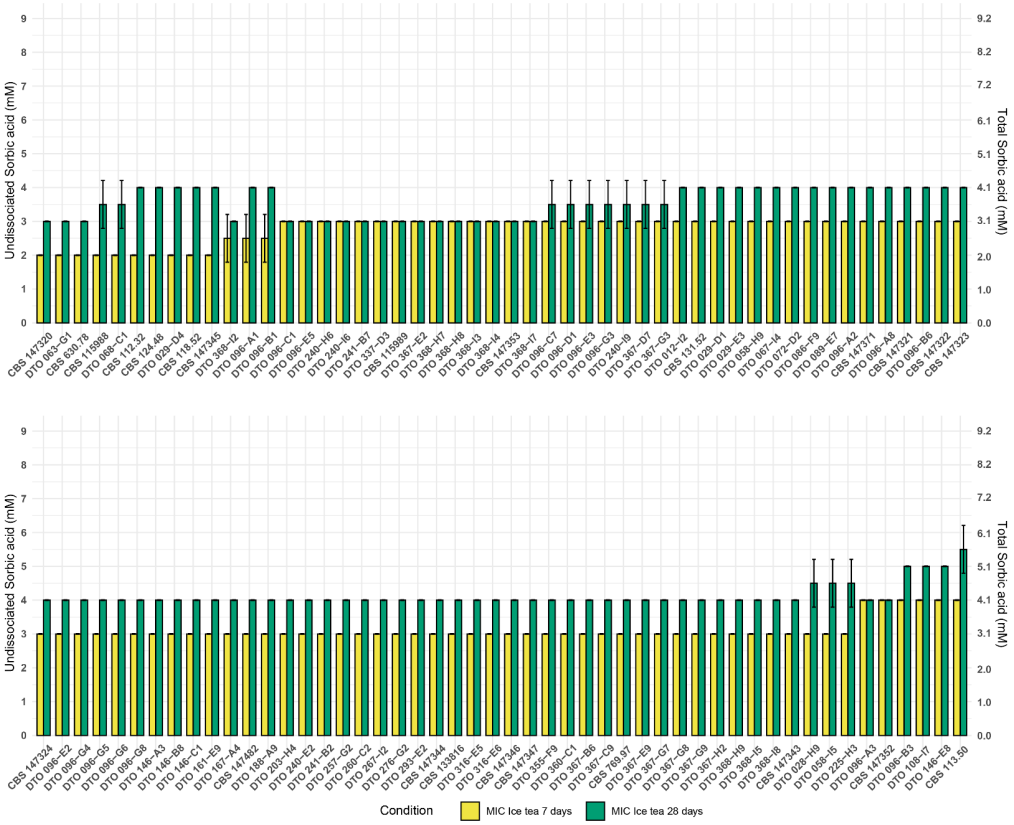

Figure 2.2. MIC assay showing strain diversity of 100 *A. niger* strains grown in ice tea. Mean MIC values for each strain was determined after 7 days (yellow), and 28 days (green) of growth at 25 °C, from biological duplicates. The error bar indicates the standard deviation between the duplicates. The primary Y-axis indicates the undissociated sorbic acid concentration, whereas the secondary Y-axis indicates the total sorbic acid concentration.

The average MIC_u of the 100 *A. niger* strains was determined in both MEB and a commercial ice tea, and shown together with the MIC_u of the most resistant and sensitive strain in Table 2.1. The fungal static effect of sorbic acid became apparent by determining the MIC after prolonged incubation (28 days) compared to 7 days. After 28 days the MIC increased 1.1 mM and 0.9 mM in MEB and Ice tea respectively. The average MIC_u of sorbic acid when tested in commercial ice tea was roughly 1 mM lower than the values obtained in MEB (Table 2.1).

Table 2.1. The average and most extreme sorbic MIC_u values (average ± SD) out of 100 *A. niger* strains grown in MEB and commercial ice tea.

Sample	MIC_u in MEB (mM)		MIC_u in ice tea (mM)	
	7 days	28 days	7 days	28 days
Average of 100 strains	3.7 ± 0.6	4.8 ± 0.8	2.9 ± 0.4	3.8 ± 0.5
CBS 147320 (sorbic acid sensitive strain)	2.5 ± 0.7	4.0 ± 1.4	2.0 ± 0.0	3.0 ± 0.0
CBS 113.50 (sorbic acid resistant strain)	6.5 ± 0.7	7.0 ± 0.0	4.0 ± 0.0	5.5 ± 0.7

Genome sequencing and SNP analysis of the most sorbic acid sensitive strain CBS147320

The genome of CBS 147320 was previously sequenced (Chapter 3). When analysing the genome of this strain, we discovered a SNP inside the *sdrA* gene (G1296A, located in the Fungal specific transcription factor domain PF04082), resulting in a premature stop codon. Therefore, the SdrA protein (originally 657 amino acids long) is truncated in the sorbic acid sensitive wild-type strain CBS 147320 and only 384 amino acids long. In order to test whether the missense mutation in *sdrA* is responsible for the high sensitivity towards sorbic acid, a complementation study was designed to restore the mutation resulting in a stop codon (TGA) back to the codon (TGG) found in the wild type *sdrA* gene found in other isolates and the N402 strain (for complementation methodology see Figure 2.S1). In short, fungal transformations were performed in which a double strand break (DSB) was introduced in the *sdrA* locus in CBS 147320 using CRISPR/Cas9. Donor DNA containing a truncated copy of *sdrA* amplified from lab strain N402 (containing the wild-type gene of *sdrA*) was provided during the transformation. Transformants with a putatively restored *sdrA* locus were created in this way, and subsequently screened for sorbic acid resistance. Thirteen transformants were obtained and analysed for their sorbic acid resistance by performing a spot-assay on MM plates containing 1, 2, 3 and 4 mM sorbic acid at pH = 4 (Figure 2.3). Parental strain CBS 147320, a *sdrA* deletion strain and sorbic acid resistant strain CBS 113.50 were taken along as controls. Trans-

formants could be grouped in three different groups based on the phenotypes seen in the sorbic acid spot-assay. Seven transformants (group 1) have similar sorbic acid sensitivity as parental strain CBS 147320, indicating that the SNP in the *sdrA* gene has not been restored in these transformants. The double-stranded break in these transformants had been most likely repaired by non-homologous end-joining and not by homologous recombination of the donor DNA. Four transformants (group 2) show increased sorbic acid resistance compared to parental strain CBS 147320, having visible growth after 4 days on MM plates containing 3 mM sorbic acid. For now, we assume that in these transformants the mutated *srdA* gene is repaired by homologous recombination of the donor DNA. Two transformants (group 3) have a higher sorbic acid resistance, comparable to the sorbic acid resistant strain CBS 113.50. We assume that in these transformants, the missense mutation in sdrA is repaired by the donor DNA and that additional copies of the donor DNA have been integrated. These results indicate that the weak acid sensitivity of wild-type strain CBS 147320 could be restored by introducing the wild-type *sdrA* gene back into the genome. An additional diagnostic PCR or Southern blot analysis is needed to further confirm the genomic alterations in all groups of transformants.

