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Tools for advanced and targeted genetic manipulation of the β-lactam antibiotic producer *Acremonium chrysogenum*

S. Bloemendal[a,b], D. Löper[a,b], D. Terfehr[a,b], K. Kopke[a,b], J. Kluge[a,b], I. Teichert[b], U. Kück[a,b,*]

[a] Christian Doppler Laboratory for Fungal Biotechnology, Universitätsstraße 150, 44780 Bochum, Germany
[b] Lehrstuhl für Allgemeine und Molekulare Botanik, Ruhr-Universität Bochum, Universitätsstraße 150, 44780 Bochum, Germany

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ABSTRACT

Acremonium chrysogenum is the major producer of the β-lactam antibiotic cephalosporin C and therefore of great importance for the pharmaceutical industry. However, this filamentous fungus is known to reproduce solely by asexual means, shows only sporadic conidiospore production, and has gradual fragmentation of the vegetative mycelium into arthrospores. Due to these peculiar growth characteristics and life style, strain improvement by recombinant technologies is much more challenging than for other biotechnologically relevant fungi. Here, we describe several molecular tools for genetic engineering of *A. chrysogenum*, including a Δ*Acku70* deletion strain for homologous recombination. No physiological or morphological changes occurred due to deletion of the *ku70* gene or integration of the *nat1* cassette in this recipient strain. We also used a xylose-inducible promoter from *Sordaria macrospora* (*Smxyl*) to demonstrate induction of the *gfp* reporter gene in *A. chrysogenum*. The *Smxyl* promoter was used for construction of a vector molecule to develop a one-step FLP/*FRT* recombination system in *A. chrysogenum*. This system was then used in the Δ*Acku70* deletion strain to construct a marker-free recipient strain for targeted DNA insertion into genomic DNA. The applicability of our tools was demonstrated by construction of a marker-free transgenic strain, lacking any foreign genes.

1. Introduction

The world's major anti-infective agents are β-lactam antibiotics, with an estimated world market of about 22 billion US$ for the dosage form (Demain, 2009). With a market share of 50%, cephalosporin C and its semisynthetic derivatives play an important role in the pharmaceutical industry. Like most β-lactam antibiotics, cephalosporin C is produced by fermentation and the filamentous fungus *Acremonium chrysogenum* is exclusively used for industrial production. This fungus was first isolated in 1948 from Sardinian coastal seawater and was shown to produce an antibiotic against gram-positive and gram-negative bacteria (Abraham et al., 1955; Newton and Abraham, 1955). Since then, strain improvement programs using several rounds of mutagenesis have resulted in industrial high production strains with highly elevated cephalosporin C titers compared to the wild-type strain (Elander, 2003).

The biotechnological relevance of cephalosporin C and, therefore, of the producer *A. chrysogenum*, makes the optimization of production processes a valuable goal for applied research; while, a deeper knowledge of the regulatory changes that lead to improvement of cephalosporin C production strains will be valuable for basic research. In filamentous fungi, secondary metabolism and morphogenesis are tightly connected processes, and several global regulators that are involved in both processes have been described. The most prominent is velvet (VeA), a component of a high molecular weight complex, which regulates both penicillin production and conidiosporogenesis in the two β-lactam producers *Aspergillus nidulans* and *Penicillium chrysogenum* (Bayram et al., 2008; Hoff et al., 2010a; Kopke et al., 2013; Stinnett et al., 2007). In *A. chrysogenum*, AcVEA, the homolog of VeA, controls the transcriptional expression of six cephalosporin C biosynthesis genes (Dreyer et al., 2007). Transcriptional expression data are consistent with Ac*veA* disruption strains having a strongly reduced cephalosporin C titer. In addition to its role in cephalosporin C biosynthesis, AcVEA is involved in hyphal fragmentation, which is an active developmental process in the cellular differentiation of *A. chrysogenum* and results in the formation of spherical cells, called arthrospores (Nash and Huber, 1971). Arthrospores show increased antibiotic production, underlining the relationship between secondary metabolism and morphology (Nash and Huber, 1971). Another important player

* Corresponding author at: Lehrstuhl für Allgemeine und Molekulare Botanik, Ruhr-Universität Bochum, ND7/131, Universitätsstraße 150, 44780 Bochum, Germany. Tel.: +49 234 3226212; fax: +49 2345 3214184.
E-mail address: ulrich.kueck@rub.de (U. Kück).

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Table 1
A. chrysogenum strains used in this study.

Strains	Relevant genotypes[a]	Source
CEM3/44	Producer strain, nat^s, hyg^s	Gsaller et al. (2013)
A3/2	Producer strain, nat^s, hyg^s	Radzio and Kück (1997)
Ac + *Smxyl – gfp*	CEM3/44, *Psmxyl::gfp::TtrpC*; *PtrpC::nat1*, nat^r	This work
Ac + *gpd – gfp*	CEM3/44, *Pgpd::gfp::TtrpC; PtrpC::nat1*, nat^r	This work
AcFRT1	A3/2, *FRT::PtrpC::nat1::FRT*, nat^r	This work
AcFRT2	A3/2, *PtrpC::Pcflp::TrpC*, *ptrA*, *FRT*, pyr^r, nat^s	This work
AcFlipFRT1	CEM3/44, *FRT::Psmxyl::Pcflp::PtrpC::hph::FRT*, hyg^r	This work
AcFlipFRT2	CEM3/44, *FRT*, hyg^s	This work
Δ*Acku70*	Δ*Acku70::PtrpC::nat1*, nat^r	This work
A3/2:nat1^{ect}	A3/2, *PtrpC::nat1* (ectopic integration), nat^r	This work
Δ*Acku70* FRT1	Δ*Acku70::FRT::Smxyl::Pcflp::PtrpC::hph::FRT*, *hyg*^{r}, nat^s	This work
Δ*Acku70* FRT2	Δ*Acku70::FRT*, hyg^s, nat^s	This work

