J Ind Microbiol Biotechnol (2009) 36:1275–1280
DOI 10.1007/s10295-009-0607-y

ORIGINAL PAPER

Gene deletion of cytosolic ATP: citrate lyase leads to altered organic acid production in *Aspergillus niger*

Susan Meijer · Michael Lynge Nielsen · Lisbeth Olsson · Jens Nielsen

Received: 21 April 2009 / Accepted: 2 June 2009 / Published online: 25 June 2009

Abstract With the availability of the genome sequence of the filamentous fungus *Aspergillus niger*, the use of targeted genetic modifications has become feasible. This, together with the fact that *A. niger* is well established industrially, makes this fungus an attractive micro-organism for creating a cell factory platform for production of chemicals. Using molecular biology techniques, this study focused on metabolic engineering of *A. niger* to manipulate its organic acid production in the direction of succinic acid. The gene target for complete gene deletion was cytosolic ATP: citrate lyase (*acl*), which had previously been identified by using genome-scale stoichiometric metabolic model simulations. The *acl* gene was deleted using the bipartite gene-targeting method, and the mutant was characterized in batch cultivation. It was found that the succinic acid yield was increased threefold by deleting the *acl* gene. Additionally, the total amount of organic acids produced in the deletion strain was significantly increased. Genome-scale stoichiometric metabolic model predictions can be used for identifying gene targets. Deletion of the *acl* led to increased succinic acid production by *A. niger*.

Keywords *Aspergillus niger* · Organic acids · ATP: citrate lyase · Succinate dehydrogenase

S. Meijer · M. L. Nielsen · L. Olsson · J. Nielsen
Department for Systems Biology,
Center for Microbial Biotechnology,
Technical University of Denmark,
Building 223, 2800 Lyngby, Denmark
e-mail: SuLM@novozymes.com

Present Address:
S. Meijer
Novozymes A/S, Krogshøjvej 36,
2880 Bagsværd, Denmark

Present Address:
L. Olsson · J. Nielsen (✉)
Department of Chemical and Biological Engineering,
Chalmers University of Technology, Kemivägen 10,
412 96 Gothenburg, Sweden
e-mail: nielsenj@chalmers.se

Introduction

The release of the genomic sequence of *Aspergillus niger* in 2006 has opened a window of opportunities in the field of biotechnology [1, 10]. However, although a genomic sequence can yield much information, genome sequencing is only the first step towards understanding a microbe's biological capabilities. It is still a challenge to interpret genomic information in terms of the functions of individual genes and, furthermore, to assess their role in overall cellular function. Because of several sequencing projects and intensive annotation programs we are now able to compare genomes among different species. The comparison helps us to identify genes and also makes it possible to assign putative function to genes. With the genome sequence available for *A. niger* it has also become possible to perform targeted deletion of genes, which is often the key to redirecting fluxes towards desirable pathways.

Tools for predicting the effect of a gene deletion in silico have already been developed for *A. niger*. David et al. [3] created a detailed genome-scale stoichiometric metabolic model of central carbon metabolism. They used the model to identify metabolic engineering targets for increased succinate production, and predicted it should be possible to obtain increased succinate yield in a double-deletion mutant (pyruvate decarboxylase and cytosolic ATP: citrate lyase) when grown under oxygen-limiting conditions. Although

genome-scale stoichiometric models do not contain regulatory information, these models have previously proven to provide strategies for metabolic engineering [5, 9, 11, 13]. However, model predictions still need to be verified experimentally, which may lead to revision of the model. In this study our objective was to verify the predictions of the stoichiometric model. In a previous study [6] investigating the physiological changes of the wild-type strain due to different aeration rates, it was shown that the pyruvate decarboxylase (PDC) enzyme was not active under oxygen-limiting conditions. Therefore, to reduce cumbersome molecular manipulations in *A. niger*, only the single deletion mutant in which cytosolic ATP: citrate lyase (cACL) was knocked out, was created. Because homologous recombination is a rare event compared with non-homologous end joining in *A. niger*, the bipartite gene-targeting method, which reduces the occurrence of ectopic integrations during transformations [8], was used to construct the deletion strain (*Δacl*).