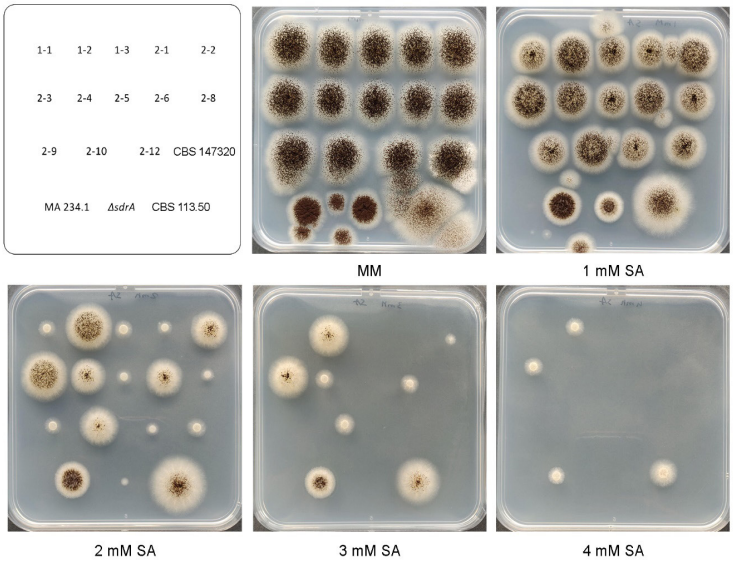

Figure 2.3. Phenotypic screen of CBS 147320 transformants with a putatively restored *sdrA* locus on sorbic acid. The transformants, JvD1-1 until JvD2-12, with a potentially restored *sdrA* locus were tested on sorbic acid resistance. The spot assay was done on MM + glucose with the

addition of sorbic acid (SA), concentrations given of undissociated sorbic acid. Conidia were spotted and plates were subsequently grown for 4 days at 30 °C. Several controls were taken along; sorbic acid sensitive wild-type strain CBS 147320, parental strain MA 234.1, knock-out strain (*ΔsdrA*) and sorbic acid resistant strain CBS 113.50. Transformants showed three phenotypes, (1) like parental strain CBS 147320, (2) semi-resistant showing growth in the presence of 3 mM SA or (3) very resistant to SA, comparable to the most resistant wild-type strain CBS 113.50.

Screening for transcription factors that are related to weak acid stress resistance

In order to identify additional transcription factors involved in weak acid stress resistance of *A. niger*, a library of 240 *A. niger* transcription factor knock-out strains was screened for sorbic acid, cinnamic acid, benzoic acid and propionic acid resistance (knock-out library made by Arentshorst, van Peij, Pel and Ram, unpublished data). A selection of transcription factor knock-out strains with interesting phenotypes were re-evaluated using smaller concentration steps (Figure 2.4).

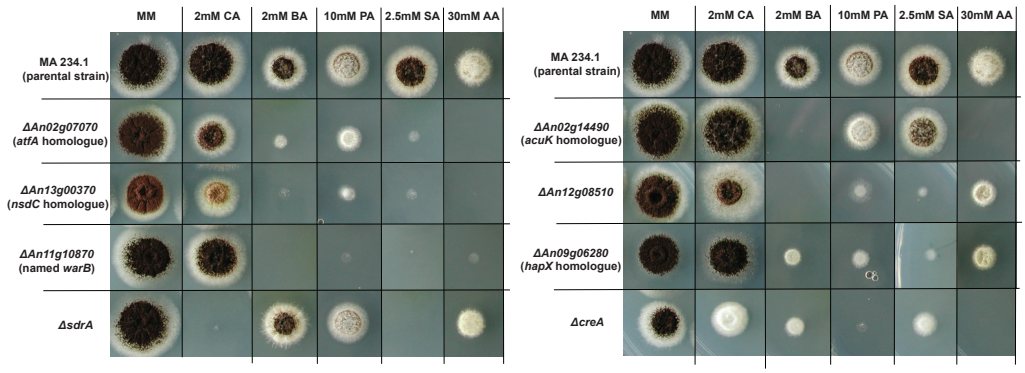

Figure 2.4. Transcription factor knock-out strains sensitive to weak acid stress. The spot assay was conducted on MM containing glucose (pH = 4). The weak acids tested were 2 mM cinnamic acid (CA), 2 mM benzoic acid (BA), 10 mM propionic acid (PA), 2.5 mM sorbic acid (SA) and 30 mM acetic acid (AA). Weak acid concentrations listed are total weak acid concentrations added, the undissociated acid concentrations (pH = 4) are 1.5 mM CA, 1.2 mM BA, 8.8 mM PA, 2.1 mM SA and 25.6 mM AA. Growth was scored and pictures were taken after 5 days of incubation at 30°C. The top row contains parental strain MA 234.1 (ΔkusA), two times the same spots, as a control for growth comparison.

The screening of 240 transcription factor knock-out strains revealed multiple candidate transcription factors involved in weak acid stress resistance in *A. niger*, including the *atfA* homologue putatively involved in the general stress response [31] the

nsdC homologue, the *hapX* homologue, the *acuK* homologue and *creA* which is the main regulator of carbon catabolite repression [32]. Additionally, knock-out strains lacking transcription factors An12g08510 and An11g10870, with no clear homologues, showed reduced growth on plates containing weak acids. The knock-out strain lacking gene An11g10870 was specifically interesting, showing a severe growth reduction on four out of the five weak acids tested: benzoic acid, propionic acid, benzoic acid and acetic acid. This gene was studied further and named WarB for 'weak acid resistance B'. WarB is a Zn_2Cys_6 transcription factor consisting of only 307 amino acids and is significantly shorter than other sorbic acid response regulators SdrA (628 amino acids) and WarA (777 amino acids). No clear homology exists between WarB and previously described transcription factors WarA, SdrA or the sorbic acid response regulator in yeast; War1p [28,30]. In order to further investigate the effects of the *warB* deletion, single and combination knock-out strains lacking *sdrA*, *warA* and *warB* were made using CRISPR/Cas9 genome editing. The proper deletion of the strain was verified by diagnostic PCR (Figure 2.S2) and these strains were tested for their resistance against weak acid preservatives, using a spot assay (Figure 2.5).

