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Short title: Characterization of AB21 protein from *A. bisporus*. (45 characters max)

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Names and affiliations of the authors:

Jan Komárek^{1,2}, Eva Ivanov Kavková^{3§}, Josef Houser^{1,2}, Aneta Horáčková³, Jitka Ždánská¹, Gabriel Demo^{1,2#}, Michaela Wimmerová^{1,2,3}

¹ Central European Institute of Technology, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic

² National Centre for Biomolecular Research, Faculty of Science, Masaryk University, Kotlarska 2, 625 00 Brno, Czech Republic

³ Department of Biochemistry, Faculty of Science, Masaryk University, Kotlarska 2, 611 37 Brno, Czech Republic

§ current address: Department of Biosciences, Faculty of Natural Sciences, University of Salzburg, Hellbrunner Straße 34, A-5020 Salzburg, Austria

current address: RNA Therapeutics Institute, University of Massachusetts Medical School, Plantation street 368, Worcester, MA, USA

The institution at which the work was performed:

Central European Institute of Technology (CEITEC), Masaryk University

Contact information for the author responsible for correspondence:

Name: Michaela Wimmerova

Address/Affiliation:

Central European Institute of Technology (CEITEC), Masaryk University,
Kamenice 5, Brno, 625 00, Czech Republic

E-mail address: michaw@chemi.muni.cz

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Abstract (250 words)

We report the characterization of the dimeric protein AB21 from *Agaricus bisporus*, one of the most commonly and widely consumed mushrooms in the world. The protein shares no significant sequence similarity with any protein of known function, and it is the first characterized member of its protein family. The coding sequence of the *ab21* gene was determined and the protein was expressed in *E. coli* in a recombinant form. We demonstrated a high thermal and pH stability of AB21 and proved the weak affinity of the protein to divalent ions of some transition metals (nickel, zinc, cadmium and cobalt). The reported crystallographic structure exhibits an interesting rod-like helical bundle fold with structural similarity to bacterial toxins of the ClyA superfamily. By immunostaining, we demonstrated an abundance of AB21 in the fruiting bodies of *A. bisporus*.

Introduction

Progress in genome sequencing over the last two decades has led to the accumulation of a huge number of protein sequences in the public databases. As of January, 2018, the Uniprot KB database¹ contained almost 108 million entries. However, a significant fraction of them are proteins that have not been experimentally characterized, and whose functions cannot be deduced from sequence comparisons. It was estimated that 30-40% of the proteins encoded by bacterial genomes had no clear function, the prevalence of unknown proteins was even higher in eukaryotic and archaeal genomes, and was well over 50% in plants and mammals.²⁻⁵ Even for *Escherichia coli*, the experimental evidence was only available for 54% of the gene products.^{3,6} Investigating the functions and properties of the unknown proteins is important to understanding the whole picture of processes that occur in living organisms. This task represents one of the greatest challenges of the post-genome era, but also an opportunity to discover new aspects of life's machinery.³

The classical strategy for predicting the functions of new proteins uses sequence or structure homology by finding a similar protein of known function or identifying the functionally important regions of the protein.^{7–9} Where there is no homology, comparative genomics methods can be highly valuable for elucidating the protein function^{3, 10}. The experimental approaches are typically based on gene expression profiles^{11, 12}, the identification of interacting partners (both small molecule ligands and macromolecules)^{13–18} or associating the protein with a particular phenotype using genetically altered organisms with down or up-regulated protein expression^{19–21}. However, in cases where no prior expectation of the function exists, investigating the biological role is often problematic and like “looking for a needle in a haystack”.

The white button mushroom *Agaricus bisporus* is one of the most widely cultivated mushrooms in the world and an important component of the human diet. Apart from its high nutritional value, it has a significant ecological role. As a saprotrophic fungus, *A. bisporus* is involved in the decay of leaf litter in temperate forests. In 2012, the genome projects of two *A. bisporus* variants (var *bisporus*, strain H97 and var *burnettii*, strain JB137-S8) were presented.²² Knowledge of the genome sequence enabled a better understanding of aspects of *A. bisporus* growth in a humic-rich environment, and provided a basis for performing targeted molecular biology and structural biology experiments. In total, 10,438 protein-coding genes were estimated in the genome of the H97 strain, of which 83% are proteins with homology to other sequences in the protein database. However, a significant number of gene products have an unassigned function.

Here we report the production and characterization of the AB21 protein (named after the source organism and molar mass of the monomer – 21 kDa). The protein shares no detectable sequence homology to any protein of known function, and was discovered during the screening of new potential lectins (sugar-binding proteins) in higher fungi (unpublished data).

However, the lectin activity was not confirmed in the following experiments, leaving AB21 as a protein of unknown function. In this study, the experiments were performed to characterize the protein and investigate its possible biological role. The coding sequence of the *ab21* gene was determined and the gene was expressed in a recombinant form in *E. coli*. AB21 was characterized in terms of quaternary structure, stability and possible interactions with physiologically-relevant ligands (small molecules and ions). The determined X-ray structure of AB21 exhibited an interesting relation to bacterial pore-forming toxins. Finally, the naturally occurring protein was immunolocalized within the fruiting bodies of *A. bisporus*.

Materials and Methods

Isolation of RNA and reverse transcription PCR

Young fruiting bodies of *A. bisporus* var *bisporus* (mushroom not fully open yet, stipe and pileus connected by the velum) were obtained from a local market and used for the RNA isolation. The whole fruiting body (pileus and stipe) was frozen in liquid nitrogen and homogenized with mortar and pestle. Total RNA was isolated with SV Total RNA Isolation System (Promega). mRNA of the gene of interest was reverse transcribed to cDNA with AMV reverse transcriptase and obtained cDNA was subsequently amplified by PCR using Tfl DNA polymerase (AccessQuick™ RT-PCR System, Promega). Primers for RT-PCR amplification of a predicted open reading frame of the *ab21* gene (Table S1) were designed based on the genome of *A. bisporus*.²² The amplified product was sequenced by Sanger method (GATC Biotech, Germany) and the coding sequence was deposited in GenBank²³ (accession number MF004389).

Gene cloning and site-directed mutagenesis

The gene was amplified by PCR with *ab21* specific primers introducing recognition sites for NdeI and XhoI restriction endonucleases. For the PCR reaction, PfuUltra II DNA polymerase was used. After digestion, the fragment was inserted into the cloning site of the pRSETa expression vector (Thermo Fischer Scientific), resulting in the plasmid pRSET-*ab21*.

