Identification of a classical mutant in the industrial host *Aspergillus niger* by systems genetics: LaeA is required for citric acid production and regulates the formation of some secondary metabolites

Jing Niu*, Mark Arentshorst*, P. Deepa S. Nair*, Ziyu Dai†, Scott Baker‡, Jens C. Frisvad§, Kristian F. Nielsen§, Peter J. Punt***, Arthur F.J. Ram*

*Leiden University, Institute of Biology Leiden, Molecular Microbiology and Biotechnology, Sylviusweg 72, 2333 BE Leiden, The Netherlands
‡Chemical and Biological Process Development Group, 2720 Crimson Way, Pacific Northwest National Laboratory, P.O. Box 999, Richland, WA 99352, USA.
†Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, P.O. Box 999, Richland, WA 99352, USA.
§Department of Systems Biology, Technical University of Denmark, Søltofts Plads 221, 2800 Kgs Lyngby, Denmark.
***Dutch DNA, P.O. Box 360, 3700 AJ Zeist, the Netherlands

Running Title: Citric acid production requires LaeA

Key words: organic acids; filamentous fungi; bulk segregant analysis; parasexual cycle; genome sequencing

Corresponding author:
Dr. Arthur F.J. Ram
Leiden University, Institute of Biology Leiden, Molecular Microbiology and Biotechnology, Sylviusweg 72, 2333 BE Leiden, The Netherlands
tel: + 0031 71 527 4914
a.f.j.ram@biology.leidenuniv.nl

© The Author(s) 2013. Published by the Genetics Society of America.
Abstract

The asexual filamentous fungus *Aspergillus niger* is an important industrial cell factory for citric acid production. In this study, we genetically characterized a UV-generated *A. niger* mutant that was originally isolated as a non-acidifying mutant, which is a desirable trait for industrial enzyme production. Physiological analysis showed that this mutant did not secrete large amounts of citric acid and oxalic acid, thus explaining the non-acidifying phenotype. As traditional complementation approaches to characterize the mutant genotype were unsuccessful, we used bulk segregant analysis in combination with high-throughput genome sequencing to identify the mutation responsible for the non-acidifying phenotype. Since *A. niger* has no sexual cycle, parasexual genetics was used to generate haploid segregants derived from diploids by loss of whole chromosomes. We found that the non-acidifying phenotype was caused by a point mutation in the *laeA* gene. *LaeA* encodes a putative methyltransferase-domain protein, which we show here to be required for citric acid production in an *A. niger* lab strain (N402) and in citric acid production strains. The unexpected link between *LaeA* and citric acid production could provide new insights into the transcriptional control mechanisms related to citric acid production in *A. niger*. Interestingly, the secondary metabolite profile of a Δ*laeA* strain differed from the wild-type strain, showing both decreased and increased metabolite levels, indicating that *LaeA* is also involved in regulating the production of secondary metabolites. Finally, we show that our systems genetics approach is a powerful tool to identify trait mutations.
1. Introduction

*Aspergillus niger* is an biotechnologically important filamentous fungus and is used as an industrial cell factory for the production of organic acids and enzymes (Pel *et al.* 2007; Andersen *et al.* 2011). A key characteristic of *A. niger* is the rapid acidification of the culture medium during exponential growth owing to the secretion of mainly gluconic acid, citric acid, and oxalic acid, resulting in a pH below 2.0 in uncontrolled batch cultures. The medium acidification has some important consequences for the behavior of *A. niger* as a cell factory because both organic acid production and enzyme production are highly dependent on the ambient pH. For further reading about the metabolic pathways involved in organic acid biosynthesis we refer to two recent reviews (Kubicek and Karaffa, 2010; Li and Punt, 2013). The genome sequence of the citric acid production wild-type strain (ATCC1015) has been determined, and a spontaneous mutant of this strain (ATCC11414) was used for subsequent studies of citric acid production (Perlman *et al.* 1946; Baker, 2006; Andersen *et al.* 2011). Organic acid production is highly dependent on medium composition and, interestingly, also on the environmental pH. Under laboratory conditions using bioreactor-controlled fermentation, the pH can be maintained at a fixed value, and this revealed that the production of specific organic acids is clearly pH-dependent. Citric acid production is optimal at low pH (2.0) (Karaffa *et al.*, 2003; Magnuson and Lasure, 2004), and requires high glucose and low manganese concentrations (de Ruijter *et al.* 1999; Andersen *et al.*, 2009). Oxalic acid production is most efficient between pH 5.0 and 8.0 and absent at pH 3.0 (de Ruijter *et al.* 1999; Andersen *et al.*, 2009). Production of gluconic acid is also pH-dependent and optimal at pH6.0, but absent at pH 2.5 (Andersen *et al.*, 2009). Gluconic acid and citric acid can be metabolized by *A. niger*, while oxalic acid is not taken up and metabolized and accumulates in the medium (Poulsen *et al.* 2012). Therefore, oxalic acid accumulation is the main cause of the acidification of the medium during the late growth phases of batch cultures. Indeed, an *A.*
A. niger mutant in which oxalic acid synthesis was abolished through inactivation of the oxaloacetate hydrolase (oahA) gene behaves as a non-acidifying mutant (Pedersen et al., 2000; Andersen et al., 2009; Li et al., 2013).

Ambient pH is also an important environmental factor influencing the expression of extracellular enzymes (van den Hombergh et al., 1996; Peñalva and Arst, 2002). As a saprophytic fungus, A. niger is well known for its ability to secrete enzymes that are required for the decay of organic plant-derived polysaccharides and proteins. The influence on ambient pH on protease production has been studied in more detail and it has been shown that at pH 4.0 and lower, protease activity is high. Protease activities are lower at pH 5.0 and decrease further at pH 6.0 (Braaksma and Punt, 2008; Braaksma et al., 2009). The genes encoding the major extracellular proteases pepA and pepB are induced under acidic conditions (Jarai and Buxton, 1994; van den Hombergh et al., 1997a, 1997b). The regulation of proteases is not only dependent on ambient pH but is also controlled by nutrient conditions, including nitrogen source and carbon availability (Braaksma et al., 2009; van den Hombergh et al., 1997a).

Several wide domain transcription factors are involved, including the nitrogen regulator AreA (MacCabe et al., 1998; Lenouvel et al., 2001), the general repressor TupA (Schachtschabel et al., 2013), the carbon repressor protein CreA, and PacC (Fraissinet-Tache et al., 1996). In A. nidulans, PacC is an transcriptional activator of alkaline-induced genes, and as a repressor of acid-induced genes (Peñalva and Arst, 2002). Analysis of the expression of protease genes in pacC mutants of A. niger has indicated the involvement of PacC in the regulation of pepA and pepB (Fraissinet-Tache et al., 1996). In addition, a gene encoding a protease-specific, positive-acting transcription factor required for the induction of several protease-encoding genes, including pepA and pepB, has been identified. A strain carrying a mutation in this gene, prtT, was identified in a mutant screen for protease-minus mutants (Punt et al., 2008). The prtT gene
encodes a Zn(II)$_2$Cys$_6$ transcription factor, and controls in combination with the wide-domain regulators (CreA, AreA, TupA and PacC) the expression of protease genes.

