Received: 24 April 2018 | Revised: 11 July 2018 | Accepted: 16 July 2018
DOI: 10.1002/jobm.201800200

RESEARCH PAPER

Journal of Basic Microbiology

Physiological background of the remarkably high Cd^{2+} tolerance of the *Aspergillus fumigatus* Af293 strain

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Funding information
National Research, Development and Innovation Office, Grant numbers: NKFIH K112181, K119494; Hungarian Research Fund co-financed by the European Union and the European Social Fund, Grant number: TÁMOP-4.2.2.A-11/1/KONV-2012-0045; European Union and the European Social Fund, Grant number: EFOP-3.6.1-16-2016-00022

The physiological background of the unusually high cadmium tolerance ($MIC_{50} > 2$ mM) of *Aspergillus fumigatus* Af293 was investigated. The cadmium tolerance of the tested environmental and clinical *A. fumigatus* strains varied over a wide range (0.25 mM < MIC_{50} < 1 mM). Only the Af293 strain showed a MIC_{50} value of >2 mM, and this phenotype was accompanied by increased *in vivo* virulence in mice. A strong correlation was found between the cadmium tolerance and the transcription of the *pcaA* gene, which encodes a putative cadmium efflux pump. The cadmium tolerance also correlated with the iron tolerance and the extracellular siderophore production of the strains. In addition to these findings, Af293 did not show the synergism between iron toxicity and cadmium toxicity that was detected in the other strains. Based on these results, we suggest that the primary function of PcaA should be acting as a ferrous iron pump and protecting cells from iron overload. Nevertheless, the heterologous expression of *pcaA* may represent an attractive strain improvement strategy to construct fungal strains for use in biosorption or biomining processes or to prevent accumulation of this toxic metal in crops.

KEYWORDS
cadmium toxicity, iron metabolism, oxidative stress, *pca1* efflux pump, siderophore production

1 | INTRODUCTION

Cadmium is a nonessential heavy metal with high toxicity. Frequent exposure to cadmium has been demonstrated to increase the risk of renal dysfunction and bone, lung and nervous system diseases, as well as cancer [1]. It has also been suggested that chronic cadmium exposure is accompanied by cardiovascular diseases, including hypertension, diabetes,

Abbreviations: FC, ferricrocin; MSB, menadione sodium bisulfate; TAF-C, triacetylfusarinine C; tBOOH, tert-butyl hydroperoxide.

and atherosclerosis [2,3]. In addition to occupational exposure, cigarette smoke and food contaminated with cadmium are among the most important health-threatening sources of cadmium [4]. Itai–itai disease, which was observed in the mid-20th century in Japan and was caused by the consumption of rice contaminated with cadmium [5,6], clearly demonstrated the risk associated with the local accumulation of cadmium in our environment. Cadmium is regularly found in ores of zinc, copper, and lead and as a consequence, volcanic activity can temporarily increase the local cadmium concentrations in nature. Significant portions of elevated cadmium levels detected in the environment are of anthropogenic origin and are caused by industrial pollution [7]. Approximately 13,000 tons of cadmium have been reported to be released annually due to human activity [8]. Batteries, anti-corrosive agents and PVC products can contain cadmium, and cadmium is also used in nuclear power plants as a neutron absorber [7]. Moreover, phosphate fertilizers may also have a high cadmium content; in addition to atmospheric deposition and water irrigation, the use of phosphate fertilizers is among the most important factors leading to increased cadmium concentrations in agricultural fields [9]. Several approaches (from physical or chemical solutions to phytoremediation) have been developed to remove cadmium from soil or waste water [9–11], and techniques based on biosorption by microbes are also being intensively investigated [12–17].

Aspergillus fumigatus is a common mold in soil and decaying organic matter. As an opportunistic pathogen, *A. fumigatus* is also responsible for the majority of invasive aspergillosis cases, which have a high (50–95%) mortality rate [18]. People continuously inhale the conidia of this fungus [18], but these conidia typically cause systemic aspergillosis only in immunocompromised individuals [19]. Several properties of the fungus make this generally saprobic mold the most successful human pathogen among the Aspergilli [20]. Most of these properties may have evolved in order to increase the fitness of *A. fumigatus* in saprobic environments, whereas they enhance virulence only incidentally. A good example of this evolution is the ability of conidia to germinate in macrophages and grow out from these immune cells, which has been suggested to have evolved in soil environments to protect conidia against free-living amoebae [21].

Previously, we observed that *A. fumigatus* Af293 had an unusually high cadmium tolerance that was accompanied by the presence of the *pcaA* gene (Afu1g16130) in the genome [22]. The *pcaA* gene is an ortholog of *Saccharomyces cerevisiae pca1*, which encodes an efficient cadmium efflux pump (a P_{1B}-type ATPase) [23,24]. More recently, Bakti et al. [25] demonstrated by gene deletion and overexpression experiments that *A. fumigatus* PcaA contributed to the remarkable cadmium tolerance of this fungus as well.

The high cadmium tolerance of *A. fumigatus* Af293 is not only an interesting feature of this strain but also has potential practical significance: first, understanding the molecular background of cadmium tolerance can help us develop *Aspergillus* strains suitable for efficient cadmium biosorption even at high Cd^{2+} concentrations or could lead to the development of new variants of cultivated plants with decreased cadmium accumulation. Second, the physiological and biochemical processes leading to high cadmium tolerance may also contribute to the pathogenesis of the fungus. Here, we studied the physiological background of the remarkable cadmium tolerance of the *A. fumigatus* Af293 strain.

2 | MATERIALS AND METHODS

2.1 | Strains

The *A. fumigatus* strains presented in Table 1 were used throughout the study. The strains were maintained on Barratt's minimal nitrate medium at 37 °C, and only fresh conidia harvested from 6-day-old cultures were used for experiments [22].

2.2 | Isolation and identification of fungal strains from soil

The origins of the soil samples, along with GPS coordinates and the date of sample collection, are listed below: 1. Szentegyháza, Romania (N46.3455232; E25.5297916) (2013/10/06); 2. Szentegyháza, Romania (N46.3455232; E25.5297916) (2013/10/06); 3. Farkaslaka, Romania (N46.393333; E25.257222) (2013/10/06); 4. Tiszadob, Hungary (N48.005686; E21.14667) (2013/10/12); 5. Tiszagyulaháza, Hungary (N47.940797; E21.143212) (2013/10/12); 6. Debrecen, Hungary (N47.561484; E21.617729) (2013/09/21); 7. Debrecen, Hungary (N47.561169; E21.617483) (2013/09/21); 8. Hortobágy, Hungary (N47.492965; E21.143861) (2013/09/22); 9. Hortobágy, Hungary (N47.464018; E21.177850) (2013/09/22); 10. Hajdúszoboszló, Hungary (N47.490558; E21.318920) (2013/09/22); 11. Debrecen, Hungary (N47.559954; E21.609243) (2013/10/02); 12. Debrecen, Hungary (N47.559087; E21.622407) (2013/10/02).