The single knock-out strain *ΔwarB* was more sensitive towards sorbic acid, benzoic acid and cinnamic acid compared to its parental strain. The *ΔwarB* single knock-out strain was more sensitive towards benzoic acid when compared to the *ΔsdrA* or *ΔwarA* single knock-out strains. The double knock-out strain *ΔsdrA, ΔwarB* showed severely reduced growth in the presence of cinnamic acid when compared to either *ΔsdrA* or *ΔwarB* single knock-out strains. All three transcription factors, *sdrA* and *warA* and *warB*, were involved in sorbic acid resistance, as shown by the higher sensitivity of the triple knock-out strain than any double knock-out strain, indicating that these three transcription factors work side-by-side to generate the regular sorbic acid stress response. Additionally, sorbic acid sensitivity of the knock-out strains was investigated in a liquid assay (MEB) in 96-wells plates, similar to the experiment performed on wild types in Figure 2.1, confirming the same impact of *sdrA*, *warA* and *warB* on sorbic acid resistance in liquid (Figure 2.S3).

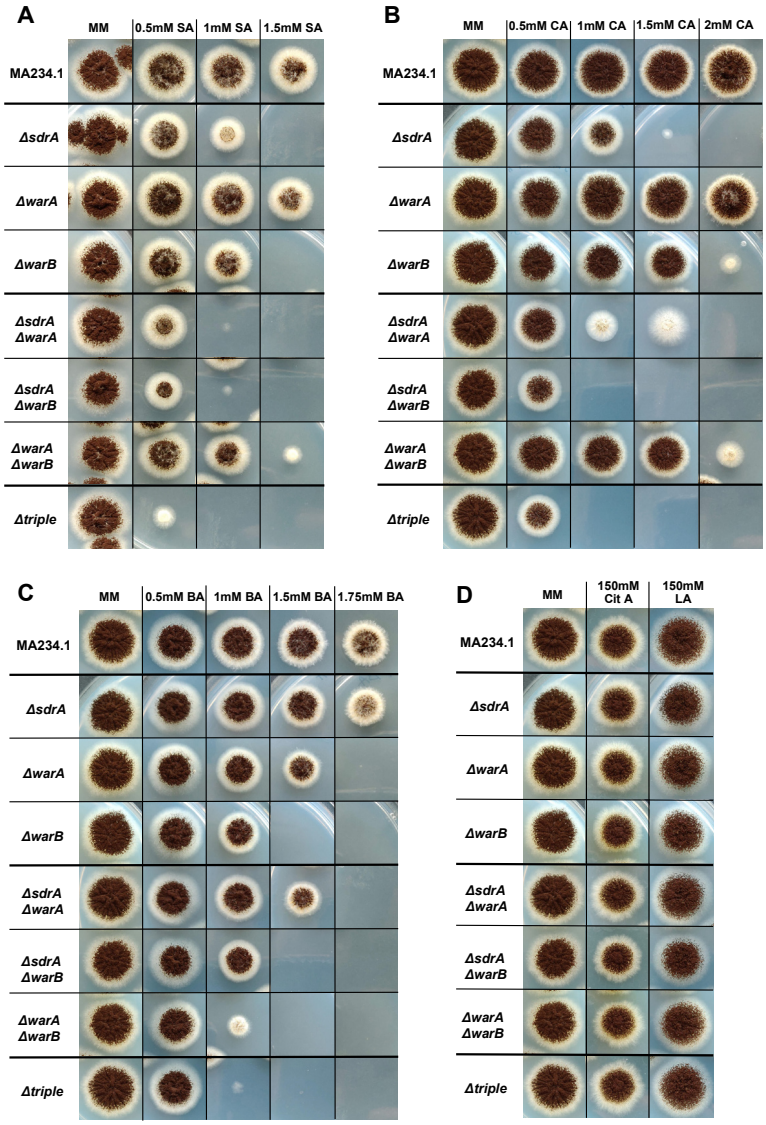

Figure 2.5. Weak acid stress resistance of knock-out strains lacking *sdrA*, *warA* and/or *warB*. Conidia are spotted on MM containing glucose and weak acid, pH 4, grown for 4 days at 30 °C. Growth was compared to the growth phenotype of the parental strain MA234.1 (*ΔkusA*). All concentrations of weak acids added listed are total weak acid concentrations. A. Sorbic acid (SA) stress resistance. The triple knock-out strain is the most sensitive for sorbic acid stress. A double knock-out strain *ΔwarA*, *ΔwarB* is less sensitive than the single knock-out strain *ΔwarB*. B. Cinnamic acid (CA) stress resistance. The Δ*sdrA, ΔwarB* strain is the most sensitive to cinnamic acid, the *warA* deletion does not seem to affect cinnamic acid stress resistance. C. Benzoic acid (BA) stress resistance. The *warB* deletion has the largest effect on the benzoic acid resistance. D. Citric acid (Cit A) and lactic acid (LA) stress resistance seemed not affected.

Discussion

Heterogeneity among different (natural) isolates of the same species in relation to weak acid stress resistance of fungi has been reported before. For example in the case of spoilage yeast *Z. bailii* [3], variation in MIC values among strains were reported to vary between 4.5 mM and 9.5 mM (mean 7.1 mM). In our study, the sorbic acid MIC_u of *A. niger* was determined for 100 strains, and showed and average of 4.8 ± 0.8 mM in MEB and 3.8 ± 0.5 mM in commercial ice tea when scored after 28 days (Table 2.1). These findings for *A. niger* are consistent with earlier reports. A recent study tested three *A. niger* strains and one *Aspergillus tubingensis* strain and reported sorbic acid MIC_u values of these strains between 2.88 mM – 4.80 mM [22]. Therefore, most *A. niger* strains will survive the maximum allowed sorbates in flavored drinks (2.67 mM), some will survive the limits allowed in fruit juices (4.46 mM) and no strains will survive limits allowed in processed cheeses (17.84 mM) [13]. However, it is important to note the outliers, specifically the most sorbic acid resistant *A. niger* strain out of the 100 reported in our study (Table 2.1), CBS 113.50, with a sorbic acid MIC_u of 7 mM. This means that, depending on the strains found in any specific food processing facility, one might need 7 mM of undissociated sorbic acid to reliably prevent growth of *A. niger*. Additionally, MIC_u values are depending on the medium used, as strains consistently showed lower MIC_u values in commercial ice tea when compared to relatively rich medium MEB. We noticed that sporulation was limited in the ice tea medium, and the 96-wells plates showing growth were not as densely packed with mycelium when compared to the same assay performed in MEB. Perhaps ice tea is a relatively poor growth medium for *A. niger*, thereby lowering the minimal concentration of sorbic acid needed to prevent outgrowth. No clear relationship between isolated source and sorbic acid resistance was found. *A. niger* strains isolated as food contaminants were not the most sorbic acid resistant strains in our study. Only two strains, CBS 113.50 and DTO 146-E8, belong consistently to the top 5 most sorbic acid resistant strains in both MEB and commercial ice tea, and these strains were isolated from leather and indoor environment, respectively (Table 2.S1). The most sorbic acid sensitive strain in both MEB and commercial ice tea, CBS 147320,

was isolated from grape.

