Molecular characterization of central cytoplasmic loop in Aspergillus nidulans AstA transporter

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AstA (alternative sulfate transporter) belongs to a large, but poorly characterized, DaS family of allantoate permeases of the Major Facilitator Superfamily. The \textit{astA} gene has been cloned from an IAM 2006 Japanese strain of \textit{Aspergillus nidulans} by complementation of a sulfate permease-deficient mutant. In this study we show that conserved lysine residues in Central Cytoplasmic Loop (CCL) of the AstA protein may participate in anion selectivity, and control kinetic properties of the AstA transporter. A three-dimensional model containing four clustered lysine residues was created, showing a novel substrate-interacting structure in Major Facilitator Superfamily transporters. The assimilation constant \((K_c)\) of wild type AstA protein is \(85 \mu M\), while \(V_{\text{max}}/\text{mg of DW}\) of AstA is twice that of the main sulfate transporter SB per mg of dry weight (DW) of mycelium (1.53 vs. 0.85 nmol/min, respectively). Amino acid substitutions in CCL did not abolish sulfate uptake, but affected its kinetic parameters. Mutants affected in the lysine residues forming the postulated sulfate-interacting pocket in AstA were able to grow and uptake sulfate, indicating that CCL is not crucial for sulfate transportation. However, these mutants exhibited altered values of \(K_c\) and \(V_{\text{max}}\), suggesting that CCL is involved in control of the transporter activity.

**Key words:** \textit{Aspergillus nidulans}, sulfate permease, alternative sulfate transporter, AstA, intracellular loop, mutagenesis

**INTRODUCTION**

Sulfate is taken up and utilized for the synthesis of organic sulfur compounds by bacteria, fungi and plants. In eukaryotes, sulfate uptake is carried out by sulfate permeases of the SulP family (Kertesz 2001; Loughlin \textit{et al.}, 2002; Piłsyk \& Paszewski 2009; Alper \& Sharma 2013). These proteins are assigned to the TC 2.A.53 class, according to the Transporter Classification (TC) system. In \textit{Aspergillus nidulans} the main sulfate permease is encoded by the \textit{SB} gene, however, an alternative sulfate transporter (AstA) is also functional in some \textit{A. nidulans} strains. The \textit{astA} gene has been cloned earlier from a genomic library of the Japanese IAM 2006 strain, as a suppressor complementing sulfate permease-deficiency of the \textit{SB} mutant (Piłsyk \textit{et al.}, 2007). The \textit{astA} gene is regulated at the transcriptional level by sulfur metabolite repression (SMR), being derepressed under sulfur limitation conditions. In the reference \textit{A. nidulans} strains of Glasgow origin \textit{astA} is non-functional, thus, SB is the only sulfate permease in these strains. Recently, it was shown that AstA transports also sulfite and choline sulfate (Holt \textit{et al.}, 2017). Orthologues of \textit{astA} occur frequently in evolutionarily distant fungi belonging to the \textit{Dikarya} phylum with common feature of plant or animal pathogenicity. An \textit{astA} orthologue identified from pathogenic \textit{Fusarium sambucinum} fungus was found to be derepressed during potato tuber infection, where the AstA protein may efficiently take up sulfate (Piłsyk \textit{et al.}, 2015).