[a] nat$^{s/r}$, nouresothricin-sensitive/-resistant; hyg$^{s/r}$, hygromycin B-sensitive/-resistant; pyr^r, pyrithiamin-resistant.

in secondary metabolism and morphogenesis in *A. chrysogenum* is the transcription factor CPCR1 (Schmitt and Kück, 2000), which not only regulates cephalosporin C biosynthesis, but also controls hyphal fragmentation (Hoff et al., 2005; Schmitt et al., 2004).

Despite the biotechnological importance of *A. chrysogenum* as the sole producer of cephalosporin C, molecular genetic analysis of this fungus is much more challenging than for other biotechnologically relevant fungal systems, such as *P. chrysogenum* or *Trichoderma reesei* (Kück and Hoff, 2010; Schuster and Schmoll, 2010). One reason is the lack of knowledge about a sexual life cycle and rare conidiospore production in *A. chrysogenum*. The fungus solely generates a vegetative mycelium that gradually fragments into arthrospores (Hoff et al., 2005; Nash and Huber, 1971). Furthermore, *A. chrysogenum* displays a very slow growth rate, reaching a colony size of only 8–15 mm after 10 days on malt extract medium (Onions and Brady, 1987). Another drawback is the lack of a freely accessible genome sequence for *A. chrysogenum*. These aspects, together with the limited resources available for the study of *A. chrysogenum*, delay the experimental progress that is easily accomplishable in other filamentous fungi. Therefore, targeted genetic engineering will not only lead to significant improvement in industrial cephalosporin C production, but also provide valuable insights into the biology of *A. chrysogenum*, allowing currently unforeseen applications. This might result in the identification of an *A. chrysogenum* sexual cycle, which would significantly facilitate both industrial and basic research, similar to other biotechnologically relevant fungi (Böhm et al., 2013; Seidl et al., 2009). Thus, the development of molecular tools, such as those already existing for other filamentous fungi (Kück and Hoff, 2010), is important for ensuring *A. chrysogenum* is amenable to current recombinant technologies and to better understand the physiology and development of this highly relevant β-lactam antibiotic producer.

Here, we describe the identification of a heterologous inducible xylose promoter for *A. chrysogenum* and its application to generate marker-free strains. Like many other filamentous fungi, *A. chrysogenum* has only a limited number of applicable selection markers, thereby hampering multiple gene deletions. We used this inducible promoter and adapted the FLP/*FRT* recombination system (Kopke et al., 2010) for *A. chrysogenum*. We further generated a *ku70* deletion strain to facilitate homologous recombination, an important prerequisite for targeted gene deletions (Kück and Hoff, 2010). Finally, the application of these newly established molecular tools resulted in the generation of a marker-free recipient strain that is suitable for the targeted integration of recombinant DNA. With these new tools in hand, analysis of the regulation of cephalosporin C biosynthesis and targeted strain improvement should be much more feasible for *A. chrysogenum*.

2. Materials and methods

2.1. Strains and culture conditions

Recombinant plasmids were generated using either standard laboratory techniques (Sambrook and Russell, 2001) or the In-fusion® Advantage PCR Cloning Kit (Clontech, USA) following the manufacturer' instructions with *Escherichia coli* strain XL1-Blue MRF′ as the host for general plasmid construction and maintenance (Bullock et al., 1987), or by homologous recombination in *Saccharomyces cerevisiae* strain PJ69-4A as described previously (Colot et al., 2006). Yeast cells were transformed by electroporation according to the method of Becker and Lundblad (1994) in a Multiporator (Eppendorf, Germany) at 1.5 kV.

All *A. chrysogenum* strains used in this study are listed in Table 1. Liquid cultures of *A. chrysogenum* strains were grown at 27 °C and 180 rpm for 3–5 days as previously described, in either complete culture medium (CCM) (Walz and Kück, 1991) or *A. chrysogenum* minimal medium (MM) (Gsaller et al., 2013) supplemented with either glucose or xylose. Transformation of *A. chrysogenum* was done according to conventional procedures (Radzio and Kück, 1997; Walz and Kück, 1993). Resulting transformants were selected on media containing either nourseothricin or hygromycin B at concentrations previously reported (Dreyer et al., 2007).

For expression analysis of xylanase genes, the *Sordaria macrospora* wild-type strain S48977 was grown as a surface culture for 2 or 5 days either on liquid cornmeal malt fructification medium (BMM) (Esser, 1982), *S. macrospora* MM (Rech et al., 2007), or synthetic Westergaards (SWG) medium (derived from synthetic crossing medium according to Nowrousian et al., 2005) supplemented with either glucose or xylose as the sole carbon source. To induce *Pcflp*-mediated site-specific recombination, appropriate strains were grown on solid MM supplemented with xylose as described by Kopke et al. (2010). Recombination events in transgenic strains were detected by growth tests on MM and CCM containing hygromycin B. All transformants and the wild-type strains were grown on CCM without selection, as positive controls.