Soil samples (1 g) were suspended in 2 ml of sterile water, and 0.1 ml aliquots of these suspensions were spread onto Rose–Bengal chloramphenicol agar plates (Sigma–Aldrich, Ltd., Budapest, Hungary). Alternatively, Barratt's minimal nitrate medium agar plates supplemented with 2 mM $CdCl_2$ and 0.1 g L^{-1} chloramphenicol were used for isolation. Plates were incubated at room temperature for 7 days, and "*A. fumigatus*-like" colonies were isolated. Isolates were identified by their partial calmodulin sequences (amplified with the cmd5 and cmd6 primers) as described earlier [27]. Only five isolates, namely, DBMCC201-205, were verified to be *A.*

fumigatus; these isolates were from samples 3, 7, 8, 10, and 12, respectively. All these strains showed 100% sequence identity with the *A. fumigatus* NRRL163 strain. Partial calmodulin sequences are available under the following GenBank accession numbers: KY421201-5.

2.3 | Testing the growth inhibitory effect of metal ions and oxidative agents

Barratt's minimal nitrate medium supplemented with 0, 0.5, 1, 1.5, 2, 2.5 or 3 mM $CdCl_2$ was spotted with 5 µl of freshly made conidial suspension (2×10^7 conidia/ml) and incubated for 5 days at 37 °C. The cadmium tolerance was characterized by the ratio of the colony diameters measured in the heavy metal-treated cultures to that measured in the untreated control cultures (relative growth) as well as by the MIC_{50} value. The MIC_{50} was defined as the lowest $CdCl_2$ concentration at which the relative growth value was less than 0.5. The growth inhibitory effect of $FeCl_3$ (0–10 mM), $CuSO_4$ (0–1 mM), $ZnSO_4$ (0–20 mM), $CoCl_2$ (0–5 mM), $NiSO_4$ (0–1 mM), menadione sodium bisulfate (MSB; 0.01 mM), diamide (0.7 mM), H_2O_2 (3 mM) and *tert*-butyl hydroperoxide (tBOOH; 0.56 mM) was characterized by measuring the relative growth only.

Relative growth values (mean ± SD) were determined from three independent experiments for each treatment. Dunnett's test ($p < 0.05$) was used to compare each of the strains with the Af293 control strain. Pairwise correlations between cadmium tolerance and oxidative stress tolerance or cadmium tolerance and metal ion tolerance were characterized by Pearson's correlation coefficient.

TABLE 1 Strains used in this study

Strain[a]	Description
Af293	Identical to FGSC1100; isolated from human systemic aspergillosis
DBMCC101	Isolated from human systemic aspergillosis
DBMCC201-5	Isolated from soil
NCAIM F.00056	Identical to NVK 33
NCAIM F.00673	Identical to CCM F-373
NCAIM F.00948	Identical to CBS 457.75; isolated from soil
SZMC3100, SZMC3102-4, SZMC3106	Isolated from human keratitis

[a]NCAIM (National Collection of Agricultural and Industrial Microorganisms; Budapest, Hungary; http://ncaim.uni-corvinus.hu); SZMC (Szeged Microbial Collection; University of Szeged, Hungary; http://www2.sci.u-szeged.hu/microbiology/collection.htm) [26]; DBMCC (Department of Biotechnology and Microbiology Culture Collection; University of Debrecen, Hungary).

2.4 | Studying pairwise interactions between growth inhibiting agents

Data for growth in the presence of one of the two selected compounds, both compounds or neither compound were determined as described in the previous section. IR values were calculated according to Abbott's formula [28]: $IR = I_o/I_e$, where I_o is the observed percentage inhibition in the presence of both compounds and I_e is the expected percentage inhibition in the presence of both compounds, assuming that the two compounds act independently. I_e was calculated as follows: $I_e = X + Y - (XY/100)$, where X and Y are the percentage growth inhibition caused by each compound alone. $IR < 0.5$ and $IR > 1.5$ are indicative of antagonistic and synergistic interactions, respectively. An IR in the range of $0.5 \leq IR \leq 1.5$ suggests no interaction (additive effect).

2.5 | Detecting the cadmium content of mycelia in surface cultures

Strains were spotted (5 µl of freshly made suspension, 2×10^7 conidia/ml) onto Barratt's minimal nitrate medium supplemented with 0–5 mM $CdCl_2$, and the plates were covered with a sterile cellophane sheet. After incubation for 5 days at 37 °C, the cellophane sheets were removed, and mycelia were harvested from the surface. Cell-free extracts were prepared by boiling mycelia in cc. HNO_3, and the cadmium concentrations of these solutions were determined at a wavelength of 228.802 nm by a microwave plasma atomic emission spectrometer (Agilent MP-AES 4100) using a Meinhard-type nebulizer and a double-pass spray chamber. The mean ± SD values were determined from three independent experiments.

2.6 | Detecting oxidative stress response elements

Erlenmeyer flasks (500 ml) containing 100 ml of Barratt's minimal nitrate medium were inoculated with 5×10^7 freshly harvested conidia and incubated at 37 °C with shaking at 220 rpm. Sterile $CdCl_2$ solutions were added to some of the cultures at 17 h of cultivation (treated cultures). The final $CdCl_2$ concentrations were 0.2, 0.5, and 1 mM, depending on the cadmium tolerance of the strains. Treated and untreated (control) cultures were further cultivated for 5 h at 37 °C with shaking at 220 rpm. Mycelia were harvested and used for measuring the specific activity of enzymes, the content of glutathione (GSH) and glutathione disulfide (GSSG), and the production of 2′,7′-dichlorofluorescein (DCF) from 2′,7′-dichlorodihydrofluorescein diacetate. The specific activity of superoxide dismutase (SOD), catalase, glutathione reductase (GR), and glutathione peroxidase (GPx) was measured with the appropriate rate assays as described earlier [29]. The

content of GSH and GSSG was determined using the DTNB (5,5′-dithio-bis(2-nitrobenzoic acid))-based recycling assay [29]. DCF formation was measured spectrofluorometrically and used to detect perturbations in redox homeostasis [29]. For further details, see Supporting Information.

For statistical analysis, the mean ± SD values were calculated from four parallel experiments. Significant differences between the cadmium-treated and control cultures of each strain were analyzed by Student's *t*-test ($p < 0.05$).

2.7 | Measuring siderophore production

Triacetylfusarinine C (TAF-C) and ferricrocin (FC) production was characterized in iron-depleted submerged cultures as described previously [30] (see also Supporting Information S2).

For statistical analysis, the mean ± SD values were calculated from three independent experiments. Pairwise correlations between cadmium tolerance and the production of TAF-C and FC were characterized by Pearson's correlation coefficient.

2.8 | Reverse transcription quantitative real-time PCR (RT-qPCR)

Lyophilized mycelia originating from submerged, control (not treated with $CdCl_2$) cultures were used for TRIzol reagent-based total RNA isolation following the protocol described by Chomczynski [31]. The expression of the *pcaA* gene was quantified with an Xceed qPCR SG 1-step 2 × Mix Lo-ROX kit (Institute of Applied Biotechnologies, Prague, Czech Republic) according to the manufacturer's recommendations; 400 ng of total RNA was used in each reaction. The following primer pairs were used to amplify the gene transcripts:

-Afu1g16130 (*pcaA*) as the target gene:
5′-TGCGGGAATGCGAGATGAG-3′ and
5′-AGTGATGTGCGAAGGGAGC-3′
-Afu7g00250 (*tub2*) as the reference gene:
5′-ACCTGCTCGGCTCTTTTCC-3′ and
5′-CATCTCGTCCATTCCCTCGC-3′.

