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RESEARCH PAPER

Increased Cd²⁺ biosorption capability of *Aspergillus nidulans* elicited by *crpA* deletion

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Abstract

The P-type ATPase CrpA is an important Cu^{2+}/Cd^{2+} pump in the Aspergilli, significantly contributing to the heavy metal stress tolerance of these ascomycetous fungi. As expected, the deletion of *crpA* resulted in Cu^{2+}/Cd^{2+} -sensitive phenotypes in *Aspergillus nidulans* on stress agar plates inoculated with conidia. Nevertheless, paradoxical growth stimulations were observed with the $\Delta crpA$ strain in both standard Cu^{2+} stress agar plate experiments and cellophane colony harvest (CCH) cultures, when exposed to Cd^{2+} . These observations reflect efficient compensatory mechanisms for the loss of CrpA operating under these experimental conditions. It is remarkable that the $\Delta crpA$ strain showed a 2.7 times higher Cd biosorption capacity in CCH cultures, which may facilitate the development of new, fungal biomass-based bioremediation technologies to extract harmful Cd²⁺ ions from the environment. The nullification of *crpA* also significantly changed the spatial distribution of Cu and Cd in CCH cultures, as

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demonstrated by the combined particle-induced X-ray emission and scanning transmission ion microscopy technique. Most important, the centers of gravity for Cu and Cd accumulations of the $\Delta crpA$ colonies shifted toward the older regions as compared with wild-type surface cultures.

K E Y W O R D S

heavy metals, ICP-OES, ion transport, stress response, PIXE

1 | INTRODUCTION

The removal of heavy metal ions, such as Cd^{2+} and Cu^{2+} , from contaminated soil or wastewater, is essential to avoid their adverse health and environmental effects observable especially at high concentrations. It is clear that Cd^{2+} ions are not transformed into less toxic products in the environment, which explains its bioaccumulation in plants and animals [1]. Cadmium entering the human body through the gastrointestinal and respiratory systems may cause serious damages to all organs including the kidneys, the skeletal muscle, and the liver, which may diminish respiratory function and also elicit nervous disorders [1,2]. Copper is a biologically active essential metal, because some important metalloenzymes like cytochrome oxidase and superoxide dismutase need Cu^{2+} ions for their activity, but exposure to high concentrations of copper may be harmful [3–5].

Various abiotic and biotic technologies have been developed and tested to remove these heavy metal ions from contaminated soil or wastewater. In recent years, research has been focused on the elaboration of novel biosorptionbased environmental technologies using promising, effective, and environment-friendly microorganisms [6–8].

Filamentous fungi belonging to the genus Aspergillus can tolerate various heavy metal ions, even when they are present at high concentration levels [5,9,10], because Aspergillus spp. have adopted several different strategies to resist heavy metal stress as well as to capture and, hence, extract these poisonous ions from the environment [5]. For example, Aspergillus spp. possess several protein classes to bind metal ions intracellularly like metallothioneins and others to stabilize and regulate metal homeostasis such as metal ion transporters and metal-binding transcription factors [5,6]. Some heavy metal resistance-associated P-type ATPases play a distinguished role in the control of intracellular concentrations of various heavy metals including Cd²⁺, Cu²⁺, and Ag⁺, and these transporters are evolutionarily conserved and ubiquitously present in bacteria, yeast cells, and filamentous fungi [11].

In the Aspergilli, *Aspergillus nidulans* and *Aspergillus fumigatus* are widespread, saprophytic, and soil-inhabiting molds, which play an essential role in recycling organic

materials in the ecological niches occupied by them [5,6]. In addition, *A. nidulans* is a well-known and widely used laboratory model organism, and *A. fumigatus* is a dreadful, opportunistic human pathogen causing serious aspergillosis in the lungs and also systemic mycosis [12]. The third *Aspergillus* species, where Cu^{2+} transporters have been functionally characterized thus far, is *Aspergillus flavus*, an aflatoxin-producing fungus. These highly carcinogenic mycotoxins may enter the feed and food chain, which may cause contaminations dangerous for both livestock and humans [13,14].

