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Flow cytometric sorting of the filamentous fungus *Trichoderma reesei* for improved strains

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ABSTRACT

Metabolic measurements and screening of *Trichoderma reesei* have conventionally been performed during the hyphal stage of fungal development. To determine if flow cytometric measurements of protein expression could be made on germinating spores we created a gene construct, placing the *Renilla reniformis* green fluorescent protein gene under control of the cellobiohydrolase I (*cbh*1) promoter and terminator of *T. reesei*. This vector was transformed into *T. reesei* and GFP expression was measured in germlings by flow cytometry. Fluorescence associated with GFP expression was observed in germlings grown under conditions known to induce cellulases in *Trichoderma*. Spores were mutated using UV light and germinating spores were screened for increased GFP expression using high-speed cell sorting, to select for strains with genetic changes associated with increased protein expression. Secondary screens for cellulase production were conducted in microtitre plates. Flow cytometric screening of germinating spores expressing GFP yielded a mutant with improved ability to hydrolyse biomass.

1. Introduction

Cellulose is an important component of plant material and the most abundant form of organic carbon on Earth. Saprophytic microorganisms including filamentous fungi produce extracellular hydrolytic enzymes, which cleave the β1, 4-glycosidic bonds present in cellulose and its derivatives [25]. Filamentous fungi catabolise cellulose into small oligosaccharides and finally glucose [20]. *Trichoderma reesei* (teleomorph *Hypocrea jecorina*) is a filamentous fungus belonging to the group Deuteromycota. It alternates morphologically between septated hyphae in its somatic stage and asexual haploid spores termed conidia during its reproductive phase [11]. *T. reesei* is widely used in industrial fermentation because of its ability to produce large amounts of endoglucanases (EGI/Cel7B, EGII/Cel5A, EGIII/Cel12A, EGIV/Cel61A, and EGV/Cel45A) and exoglucanases (the cellobiohydrolases, CBHI/Cel7A, and CBHII/Cel6A) that act in synergy to break down cellulose into cellobiose (glycosyl b-1,4-glucose) and subsequent hydrolysis to glucose by β-glucosidase [29,33,38,42]. These enzymes are used commercially in the textile and paper industries, and increasingly for the production of biofuels, which requires the hydrolysis of plant matter to fermentable sugars. Several cellulase and hemicellulase genes have been cloned from *T. reesei*. The expression and regulation of these genes in *Trichoderma* have been studied in detail at the level of transcription, but have not been monitored until now on a single cell basis for levels of protein expression using flow cytometry [15,19,20,37].

Strain improvement is a fundamental aspect of industrial microbiology, which is aimed at increasing enzyme yields, and reducing production costs [22]. Screening filamentous fungi for improved strains is complicated by difficulties in manipulating the branched hyphal mats known as mycelia. Improved strains are typically isolated using low-to medium-throughput screening based upon agar plate or microtitre plate assays [3,12]. While these screens may be automated using robotics, only a limited number of variants (thousands to hundreds of thousands) can be screened for improved strains using the microtitre plate format. In contrast, it is feasible to screen 10^9 mutants per day using high-speed fluorescent activated cell sorting (FACS). Fluorescence-based flow cytometric assays are widely used to analyze and screen populations of cells [9,10,16,30,32,36,44]. Gene expression studies using fluorescent reporter proteins and flow cytometry (FCM) have elucidated metabolic aspects of a wide range of prokaryotes and eukaryotes[7,8,18]. This study was aimed at exploiting the window of opportunity for flow cytometric analysis and sorting, when germinating spores are metabolically active, yet still small enough to pass through the nozzle of the cell sorter [6]. FACS analysis to measure carbon-regulated cellulase expression and high-throughput FACS screening for isolation of *Trichoderma*

