Genome Editing in Filamentous Fungus *Penicillium Verruculosum* Using the CRISPR/Cas9 System

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Abstract

Purpose

To adapt CRISPR/Cas9 genome editing method for use in filamentous fungus *Penicillium verruculosum*, which is industrial producer of carbohydrases.

Results

For the first time the CRISPR/Cas9 method was adapted for genome editing in the filamentous fungi *Penicillium verruculosum*. Using the nitrate reductase gene (*niaD*) as a selective marker with the CRISPR/Cas9 system we performed double knockout of *niaD* and cellobiohydrolase 1 (*cbh1*) genes. The efficiency of double editing was 50%. At the same time, it was unexpected that the specific cellobiohydrolase activity rised after knockout of *cbh1* gene due to the increase CBH2 expression.

Conclusion

We developed effective method for genome editing in *P. verruculosum*, which can be used to improve qualities of industrial strains.

Introduction

P. verruculosum is a filamentous fungal strain with high cellulase expression level. It was developed from wild-type *P. verruculosum* WA30 cellulolytic strain via stepwise mutagenesis followed by selection. The recipient *P. verruculosum* B1-537 (*niaD*⁻) strain that retained a high secretory activity at the level of 50–60 g/L of extracellular protein was developed via mutagenesis from *P. verruculosum* B1-221-151 (Sinitsin et al. 2020). B1-537 strain was used to develop recombinant strains for high productive expression of heterologous enzymes by cotransformation of the recipient strain with genes of interest and *niaD* gene, which leads to the formation of prototrophic strains (Sinitsin et al. 2020). At the same time, in order to further improve the qualities of the fungal strain *P. verruculosum* B1-221-151, it is necessary to adapt the method of knockout or deletion of various genes. As it is known, the frequency of homologous recombination in filamentous fungi is rather low, which hampers to use it for gene deletions. However, recently the CRISPR/Cas9 genome editing method became widely used for mycelial fungi at the last few years (Nødvig et al. 2015; Song et al. 2019). As it known, Cas9:sgRNA ribonucleoprotein complex can be either assembled *in vitro* and delivered to the cell or expressed from the genes directly within the cell, both methods are used for editing the genomes of mycelial fungi (Song et al. 2019). The absence of Cas9 nuclease and guide RNA genes in the final product is one of the advantages of genome editing by the assembled *in vitro* complex. However, this experimental setup requires additional efforts for its synthesis, purification and delivering into the fungal cell. At the same time, transcription of the gRNA in filamentous fungi is often hampered by the absence of described promoters for a RNA-polymerase III. Therefore, for the sgRNA expression we chose the highly conservative promoter of 5S rRNA of *Aspergillus niger*, as it was shown to be effective to drive sgRNA expression by Zheng et al. (2018).

Here we have adapted CRISPR/Cas9 genome editing method to make recipient (*niaD*⁻) strains in filamentous fungus *P. verruculosum* and knocked out nitrate reductase (*niaD*) and cellobiohydrolase 1 (*cbh1*) genes. In addition, we identified the impact of *cbh1* gene knockout, which is a major component of *P. verruculosum* cellulase complex, for its enzyme activity.

Materials And Methods

Fungal strains transformation and mutants selection

P. verruculosum B1-221-151 (*niaD*⁺) was used for the *niaD* and *cbh1* genes knockout by CRISPR/Cas9 system. For single knockout of *niaD* gene it was transformed with pGpdCas9 and p5SniaD plasmids as it was described in Aleksenko et al. (1995) with followed selection of transformants on the selective medium (SM) with addition of NH₄Cl as nitrogen source and NaClO₃ as a selective agent. For double knockout of *niaD* and *cbh1* genes B1-221-151 stain was transformed with plasmids pGCC and p5SniaD by the same way. Colonies grown on this medium were reseeded on the minimal media with NH₄Cl or NaNO₃ as nitrogen sources. Clones able to grow on NaNO₃ were rejected from further analysis.

Plasmid construction

Plasmid p5SniaD (Fig 1) contains *A. niger* 5S rRNA promoter and the sgRNA scaffold with specific spacer for *niaD* gene knock out. In order to develop it 5S rRNA promoter was amplified from *A. niger* genomic DNA using primers: 5SAn160HindIIIIF and 5SAnR (Supplementary Table 1) designed according to Zheng et al. (2018). The sgRNA scaffold with linker and spacer on the 5'-end was synthesized and cloned into pAL2-T vector by EVROGEN (Russia). sgRNA was amplified using sgRNAF and sgRNABamHIR primers pair (Supplementary Table 1) designed according to Zheng et al. (2018). After these amplifications PCR-products were used for overlap-PCR with 5SAn160HindIIIIF and sgRNABamHIR primers (Sup. Table 1). Then, amplified fragment was cloned by HindIII and BamHI restriction sites into pBluescript SK II (+) plasmid. At the next stage, spacer specific for *niaD* gene in p5SniaD plasmid was changed to knockout *cbh1* gene was by PCR mutagenesis using sgRcbh1F and sgRcbh1R primers resulting plasmid p5Scbh1.