Primer pairs were designed according to the sequences available in the *Aspergillus* Genome Database (http://www.aspergillusgenome.org).

Relative transcription levels were characterized by the ΔCP values, where $\Delta CP = CP_{tub2} - CP_{pcaA}$ and CP stands for the qRT-PCR cycle numbers corresponding to the crossing points. For statistical analysis, the mean ± SD values were calculated from three independent experiments. Pairwise correlations between the relative transcription level of *pcaA* and the cadmium tolerance or the TAF-C production were characterized with Pearson's correlation coefficient.

2.9 | Animal studies

Animal experiments were carried out in our Experimental Animal Facility (reg. no: III/3.-KÀT./2015) under the supervision of the Animal Care Committee, University of Debrecen. The experimental protocol was approved by the Animal Care Committee (license number: 2/2014 DEMAB, to coauthor GN). Animal experiments conformed to the general guidelines of the European Community (86/609/EEC) and the special BSL2 guidelines (200/54/EC 16(1)). Every group ($n = 10$) of mice were housed in PI plastic cages (425 × 135 × 120 mm, 573.75 cm^2) with mesh covers, according to the 2010/ 63/EU guidelines. Animals were fed pelleted mouse chow (Purina, LabDiet, St. Louis, MO, USA) and tap water ad libitum. Automated room illumination in 12/12 h light/dark cycles and room temperatures between 22 and 25 °C were maintained.

BALB/c mice (both sexes; 8–10 weeks old) were used for all experiments. Mice were immunosuppressed with 200 mg kg^{-1} cyclophosphamide 3 days before infection and 1, 4, and 7 days after infection. Infection was carried out by the instillation of 50 µl of freshly made suspension containing 7×10^6 conidia [32]. The survival of the mice was monitored for 10 days. After 10 days, animals were killed by cervical dislocation. The lungs of the mice were examined histologically to detect infection [32].

For evaluation of the data, Kaplan-Meier survival analysis was used, and survival curves were compared by a log-rank test using MedCalc statistical software (https://www.medcalc.org/).

3 | RESULTS

Ten *A. fumigatus* strains obtained from different culture collections and five novel *A. fumigatus* isolates were tested in order to characterize the distribution of the high cadmium tolerance phenotype among the *A. fumigatus* species (Table 2). Although we found variability in the cadmium tolerance of the tested strains, none of the other strains showed a cadmium tolerance as high as that of Af293 (Table 2).

All the tested strains contained a transcriptionally active *pcaA* gene, and a positive correlation ($R = 0.84$; $p = 0.002$; $n = 10$) was found between *pcaA* transcription levels and cadmium tolerance (Fig. 1A).

When supplied at appropriate concentrations, $CdCl_2$ caused oxidative stress in all tested strains independent of their cadmium tolerance (Table 3). Cadmium treatment enhanced the specific activity of superoxide dismutase (and in

TABLE 2 Cadmium tolerance of selected *A. fumigatus* strains

Strain	Growth on 2 mM $CdCl_2$[a]	MIC_{50} (mM)
Af293	+	>2
SZMC3102-4 and SZMC3106	+	1
DBMCC101	-	1
F.00673, DBMCC205 and SZMC3100	+	0.5
DBMCC201-4, F.00056, and F.00948	-	0.25

[a]A "+" symbol means that the colony diameter on plates containing 2 mM $CdCl_2$ was greater than 10% of that measured in the absence of $CdCl_2$.

some cases that of glutathione reductase), caused an elevation in GSH and GSSG concentrations and decreased the GSH/GSSG ratio (Table 3). In addition, the increased DCF production in the cadmium-treated cultures demonstrated the perturbation of redox homeostasis (Table 3). Interestingly, cadmium treatment did not affect the specific activities of GPx and catalase. Furthermore, cadmium treatment influenced the transition metal homeostasis of cells. Synergism was found between Cd^{2+} and Cu^{2+} and Cd^{2+} and Zn^{2+}, and for the F.00056 and SZMC3106 strains (but not for Af293), between Cd^{2+} and Fe^{3+} in the surface culture experiments when the growth inhibitory effect of these transition metals was studied (Table 4).

Although $CdCl_2$ induced oxidative stress in all tested strains, we did not find any correlation between the cadmium tolerance and oxidative stress tolerance (tested with diamide, MSB, tBOOH or H_2O_2) of the *A. fumigatus* strains (Table 5). There was no strong correlation between the cadmium tolerance and metal ion (Cu^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+}) tolerance, except for a moderate positive correlation ($R = 0.64$; $p = 0.046$, $n = 10$) between the Cd^{2+} and Fe^{3+} tolerance (Table 6). Among the tested strains, Af293 showed good tolerance to Fe^{3+} and Ni^{2+} in addition to its outstanding tolerance to Cd^{2+}, but it was poorly tolerant to Cu^{2+}, Zn^{2+} and Co^{2+} (Table 6).

Regarding iron metabolism, a very detectable positive correlation was found between the cadmium tolerance and the production of TAF-C ($R = 0.70$; $p = 0.024$; $n = 10$) but not the production of FC ($R = 0.40$; $p = 0.246$; $n = 10$) (Figs. 1B and 1C). It is worth mentioning that we also found a positive correlation between TAF-C production and *pcaA* transcription ($R = 0.74$; $p = 0.014$; $n = 10$).

In vivo virulence experiments demonstrated that Af293, which is characterized as having a high cadmium tolerance, an elevated *pcaA* transcription level and good TAF-C production, was significantly more virulent in a mouse infection system than the SZMC3102, SZMC3104, SZMC3106, F.00056, and F00948 strains, which are characterized by moderate or weak cadmium tolerance, a low *pcaA* transcription level and moderate TAF-C production (Fig. 2).

At low concentrations of cadmium, the biosorption capacities of the tested strains were similar despite their