The \textit{AstA} protein (GenBank\textsuperscript{\textregistered} accession number AB28286) is a member of the poorly characterized DaS allantoate permease family belonging to the Major Facilitator Superfamily (MFS, TC 2.A.1). The MFS transporters are localized in the cell membrane or in organelar membranes facilitating the transport of various substrates (ions, sugars, drugs, neurotransmitters, amino acids, or peptides). Contrary to ATP-dependent SulP family of sulfate permeases (Tweedie \& Segel 1970, Woodin \& Wang 1989), three different mechanisms of transport via MFS transporters are distinguished: uniporters transporting one type of substrate and energized by its gradient, symporters translocating simultaneously two or more substrates and drawing energy from the electrochemical gradient of one of them, and antiporters transporting two or more substrates in opposite directions (Law \textit{et al.}, 2008; Iancu \textit{et al.}, 2013; Ethayathulla \textit{et al.}, 2014; Patron \textit{et al.}, 2014). The AstA transporter can uptake sulfate in proton symport-driven energy (Holt \textit{et al.}, 2017). Until now, only one report presented experimental evidence for ATP-independence of MFS - the gene encoding Hxt13p hexose transporter was cloned while screening the yeast genomic library for resistance to antifungal drug miltefosine (Biswa \textit{et al.}, 2013). The authors have shown that Hxt13p acts as an ATP-independent efflux pump for miltefosine. On the other hand, it was shown that GLUT1 glucose transporter in human erythrocytes binds ATP, which triggers significant conformational changes in GLUT1 that lead to its inactivation (Blodgett \textit{et al.}, 2007). In this case ATP serves as a low molecular weight effector rather than energy source.

Crystal structures of bacterial MFS transporters (FucP, LacY, GlpT, EmrD) have revealed the presence of 12 transmembrane (TM) alpha helices. These helices are clustered in the N- and C-terminal domains, each comprising six helices (Fig. 1) (Abramson \textit{et al.}, 2003; Lemon \textit{et al.}, 2004; Yin \textit{et al.}, 2006; Dang \textit{et al.}, 2010). A similar structure of 12 TM helices has also been identified in another MFS phosphate transporter, the PiPT protein of the \textit{Piriformospora indica} fungus (Pedersen \textit{et al.}, 2010).
The substrate-binding residues are found in various TM helices: TM5 in the Candida albicans Mdr1p protein (Pasrija et al., 2007), TM7 in human GLUT-1 (Kasahara et al., 2009), TM1, TM5 and TM7 in E. coli GlpT and FucP (Lemieux et al., 2004; Dang et al., 2010), and TM5, TM 7, TM10 and TM11 in E. coli XylE (Sun et al., 2012). Between the N- and C-terminal domains of all MFS proteins, a third domain is present, called the Central Cytoplasmic Loop (CCL).

Substrate uptake mechanism of MFS transporters involves a rocker-switch type of movement, followed by transient formation and breakage of a salt bridge located opposite in the apical surface of the TM helices (Law et al., 2008). Thus, CCL transiently forms a latch-like structure in the outward-facing open conformation, while in the inward-facing open conformation, it unfolds into a partially disordered chain and swings away from the substrate translocation pathway (Wischedehaisri et al., 2014). Additionally, CCL acts as a constraint for movement of the N- and C-terminal domains and permits a relatively large interdomain movement, which has implications for the substrate translocation mechanism (Law et al., 2008). It has also been shown that CCL is critical for efficient substrate transport by the C. albicans Mdr1 multidrug transporter (Mandal et al., 2012) and likely participates in formation of a substrate translocation pore.

In this study, we demonstrate that CCL of the A. nidulans AstA protein controls sulfate transport. The role of four conserved lysine residues in the A. nidulans AstA protein was analyzed using site directed mutagenesis. Interestingly, four single amino acid substitutions (K260A, K263A, K263L and K264L), introduced in the substrate-interacting region of AstA, led to more selective uptake of sulfate compared to selenate.

### MATERIALS AND METHODS

**Strains and media.** The Aspergillus nidulans strains from our collection, carrying standard markers (Clutterbuck 1994; Martinelli 1994), together with the Escherichia coli strain used in this study, are listed in Table 1. The M111 strain, derived from Glasgow wild-type, bearing the sB43 mutation complemented with the sB gene and carrying a non-functional astA gene, was used as the A. nidulans reference strain.