A different combination of primers was used for the cloning of *ab21* into the pET29a vector (Merck Biosciences). The reverse primer introduced a HindIII restriction site and removed the stop codon, allowing the His-tag to be added at the C-terminal part of the recombinant protein. Sequences of the primers used for gene cloning are listed in Table S1.

For protein production, pRSET-*ab21* and pET29-*ab21* plasmids were transformed into *E. coli* BL21(DE3) cells (Agilent Technologies) by heat shock. The sequence of the plasmids and their presence in transformed cells were confirmed by restriction cleavage of the re-isolated plasmid and Sanger sequencing.

Mutated versions of AB21 (H11A, H37A, H41A and H84A) were generated by site-directed mutagenesis using a modified QuikChange protocol (Agilent Technologies). The modifications involved the use of PfuUltra II DNA polymerase and 72 °C elongation (instead of manufacturer's 68 °C). As a template, pRSET-*ab21* plasmid (50 ng) was used. Oligonucleotide primers used to introduce the mutations are listed in Table S1. After PCR the parental DNA was digested with DpnI. The introduction of mutations was confirmed by Sanger sequencing.

Protein expression and purification

E. coli BL21(DE3) cells harbouring pRSET-*ab21* or pET29-*ab21* were cultivated at 37 °C in standard low-salt LB medium with 100 µg·ml⁻¹ ampicillin or 15 µg·ml⁻¹ kanamycin, respectively. At OD₆₀₀ of ~ 0.5, the protein expression was induced by 0.2 mM isopropyl-β-

D-thiogalactopyranoside. After additional 20 hours of cultivation at 16 °C, the cells were harvested by centrifugation (15 min/9000 g/4 °C) and the pellet was resuspended in 20 mM Tris, 150 mM NaCl, pH 7.4.

AB21 protein was purified by metal affinity chromatography on Ni-NTA Superflow Cartridge (Qiagen) using ÄKTA FPLC system (GE Healthcare). Prior to purification the cells were disintegrated by sonication (Soniprep 150, Sanyo) and the sample was centrifuged (45 min/21000 g/4 °C) and 0.45 µm filtered. Cleared supernatant was loaded on the column equilibrated with 20 mM Tris, 150 mM NaCl, pH 7.4. After washing out the unbound proteins, the protein was eluted by step gradient of imidazol. His-tagged AB21 was eluted by 100-250 mM imidazol. The untagged AB21 was purified on Ni-NTA due to its natural affinity to divalent nickel ions and was eluted by 25 mM imidazol.

Fractions containing pure protein (verified by SDS-PAGE) were extensively dialyzed against 20 mM Tris, 150 mM NaCl, pH 7.4 for short-term storage at 4 °C, or against an appropriate buffer for further studies. The His-tagged AB21 was used for biophysical characterization, ligand screening with small molecules, crystallization and production of anti-AB21 antibodies. The untagged AB21 was used for the metal binding studies (DSF and ITC experiments) only. Protein concentration was determined spectrophotometrically at 280 nm (ND-1000 Spectrophotometer, NanoDrop Technologies). If desired, the protein was concentrated using Amicon ultrafiltration device with a 3 kDa cut-off membrane (Merck Millipore).

Analytical ultracentrifugation

Analytical ultracentrifugation (AUC) experiments were performed using ProteomeLab XL-I analytical ultracentrifuge (Beckman Coulter) equipped with An-60 Ti rotor. Before

analysis, purified protein was brought into the appropriate buffer by dialysis and the dialysate was used as an optical reference.

Sedimentation velocity experiments were conducted in standard double-sector centerpiece cells loaded with 425 μl of both protein sample ($0.2\text{--}1.0\text{ mg}\cdot\text{ml}^{-1}$) and reference solution. pH stability experiments were performed at $1\text{ mg}\cdot\text{ml}^{-1}$ protein concentration and the pH range of 3.0 to 12.0 (Table S2). Data were collected at $20\text{ }^{\circ}\text{C}$ at a rotor speed of 48,000 rpm. Absorbance scans were performed at 280 nm at 5 min intervals and 0.003 cm spatial resolution in continuous scan mode. The partial specific volume of protein together with solvent density and viscosity was calculated from the amino acid sequence and buffer composition, respectively, using the software Sednterp (<http://bitcwiki.sr.unh.edu>). The sedimentation profiles were analyzed with the program Sedfit 15.01.²⁴ Continuous $c(s)$ distribution model was used for the analysis.

Sedimentation equilibrium experiments were performed at $20\text{ }^{\circ}\text{C}$ in a six-channel centerpiece cell loaded with 100 μl of AB21 ($0.1\text{ mg}\cdot\text{ml}^{-1}$, $0.25\text{ mg}\cdot\text{ml}^{-1}$ and $0.5\text{ mg}\cdot\text{ml}^{-1}$) and 110 μl of the reference solution. The sample was gradually spun at rotor speeds of 12,500 rpm, 15,000 rpm, and 26,000 rpm, respectively. After the equilibrium was achieved, data were collected at 280 nm by averaging 20 replicates with 0.001 cm spatial resolution in a step mode. Data from the multi-speed experiment obtained at various protein concentrations were globally analyzed with SEDPHAT 14.0 using non-interacting discrete species model with mass conservation constraint.²⁵

Analytical ultracentrifugation was also used to test AB21 interaction with lipid membranes. The membrane mimicking was achieved by *n*-dodecyl β -D-maltoside (DDM) micelles. The sedimentation velocity experiment was performed with $0.8\text{ mg}\cdot\text{ml}^{-1}$ protein in 20 mM Tris, 150 mM NaCl, pH 7.5 in the presence and absence of 1.95 mM DDM (concentration above the CMC value).

Circular dichroism spectroscopy

CD spectra were acquired on J-815 spectrometer (Jasco) in the far-UV region (200-250 nm). Measurements were performed at 20 °C and 20 nm·s⁻¹ scan speed using a cell with 1 mm path length. For the experiments, 0.3 mg·ml⁻¹ protein in the appropriate buffer (Table S2) was used. All spectra were obtained by averaging of three following scans and the spectrum of the buffer was subtracted.