Materials and methods

Strains

The *A. niger* strains used in this study were:

- AB4.1 derived from strain N402 containing a mutation in *pyrG1* [14].
- As reference the N402 strain (*cspA1* mutation) was used (A733 at the fungal genetics stock center).

Deletion strategies

The deletion strategy applied was the deletion of the target gene (*acl*) in the *pyrG1*$^{-}$ AB4.1 strain, where *pyr-4* from *Neurospora crassa* is used as marker gene [2]. Protoplasting and gene-targeting procedures for *A. niger* were used, as has been described previously for *Aspergillus nidulans* [8]. The deletion strains were constructed using PCR-generated bipartite gene targeting substrates. Each part of the bipartite substrate consists of a targeting fragment and a marker fragment, which are amplified individually by PCR using the primer pairs presented in Table 1.

To achieve adequate sequence overlap to favor homologous recombination, 2.5-kb flanking regions were used, flanking both sides of the coding region. The upstream flanking region of the gene was fused to the *pyr-4* upstream marker fragment and the downstream flanking region of the gene to the downstream part of *pyr-4*. The *pyr-4* fragments were made without direct repeats (see primers Table 2). The fusion is facilitated by using oligonucleotides that have fusion tags (adaptamers) [4, 8]. Each purified fusion PCR fragment (2–3 μg) was used for the transformation in 100 μL protoplast solution of *A. niger*. The transformation was performed as described by Nielsen et al. [8]. Selection of transformants was done on minimal medium (MM) plates, to select for prototrophs. The auxotrophic AB4.1 strain on the other hand needs 10 mM of both uracil and uridine for survival. This strain is used as negative control as it is unable to grow on MM.

Table 1 Organic acid yields in C-mmol C-mol^{-1} carbon source

	Succinate	Acetate	Glycerol	Citrate	Fumarate	Oxalate	Pyruvate
WT xylose	n.d.	n.d.	14	1	0.18	27	1.1
Δacl xylose	n.d.	n.d.	16	5	0.74	24	2.3
WT glucose	2.1	n.d.	12	25	0.40	26	n.d.
Δacl glucose	7.1	51	11	40	0.30	29	n.d.

n.d. not detected

Table 2 Primers used for bipartite PCR fragments

Primer name	Sequence[a]
pDEL1-pyr4	
Upst-pDEL1-F-Ad	cat ggc aat tcc cgg gga tc TGG ATA ACC GTA TTA CCG CC
Dst-pDEL1-R-Ad	cat ggt ggt cag ctg gaa tt TGC CAA GCT TAA CGC GTA CC
pyr_R	cat ggt ggt cag ctg gaa tt CCT CCG CCA TTT CTT A
pyr_F2	cat ggc aat tcc cgg gga tc GCC GGC AAT TCT TTT T
ACL	
ACL2_edge_R*	aat tcc agc tga cca cca tg GCC TGT TTT CTT TTC ACC CA
ACL2_edge_F2*	gat ccc cgg gaa ttg cca tg GGC TGA ATA CTC TGT GAA TCG
ACL_fl_F3	CGG GAA GTC AAC CTA CTG CA
ACL_fl_R3	GAA TCG ACT TGG CGG ACA TG

[a] The capital letters are the homologous regions with the target DNA, the small letters are the adaptamer sequences used for the fusion PCR

Southern blot analysis of transformants

For each strain (N402 and *Δacl*), 2 μg genomic DNA was isolated and digested with appropriate restriction enzymes (*Mlu*I and *Dra*III). Sequence information for restriction digest of the target loci (*acl*) were obtained from the *A. niger* ATCC 1,015 genome sequence from the US Department of Energy Joint Genome Institute (http://genome.jgi-psf.org/Aspni1/). Blotting was done according to the method described by Sambrook and Russell [12], using RapidHyb hybridization buffer (Amersham Pharmacia) for probing. The target locus was detected by probing with the labeled upstream target gene PCR fragment. The probes were radioactively labeled with α-32P-dCTP by random priming using Rediprime II kit (GE Healthcare).

