Deactivation of the autotrophic sulfate assimilation pathway substantially reduces high-level \( \beta \)-lactam antibiotic biosynthesis and arthrospore formation in a production strain from \textit{Acremonium chrysogenum}

Dominik Terfehr and Ulrich Kück*

Abstract

The filamentous ascomycete \textit{Acremonium chrysogenum} is the only industrial producer of the \( \beta \)-lactam antibiotic cephalosporin C. Synthesis of all \( \beta \)-lactam antibiotics starts with the three amino acids \( \text{L-\alpha-aminoadipic acid, L-cysteine and L-valine} \) condensing to form the \( \delta-(\text{L-\alpha-aminoadipyl-L-cysteinyl-D-valine)} \) tripeptide. The availability of building blocks is essential in every biosynthetic process and is therefore one of the most important parameters required for optimal biosynthetic production. Synthesis of L-cysteine is feasible by various biosynthetic pathways in all eusacomyctes, and sequencing of the \textit{Acr. chrysogenum} genome has shown that a full set of sulfur-metabolizing genes is present. In principle, two pathways are effective: an autotrophic one, where the sulfur atom is taken from assimilated sulfide to synthesize either L-cysteine or L-homocysteine, and a reverse transsulfuration pathway, where L-methionine is the sulfur donor. Previous research with production strains has focused on reverse transsulfuration, and concluded that both L-methionine and reverse transsulfuration are essential for high-level cephalosporin C synthesis. Here, we conducted molecular genetic analysis with A3/2, another production strain, to investigate the autotrophic pathway. Strains lacking either cysteine synthase or homocysteine synthase, enzymes of the autotrophic pathway, are still autotrophic for sulfur. However, deletion of both genes results in sulfur amino acid auxotrophic mutants exhibiting delayed biomass production and drastically reduced cephalosporin C synthesis. Furthermore, both single- and double-deletion strains are more sensitive to oxidative stress and form fewer arthrospores. Our findings provide evidence that autotrophic sulfur assimilation is essential for growth and cephalosporin C biosynthesis in production strain A3/2 from \textit{Acr. chrysogenum}.

INTRODUCTION

In the first half of the last century, the discovery of \( \beta \)-lactam antibiotics revolutionized the treatment of bacterial infections, and today these antibiotics constitute the largest proportion of drugs used in human and veterinary medicine [1]. \( \beta \)-lactam antibiotics are synthesized by bacteria and filamentous fungi and are characterized by a four-membered lactam, which is also called the \( \beta \)-lactam ring. Despite the fact that \( \beta \)-lactam-antibiotic-producing microbes are not taxonomically related, the first two steps of \( \beta \)-lactam antibiotic synthesis are identical in all investigated organisms (for a review see [2]). In the first step, catalysed by the \( \delta-(\text{L-\alpha-aminoadipyl-L-cysteinyl-D-valine)} \) synthetase, the two amino acids L-cysteine and L-valine, as well as \( \alpha \)-aminoadipic acid, which is a precursor of L-lysine, are condensed. Subsequently the product of this reaction, the \( \delta-(\text{L-\alpha-aminoadipyl-L-cysteinyl-D-valine)} \) tripeptide, is converted into the bicyclic intermediate isopenicillin N by isopenicillin N synthase. The three amino acids are essential for product formation.

In filamentous fungi, different biosynthetic routes generate the sulfur-containing amino acid L-cysteine (Fig. 1). One biosynthetic route is the autotrophic pathway, where inorganic sulfate is taken up from the extracellular medium and further assimilated to sulfide, which is used to convert O-acetylserine to L-cysteine. An alternative route is reverse transsulfuration, originating from L-methionine, the second sulfur-containing proteinogenic amino acid, which can be converted into S-adenosylmethionine, S-adenosylhomocysteine, L-homocysteine and L-cystathionine to finally form L-cysteine. In addition to these two pathways, many fungi possess a third option to form L-cysteine. In this alternative route, the assimilated sulfide is not used to convert O-acetylserine to L-cysteine, but to...
convert O-acetylhomoserine to L-homocysteine, which can be used to form L-cysteine through the final steps of the reverse transsulfuration pathway.

So far, the preferred pathways to synthesize L-cysteine can vary in fungi and seem to be organism-specific. For example, the well-studied yeast *Saccharomyces cerevisiae* only possesses the ability to use the assimilated sulfide for L-homocysteine production, which is then further converted to L-cysteine [3, 4]. *Penicillium chrysogenum*, the main producer of the β-lactam antibiotic penicillin, mainly synthesizes the required L-cysteine through sulfate assimilation and condensation with O-acetylserine [5].