The *prtT* mutant was isolated by a classical forward genetic mutant screen for protease mutants (Mattern *et al.* 2002). Such screens are still powerful tools to identify new and unexpected gene functions. To identify the gene mutated in a particular mutant, complementation analysis with genomic libraries is traditionally used. Such a genomic cosmid library is also available for *A. niger* and has been successfully used before (e.g. Punt *et al.* 2008; Damveld *et al.* 2008; Meyer *et al.* 2009). However, several problems are encountered in identifying complementation mutants either because the gene might be lacking in the library or because of complications in screening thousands of transformants for complementation. Whole genome sequencing is an alternative method for identifying the mutation that is responsible for a particular phenotype. As classical mutagenesis might also result in mutations unrelated to the phenotype, several researchers have used bulk segregant analysis to identify the relevant mutations. This method was first developed in plant genetics (Michelmore *et al.* 1991) and, subsequently, used in combination with next-generation sequencing in various other organisms including *S. cerevisiae* (Wenger *et al.* 2010; Dunham, 2012), filamentous fungi (Pomraning *et al.* 2011, Nowrousian *et al*., 2012, Bok *et al*., 2014), and insects (Park *et al.* 2014). In this approach, the mutant of interest is crossed to a wild-type strain, haploid segregants displaying the phenotype of interest are pooled, and DNA from this pool of segregants is sequenced using deep sequencing (e.g. Illumina). In addition to the pooled segregants, the two parental strains from the cross are sequenced, and single-nucleotide polymorphism (SNP) analysis between the two parental strains is performed. The SNP that causes the phenotype will be conserved in all the progeny displaying the phenotype (homozygous SNP), while mutations that are not related to the phenotype will have a 50% chance to be present in the genomic DNA of the pool (heterozygous SNPs). SNPs that are
located close to the mutations of interest will co-segregate and can only separate via recombination. Since *A. niger* lacks a sexual cycle, we have used the parasexual cycle of *A. niger* (Pontecorvo *et al.* 1953; Bos *et al.* 1988) to generate a pool of segregants. In this study we have used bulk segregant analysis combined with Illumina sequencing to characterize the non-acidifying mutant D15 mutant of *A. niger*. Here we show that a mutation in the *laeA* gene causes the non-acidifying phenotype of the D15 mutant and that the loss of *laeA* strongly affects the production of secondary metabolites in *A. niger*.

2. Materials and methods

2.1. Strains, media, and molecular methods

*A. niger* strains used in this study are listed in Table 1. Because of the complexity of the strain background of the D15 mutant, a schematic overview of the strain lineages is given in Fig 1. Strains were grown on minimal medium (MM) (Bennett and Lasure, 1991), containing 1 % (w v\(^{-1}\)) glucose, or on complete medium (CM), containing 2 % (w v\(^{-1}\)) glucose, 0.5 % (w v\(^{-1}\)) yeast extract and 0.1 % (w v\(^{-1}\)) casamino acids in addition to MM. When required, plates or medium were supplemented with 10 mM uridine or 0.2 mg/ml arginine. Plates were incubated at 30°. Skim milk, MacConkey agar plates to assay acidification contain MM+glucose medium without nitrate (ASP-N) (Arentshorst *et al.* 2012) supplemented with 1% skim milk (Difco) and 2% MacConkey agar. Pre-acidified (pH 3.0) skim milk, MacConkey agar plates were used to assay protease activity. The pH was set at 3.0 by the addition of hydrogen chloride. Citric acid production (CAP) medium was prepared as described previously (Dai *et al.* 2004).

Amplification of plasmid DNA was performed using the XL1-Blue strain, which was transformed using the heat-shock protocol as described (Inoue *et al.* 1990). Transformation of *A. niger* was performed as described by (Arentshorst *et al.* 2012), using 40 mg lysing enzyme
isolated as described previously (Arentshorst et al. 2012). The (α-32P)dCTP-labeled probes were synthesized using the Rediprime II DNA labeling system (Amersham Pharmacia Biotech, Piscataway, NJ), according to the instructions of the manufacturer. All molecular techniques were carried out as described previously (Sambrook et al. 1989). Sequencing was performed by Macrogen Europe (Amsterdam, The Netherlands).

2.2. Construction of plasmids and strains

D15#26 was transformed with the fwnA::hygB disruption plasmid (Jørgensen et al. 2011a) to generate MA273.1 (prtT-13, pyrG378, ΔfwnA::hygB, non-acidifying). Strain JN3.2 (olvA::pyrG, argB::hygB) was obtained by disrupting the argB gene of A. niger (Lenouvel et al. 2001) in AW8.1 (Jørgensen et al. 2011a). Details for the disruption of argB in JN6.2 will be published elsewhere (J. Niu and A.F.J. Ram, unpublished results).

Disruption of the laeA gene (An01g12690) in the N402 background was carried out using the split-marker approach (Arentshorst et al. 2015). The 910-bp-long 5’-flank and 901-bp-long 3’-flank regions were amplified using the primers listed in Table S1. These PCR fragments were used in a fusion PCR with the A. oryzae pyrG gene (pAO4-13) (de Ruiter-Jacobs et al. 1988) to generate the split-marker fragments. After amplification, the 5’flank-pyrG and 3’flank-pyrG fragments were purified from the agarose gel and simultaneously transformed to the recipient A. niger strain AB4.1. Putative laeA disruptions strains were purified by two consecutive single colony streaks. Genomic DNA was isolated as described (Arentshorst et al. 2012) and Southern blot analysis was performed to confirm proper deletion. JN24.6 was used for further experiments.

The ΔlaeA mutant strain in the ATCC11414 background was generated by homologous replacement of laeA in the ATCC11414 ΔkusA derivative (Chiang et al. 2011). The laeA deletion cassette was constructed by PCR amplification of upstream and
downstream regions of the *Aspergillus niger laeA* gene using primers listed in Table S2. The hygromycin resistance marker was amplified from pCB1003 (Fungal Genetics Stock Center) by PCR using the oligonucleotides hph5 and hph3 (Table S2). The DNA fragments were assembled into the backbone plasmid vector of pBlueScript II SK(-), linearized with restriction endonucleases *HindIII* and *PstI* using the Gibson assembly cloning kit (New England Biolabs). The assembled plasmid DNA was transferred into the Top10 *E. coli* competent cells by lithium acetate-mediated transformation (Life Technologies). The transformed bacterial colonies were screened for the DNA fragment insertion by restriction endonuclease digestion with *PvuII* and *XhoI*. The $\Delta$laeA cassette was isolated from plasmid DNA by digestion with endonucleases *HindIII* and *XbaI* for *A. niger* transformation. After purification of hygromycin-resistant transformants, proper laeA deletion strains were identified via diagnostic PCR using primers laeAsc5 and laeAsc3 (Table S2).