In the opportunistic human pathogenic fungus Candida albicans, the CaCRP1 gene encodes a P-type ATPase, which was assigned to the detoxification of Cu^{2+} , Cd^{2+} , and Ag⁺ ions, and which transporter is also required for the virulence of C. albicans [15]. More recently, an ortholog of C. albicans CRP1 has been identified and functionally characterized in A. fumigatus (crpA; locus ID: AfuA_3g12740), which encodes an efficient Cu²⁺ efflux pump [16,17] and which is also involved in Zn^{2+} detoxification [18]. CrpA is an essential element of the defense mechanism of A. fumigatus against alveolar macrophages [16]. Most important, the expression of both A. nidulans (locus ID: AN3117) and A. fumigatus crpA is under the control of the Cu²⁺-responsive transcription factor AceA [17-19]. The genome of the crossover pathogen A. flavus harbors two A. fumigatus crpA homologs called crpA (locus ID: AFL2T 03712) and crpB (locus ID: AFL2T 10544), whose deletion caused higher Cu²⁺sensitivity than the lack of AceA [20]. The double gene deletion strain A. flavus $\Delta crpA \Delta crpB$ colonized maize, as effectively as the wild-type control strain, but a significantly reduced virulence was recorded in a mouse invasive aspergillosis model [20].

In *A. nidulans*, the loss of *crpA* also causes distorted colony morphology under Cu^{2+} stress, with low cellular density in the central regions and, at higher copper concentrations, even extremely thin mycelial mats with individually growing hyphae, which was previously called as "copper phenotype" by Antsotegi-Uskola et al. [19]. *A. nidulans* CrpA localizes primarily in the plasma membrane, but at longer Cu²⁺ exposures, it also appears

⁵⁷⁶ Journal of Basic Microbiology-

in versatile dispersed subcellular structures [19]. CrpA seems to be a Cu²⁺ transporter with some Ag⁺ pumping capability, and it can also export Cd²⁺ ions into the extracellular space when the primary Cd²⁺ detoxification systems (e.g., glutathione-dependent detoxification) are exhausted [19].

In the present study, we further investigated the physiological roles of the CrpA transporter via generating A. nidulans $\Delta crpA$ strains and exposing them to Cu²⁺ and Cd^{2+} stress. In mycelial mats of a selected $\Delta crpA$ mutant, which were pregrown on cellophane sheets under unstressed conditions, we found a remarkably increased Cd²⁺ biosorption capability after transferring them to stress agar plates supplemented with a high 0.3-mM CdCl₂ concentration. We also mapped and visualized the altered spatial distributions of the Cu²⁺ and Cd²⁺ ions within cellophane surface cultures of the A. nidulans wild-type and $\Delta crpA$ mutant strains, using particle-induced X-ray emission (PIXE) and scanning transmission ion microscopy (STIM) accelerator-based ion beam analytical methods.

2 **MATERIALS AND METHODS**

Strains and culture conditions 2.1

In this study, the A. nidulans TNJ36 (pyrG89 $AfpyrG^+$ *pyroA4 veA*⁺) control strain as well as $\Delta crpA$ (*pyrG89*; $\Delta crpA$: AfupyrG⁺; pyroA4; veA⁺) gene deletion mutants were used. The strains were cultured on Barratt's minimal nitrate medium (MNM, supplemented with 0.05% [vol/vol] pyridoxin) at 37°C for 6 days, and the freshly grown conidia were harvested and used in the forthcoming experiments [21]. Most important, MNM is a chemically defined medium, which contains only 0.006mM Cu^{2+} supplemented with a complex trace element solution [21].

Construction of the A. nidulans and 2.2 A. fumigatus Δ crpA gene deletion strains

The A. nidulans AN3117 locus encoding the CrpA transporter in this fungus was deleted by the double-joint polymerase chain reaction (PCR) method of Yu et al. [22,23], using the primers listed in Table S1. The amplified deletion cassette was used to transform RJMP1.59 strain using the Vinoflow FCE lysing enzyme [24]. Single-copy transformants were selected after PCR analysis [25], and three of the transformants lacking the crpA gene (MKL5, MKL10, and MKL14) were chosen for stress physiological experiments.

Testing the growth inhibitory 2.3 effects of metal ions

Metal stress sensitivity assays were performed on solid MNM plates, which were supplemented with 0.05-0.5 mM CuCl₂ or 0.1-6.0 mM CdCl₂, and which were then inoculated with 5 μ l of conidial suspension (1 \times 10⁵ conidia) and incubated at 37°C for 5 days as per previous standardized protocols [9,10]. Fungal growth values (mean $\pm SD$) were determined from three independent experiments for each heavy metal treatment. Fungal growth was characterized by colony diameters, and metal stress sensitivities were quantified by percentage decreases in the colony growth as compared with the appropriate control cultures [9,10].