*Acremonium chrysogenum*, the producer of the broad-spectrum antibiotic cephalosporin C, can use all three possible pathways to metabolize sulfur-containing amino acids. However, reverse transsulfuration was thought to be the major route supplying L-cysteine for cephalosporin biosynthesis [6–8]. Therefore, applied microbial research has previously focused on the reverse transsulfuration pathway, and L-methionine was assigned an important role in L-cysteine synthesis (reviewed in [9]).

Here, we asked how sulfate assimilation and subsequent synthesis of either L-cysteine or L-homocysteine affect cephalosporin C production in production strain A3/2. For this purpose, we generated strains deficient in the condensation of sulfide with O-acetylserine, O-acetylhomoserine or both, and analysed the impact on primary and secondary metabolism. Our investigation demonstrated that production strain A3/2 uses sulfate for biosynthesis of L-cysteine, a precursor amino acid for cephalosporin C production.

**METHODS**

**Strains and culture conditions**

Recombinant plasmids were generated using either standard laboratory techniques [10] or the Infusion Advantage PCR cloning kit (Clontech) following the manufacturer’s instructions. *Escherichia coli* strain XL1-Blue MRF’ was used as the host for general plasmid construction and maintenance [11]. Plasmid construction via homologous recombination was performed using *Sacch. cerevisiae* strain P69-4A as described previously [12]. Yeast cells were transformed by electroporation according to the method of Becker and Lundblad [13] in a multiporator (Eppendorf) at 1.5 kV.

Liquid cultures of *Acr. chrysogenum* strains were grown at 27 °C and 180 r.p.m. for 3–5 days as previously described in rich medium (CCM) [14]. Strains on either solid CCM medium or minimal medium (MM) [15] were grown at 27 °C and in constant light. Transformation of *Acr. chrysogenum* was performed according to conventional procedures [16, 17]. The resulting transformants were selected on media containing either nourseothricin or hygromycin B at concentrations previously reported [18]. Transformants from complementation experiments were selected for prototrophy on MM. The *Acr. chrysogenum* strains used in this study are listed in Table 1.

**Construction of plasmids and strains**

All of the plasmids and oligonucleotides used in this study are listed in Tables 2 and S1 (available in the online Supplementary Material), respectively. To generate the ΔAccysD strain, the AccysD open reading frame was replaced by a hygromycin B (hph) resistance cassette under control of the trpC promoter.

**Fig. 1.** Simplified overview of predicted sulfur biosynthetic routes for sulfur metabolism in *Acr. chrysogenum*. Sulfur amino acids can be synthesized in different ways in filamentous fungi. Within the autotrophic pathway (blue arrows) inorganic sulfate is taken up, assimilated and finally used to synthesize either L-cysteine or L-homocysteine. Another option is the reverse transsulfuration pathway (brown arrow) in combination with the transmethylation pathway (red arrow). During transmethylation, L-methionine is converted through S-adenosylmethionine (Ado-Met) and S-adenosylhomocysteine (Ado-Hcy) to L-homocysteine. Subsequently L-homocysteine can be converted into cystathionine to finally generate L-cysteine by reverse transsulfuration. Starting from L-cysteine, L-methionine is generated by the transsulfuration pathway (grey arrow). (Adapted from [47].)