The vector for complementing the non-acidifying phenotype of the D15 mutant (pJN32) was made by amplifying the laeA gene including promoter and terminator sequences with primers laeA(EcoRI)5f and laeA(EcoRI)6r. The 3139-bp-long PCR fragment was then cloned into pJet1.2 (blunt-end cloning vector) and this was verified by DNA sequencing. Subsequently, the PCR fragment was excised from pJet1.2-laeA using *EcoRI* and inserted into *EcoRI*-digested plasmid pAO4-13 to give pJN33 and transformed to the recipient *A. niger* strains MA273.1 and D15#26. JN24.6 was complemented using the same vector by performing co-transformation with the hygromycin resistance gene-containing plasmid pAN7.1.

To sequence the oahA gene in the D15 mutants, two primers (Table S1) were designed to amplify the open reading frame including 1-kb flanking regions. The PCR fragment was cloned in pJet2.1 and fully sequenced.
2.3. *A. niger* genetics and analysis of segregants

Parasexual crossings were performed as described (Bos *et al.* 1988), with minor modifications. Selecting of a balanced heterokaryon of a cross between MA273.1 (*prtT*-13, *pyrG*378, *AfwnA::hygB*, non-acidifying) and JN3.2 (*olvA::pyrG, argB::nicB*) was performed on MM after pre-growth of both strains for 36 h in 0.5 ml CM containing uridine and arginine. The mycelial mat was fragmented using toothpicks and incubated for 7 days on MM. Spores from heterokaryotic mycelium were carefully isolated to prevent fragmentation of the mycelia, filtered over a double miracloth filter and plated out on selective MM. Using two color marker-containing, haploid strains, we could identify diploids visually by selecting colonies that exclusively formed black spores. A resulting diploid (JN20), was haploidized by adding benomyl (0.6 µg/ml) to CM supplemented with uridine and arginine. Haploid segregants (fawn- or olive-colored sectors) were purified and genotypically analyzed for conidial spore color, *pyrG* and *argB* auxotrophies, acidification, and protease production. Non-acidifying segregants were collected and in total 140 non-acidifying segregants were obtained. Seventy-eight segregants were individually grown in complete medium and from each strain 200 mg fresh weight mycelium were collected for genomic DNA isolation. Mycelium of ~20 strains (4 gram of mycelium) was mixed and grinded, and genomic DNA was isolated. Equal amounts of DNA of each of the four pools was pooled together to obtain the genomic DNA pool for sequencing. Genomic DNA from D15#26 and JN3.2 and the pools was further purified using Macherey-Nagel NucleoBond Xtra columns and used for DNA sequencing.

2.4 DNA sequencing and data analysis

Illumina Paired-End sequencing was performed by ServiceXS using Illumina kits (cat# 1001809 and 1005063) and protocols according to the instructions provided by the supplier.
The quality and yield after sample preparation were checked and were consistent with the expected size of 300 bp after excision from the gel. Clustering and DNA sequencing using Illumina cBot and HiSeq 2000 were performed according to manufacturer’s protocols. Two sequencing reads of 100 cycles each using Read1 sequencing and Read2 sequencing primers were performed with the flow cell. For strains MA273.1 and JN3.2, 4.0 Gb of DNA sequence was obtained. Two separate pools of segregant DNA, consisting of 10.3 and 13.4 Gb of DNA sequence, respectively, were separately sequenced. All raw high-throughput sequence data will be deposited in the SRA database. Image analysis, base calling, and quality check were performed with the Illumina data analysis pipeline RTA v1.13.48 and/or OLB v1.9/CASAVA v1.8.2. Based on the mapped reads, variants in the sample data were detected by comparison with the reference genome of ATCC1015 (http://genome.jgi-psf.org/pages/search-for-genes.jsf?organism=Aspni5), and between the samples by using an in-house SNP pipeline v3.2 (ServiceXS). Validated variants must be consistently found in one location in at least one sample with a frequency of 0.7 or higher, in at least 20 overlapping reads (minimum coverage) with no quality filtering, before it is reported as a SNP. The combined pool sample (23.7 Gb) was processed with a minimal variant frequency of 0.3. For each SNP, it was verified whether the SNP was in a predicted protein-encoding region using the A. niger 3.0 genome at JGI and the SNP coordinates.

2.5 Culture conditions and metabolite analysis

Controlled bioreactor cultivations for A. niger N402 and D15#26 were performed as previously described, using fixed pH values varying from pH 2 to pH 7 (Braaksma et al. 2009). Organic acid analyses were performed as described previously (Li et al. 2013). Shake flask cultures containing 50 ml of MM were inoculated with 5 x 10⁷ spores and incubated at 30⁰ at 150 rpm. For each sampling time point an individual flask was inoculated to determine
biomass accumulation, and culture pH, and to sample medium for acid and metabolite analysis. Protease activities of culture medium samples were measured using the P-check assay at pH 2.7 according to the instructions by the supplier (Jena BioScience). Broth samples of N402, AB1.13, D15 and ΔlaeA taken at 96 h were analyzed for secondary metabolite production. A 5.0 ml sample of fermentation broth (including biomass) was diluted with 5.0 ml isopropanol (LC-MS grade, Sigma-Aldrich), placed in an ultrasonication bath for 20 min, and centrifuged at 4,000 x g for 5 min. A 1-mL subsample was transferred to a 2-ml HPLC vial. For secondary metabolite analysis, N402, AB1.13, D15 and ΔlaeA were grown on YES or CYA agar in darkness at 25˚C for 7d, three plugs of approx. 0.6 cm² culture were sampled and extracted using ethyl-acetate-dichloromethane-methanol, evaporated to dryness, and redissolved in methanol (Nielsen et al. 2009).

Samples were then analyzed by liquid chromatography-high resolution mass spectrometry on Agilent 1290 infinity UHPLC (Agilent Technologies, Torrence, CA) equipped with an Agilent Poroshell 120 phenyl-hexyl column (250 mm × 2.1 mm, 2.7 µm particles), running an acidic water/acetonitrile gradient. This was coupled to an Agilent 6550 Q-TOF-MS equipped with an ESI source and operated in positive polarity and sampling m/z 50-1700 in full scan and auto MS/MS mode (Kildgaard et al. 2014). Compounds were then identified by MS/HRMS spectra and retention time (Kildgaard et al. 2014), and peaks integrated using Agilent Quant Analysis 6.0 as described (Nielsen and Larsen, 2015).

Cultivation to measure citric acid production under citric acid production conditions were performed in glass baffled shake flasks of 250 ml, which were silanized with 200 ml of a 5% solution of dichlorodimethylsilane in heptane to minimize leaching of metals. A. niger strains were grown in 75 ml CAP media containing 10 ppb Mn²⁺ at 30º and 200 rpm. Samples for citric acid analysis were taken after 5 days of growth. Citric acid concentrations
were determined with an end-point spectrophotometric enzyme assay as described previously (Bergmeyer, 1985) using five microliters of each culture supernatant.