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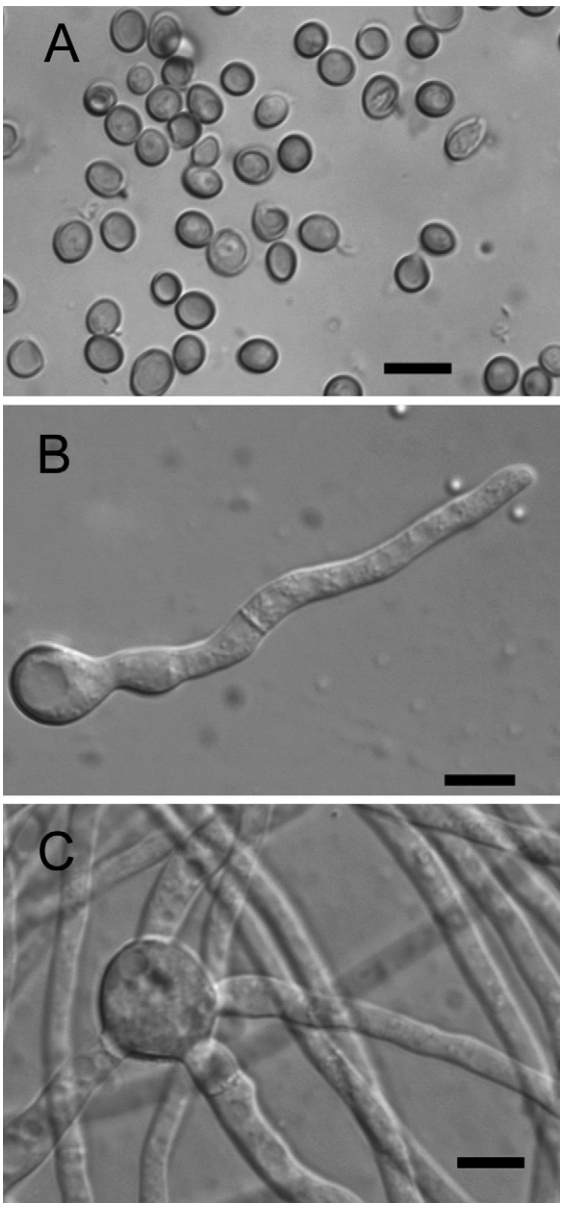

Fig. 1. Differential Interference Contrast light micrographs stages of growth. Freshly harvested spores **A**; germlings grown in media supplemented with glucose for 12 h **B**; and mycelia 48 h after inoculation of spores into glucose containing media **C**. Reference bar is 10 μM.

strains with improved ability to hydrolyse biomass were evaluated (Fig. 1).

2. Materials and methods

2.1. Strain creation and culture conditions

The *T. reesei* strain 41G-hrGFP used in this study was originally derived from the wild-type isolate QM6a. The QM6a strain has been improved over many years for cellulase production through several rounds of conventional mutagenesis and screening, resulting in a strain (41G) producing relatively large amounts of hydrolytic enzymes. Strain 41G-hrGFP was created by random insertion of the hrGFP gene into strain 41G [24]. The hrGFP gene was amplified from phrGFP-1 (Stratagene, La Jolla, CA) using primers 5'CACCATGGTGAGCAAGCAGATCCT3'(forward), and 5'TTACACCCACTCGTGCAGGCT3' (reverse). The PCR amplification conditions consisted of an initial denaturation step at 94 °C for 30 s, followed by 23 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s, and ending with a final extension at 72 °C for 5 min. The resulting PCR fragment of 724 bp was purified using Qiagen PCR purification kit (Qiagen, Valencia, CA), and cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA), and transformed into *E. coli* TOP 10 competent cells (Invitrogen). Plasmid DNA from the resulting clones was sequenced using M13F and M13R primers. The pENTR/D-TOPO vector containing the hrGFP was then recombined into the pTrex2g vector containing the Gateway cassette (Invitrogen) using the LR clonase reaction (Invitrogen), and transformed into *E. coli* TOP 10 competent cells. The vector map in Fig. 2 shows that GFP is under control of the *cbh*1 promoter and terminator and contains a *pyr4* selectable marker.

A 41G *pyr4*⁻ variant was obtained by selection on 5-fluoroorotic acid (5-FOA) plates as described by Boeke et al. [4].