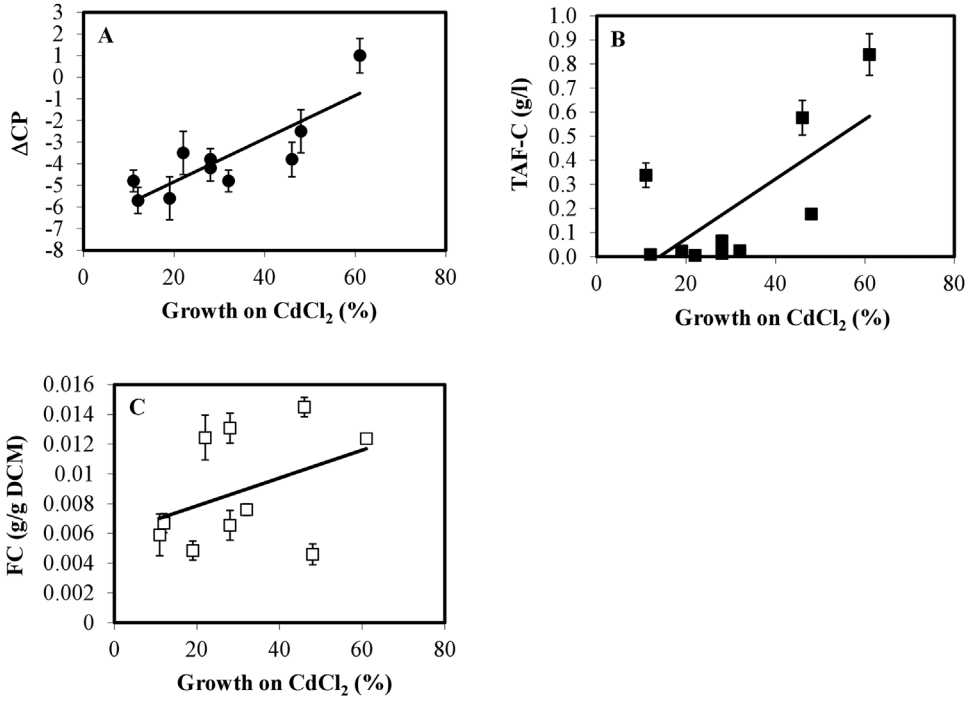

FIGURE 1 Correlation between cadmium tolerance and the relative transcription level of the *pcaA* gene (A) as well as the production of TAF-C (B) and FC (C) in certain *A. fumigatus* strains

TABLE 3 Cadmium induced changes in redox homeostasis in selected *A. fumigatus* strains

	F.00056[a]		SZMC3106		Af293	
	Control	0.2 mM $CdCl_2$	Control	0.5 mM $CdCl_2$	Control	1 mM $CdCl_2$
GR (mkat/kg protein)	4.6 ± 0.5	6.0 ± 0.6[c]	4.6 ± 0.5	5.5 ± 0.6[c]	4.5 ± 0.5	5.1 ± 0.5
GPx (mkat/kg protein)	2.3 ± 0.2	1.9 ± 0.2	2.6 ± 0.2	2.2 ± 0.2	2.3 ± 0.2	2.0 ± 0.2
Catalase (kat/kg protein)	1.2 ± 0.2	1.0 ± 0.2	2.0 ± 0.2	2.0 ± 0.2	1.6 ± 0.2	1.4 ± 0.2
SOD (U/mg protein)	22 ± 5	42 ± 6[c]	33 ± 5	55 ± 7[c]	28 ± 5	49 ± 7[c]
GSH (nmol/mg DCM)	1.8 ± 0.3	3.2 ± 0.5[c]	2.8 ± 0.4	5.1 ± 0.7[c]	3 ± 0.3	4.8 ± 0.7[c]
GSSG (nmol/mg DCM)	0.1 ± 0.03	0.8 ± 0.1[c]	0.3 ± 0.04	1.5 ± 0.3[c]	0.2 ± 0.04	1.1 ± 0.2[c]
GSH/GSSG	18 ± 6	4 ± 1[c]	9 ± 2	3 ± 1[c]	10 ± 2	4 ± 1[c]
DCF[b] (pmol/mg DCM)	0.3 ± 0.05	1.2 ± 0.2[c]	0.4 ± 0.06	2 ± 0.4[c]	0.3 ± 0.05	1 ± 0.3[c]

[a]Similar redox changes were observed with the F.00948, SZMC30100, and SZMC31104 strains (data not shown).
[b]The onset of redox homeostasis was characterized by the production of DCF [28].
[c]A significant difference was found between the appropriate control and cadmium-treated cultures.

TABLE 4 Effect of certain transition metals on the toxicity of $Cd2^+$ in selected strains

	Relative growth (%)[a]		
Tested compounds	F.00056	SZMC3106	Af293
0.2 mM $CdCl_2$	57 ± 3	84 ± 5	91 ± 3
2 mM $CdCl_2$	0	0	56 ± 3
0.3 mM $CuSO_4$	92 ± 3	78 ± 4	96 ± 4
5 mM $ZnSO_4$	99 ± 4	91 ± 4	94 ± 3
5 mM $FeCl_3$	82 ± 3	79 ± 3	84 ± 3
0.3 mM $CuSO_4$ + 0.272 mM $CdCl_2$	0	0	72 ± 4
IR[b]	2.10	2.90	2.22
5 mM $ZnSO_4$ + 0.2 mM $CdCl_2$	0	32 ± 3	67 ± 4
IR	2.29	2.88	2.28
5 mM $FeCl_3$ + 0.2 mM $CdCl_2$	0	0	72 ± 4
IR	1.88	2.97	1.18
0.3 mM $CuSO_4$ + 2 mM $CdCl_2$	-	-	10 ± 2
IR			1.95
5 mM $ZnSO_4$ + 2 mM $CdCl_2$	-	-	28 ± 3
IR			1.52
5 mM $FeCl_3$ + 2 mM $CdCl_2$	-	-	51 ± 3
IR			0.93

[a]The colony diameters of the untreated cultures were 74 ± 3, 77 ± 4, and 67 ± 4 mm for the F.00056, SZMC3106, and Af293 strains, respectively.
[b]IR < 0.5 and IR > 1.5 are indicative of antagonistic and synergistic interactions, respectively. An IR in the range of 0.5 ≤ IR ≤ 1.5 suggests no interaction (additive effect).

different cadmium tolerances (Table 7). However, owing to its high cadmium tolerance, the Af293 strain could be tested at a high (2 mM) cadmium concentration and could absorb an amount of cadmium as high as 850 mg kg^{-1} dry cell mass (Table 7).

4 | DISCUSSION

In a previous large-scale comparative stress tolerance study performed with 17 *Aspergillus* spp., we found that although cultivation conditions (e.g., the incubation time and temperature and the origin of the conidia) highly influenced the results, *A. fumigatus* Af293 showed a high $CdCl_2$ tolerance under all the tested conditions [22]. The $CdCl_2$ MIC_{50} value for Af293 (measured at 10 days on plates incubated at 25 °C) was 1.5 mM, while the $CdCl_2$ MIC_{50} value detected under the same conditions for other *Aspergillus* species varied between 0.15 and 3 mM [22]. The species/strains characterized as having the lowest MIC_{50} values (*A. carbonarius* DTO 115-B6, 0.15 mM; *A. aculeatus* CBS 172.66, 0.19 mM; and *A. glaucus* CBS 516.65, 0.19 mM) have no *pcaA* ortholog, while the strains/species characterized as having the highest MIC_{50} values (*A. versicolor* CBS 795.97, 3.0 mM; *A. sydowii* CBS 593.65, 2.9 mM; and *A. fumigatus* Af293, 1.5 mM) have either one or two (for *A. sydowii*) *pcaA* orthologs [22]. Nevertheless, the Kruskal-Wallis test did not show a significant difference ($p = 0.068$) between the three ("no *pcaA* ortholog," "one *pcaA* ortholo," and "two *pcaA* orthologs") groups (Supporting Information Fig. S1). Fazli et al. isolated several fungal strains from cadmium-polluted soil [33], and two of the strains belonged to the genus *Aspergillus*: one isolate represented the species *A. versicolor* and the other, *A. fumigatus*. Both strains were characterized as having a MIC_{50} of >1.6 mM [33], and remarkably, both species had a *pcaA* ortholog [22]. In contrast, Chakraborty et al. reported an *A. foetidus* strain isolated from polluted soil that could grow even in the presence of 63 mM