Characterization of *Δacl*

The deletion strain *Δacl* was investigated in separate batch fermentations with 50 g L^{-1} glucose and 50 g L^{-1} xylose as initial carbon source concentrations and compared with the wild-type strain N402. The medium composition used was the same as described in Meijer et al. [6] and this medium was also used for the pre-culture.

Before inoculation of the batch fermentors, spores were harvested with 0.01% *w/w* Tween-80 from pre-culture plates that were incubated for 4–7 days at 30°C. Subsequently, 10^9 spores L^{-1} were added to the bioreactors. All strains were cultivated under oxygen limitation (0.1 vvm) in 5-L in-house-manufactured bioreactors with a working volume of 4 L. The bioreactors were equipped with two disk turbine impellers rotating at 700 rpm. The initial pH was set at 2.5 and after 16 h it was increased to 5 with $NaHCO_3$. The temperature was maintained constant at 30°C and the pH was controlled by automatic addition of 1 M $NaHCO_3$ or 2 M HCl.

Cell dry weight determination

The cell mass concentration on a dry weight basis was determined by the use of nitrocellulose filters with a pore size of 0.45 μm (Osmonics, Minnetonka, MN, USA). 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 residue was washed with distilled water. Finally, the filter was dried in the microwave at 150 W for 15 min and the dry weight was determined.

Extracellular metabolite quantification

For determination of the extracellular metabolites, a culture sample was taken and immediately filtered through a 0.45 μm-pore-size nitrocellulose filter (Osmonics). The filtrate was frozen and kept at −20°C until analysis. Glucose, acetate, pyruvate, citrate, ethanol, succinate, fumarate, malate, glycerol, and oxalate concentrations were determined using an Aminex HPX-87H cationic-exchange column (BioRad, Hercules, CA, USA) eluted at 60°C with 5 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1}. Metabolites were detected by refractive index and UV.

Results and discussion

The objective of the metabolic engineering strategy tested in this study was to divert carbon fluxes in the direction of succinate. The cytosolic ATP: citrate lyase gene locus was targeted for complete gene deletion in order to divert the fluxes of the primary metabolism towards succinate. The gene-deletion strategy was based on an in-silico model prediction that has shown an increase in succinate production when both *pdc* and *acl* were deleted [3]. In a previous study it was shown that PDC has no detectable activity under the cultivation conditions imposed on the system [6], so it was decided to delete only *acl*.

Strain construction

The recovery of transformants was high in the transformations performed, but only one correct deletion mutant could be retrieved (over 200 were checked). The Southern blot (Fig. 1) clearly shows that the *acl* gene is replaced by the marker gene *pyr-4*. The other transformants that were retrieved from these transformations still showed a high number of ectopic integrations of the bipartite fragments, resulting in several bands on the Southern blots (data not shown).

The fact that only one transformant was successfully deleted in the target locus, indicates that the transformation procedures in *A. niger* are far from optimum, because of non-homologous endjoining. Efforts towards deleting the non-homologous end joining pathways have been made [7]. This has been shown to be a good strategy for deleting target-specific genes.

Strain characterization

The *acl* deletion strain was compared with the wild-type N402 strain in two different batch set-ups. The carbon sources tested were glucose and xylose and the cultivations were run under oxygen-limiting conditions. $NaHCO_3$ was used as base control, because it can stimulate carboxylation reactions. An overview of the batch profiles of the different fermentations is shown in Fig. 2. The first observation during the fermentation was that the *Δacl* strain consumed