2.4 Measuring cadmium and copper contents of A. nidulans TNJ36 control and MKL14 Δ crpA mutant strains using inductively coupled plasma optical emission spectrometry

The cellophane colony harvest (CCH) method [26,27] was used for heavy metal elemental analyses. Both the TNJ36 control and the MKL14 $\Delta crpA$ strains were point-inoculated (with 1×10^5 conidia in 5 µl suspensions) into MNM agar plates covered by sterile and semipermeable cellophane sheets. After 24-h incubation at 37°C, the pregrown mycelial mats were transferred into freshly prepared stress agar plates supplemented with 0.1-0.4 mM CuCl₂ or 0.1-0.5 mM CdCl₂, as required, and then incubated further at 37°C for 5 days. Then, relative growth values (means \pm SD values calculated from three independent measurements) were determined, as described above for the stress experiments.

Cellophane sheets with heavy metal ion-exposed mycelial mats were removed from the surface of MNM agar, and fungal mycelia were harvested with spatula, frozen immediately at -70°C, and freeze-dried overnight in a CHRIST Alpha 1-2 LDplus lyophilizer (Osterode, Germany) [27]. Then, the metal contents of the biomass samples were determined by inductively coupled plasma optical emission spectrometry (ICP-OES; 5100 Agilent Technologies, Santa Clara, CA) following atmospheric wet digestion in 65% (M/M) HNO₃ and 0.5-ml, 30% (M/M) H₂O₂ in glass beakers. Using ICP-OES elemental analysis data, metal contents of the biomasses were calculated and expressed in mg/kg units, as described elsewhere [28].

2.5 | Elemental analysis of fungal biomass samples by PIXE-STIM

For PIXE-STIM elemental analyses, we also used the CCH cultures of the TNJ36 control and the MKL14 $\Delta crpA$

strains [26,27]. Nevertheless, in this case, we did not separate mycelial mats from the cellophane sheets. Instead, whole CCH specimens (mycelia on sheets) were frozen $(-70^{\circ}C)$, lyophilized, and Petri dishes covered by sealing film were transported at 4°C to the PIXE–STIM laboratory without delay.

The PIXE measurements were carried out at the scanning nuclear microprobe facility at the 0° beamline of the 5-MV Van de Graaff accelerator located at ATOMKI, Debrecen, Hungary [29,30]. Narrow rectangles (0.5 cm) of the mycelial mats were cut out by scalpels, starting from the point of inoculation to the edges of the colonies, and they were fit into grids (2.0 cm); a proton beam of 2.5-MeV energy focused down to $\sim 2 \,\mu\text{m} \times 2 \,\mu\text{m}$, and 200–300 pA current was used to irradiate the samples.

For the characterization of the fungal mycelial mats, a measurement setup developed for biomedical applications [31], using a combination of ion beam analytical techniques, was applied. PIXE technique was used to measure the elemental composition of the samples for Z > 5, whereas STIM method provided information about the morphology, surface density, and thus the thickness of the samples. The measurement setup consisted of two X-ray detectors, placed at 135° geometry to the incidence beam, a surface barrier PIPS particle detector, and a beam chopper. An SDD detector with AP3.3 ultra-thin polymer window (SGX Sesortech) with 30-mm² active surface area was used to measure low- and mediumenergy X-rays (0.2–12 keV, Z > 5), whereas a Greshamtype Be Windowed Si(Li) X-ray detector with 30-mm² active surface area equipped with an additional Kapton filter of 125-µm thickness was applied to detect the medium- and high-energy X-rays (3-30 keV, Z > 19) [30]. For STIM measurements, a Canberra-type PIPS particle detector (11-keV nominal energy resolution) with 50-mm² active area was used both in on-axis and off-axis geometries. The beam dose was measured with a beam chopper and with a Faraday cup behind the sample [32]. Signals from all detectors were recorded event by event in list mode by the Oxford-type OMDAQ data acquisition system [33]. Elemental maps of a 1.5-mm $\times 1.5$ -mm area were recorded through the entire length of the freezedried samples. Such a 1.5-mm \times 1.5-mm area represented one spot. In a sample, 6-9 spots were selected for measurement, equally distributed between the edges and centers of the colonies. During the analysis, we excluded spots at the very edges where the cellophane sheets were not fully covered by mycelial mats yet, that is, where the confluency was less than 100%. To protect the particle detector from radiation damage, STIM measurements in on-axis geometry were performed with a beam current of 500-1,000 protons/s.