**Growth conditions.** The following media (solid and liquid) were used: complete medium (CM) (Cove, 1966) for protoplasts or DNA isolation, minimal medium (MM) or minimal sulfur free medium (MM-S) (Lukasziewicz & Paszewski 1976), the latter supplemented either with sulfate (1 mM, inorganic sulfur) or L-methionine (0.1 mM, organic sulfur). The minimal media were also supplemented according to the auxotrophic requirements of the cultured strain. Liquid cultures were grown at 37°C for 16 hours in a rotary shaker (200 rpm). The culture doubling times were calculated using the Doubling Time Computing web page (Roth 2006). Escherichia coli was grown in the standard LB medium supplemented with antibiotics as described before (Sambrook et al., 1989).

### Table 1. Growth rates of AstA transformants on solid and liquid MM-S media supplemented with 1 mM sulfate, transportation constant and maximum velocity of sulfate transport to mycelia expressing SB or AstA variants. The results are average values from at least three biological replicates.

<table>
<thead>
<tr>
<th>Transformant</th>
<th>Growth on solid medium [mm/h]</th>
<th>Doubling time in liquid medium [h]</th>
<th>Sulfate uptake [nmol/min/mg DW] av.</th>
<th>$K_T$ [μmol]</th>
<th>$V_{max}$ [nmol/min/mg DW]</th>
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</thead>
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<td>0.23</td>
<td>6.70</td>
<td>1.50</td>
<td>108</td>
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<td>0.74</td>
<td>85</td>
<td>1.53</td>
</tr>
<tr>
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<td>5.16</td>
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<td>25</td>
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<td>TRAstAK262-264A</td>
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<td>2.86</td>
<td>0.89</td>
<td>137</td>
<td>1.36</td>
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</table>
Nucleic acids manipulations, plasmid construction and mutagenesis. The plasmids used in this study are listed in Table 1. Plasmids were propagated and isolated according to the standard procedures (Sambrook et al., 1989). A. nidulans DNA was isolated using a salting out method. The frozen mycelia were disrupted by grinding in liquid nitrogen, followed by immediate suspension in warm STEN buffer (1% SDS, 100 mM Tris pH 7.5, 50 mM EDTA pH 8, 100 mM NaCl) (Sambrook et al., 1989). Polymerase chain reactions (PCR) were performed in a Tecneh Thermocycler. DNA was sequenced, and primers were synthesized by the DNA Sequencing and Oligonucleotide Synthesis Laboratory, Institute of Biochemistry and Biophysics, PAS. The sequences of the primers used are provided in supplementary Table S1. The kPMS11-52 plasmid carrying the wild-type astA gene (Pilsyk et al., 2007) was modified by an insertion of the Neurospora crassa pyr-4 selection cassette into the PvuI restriction site yielding the kPMS11-524 plasmid. The latter could be selected by uridine prototrophy and was used for construction of a series of plasmids bearing the mutated astA alleles. Selected lysine residues were changed to alanine or leucine using Overlap Extension PCR (OE-PCR) with Pfu polymerase (Thermo Scientific). Obtained PCR products were cleaved with the SnaBI and MunI restriction enzymes (Thermo Scientific) and ligated into the kPMS11-524 vector digested with the SnaBI and MunI restriction enzymes to replace the wild type astA. These mutations were verified by DNA sequencing and introduced into the A. nidulans sulfate permease-deficient M111 strain bearing the sB43 mutation (Table 1).

A 3.3 kb PstI fragment of the non-functional WastA gene was excised from the kpB6-7 plasmid (Pilsyk et al. 2007) and inserted into kpG23B yielding kpG23WPastA plasmid. For reconstitution of the missing functional TM11-TM12 domains in WastA, a 3.2 kb SpeI fragment from kPMS11-524 plasmid (last 586 nucleotides of astA 3' ORF encoding TM11-TM12 together with N. crassa pyr-4 selection cassette) was ligated between SpeI restriction sites of kpB6-7 plasmid, yielding kpB611-12.

The Green Fluorescent Protein-labeled astA allele was constructed with OE-PCR of PCR-generated GF into PCR-generated astA protein vector using overlapping primers. The hybrid product was initially cloned into pGEM®-T Easy vector (Promega), then excised with MunI-Van911, and inserted into MunI-VaN911 sites of kPMS11-524, yielding the kPMS11-524 plasmid. Similarly, the same MunI-Van911 fragment was introduced into a series of kPMS11-524 derivatives bearing the mutated astA alleles.