The most sorbic acid sensitive strain, CBS 147320, had a SNP inside the *sdrA* gene resulting in a premature stop codon. The sorbic acid resistance could be increased again by replacing the SNP with the 'normal' base present in all other *A. niger* strains (Figure 2.3). Therefore, the most sorbic acid sensitive wild-type strain found, originally isolated from a grape in Australia, was in fact a strain lacking SdrA activity. This indicates that the transcription factors involved in the sorbic acid response are important, not solely for our understanding of the molecular mechanisms behind fungal sorbic acid resistance, but are also an important factor within the observed strain variability of *A. niger*.

A spot-assay testing weak acid resistance of 240 *A. niger* knock-out strains, each lacking a single transcription factor, revealed transcription factors that are potentially involved in the weak acid stress response. One transcription factor is a homologue of general stress response regulator AtfA [31]. Three transcription factors which upon deletion reduced the resistance towards sorbic acid are homologues of genes regulating siderophores and iron uptake, HapX, NsdC and AcuK [33–35]. AcuK is known to be essential for growth on gluconeogenic carbon sources and its reduced resistance could possibly be caused by a metabolic imbalance an reduced catabolism of sorbic acid. However, AcuK is also required for iron uptake in *Aspergillus fumigatus* and regulates a set of genes involved in iron homeostasis, including gene *hapX* [33]. Recently, researchers have shown that NsdC regulates many genes in *A. fumigatus* and impacts stress resistance against cell wall damaging agents, however NsdC also regulates expression of siderophores and genes involved in iron-uptake, again including *hapX* [35]. Transcription factor HapX is best known for its role in iron homeostasis, however researchers have shown that the HapX protein also has a putative role in mitochondrial metabolism in *A. fumigatus*, more than 30% of the target genes of HapX have a function in the mitochondria [36]. As discussed in the introduction, a recent publication has disputed the classical weak acid theory of cytosolic acidification, and instead proposes that weak acids disrupt mitochondrial respiration by localizing in the mitochondrial membrane [20]. It is interesting to note that the effectivity of weak acids in the mitochondrial membrane

could perhaps explain why *acuK*, *nsdC* and *hapX* knock-out strains were linked to weak acid stress sensitivity. The *ΔcreA* strain also seems impacted by weak acids. Since the *ΔcreA* strain is impacted in the carbon catabolite repression, the strain does not limit itself to glucose uptake and metabolism. Perhaps the active uptake of the weak acids as a potential carbon source in the *ΔcreA* strain is causing its weak acid sensitive phenotype.

Another interesting gene was putative transcription factor An11g10870, dubbed WarB. We analysed available expression data of *A. niger* growing in the presence of sorbic acid to investigate the expression of the *warB* gene [28]. The *warB* gene shows induction (logFC = 5.5) in the presence of sorbic acid compared to the control, indicating the possible involvement of WarB in the sorbic acid stress response. The deletion of *warB* resulted in increased sensitivity towards benzoic, sorbic, cinnamic, propionic and acetic acid (Figure 2.3 and Figure 2.4). Double and triple knock-out strains were made in *A. niger* lacking *sdrA*, *warA* and/or *warB* to investigate the relative importance of each transcription factor in the weak acid stress response. All three transcription factors seem to contribute to the sorbic acid resistance of *A. niger*, as indicated by the high sorbic acid sensitivity of the triple knock-out strain. Interestingly, the *ΔwarA*, *ΔwarB* double knock-out strain seems to be slightly more resistant to both sorbic acid and cinnamic acid than the *ΔwarB* single knock-out strain. Perhaps this finding indicates a compensatory effect, where *sdrA* is upregulated in the absence of *warA* and *warB*, but further research is needed to confirm this hypothesis. Future research could focus on the target genes regulated by WarB, thereby expanding our knowledge on the weak acid stress response of *A. niger*.

Material and Methods

Strains and growth conditions

All strains used in this study are listed in Table 2.S1. The 100 *A. niger* wild-type strains were obtained from the CBS strain collection of the Westerdijk Institute of Fungal Biodiversity, Utrecht, the Netherlands. The *A. niger* strains were cultivated on malt extract agar (MEA, CM0059, Oxoid) plates for 7 days at 30 °C to harvest conidia for weak acid stress resistance assays. Conidia were harvested by adding saline solution, consisting of 0.9% NaCl + 0.02% Tween 80 in demi water, to the plates and gently scraping the spores with a sterile cotton swab, after which the spore solution is filtered through a sterile filter (Amplitude EcoCloth, Contec).

Sorbic acid sensitivity screening by liquid assay

The sorbic acid (SA) minimal inhibitory concentration (MIC) of 100 *A. niger* strains was determined using a liquid assay using 96-wells plates based on previous research (van den Brule *et al.* unpublished results). In short, 96-wells plates contained malt extract broth (MEB, CM0057, Oxoid) and a concentration range of undissociated sorbic acid (0 – 9 mM in steps of 1 mM). MEB was adjusted to pH 4 by the addition of NaOH/HCl after autoclaving. The sorbic acid stock of 10 mM undissociated sorbic acid was made by dissolving 11.78 mM sorbic acid in warm MEB after autoclaving and subsequently adjusted to pH 4 with NaOH/HCl and filter sterilized. The undissociated sorbic acid concentrations were calculated with the Henderson-Hasselbalch equation as defined by pH = pKa + log ($[A^-]/[HA]$) [37]. Each well contained a total volume of 200 µL with a total of 10^4 spores, by adding 10 µL of 10^6 spores/mL spore stock, counted and diluted by using TC20 automated cell counter (Bio-Rad). Growth was scored after 7 days and 28 days of growth at 25 °C using biological duplicates. The wells at the borders of the 96-well plates are used as water reservoirs and filled with 200 µL Milli-Q in order to prevent dehydration. To further limit dehydration and cross-contamination, the lids of the 96-wells plates are kept closed during the experiment. Additionally, the 96-well plates are kept inside a closed box con-

taining a falcon tube of water that functions as an additional water reservoir preventing dehydration of the wells.

The same assay was also performed to determine the MIC of 100 *A. niger* strains in ice tea peach. Filter sterilized and uncarbonated ice tea peach (Lipton) was used. The pH of the ice tea was measured at 3.1 and not adjusted. A total of 10.2 mM sorbic acid was added to ice tea and was subsequently filter sterilized, resulting in a sorbic acid stock with a concentration of 10 mM undissociated sorbic acid.