Differential scanning fluorimetry

Thermal stability of AB21 was studied using Prometheus NT.48 instrument (NanoTemper Technologies) and nanoDSF grade standard capillaries filled with the samples. The measurements were done with the protein concentration of 0.5 mg·ml⁻¹. Thermal unfolding (refolding) was detected in a thermal ramp between 25-90 °C with a heating (cooling) rate of 1 °C·min⁻¹ and the excitation power of 20%. Transition points were determined from the ratio of tryptophan emission at 330 and 350 nm. Buffers used for testing the influence of pH are listed in Table S2. For ligand screening a set of 14 different metal chlorides and 85 other small molecules (e.g. coenzymes, nucleotides, amino acids and sugars, see Table S3 for details) was used. Metal ions were tested at 1.5 mM concentration, small molecules were used at 0.5 mM concentration.

Isothermal titration calorimetry

ITC experiments were carried out at 25 °C on an iTC200 microcalorimeter (Malvern Instruments). The protein in a cell was titrated with the metal chlorides in a syringe while stirring at 750 rpm. The concentration of protein and ligands and the buffer composition

differed for individual measurements, details of the experiments are described in Results. Metal ion titrants were dissolved in the identical buffer as protein and prepared fresh before each experiment. The titrations consisted of 20 injections of 2 μ l volume of the ligand (0.4 μ l for the initial injection). Usually, second titration was performed to provide a complete reaction profile and account for the heat of dilution. The raw data sets obtained in the successive titrations were linked together prior to data analysis using ConCat32 software (MicroCal of Malvern Instruments). The raw calorimetry data were integrated and the heat of dilution (heat associated with the last few injections) was subtracted. The data were analyzed by nonlinear regression in Origin 7 (MicroCal). The binding isotherms were fitted to the one-site binding model, which brought up stoichiometry (n), association constant (K_a) and the enthalpy of binding (ΔH). The free energy (ΔG) and entropy (ΔS) were calculated from the equation $\Delta G = \Delta H - T \cdot \Delta S = -RT \ln K_a$, where T is the absolute temperature and R is the molar gas constant ($8.314 \text{ J} \cdot \text{mol}^{-1} \text{ K}^{-1}$).

Diethylpyrocarbonate-treated sample for ITC was prepared in the following way. The untagged AB21 ($3 \text{ mg} \cdot \text{ml}^{-1}$) was incubated in 0.1 M MES, pH 6.0 with 30 mM DEPC for 2 hours at 20 $^{\circ}\text{C}$, then the reaction was terminated by adding 60 mM imidazole to quench the unreacted DEPC. The sample was buffer exchanged into 0.1 M Hepes, pH 7.5 and used for ITC titration. The control sample was prepared with the same procedure, except that no DEPC was added.

Crystallization and data collection

Initial crystallization experiments were performed with $12 \text{ mg} \cdot \text{ml}^{-1}$ protein (His-tagged form) dissolved in 20 mM MES, 150 mM NaCl, pH 6.2. Commercial kits Classics, Classics II, Classics Lite, PACT (all Qiagen), Structure Screen 1+2 and Morpheus (both Molecular

Dimensions) were used for the screening. Crystallization screening was performed with the sitting drop vapour diffusion technique and the crystallization drops (0.2 μ l) were equilibrated at 17 °C over a 35 μ l reservoir of the precipitant.

Positive hits from the screening were further optimized with hanging drop technique by varying the concentration of protein and individual precipitant's components. Well-diffracting crystals were obtained with 10 mg·ml⁻¹ protein under the following condition: 0.15 M potassium-sodium tartrate, 1.8 M ammonium sulphate and 0.1 M sodium citrate, pH 5.6. The drops were prepared by mixing the protein and precipitant in the volumes of 1.5:1.5 μ l, 1.5:3 μ l and 3:1.5 μ l and equilibrated at 17 °C over a 1 ml reservoir of the precipitant.

Heavy atom derivative crystals were prepared by direct addition of powdered ammonium tetrathiotungstate (Heavy Atom Screen M2, Hampton Research) into drops with AB21 crystals and subsequent soaking for one week.

All crystals were cryo-protected in 40% PEG 400 and frozen in liquid nitrogen. Diffraction data of native crystals were collected at 100 K at the BESSY II synchrotron, beamline 14.2 (Helmholtz-Zentrum Berlin, Germany)²⁶. The data for heavy atom derivative was measured at the PETRA III synchrotron, beamline P14 (Hamburg, Germany).

Structure determination

Collected images for heavy atom derivative and native crystal were processed by XDS²⁷ and XDSAPP²⁸, respectively, and converted to structure factors using the CCP4 6.5 program package²⁹, with 5% data reserved for R_{free} calculation. The initial model of AB21 was prepared by Auto-Rickshaw web server^{30, 31} using MAD data collected at remote, inflection and peak wavelength of tungsten edge (1.215 Å). The initial model was supplied for molecular replacement with Molrep³² using the data of native AB21 crystal. Refinement was

performed using Refmac5³³ alternated with manual model building in Coot 0.8.4³⁴. Other compounds that were present were placed manually using Coot. Water molecules were added by Coot and checked manually. The quality of the model was validated by Molprobity³⁵ and the model was deposited in the PDB database with accession number 6fpd.

Preparation of anti-AB21 antibodies

The anti-AB21 polyclonal antibodies were custom produced in EXBIO Prague by immunization of rabbits with pure AB21 (C-His6) protein and delivered as a purified IgG fraction. The activity and specificity of anti-AB21 antibodies were verified by Western blotting. The cross-reactivity of anti-AB21 antibodies with the non-homologous His-tagged protein (tested by immunodotting) was negligible.

Immunohistochemical staining

A. bisporus tissue for fluorescence microscopy was sectioned on CM1950 cryostat (Leica) at 10 μm thickness. The sections were mounted onto PolyPrep slides (Sigma), allowed to air dry and fixed for 10 min in methanol (-20 °C). The sections were rinsed in wash buffer (20 mM Tris, 500 mM NaCl, 0.1% Tween 20, pH 7.5) and processed for 1 hour in blocking solution (1% BSA in wash buffer). The tissue sections were incubated overnight at 4 °C with the primary antibody (rabbit anti-AB21 IgG, 1.5 $\mu\text{g}\cdot\text{mL}^{-1}$), then incubated with the secondary antibody (goat anti-rabbit IgG labeled with DyLight488, Thermo Scientific, dilution 1:500) for 1 hour at room temperature in darkness. After each incubation the sections were rinsed in wash buffer. Sections were examined under the IX81 inverted fluorescence microscope (Olympus). For accurate interpretation of the results, a set of controls was included. The sections were tested for autofluorescence and two types of negative controls were utilized. In

the first control, the sections were incubated in the absence of primary antibody (incubation with the secondary antibody only), the other one used rabbit anti-GFP IgG (Thermo Scientific, dilution 1:800) as primary antibodies.