Transformation of A. nidulans strains. The mycelia for transformation were collected using the PEG method (Kuwano et al., 2008). To increase transformation efficiency, 5 μg of the HELP helper plasmid (Gems & Clutterbuck 1993) was added. Transformants were selected for uracil prototrophy on MM-S medium supplemented with 2 μM sulfate and 1.2 M sorbitol.

Sulfate uptake assay. The sulfate uptake analysis was performed as described previously (Pilsyk et al., 2015) using 0.1 mM methionine — supplemented MM-S for mycelia growing under derepressing conditions. Ten ml aliquots were taken after 2, 3, 5, 10, 15 and 20 min of incubation, and the sulfate transport rates [nmol/min/mg dry weight] were calculated based on at least three measurements taken at different times.

For the estimation of the assimilation constant (K), a cold sulfate was added to the final concentrations of 0.166, 0.2, 0.25, 0.33, 0.5 and 1 mM, and incubations were carried out for 1 h. Mycelia were treated as above and the initial rate of transport [nmol/min/mg dry weight] was calculated for each concentration of the sulfate, based on at least four measurements. These data were rendered as Hanes-Woolf plots, and linear regression was used for calculation of V_max from the slope, and K from the ordinate axis intercept.

Confocal microscopy. The mycelia for microscopic observations were grown in liquid MM-S medium supplemented with 0.1 mM methionine (derepressing conditions) for 18 h at 37°C in a rotary shaker (200 rpm). The mycelia were harvested by filtration, suspended in fresh MM-S medium supplemented with 1 mM sulfate and kept at 37°C in a rotary shaker (200 rpm). The mycelial samples were collected at time of the medium shift (t = 0) and 5 h after the shift, and examined under a Nikon Eclipse TE2000-E inverted microscope (Nikon, Tokyo, Japan).

Membrane protein isolation. For protein isolation, the mycelia were grown as above (Confocal microscopy), and the samples were harvested just before and 5 h after a shift to repressing conditions. Next, the collected samples were ground in TM isolation buffer (30 mM Tris-HCl pH 7.4, 50 mM EDTA pH 8, 1% sorbitol, 0.05% Tween-20, 200 mM NaCl, 20 mM dithiothreitol, 1 mM PMSF, protease inhibitors Complete™-Roche), and the homogenate was centrifuged at 4000×g for 10 min. The supernatants were centrifuged for 1 h at 50000×g and the pelletted membrane fractions were used for Western dot-blots.

Immunodetection of AstA-GFP protein. Membrane proteins suspended in TM isolation buffer were subjected to the Immobilon P membrane (Millipore), and AstA-GFP protein was detected with rabbit anti-GFP polyclonal antibodies (Life Technologies). Membrane protein isolation was used for calculation of V_max, based on at least four measurements. These data were rendered as Hanes-Woolf plots, and linear regression was used for calculation of V_max from the slope, and K from the ordinate axis intercept.

Bioinformatics. Multiple alignments of protein sequences were generated using the MUSCLE software (URL: http://www.drive5.com/muscle/) (Edgar, 2004). Homology searches of the GenBank database (release 95.0) were carried out using the BLAST program (Altschul & Lipman, 1990). Transmembrane topology was predicted basing on four independent algorithms: the PredictProtein server ver. 10.20.04 (https://www.predictprotein.org/) (Rost et al., 2004), TMPred (https://embnet.vital-it.ch/software/TMPRED_form.html) (Hofmann & Stoffel 1993), PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) (Buchan et al., 2013) and SMART (http://smart.embl-heidelberg.de/) (Letunic & Bork 2017). Modeling of 3D structure and ligand binding were performed with the I-TASSER metaserver (Zhang 2008; Roy et al., 2010; Roy et al., 2012). The image of CCL was created with RasMol (Sayle & Milner-White, 1995).
RESULTS