Weak acid sensitivity screening by spot assays

Spot assays were performed on minimal medium plates (MM) containing 27,75 mM glucose and varying concentrations of weak acids. For the initial spot assay testing 240 transcription factor knock-out strains, the following concentrations of (total) weak acids (pH = 4) were used: 4.5 mM sorbic acid, 2 mM and 3 mM benzoic acid, 2 mM and 3 mM cinnamic acid and 10 mM and 20 mM propionic acid. Minimal medium is prepared as described before [38], and set to pH 4 by the addition of NaOH/HCl after autoclaving. The weak acids tested were sorbic acid (Fluka chemika), cinnamic acid (Fluka chemika), benzoic acid (p-hydroxy-benzoic acid, Sigma), propionic acid (Propionic acid sodium salt, Sigma), lactic acid (Sigma Aldrich), citric acid (tri-sodium citrate dihydrate, VWR chemicals) and acetic acid (acetic acid glacial, Biosolve chemicals). Sorbic acid and cinnamic acid stock solutions were made in 70 % ethanol. Acetic acid was used directly from the liquid stock solution. All other weak acids stocks were made in Milli-Q and filter sterilized before use.

Spot assays on MM containing weak acids were performed by spotting 5 µL of a 10^6 conidia/mL spore stock solution, concentration determined by using TC20 automated cell counter (Bio-Rad), thereby inoculating a total of 5000 conidia per spot. Spot assay plates were cultivated for 4 days at 30 °C after which pictures were taken and growth was determined unless noted otherwise.

CRISPR/Cas9 genome editing in *A. niger*

Knock-out strains were constructed using a marker-free CRISPR/Cas9 genome editing approach as described previously [39]. All primers and plasmids used in this study are listed in Table 2.S2 and Table 2.S3, respectively. Single knock-out strains lacking the genes *sdrA* (An03g06580), *warA* (An08g08340) and *warB (*An11g10870) were made in MA234.1. Additionally, all possible combination knock-out strains were made; (*ΔsdrA*, *ΔwarA*), (*ΔwarA, Δwar*B), (*ΔsdrA, ΔwarB*) and (*ΔsdrA, ΔwarA, ΔwarB*) (Table 2.S1).

A schematic overview of the technique used for complementation to replace *sdrA* in wild-type strain CBS 147320 by the *sdrA* locus obtained from laboratory strain N402 is shown in Figure 2.S1. CRISPR/Cas9 plasmid SdrA gRNA2 in pFC332 (Figure 2.S1B) was used to obtain a double strand break in *sdrA*. The repair DNA was constructed by amplifying the gene as present in N402 by PCR. The donor DNA contained two newly introduced silent point mutations in order to eliminate further Cas9 endonuclease activity after a homology directed repair event has taken place. In short, the reverse primer of 5' part of the gene (p2r sjs28) and the forward primer of the 3' part of the gene (p3f sjs28) were designed to contain an overlapping sequence. This overlapping sequence contained the two newly introduced silent point mutations. The repair DNA was subsequently constructed by fusion PCR (Figure 2.S1C). PCR reactions to obtain repair DNA for the complementation were performed using Phusion™ High-Fidelity DNA Polymerase (Thermo scientific) with its appropriate buffer and protocol as prescribed by the manufacturer. Transformation was performed using a PEG-mediated protocol described previously [38], with few exceptions. Protoplast formation of wild-type *A. niger* strain CBS 147320 was seen after 2.5 hours of incubating. After 5 days of growth, transformants were single streaked on MM+ hygromycin (100 µg/mL) for purification and afterwards on MM, and MM +hygromycin for subsequent removal of selection pressure to select for transformants that lost the CRISPR/Cas9 containing plasmid.

Data availability

All data is included in the manuscript. Knock-out strains and plasmids used are available

upon request. Wild-type strains are available as part of the CBS collection, Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands. Figure 2.S1 contains a detailed overview the SNP complementation methodology in wild-type *A. niger* strain CBS 147320. Figure 2.S2 contains the diagnostic PCRs performed to confirm *warA, sdrA* and *warB* deletions. Figure 2.S3 contains the MICu values of transcription factor knock-out strains lacking any combination of *sdrA*, *warA* and/or *warB* determined in MEB.

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Additional files

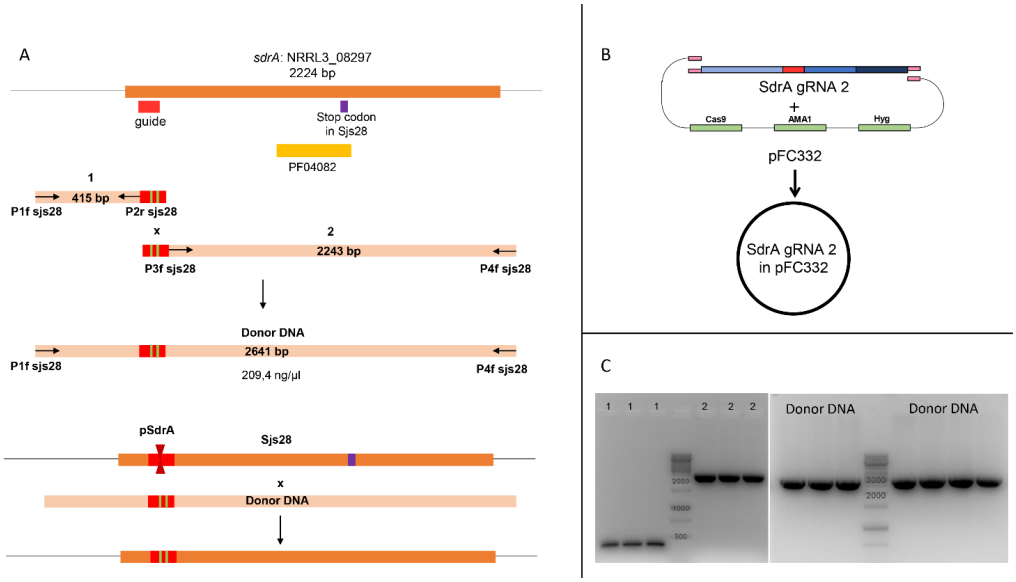

Figure 2.S1. Complementation of the premature stop codon of *sdrA* in CBS 147320 using CRISPR/Cas9. A. Schematic overview of the complementation methodology with *sdrA* (orange), the location of the guide of sdrA gRNA2 (red), the location of the stop codon in CBS 147320 (purple), and the location of the PFAM domain PF04082, fungal transcription factor domain (yellow). Also includes a schematic overview of the amplification method of the repair DNA (pink), with SNPs in the guide indicated (green). B. A schematic overview of the construction of the CRISPR/Cas9 containing plasmid pSdrA containing a guideRNA specifically targeting the *sdrA* locus. C. A PCR showing the fragments 1 (415 bp) and 2 (2243 bp), with fusion PCR obtaining the complete Donor DNA (2641 bp). Gels are run on 1% agarose gel with GeneRuler 1 kb DNA ladder.