Results

Identification of exons and introns and sequence analysis

The coding sequence of the *ab21* gene was amplified by reverse transcription PCR. The RT-PCR reaction with the total *A. bisporus* RNA and the specific *ab21* primers resulted in a single-size product with 588 bp long open reading frame (ORF), encoding a protein of 194 amino acids and a deduced molecular mass of 20.9 kDa. The cDNA sequence exhibited 100% identity to the corresponding regions of genomic DNA of *A. bisporus* var *bisporus* (H-97).²² Comparison of the genomic sequence with the sequence of cDNA revealed the presence of six exons and five introns. All the splice donor and acceptor sites conform to the general GT/AG rule. The determined coding sequence of the *ab21* gene is different from the sequence in the NCBI database.³⁶ The sequence in the database (accession number XP_006461442), which is based on the automatic splicing algorithms, is incorrect due to an erroneous prediction of the acceptor splice sites of introns 4 and 5. These errors shifted the reading frame and resulted in an incorrect sequence in the C-terminal part of the protein and an increased length of the translated product (Figure 1 and Figure S1).

To compare the sequence of AB21 with the protein sequence database, a BLAST⁷ search was performed. Several paralogs of AB21 were identified in the genome of *A. bisporus*. Three homologous genes were present in the variant *bisporus*, while four genes were found in the variant *burnettii* (Figure S2). Outside of *Agaricus* spp., the algorithm identified no hits with significant homology (> 30% sequence identity) in the UniProtKB database.¹ More distant

sequence homologs with E-values between 10^{-4} and 10^{-8} were found in other organisms from the Agaricomycetes class. These matches share a sequence identity of $\sim 20\text{-}25\%$ (Figure S3) and they correspond to hypothetical proteins with unknown function from several fungi (*Hydnomerulius pinastri*, *Rhizoctonia solani*, *Fibrosporia radiculosa*, *Serpula lacrymans*). Surprisingly, no homologs were found in *Pleurotus ostreatus*, *Laccaria bicolor* or *Coprinopsis cinerea* – the organisms that are taxonomically more related to *A. bisporus*.

Biophysical characterization and stability of AB21

The intact molecular mass of AB21 was determined by mass spectroscopy (MALDI-TOF, performed at the Proteomics Core Facility of CEITEC). The spectrum revealed a peak of 22538 Da and proved the cleavage of the initial methionine in the recombinant AB21 protein.

The oligomeric state of AB21 was investigated using analytical ultracentrifugation. Analysis of sedimentation velocity data (Figure 2A) showed a single peak with a sedimentation coefficient of 2.8 S ($s_{20,w} = 2.9$ S) which corresponds to a dimeric species present in the solution. The results were identical for all protein concentrations tested (0.2-1.0 mg·ml⁻¹). The higher value of the refined frictional ratio ($f/f_0 = 1.6$) indicated an elongated shape of the protein or higher protein flexibility. The dimeric state was confirmed by the sedimentation equilibrium technique (Figure 2B). The determined molecular weight (45.6 kDa) is in good agreement with the theoretical value of the dimer (45.1 kDa).

The dimer was found to be stable over a range of pH values of 4.5-10.5 (Figure S4A). Under more acidic conditions, the protein tended to aggregate. At pH 3.0, $\sim 50\%$ of the protein was present in the form of small aggregates. In the alkaline region (pH 12.0), the protein was mostly unfolded monomer with a sedimentation coefficient of 1.3 S and frictional ratio of ~ 2.2 .

CD spectroscopy of AB21 showed a typical spectrum of α -helical proteins with negative bands at 209 and 221 nm. The protein preserved the helical structure between pH 3.0 and 10.5 (Figure S4B), suggesting no significant influence of pH on the secondary structure. The lower signal observed at pH 3.0 can be attributed to the presence of a small extent of aggregates. At pH 12.0, the protein loses its helical structure and becomes partially unfolded. This unfolding was found to be reversible. Most of the protein was successfully refolded after the sample was dialyzed into a buffer with pH 7.5 (data not shown).

The thermal stability of AB21 was studied by differential scanning fluorimetry. The results showed that AB21 is stable and unfolds at relatively high temperatures. The highest thermal stability was observed between pH 6.0 and 9.0 (68-69 °C), and the melting temperature (T_m) gradually decreased with pH above 9.0 or below 6.0 (Figure S4C). At pH 12.0 (unfolded protein), no transition point was observed. Similarly to pH-dependent unfolding, the thermal unfolding was found to be a partially reversible process. At pH 7.5 the protein unfolded at 69.0 °C and refolded below 36.0 °C, and it was able to withstand several unfolding/refolding cycles in the temperature range of 25 to 90 °C. However, with every other cycle the percentage of refolded protein decreased (Figure S5).

Ligand and metal screening

The ligand screening using DSF was performed on a set of physiologically-relevant small molecules (e.g. coenzymes, nucleotides, amino acids, sugars, enzyme inhibitors, metabolic intermediates) to identify the potential natural ligands for the AB21 protein (Table S3). The compounds that increased the thermal stability of the protein by more than 0.5 °C (NADPH, hemin) were further tested on ITC, however, no evidence of binding was observed.

In parallel, AB21 was screened against a set of metal cations. To avoid the interference of the oligohistidine tag with the assay, the untagged variant of AB21 was expressed and purified on Ni-NTA (see Materials and Methods), which by itself demonstrated the inherent ability of the protein to interact with divalent nickel ions. No protein was captured on uncharged NTA, which excluded the possibility of unspecific interaction with the matrix. The metal screening was performed in two buffer systems (20 mM Tris and Hepes, pH 7.5), the results are shown in Figure 3. No stabilization effect was observed for monovalent and trivalent metals. Interestingly, divalent metals Ni^{2+} , Co^{2+} , Cd^{2+} (in both buffers) and Zn^{2+} and Cu^{2+} (Tris buffer only) increased the T_m value of the protein by 2-4 °C, suggesting their binding to AB21. The contradictory effect of Zn^{2+} and Cu^{2+} observed in Tris and Hepes buffers is probably related to the effective concentration of the cation present in the particular buffer system and higher tendency of the protein to precipitate at higher concentrations of Zn^{2+} and Cu^{2+} ions.