-Journal of Basic Microbiology

The obtained PIXE spectra were evaluated with the GUPIXWIN program code [34]. The simplest case for the evaluation of PIXE spectra is when the major elemental content (i.e., those that constitute ~90–99% of the sample, the so-called matrix) is known, and GUPIX solves in a simple and direct fashion to determine the desired trace element concentrations in that known matrix. The thickness of the samples and the energy loss of the irradiating beam due to the transmission through the samples were determined from the STIM measurements. The samples were treated as "intermediate thick" samples. The light element components (H, C, N, O, and S—the matrix) of the dried and harvested biomass samples were determined by an Elementar Vario Micro analyzer (Hanau, Germany; Table S2).

On the spectra of both PIXE detectors, the X-ray energy range of 3.0–8.5 keV is common; therefore, intensive X-ray lines within this range (e.g., Ca K_{α}, K K_{α}), were used to normalize the elemental concentrations. In most cases, the differences of the concentration values of the two detectors were between 0% and 5%. The following elements were fitted: O, Na, Mg, Al, Si, P, S, Cl, K, Ca, Ti, V, Cr, Mn, Fe, Cu, Zn, and Cd. The uncertainty of the PIXE measurement for the main components is ~25%, whereas for the trace elements, it is ~10–15%. Concentration values were expressed in μ g/(g DCM).

To check the quality and precision of the dose measurement and the determination of the concentrations, measurements on standard reference materials (SRMs) were carried out. SRMs used for the validation were the following: A series of pure metals (Zn, Sn, Ti, Ta) and layered samples ($6-\mu$ m-thick Ti foil on $50-\mu$ m Ni and $6-\mu$ m-thick Ti foil on $8-\mu$ m Ta). Layered standards were also used for calibration of the STIM measurements. The calibration of the beam chopper was also done on each measurement day. These measurements help determine the exact measurement conditions (e.g., solid angles of detectors).

3 | RESULTS

Not surprisingly, all *A. nidulans* $\Delta crpA$ gene deletion strains, constructed and tested (MKL5, MKL10, and MKL14), showed an increased sensitivity to Cu²⁺ ions, when added at least at 0.150-mM concentrations (Figures S1 and S2). Most important, all colonies at and above 0.125-mM Cu²⁺ concentrations showed characteristic "copper phenotype" colony morphologies with thin mycelial mats [19]. In addition, all $\Delta crpA$ strains showed a significantly increased sensitivity to Cd²⁺ ions, even at as low as 0.1-mM concentration (Figures S3 and S4). It is worth mentioning that each

-Journal of Basic Microbiology-

 $\Delta crpA$ strain including MKL14 showed a significantly increased tolerance to Cu²⁺ stress elicited by 0.125-mM or lower CuCl₂ concentrations (e.g., $14.7 \pm 4.2\%$ growth stimulation was recorded with the MKL14 strain at 0.1-mM Cu²⁺ concentration: Figure 1). These observations may be indicative that other effective Cu^{2+} detoxification systems were activated in the absence of *crpA*, overcompensating the loss of this important Cu^{2+} pump (Figures 1, S1, and S2).

In CCH cultures of the A. nidulans strains, both Cu^{2+} and Cd²⁺ tolerances of the TNJ36 control and the MKL14 $\Delta crpA$ strains increased considerably as compared with stress agar experiments, which were inoculated by conidia (Figures 1 and 2). It was surprising that no "copper phenotype" [19] appeared under these experimental conditions (Figure 1). Quite unexpectedly, exposures to 0.1-0.5 mM CdCl₂ in CCH cultures slightly stimulated the colony growth of the A. nidulans MKL14 $\Delta crpA$ mutant as compared with the control strain; meanwhile, no such phenomenon was observed in standard stress agar experiments where agar plates were inoculated with spore suspensions (Figure 2).

Considering the total cadmium uptakes by the A. nidulans TNJ36 control and MKL14 $\Delta crpA$ gene deletion strains, the Cd-accumulating capability of the mutant in the presence of 0.3-mM CdCl₂ was approximately 2.7 times higher than that of the wild-type strain $(1672.7 \pm 104.7 \text{ vs. } 612.5 \pm 189.3 \text{ mg}/(\text{kg DCM}); \text{ Table 1}).$ Most important, the increased Cd²⁺ biosorption potential of the A. nidulans MKL14 $\Delta crpA$ strain seemed to be specific to heavy metal, because the Cu accumulations by the tested TNJ36 and MKL14 strains were comparable with each other at 0.3-mM CuCl₂ concentration (Table 1).

