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Functional analysis of OAT gene in *Aspergillus Niger*[*]

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ABSTRACT

Mitochondrial oxaloacetate transporter protein encoded by OAT gene transports oxaloacetate from cytoplasm into mitochondria. To investigate the primary effects of OAT gene on relative metabolism in *Aspergillus niger*, a oat-deleted mutant was derived from wild-type *A. niger* ATCC1015 using the double-crossover chromosome replacement technique. Then batch fermentation was performed to evaluate the mutant. Compared with the wild type, the mutant showed lower organic acids production, with the experimental data that the production of pyruvate and 2-ketoglutarate decreased by 31.6% and 35.7%, respectively, and by contrast, the mutant showed higher glycerol formation. The results suggest that OAT gene plays significant roles on metabolism in *A. niger*.

Keywords: Aspergillus Niger; Oxaloacetate Transporter; Organic Acids; Glycerol Formation

1. Introduction

The filamentous fungus *Aspergillus niger* was widely applied to industrial enzymes and organic acids production [1]. Despite decades of study, the production process is not well understood in details yet [2]. The release of the genomic sequences of *Aspergillus niger* ATCC1015 in 2006 [3-4] provided a very good opportunity to characterize the function of genes in *A. niger*. Additionally, the deletion of non-homologous end-joining gene *Ku*80 showed high recombination frequency, which provided a useful tool for gene targeting in *A. niger* [5-7].

In the fugus strain *A. niger*, citrate can be synthesed from oxaloacetate and acetyl-CoA by citrate synthase. The progress of citrate synthesis constantly accompanied with oxaloacetate consumption, so it is important to ensure adequate oxaloacetate for the production of citric acid [8]. Formers' researches verified that oxaloacetate transporter delivers oxaloacetate from the cytoplasm to the mitochondria in *A. niger* [3]. In this study, oxaloacetate transporter OAT null mutant was generated, and the effect of deletion of OAT on the metabolites of *A. niger* was investigated.

2. Materials and Methods

2.1. Strains

Aspergillus niger strain ATCC 1015 used in this study was kindly provided by Dr Sun Ji Bin, Tianjin Institute of Industrial Biotechnology, CAS.

Escherichia coli DH5α was used for routine cloning experiments.

Agrobacterium tumefaciens AGL1 was used for the transformation of *A. niger*.

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2.2. Strain Growth

A. niger strains were grown at 30 ℃ on potato dextrose agar (PDA) slant for sporulation and stock [9].

Screening of transformants was performed on fungal complete media (CM) containing 200 μg/mL hygromycin B and 200 μM cefotaxim [10].

E. coli DH5α was grown at 37 °C on LB medium. *A. tumefaciens* AGL1 was grown at 28°C on LB medium.

The batch cultivation medium composed of 50 g/L glucose, 5 g /L NH_4NO_3, 2 g/L KH_2PO_4, 1 g/L $MgSO_4 \cdot 7H_2O$, 1 g /L NaCl, 0.1 g/L $CaCl_2 \cdot 2H_2O$, and 1 ml/L trace element solution.

Trace element solution consisted of 0.8 g/L $ZnSO_4 \cdot 7H_2O$, 0.8 g/L $CuSO_4 \cdot 5H2O$, 0.8 g /L $FeSO_4 \cdot 7H_2O$.

2.3. OAT Gene Deletion

E-mail address: liuhao@tust.edu.cnThe relative sequences of OAT gene were obtained from the genome sequences database DOE Joint Genome Institute (http://genome.jgi.doe.gov/Aspni5/Aspni5.home.html). The plasmid p44 containing *hph* gene was derived from pCAMBIA1300. The *oatΔ* strain was constructed using the standard one-step gene replacement strategy in which *hph* gene was used as selective marker [11]. Briefly, about 1 kb of 5' UTR and 3' UTR fragments were amplified by PCR using the primer pairs oat-upF/oat-upR and oat-downF/oat-downR, respectively. The two fragments Were ligated to flank the hygromycin resistance cassette in p44, sequentially. The gene replacement construct was introduced into *A.niger* ATCC 1015 via *Agrobacterium tumefaciens*-mediated transformation. CM containing 200 μg/mL hygromycin was used for selecting transformants. Correct gene replacement event was confirmed by PCR using primer pairs oat-upF1/oat-downR1. The sequences of the pimers are presented in **Table 1**.

Table 1. PCR pimer.

Primer name	sequnece
oat-upF	CTGTAAGCTTCAAGCCAGACAGATCCGTTTG
oat-upR	CTGGCTGCAGTGCAGACTTTCGTATAGATAC
oat-downF	GTCTGGATCCGTATTCTGCCTCACACGGTAAG
oat-downR	GTCAGGTACCCAGTGTGGTGATGACGTTACAG
oat-upF1	GTCCGAGACCTTCTGGCATTGTAG
oat-downR1	CTTCATGCACATTGGCAGCTGTC

2.4. Cultivation Conditions

For inoculation of batch cultivations, spores of *A. niger* incubated at 30 °C on PDA solid medium for 5–7 days were harvested with 0.05% （w/w） Tween-80.