<table>
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<tr>
<th>Strains</th>
<th>Relevant genotypes</th>
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<tbody>
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<td>ΔAcss70 FRT2</td>
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<td>ΔAccysB</td>
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| Table 1. *Acr. chrysogenum* strains used in this investigation |
|------------------|------------------|------------------|
| Strains          | Relevant genotypes | Source |
| ΔAcss70 FRT2     | ΔAcss70::FRT     | [19] |
| ΔAccysB          | ΔAccysB::natI    | This study |
| ΔAccysD          | ΔAccysD::hph     | This study |
| ΔAccysB ΔAccysD  | ΔAccysB::natI; ΔAccysD::hph | This study |
| ΔAccysB          | ΔAccysB::AccysD::hph | This study |
| ΔAccysD          | ΔAccysD::AccysB::hph | This study |
| ΔAccysB          | ΔAccysB::natI; ΔAccysD::hph | This study |
| ΔAccysD ΔAccysB  | ΔAccysD::AccysB::hph | This study |
from *Aspergillus nidulans*. The AccysD deletion vector was constructed using yeast recombination. Therefore, *EcoRI/XhoI* linearized plasmid pRS426 was transformed together with AccysD flanking regions amplified using AccysD_5-fw/AccysD_5-rv and AccysD_3-fw/AccysD_3-rv and a hygromycin B resistance cassette cut out from plasmid pSF27-34 using *EcoRI*. For homologous recombination in *Acr. chrysogenum*, the split-marker technology was used as described previously [19] using AccysD_5-fw/hph-split-5’-rv and hph-split-3’-fw/AccysD_3-rv to amplify the overlapping fragments for transformation of *Acr. chrysogenum*. ΔAccysB strains were generated by replacing the AccysB open reading frame by a nourseothricin (*nat1*) resistance cassette driven by the *trpC* promoter from *Asp. nidulans*. The AccysB deletion constructs were assembled using the Infusion Advantage PCR cloning kit. For this purpose, AccysB flanking regions were amplified using AccysB_5-fw/AccysB_5-rv and AccysB_3-fw/AccysB_3-rv and used together with plasmid pDNA1 cut with *HindIII* for integration of the 5’ flanking region and with *BamHI* for integration of the 5’ flanking region for Infusion reaction. Split-marker fragments for the transformation of *Acr. chrysogenum* were amplified using AccysB_5-fw/nat-split-rv and nat-split-fw/AccysB_3-rv. Double-deletion strains (ΔAccysB ΔAccysD) were generated by replacing the AccysB open reading frame by a *nat1* resistance cassette driven by the *trpC* promoter from *Asp. nidulans* in a ΔAccysD background. All constructed plasmids were verified via Sanger sequencing. Complementation of AccysB and AccysD deletions was achieved by exchanging the respective resistance cassettes with the wild-type open reading frames. The DNA fragments used for complementation were amplified from Aku70 FRT2 gDNA using oligonucleotides AccysB_5-fw/AccysB_5-rv and AccysB_fw/AccysB_3-rv for AccysB complementation and oligonucleotides AccysD_5-fw/AccysD_5-rv and AccysD-fw/AccysD_3-rv for AccysD complementation. In this context, the DNA fragments used overlap within the AccysB or AccysD instead of within a resistance cassette. Double-deletion strains were transformed with these fragments and selected for prototrophy on MM.

### Preparation of nucleic acids and hybridization protocols

DNA extraction and Southern blot analysis methods were performed as described previously [20]. Hybridization of Southern blots was done as described previously using 32P-labelled DNA probes [10].

### Analysis of cephalosporin C and biomass production

Strains were grown for 72 to 120 h at 27°C and 180 r.p.m. in liquid CCM medium. The qualitative cephalosporin bioassay was performed as described previously [21], with minor modifications. After harvesting, supernatants were used for the cephalosporin C bioassay, with *Alcaligenes faecalis* as the indicator organism. The obtained mycelia were used to measure the dry weight. All experiments were performed in triplicate and Student’s t-test was applied to analyse significant differences.

### Growth assays

Cell suspensions were generated for all strains with 100 mg mycelium per ml. For all tests, 2 µl of these suspensions was dropped on the corresponding plate and incubated for 7 d at 27°C. For oxidative stress tests, we used CCM with 0.005, 0.01, 0.02, 0.03, 0.04 and 0.05% H2O2. Dimethyl-diselenide (DMDSe) stress tests were performed as described by Brzywczy and Paszewski [22], with minor modifications. Equivalent DMDSe concentrations of 1, 2 and 5µM were used and strains were cultivated for 7 d at RT. Auxotrophy growth tests were performed on MM either without or with addition of 2 µM L-cysteine or L-methionine.

### Microscopic analysis and quantification of arthrospore formation

Hyphal morphology was analysed using an Axiophot microscope (Zeiss) at different time points (72–144 h). For microscopic quantification, we took 100 µl aliquots from each 100 ml liquid medium. Arthrospores were counted in appropriate dilutions in triplicate using a Thoma cell-counting chamber. Images were captured with an Axiom digital imaging system using MetaMorph software (version 7.7.5.0; Universal Imaging) in combination with a Photometrix Cool SnapHQ camera (Roper Scientific) and were processed with Adobe Photoshop CS6 software.

### Calculation of expression levels

Read counts were calculated using R [23] and summarize Overlaps from the GenomicAlignments package [24] in union mode and were normalized using DEseq2 [25]. Expression levels are given as normalized read counts per 1 kb coding sequence (CDS) length.
RESULTS

In silico reconstruction of sulfur metabolism in Acr. chrysogenum

Previously, the sulfur metabolism from filamentous fungi was characterized biophysically and biochemically by a number of investigators (reviewed in [9]). At the molecular level, so far only mecB, encoding a cystathionine-γ-lyase, has been characterized using targeted gene deletion by homologous recombination in Acr. chrysogenum [26]. This enzyme is responsible for the last step of the reverse transsulfuration pathway, namely the conversion of L-cystathionine to L-cysteine.