The plasmid pGpdCas9 for *cas9* gene delivery (Fig 2) was constructed by PCR excision of the eGFP gene from plasmid pGpdCas9GFP (Kislitsin et al. 2020) using CasStopD2 and CasStopR2 PCR-primers (Supplementary Table 1). The 5

- region to the beginning of the transcriptional terminator of the *cbh1* gene. CasStopR2 reverse primer was complementary to the forward primer. The PCR

product was transformed into *E. coli* cells. Isolated plasmid DNA was checked for correct assembly and the absence of mutations by sequencing using follow PCR primers: CasGFPSR1, CasGFPSD1, CasGFPSD2, CasGFPSD3, CasGFPSD4, CasGFPSD5, CasGFPSD6 и CasGFPSD7 (Supplementary Table 1).

The pGCC1 plasmid was constructed by ligation of *Bam*HI-*Sal*I fragment from p5Scbh1 plasmid to pGpdCas9.

Design of sgRNA spacers

The conserved regions of *niaD* gene were determined by aligning the follow whole genome shotgun sequences from GenBank for the genera *Penicillium* and *Talaromyces*: (NW_002196662.1:1674201-1676795 *Penicillium marneffe* ATCC 18224, NW_002990114.1:2133873-2135844 *Talaromyces stipitatus* ATCC 10500, NW_017971432.1:2233740-2236407 *Talaromyces atrovirens* strain IBT 11181). Primers pair niaDF252 and niaDR698 was selected for conserved regions in order to amplify *niaD* gene fragment with the approximate length of 450 bp (Supporting information) . An internal fragment of *niaD* gene was amplified from the genomic DNA of *P. verruculosum* B1-221-151 using these primers. This fragment was sequenced and used as well as *cbh1* gene sequence for sgRNA spacers design by the ChopChop tool (<https://chopchop.cbu.uib.no/>).

Amplification of *niaD* and *cbh1* genes fragments in the transformants

DNeasy Plant Mini Kit (Qiagen) was used for DNA isolation from selected colonies after transformation. Fragments of *niaD* and *cbh1* genes were amplified from isolated DNA with primers pair niaDF252, niaDR698 and cbh1sd008, cbh1R593 respectively.

MALDI-TOF mass spectrometry fingerprinting of peptide fragments

The in-gel tryptic digestion of protein bands after the SDS-PAGE was carried out essentially as described by Smith (1997). Trypsin (Promega, Madison, WI, USA, modified, 5µg/mL) in 50 mM NH₄HCO₃ was used for a protein digestion. The resulting peptides were extracted from a gel with 20% aqueous acetonitrile containing 0.1% trifluoroacetic acid and subjected to MALDI-TOF mass spectrometry on an UltrafleXtreme II TOF/TOF mass spectrometer (BrukerDaltoniks GmbH, Bremen, Germany). Enzyme identification was carried out using Mascot peptide mass fingerprint server (<http://www.matrixscience.com/>).

Enzymatic activities assays, protein concentration measurement

These were performed as described in Supplementary Methods.

Results And Discussion

NiaD knockout by CRISPR/Cas9 system

The choice of an appropriate selection marker for genome editing experiments is an important step. The knockout of *pyrG* provides the resistance towards 5'-fluoroorotic acid (5'-FOA) and is used widely as a marker gene (Katayama et al. 2019). Knockout of the *wA*, *pksP*, and *albA* genes leads to the formation of colorless mycelium (Fuller et al. 2015, Katayama et al. 2019). However, the *P. verruculosum* B1-221-151 strain is highly resistant to 5'-FOA and has an unstained mycelium. The nitrate reductase gene was chosen as a marker gene because its knockout leads to the loss of the ability to use nitrates as a nitrogen source and to chlorate resistance (Cove 1976). The nucleotide sequence of the *P. verruculosum niaD* gene is essential for selection of the guide RNA spacer sequence. Therefore, we amplified and sequenced an internal fragment of the *P. verruculosum niaD* gene (Supplementary 1). High homology (91%) of *niaD* region from *P. verruculosum* and corresponding region of *niaD* gene from *P. marneffe* was revealed. The sequence of guide RNA spacer for the *niaD* gene was selected using the ChopChop program and this spacer was used to construct p5SniaD plasmid.