TABLE 5 Oxidative stress tolerance of different *A. fumigatus* strains

	Relative growth (%)[a]										
Additives	**F.00673**	**F.00056**	**F.00948**	**DBMCC101**	**Af293**	**SZMC3100**	**SZMC3102**	**SZMC3103**	**SZMC3104**	**SZMC3106**	**Correlation coefficient[b]**
1 mM $CdCl_2$	19 ± 2[c]	11 ± 1[c]	12 ± 1[c]	48 ± 3[c]	61 ± 2	22 ± 2[c]	28 ± 1[c]	28 ± 2[c]	32 ± 2[c]	46 ± 3[c]	
1 mM diamide	60 ± 4[c]	77 ± 3	59 ± 2[c]	56 ± 4[c]	71 ± 4	60 ± 2[c]	60 ± 4[c]	64 ± 3	58 ± 3[c]	89 ± 3[c]	0.24
0.01 mM MSB	83 ± 3	81 ± 3	90 ± 4	92 ± 4	89 ± 3	87 ± 4	87 ± 4	86 ± 3	85 ± 5	67 ± 3[c]	−0.02
0.8 mM tBOOH	60 ± 4	63 ± 4	87 ± 3[c]	93 ± 4[c]	64 ± 4	73 ± 3[c]	67 ± 4	67 ± 4	67 ± 3	60 ± 3	−0.01
3 mM H_2O_2	49 ± 4	34 ± 5[c]	79 ± 3[c]	84 ± 5[c]	51 ± 4	59 ± 5	51 ± 4	47 ± 2	48 ± 6	48 ± 2	0.13

[a]The colony diameters of the untreated (control) cultures were between 66 and 78 mm, depending on the strain.

[b]Pearson's correlation coefficients between the $CdCl_2$-treated culture and each of the oxidative stress generating agent-treated cultures.

[c]A significant difference was found between Af293 and the appropriate strain.

TABLE 6 Metal ion tolerance of different *A. fumigatus* strains

	Relative growth (%)[a]										
Additives	**F.00673**	**F.00056**	**F.00948**	**DBMCC101**	**Af293**	**SZMC3100**	**SZMC3102**	**SZMC3103**	**SZMC3104**	**SZMC3106**	**Correlation coefficient[b]**
1 mM $CdCl_2$	19 ± 2[c]	11 ± 1[c]	12 ± 1[c]	48 ± 3[c]	61 ± 2	22 ± 2[c]	28 ± 1[c]	28 ± 2[c]	32 ± 2[c]	46 ± 3[c]	
5 mM $FeCl_3$	68 ± 3[c]	74 ± 3	64 ± 2[c]	76 ± 3	79 ± 3	79 ± 2	71 ± 4[c]	68 ± 2[c]	76 ± 3	84 ± 3	0.64
0.5 mM $CuSO_4$	42 ± 2	46 ± 3[c]	61 ± 2[c]	67 ± 2[c]	37 ± 2	55 ± 2[c]	45 ± 2[c]	42 ± 3	39 ± 2	47 ± 2[c]	−0.14
10 mM $ZnSO_4$	60 ± 4[c]	59 ± 3[c]	48 ± 2	58 ± 2[c]	46 ± 2	57 ± 3[c]	50 ± 4	46 ± 3	53 ± 2[c]	56 ± 2[c]	−0.24
3 mM $CoCl_2$	68 ± 3[c]	65 ± 2[c]	85 ± 4[c]	87 ± 3[c]	46 ± 3	80 ± 3[c]	82 ± 4[c]	92 ± 3[c]	65 ± 2[c]	85 ± 4[c]	−0.26
1 mM $NiSO_4$	82 ± 3	82 ± 2	79 ± 2	60 ± 2[c]	86 ± 4	58 ± 3[c]	67 ± 3[c]	70 ± 2[c]	72 ± 2[c]	79 ± 3	−0.01

[a]The colony diameters of the untreated (control) cultures were between 66 and 78 mm depending on the strains.

[b]Pearson's correlation coefficients between the $CdCl_2$-treated culture and each of the other metal ion-treated cultures.

[c]A significant difference was found between Af293 and the appropriate strain.

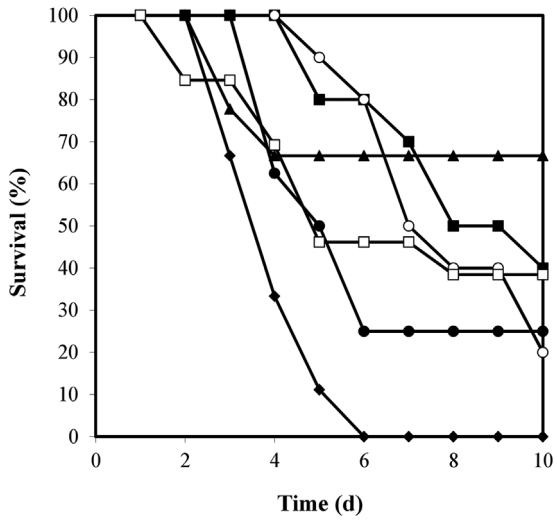

FIGURE 2 *In vivo* virulence of selected *A. fumigatus* strains. Mice were infected with the *A. fumigatus* Af293 (♦; "high cadmium tolerance"), F.00056 (●; "weak cadmium tolerance"), F.00948 (○; "weak cadmium tolerance"), SZMC3102 (■; "moderate cadmium tolerance"), SZMC3104 (□; "moderate cadmium tolerance") and SZMC3106 (▲; "moderate cadmium tolerance") strains, and the survival of the mice was tracked for 10 days

cadmium [15] despite the fact that the genome of this species does not harbor any *pcaA* ortholog [22]. These data, along with the data presented in Table 2, demonstrate that i) the high cadmium tolerance of Af293 is neither unique within the genus *Aspergillus* nor characteristic of the *A. fumigatus* species, and ii) the presence of the *pcaA* gene in the genome of Aspergilli is generally neither sufficient nor essential for a high cadmium tolerance.

Nevertheless, the expression of *pcaA* can be an important factor that determines cadmium tolerance because a strong positive correlation was found between the relative transcription level of the *pcaA* gene and the cadmium tolerance in the studied *A. fumigatus* strains (Fig. 1A). Furthermore, it has also been reported that the deletion of *pcaA* decreases the cadmium susceptibility of the Af293 strain, while *pcaA* overexpression significantly increases this susceptibility [25].

The deleterious heavy-metal cadmium affects cell homeostasis at multiple levels. Cd^{2+} ions can interact with neighboring thiol groups to inactivate enzymes and other proteins [34]. Cd^{2+} can also replace zinc in various metalloproteins, and this effect—along with its action on thiol groups—can explain the observed inhibition of DNA repair enzymes in the presence of this metal, which increases the sensitivity of cells to genotoxic agents [35]. Although cadmium is not a Fenton metal, it can induce oxidative stress indirectly in several ways [3,36]: 1) in addition to replacing zinc, cadmium can replace iron and copper in proteins and consequently liberate these redox-active metals, which can cause oxidative stress via Fenton reactions; 2) the inhibition of elements in the electron transport chain leads to uncoupled electron flow and to further increases in the ROS burden of the cells; and 3) the depletion of the GSH pool as well as 4) the inactivation of antioxidative enzymes in the presence of cadmium will also contribute substantially to the oxidative stress-generating effect of this metal. The generation of oxidative stress during cadmium exposure was also demonstrated in *A. fumigatus* (Table 3) and *A. foetidus* [15], and the oxidative stress was not the consequence of either GSH depletion or the strong suppression of catalases, SODs, GPx or GR in these cases (Table 3; [15]). In contrast, the observed synergism between the growth inhibitory effect of Cd^{2+} and Cu^{2+}, Fe^{2+}/Fe^{3+} or Zn^{2+} (Table 4) suggests that cadmium may act in *A. fumigatus* primarily via perturbing cellular metal homeostasis. Interestingly, neither the oxidative stress tolerance nor the metal ion tolerance (with the exception of iron tolerance) of the *A. fumigatus* strains correlated with their ability to grow in the presence of cadmium (Tables 5 and 6).