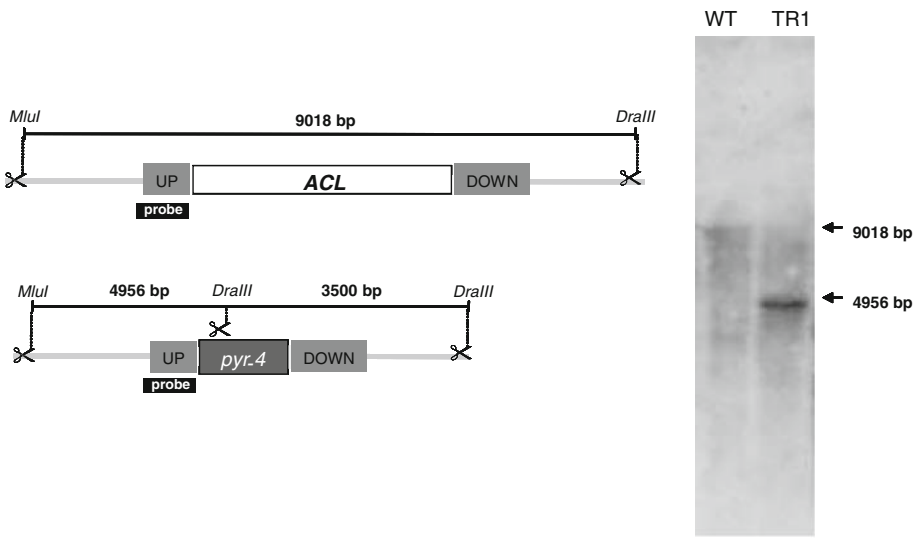

Fig. 1 On the *left side* the genomic region of the target locus, its length, and the restriction enzymes used for Southern blot analysis are shown before and after the deletion. No direct repeats were used in this deletion. On the *right side* the Southern blot is presented with the *first lane* containing the WT band and the *second lane* containing the deletion band

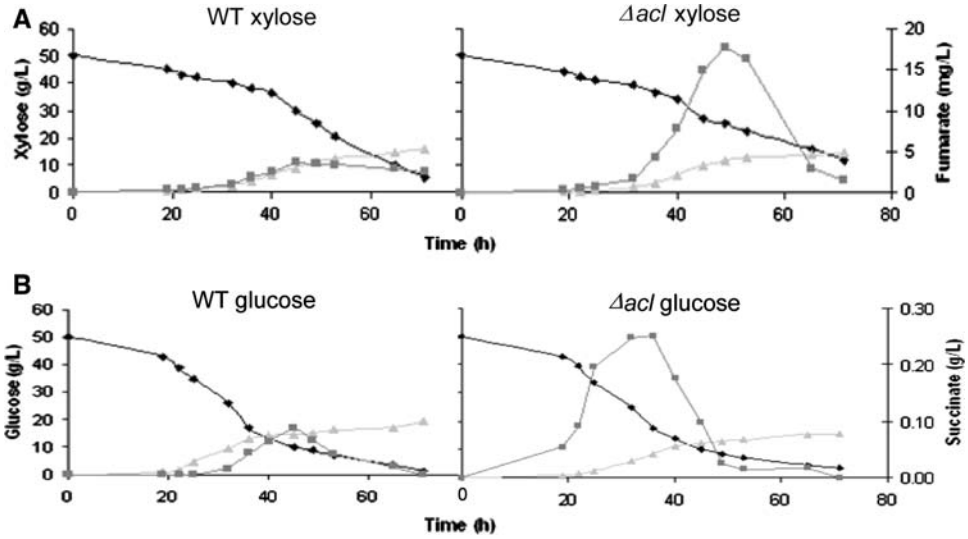

Fig. 2 Batch profiles of carbon source consumption, biomass, and organic acid production. **a** *Filled diamonds*, xylose consumption; *filled triangles*, biomass production; *filled squares*, fumarate production. **b** *Filled diamonds*, glucose consumption; *filled triangles*, biomass production; *filled squares*, succinate production

twice as much base as the wild-type during growth on both glucose and xylose. This indicated that more acids were produced. The increased acid production could be verified by HPLC analysis (Table 1). It was observed that the ratio of the acids changed when the carbon source was changed. The high acetate yield obtained in the deletion strain grown on glucose is likely to be caused by activation of the PDC enzyme. The enzyme activity of PDC was measured in a previous study, where it was shown to be inactive under oxygen-limiting conditions [6]. The stoichiometric metabolic model indicated a deletion of both *pdc* and *acl* in order to improve the succinate yield. This indicates the value of the stoichiometric model, because rationally it was decided to delete only *acl*, because PDC has shown no activity under the oxygen-limited cultivation conditions. However, as soon as *acl* is deleted the metabolism needs to redirect its fluxes and this study indicates that the PDC enzyme might be activated thereby forming acetate.