Interaction with divalent metal ions

Isothermal titration calorimetry (ITC) revealed the specific binding of divalent metal ions to untagged AB21. In 0.1 M Tris, pH 7.5, the binding was confirmed for Zn^{2+} , Ni^{2+} , Cd^{2+} and Co^{2+} ions, no detectable heat was observed for Mn^{2+} , Ca^{2+} , Mg^{2+} , Al^{3+} and Fe^{3+} (in agreement with the previously mentioned metal screening results). However, the apparent binding affinity in Tris buffer was decreased ($K_a \sim 10^3 \text{ M}^{-1}$) due to the well-known ability of Tris buffer to compete with the protein for the binding of metal ions³⁸⁻⁴⁰, and the thermodynamic parameters could not be determined accurately (c value < 1).

To avoid the complications associated with measuring the interactions in Tris buffer, ITC titrations were performed in 0.1 M Hepes, pH 7.5. The thermodynamic parameters were

determined for the interaction of AB21 with Ni^{2+} and Co^{2+} ions, titrations with Zn^{2+} and Cd^{2+} resulted in the precipitation of the protein in the cell (inspected visually) and the measured ITC thermogram was not interpretable. The raw data for Ni^{2+} and Co^{2+} were integrated and fitted with a one-site binding model (Figure 4 and Table 1). The data indicated the binding of protein and metal ion with a stoichiometry close to 1:1 and the binding was characterized by negative enthalpy and positive entropy change. The relative affinities ($\text{Co}^{2+} < \text{Ni}^{2+}$) follow the Irving-Williams series for stabilities of complexes formed by divalent first-row transition metals.⁴¹ The binding site is common to both ions, because no response was observed for titration of Co^{2+} to AB21 pre-titrated with Ni^{2+} and, vice versa, the heat associated with Ni^{2+} (stronger binder) binding to protein pre-titrated with Co^{2+} (weaker binder) was reduced (Figure S6).

Identification of essential residues for metal binding

The key residues involved in metal binding were identified by ITC and site-directed mutagenesis. At first, the experiments were performed at various pH levels, and the binding of nickel ions to AB21 was found to be pH-dependent (Figure S7). A continuous decrease of the pH from 7.0 to 5.5 caused the complete disappearance of the binding event. Since the binding of a metal ion is often coupled to deprotonation, acidic pH prevents the proton release and inhibits the metal binding. The fact that AB21 contains no cysteine residues and the protonation of carboxylates would occur at a much lower pH (pK_a value of 3.9 for aspartates and 4.3 for glutamates)⁴² suggests that histidine residues (pK_a of 6.5)⁴² are involved in the metal binding.

To further verify the suggested role of histidines, the protein was treated with diethylpyrocarbonate (DEPC). The reagent is reasonably specific for the modification of

histidine residues in the pH range 5.5–7.0.⁴³ Pre-incubation of AB21 with DEPC abolished the binding of nickel ions during ITC titrations (Figure S8) and confirmed that histidines are involved in the binding of metal ions. The protein contains four histidine residues (His11, His37, His41 and His84), which restricts the number of potential locations of the metal binding site. To identify the essential histidine(s), a set of single-point mutants, each with one histidine replaced by alanine, was generated. The binding of H11A and H84A mutants onto an Ni-NTA column was not affected, and the mutants were eluted using imidazole. In contrast, the H37A and H41A mutants did not bind to Ni-NTA and were identified in the flow-through (unbound) fractions. The results showed that two nearby histidine residues His37 and His41 are responsible for the metal binding.

Structure analysis

The structure of AB21 was determined by X-ray crystallography. The protein crystallized in the P6(4) spacegroup with one monomer per asymmetric unit. The structure of the initial model was determined using multiple-wavelength anomalous dispersion (MAD) with a tetrathiotungstate-derivative crystal. A single ion of tetrathiotungstate was detected in between two adjacent AB21 monomers being coordinated by Thr90 and Arg188 side chains. The initial model served for molecular replacement with the native AB21 crystal. The data collection and structure refinement details are listed in Table 2. The C- and N- termini are not well resolved in the electron density as well as $\alpha 4$ - $\alpha 5$ loop what results in a higher final R_{free} factor.

The structure of the AB21 monomer has a helical bundle fold, consisting of five α -helices, $\alpha 1$ (residues 12-48), $\alpha 2$ (55-102), $\alpha 3$ (107-122), $\alpha 4$ (132-155) and $\alpha 5$ (163-197, with a distinct 52° kink induced by Pro175). The helices are arranged with up-down-up-down-up topology and stabilized by the hydrophobic core. The structure of the monomer is elongated,

consisting of an AB-domain formed by two tetrahelical bundles of $\alpha 1$, $\alpha 2$, $\alpha 4$, $\alpha 5$ and $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, and a C-domain (C-terminus of $\alpha 1$ and N-terminus of $\alpha 2$), respectively (Figure 5).

The protein crystallized as a dimer in 2-fold axial symmetry (Figure 5). The monomers interact via C-domains, forming an additional tetrahelical bundle. The resulting dimer is a long, rod-shaped molecule, with a total length of ~ 130 Å and maximal width of ~ 25 Å. The dimer is stabilized predominantly by the hydrophobic core formed by helices $\alpha 1$ and $\alpha 2$ of both protomers. A top view of the dimer (long axis oriented horizontally and $\alpha 2$ and $\alpha 4$ helices in the back) shows the $\alpha 2$ and $\alpha 4$ helices of one protomer locked into the adjacent $\alpha 2'$ and $\alpha 4'$ helices of the other one, thus forming two long parallel helical structures. Four helices in the front (arranged in the order $\alpha 5$ - $\alpha 1$ - $\alpha 1'$ - $\alpha 5'$), nearly parallel to each other, cross the long $\alpha 2/\alpha 4'$ and $\alpha 2'/\alpha 4$ helices located behind in a diagonal direction. From the front view, the structure is slightly bent, with the narrowest place found in the dimerization interface.