In addition to replacing iron intracellularly in metalloproteins, Cd^{2+} can also interfere with iron metabolism in earlier stages such as during its uptake. Cd^{2+} enters cells via nonspecific metal transporters, including Fet4 [37], which also acts as a low affinity Fe^{2+} transporter in *S. cerevisiae* [38]. However, in plants, Cd^{2+} enters root cells predominantly via Fe^{2+} transporters, for example through OsIRT1 and OsNRAMP5 in rice [39,40]. The upregulation of iron transport mechanisms specific to iron but not to cadmium—for example (phyto)siderophore-mediated iron transport or certain metal transporters—can reduce cadmium uptake by reducing the activity of iron transporters also used by Cd^{2+} [41,42]. Therefore, the observed correlation between the ability for TAF-C production and for growth in the presence of $CdCl_2$ in *A. fumigatus* (Fig. 1B) may suggest another explanation for the cadmium tolerance of Af293 in addition to that suggested by the elevated *pcaA* transcription level.

Although the reason that a high cadmium tolerance has developed in *A. fumigatus* Af293 is far from obvious, the simplest explanation of this phenotype is that this strain evolved to cope with a high cadmium concentration in a polluted area. Unfortunately, the natural habitat of *A. fumigatus* Af293 is not known because it is a clinical strain isolated from a case of invasive aspergillosis in a human lung (http://aspergillusgenome.org/Strains.shtml). However, other explanations may also be reasonable. The *S. cerevisiae* Pca1 P_{1B}-type ATPase is not only an efficient cadmium efflux

TABLE 7 Cadmium adsorption (biosorption) of different *A. fumigatus* strains

	Cd content (mg kg^{-1} DCM)		
Treatment	**F.00056**	**SZMC3106**	**Af293**
0.2 mM $CdCl_2$	55 ± 6	60 ± 4	56 ± 5
2 mM $CdCl_2$	-	-	850 ± 110

pump [22–24,43] but may also be involved in the metabolism of other heavy metals [24,44]. Although this transporter is unable to transport Cu^{2+}, it most likely sequesters copper in its cysteine-rich region, which explains its contribution to copper resistance [24,44]. Moreover, the deletion of *aft1* (which codes for iron utilization and homeostasis transcription factor) down-regulated *pca1* transcription in *S. cerevisiae*, suggesting that Pca1 is important in the regulation of iron homeostasis in this species also [44]. In *A. fumigatus*, treatment with either Fe^{2+} or Cu^{2+}, in addition to treatment with Cd^{2+}, induced the expression of the *pcaA* transporter gene [25]. A *ΔpcaA* strain also showed increased MSB sensitivity, while a *pcaA*-overexpressing strain showed reduced MSB sensitivity [25]. This finding is interesting because superoxide radical anions generated by MSB can efficiently destroy Fe-S cluster proteins and, as a consequence, strongly affect iron metabolism [45,46]. The activity of *pcaA* also correlated positively with the *in vivo* virulence of *A. fumigatus* in the wax moth (*Galleria mellonella*) virulence model [25], and the high transcriptional activation of *pcaA* in Af293 was accompanied by the superior *in vivo* virulence of Af293 in mice (Fig. 2). These data, along with the presence of *pcaA* in several cadmium-sensitive strains and species (Table 2, Supporting Information Fig. S1), suggest that the function of PcaA could be much more complex than simply pumping Cd^{2+} out from cells when necessary.

In our experiments, compared with that of the other tested strains, Af293 showed poor Cu^{2+} tolerance (Table 6). Therefore, it is very unlikely that PcaA could be a key player in the Cu^{2+} homeostasis of *A. fumigatus*. However, Af293 had an iron tolerance superior to that of the other tested strains (Table 6), and Af293 was the only strain in which there was no synergism between the toxicities of cadmium and iron (Table 4).

Iron efflux proteins are widely distributed among bacteria, whose iron protection systems include cation diffusion facilitator proteins, major facilitator superfamily proteins, ferritin-like proteins and P_{1B}-type ATPases [47]. The importance of ferrous iron P_{1B}-type ATPases lies in protecting bacterial cells from excess iron, which catalyzes detrimental redox-cycling reactions [47]. In human pathogenic bacteria (e.g., *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *Streptococcus* sp.), P_{1B}-type ferrous iron ATPases (FrvA, CtpD, and PmtA, respectively) are described as virulence factors [48–51]. However, the contribution of these ATPases to virulence is unclear. They may protect cells against iron overload upon cellular escape from iron-limited phagocytic vacuoles to the relatively iron-rich cytosol [47]. It is also possible that these ATPases secrete iron liberated within bacterial cells by oxidative attack from the host immune system [47]. Similar mechanisms may also exist in human pathogenic fungi, and PcaA is a good candidate for being a part of these mechanisms.

A. fumigatus Af293 has a remarkable potential to acquire iron due to its intensive TAF-C production (Fig. 1B), and intensive iron uptake requires mechanisms (e.g., P_{1B}-type ferrous iron ATPase activity) that protect the cells against excess iron. Therefore, we hypothesize that the Af293 strain has adapted to iron-limited (instead of cadmium-rich) conditions in its original environmental habitat. Iron is an abundant element in soil, with an average total concentration of 20–40 g kg^{-1} [52]. However, due to the formation of weakly soluble ferric oxides, its availability is often extremely low. The iron concentration in soil solution is approximately 10^{-10} mol Lunder aerated conditions at pH > 7; therefore, iron availability is a critical factor that determines the growth of microbes and plants in most soils [53]. On the other hand, iron availability can be increased locally and temporally under certain conditions (reviewed in [54]), which even leads to iron toxicity and causes serious problems e.g., during rice production [54]. Therefore, coping with both iron shortages and iron overloads can be a real challenge for soil microbes, including fungi. Under these ecological conditions, the overproduction of TAF-C [41,42] and the overexpression of *pcaA* [25] would also lead to a high cadmium tolerance merely by accident, but this phenotype would then be independent of the Cd^{2+} burden of the habitat. Interestingly, in manure, animal litter, sewage sludge and compost, which are typical habitats for *A. fumigatus*, the fungus typically seems to face high-iron, low-cadmium environments [55–57], which also makes the evolution of highly specific Cd^{2+}-specific pumps rather unlikely on these substrates.