Based on the available genome sequence of Acr. chrysogenum, we searched for other genes predicted to be involved in sulfur metabolism [27]. We identified at least 23 genes for proteins putatively involved in the three predicted pathways involved in sulfur metabolism and calculated the relative expression levels based on RNA-seq data (Fig. S1). Among these, we identified cysteine synthases as well as a homocysteine synthase, which led us to hypothesize that Acr. chrysogenum possesses the enzymes to incorporate sulfide into O-acetylserine or O-acetylhomoserine to form L-cysteine or L-homocysteine. A cysteine synthase is encoded by ACRE_002155 (AccysB), a 1183 bp long gene with one 70 bp intron near the 5’ end. The encoded protein is predicted to be 370 amino acids long, with 83 % identity to the already-described cysteine synthase from Asp. nidulans and 87 % identity to the predicted cysteine synthase Cys-17 from Neurospora crassa [28]. The predicted homocysteine synthase is encoded by ACRE_045250 (AccysD), a 1750 bp gene, carrying five introns with lengths ranging from 61 to 151 bp. It encodes a 433-amino-acid protein with 83 % identity to the known orthologue from Asp. nidulans [29]. Gene models from both genes were verified using RNA-seq data (Figs S2 and S3).

Deletion and complementation of cysteine and homocysteine synthase-encoding genes

To verify that the two assimilation pathways are active in Acr. chrysogenum and investigate how they affect β-lactam synthesis, both genes were deleted in a parent A3/2 strain (ΔAccu70 FRT2) using the split-marker approach [19]. Furthermore, we used a ΔAccysD strain as a recipient for an additional AccysB deletion to obtain a double mutant deficient for both sulfide incorporation routes. Complementation of either gene deletion was achieved by reintegrating the wild-type genes, flanked by their native genomic regions, as confirmed by growth tests on selection media containing hygromycin B and/or nourseothricin (Fig. 2). The single-deletion strains could only grow on media containing either hygromycin B (ΔAccysD) or nourseothricin (ΔAccysB), while the double-deletion strain was able to grow on media containing both. The single complementation strains restored the wild-type antibiotic sensitivity of the complemented gene and resembled the reciprocal single-deletion strains. Sulfur amino acid auxotrophy of the double-deletion strains, as discussed below, was used to select complementation strains. Two independent strains from every gene disruption and complementation experiment were chosen for further analysis. In addition, all deletion and complementation strains were characterized by PCR and Southern blot analysis (Fig. S4).

ΔAccysD strains are resistant to dimethylselenide

DMDSe is a volatile selenium species that is reduced in vivo to methylselenide. This selenide is converted by homocysteine synthase into the toxic compound selenomethionine, providing a means to select homocysteine synthase-deficient strains in various yeast species [22]. To prove our hypothesis that AccysD encodes an active homocysteine synthase, we grew the parent and deletion strains on media with increasing concentrations of DMDSe (0–5 µM). DMDSe inhibited the growth of the parent strains, ΔAccysB and ΔAccysB ΔAccysD :: AccysD, whereas all homocysteine synthase-deficient strains were able to grow under all conditions (Fig. 3).

Double mutants are auxotrophic for sulfur amino acids

Sulfur amino acids are essential for every organism, since they play important roles in cellular processes, such as the redox cycle, stress response and enzymatic reactions, and they are also important for protein structure and folding. To check whether deletions of either AccysB or AccysD, or the deletion of both AccysB and AccysD, influence primary metabolism, we conducted growth tests on minimal and rich media. While sulfate is the only sulfur source in minimal medium, rich medium also contains complex sources such as yeast and...
meat extract, which in addition to sulfate supply a wide range of sulfur sources. All strains tested were able to grow on rich medium, while only the parent strain and the single-deletion strains \( \text{AccysB} \) and \( \text{AccysD} \) were able to grow on minimal medium (MM) (Fig. 4). MM supplemented with either \( \text{L-cysteine} \) or \( \text{L-methionine} \) restored growth of the double-deletion strains. Complementation of double-deletion strains with either \( \text{AccysB} \) or \( \text{AccysD} \) resulted in growth on minimal medium and therefore restored sulfate metabolism.