Verification of astA allele functionality

Our previous studies showed that sB mutants in A. nidulans could be complemented by the astA gene only when it was derived from the Japanese IAM2006 strain. Numerous attempts to complement the auxotrophic phenotype of sB mutants by a copy of astA derived from a reference wild-type strain of Glasgow origin, have failed (Piłsyk et al., 2007). Northern blot analysis of the astA expression in the reference strain showed no detectable signal. Moreover, comparison of the nucleotide sequences of astA ORFs in the Japanese and Glasgow strains suggested numerous mutations and deletions in the latter (Piłsyk et al., 2007), including lack of 187bp sequence putatively encoding TM11 and TM12 (Suppl. Fig. S1 at www.actabp.pl). These mutations might abolish the function of the putative AstA protein in the reference strain. Hence, we concluded that this allele exists as a pseudogene (Ψ astA). However, RNA-seq results allowed for a distinction of transcriptional units in AspGD, and transcriptomic data indicate that the astA locus (AN10387) in the reference strain is transcribed (Cerqueira et al., 2014; Sierenko et al., 2014).

To verify functionality of the Ψ astA gene, we generated two constructs bearing selectable N. crassa pyr-4 cassette and the Ψ astA gene or Ψ astA fused with functional sequence coding for TM11-TM12 from the Japanese astA allele. However, transformation of sB mutant with these constructs did not restore sulfate prototrophy (see: Suppl. Fig. S1 at www.actabp.pl). Thus, the lack of TM11 and TM12 helices was not the only reason for abolishing function of the astA gene in strains of Glasgow origin, but the other mutations of the astA ORF present in these strains were sufficient to abolish protein activity. Hence, in further studies a copy of the astA gene derived from the Japanese IAM2006 strain was used as a reference.

In silico analysis of the Central Cytoplasmic Loop.

A predicted topology of the A. nidulans AstA transporter revealed twelve transmembrane domains and a centrally located CCL (Fig. 1), the latter domain containing four lysine residues clustered into a tight bundle located in the middle of CCL – K_260, K_262, K_263 and K_264. These four lysine residues, together with the fifth basic amino acid (arginine or lysine), are conserved in AstA orthologs from various fungal pathogens (Fig. 2A). We previously suggested that the positively charged CCL of AstA could be responsible for interaction with a negatively charged sulfate anion during translocation (Piłsyk et al., 2007).

The AstA region from D_225 through A_275 was modeled using the I-TASSER meta-server (Zhang, 2008; Roy et al., 2010; 2012) (Fig. 2B). Five similar models of the AstA CCL bound with sulfate were obtained, and the highest-scoring one (Fig. 2B) was used in further studies. This model exhibits a small ligand-binding region comprising amino acids 260 through 264 and resembling the calcium-binding pocket of human frequenin (PDB ID: 1G8I, Bourne et al., 2001). The predicted sulfate-interacting pocket contains the four conserved lysine residues (K_260, K_262, K_263 and K_264, see Fig. 2B), therefore, it seems likely that these lysine residues directly interact with the sulfate.
Central Cytoplasmic Loop mutants can take up sulfate

Sulfate uptake was measured in transformants of the M111 strain bearing the sB43 mutation and various mutated astA alleles (Table 1), and compared with the TRAstAWT and TRSBWT strains serving as positive controls. The SB transporter in TRSBWT was more effective in sulfate uptake than the AstA mutants or wild type AstA (Fig. 4, Table 1). The K260A, K263A and K260,263A mutations did not affect the uptake of sulfate significantly, while the K260A, K263A, K263L, K264A and K264L mutations increased the uptake by 38, 51, 71.6, 64.9 and 79.7%, respectively, compared to TRAstAWT (Fig. 4, Table 1). K263L decreased sulfate uptake by 16%. Altogether, the mutations affecting individual lysine residues in CCL did not abolish the sulfate uptake, moreover, most of the tested mutations led to even faster sulfate transport.