Additionally, the total amount of acid being produced differed between the two strains. Looking at the total amount of organic acid measured, it is observed that during growth on xylose the *Δacl* strain produced more acids than the wild-type (48 and 43 C-mmol, respectively). During growth on glucose, the difference in amount of organic acids produced was more pronounced. Here a total acid production of 66 C-mmol was observed in the wild-type compared with 138 C-mmol in the *Δacl* strain. Thus, using glucose as carbon source increased acid production in the deletion strain more than twofold. When using glucose as C-source no pyruvate could be detected, indicating that there is no limitation at the interface between the glycolysis and the TCA cycle, which might have resulted in the production of organic acids. One of the organic acids produced was citrate, which is known to be preferentially produced during growth on glucose. The *acl* deletion also seemed to stimulate citrate production during growth on xylose, but

the yield was significantly lower than the yields achieved during growth on glucose. During growth on xylose, pyruvate could be measured, indicating that there is flux control at the entry into the TCA cycle, and this may explain the reduced amount of total organic acid production compared with cultivation on glucose.

It was observed that succinate was not produced when xylose was used as carbon source. On the other hand, using glucose as C-source resulted in production of small amounts of succinate (0.10–0.25 g L^{-1}) (Fig. 2). The deletion of *acl* increased the succinate yield more than threefold, supporting the model prediction.

Although the succinate yield was significantly increased, it did not reach the maximum yields (>0.7 g g^{-1} carbon source) predicted by the model. One explanation might be that only *acl* was deleted and not both *acl* and *pdc*, but it is likely also to be a limitation in the model to predict sufficiently correctly the transport of organic acids between the different cellular compartments. Another explanation might be that some reactions are under-estimated and that regulatory effects that are not incorporated in the model affect the metabolic state. Modeling is therefore an iterative process, i.e., the model needs to be adjusted to the experimental results obtained in order to obtain better predictions on cellular functions.

Finally, during growth on xylose, the fumarate yield increased significantly in the deletion strain compared with the wild-type strain, which was not observed during growth on glucose. This is an unexpected result, because in other cases (*E. coli* or *M. succiniciproducens*) succinate and fumarate production are closely related. If succinate is produced through the reductive TCA cycle, fumarate must be produced before succinate. In this study it was observed that fumarate and succinate production were not correlated (Fig. 2), indicating that *A. niger* does not use the reductive TCA cycle for succinate production. More likely it will use the oxidative TCA cycle or the glyoxylate shunt, whereas fumarate production is possibly obtained by the reductive pathway. The fumarate reductase is most likely to be missing or not active in *A. niger*, making it impossible to produce succinate via the reductive pathway under these experimental conditions. No large differences in glycerol and oxalate yield were observed between the different carbon sources and strains. The most prominent change occurred in the TCA cycle and not in other pathways.

Conclusion

A deletion strategy based on a genome-scale stoichiometric metabolic model prediction was investigated in an attempt to increase succinate production by *A. niger* under oxygen-limited conditions. The cytosolic ATP: citrate lyase gene was completely deleted using the bipartite gene-targeting method, thereby removing the cytosolic reaction from citrate towards oxaloacetate and acetate.

The *acl* deletion strain shows increased organic acid production on both glucose and xylose as carbon sources. On glucose a threefold higher succinate yield was observed in the deletion strain compared with the parent strain. This shows that stoichiometric models are valuable for identification of metabolic engineering strategies.

Although *A. niger* can be used as a cell factory for succinate production, further optimization is needed in order to reach levels of industrial relevance. The gene-targeting methods used for *A. niger* in this study were, although successful, not very efficient and disruption of multiple loci would most certainly have been very cumbersome and time-consuming. Since the completion of this work, end-joining deficient strains (Ku mutants) in which gene targeting is highly efficient have been made available for *A. niger*. If multiple genes are to be knocked out, it would be advisable to use these strains if possible. Additionally, there is still a lack of physiological knowledge, which complicates the optimization process. However, through a focused systems biology analysis of the metabolism of *A. niger*, it will be possible to obtain detailed mapping of biochemical reactions that can be used to improve the models that can further enhance metabolic engineering of this fungus.

Acknowledgments The study was kindly supported by The Research School for Biotechnology (FOBI), The Royal Veterinary and Agricultural University (KvL), and Novozymes funding.