The *P. verruculosum* B1-221-151 strain was transformed with the pGPDcas9 and p5SniaD, the resulting clones were plated on selective medium (SM). A number of colonies were observed on the SM with 0.6 M sodium chlorate on the 6th day after transformation. Sixteen colonies were further were reseeded on the minimal media with NH₄Cl or NaNO₃ as nitrogen sources. Further, eight clones which were not able to grow on MM with NaNO₃, were randomly selected for *niaD* gene sequencing. The presence of mutations leading to a shift in the reading frame was identified in four clones in the region where the double-stranded break of genomic DNA was supposed (Fig. 5)

Thus, clone #4 as a one of obtained niaD⁻ mutants was randomly selected and cultivated in Erlenmeyer flasks on fermentation medium (FM) (Supporting methods) in order to compare its activities with *P. verruculosum* B1-537 strains. The results of avicelase, β-glucosidase and xylanase activity measurements are presented in Table 1. These results show that enzyme activities of clone #4 and B1-537 strain are similar and consistent with previously obtained results (Sinitsin et al. 2020).

Table 1. Enzymatic activities in the culture liquids of *P. verruculosum* B1-2743 and B1-537 strains.

Strain	Protein concentration (mg/ml)	Enzymatic activity (u/mg of protein)		
		avicelase	β-glucosidase	xylanase
B1-537	6±0.2	0.6±0.04	2±0.36	10±2.1
B1-2743	6±0.1	0.7±0.06	2±0.23	10±2.3

Thereby, *niaD* gene knockout efficiency was 50% because in four out of eight analyzed clones mutations were detected. The absence of mutations in desired region in half of selected clones could be explained of low NaClO₃ and their adaptation for NaClO₃ by the other mechanisms. Nevertheless, we considered this result as comparable with single gene knockout efficiency by CRISPR/Cas9 system in other filamentous fungi (Table 2)

Table 2. Single knockout efficiency using CRISPR/Cas9 system in various fungal strains

Fungal species	Target gene(s)	Efficiency	Reference
<i>A. fumigatus</i>	<i>pksP</i>	25-53%	Fuller et al. 2015
<i>A. oryzae</i>	<i>wA</i> and <i>pyrG</i>	10-20%	Katayama et al. 2016
<i>P. chrysogenum</i>	<i>pks17</i>	33-100%	Pohl et al. 2016
<i>P. subrubescens</i>	<i>ku70</i>	17%	Salazar-Cerezo et al. 2020
<i>P. verruculosum</i>	<i>niaD</i>	50%	This study

Double knockout of *cbh1* and *niaD* genes

At the next stage, we aimed to investigate the possibility to use CRISPR/Cas9 system to make double knockouts in filamentous fungus *P. verruculosum*. The *niaD* gene was used as a marker, and the gene cellobiohydrolase 1 (*cbh1*) was chosen as the main target. This enzyme is the main component of the enzymatic complex and makes up 60% of all proteins (Sinitsin et al. 2020), although there is only one copy of *cbh1* gene in the genome of *P. verruculosum* (Chulkin et al. 2019). Thus, its knockout can be conveniently detected by the absence CBH1 expression. In addition, *P. verruculosum* strains with knockout *cbh1* have not been obtained previously, therefore, such a strain may be used for studying its physiological role in the growth on cellulose-containing media.

To knockout the *cbh1* and *niaD* genes simultaneously, *P. verruculosum* B1-221-151 strain was transformed with plasmids pGCC1 and p5SniaD. The plasmid pGCC1 contained the *cas9* gene and sgRNA scaffold with a spacer specific for the *cbh1* gene, which would increase the frequency of a double knockout. Eight clones were selected like mutants with single *niaD* gene mutation, which were tested for the presence of mutations in the *cbh1* and *niaD* genes. As a result, at the site of a double-strand break in the *niaD* gene were found in all eight clones, while mutations in the *cbh1* gene were just in four of them (Fig. 5).

Clones with mutations in *cbh1* gene were cultivated in Erlenmeyer flasks (in batch fermentation conditions) on the FM medium for six days. The SDS-PAGE of their culture liquids revealed increase of CBH2 rate in clones #2, 3, 5. (Fig. 6).

Although, there are protein bands at 66 kDa in clones #2, 3 and 5, MALDI-TOF MS does not revealed their similarity with CBH1, however we were not able to identify these proteins. In case of clone #8 the rate of CBH2 has not increased, which is explicable since the sequence of *cbh1* gene revealed deletion of nine nucleotides, which lead to deletion of three amino acid residues, but not to the frameshift and stop codon formation.

Although we have not used donor DNA, double knockout efficiency was about 50% (four double mutations in eight analyzed clones). At the same time, Chen et al. (2020) reported that double gene disruption by CRISPR/Cas9 system without addition of donor DNA in *Acremonium chrysogenum* was lethal. Thereby, we consider, that CRISPR/Cas9 genome editing method was successfully adapted to make double knockouts in *P. verruculosum*.