A number of fungi have an outstandingly high biosorption capacity that can be exploited to remove polluting metal ions (including Cd^{2+}) from wastewater [10–12] or even to biomine valuable transition metals [58]. In addition to direct biosorption by cell wall biopolymers, other extracellular matrices ("extracellular polymeric substances") [16] and the formation of metal-oxalate precipitate on the cell wall contribute to their good biosorption potential [15]. The high relative transcription level of *pcaA* did not decrease the biosorption capacity of the Af293 strain in comparison to that of strains with lower *pcaA* relative transcription levels (Table 7). Moreover, owing to its high cadmium tolerance, the Af293 strain grew well in the presence of a high (2 mM) cadmium concentration and adsorbed amounts of Cd of up to 850 mg kg^{-1} dry cell mass (Table 7). Therefore, the heterologous expression of the *pcaA* gene will be able to increase the cadmium tolerance of fungal strains with bioremediation and biomining potential, and this approach may represent an attractive future strain improvement strategy when the application of living fungal biomass is considered in various environmental biotechnological processes. Moreover, our results also suggest that strategies based on the inhibition of cadmium uptake by plants [41] can be efficiently combined with strategies aimed at increasing the cadmium efflux of plant cells to prevent the accumulation of this toxic metal in crops.

ACKNOWLEDGMENTS

This work was supported financially by the National Research, Development and Innovation Office grants NKFIH K112181 and K119494, by the Hungarian Research Found TÁMOP-4.2.2.A-11/1/KONV-2012-0045 project, which was co-financed by the European Union and the European Social Fund, and also by the European Union and the European Social Fund through the project EFOP-3.6.1-16-2016-00022. RT-qPCR experiments were carried out at the Genomic Medicine and Bioinformatics Core Facility, University of Debrecen, Debrecen, Hungary. We acknowledge the Agilent Technologies and the Novo-Lab Ltd. (Hungary) for providing us with the MP-AES 4100 equipment.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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REFERENCES

[1] Jaishankar M, Tseten T, Anbalagan N, Mathew BB. Toxicity, mechanism and health effects of some heavy metals. Interdiscip Toxicol 2014;7:60–72.

[2] Satarug S, Moore MR. Emerging roles of cadmium and heme oxygenase in type-2 diabetes and cancer susceptibility. Tohoku J Exp Med 2012;228:267–88.

[3] Kukongviriyapan U, Apaijit K, Kukongviriyapan V. Oxidative stress and cardiovascular dysfunction associated with cadmium exposure: beneficial effects of curcumin and tetrahydrocurcumin. Tohoku J Exp Med 2016;239:25–38.

[4] Järup L, Akesson A. Current status of cadmium as an environmental health problem. Toxicol Appl Pharmacol 2009;238:201–8.

[5] Horiguchi H, Aoshima K, Oguma E, Sasaki S. Latest status of cadmium accumulation and its effects on kidneys, bone, and erythropoiesis in inhabitants of the formly cadmium-polluted Jinzu River Basin in Toyama, Japan, after restoration of rice paddies. Int Arch Occup Environ Health 2010;83:953–70.

[6] Uraguchi S, Kamiya T, Sakamoto T, Kasaki K. Low-affinity cation transporter (OsLCT1) regulates cadmium transport into rice grains. Proc Natl Acad Sci USA 2011;108:20959–64.

[7] Godt J, Scheidig F, Grosse-Siestrup C, Esche V. The toxicity of cadmium and resulting hazards for human health. J Occup Med Toxicol 2006;1:22.

[8] Gallego SM, Pena LB, Barcia RA, Azpilicueta CE. Unraveling cadmium toxicity and tolerance in plants: insight into regulatory mechanisms. Environ Exp Bot 2012;83:33–46.

[9] Tang X, Li Q, Wu M, Lin L. Review of remediation practices regarding cadmium-enriched farmland soil with particular reference to China. J Environ Manage 2016;181:646–62.

[10] Zhao FJ, Huang XY. Cadmium phytoremediation: call rice CAL1. Mol Plant 2018;11:640–42.

[11] Jiang Y, Huang R, Jiang S, Qin Z. Adsorption of Cd(II) by rhizosphere and non-rhizosphere soil originating from mulberry field under laboratory condition. Int J Phytoremediation 2018;20:378–83.

[12] Abdel-Aty AM, Ammar NS, Abdel Ghafar HH, Ali RK. Biosorption of cadmium and lead from aqueous solution by fresh water alga *Anabaena sphaerica* biomass. J Adv Res 2013;4:367–74.

[13] Bano A, Hussain J, Akbar A, Mehmood K. Biosorption of heavy metals by obligate halophilic fungi. Chemosphere 2018;199:218–22.

[14] Abbas SZ, Rafatullah M, Ismail N, Lalung J. Isolation, identification, characterization, and evaluation of cadmium removal capacity of *Enterobacter* species. J Basic Microbiol 2014;54:1279–87.

[15] Chakraborty S, Mukherjee A, Khuda-Bukhsh AR, Das TK. Cadmium-induced oxidative stress tolerance in cadmium resistant *Aspergillus foetidus*: its possible role in cadmium bioremediation. Ecotoxicol Environ Saf 2014;106:46–53.

[16] Yin Y, Hu Y, Xiong F. Biosorption properties of Cd(II), Pb(II), and Cu(II) of extracellular polymeric substances (EPS) extracted from *Aspergillus fumigatus* and determined by polarographic method. Environ Monit Assess 2013;185:6713–8.

[17] Siñeriz ML, Kothe E, Abate CM. Cadmium biosorption by *Streptomyces* sp. F4 isolated from former uranium mine. J Basic Microbiol 2009;49:S55–62.

[18] Balloy V, Chignard M. The innate immune response to *Aspergillus fumigatus*. Microbes Infect 2009;11:919–27.

[19] Park SJ, Mehrad B. Innate immunity to *Aspergillus* species. Clin Microbiol Rev 2009;22:535–51.

[20] Abad A, Fernández-Molina JV, Bikandi J, Ramírez A. What makes *Aspergillus fumigatus* a successful pathogen? Genes and molecules involved in invasive aspergillosis. Rev Iberoam Micol 2010;27:155–82.

[21] van Waeyenberghe L, Baré J, Pasmans F, Claeys M. Interaction of *Aspergillus fumigatus* conidia with *Acanthamoeba castellanii* parallels macrophage-fungus interactions. Environ Microbiol Rep 2013;5:819–24.

[22] de Vries RP, Riley R, Ad Wiebenga A, Aguilar-Osorio G. Comparative genomics reveals high biological diversity and specific adaptation in the industrially and medically important fungal genus *Aspergillus*. Genome Biol 2017;18:28.

[23] Shiraishi E, Inouhe M, Joho M, Tohoyama H. The cadmium-resistant gene, CAD2, which is a mutated putative copper-transporter gene (PCA1), controls the intracellular cadmium-level in the yeast *Saccharomyces cerevisiae*. Curr Genet 2000;37:79–86.

[24] Adle DJ, Sinani D, Kim H, Lee J. A cadmium-transporting P1B-type ATPase in yeast *Saccharomyces cerevisiae*. J Biol Chem 2007;282:947–55.

[25] Bakti F, Sasse C, Heinekamp T, Pócsi I. Heavy metal-induced expression of PcaA provides cadmium tolerance to *Aspergillus fumigatus* and supports its virulence in the *Galleria mellonella* model. Front Microbiol 2018;9:744.