3. Results

3.1 Isolation of a non-acidifying A. niger strain D15#26

In a gene-expression study aimed at overproduction of bacterial levansucrase using cotransformation of low-protease mutant A. niger AB1.13 (Mattern et al. 1992; Punt et al. 2008) with the A. niger pyrG gene, a non-acidifying A. niger transformant showing increased growth on medium with inulin as sole carbon source was isolated (E. Wanker and P. Punt, unpublished results). Acidification of the medium by A. niger can be easily visualized by using MacConkey agar milk plates. These plates contain dissolved milk powder; they are clear at the initial pH of about 5, but form a white precipitate when the pH in the plate decreases to below 4.0. Growth of the wild-type strain and accompanying acidification of the medium results in a white precipitate around the colony while in the D15 mutant no precipitate is formed (Fig 2).

The mutant, displaying a non-acidifying phenotype, was crossed to A. niger strain N879 and a non-acidifying pyrG, prtT segregant (D15#26) was selected for further studies. Southern analysis of this segregant showed that this segregant did not carry any additional remnants of the pyrG gene copies used in the transformant experiment that gave rise to strain D15 (data not shown). Another effect of the reduced acidification of this strain was that also the total protease activity was further reduced compared to the low-protease host strain AB1.13. Culture pH and total proteolytic activities of batch-cultured N402, AB1.13 and D15 strains were analyzed in time. As shown in Table 2, the pH of the culture medium of the D15 strain remains around 6.5 whereas the N402 and the AB1.13 strains show the typical acidification of the medium. Proteolytic activity in the culture medium was assayed using the P-check assay.
Proteolytic activity was reduced in the AB1.13 mutant and further reduced to about 10% of the wild-type level in the D15 mutant (Table 2).

3.2. The non-acidifying phenotype in D15 is not caused by a mutation in the oahA gene

A low-protease, non-acidifying *A. niger* mutant was previously isolated by van den Hombergh and co-workers (van den Hombergh *et al*. 1995). This mutant, named prtF, lacks oxaloacetate acetylhdrolase activity and it was shown that this strain was mutated in the oahA gene (Ruijter *et al*. 1999). Linkage analysis assigned the prtF mutation to linkage group V (van den Hombergh *et al*. 1995). Linkage analysis of the D15 mutant by carrying out a parasexual cross with tester strain N879 (Table 1) revealed that the non-acidifying phenotype was linked to the argH12 marker on linkage group II (12.5% recombination) (P. J. Punt, unpublished results), indicating that the two mutants are affected in different loci. To make sure that the oahA gene was not mutated in the D15 mutant, the oahA gene (An10g00820) including 1,000-nucleotide-flanking regions was PCR-amplified from D15 and sequenced. No mutation in the gene was found, indicating that the mutation in D15 is not located in the oahA locus.

3.3. Physiological analysis of the D15 mutant

The non-acidifying phenotype of the D15 mutant was compared with N402 and a ΔoahA mutant (Li *et al*. 2013) during batch growth using shake flask cultures. During growth, the unbuffered medium of the wild-type strain acidified quickly to reach a pH value of 3.5. At later time points (72 h after inoculation), the pH of the wild-type strains stabilized around 5.4. The pH of the culture medium of the D15 mutant remained between 5.5 and 6.5 during the cultivation period (Table 2), while the pH of the oahA mutant strain increased from pH 4.5 to pH 8 (data not shown). HPLC analysis of the medium samples at different time points
confirmed that the levels of citric acid and oxalic acid were reduced at the different time points in the D15 strain, whereas in the oahA strain citric acid was produced at even higher levels than in N402, and no oxalic acid was produced (data not shown). These physiological results also show that the genotype of D15 differs from the oahA mutant.

To analyze the profile of organic acids produced during controlled batch growth, the N402 strain and the D15 strain were cultivated in bioreactors at fixed pH values under the conditions described in Materials and Methods. Since gluconic acid, oxalic acid, and citric acid are the main organic acids secreted into the medium, these acids were quantified by HPLC analysis. As shown in Table 3, the production of organic acids in the D15 mutant is strongly reduced. Production of citric acid was low in all samples and probably caused by high manganese concentrations and low glucose concentrations, both of which are known to diminish citrate production (Dai et al. 2014). Citric acid secretion was observed in N402 at all pH values, whereas no citric acid could be detected in the medium of the D15 mutant. At pH 3 and 4, oxalic acid was not detected in D15 medium whereas gluconic acid levels were either similar (at pH 3.0) or reduced (at pH 4.0) compared to the wild-type (N402). At pH 5.0, 6.0, and 7.0, oxalic acid was again reduced in D15 medium compared to N402 medium. At these higher pH values, the D15 mutant produced similar amounts of gluconic acid. Growth of the N402 strain is severely reduced at pH 5.0 (2.8 g biomass/liter) in comparison to D15. At pH 5.0 the N402 strain produced high amounts of gluconic acid, and the base had to be added to maintain the pH at 5.0. At pH 5.0 the D15 mutant still grew relatively well (13 g biomass/liter) and did not secrete high amounts of gluconic acid. It should be noted that at pH 4.0 and 5.0 both strains also produced detectable levels of other unidentified acids. Based on these results it is clear that the mutation in D15 caused considerable and complex physiological alterations in organic acid production, suggesting a mutation in a regulatory
circuit governing primary metabolism. This hypothesis encouraged further research to elucidate the genetic background of the mutant strain.

3.4 Isolation of segregants for bulk segregation analysis using next generation sequencing

To facilitate the isolation of a diploid strain to generate segregants for bulk sequencing analysis, mutant D15#26 was first transformed with the \textit{fwnA::hygB} deletion cassette (Jørgensen et al. 2011a). The \textit{fwnA} gene encodes the polyketide synthase involved in conidial melanin synthesis, and a fawn-colored transformant was purified. This strain (MA273.1) produces fawn-colored conidiospores and also contains the \textit{pyrG} auxotrophic marker. MA273.1 was crossed with JN3.2 (\textit{olvA::pyrG, argB::hygB}). Using the complementary color markers (\textit{fwnA} and \textit{olvA}) and the complementary auxotrophies (\textit{pyrG} and \textit{argB}), a diploid was isolated from heterokaryotic mycelium. The resulting black-conidiating, prototrophic, diploid strain (JN20) acidified the medium, showing that the non-acidifying trait in D15 was recessive (Fig 2).