The Trex2g-hrGFP construct was introduced into spores of the *pyr4*⁻ hypercellulase producing *T. reesei* mutant strain 41G (Genencor proprietary), using the PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA) as described by Toffaletti et al. [40]. For the transformation, 150 μl of prepared spore suspension (10% glycerol solution) of strain 41G *pyr4*⁻ was plated on Vogel's agar [41] supplemented with uridine (0.1% wt/vol), at a concentration of approximately 10^7 spores/mL, and the plates were incubated at 28 °C. The resulting transformants were serially transferred onto new plates with Vogel's agar to check for stable morphology. The expression of GFP in stable transformants was confirmed by growing germlings in 250 mL shake flasks containing 0.2% (vol/vol) lactose at 28 °C for 48 h, and visualizing mycelia using fluorescence microscopy. A highly expressing strain (41G-hrGFP) was chosen, and maintained on potato dextrose agar (PDA) plates for all subsequent experiments.

2.2. Flow cytometric monitoring of GFP expression

For FCM analysis of germlings of strain 41G-hrGFP, approximately 2.5 X 10^7 spores were inoculated into 25 mL liquid minimal medium as described previously [20], except that 100 mM piperazine-*N*,*N*-bis(3-propanesulfonic acid) (Calbiochem, San Diego, CA) was included in the medium to maintain the pH at 5.5. The medium was supplemented with 0.1% (vol/vol) lactose, 0.1% glucose, 0.1% glycerol, or 0.1% glucose containing 0.003% sophorose [14] and grown at 28 °C in 250 mL shake flasks with vigorous agitation. All cultures in this study used the same batch of glucose–sophorose at appropriate dilutions. Analysis for P*cbh*1-GFP and scattered light was performed on a FACS Calibur (Becton Dickinson, San Jose, CA) equipped with the standard emission filter for green (FL1) fluorescence. All samples were carefully filtered through 50 μm mesh filters (DakoCytomation, Fort Collins, CO) prior to analysis (Fig. 3a–c). With careful handling, almost all of the germlings passed through the filter until after 16 h, when a significant percentage of the germlings grown in glucose were too large to pass through the filter.

2.3. FACS screening

Spores (~5×10^8) from PDA plates were suspended into 40 mL of sterile deionized water. The volume was split into two 100 mm Petri dishes and placed on a magnetic stirrer inside a Stratalinker 1800 UV Crosslinker (Stratagene, La Jolla, CA).

The two 20 mL volumes of spores were irradiated while stirring with 50,000 and 100,000 μJ of 254 nm light, respectively. The aliquots were recombined and a small volume was used for serial dilutions to determine the kill percentage. Dilutions of irradiated and untreated spores were plated on PDA. The remaining spores were stored in water overnight at 4 °C. Spores were inoculated into liquid minimal medium containing 6% (w/v) glucose–sophorose and incubated at 30 °C with vigorous shaking overnight (14–16 h). High-speed sorting was performed on a MoFlo sorter at an event rate15,000 events per second, 60 psi with a 70 μm nozzle. The germlings with the brightest (top 0.01%) GFP signal were sorted and pooled. The (approximately 20,000) sorted germlings were plated on PDA and incubated at 28 °C for one week. Spores were recovered in sterile medium and the sorting procedure was repeated as above but without mutagenesis. The spores from the second round

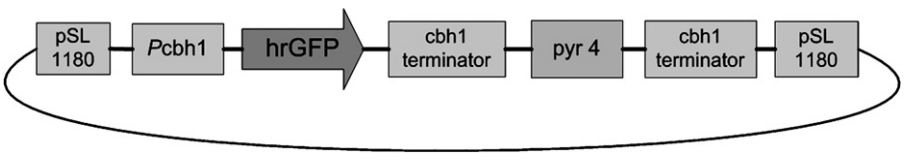

Fig. 2. Vector map of the pTrex2g/hrGFP construct used for creation of the green fluorescent protein-cellobiohydrolase I strain of *T. reesei*.