All strains were cultivated in 5L-Braun bioreactors with a working volume of 4 L. The bioreactors were equipped with two Rushton four-blade disc turbines used for culture broth mixing. No baffles were incorporated in the bioreactors thereby reducing the surface area available for wall growth. Subsequently, Spores were added to the bioreactors to an initial concentration of 5×10^8/L. The temperature was maintained at 30 °C. The initial aeration rate, rotate speed and pH were 0.3 vvm, 200 rpm and 2.5, respectively. After germination (approximately 16 h), the culture condition described above were gradually adjusted to 1 vvm, 600 rpm and pH 4.5 and kept constantly throughout the rest of the fermentation time. The pH was controlled by automatic addition of 1 M $NaHCO_3$ or 2 M HCl.

2.5. Biomass Determination

The biomass was determined by the use of 0.45 μm nitrocellulose filter. Initially, the filters were pre-dried in a microwave oven at 150 W for 10 min, and then weighed. A known weight of cell culture was filtered, and the cells were washed with distilled water. Finally, the filter was dried in the micro-wave at 150 W for 15 min and the dry weight was determined.

2.6. Extracellular Metabolites Quantification

To determine the extracellular metabolites, a culture sample was taken and immediately filtered through a 0.45 μm nitrocellulose filter. The filtrate was frozen and kept at -20 °C until analysis. Glucose, pyruvate, citrate, succinate, oxalate and glycerol concentrations were determined using an cationic- exchange column (Aminex HPX-87H, BioRad, USA) eluted with 5mM H_2SO_4 with the flow rate of 0.6 mL min^{-1} at 65 °C. Metabolites were detected by refractive index and UV.

3. Results and Discussion

3.1. The Construct of p 44-oat::hph Gene Deletion Cassette

The 3’ UTR fragment (966 bp) was amplified and inserted into *Bam*H I and *Kpn* I sites of p44 to construct the plasmid p44-oat-dw. The 5’ UTR fragment (855 bp) was amplified and inserted into *Hin*d III and *Pst* I sites of p44-oat-dw to obtain the plasmid p44-oat::hph (**Figure 1**). The result of double digestion of p44-oat::hph to confirm the successful cloning being constructed (**Figure 2**).

3.2. The Generation of the OAT Gene Deletion in A. Niger

T-DNA fragment containing the 5’ UTR (855 bp) and 3’ UTR (966 bp) fragments of the OAT gene was transferred into the strain *A. niger* ATCC 1015 and the transformants were selected with hygromycin.

The resistant colonies grown in the plates were picked out and total DNA were extracted from the transformants, and then used as the template for PCR amplification and examination. More than 30 transformants were examined, and two gene deletion candidates were obtained. When the OAT gene was replaced by *hph* gene, the length of the DNA fragment amplified by PCR using primer pairs oat-upF1/ oat-downR1 is about 3.5 kb (**Figure 3**, Lane 3) compared to 3.0 kb obtained from the wild type strain(**Figure 3**, Lane 1). The length of the PCR product obtained from transformants1 (**Figure 3**, Lane 2) is the same as that obtained from the wild type strain, indicating that the transformants1 was not the *oatΔ* strain. All the data suggested that the transformants2 was an OAT gene deletion strain.

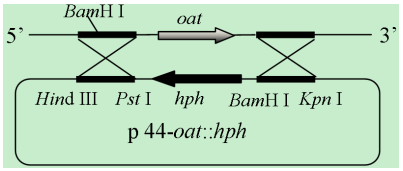


Figure 1. Map of p 44-*oat::hph*.

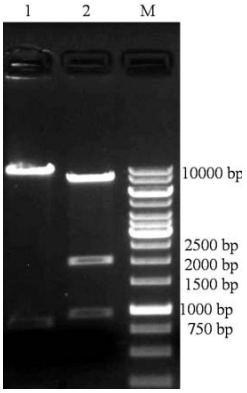
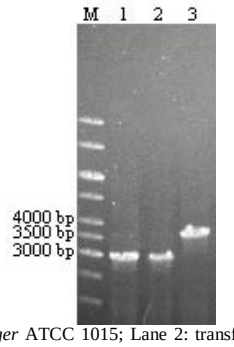


Figure 2. Double digestion of p 44-*oat::hph*



ane 1: *A.niger* ATCC 1015; Lane 2: transformants1; Lane 3: transformants2

Figure 3. PCR detection of OAT gene deletion

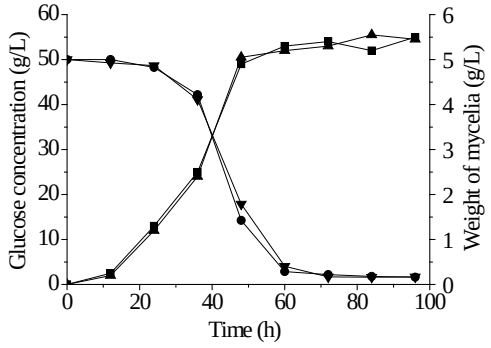


Figure 4. Residual glucose and biomass of the strains. ▲: glucose of wild type ■: glucose of *oatΔ* ▼: biomass of wild type ●: biomass of *oatΔ*.