To obtain a collection of D15-derived segregants, diploid strain JN20 was point-inoculated on complete medium, supplemented with uridine and arginine, in the presence of benomyl. Benomyl affects microtubule dynamics, and growth of an \textit{A. niger} diploid strain in the presence of sub-lethal concentrations of benomyl results in spontaneous haploidization by the loss of one of each pair of the eight chromosomes. The use of complementary spore color mutants allows easy identification of haploid sectors as these sectors display the spore color marker (Bos et al. 1998). From each point-inoculated diploid, a maximum of two segregants with different colors (fawn or olive) were purified. In total, 140 segregants were collected, purified, and analyzed for their spore color, \textit{pyrG} and \textit{argB} auxotrophies, acidification phenotype, and their protease production phenotype (Table S2). The possible genotypes of
First, we determined if all markers were more or less equally represented in the segregants. As shown in Table 4, roughly equal numbers of segregants were found for both alleles of the markers. The conidial color markers \textit{fwnA} and \textit{olvA} are localized on different arms of linkage group I and no haploid recombinants producing black spores were isolated in our segregants. Two possible \textit{fwnA}/\textit{olvA} double mutants were detected in the segregants since such \textit{fwnA} mutants are \textit{pyrG}%, indicating that they might also harbor the \textit{olvA::pyrG} disruption (Table S2). Table 5 presents the results from the marker linkage analysis. Because the \textit{olvA} gene is disrupted by the \textit{pyrG} gene, all \textit{olvA} strains are \textit{pyrG}%. The \textit{argB} gene is on the same linkage group as the \textit{olvA} marker (linkage group I), explaining the observed linkage of \textit{olvA} and \textit{argB}. We also noticed the strong coupling of the \textit{fwnA::hygB} disruption with the \textit{pyrG} gene. The \textit{pyrG} is reported to be localized on the left arm of linkage group III, but our data show strong linkage between the \textit{pyrG} marker and the \textit{fwnA} marker (Table 5). Possibly, the \textit{pyrG} gene in our strain is translocated to linkage group I, which could explain the linkage. Further research is required to clarify this, but the possible translocation has no effect on linkage analysis of the non-acidifying mutation in D15. The linkage analysis also showed that the non-acidifying phenotype is not linked to linkage group I (\textit{fwnA}, \textit{olvA} and \textit{argB}) and is not linked to the \textit{prtT} mutation (linkage group VI) (Punt \textit{et al.}, 2008) as expected. From the 140 segregants, 78 displayed the non-acidifying phenotype, indicating that this phenotype is caused by a single mutation. These 78 segregants were individually grown and fresh weight mycelium of each strain was collected. Pooled mycelium of about 20 strains was used for genomic DNA purification. An equal amount of DNA of each of the four pools was combined to obtain the genomic DNA pool for sequencing.

### 3.5 SNP analysis of parental strains and bulk segregants
The genomes of parental strains (MA273.1 and JN3.2) were sequenced and SNP analysis was performed as described in Materials and Methods. The reads were mapped to the genome sequence of the *A. niger* strain ATCC1015 as this strain is most similar to the N400/N402 background (Andersen et al. 2011). In total, 52 SNPs were identified between the two parental strains. We also expected to identify the mutation in the *prtT* gene, which was previously shown to be a single point mutation (T to C), causing an amino acid change (leucine (CTA) to proline (CCA)) in the PrtT protein (Punt et al. 2008). Indeed, as indicated in Table S4, we found again the SNP in the D15 mutant that is responsible for the *prtT* phenotype. Subsequently, we looked for homozygous SNPs within the pool of segregants.

Theoretically, the mutation responsible for the phenotype should be completely conserved in the pools of segregants, whereas SNPs not related to the phenotype should have a 50% chance to be present. As shown in Table S4, three SNPs were found to be completely conserved. All three mapped to the right arm of linkage group II. Three other SNPs in linkage group II showed a high (~98%), but not absolute conservation. Apparently, these SNPs are linked to our trait of interest, but a few segregants have been recombined between the SNP and our gene of interest and therefore have lost complete conservation. The linkage of the conserved SNPs to linkage group II is consistent with the observed linkage to linkage group II when crossed to marker strain N879 (see above). Further examination of the three SNPs that were fully conserved showed that only a single SNP at position 1762101 (G to C) was present in a protein-encoding region, corresponding to gene An01g01260. The protein encoded by this gene is the predicted ortholog of LaeA, a well-studied putative methyltransferase in several fungal species (see discussion). The mutation results in an amino acid change at position 327 (alanine (GCC) to proline (CCC)) in the *A. niger* LaeA protein. The alanine residue at this position is conserved among twenty *Aspergillus* species (www.aspgd.broadinstitute.org).
3.6 Complementation and disruption analysis

To show that the non-acidifying phenotype of the D15 mutant was caused by the mutation in \textit{laeA}, the D15#26 mutant was complemented with the \textit{laeA} gene. The \textit{laeA} gene, including an ~1,000-nucleotide promoter and terminator region was PCR-amplified and cloned into pAO4-13 containing the \textit{pyrG} gene from \textit{A. oryzae}. Transformation of the plasmid to MA273.1 or D15#26 restored the ability to acidify the medium, indicating that \textit{laeA} complements the non-acidifying phenotype of the D15 mutant (Fig 2).

The \textit{laeA} gene was also inactivated by targeted deletion. Bipartite gene deletion fragments were generated as described in Materials and Methods and transformed to AB4.1. Transformants were purified and analyzed for their acidification phenotype on MacConkey agar plates. Several transformants were isolated that did not acidify the medium and these mutants were shown to be deleted in the \textit{laeA} gene by Southern blot (Fig S1). The \textit{ΔlaeA} mutant was also cultivated in shake flask cultures as described for the D15 and the N402 strains (see above). Similar as the D15 mutant, the pH of the \textit{ΔlaeA} culture remained between 5.5 and 6.5 during the entire cultivation period. Organic acid analysis of the medium samples of the \textit{ΔlaeA} also confirmed that the levels of citric and oxalic acid were reduced (data not shown). Both the complementation experiment and the targeted deletion of \textit{laeA} show that the mutation in \textit{laeA} in the D15 mutant is responsible for the acidification defect in the D15 mutant.

In order to assess the effect of \textit{laeA} deletion under classical citric acid production conditions, we used strain ATCC 11414, which is a spontaneous derivative of ATCC 1015 (Dai et al, 2004; Baker, 2006). Proper deletion of \textit{laeA} in the ATCC11414 background was verified via diagnostic PCR (data not shown). Under low-manganese, high-glucose conditions, parent strain can produce significant amounts of citrate. Deletion of \textit{laeA} in this background resulted in a complete absence of citrate production in comparison to the parental
strain, which made 30g/l citric acid, indicating that LaeA is also required under high-citrate production conditions (Fig. 3A). Deletion of laeA in the ATCC11414 grown under citric acid-producing conditions altered the morphology of the culture. Whereas ATCC11414 formed pellets, which is the typical morphology during citric acid-producing conditions, pellets in the ΔlaeA strain were smaller and the mycelium was much more dispersed (Fig. 3B).