**Shut-down of the autotrophic pathway leads to delayed growth and drastically reduced cephalosporin C production**

To better understand the growth changes in single- and double-deletion strains we performed time series experiments with rich medium in shaking cultures (Fig. 5). The parent strain \( \Delta \text{Acku70 FRT2} \) generated a biomass of 646 mg (±25) after 3 d growth. While biomass production of the \( \text{AccysB} \)-deficient strain (552±40 mg) and the \( \Delta \text{AccysD} \) strain (689 ±12 mg) was not significantly altered, biomass production of the double-deletion strain was significantly reduced (\( P<0.005 \)), with a biomass of 310 mg (±18). Complementation of the double-deletion strain by reintroduction of either \( \text{AccysB} \) (642 ±30 mg) or \( \text{AccysD} \) (543±25 mg) restored the parent-strain phenotype. After 5 d inoculation all analysed strains had a biomass of about 400 mg (\( \Delta \text{Acku70 FRT2} \) 381±35; \( \Delta \text{AccysB} \) 416 ±19; \( \Delta \text{AccysD} \) 434±23; \( \Delta \text{AccysB} \Delta \text{AccysD} \) 362±11; \( \Delta \text{AccysB} \Delta \text{AccysD} \Delta \text{AccysD} \) 463±21; \( \Delta \text{AccysB} \Delta \text{AccysD} \Delta \text{AccysB} \) 370 ±46) and showed no significant alterations.

Since sulfur metabolism supplies \( \text{L-cysteine} \) for cephalosporin C synthesis, we were interested in the impact of single and double deletions on cephalosporin C production (Fig. 6). The single-deletion strains showed no significant alteration in antibiotic production compared to the parent strain after 72 h of cultivation, as determined by halo assays. The culture supernatant from the parent strain produced a halo area of 3.2 cm\(^2\) (±0.16) per gram biomass. With halo areas per gram dry weight of 3.4 cm\(^2\) (±0.36) and 2.7 cm\(^2\) (±0.01), the \( \Delta \text{AccysB} \) and \( \Delta \text{AccysD} \) strains were not significantly different from the parent strain. However, the supernatant from the double-deletion strain, with a halo area of 0.9 cm\(^2\) (±0.23) per gram, produced a significantly reduced (\( P<0.01 \)) amount of antibiologically active compounds. This reduction could be restored by reintroduction of one of the genes. The culture supernatant from the \( \Delta \text{AccysB} \Delta \text{AccysD} \Delta \text{AccysD} \) strain produced a halo area of 2.9 cm\(^2\) (±0.27) per gram and the \( \Delta \text{AccysB} \Delta \text{AccysD} \):
AccysB strain supernatant produced a halo area of 3.0 cm² (±0.37) per gram.

The differences between the double-deletion strain and all other analysed strains were even more apparent 120 h after inoculation. While the supernatants from the parent, single-deletion and complementation strains produced similar halo areas of between 4 and 5 cm² (Accu70 FRT2 5.2±0.75; ΔAccysB 4.9±0.35; ΔAccysD ΔAccysD :: AccysD 4.2±0.14; ΔAccysB ΔAccysD :: AccysB 3.8±0.58) per gram, the supernatant from the double-deletion strain produced a halo area of only 0.5 cm² (±0.08) per gram, significantly (P<0.01) smaller than the halo produced by all the other strains. Reintroduction of either AccysB or AccysD into the double deletion restored the parental phenotype.

**Single and double deletion of AccysB and AccysD has severe effects on oxidative stress tolerance**

A large proportion of synthesized L-cysteine is stored as glutathione to maintain the redox state of the cells. Since glutathione is also an important antioxidant, we were interested in how single- and double-deletion strains respond to H₂O₂. Therefore, we conducted stress tests on rich medium plates containing zero to 0.02 % H₂O₂ (Fig. 7). Whereas the parent strain was able to grow under all conditions, ΔAccysB and, interestingly (see the Discussion), the double-deletion strains showed a slight reduction in H₂O₂ tolerance, and were only able to grow on medium containing up to 0.03 % hydrogen peroxide. In contrast, the single-deletion strain ΔAccysD showed a more severe reduction in oxidative stress tolerance, and growth was only observed on plates containing up to 0.01 % H₂O₂. Complementation of AccysB or AccysD in the double-deletion mutant derivatives led to similar phenotypes to the reciprocal single-deletion strains. The supplementation of either L-cysteine or L-methionine in concentrations ranging from 2 mM to 10 mM did not alter stress tolerance of the ΔAccysB and double-deletion strains, while stress tolerance of ΔAccysD was raised. ΔAccysD supplemented with 10 mM L-cysteine can grow on plates containing up to 0.02 % H₂O₂ and supplementation of 10 mM L-methionine enables growth on plates containing up to 0.03 % H₂O₂ (Fig. S5).