The spatial distributions of Cu^{2+} and Cd^{2+} in A. nidulans colonies were mapped by PIXE. As indicated above, the TNJ36 control strain grew somewhat better in CCH cultures in the presence of 0.3-mM CuCl₂ and the CdCl₂ tolerance of MKL14 $\Delta crpA$ strain exceeded that of the control strain (Figures 1, 2, and S5).

In the case of the 0.3-mM CuCl₂-exposed TNJ36 control strain, copper was accumulated evenly after the very edge of the colony (4.5-6 mm from the edge) at a concentration of $1,100 \,\mu g/(g \text{ DCM})$ (the uncertainties of the Cu concentrations were about 10%), yet in the middle of

100 100 80 Growth (%) Growth (%) 60 ;0 40 20 20 0.05 mM CuCh 0.075 mM CuCh 0.125 mM CuCh 0 0.2 mM CuCh 0.15 mM CuCh 0.175 mM CuCh 0.1 mM CuCh 0.3 mM CuCh 0.1 mM CuCh 0.4 mM CuCh control control TNJ36 TNJ36 MKL14 MKL14 (AcrpA) (AcrpA) Cu^{2+} stress sensitivities of the Aspergillus nidulans TNJ36 control and the MKL14 $\Delta crpA$ gene deletion mutant FIGURE 1 strains. Stress sensitivities observed in surface cultures on minimal nitrate medium (MNM) stress agar plates (a) and on cellophane-





FIGURE 2 Cd^{2+} stress sensitivities of the *Aspergillus nidulans* TNJ36 control and the MKL14 $\Delta crpA$ gene deletion mutant strains. (a) Stress sensitivities observed in surface cultures on minimal nitrate medium (MNM) stress agar plates are presented, and (b) stress sensitivities recorded on cellophane-covered MNM stress agar plates (cellophane colony harvest [CCH] cultures; b) are shown. The experimental arrangements were the same as described for Cu^{2+} exposures in the legend of Figure 1. *, [#], and [†] indicate significant differences at p < .05 significance level (calculated by Student's *t* test) between treated TNJ36 control versus nontreated TNJ36 control, between treated MKL14 $\Delta crpA$ versus reated TNJ36 control strains, respectively

the colony (13.5–15 mm), it showed a reduced concentration, 590 μ g/(g DCM), toward the aging region (Figure 3). The MKL14 Δ *crpA* gene deletion mutant strain showed a slower, but increasing, copper accumulation dynamics, with the maximum of 1,200 μ g/(g DCM) in the metabolically inactive regions (10–13.5 mm), but, similar to the control, the concentration of copper continued to

TABLE 1 Copper and cadmium adsorptions by *Aspergillus nidulans* TNJ36 control and the $\Delta crpA$ gene deletion mutant strains

	Metal contents ^a	
Treatment	TNJ36	MKL14 (ΔcrpA)
+0.3-mM CuCl ₂	665.6 ± 182.9 (mg/kg)	697.1 ± 106.9 (mg/kg)
	$10.5 \pm 2.9 \text{ (mM/kg)}$	$10.9 \pm 1.7 \text{ (mM/kg)}$
+0.3-mM CdCl ₂	612.5 ± 189.3 (mg/kg)	1672.7 ± 104.7 (mg/kg)*
	5.4 ± 1.7 (mM/kg)	14.9 ± 0.9 (mM/kg)*

^aMean metal content \pm *SD* values, calculated from three independent experiments, are presented.

*Significant difference at p < .05 (calculated by Student's *t* test) between cadmium-exposed control (TNJ36) and $\Delta crpA$ gene deletion (MKL14) strains.

decrease, with the minimum of $870 \,\mu\text{g}/(\text{g DCM})$, inside the colony (Figure 3).

The cadmium concentrations were higher, with the maximum of 850 μ g/(g DCM) (the uncertainties of the Cd concentrations were about 15%), between the center and the edge of the colony (7.5–9.0 mm; Figure 4). The cadmium concentration showed an increasing tendency from the edge of the colony to the aging region, with the maximum of 1,300 μ g/(g DCM) (16.5–19.5 mm from the edge; Figure 4).