3.7. Secondary metabolite profile of the laeA mutant in A. niger

Previous studies have shown that the putative protein methyltransferase LaeA affects the expression of multiple secondary metabolite gene clusters in several fungi (55-60). We observed when working with the D15 or ΔlaeA strains that plate-grown mycelium is yellowish and not greyish as seen in the wild-type and that in submerged cultures of the ΔlaeA mutant the medium turns purple (Fig. 4). It is also apparent from Fig. 4 that deletion of laeA did not result in an obvious growth defect under these conditions. To determine the role of laeA in A. niger in relation to secondary metabolite production, the production of secondary metabolites in wild-type and laeA mutants on three different media and culture conditions was analyzed. These conditions include submerged cultivation in nitrate-based minimal medium (subMM), and cultivation on solid media (Yeast Extract Sucrose (YES) agar) and Czapek Yeast Autolysate (CYA) agar (see Material and Methods). We tested different media because it has been shown that these can have a pronounced effect on the production of secondary metabolites (Nielsen et al. 2011, Andersen et al. 2013). From this analysis, we could consistently identify seventeen compounds in the wild-type strains (Table 6). Nine of the seventeen of the compounds were detected under all three growth conditions, five compounds were detected on both YES and CYA agar, two compounds were detected on YES agar only, and one compound was only detected in subMM (Table 6). After establishing the secondary metabolite profile in the wild-type, it was possible to identify secondary metabolites whose...
production is affected by the absence of laeA by comparing the profiles of the D15 strain and the \( \Delta laeA \) strain (both \( laeA^- \)) to the profiles of the original parental strain (N402) and the AB1.13 strain (\( laeA^+ \)). As indicated in Table 4, the presence or abundance of the majority of the secondary metabolites (11 out of 17) was not dramatically altered in the \( \Delta laeA \) or D15 strain compared to the wild-type strains. Table S5 presents the identified compounds, including peak areas for each compound. Two compounds, BMS-192548 and aspernigrin A are produced in much higher amounts in the \( laeA \) mutants compared to the wild-type strains, indicating that \( laeA \) has a repressive function for the expression of genes related to the production of these secondary metabolites. Three compounds, asperrubrol, atromentin and JBIR86 require LaeA, indicating that LaeA is involved in activating expression of the gene clusters responsible for the synthesis of these compounds. Interestingly, the requirement of LaeA for the production of these compounds is conditional and growth on YES agar medium bypasses the requirement of LaeA as also observed by us for \( A. fumigatus \) (K.F. Nielsen and J.C. Frisvad, unpublished). The production of tensidol B on CYA was absent in the \( \Delta laeA \) mutant, while in the D15 mutant, which contains a point mutation in the \( laeA \) gene, tensidol B was still produced. The results indicate that the LaeA protein of \( A. niger \) can affect the expression of secondary metabolite gene clusters both positively and negatively.
4. Discussion

Owing to its low production of proteases the \textit{A. niger} D15 mutant has been used in various studies of the production of heterologous proteins (Rose \textit{et al.} 2002; Record \textit{et al.} 2003; Benoit \textit{et al.} 2007; Chimphango \textit{et al.} 2012; Turbe-Doan \textit{et al.} 2013; Benghazi \textit{et al.} 2014; Piumi \textit{et al.} 2014; Zwane \textit{et al.} 2014). The D15 strain does not only contain a mutation in the protease regulator gene \textit{(prtT)} (Punt \textit{et al.} 2008) but also a mutation leading to a non-acidifying phenotype and, consequently, low levels of acid-induced proteases in the medium.

Several attempts have been made to identify the mutation in the D15 mutant by complementation analysis, using a specific \textit{A. niger} genomic cosmid library that has been successfully used before (Punt \textit{et al.} 2008; Damveld \textit{et al.} 2008; Meyer \textit{et al.} 2009). However, complementation of the D15 non-acidifying phenotype was not successful, partly due to the problems involved in screening for complementation. We therefore decided to use whole genome sequencing to identify the responsible mutation. The strain lineage of the D15 mutant is rather complex (Fig. 1) and therefore, we used a bulk segregant approach, which narrows down the genomic region responsible for the phenotype. Whole genome sequencing in combination with genetic crosses to reduce the number of SNPs for further investigation has recently been used for mutant identification in \textit{N. crassa} (Pomraning \textit{et al} 2011), \textit{Sordaria macrospora} (Nowrousian \textit{et al.}, 2012), and \textit{A. nidulans} (Bok \textit{et al.}, 2014), either via a pooled-segregant approach (Pomraning \textit{et al} 2011, Nowrousian \textit{et al.}, 2012), or via successive backcrossings (Bok \textit{et al.}, 2014) using the sexual cycle. Since \textit{A. niger} does not have a sexual cycle, which is normally used to obtain segregants, we employed the parasexual cycle of \textit{A. niger} to generate segregants (Pontecorvo \textit{et al.} 1953). For bulk segregant analysis, a pool of 78 non-acidifying segregants was used. The size of the pool turned out to be sufficient to narrow down the homozygous SNPs to a 1.6-MB DNA region on chromosome II. This region contained only three fully homozygous SNPs (S4 Table). Three other SNPs on chromosome
II were clearly genetically coupled to the three fully conserved SNPs, but the coupling up to 97 to 98 % indicated the occurrence of mitotic recombination in the diploid or during haploidization of the diploid. Since the occurrence of mitotic recombination is low, a mitotic cross-over involving the SNPs on chromosome II in a 1.4-MB region probably occurred only in a single segregant (out of 78). To further narrow down the number of relevant SNPs, a larger pool of segregants or the use of chemicals such as neomycin or 5-azacytidine (van de Vondervoort et al. 2007) to induce mitotic recombination might be used. However, in view of the relatively low number of SNPs found in the D15 mutant (52 in total), we were left with only a few candidate genes. It is interesting to note that the mutation at position 1762101 at chromosome II is located in gene An01g06900. This gene encodes a Zn(II)$_2$Cys$_6$ transcription factor (FumR), which is located in the fumonisin gene cluster. In the orthologous fumonisin gene cluster in *Fusarium verticillioides* this transcription factor is required for fumonisin production (Brown et al. 2007). Secondary metabolite analysis of the AB1.13 and D15 revealed the absence of fumonisin in the AB1.13 and D15 mutants and its presence in N402 and ΔlaeA (Table S5). It is tempting to speculate that the mutation in the intron sequence of An01g06900 (already present in the AB1.13 mutant and its derivative D15) affects proper processing of mRNA leading to a truncated and inactive FumR protein and inability to produce fumonisin.

The role of LaeA in organic acid production as shown in this paper is not completely unprecedented. In *A. oryzae* it has been shown that deletion of the *laeA* homolog resulted in the loss of kojic acid production (Oda et al. 2011). The gene cluster likely to be involved in the synthesis of kojic acid production (AO09113000136, FDA-dependent oxidoreductase; AO09113000137, transcription factor; and AO09113000138, transporter protein) is severely down-regulated in the ΔlaeA mutant of *A. oryzae*. A role for LaeA in citric acid production in *A. niger* is supported by the observation that overexpression of *A. nidulans laeA* in *A. niger*
results in a 40% increase in citric acid production (Dai and Baker, 2015). The increased
production of citric acid upon laeA overexpression and the reduced production in the laeA
deletion strain offer interesting possibilities to identify genes directly involved in citric acid
production by transcriptomic or proteomic studies. Whether LaeA directly regulates genes
involved in citric acid production, or whether its role is more indirect e.g. by affecting fungal
morphology or by sensing the triggers that induce citric acid formation (low manganese, high
glucose, etc) is still not clear.