**Arthrospore formation is affected by autotrophic sulfur assimilation**

High-level biosynthesis of cephalosporin C and differentiation into arthrospores are thought to be connected. Therefore, we microscopically analysed the parent, single-deletion, double-deletion and complementation strains after 72, 120 and 144 h cultivation in rich medium (Fig. 8a). After 72 h of growth, all strains showed hyphal growth and only few arthrospores. The parental, single-deletion and complementation strains generated about 3×10⁶ arthrospores ml⁻¹, while the double-deletion mutant showed only 7×10⁵ arthrospores ml⁻¹ (Fig. 8b). One hundred and twenty hours after inoculation the parental strain formed swollen hyphae, which subsequently disintegrated into free arthrospores (6.9×10⁷ arthrospores ml⁻¹). With a smaller number of arthrospores,
**DISCUSSION**

**Sulfur metabolism in Acr. chrysogenum is comparable to other euascomycetes**

Sulfur metabolism is diverse across fungal species. In the well-studied yeast *Sacch. cerevisiae*, the autotrophic pathway ends with the incorporation of sulfide into *L*-homocysteine to form *L*-homocysteine [30]. This has to be interconverted via reverse transsulfuration to form *L*-cysteine. In contrast, *Hansem. polymorpha* only has enzymes to condense sulfide with *O*-acetylserine to form *L*-cysteine and the other sulfur amino acids are synthesized through transsulfuration [31]. However, other yeasts, such as the fission yeast *Schiz. pombe*, are able to condense sulfide with both *O*-acetylserine and *O*-acylamino- 

*Fig. 6.* Analysis of antibiotic production. Strains were grown for 72 to 120 h in shaking cultures. Culture supernatants from strains grown in the time-series experiments were used for halo assays to determine the titre of antibacterial active compounds. The measured halo areas were normalized to the dry weights obtained. Representative values from one of two independent strains for each deletion and complementation experiment are shown. Mean values and standard deviations are derived from three independent experiments and Student’s *t*-test was applied to analyse significant differences. Double-deletion strains (dark grey bars) show a significantly reduced (*P*<0.01) halo/dry weight compared to all other strains (parent strain ∆Acku70 FRT2, black bars; single-deletion strains AccysB and AccysD, light grey). Complementation strains derived from the double-deletion strain are shown as white bars next to the corresponding single-deletion strain.

**∆**AccysB (4.2×10⁷ arthrospores ml⁻¹) and **∆**AccysD (5.3×10⁷ arthrospores ml⁻¹) mostly exhibited intact hyphae. This reduction of arthrospore formation was even more severe in the double-mutant **∆**AccysB **∆**AccysD (3.3×10⁸ arthrospores ml⁻¹). When we used the double mutant for complementation experiments, we found that the number of arthrospores resembled the phenotype of the corresponding single mutants. The same tendencies were observed when we quantified arthrospores in 144 h cultures. For example, the double-deletion strain mainly exhibited intact long hyphae and had only a few swollen cells and arthrospores (5.6×10⁸ arthrospores ml⁻¹) (Fig. 8b). The drastically reduced forma- 

**AccysB and AccysD encode the main sulfur-incorporating enzymes**

Publicly available genome sequences facilitate functional genomic approaches to provide deeper insights into various metabolic pathways. *Acr. chrysogenum* strains that are deficient in one of the sulfur-incorporating enzymes show only
slight alterations in the growth and production of secondary metabolites. This led us to assume that these pathways are redundant: when one sulfide incorporation pathway is non-functional, this is compensated by the alternative route. This also applies to *A. nidulans*, although the pathway using homocysteine synthase is described as a less efficient alternative pathway [35]. Indeed, in *N. crassa* and *A. nidulans*, the incorporation of sulfide into O-acetylserine is considered to be the favoured mode to produce sulfur amino acids based on the autotrophic pathway [36, 37].

Using DMDSe, we showed that AccysD encodes the only active homocysteine synthase within the genome of *Acr. chrysogenum* and that AccysD is active under normal growth conditions. Based on these data and our findings that AccysB AccysD double-deletion strains are strict auxotrophs for sulfur amino acids, we concluded that AccysB is the only cysteine synthase that is active under the conditions used in our investigation. Our findings resemble the results for *A. nidulans*, where the double-deletion strains of the orthologues cysB and cysD are also tight auxotrophs for sulfur amino acids [38].