Feed-bath cultivation of double *niaD*⁻ and *cbh1*⁻ mutants and analysis of their enzymatic complex

Clones #3 and 5 were named as B1-Z3 and B1-Z6 and chosen for feed-bath cultivation in three-liter fermenters in order to reveal influence of *cbh1* knockout on their growth and enzymatic activity of *P. verruculosum* in industrial cultivation conditions. The results of enzymatic activities and protein concentration measurements of the culture liquids (Fig. 7) showed, that, although, the level of protein secretion in such strains decreased, the unit cellobiohydrolase, β -glucosidase, endoglucanase and xylanase activities rose.

The fact that the unit cellobiohydrolase activity for even increased was unexpected, since although the expression of *cbh2* was also increased in *cbh1*⁻ *Trichoderma reesei* strains, the cellobiohydrolase activity of its complex significantly decreased (Suominen et al. 1993; Ren et al. 2020)

Conclusions

In the present study, CRISPR/Cas9 system was successfully adapted for genome editing in filamentous fungus *P. verruculosum*. We demonstrated its abilities by knockout of *cbh1* as target gene and *niaD* as marker one. This method will expand the possibilities to study gene functions in *P. verruculosum* and to develop new industrial strains.

Declarations

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Research involving human and animal participants This article does not contain any studies with animals performed by any of the authors.

Availability of data and material

Code availability: SnapGene 3.2.1., CHOPCHOP web tool.

Author Contributions VYuK designed and carried out experiments and measurements, interpreted experimental data. AMC designed interpreted experimental data. INZ, YuAD interpreted experimental data and reviewed manuscript. AMR initiated, and coordinated the study and supported the research funding. APS reviewed the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

The map of sgRNA delivering plasmids.

Figure 2

The map of the pGpdCas9 plasmid.

Figure 3

The map of the pGCC1 plasmid.

	PAM	spacer	
<i>niaD</i> wt	TGGGCCCCGTCCCACGAGTCCGCGATGAAGACATCCCAACCTGGGA		
clone 1	TGGGCCCCGTCCCACGAGTCCGCGATTGAAGACATCCCAACCTGGG		
clone 2	TGGGCCCCGTCCCACGAGTCCGCGAGCAAGGACATCCCAACCTGGG		
clone 3	TGGGCCCCGTCCCACGAGTCCGCGA-GAAGACATCCCAACCTGGGA		
clone 4	TGGGCCCCGTCCCACGAGTCCGCGATTGAAGACATCCCAACCTGGG		

Figure 4

Comparison of mutant nitrate reductase gene sequences with the native *P. verruculosum* *niaD* gene.

a

		PAM	spacer	
<i>niaD</i> wt	TGGGCCCGTCCCACGAGTCCGCGATGAAGACATCCCAACCTGGGA			
clone 1	TGG-----ACATCCCAACCTGGGA			
clone 2	TGGGCCCGTCCCACGAGTCCGCGA-GAAGACATCCCAACCTGGGA			
clone 3	TGGGCCCGTCCCACGAGTCCGCGA-GAAGACATCCCAACCTGGGA			
clone 4	TGGGCCCGTC-----GAAGACATCCCAACCTGGGA			
clone 5	TGGGCCCGTCCCACGAGTCCGCGA-GAAGACATCCCAACCTGGGA			
clone 6	TGGGCCCGTCCCACGAGTCCGCGA-GAAGACATCCCAACCTGGGA			
clone 7	TGGGCCCGTCCCACGAGTCCGCGA-GAAGACATCCCAACCTGGGA			
clone 8	TGGGCCCGTCCCACGAGTCCGCGA-GAAGACATCCCAACCTGGGA			

b

			spacer		PAM
<i>cbh1</i> wt	TCTTTGAGCTGGTCTACTTGCAAATCAGGTGGTAGCTGC				
clone 2	TCTTTGAGCTGGTCTACTTGCAAATAGGTGGTAGCTGC				
clone 3	TCTTTGAGCTGGTCTACTTGCAAATGACGTCTAGCTGC				
clone 6	TCTTTGAGCTGGTCTACTTGCAAATCCAGGTGGTAGCTG				
clone 8	TCTTTGAGCTGGTCTACTTGCAAATCC-----TGC				

Figure 5

Comparison of the *niaD* (a) and *cbh1* (b) genes sequenced fragments with the native *P. verruculosum* genes.

Figure 6

The SDS-PAGE of the culture liquids of *P. verruculosum* clones with knockouts of *cbh1* gene after six day of cultivation on FM media in Erlenmeyer flasks.

Figure 7

Protein concentration and enzyme activities (u/mg of protein) of the culture liquids of *P. verrucosum* cbh1- strains after bath fermentation. a Dynamics of protein concentrain. b Dynamics of cellobyohydrolase activity. c Dynamic of β -glucosidase activity. d Dynamic of xylanase activity.