Mutations in Central Cytoplasmic Loop affect kinetics of sulfate uptake

Since the mutations affecting CCL did not impair sulfate uptake, it seemed possible that CCL could control its kinetics. Therefore, the effects of these mutations on the kinetic parameters of AstA were next studied. The $K_r$ values determined for wild type AstA and the main sulfate permease SB were 85 μM and 108 μM, respectively (Fig. 5, Table 1). Each of the four substitutions of a single lysine with alanine or leucine decreased the $K_r$ with the most pronounced decrease observed in transformants bearing the K260A, K263L and K264L mutations, by 70.6, 57.6 and 56.4%, respectively (Table 1). Conversely, the quadruple substitution of the four lysine residues in CCL with alanine led to an increase of the $K_r$ by almost 40%.

The $V_{max}$ for wild type transporters was 0.85 (nmol/min) for SB, and twice as much (1.53) for AstA, measured per mg of dry weight (DW) of mycelium (Fig. 5, Table 1). Each substitution of lysine with alanine in AstA led to a lowering of $V_{max}$ – a high decrease (by 48%) was observed in the K263L mutant. Meanwhile, K263L substitution led to the strongest decrease of transport velocity (by 57.5%), while two other mutations (TRAstAK260,L and TRAstAK263,L) had significantly increased velocity of transportation (by 82.4 and 142%, respectively). A single K263L mutation, or a quadruple substitution of all four lysine residues in CCL with alanine, did not affect $V_{max}$ significantly. In summary, the substitutions of the lysine residues located in CCL resulted in various changes of both $V_{max}$ and $K_r$, suggesting that these residues are important for controlling activity of the AstA transporter. The highest increase of $K_r$ (up to 161%) was observed in the quadruple TRAstAK260,263,A mutant, in which all four lysine residues in CCL were replaced with alanine.
To verify if the mutated AstA proteins were properly located in the cell membrane during the kinetic studies, each mutated AstA variant fused with GFP was expressed in the M111 recipient strain and examined under microscope. In all the transformants, both the GFP-tagged wild type AstA protein and its mutated variants were correctly located in the cytoplasmic membrane, both before and after the shift to repressing conditions (Suppl. Fig. S2A at www.actabp.pl). Additionally, the amount of AstA-GFP or its mutated variants was verified by Western dot-blot analysis (Suppl. Fig. S2B at www.actabp.pl), indicating that the AstA protein and its mutant versions were stable in the applied experimental conditions.

Sulfate uptake by AstA is ATP-independent, in contrast to SB (SulP) permease

In order to study AstA transport mechanism, we compared the effect of 100 μM sodium azide on the radioactive sulfate uptake by the TRSBWT or TRAstAWT strains (Fig. 6). Sulfate uptake by the SB permease was significantly decreased by azide while AstA was resistant to the azide treatment. Since azide inhibits ATP-synthase (Bowler et al., 2006), it leads to a decreased intracellular ATP level. Thus, azide-insensitive transport exhibited by the AstA transformant indicates an ATP-independent transport mechanism, contrary to the SB sulfate permease, which is ATP-dependent (Fig. 6 and earlier published results by Tweedie & Segel 1970; Woodin & Wang, 1989).

DISCUSSION

Alternative sulfate transporter AstA, a member of the MFS transporters, is found in some fungi, particularly frequently in pathogenic species. Numerous mutations present in the astA locus (AN10387) in the A. nidulans reference strain abolish its functionality, as it was postulated earlier (Pilsyk et al., 2007) and confirmed in this study. Despite of detectable transcription of the ΨastA locus (Cerqueira et al., 2014, Sierko et al., 2014), the protein product is either not produced or non-functional. We showed that truncated ΨastA was unable to complement the sulfate permease-deficient mutants, even when its ORF was reconstituted with a missing 3’ sequence encoding the last two TM helices 11 and 12. This result supports our earlier conclusion that the astA allele derived from the Japanese IAM2006 strain is functional, while A. nidulans strains of Glasgow origin contain pseudogene sequences.