The determined structure of AB21 confirmed the spatial proximity of His37 and His41 (essential residues for binding of divalent metal ions). The histidines are found on the $\alpha 1$ helix, located 6.1 Å apart. Due to the presence of a high concentration of metal-complexing compounds in the precipitant, the structure of the complex with the heavy metal cations was not obtained.

A Dali search⁸ was performed with the AB21 structure to identify structurally similar proteins in the PDB database (Table S4). The closest structural homologs belong to the ClyA⁴⁴⁻⁴⁶ superfamily of bacterial pore-forming toxins (Figure 6). The best matches were two enterotoxins secreted by *Bacillus cereus* - HBL-B (subunit B of hemolysin BL)⁴⁷ and NheA (subunit A of Nhe toxin)⁴⁸, aligned with a Z-score of 10.1 and 8.9, respectively, and the insecticidal Cry6Aa toxin⁴⁹ from *Bacillus thuringiensis*, aligned with a Z-score of 9.4. The toxins consist of two sub-domains: the tail domain made of a bundle of helices and the head domain. In HBL-B and NheA, the head domain is a small α/β domain with a β -hairpin flanked

by the two α -helices, located between the third and fourth helix of the bundle. The tail part of the toxins (helical bundle) shares the same structural arrangement with AB21 in terms of topology and in case of HBL-B and NheA, also the number of helices and their relative orientation is the same. On the other hand, the $\alpha 3$ and $\alpha 4$ helices of AB21 are significantly shorter than the corresponding helices in the toxins and the head domain, indicated as a region that interacts with the target membranes,⁴⁴ is absent in AB21. The expected inability of AB21 to interact with the membranes is in agreement with the experimental data. Analytical ultracentrifugation showed that the s-value of AB21 was not affected in the presence of DDM micelles (membrane mimicking), suggesting that unlike ClyA, HBL-B and NheB,^{45–47, 50} AB21 does not form oligomers when exposed to the micelles of non-ionic detergents and does not interact with the membranes (data not shown).

Other Dali matches share the overall helical fold, but their structures differ significantly from AB21 in the overall shape, topology, number, length and orientation of helices and the presence of additional structural features.

Analysis of ab21 genomic islands

To further investigate the relation of AB21 to bacterial pore-forming toxins, the neighbourhood of the *ab21* gene in the *A. bisporus* genome (~9 kb both upstream and downstream) was inspected. At the sequence level, no relationship was found between proteins coded by the neighbouring genes and bacterial toxins. To assess the relationship at the structural level, the structures of the encoded proteins were predicted by homology modelling in Phyre2.⁵¹ Some of them were modeled according to the structure of Cry6Aa⁴⁹ (as the most preferred template), even though the sequence identity with Cry6Aa was only ~17-19%. The results showed that several genes coding for toxin-like proteins are co-localized with the *ab21* gene in the genome (Figure 7). They share 19-22% sequence identity

with AB21. Surprisingly, these proteins preserve the functionally important head domain (Figure S9). For each *ab21* paralog, one or two copies of genes encoding toxin-like proteins were found in the neighbourhood. Since clustered genes in the genome are frequently functionally-related, the results indicate that despite the low Z-score identified by Dali and the missing head domain in AB21, the relationship between AB21 and bacterial pore-forming toxins is not random.

Immunolocalization of AB21

The localization of AB21 within the mature basidiocarp of *A. bisporus* was determined by immunohistochemical staining with rabbit polyclonal anti-AB21 IgG (primary antibody) and goat anti-rabbit IgG conjugated to DyLight488 (secondary antibody). The specificity of anti-AB21 antibodies was confirmed by Western blotting (Figure S10). The labeling was observed throughout the whole fruiting body and the protein was abundant in both the pileus and stipe (Figure 8). A higher level of fluorescence (compared to pileus and stipe) was observed in the gills. Basidiospores, in contrast, exhibited no fluorescence signal. In the control experiments, the sections exhibited no autofluorescence at the emission wavelength of the dye (518 nm) and the negative controls (see Materials and Methods) gave a negligible fluorescence signal.

Discussion

In this work we used a combination of different biophysical, structural and bioinformatical techniques to characterize the sequentially unique protein AB21 from *A. bisporus*. It was shown that AB21 is a dimer with high pH and thermal stability, which can undergo reversible unfolding. AB21 is the first characterized member of a new protein family of fungal proteins structurally related to the ClyA family of bacterial pore-forming toxins.

We identified the ability of AB21 to bind divalent ions of some transition metals (Cd^{2+} , Co^{2+} , Ni^{2+} and Zn^{2+}). The untagged protein was purified on Ni-NTA due to its natural affinity to divalent metal ions (by an approach which has been used for the purification of several metal-binding proteins, e.g. zinc finger domains)^{52–55} and the thermodynamic parameters of Ni^{2+} and Co^{2+} binding were determined by ITC. The pH-dependent binding assay and DEPC treatment showed that histidine residues are directly involved in metal ion binding. Single-point mutants of AB21 documented the essential role of the His37 and His41 residues in coordinating the metal ion. In the living organisms, metal-binding proteins play various roles. For example, they are involved in metal homeostasis (uptake, transport, delivery or storage of metals),^{56–59} regulational pathways (e.g. metal-dependent transcription factors)⁶⁰ or detoxification processes (sequestration and removal of toxic metals).^{61–64} In some proteins and enzymes, metal ions play structural or catalytic roles.^{65, 66} The presence of a heavy-metal binding protein in the fruiting bodies of *A. bisporus* would not be surprising, since higher fungi (including *Agaricus* spp.) are well known for their ability to accumulate heavy metals.^{67–71} However, the affinity of AB21 for metal ions is relatively weak (apparent K_d of 55 μM for Ni^{2+} and 180 μM for Co^{2+}) and it is not clear whether the observed binding has a biological relevance, or whether the interaction merely results from the close proximity and favourable orientation of two histidine residues. Moreover, His41 is not conserved across all AB21 paralogs in *A. bisporus*. (Figure S2) and we can assume that the binding of metal ions is not the primary function of these proteins. On the other hand, metal ion binding might play a role in the studied protein variant (AB21) and not in the others.