Table 2. Product yields (g/L).

	Glycerol	Citrate	Oxalate	Pyuvate	2-ketoglutarate	succinate
WT	0.98	8.21	17.60	0.19	0.14	0.73
oatΔ	1.15	8.04	15.40	0.13	0.09	0.69

3.3. Characterization of OAT Deletion Strain

The biomass of the *oatΔ* strain and the wild type strain were almost the same along with the culture time (**Figure 4**). Moreover, the glucose consumptions of the two strains were also similar, indicating that the deletion of the OAT gene has little influence on the growth of *A. niger* (**Figure 4**).

Organic acids production of the *oatΔ* strain and the wild type strain were tested and compared with each other. The results are shown in **Table 2**. Generally, *oatΔ* strain produced less quantity of organic acids than the wild type strain. Oxalate and citrate were the most abundant acids produced at pH 4.5. It was observed that the productions of oxalate and citrate in the *oatΔ* strain were reduced compared to the wild type strain. When the OAT gene was deleted, pyuvate production decreased, less pyuvate was transferred into the mitochondria which caused the synthese of citrate blocked (The data was shown in **Table 2**). pyruvate and α-ketoglutarate of the *oatΔ* strain was reduced by 31.6% and 35.7% respectively compared to the wild type strain. In contrast, the glycerol production in the *oatΔ* strain was increased than the wild type strain.

4. Conclusion

It is concluded that deletion of OAT has significant effect on the metabolic flux of oxaloacetate. For the reason that oxaloacetate in mitochondria can be supplemented by muliple pathways [12], so it is necessary to further study the details of oxaloacetate metabolic processes in *A.niger* in organic acids metabolism.

REFERENCES

[1] S. Meijer, W. A. de Jongh, L. Olsson and J. Nielsen, "Physiological characterisation of *acuB* deletion in *Aspergillus niger*," Applied Microbiology and Biotechnology, vol. 84, pp. 157–167, 2009.

[2] W.A. de Jongh, J. Nielsen, "Enhanced citrate production through gene insertion in *Aspergillus niger*," Metabolic Engineering, vol.10, pp. 87-96, 2008.

[3] H. J Pel, J. H de Winde, D. B Archer, P. S Dyer, G. Hofmann, P. J Schaap, et al, "Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88," Nature Biotechnology, vol. 25, pp. 221-231, 2007.

[4] Baker SE, "*Aspergillus niger* genomics: past, present and into the future," Medical Mycology, vol. 44, pp. 17–21, 2006.

[5] V. Meyer, M. Arentshorst, A. El-Ghezal, A. C. Drews, R. Kooistra, van den Hondel CAMJ, et al, "Highly efficient gene targeting in the *Aspergillus niger kus*A mutant," Journal of Biotechnology, vol. 128, pp. 770–775, 2007.

[6] Z. Jinxiang, M. Zhihui, X. Wei, L. Ying, T. Guomin, W. Aoquan, et al, "Ku80 gene is related to non-homologous end-joining and genome stability in *Aspergillus niger*," Current Microbiology, vol. 62, pp. 1342–1346, 2011.

[7] N. D. S. P. Carvalho, M. Arentshorst, M. J. Kwon, V. Meyer, A. hur, F. J. Ram, "Expanding the *ku*70 toolbox for filamentous fungi: establishment of complementation vectors and recipient strains for advanced gene analyses," Applied Microbiology and Biotechnology, vol. 87, pp. 1463–1473, 2010.

[8] M. Yigitoglu, "Production of citric acid by fungi," Journal of Islamic Academy of Sciences, vol. 5, pp. 100-106, 1992.

[9] M. J. A. de Groot, P. Bundock, P. J. J. Hooykaas, A. G. M. Beigersbergen, "*Agrobacterium tumefaciens*-mediated transformation of filamentous fungi," Nature Biotechnology, vol. 16, pp. 839-842, 1998.

[10] C. B Michielse, P. J J Hooykaas, C. A M J J van den Hondel, A. F J Ram, "*Agrobacterium*-mediated transformation of the filamentous fungus *Aspergillus awamori*," Nature Protocols, vol. 3, pp. 1671-1678, 2008.

[11] H. Liu, A. Suresh, F. S Willard, D. P Siderovski, S. Lu and N. I Naqvi, "*Rgs*1 regulates multiple Ga subunits in *Magnaporthe pathogenesis*, asexual growth and thigmotropism," The EMBO Journal, vol. 26, pp. 690–700, 2007.

[12] K. Kapoor，K. Chaudhary，P. Tauro, "Citric acid," Prescott and Dunn's Industrial Microbiology， vol. 4, pp. 709-742, 1982.