Structural studies of various MFS transporters have shown the presence of two transmembrane domains, each one comprising six membrane-spanning helices. The two domains are connected by the central cytoplasmic loop (CCL) (Fig. 1), predictably localized in the way of the transported substrate (Law et al., 2008).

Looking for the role of CCL in AstA, we focused on a highly conserved motif of four lysine residues present in AstA orthologs from various fungi (Fig. 2A). These positively charged residues seemed to interact with a negatively charged sulfate ion during translocation, and this assumption was confirmed by the predicted structure of CCL bound with sulfate. Therefore, the role of
these four lysine residues (K_{260}, K_{262}, K_{263}, and K_{264}) was subjected to analysis using site-directed mutagenesis.

Surprisingly, neither substitution of each individual lysine with alanine or leucine, nor substitution of all of them with alanine resulted in growth inhibition. Radiolabeling experiments confirmed that the mutated proteins possessed the ability to take up sulfate, albeit with significantly affected kinetics. Except of the quadruple TRAstAK_{260-264}A mutant, all variants of AstA with a single lysine in CCL mutated had lower transportation constants (K) for sulfate, which indicated enhanced affinity for sulfate. However, we did not find any similar potentially beneficial substitution in sequences of AstA orthologues from other fungi. This apparently surprising finding may be explained by AstA’s ability to transport sulfate and choline sulfate ester (Holt et al., 2017), which implies that under physiological conditions CCL of AstA should be flexible enough to conform to all these substrates. This versatility of AstA might also prevent substitution of K_{264} by a hydrophobic amino acid, which does not occur in nature. While all four single lysine substitutions into alanine resulted in the decreased rate similar to that of the wild type TRAstAWT strain. None of the K_{263} substitutions studied here altered K_{r}, indicating that this lysine 262 is not involved in direct interaction with the sulfate.

Table 2. List of strains and plasmids

<table>
<thead>
<tr>
<th>Designation</th>
<th>Genotype or relevant features</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
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<td>E. coli strain XL1 Blue</td>
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<td>A. nidulans strains</td>
<td></td>
<td></td>
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Vol. 65 Central cytoplasmic loop in Aspergillus nidulans AstA transporter

CCL flexibility during MFS transporter movements. Regarding the XylE transporter CCL forms transient latch-like structure (Wisedchaisri et al., 2014), it might be difficult to verify the transient hydrophobic enzyme-substrate interactions relying solely on solid crystal structures.

Among the single amino-acid substitution mutants, TRAstAK_{264}L had the most affected sulfate uptake rate and velocity of transportation (V_{max}). Its growth on solid minimal media was also significantly affected after 48 h, however, in a longer time TRAstAK_{260}L reached growth rate similar to that of the wild type TRAstAWT strain. None of the K_{263} substitutions studied here altered K_{r}, indicating that this lysine 262 is not involved in direct interaction with the sulfate.

The quadruple TRAstAK_{260-264}A mutant with all four lysine residues substituted, exhibited the most visibly altered substrate transportation, which could also be observed as a decreased selenate resistance on a minimal solid medium. This result suggests that negatively charged lysine residues in wild type CCL of AstA are necessary for the maximum efficiency of the transporter. However, the quadruple K_{260-264}A mutation in CCL did not abolish AstA function completely, as opposed to mutations in CCL of C. albicans Mdr1 multidrug transporter (Mandal et al., 2012). Shortening of CCL in Mdr1 eliminated the drug resistance, suggesting an important
role of the loop for the functioning of this transporter. Since insertional mutagenesis restoring the loop length did not result in a functional transporter, one can infer that the amino acid composition of CCL is more relevant to substrate transport than its mere length.