The X-ray structure of the protein was solved to a final resolution of 2.5 Å. The refined AB21 structure revealed the monomer adopting a helical bundle fold. The protein crystallized as a dimer, with two protomers interacting via their C-domains, resulting in the overall rod-like shape of the molecule. Although helical bundles are very common and found in a wide

variety of proteins, we believe that the reported structure of the protein dimer, formed by locking two monomers inside each other, is unique and has not been described before. The crystallographic structure of AB21 presented here adds to the small number of structures of proteins from *A. bisporus* that have been determined so far, namely the lectin ABL⁷², mannitol 2-dehydrogenase⁷³, tyrosinases abPPO3⁷⁴, abPPO4^{75, 76} and the tyrosinase-binding protein MtaL^{74, 77}.

Enterotoxin subunits HBL-B⁴⁷ and NheA⁴⁸ secreted by *B. cereus* and the insecticidal Cry6Aa toxin⁴⁹ from *B. thuringiensis* were identified as the closest structural homologs to AB21. Despite the evident structural similarity of AB21 to the tail domain of bacterial toxins, AB21 lacks the functionally important head domain. Also, unlike HBL-B, NheB and ClyA,^{45–47, 50} the exposure of AB21 to lipid membrane mimicking (DDM micelles) did not induce protein oligomerization. On the other hand, the clustering of the *ab21* gene with genes encoding toxin-like proteins with a preserved head domain shows AB21 to be more closely related to the bacterial toxins. Also, the high pH and thermal stability of AB21 and the observed reversible unfolding is in agreement with the expected properties of the toxins or toxin-like proteins. Nevertheless, it remains to be elucidated whether AB21 (and the related toxin-like proteins expressed by the neighbouring genes in the *A. bisporus* genome) keep their toxin properties and might be involved, e.g. in the defense against nematodes or insect predators or if they adapted in *A. bisporus* to different physiological roles.

The presence of proteins structurally related to bacterial toxins in the genome of *A. bisporus* may be a result of the ecological associations between *A. bisporus* and the species of the *Bacillus* genus, which might have provided an opportunity for a horizontal gene transfer. The evidence of structural homologs of bacterial toxins in *A. bisporus* is remarkable, however toxin proteins have already been reported in some other edible mushrooms. Aegerolysins, a family of proteins with hemolytic activity, were identified in *Agrocybe aegerita*, *Pleurotus*

ostreatus and *Pleurotus eryngii*.^{78–81} Their function in fungi remains unclear, even though they were shown to be specifically expressed during the development of fruiting bodies and their involvement in the compaction of hyphae in primordia was proposed.^{80, 81}

Despite these efforts, the exact biological role of AB21 remains unclear. However, the fact that the fungus uses its energy and metabolic resources to express the gene (proved at the transcript and protein level) means that it has a specific biological role. According to immunostaining, AB21 is abundant in all parts of *A. bisporus* fruiting bodies. In the future, the biological aspects of AB21 (potential toxicity, hemolytic and insecticidal activity) should be investigated. Also, further approaches should be used to shed more light on the function of the protein *in vivo*. Investigating the essentiality of the protein or associating the protein with a particular phenotype using genetically altered organisms with down-regulated protein expression would provide further insight into the protein's function. The classical methods based on insertional mutagenesis or targeted gene disruption (knock-out) are difficult to apply in practice, since *A. bisporus* is a multinucleate, heterokaryotic species.⁸² RNA interference-mediated silencing is an alternative approach that was used in several basidiomycetes to down-regulate the expression of target genes.^{83–89} The importance of RNAi in fungi is growing, but based on the number of research papers in public databases, it has not become a routinely used technique yet. However, future research on AB21 might go in this direction.

Conclusion

In this study, we reported the characterization of the protein AB21, which has no detectable sequence similarity to any protein of known function. We determined the correct coding sequence of the gene and characterized the protein in terms of stability and oligomeric state. We identified and characterized its interaction with divalent heavy metal ions, however, the biological relevance of the observed binding is not clear. The solved X-ray structure

showed a unique rod-like helical bundle fold with structural similarity to bacterial pore-forming toxins. The analysis of *ab21* genomic islands provided an evidence that the structural similarity between AB21 and bacterial toxins is - despite the missing head domain in AB21 - not random. Finally, the protein existence was proved at the transcript and protein level, and AB21 was immunolocalized in different parts of *A. bisporus* fruiting bodies (pileus, stipe, gills).

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Additional Supporting Information may be found in the online version of this article.

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Abbreviations:

AUC, analytical ultracentrifugation; AB21, protein from *A. bisporus* (monomer 21 kDa); CD, circular dichroism; DDM, *n*-dodecyl β -D-maltoside; DEPC, diethylpyrocarbonate; DSF, differential scanning fluorimetry; ITC, isothermal titration calorimetry; NTA, nitrilotriacetic acid

Figure legends:

Figure 1 Schematic representation of *ab21* gene structure. Exons are shown in black and grey boxes, introns are white. For comparison, the structure of the XP_006461442 gene with the erroneously predicted introns (highlighted with stars) is shown.

Figure 2: Oligomeric state of AB21 (C-His6) determined by analytical ultracentrifugation. The experiments were performed in 20 mM Tris, 150 mM NaCl, pH 7.5 **A** Sedimentation velocity profiles (left upper panel) and the distribution of sedimentation coefficients (right panel) for the AB21 protein (1 mg·ml⁻¹). For clarity, every fourth scan is shown. The residual

plot (left lower panel) reflects the quality of the fit. **B** Sedimentation equilibrium experiment. Equilibrium distributions of AB21 ($0.5 \text{ mg} \cdot \text{mL}^{-1}$) were obtained at rotor speeds of 12,500 rpm (circles), 15,000 rpm (triangles) and 26,000 rpm (squares). The figures were created in GUSI.³⁷

Figure 3 Screening of metal ions performed by DSF. The assay was carried out in 20 mM Tris, pH 7.5 (left panel) and 20 mM Hepes, pH 7.5 (right panel) and AB21 protein (untagged, $0.5 \text{ mg} \cdot \text{mL}^{-1}$) was screened against a set of 14 metal cations (1.5 mM). The plots show mean ΔT_m values calculated by subtracting the control T_m (no ligand added) from the measured T_m value (added ligand). The error bars represent standard deviations obtained from measuring the samples in triplicates.