References

1. Baker SE (2006) *Aspergillus niger* genomics: past, present and into the future. Med Mycol 44:S17–S21. doi:10.1080/13693780600921037
2. Ballance DJ, Buxton FP, Turner G (1983) Transformation of *Aspergillus nidulans* by the orotidine-5′-phosphate decarboxylase gene of *Neurospora crassa*. Biochem Biophys Res Commun 112(1): 284–289
3. David H, Akesson M, Nielsen J (2003) Reconstruction of the central carbon metabolism of *Aspergillus niger*. Eur J Biochem 270(21):4243–4253. doi:10.1046/j.1432-1033.2003.03798
4. Erdeniz N, Mortensen UH, Rothstein R (1997) Cloning-free PCR-based allele replacement methods. Genome Res 7(12):1174–1183. doi:10.1101/gr.7.12.1174
5. Ideker T, Galitski T, Hood L (2001) A new approach to decoding life: systems biology. Annu Rev Genomics Hum Genet 2:343–372
6. Meijer S, Panagiotou G, Olsson L, Nielsen J (2007) Physiological characterisation of xylose metabolism in *Aspergillus niger* under oxygen-limited conditions. Biotechnol Bioeng 98(2):462–475. doi:10.1002/bit.21397
7. Meyer V, Arentshorst M, El-Ghezal A, Drews AC, Kooistra R, van den Hondel CAMJ, Ram AFJ (2007) Highly efficient gene targeting in the *Aspergillus niger kusA* mutant. J Biotechnol 128(4): 770–775. doi:10.1016/j.jbiotec.2006.12.021

8. Nielsen ML, Albertsen L, Lettier G, Nielsen JB, Mortensen UH (2006) Efficient PCR-based gene targeting with a recyclable marker for *Aspergillus nidulans*. Fungal Genet Biol 43(1):54–64. doi:10.1016/j.fgb.2005.09.005
9. Patil KR, Akesson M, Nielsen J (2004) Use of genome-scale microbial models for metabolic engineering. Curr Opin Biotechnol 15(1):64–69. doi:10.1016/j.copbio.2003.11.003
10. Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, de Vries RP, Albang R, Albermann K, Andersen MR, Bendtsen JD, Benen JAE, van den Berg M, Breestraat S, Caddick MX, Contreras R, Cornell M, Coutinho PM, Danchin EGJ, Debets AJM, Dekker P, van Dijck PWM, van Dijk A, Dijkhuizen L, Driessen AJM, d'Enfert C, Geysens S, Goosen C, Groot GSP, de Groot PWJ, Guillemette T, Henrissat B, Herweijer M, van den Hombergh JPTW, van den Hondel CAMJ, van der Heijden RTJM, van der Kaaij RM, Klis FM, Kools HJ, Kubicek CP, van Kuyk PA, Lauber J, Lu X, van der Maarel MJEC, Meulenberg R, Menke H, Mortimer MA, Nielsen J, Oliver SG, Olsthoorn M, Pal K, van Peij NNME, Ram AFJ, Rinas U, Roubos JA, Sagt CMJ, Schmoll M, Sun JB, Ussery D, Varga J, Vervecken W, de Vondervoort PJJV, Wedler H, Wosten HAB, Zeng AP, van Ooyen AJJ, Visser J, Stam H (2007) Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat Biotechnol 25(2):221–231. doi:10.1038/nbt1282
11. Price ND, Papin JA, Schilling CH, Palsson BO (2003) Genome-scale microbial in silico models: the constraints-based approach. Trends Biotechnol 21(4):162–169. doi:10.1016/S0167-7799(03)00030-1
12. Sambrook J, Russell DW (2001) Molecular cloning a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
13. Stephanopoulos G, Alper H, Moxley J (2004) Exploiting biological complexity for strain improvement through systems biology. Nat Biotechnol 22(10):1261–1267. doi:10.1038/nbt1016
14. Vanhartingsveldt W, Mattern IE, Vanzeijl CMJ, Pouwels PH, Vandenhondel CAMJ (1987) Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene. Mol Gen Genet 206(1):71–75