4 | DISCUSSION

As expected, all $\Delta crpA$ gene deletion mutants constructed and tested by us (MKL5, MKL10, MKL14) showed increased sensitivities to Cu²⁺ and Cd²⁺ heavy metal stresses (Figures S1–S4). All these observations were in line with previous experimental data published by Antsotegi-Uskola et al. [19], and the MKL14 $\Delta crpA$ strain was chosen for further stress and analytical studies.

"Copper phenotype" colony morphology (very thin, loose mycelial mats) [19] was only visible in standard stress agar experiments with the $\Delta crpA$ strains (Figures 1



FIGURE 3 The particle-induced X-ray emission (PIXE) elemental analysis of copper distributions in *Aspergillus nidulans* TNJ36 control (a) and MKL14 $\Delta crpA$ (b) colonies. Changes in Cu distribution observed in colonies of the *A. nidulans* TNJ36 control (a) and the MKL14 $\Delta crpA$ gene deletion mutant (b) strains. Elemental contents ($\mu g/(g DCM)$), filled symbols) of 1.5 mm × 1.5 mm areas were measured with PIXE and scanning transmission ion microscopy (STIM) techniques through the freeze-dried samples (in 9 spots), where the origin (point 0) of the *x*-axis was set at the very edge of the colony. Elemental distribution maps (Cu, C) and STIM images recorded at spots 1 (edge, where the cellophane sheet was covered by the mycelial mat with 100% confluency), 7 (between the edge and the middle), and 9 (middle of the colony) are presented. The color scales indicate the variation in elemental contents from the highest concentration in red and white to lowest in blue and black

and S1), and it was absent in CCH cultures of the MKL14 strain (Figure 1), clearly indicating that the appearance of this special phenotype was organically coupled to the germination of Cu^{2+} -exposed mutant conidiospores under high Cu^{2+} concentration.

Most important, CCH cultures of both the TNJ36 and MKL14 strains were more tolerant to both Cu²⁺ and Cd²⁺ stresses than their standard stress agar cultures (Figures 1 and 2). Similar phenomena have been reported previously with *A. nidulans* $\Delta atfA$ mutants and appropriate control strains in the presence of the lipid peroxidation-initiating compound *tert*-butyl hydroper-oxide [26]. The bZIP-type transcription factor AtfA is one of the key regulators of environmental stress response in *A. nidulans* [26,35–37].

Paradoxical stress sensitivity phenotypes with unexpectedly increased heavy metal tolerances were observed in Cu²⁺-exposed (at concentrations equal to or less than 0.125 mM) standard stress agar cultures of the MKL14 $\Delta crpA$ strain (Figures 1, S1, and S2), and in Cd²⁺exposed MKL14 CCH cultures (Figure 2). According to Antsotegi-Uskola et al. [19], CrpA is primarily a Cu²⁺ transporter, which may also transport Cd^{2+} ions when other major Cd²⁺ detoxification systems (other Cd²⁺ pumps and the glutathione/phytochelatin system) are saturated. In agreement with this hypothesis, overcompensation mechanisms for the loss of the CrpA pump were more effective for Cd^{2+} than for Cu^{2+} ions at least in CCH cultures, resulting in a significantly increased Cd²⁺ tolerance and biosorption (Figure 2 and Table 1). Furthermore, similar overcompensations for lost elements of fungal stress response systems and consequently emerging paradoxical stress tolerance phenotypes have already been published in the literature [38–40]. Further studies are needed to shed light on the molecular background of the overcompensation mechanisms operating in the MKL14 $\Delta crpA$ mutant under Cd²⁺ exposures, but the upregulation of the biosynthesis of thiol-containing compounds like glutathione and metallothioneins [41-45] and/or alternative Cd²⁺ transporters like PcaA [27,46] should be examined.