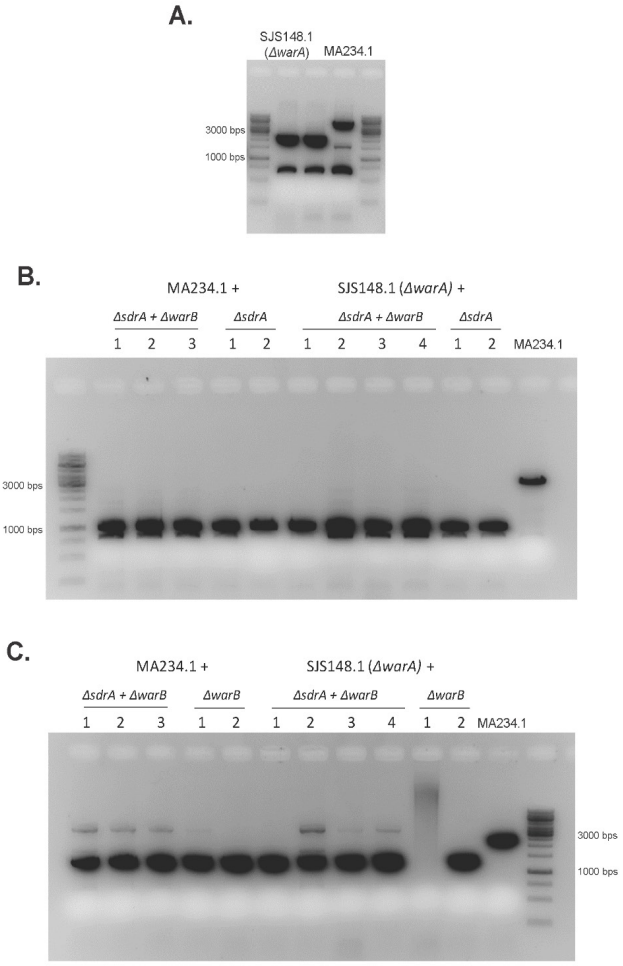

Figure 2.S2. Diagnostic PCR confirming the *warA*, *sdrA* and *warB* deletions. A. First, the *ΔwarA* deletion strain SJS148.1 was created. Diagnostic PCR was performed using forward primer DIAG_warA_fw and reverse primer DIAG_warA_rv to amplify the gene and flanking regions. A bandsize of 4800 bps is expected when *warA* is present, and a bandsize of 2166 bps is expected when *warA* is deleted. B. Diagnostic PCR was performed using forward primer DIAG_sdrA_fw and reverse primer DIAG_sdrA_rv to amplify the gene and flanking regions. A bandsize of 3181 bps is expected when *sdrA* is present, and a bandsize of 964 bps is expected when *sdrA* is deleted. C. Diagnostic PCR was performed using forward primer DIAG_warB_fw and reverse primer DIAG_warB_rv to amplify the gene and flanking regions. A bandsize of 2345 bps is expected when *warB* is present, and a bandsize of 1299 bps is expected when *warB* is deleted.

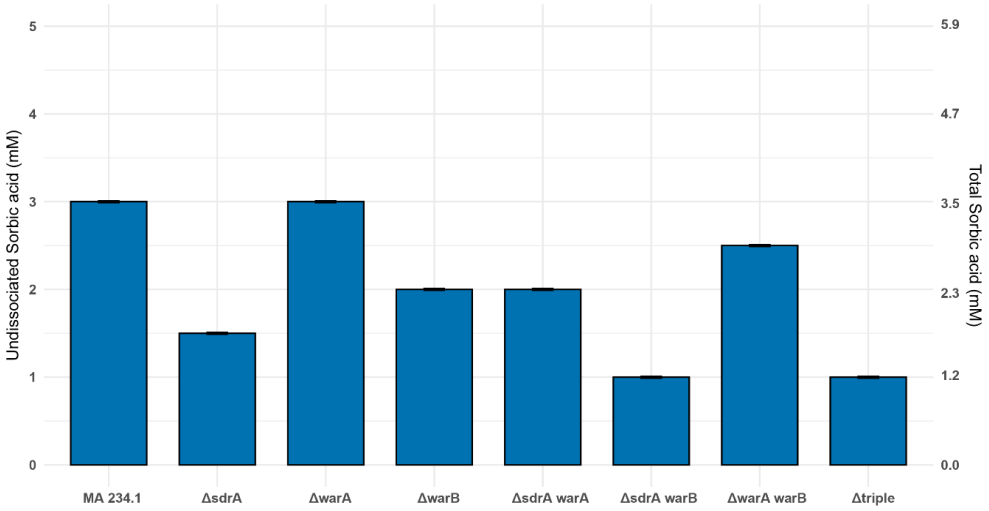

Figure 2.S3. The sorbic acid MIC values in liquid MEB assay of the knock-out strains lacking *sdrA*, *warA* and/or *warB*. Average MIC values of transcription factor knock-out strains in liquid MEB (pH = 4). MIC of each strain was identified in biological duplicates, the mean MIC is visualized in blue, with the error bar indicating the standard deviation (all duplicates gave the same MIC so the standard deviations are 0 in all cases). The growth was scored after 4 days of growth at 30 °C. The primary Y-axis indicates the undissociated sorbic acid concentration, while the secondary Y-axis indicates the total sorbic acid concentration added.

Table 2.S1. Strains used in this study

CBS number strain	DTO number strain	Genotype	Parental strain	Isolated from	Species	Obtained from
CBS 113.50	DTO 008-C3	wild type	-	Leather	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 554.65	DTO 012-I2	wild type	-	Tannic-gallic acid fermentation, Connecticut, USA	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 110.30	DTO 028-H9	wild type	-	Göttingen, Germany	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 112.32	DTO 028-I3	wild type	-	Japan	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 124.48	DTO 029-B1	wild type	-	Unknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 131.52	DTO 029-C3	wild type	-	Leather	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 263.65	DTO 029-D1	wild type	-	Copenhagen, Denmark	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 103.66	DTO 029-D4	wild type	-	Unknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 623.78	DTO 029-E3	wild type	-	France	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 117.52	DTO 058-H9	wild type	-	Unknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 118.52	DTO 058-I1	wild type	-	Unknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 139.52	DTO 058-I5	wild type	-	Kuro-koji, Japan	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 115988	DTO 059-C7	wild type	-	Unknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 123906	DTO 063-G1	wild type	-	Ryuku, Japan	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 630.78	DTO 067-H7	wild type	-	Army equipment, South Pacific Islands	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 118.36	DTO 067-I4	wild type	-	Chemical, USA	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 126.49	DTO 068-C1	wild type	-	Unknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 072-D2	wild type	-	Indoor air of archive, the Netherlands	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 086-F9	wild type	-	Filter flow cabinet, Westerdijk institute, Utrecht, the Netherlands	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 089-E7	wild type	-	Air in crawling space, Eindhoven, the Netherlands	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 096-A1	wild type	-	Wall down in the Lechuguilla Cave, Carlsbad, New Mexico, USA	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute

CBS number strain	DTO number strain	Genotype	Parental strain	Isolated from	Species	Obtained from
-	DTO 096-A2	wild type	-	Soil from dirt road, Isla Santa Cruz, Galapagos islands, Ecuador	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 096-A3	wild type	-	Spent coffee (mouldy growth), Denmark	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 147371	DTO 096-A5	wild type	-	Green coffee bean, Coffee Research Station, Netrakonda, India	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 147320	DTO 096-A7	wild type	-	Grape, Australia	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 096-A8	wild type	-	Artic soil, Svalbard, Norway	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 147321	DTO 096-A9	wild type	-	Artic soil, Svalbard, Norway	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 096-B1	wild type	-	Rice starch, imported to Denmark	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 096-B3	wild type	-	Pepper, imported to Denmark	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 096-B6	wild type	-	Saffron powder, from Kenya imported to Denmark	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 096-C1	wild type	-	Unknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 147322	DTO 096-C6	wild type	-	Coffee, Brazil	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 096-C7	wild type	-	Unknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 096-D1	wild type	-	Unknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 147323	DTO 096-D7	wild type	-	Raisin, Fabula, Turkey	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 147324	DTO 096-E1	wild type	-	Unknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 096-E2	wild type	-	Unknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 096-E3	wild type	-	Unknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 096-E5	wild type	-	Unknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 101700	DTO 096-G3	wild type	-	Japan	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 101706	DTO 096-G4	wild type	-	Soy bean	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 101707	DTO 096-G5	wild type	-	Broiler mixed feed	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 101708	DTO 096-G6	wild type	-	Uknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute

CBS number strain	DTO number strain	Genotype	Parental strain	Isolated from	Species	Obtained from
CBS 121047	DTO 096-G8	wild type	-	Coffee bean, Thailand	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 108-I7	wild type	-	Indoor environment, Thailand	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 120.49	DTO 146-A3	wild type	-	USA	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 101698	DTO 146-B8	wild type	-	Mesocarp finga – coffee bean, Kenya	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 101705	DTO 146-C1	wild type	-	Carpet dust from school, Canada	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 146-E8	wild type	-	Indoor environment, Hungary	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 161-E9	wild type	-	Bamboo sample, Ho Chi Minh city, Vietnam	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 167-A4	wild type	-	Margarine, Belgium	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 147482	DTO 175-I5	wild type	-	Surface water, Portugal	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 188-A9	wild type	-	Cinnamon, imported to the Netherlands	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 203-H4	wild type	-	Soil, Kabodan island, Iran	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 225-H3	wild type	-	Raisins, imported to Denmark	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 240-E2	wild type	-	Breakfast cereal, Turkey	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 240-H6	wild type	-	Muesli, Turkey	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 240-I6	wild type	-	Dried fig, Turkey	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 240-I9	wild type	-	Dried fruit, Turkey	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 241-B2	wild type	-	Breakfast cereal, Turkey	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 241-B7	wild type	-	Muesli, Turkey	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 257-G2	wild type	-	Filling, the Netherlands	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 260-C2	wild type	-	Indoor, school, Turkey	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 267-I2	wild type	-	House dust, Thailand	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 276-G2	wild type	-	BAL, Iran	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 147343	DTO 291-B7	wild type	-	Coffee bean, Thailand	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 293-E2	wild type	-	Coffee beans (Arabica), Thailand	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute

CBS number strain	DTO number strain	Genotype	Parental strain	Isolated from	Species	Obtained from
CBS 147344	DTO 293-G7	wild type	-	Coffee beans (Robusta), Thailand	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 133816	DTO 316-E3	wild type	-	Black pepper, Denmark	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 147345	DTO 316-E4	wild type	-	USA	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 316-E5	wild type	-	Raisins, California, USA	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 316-E6	wild type	-	Raisins, California, USA	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 147346	DTO 321-E6	wild type	-	CF patient material, the Netherlands	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 147347	DTO 326-A7	wild type	-	Petridish in soft drink factory, the Netherlands	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 337-D3	wild type	-	Fruit, Belgium	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 355-F9	wild type	-	Patient material, the Netherlands	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 360-C1	wild type	-	Liquorice solution, the Netherlands	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 115.50	DTO 367-B6	wild type	-	Unknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 281.95	DTO 367-C9	wild type	-	Unknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 769.97	DTO 367-D1	wild type	-	Leather	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 115989	DTO 367-D6	wild type	-	Unknown	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 116681	DTO 367-D7	wild type	-	Imported kernels of apricots, the Netherlands	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 119394	DTO 367-E2	wild type	-	USA	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 121997	DTO 367-E9	wild type	-	Coffee bean, Chiangmai, Thailand	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 129379	DTO 367-G3	wild type	-	Soil, Cedrus deodar forest, Mussoorie, India	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 132413	DTO 367-G7	wild type	-	Soil, 200m from W. mirabilis, Swakop, Namibia	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 133817	DTO 367-G8	wild type	-	Black pepper, Denmark	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 133818	DTO 367-G9	wild type	-	Raisins, Denmark	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 140837	DTO 367-H2	wild type	-	Soil, Rudňany, Slovakia	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute

CBS number strain	DTO number strain	Genotype	Parental strain	Isolated from	Species	Obtained from
-	DTO 368-H7	wild type	-	K-sorbate free margarine, the Netherlands	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 368-H8	wild type	-	Beverages factory, India	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 368-H9	wild type	-	Ice Tea Red, Philippines	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 147352	DTO 368-I1	wild type	-	Air next to bottle blower, Mexico	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 368-I2	wild type	-	Decaffinated tea bags, Belgium	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 368-I3	wild type	-	Environment in factory, Uzbekistan	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 368-I4	wild type	-	Potassium sorbate containing margarine, Ghana	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 368-I5	wild type	-	Foods factory of Sanquinetto, Italy	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 147353	DTO 368-I6	wild type	-	Foods factory of Sanquinetto, Italy	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
-	DTO 368-I7	wild type	-	Used in soy sauce fermentation process, China	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
CBS 554.65	DTO 368-I8	wild type	-	Connecticut, USA	*Aspergillus niger*	Westerdijk Fungal Biodiversity Institute
JvD 1-1		putative *sdrA* G1296A	CBS 147320	-	*Aspergillus niger*	This study
JvD 1-2		putative *sdrA* G1296A	CBS 147320	-	*Aspergillus niger*	This study
JvD 1-3		putative *sdrA* G1296A	CBS 147320	-	*Aspergillus niger*	This study
JvD 2-1		putative *sdrA* G1296A	CBS 147320	-	*Aspergillus niger*	This study
JvD 2-2		putative *sdrA* G1296A	CBS 147320	-	*Aspergillus niger*	This study
JvD 2-3		putative *sdrA* G1296A	CBS 147320	-	*Aspergillus niger*	This study
JvD 2-4		putative *sdrA* G1296A	CBS 147320	-	*Aspergillus niger*	This study
JvD 2-5		putative *sdrA* G1296A	CBS 147320	-	*Aspergillus niger*	This study
JvD 2-6		putative *sdrA* G1296A	CBS 147320	-	*Aspergillus niger*	This study