[26] Manikandan P, Varga J, Kocsubé S, Anita R. Epidemiology of *Aspergillus* keratitis at a tertiary care eye hospital in South India and antifungal susceptibilities of the causative agents. Mycoses 2013;56:26–33.

[27] Tóth V, Nagy CT, Miskei M, Pócsi I. Polyphasic characterization of "*Aspergillus nidulans var. roseus*" ATCC 58397. Folia Microbiol 2011;56:381–8.

[28] Galgóczy L, Papp T, Pócsi I, Hegedűs N. *In vitro* activity of *Penicillium chrysogenum* antifungal protein (PAF) and its combination with fluconazole against different dermatophytes. Antonie van Leeuwenhoek 2008;94:463–70.

[29] Sámi L, Emri T, Pócsi I. Autolysis and aging of *Penicillium chrysogenum* cultures under carbon starvation: glutathione metabolism and formation of reactive oxygen species. Mycol Res 2001;105:1246–50.
[30] Szigeti MZ, Szaniszló S, Fazekas E, Gyémánt G. Optimization of triacetylfusarinine C and ferricrocin productions in *Aspergillus fumigatus*. Acta Microbiol Immunol Hung 2014;61:107–19.
[31] Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. Biotechniques 1993;15:532–4, 536–7.
[32] Palicz Z, Gáll T, Leiter É, Kollár S. Application of a low molecular weight antifungal protein from *Penicillium chrysogenum* (PAF) to treat pulmonary aspergillosis in mice. Emerg Microbes Infect 2016;5:e114.
[33] Mohammadian Fazli M, Soleimani N, Mehrasbi M, Darabian S. Highly cadmium tolerant fungi: their tolerance and removal potential. J Environ Health Sci Eng 2015;13:19.
[34] Wu X, Cobbina SJ, Mao G, Xu H. A review of toxicity and mechanisms of individual and mixtures of heavy metals in the environment. Environ Sci Pollut Res Int 2016;23:8244–59.
[35] Witkiewicz-Kucharczyk A, Bal W. Damage of zinc fingers in DNA repair proteins, a novel molecular mechanism in carcinogenesis. Toxicol Lett 2006;162:29–42.
[36] Cuypers A, Plusquin M, Remans T, Jozefczak M. Cadmium stress: an oxidative challenge. Biometals 2010;23:927–40.
[37] Dix D, Bridgham J, Broderius M, Eide D. Characterization of the FET4 protein of yeast. Evidence for a direct role in the transport of iron. J Biol Chem 1997;272:11770–7.
[38] Caetano SM, Menezes R, Amaral C, Rodrigues-Pousada C. Repression of the low affinity iron transporter gene fet4: a novel mechanism against cadmium toxicity orchestrated by *yap1* via *rox1*. J Biol Chem 2016;290:18584–95.
[39] Nakanishi H, Ogawa I, Ishimaru Y, Mori S. Iron deficiency enhances cadmium uptake and translocation mediated by the Fe^{2+} transporters OsIRT1 and OSIRT2 in rice. Soil Sci Plant Nutr 2006;52:464–9.
[40] Sasaki A, Yamaji N, Yokosho K, Ma JF. Nramp5 is a major transporter responsible for manganese and cadmium uptake in rice. Plant Cell 2012;24:2155–67.
[41] Banakar R, Alvarez Fernández Á, Abadía J. The expression of heterologous Fe(III) phytosiderophore transporter HvYS1 in rice increases Fe uptake, translocation and seed loading and excludes heavy metals by selective Fe transport. Plant Biotechnol J 2016;15:423–32.
[42] Dimkpa CO, Merten D, Svatos A, Büchel G. Siderophores mediate reduced and increased uptake of cadmium by *Streptomyces tendae* F4 and sunflower (*Helianthus annuus*), respectively. J Appl Microbiol 2009;107:1687–96.
[43] Rad MR, Kirchrath L, Hollenberg CP. A putative P-type $Cu(^{2+})$-transporting ATPase gene on chromosome II of *Saccharomyces cerevisiae*. Yeast 1994;10:1217–25.
[44] De Freitas JM, Kim JH, Poynton H, Su T. Exploratory and confirmatory gene expression profiling of mac1Delta. J Biol Chem 2004;279:4450–8.
[45] Popović-Bijelić A, Mojović M, Stamenković S, Jovanović M. Iron-sulfur cluster damage by the superoxide radical in neural tissues of the SOD1(G93A) ALS rat model. Free Rad Biol Med 2016;96:313–22.
[46] Orosz E, Antal K, Gazdag Z, Szabó Z. Transcriptome-based modeling reveals that oxidative stress induces modulation of the AtfA-dependent signaling networks in *Aspergillus nidulans*. Int J Genomics 2017;2017:6923849.
[47] Pi H, Helmann JD. Ferrous iron efflux systems in bacteria. Metallomics 2017;9:840–51.
[48] McLaughlin HP, Xiao Q, Rea RB, Pi H. A putative P-type ATPase required for virulence and resistance to haem toxicity in *Listeria monocytogenes*. PLoS ONE 2012;7:e30928.
[49] Pi H, Patel SJ, Arguello JM, Helmann JD. The *Listeria monocytogenes* Fur-regulated virulence protein FrvA is an Fe(II) efflux P_{1B4}-type ATPase. Mol Microbiol 2016;100:1066–79.
[50] Patel SJ, Lewis BE, Long JE, Nambi S. Fine-tuning of substrate affinity leads to alternative roles of *Mycobacterium tuberculosis* Fe^{2+}-ATPases. J Biol Chem 2016;291:11529–39.
[51] VanderWal AR, Makthal N, Pinochet-Barros A, Helmann JD. Iron efflux by PmtA is critical for oxidative stress resistance and contributes significantly to Group A *Streptococcus* virulence. Infect Immun 2017;85:e00091-17.
[52] Colombo C, Palumbo G, He JZ, Pinton R. Review on iron availability in soil: interaction of Fe minerals, plants, and microbes. J Soil Sediment 2014;14:538–48.
[53] Boukhalfa H, Crumbliss AL. Chemical aspects of siderophore mediated iron transport. Biometals 2002;15:325–39.
[54] Becker M. Iron toxicity in rice—conditions and management concepts. J Plant Nutr Soil Sci 2005;168:558–73.
[55] Wong JWC, Ma KK, Fang KM, Cheung C. Utilization of a manure compost for organic farming in Hong Kong. Bioresource Technol 1999;67:43–6.
[56] Sistani KR, Brink GE, McGowen SL, Rowe DE. Characterization of broiler cake and broiler litter, the by-products of two management practices. Bioresour Technol 2003;90:27–32.
[57] Sager M. Trace and nutrient elements in manure, dung and compost samples in Austria. Soil Biol Biochem 2007;39:1383–90.
[58] Johnson DB. Biomining-biotechnologies for extracting and recovering metals from ores and waste materials. Curr Opin Biotechnol 2014;30:24–31.

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How to cite this article: Kurucz V, Kiss B, Szigeti ZM, et al. Physiological background of the remarkably high Cd2+ tolerance of the *Aspergillus fumigatus* Af293 strain. *J Basic Microbiol*. 2018;58:957–967. https://doi.org/10.1002/jobm.201800200