The remarkably increased Cd-accumulating capability (1,672.7 \pm 104.7 mg/(kg DCM) at 0.3 mM CdCl₂; Table 1) of the MKL14 $\Delta crpA$ strain is outstanding among



FIGURE 4 The particle-induced X-ray emission (PIXE) elemental analysis of cadmium distributions in Aspergillus nidulans TNJ36 control (a) and MKL14 \(\Delta crpA\) (b) colonies. Changes in Cd distribution observed in colonies of the A. nidulans TNJ36 control (a) and the MKL14 $\Delta crpA$ gene deletion mutant (b) strains. Elemental contents ($\mu g/(g \text{ DCM})$, filled symbols) measured in 1.5 mm × 1.5 mm areas using PIXE and scanning transmission ion microscopy (STIM) techniques in 8 (strain TNJ36) or 6 (strain MKL14) spots are presented, where the origin (point 0) of the x-axis was set at the very edge of the colony. Elemental distribution maps (Cd, C) and STIM images recorded at spots 1 (edge, where the coverage of the cellophane sheet by mycelia reached 100%), 6 (strain TNJ36) or 4 (strain MKL14; between the edge and the middle), and 8 (strain TNJ36) or 6 (strain MKL14; middle of the colony) are shown. The color scales indicate the variation in elemental contents from highest concentration in red and white to lowest in blue and black

fungi, because the A. fumigatus Af293 strain characterized before with a notable Cd^{2+} tolerance [9,10,23,27] accumulated less, $850 \pm 110 \text{ mg/(kg DCM)}$, Cd when exposed to 2-mM CdCl₂ in CCH cultures [27]. It is important to note that Kurucz et al. [27] inoculated cellophane sheets placed on Cd²⁺ supplemented stress agar plates directly with 5- μ l suspensions of 1×10^5 A. fumigatus spores without any preincubation under unstressed conditions. Nevertheless, the observed remarkable Cd²⁺ tolerance and accumulation of the A. nidulans MKL14 $\Delta crpA$ mutant makes this strain a promising candidate for the development of novel fungal biomass-based Cd²⁺ biosorption technologies [47-49]. In future filamentous fungus-based bioremediation technologies, A. nidulans could be superior to A. fumigatus, because the latter species is known as the most widespread and most dangerous opportunistic filamentous fungus pathogen to humans [12].

From the spatial heavy metal accumulation patterns based on PIXE-STIM measurements (Figures 3 and 4), we concluded that both heavy metals distributed mostly between the edges and the middle parts of the TNJ36 control strain colonies. It is clear that CrpA, together with other heavy metal ion transporters [16-20,23], actively pumped Cu^{2+} and Cd^{2+} ions out of the cells at apical and subapical regions of hyphae [19,23], which resulted in low Cu²⁺ and Cd²⁺ concentrations at the colony edges (Figures 3 and 4). Alternative detoxification mechanisms like complexation of these ions with metallothioneins (e.g., for Cu^{2+}) [18,19] and glutathione (e.g., for Cd^{2+}) [19,50,51] also contributed to the control and neutralization of these ions. In the MKL14 $\Delta crpA$ strain, the edges contained less heavy metal ions than the aging parts (Figures 3 and 4), indicating the onset of compensating mechanisms for the loss of CrpA, but the center of gravity for heavy metal accumulation was shifted clearly toward older regions. This is indicative of the activation of alternative intracellular detoxification processes in the MKL14 $\Delta crpA$ strain, transporting heavy metal ions and their metallothionein and glutathione complexes most likely to the vacuoles of older hyphal segments in addition to biosorption by the cell wall [42,52–55].

By using PIXE-STIM analysis, it is possible to perform quantification of the amounts and distribution of elements in biological and environmental samples [56]. Various types of bacteria, fungal, plant, and human

Journal of Basic Microbiology-

material have been analyzed using PIXE. Earlier studies have reported the elemental compositions of magnetotactic bacterium [57], heavy metal-accumulating *Citrobacter* sp. [58], *Fusarium culmorum* hyphae exposed to chemical agents [59], fruit bodies of the woodrotting fungus *Fomes fomentarius* [60], mycorrhizal roots and arbuscular mycorrhizal fungal hyphae and spores [61–67], mycothallic fern [68], living xylem infected by pathogenic fungi [69], and also human samples [70–72].

The clear-cut differences observed in the heavy metal distribution patterns of the *A. nidulans* TNJ36 control and MKL14 $\Delta crpA$ gene deletion strains using the PIXE–STIM technique in CCH stress agar plate surface cultures (Figures 3 and 4) helped us to gain a deeper insight into the molecular mechanisms behind the increased Cd²⁺ tolerance and biosorption phenotypes. We suggest that such PIXE–STIM measurements could be employed in the future in both basic research and applied research projects when the heavy metal-accumulating potential and mechanism of saprophytic fungi are of paramount interest.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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-Journal of Basic Microbiology-

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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