Figure 4 ITC binding isotherms and best fits for titration of 3.2 mM Ni^{2+} into 190 uM AB21 (untagged, left panel) and 4.0 mM Co^{2+} into 420 uM AB21 (untagged, right panel) in 0.1 M Hepes , pH 7.5 and 25°C . The data were analyzed using a one-site model.

Figure 5 X-ray structure of AB21 dimer. Top view (upper panel) and front view (lower panel). One polypeptide chain is shown in rainbow, the symmetry-related chain is grey. For the AB21 monomer, two intra-monomeric tetrahelical bundles (A and B) and the part forming the helical bundle upon dimerization (C) are marked. The central axis of symmetry is shown as an ellipse in the upper panel, the kink in the $\alpha 5$ helix (Pro 175) is marked with a star. The histidine residues involved in binding of heavy metals are shown in purple. Tetrathiotungstate ion is shown in position observed in AB21- WS_4^{2-} complex together with its corresponding electron density ($2\text{Fo}-\text{Fc}$ at $\sigma = 1.0$) shown as grey mesh.

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Figure 7 Genes located in vicinity of *ab21* gene in genome of *A. bisporus* var *bisporus*.²² Three *ab21* paralogs are black, the genes coding for associated toxin-like proteins are grey, the genes coding for structurally unrelated proteins are white.

Figure 8 Localization of AB21 in *A. bisporus* tissue determined by immunohistochemical staining with polyclonal rabbit anti-AB21 IgG and goat anti-rabbit IgG conjugated with DyLight488 (green color). Horizontal sections (10 µm) of the pileus (A), stipe (B) and gill (C). In the gill, the central region (trama, T), subhymenium (SH), hymenium (H) and basidia carrying basidiospores (S) are highlighted. Merged fluorescence (200 ms exposition) and phase-contrast images (500 µs exposition), magnification X20.

Table 1 The apparent thermodynamic parameters for metal ion binding to untagged AB21 (25 °C) in 0.1 M Hepes, pH 7.5. The standard deviations from triplicate determinations are indicated.

	n	$K_{d,ITC}$ [μ M]	ΔH_{ITC} [kJ/mol]	ΔS_{ITC} [J/mol/K]	$-T\Delta S_{ITC}$ [kJ/mol]	ΔG_{ITC} [kJ/mol]
Co ²⁺	1.02±0.02	180±16.0	-8.3±0.2	44.1±1.2	-13.1±0.4	-21.4±0.2
Ni ²⁺	1.17±0.01	55.0±5.0	-17.6±0.6	22.4±2.6	-6.7±0.8	-24.3±0.2

Please note that the experimental thermodynamic parameters reported in the table are the apparent values and do not take into account other phenomena that accompany the metal binding (displaced protons binding to the buffer, metal-buffer interaction).

Table 2 Data-collection and refinement statistics. Values in parentheses corresponds to the highest resolution shell.

Molecule	AB21 WS ₄ ²⁻ derivative			Native AB21
	Inflection	Peak	Remote	
PDB ID	-	-	-	6fpd
<i>Data collection</i>				
Beamline	PETRA III P14	PETRA III P14	PETRA III P14	BESSY II 14.2
Wavelength (Å)	1.2151	1.2146	1.2110	0.9180
Space group	P6 ₄	P6 ₄	P6 ₄	P6 ₄
Unit-cell parameters				
a and b (Å)	73.85	73.80	73.88	73.94
c (Å)	69.43	69.32	69.49	70.59
α = β [°]	90	90	90	90
γ [°]	120	120	120	120
Resolution range (Å)	69.43-1.76	69.32-1.75	69.49-1.76	36.96-2.50
	(1.85-1.76)	(1.85-1.75)	(1.86-1.76)	(2.64-2.50)
Reflections measured	412116 (37918)	411859 (37529)	408910 (38198)	88783 (13330)
Unique reflections	21304 (2850)	21291 (2851)	21133 (2848)	7689 (1113)
Completeness (%)	98.6 (90.6)	98.6 (90.6)	98.8 (91.8)	99.9 (100.0)
CC1/2	100 (78.5)	100.0 (83.8)	100.0 (66.0)	99.9 (99.8)
R _{merge} [†]	0.051 (1.212)	0.055 (1.077)	0.059 (1.690)	0.043 (0.070)
<I/σ (I)>	30.6 (2.4)	29.7 (2.8)	26.6 (1.8)	40.6 (26.7)
Multiplicity	19.3 (13.3)	19.3 (13.2)	19.3 (13.4)	11.5 (12.0)
Wilson B factor (Å ²)	27.51	26.14	28.13	20.53
Anomalous completeness (%)	97.9 (86.0)	97.8 (85.6)	98.0 (86.9)	-
Anomalous multiplicity (%)	9.9 (7.0)	9.9 (7.0)	9.9 (7.1)	-
<i>Refinement</i>				
Reflections used				7274
Reflections used for Rfree				383
R factor [‡] (%)				24.35
R _{free} [‡] (%)				31.72
R.m.s.d. bond lengths (Å)				0.009
R.m.s.d. bond angles (°)				1.25
No. of non-H atoms (total)				1468
No. of water molecules				41
No. of ligand atoms				4
No. of ions				1
Mean B factor (Å ²)				
Non-H atoms				29.89
Water molecules				24.06
Ligands				42.99
Ions				38.37
Ramachandran plot				
Residues in most favorable regions (%)				96.3
Residues in allowed regions (%)				3.2
Rotamer outliers (%)				4.1

[†] $R_{\text{merge}} = \sum |I_i - \langle I \rangle| / \sum I_i$, where I_i is the intensity of observation and $\langle I \rangle$ is the mean value for that reflection.

[‡] $R \text{ factor} = \sum ||F_o(h)| - |F_c(h)|| / \sum_h F_o(h)$, where F_o and F_c are the observed and calculated structure-factor amplitudes, respectively.

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83x34mm (600 x 600 DPI)

Accepted

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69x19mm (600 x 600 DPI)

Accepted

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74x25mm (600 x 600 DPI)

Accepted

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111x78mm (600 x 600 DPI)

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153x118mm (300 x 300 DPI)

Acce

Accepted

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117x61mm (300 x 300 DPI)

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106x70mm (600 x 600 DPI)

Accept

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76x55mm (300 x 300 DPI)

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