LaeA was initially identified as a regulator of secondary metabolism in A. nidulans
(Bok and Keller, 2004). Deletion of laeA in A. nidulans blocks the expression of several
metabolic gene clusters, including gene clusters involved in sterigmatocystin, penicillin, and
lovastatin biosynthesis, as grown on minimal media (Bok and Keller, 2004). Its role as a
global regulator of secondary metabolism has been established in various filamentous fungi,
including A. flavus (Kale et al. 2008), A. oryzae (Oda et al. 2011), A. fumigatus (Perrin et al.
2007), Penicillium chrysogenum (Kosalková et al. 2009), Penicillium citrinum (Xing et al.
2010), Fusarium fujikuroi (Wiemann et al. 2010), F. verticillioides (Butchko et al. 2012),
Trichoderma reesei (Karimi-Aghcheh et al. 2013) and Cochliobolus heterostrophus (Wu et
al. 2012). Yet another, but probably related function of LaeA, has to do with its role in A.
nidulans as a member of the Velvet Complex, which consists of the LaeA, VeA, and VeB
proteins, and controls asexual and sexual developmental pathways (Bayram and Braus, 2012).
Under light, LaeA is required for reduction of the VeA and VeB levels in order to stimulate
asesexual development. Conversely, in the absence of LaeA, VeA and VeB protein levels are
not repressed, leading to sexual development and the formation of cleistothecia (Bayram and
Braus, 2012). The role of LaeA in controlling gene expression is not necessarily restricted to
secondary metabolites and development. Transcriptome analysis of the laeA mutant in T.
reesei revealed that LaeA also controls the expression of extracellular enzymes (Seiboth et al,
2012), while LaeA in *P. chrysogenum* was found to affect chitinase expression (Kamereward *et al.*, 2011). Whether and to which extent LaeA is involved in extracellular protein production in *A. niger* remains to be determined. As many of the extracellular enzymes in *A. niger* are highly expressed in an acidic environment, it is important to conduct these studies under pH-controlled conditions. It is further important to establish to which extent the differences in secondary metabolite production in *A. niger* are directly caused by *laeA* deletion or whether the differences in secondary metabolite production are an indirect consequence of a different ambient pH.

Deletion of *laeA* in *A. niger* affects the production of several secondary metabolites. From the seventeen identified secondary metabolites, the production of six secondary metabolites was affected. Three compounds (aperrubrol, atromentin and JBIR86), from three very different pathways (aperrubrol is from the mixed polyketide-terpene pathway, atromentin from the shikimic acid pathway, whereas JBIR86 is amino acid-derived) were found to be produced in lower amounts in the *laeA* mutant, in agreement with the role of LaeA as a global regulator required for the biosynthesis of secondary metabolites (Bok and Keller, 2004, Bok *et al* 2006). Interestingly, deletion of *laeA* also leads to increased production of two secondary metabolites (BMS-192548 and aspernigrin A). A similar role for LaeA as a negative regulator of the production of some secondary metabolites has also been reported for *C. heterostrophus* and *F. fujikuroi*, in which deletion of *laeA* is resulted in increased melanin and bikaverin production, respectively, (Wu *et al*., 2012, Wiemann *et al*., 2010).

The link between LaeA and production of citric acid or secondary metabolites changes our view of citric acid production in *A. niger* as a process belonging to the primary metabolism. Both citrate and oxalic acid precursor oxaloacetate play essential roles in the tricarboxylic acid cycle and are therefore genuine primary metabolites. However, our results point to a possible uncoupling of citric acid and oxalic acid production by alternative, LaeA-
controlled metabolic pathways. Since growth of the ΔlaeA mutant is not severely reduced, it is clear that primary metabolism in ΔlaeA is not dramatically affected. LaeA’s specific role in citric acid production further suggests considering the production of citric acid in A. niger as a process belonging to the secondary metabolism. Oxalic acid production from oxaloacetate without involvement of the tricarboxylic acid cycle has also been previously reported (Kunbichek et al., 1988). The chelating properties of both oxalic acid and citric acid and the corresponding ecological role of these acids in their natural habit as well as the highly specific stress conditions that are required for citric acid production, support such a view. In addition, the gene clusters are frequently involved in the production of secondary metabolites. Interestingly, the gene clusters responsible for the production of itaconic acid and kojic acid have been found in A. terreus (Li et al. 2011) and A. oryzae, respectively (Oda et al. 2011) and this has supported the view that itaconic acid and kojic acids are secondary metabolites. With the laeA mutant and the laeA-overexpressing strain now available, we can search further for LaeA target genes involved in organic acid production in A. niger and other fungi.

5. Acknowledgements

We thank Idriss Iziyi, Abeer Hossain, Ulrike Gericke, and Karin Overkamp for technical assistance. Peter van de Vondervoort, Fons Debets, and Kees van den Hondel are acknowledged for advices and stimulating discussions. We thank Frans Klis for helpful comments on the manuscript. We are grateful to Agilent technologies for the Thought Leader Donation of the Agilent UHPLC-QTOF system. Jing Niu is supported by a grant from the China Scholarship Council.

6. References
   *Aspergillus niger* to ambient pH. Genome Biol. 10: R47

   Thykaer, *et al.*, 2011, Comparative genomics of citric-acid-producing *Aspergillus niger*
   ATCC 1015 versus enzyme-producing CBS 513.88. Genome Res. 21: 885–897.

   Hansen, *et al.*, 2013, Accurate prediction of secondary metabolite gene clusters in

   835: 133–150.

5. Arentshorst, M., J. Niu, A. F. Ram, 2015, Efficient generation of *Aspergillus niger*
   knock out strains by combining NHEJ mutants and a split marker approach. In: van den
   Berg M.A. and Maruthachalam K., editors. Genetic Transformation Systems in Fungi,

   Mycol Suppl 1 44: S17–S21.

7. Bayram, O., G. H. Braus, 2012, Coordination of secondary metabolism and
   development in fungi: the velvet family of regulatory proteins. FEMS Microbiol Rev.
   36: 1–24.

8. Benghazi, L., E. Record, A. Suárez, J.A. Gomez-Vidal, J. Martínez, T. de la Rubia,
   2014, Production of the *Phanerochaete* flavido-alba laccase in *Aspergillus niger* for
   synthetic dyes decolorization and biotransformation. World J Microbiol Biotechnol. 30:
   201–211.