CBS number strain	DTO number strain	Genotype	Parental strain	Isolated from	Species	Obtained from
JvD 2-8		putative *sdrA* G1296A	CBS 147320	-	*Aspergillus niger*	This study
JvD 2-9		putative *sdrA* G1296A	CBS 147320	-	*Aspergillus niger*	This study
JvD 2-10		putative *sdrA* G1296A	CBS 147320	-	*Aspergillus niger*	This study
JvD 2-12		putative *sdrA* G1296A	CBS 147320	-	*Aspergillus niger*	This study
MA234.1		Δ*kusA*	N402	-	*Aspergillus niger*	[40]
SJS148.1		*ΔwarA*	MA234.1	-	*Aspergillus niger*	This study
SJS157.1		*ΔsdrA*	MA234.1	-	*Aspergillus niger*	This study
SJS158.1		*ΔwarB*	MA234.2	-	*Aspergillus niger*	This study
SJS159.1		*ΔsdrA*, *ΔwarA*	SJS148.1	-	*Aspergillus niger*	This study
SJS160.2		*ΔwarA*, *ΔwarB*	SJS148.2	-	*Aspergillus niger*	This study
SJS161.1		*ΔsdrA*, *ΔwarB*	MA234.1	-	*Aspergillus niger*	This study
SJS162.1		*ΔsdrA*, *ΔwarA*, *ΔwarB*	SJS148.1	-	*Aspergillus niger*	This study

Table 2.S2. Primers used in this study

Primer name	Sequence	Function
p1f sjs28	TCCCGCATCGGCTAAGTCTCCA	*sdrA repair DNA 1 for CBS 147320*
p2r sjs28	CTGATTCCGCTTCATTCGCAGCACGCGGT-CAATCTCT	*sdrA repair DNA 1 for CBS 147320*
p3f sjs28	GAATGAAGCGGAATCAGCGCGAGGCTCGAGCGT-GTTA	*sdrA repair DNA 2 for CBS 147320*
p4r sjs28	AGTCCGAGGCCTCCGAACCA	*sdrA repair DNA 2 for CBS 147320*
TS1_sdrA_fw	TCCCGCATCGGCTAAGTCTCCA	*Creation of 5' sdrA flank, 367 bp*
TS1_sdrA_rv	GGAGTGGTACCAATATAAGCCGGCGGTGTGTCG-GAACCTCAAAAGC	*Creation of 5' sdrA flank, 367 bp*
TS2_sdrA_fw	CCGGCTTATATTGGTACCACTCCCCATGACGTTATG-CGGCCCCTC	*Creation of 3' sdrA flank, 502 bp*
TS2_sdrA_rv	AGTGGCACCCGTCATGGCTACT	*Creation of 3' sdrA flank, 502 bp*
sdrA_sgRNA2_fw	AATGAAACGCAATCAGCGCGGTTTTAGAGCTAGAAAT	*Create the sdrA target for the CRISPR/Cas9 plasmid*
sdrA_sgRNA2_rv	CGCGCTGATTGCGTTTCATTGACGAGCTTACTCGTTT	*Create the sdrA target for the CRISPR/Cas9 plasmid*
diag_sdrA_fw	ACTTAGGGGGTGGGACCAGTGG	*diagnostic PCR sdrA deletion*
diag_sdrA_rv	GGACTTTGATGCCGAGCATGGC	*diagnostic PCR sdrA deletion*
5_warA_fw	GGCGTCCTCCAGGGTCTCATCT	*Creation of 5' warA flank, 368 bp*
5_warA_rv	GGAGTGGTACCAATATAAGCCGGTGGCTTGCTGT-TATTCTAGAGAGGG	*Creation of 5' warA flank, 368 bp*
3_warA_fw	CCGGCTTATATTGGTACCACTCCTGTGTATTTGTCTG-GAGTGGATGT	*Creation of 3' warA flank, 1002 bp*
3_warA_rv	AGCTCCCGCTCAATCCTCGAGA	*Creation of 3' warA flank, 1002 bp*
warA_sgRNA_fw	CGATAGACGATGCTTACCTGGTTTTAGAGCTAGAAAT	*Create the warA target for the CRISPR/Cas9 plasmid*
warA_sgRNA_rv	CAGGTAAGCATCGTCTATCGGACGAGCTTACTCGTTT	*Create the warA target for the CRISPR/Cas9 plasmid*
diag_warA_fw	CACAATGCCATGTAGCGCGCAA	*diagnostic PCR warA deletion*
diag_warA_rv	ACACGATCTGACCGCGATGACG	*diagnostic PCR warA deletion*
TS1_warB_fw	TCGACCCTCCCGGTTTGGTCAA	*Creation of 5' warB flank, 599 bp*

Primer name	Sequence	Function
TS1_warB_rv	GGAGTGGTACCAATATAAGCCGGTGAAGGAG-GTTTGGTTGCGGGT	*Creation of 5' warB flank, 599 bp*
TS2_warB_fw	CCGGCTTATATTGGTACCACTCCACGATACGAC-GAAGTTCAGCAT	*Creation of 3' warB flank, 544 bp*
TS2_warB_rv	AGTTCGGCCACTTCTCGGACCA	*Creation of 3' warB flank, 544 bp*
warB_sgRNA2_rv	CGGTGTTCTCTTCGAAGCGCGACGAGCTTACTC-GTTT	*Create the warB target for the CRISPR/Cas9 plasmid*
warB_sgRNA2_fw	GCGCTTCGAAGAGAACACCGGTTTTA-GAGCTAGAAAT	*Create the warB target for the CRISPR/Cas9 plasmid*
diag_warB_fw	TCGCCCTCGTCTTACTCCTCCC	*diagnostic PCR warB deletion*
diag_warB_rv	CCATGACGTCCTCCATCACCGC	*diagnostic PCR warB deletion*

Table 2.S3. Plasmids used in this study

Plasmid name	Target sequence	Function	Origin
pTLL108.1	-	Template for the amplification of guide RNA	[39]
pTLL109.2	-	Template for the amplification of guide RNA	[39]
pFC332	-	Vector containing CRISPR/ Cas9	[41]
sdrA gRNA2 in pFC332	AATGAAACGCAATCAGCGCG	Targeted double stranded break in *sdrA* gene	This study
warA gRNA in pFC332	CGATAGACGATGCTTACCTG	Targeted double stranded break in *sdrA* gene	This study
warB gRNA2 in pFC332	GCGCTTCGAAGAGAACAC-CG	Targeted double stranded break in *sdrA* gene	This study