**Autotrophic pathway is important for primary and secondary metabolism**

The supply of sulfur derived from sulfate is prevented in the AccysB AccysD double-deletion strains, which leads to delayed growth and a strongly reduced antibiotic titre. Sulfur amino acids are the only usable sulfur source left for the double-deletion strains and these substrates have to supply sulfur for primary as well as secondary metabolism. Since the double-deletion strains show reduced biomass production after 72 h of growth, this indicates that primary metabolism is affected by the reduced availability of sulfur. Thus, the autotrophic pathway is necessary for optimal growth, although alternative sulfur sources in the rich medium can also be used.

Secondary metabolism is more severely affected by the reduced availability of sulfur. Even 120 h after of inoculation, when the biomass levels of the ΔAccysB ΔAccysD strains are comparable to those of the parent strain, the synthesis of active antibiotic compounds is drastically reduced. Under these conditions, cephalosporin C synthesis seems to depend strongly on the autotrophic pathway. This conflicts with a recent report using production strain C10 (ATCC 48278). There, it was suggested that L-methionine as well as the reverse transsulfuration pathway are essential for high-level cephalosporin C production in *Acr. chrysogenum* [26]. However, our results with A3/2 indicate that this strain depends almost completely on sulfate. Our findings are consistent with a previous publication that showed that L-methionine is less important for cephalosporin biosynthesis in A3/2 [39]. Mainly using sulfate and using only a small amount of L-methionine would be favourable for large-scale fermentation, since sulfate is a much cheaper component than methionine. Thus, both production strains seem to differ in their preference for the biosynthesis of L-cysteine for cephalosporin C production and therefore results should be generalized carefully when production strains from different strain improvement pipelines are compared.

**Autotrophic pathway-deficient strains are more sensitive to oxidative stress**

In oxidative stress tests, all of the deletion strains were less tolerant of stress. Microbes use different modes to prevent damage from reactive oxygen species (ROS). For example, glutathione is an important antioxidant, which can react with a wide spectrum of ROS, such as "OH, HOCl, RO", RO' 2 and $^1$O2, and is able to initiate detoxification [40]. This low-molecular-weight thiol is present in diverse biological systems and appears to be the most abundant [41, 42]. It is synthesized within the γ-glutamyl cycle and is basically built from L-glutamate, L-cysteine and glycine [43]. Besides its
important function as an antioxidant, glutathione is also important for the storage of intracellular L-cysteine, and different fungi use it as a sulfur source under depleted conditions [44, 45]. Consequently, impaired sulfur metabolism might lead to decreased L-cysteine pools, which will also lower the amount of available glutathione.

While ΔAccysB and paradoxically ΔAccysB ΔAccysD have slightly lowered tolerance against oxidative stress, deletion-mutant AccysD exhibits severely reduced oxidative stress tolerance. In general, L-cysteine levels are considered to be the sensor for the sulfur status of the cell [46]. Therefore, we hypothesize that ΔAccysB strains, where L-cysteine cannot be directly produced from O-acetylserine and sulfide, sense sulfur starvation and thus activate alternative routes to generate sulfur-containing amino acids. For example, this was demonstrated for a cysteine synthase mutant of Asp. nidulans, which also showed reduced growth under oxidative stress conditions [47]. In this Asp. nidulans mutant, genes encoding homocysteine synthase as well as other enzymes

**Fig. 8.** Microscopic analysis and quantification of arthrospore formation. (a) Microscopic analysis after 72, 120 and 144 h of growth. Arrows indicate the formation of arthrospores. (b) Quantification of arthrospore formation. The number of arthrospores produced by the analysed strains is illustrated as follows: parental strain ΔAcku70 FRT2, black bars; single-deletion strains AccysB and AccysD, light grey bars; double-deletion strain, dark grey bars; complementation strains derived from the double-deletion strains, white bars. One representative strain for each deletion and complementation experiment is shown. Mean values and standard deviations are derived from three independent experiments.
involved in reverse transsulfuration are overexpressed in order to convert homocysteine to cysteine [48].

So far, the ΔAccysD phenotype described here has not been reported for other euascomycetes. We propose that the strong reduction in stress tolerance is a result of not sensing sulfur depletion in the cell. In this deletion strain, incorporated and assimilated inorganic sulfate can only be used by cysteine synthase to form sulfur amino acids. Using all available sulfide to synthesize l-cysteine might lead to elevated cysteine levels, which prevent the sensing of sulfur depletion. Therefore, alternative sulfur metabolism is not activated, as in the ΔAccysB strains, and the lowered overall amount of sulfur amino acids might also lead to a decreased glutathione pool and thus a more severe stress phenotype.