Altogether, our results indicate that CCL is not essential for the function of the entire transporter, but seems to be engaged in controlling of AstA activity. Interestingly, it has been found earlier that CCL of the E. coli lactose permease is involved in allosteric regulation by the glucose-specific enzyme IIA of the phosphoenolpyruvate: sugar phosphotransferase system (Hoischen et al., 1996, Seok et al., 1997). Similarly, CCL in the human GLUT1 transporter mediates ATP-dependent inhibition of glucose transport (Blodgett et al., 2007). In similar way, the AstA CCL could interact with the sulfate and form an allosteric site controlling the transporter’s activity. Since AstA is ATP-independent, as we have shown (Fig. 6), fungi bearing this gene may have a higher evolutionary fitness under the low oxygen conditions, when ATP synthesis is limited, for instance in a situation of evolutionary fitness under the low oxygen conditions, when ATP synthesis is limited, for instance in a situation of...
contrast to *E. coli* LacY, the fungal AstA mutants: single substituted K260, K264 or quadruple K260,264A led to a competitive inhibition of sulfate transportation by altering $K_v$.

Since the lysine substitutions in CCL resulted in the altered sulfate transportation constants ($K_v$) in the AstA transporters, it seems likely that CCL limits the AstA affinity for substrate under the conditions of high intracellular sulfate concentration. Consequently, the substitutions of the lysine residues in CCL could abolish that allosteric regulation, resulting in an altered sulfate affinity of the mutated AstA variants. The most pronounced shift in $K_v$ was observed for quadruple TRAstAK260,264A mutant, which was also susceptible to selenate, a toxic analog of sulfate. Since an alteration of a substrate binding constant usually refers to a competitive inhibition of the enzyme binding site (Strelow et al., 2012), our results indicate that CCL indeed interacts with the sulfate. Whether the proposed binding of sulfate by CCL would be assisted by interaction with other proteins, as it is in the case of the *E. coli* lactose permease, remains an open question.

The observed physiological effects of the mutations affecting CCL could be alternatively explained, if one assumed that CCL formed a proofreading centre (or a checkpoint), controlling the parameters of the incoming substrate. Such possibility is supported by our observation that the $K_m$ of AstA, $K_{mL}$, $K_{mA}$ and $K_{mL}$ mutants of AstA with decreased $K_v$ (Table 2) were more resistant to selenate (Fig. 5), suggesting that these mutations could affect interaction with selenate rather than with the sulfate. Hence, CCL could be a flexible goalkeeper of the AstA transporter, forming a transient substrate-interactive pocket, acting as a checkpoint for the molecules entering the cell.

Numerous biochemical studies characterizing the MFS transporters were focused on substrate translocation pore, located within TM helices. However, $K_v$ or $K_m$ values are still poorly studied, and CCL has not been well investigated. The mutations introduced in substrate binding site of MFS transporters usually lead to a pronounced 10-fold $K_m$ alteration (Will et al., 1998; Sahin-Töth et al., 1999). Among some previously described enzymes, S-1,2-propanediol oxidoreductase (FucO) alters its substrate specificity substantially upon just a single point mutation $K^V_259$ in the substrate-binding site, while its $K_v$ increases just two-fold (Blikstad et al., 2014). In case of the higher eukaryotes, some mutated enzymes, it was shown that human galactokinase, point-mutated in its substrate-binding site (AstA), has $K_m$ only weakly altered (30% decrease) and its kinetic parameters are almost comparable to the wild type (Timson & Reece, 2003). However, the $A_v$ mutation in galactokinase is severely manifested on the cellular and organism level, with a clinical phenotype of a tendency to develop cataracts (Okano et al., 2001). Since there is no data referring to kinetic parameters of eukaryotic CCL, it was not possible to compare our results to published values.

Crystal structures of the bacterial XyIE transporter revealed that CCL can act as a structural linker between the N- and C-terminal domains of MFS proteins, transiently forming a latch-like structure (Wischedehausi et al., 2014). Till now, CCL was regarded as a structural linker only, but we have shown that this loop can affect the substrate uptake. Considering that some mutations located within the linker resulted in the significant $K_v$ shifts, CCL may also participate in substrate recognition. Summarizing, the presented results suggest that the four lysine residues located within the CCL linker region are not necessary for the sulfate transport, but they select for the incoming substrate and facilitate its uptake.

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