Interestingly, the AccysB AccysD double-deletion strains show an oxidative stress tolerance that is comparable to that of the parent strain. In this case, sulfate cannot be used to synthesize sulfur amino acids, and thus corresponding cellular levels are low. Therefore, sulfur starvation leads to the activation of sulfur metabolism and available sulfur sources are used to produce sulfur amino acids. Furthermore, the accumulation of sulfide from sulfate assimilation might protect the cells from oxidative stress to a certain extent, since sulfide is a known reducing agent. In addition to this, expression changes in the oxidoreductase-encoding genes might also contribute to the observed phenotype. A similar link was shown in a recent report in which the deletion of the autophagy-related gene Acatg1 led to the induction of genes encoding a glutathione peroxidase (Acglp), a glutathione reductase (AcgrA), a peroxidase (Acper) and a thioredoxin reductase (Actra) [49]. This induction led to elevated oxidative stress tolerance for the analysed strain.

While the supplementation of 2 mM l-cysteine or l-methionine was sufficient to restore growth on minimal medium and to partially restore arthrospore formation for the double-deletion strains, the stress tolerance was not increased by the addition of 2–10 mM of these sulfur amino acids in the ΔAccysB and double-deletion strains. We hypothesize that the incorporation rates of l-cysteine and l-methionine might not be sufficient to raise the intracellular amino acid pools required for oxidative stress tolerance. In contrast to the unaltered stress tolerance of the above-mentioned strains, the stress tolerance of ΔAccysD was increased by adding 10 mM l-methionine. This is consistent with the assumption discussed above that this strain has lower intracellular levels of sulfur amino acids compared to the other deletion strains.

**Deficiencies in autotrophic sulfur assimilation lead to morphological changes**

Single deletion of AccysB and AccysD, and double deletion of both AccysB and AccysD, lead to reduced fragmentation into arthrospores, swollen uni- or bicellular metabolically active cells enriched with lipid-containing vacuoles that result from the fragmentation of hyphae [50]. It has been reported that arthrospores exhibit a 40 % increase in cephalosporin C biosynthesis [51]. Arthrospore formation can be induced by the addition of methionine in Acr. chrysogenum and intracellular levels of l-methionine rise in advance of the main cephalosporin C production phase [51–53]. Homologues of the cysteine synthase AccysB are the main enzymes for l-cysteine synthesis in other filamentous fungi, and l-cysteine can be used to synthesize l-methionine through transsulfuration. We propose that the reduced supply of sulfur amino acids due to inactivation of the autotrophic pathway leads to lower intracellular concentrations of sulfur amino acids, perhaps negatively affecting arthrospore formation. This is supported by supplementation studies using 2 mM l-cysteine or l-methionine, in which arthrospore formation was partially restored in the double-deletion strain.

The observed differences in arthrospore formation between the two single-deletion strains can be explained by the fact that AccysB catalyses a key reaction in the main path of autotrophic sulfur assimilation, whereas AccysD catalyses a key reaction in the alternative route. In addition, deletion of AccysB can lead to l-cysteine depletion, promoting the conversion of available sulfur sources into l-cysteine, as indicated by transcriptomic data from Asp. nidulans [47]. Interestingly, arthrospore formation seems to be more sensitive to changes in sulfur metabolism than other analysed processes, such as biomass or cephalosporin C production.

Taken together, our results demonstrate the importance of autotrophic sulfur assimilation for growth and cephalospo- rin C production in strain A3/2. Our findings moderate the previous view that l-methionine and reverse transsulfuration are the essential pathways to generate cysteine for cephalosporin biosynthesis. Obviously, distinct industrial strain-improvement programmes generate diverse production strains that differ substantially in the mode they use to supply sulfur-containing amino acids for cephalosporin C biosynthesis. Better understanding of the favoured pathways can help to optimize production strategies, e.g. modulating sulfate and l-methionine amounts to improve cephalosporin C biosynthesis.

**Funding information**
The authors received no specific grant from any funding agency.

**Acknowledgements**
We thank Dr I. Zadra, Dr H. Kürnsteiner, Dr E. Friedlin and Dr T. Specht for their ongoing interest and support. The early work in this study was funded by Sandoz GmbH and the Christian Doppler Society. D. T. is grateful for a PhD scholarship from the Studienstiftung des Deutschen Volkes (German Academic Scholarship Foundation).

**Conflicts of interest**
The authors declare that there are no conflicts of interest.

**References**


Edited by: B. Gasser and V. J. Cid

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