Table 1. *Aspergillus niger* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N402</td>
<td>cspA1 derivative of ATCC9029</td>
<td>Bos <em>et al.</em> 1988</td>
</tr>
<tr>
<td>N879</td>
<td>fwnA1, argH12, pyrA5, leuA1, pheA1, lysD25, oliC2, crnB12</td>
<td>Bos <em>et al.</em> 2003</td>
</tr>
<tr>
<td>AB4.1</td>
<td>pyrG378 in N402</td>
<td>van Hartingsveldt <em>et al.</em> 1987</td>
</tr>
<tr>
<td>AB1.13</td>
<td>prtT-13, pyrG378</td>
<td>Punt <em>et al.</em> 2008</td>
</tr>
<tr>
<td>AB1.13-<em>pyrG</em></td>
<td>prtT-13</td>
<td>Punt, unpublished</td>
</tr>
<tr>
<td>MA169.4</td>
<td>ΔkusA::DR_amdS_DR, pyrG378</td>
<td>Carvalho <em>et al.</em> 2010</td>
</tr>
<tr>
<td>D15#26</td>
<td>prtT-13, pyrG378, non-acidifying</td>
<td>this study</td>
</tr>
<tr>
<td>D15#26-<em>pyrG</em></td>
<td>prtT-13, non-acidifying</td>
<td>Punt, unpublished</td>
</tr>
<tr>
<td>MA273.1</td>
<td>prtT-13, pyrG378, ΔfwnA::hygB, non-acidifying</td>
<td>this study</td>
</tr>
<tr>
<td>JN26.1</td>
<td>prtT-13, pyrG378, ΔfwnA::hygB non-acidifying, pAO4-13-LaeA</td>
<td>this study</td>
</tr>
<tr>
<td>AB1.13ΔoahA#76</td>
<td>ΔoahA::pyrG#76 in AB1.13</td>
<td>Li <em>et al.</em> 2013</td>
</tr>
<tr>
<td>AW8.4</td>
<td>olvA::pyrG in MA169.4</td>
<td>Jørgensen <em>et al.</em> 2011a</td>
</tr>
<tr>
<td>JN3.2</td>
<td>argB::hygB in AW8.4</td>
<td>Jing, unpublished</td>
</tr>
<tr>
<td>JN20</td>
<td>diploid MA273.1 x JN3.2</td>
<td>this study</td>
</tr>
<tr>
<td>JN21.1</td>
<td>D15#26 pAO4-13</td>
<td>this study</td>
</tr>
<tr>
<td>JN22.7</td>
<td>D15#26 pAO4-13-LaeA</td>
<td>this study</td>
</tr>
<tr>
<td>JN24.6</td>
<td>ΔlaeA in AB4.1kusA::AfpyrG</td>
<td>this study</td>
</tr>
<tr>
<td>KB1001</td>
<td>kusA::pyrG</td>
<td>Chiang <em>et al.</em> 2011</td>
</tr>
<tr>
<td>KB1001ΔlaeA</td>
<td>ΔlaeA::hygB in KB1001</td>
<td>this study</td>
</tr>
</tbody>
</table>
Table 2. Culture pH and relative protease activity during batch growth

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>24h</th>
<th>48h</th>
<th>72h</th>
<th>96h</th>
<th>120h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pH</td>
<td>pH</td>
<td>pH</td>
<td>pH</td>
<td>pH</td>
</tr>
<tr>
<td></td>
<td>Relative</td>
<td>protease activity*</td>
<td>Relative protease activity</td>
<td>Relative protease activity</td>
<td>Relative protease activity</td>
<td></td>
</tr>
<tr>
<td>N402</td>
<td>-</td>
<td>3.7</td>
<td>39%</td>
<td>4.4</td>
<td>57%</td>
<td>4.6</td>
</tr>
<tr>
<td>AB1.13</td>
<td>*</td>
<td>3.5</td>
<td>23%</td>
<td>4.1</td>
<td>25%</td>
<td>4.0</td>
</tr>
<tr>
<td>D15#2</td>
<td>prtT, nac'</td>
<td>6.3</td>
<td>5%</td>
<td>6.6</td>
<td>5%</td>
<td>6.3</td>
</tr>
</tbody>
</table>

*Relative protease activity expressed as percentage of the protease activity in the culture fluid of wild type (N402) after 120 h of growth. Protease acidity was determined using the P-check assay. For the growth experiments pyrG+ (uridine-prototrophic strains) were cultivated. nac' = non-acidifying.
Table 3. Physiological parameters of pH-controlled bioreactor cultivations of *A. niger* strains, and medium levels of the main three organic acids (gluconic, oxalic, and citric acid)

<table>
<thead>
<tr>
<th>Strain</th>
<th>pH</th>
<th>Cultivation</th>
<th>Use of acid/base</th>
<th>EFT hours</th>
<th>dwt g/L</th>
<th>gluconic acid g/L</th>
<th>oxalic acid g/L</th>
<th>citric acid g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>D15</td>
<td>3</td>
<td>acid</td>
<td></td>
<td>45</td>
<td>15</td>
<td>1.3</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>N402</td>
<td>3</td>
<td>acid</td>
<td></td>
<td>42</td>
<td>10.9</td>
<td>3.1</td>
<td>1.2</td>
<td>0.16</td>
</tr>
<tr>
<td>D15</td>
<td>4</td>
<td>acid</td>
<td></td>
<td>49</td>
<td>17</td>
<td>1.0</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>N402</td>
<td>4</td>
<td>acid</td>
<td></td>
<td>79</td>
<td>15</td>
<td>0.9</td>
<td>1.9</td>
<td>0.7</td>
</tr>
<tr>
<td>D15</td>
<td>5</td>
<td>acid</td>
<td></td>
<td>42</td>
<td>13</td>
<td>12.6</td>
<td>0.5</td>
<td>n.d</td>
</tr>
<tr>
<td>N402</td>
<td>5</td>
<td>base</td>
<td></td>
<td>49</td>
<td>2.8</td>
<td>30.5</td>
<td>4.3</td>
<td>1.6</td>
</tr>
<tr>
<td>D15</td>
<td>6</td>
<td>base</td>
<td></td>
<td>42</td>
<td>2.4</td>
<td>36.4</td>
<td>0.8</td>
<td>n.d</td>
</tr>
<tr>
<td>N402</td>
<td>6</td>
<td>base</td>
<td></td>
<td>48</td>
<td>1.8</td>
<td>32.2</td>
<td>4.4</td>
<td>0.16</td>
</tr>
<tr>
<td>D15</td>
<td>7</td>
<td>base</td>
<td></td>
<td>48</td>
<td>2</td>
<td>39.0</td>
<td>1.6</td>
<td>n.d</td>
</tr>
<tr>
<td>N402</td>
<td>7</td>
<td>base</td>
<td></td>
<td>63</td>
<td>1.7</td>
<td>43.2</td>
<td>3.4</td>
<td>1.2</td>
</tr>
</tbody>
</table>

EFT = elapsed fermentation time; dwt = dry weight; n.d = not detected
Table 4. Distribution of marker alleles among the 140 segregants

<table>
<thead>
<tr>
<th>Marker</th>
<th># of segregants</th>
<th># of segregants</th>
</tr>
</thead>
<tbody>
<tr>
<td>fwnA/olvA *</td>
<td>64 fwnA -</td>
<td>76 olvA -</td>
</tr>
<tr>
<td>pyrG</td>
<td>78 pyrG +</td>
<td>62 pyrG -</td>
</tr>
<tr>
<td>argB</td>
<td>64 argB +</td>
<td>76 argB -</td>
</tr>
<tr>
<td>non-acidifying</td>
<td>62 acidifying</td>
<td>78 non-acidifying</td>
</tr>
<tr>
<td>prtT</td>
<td>68 prtT +</td>
<td>72 prtT -</td>
</tr>
</tbody>
</table>

* Segregants are either fwnA - or olvA - due to the tight coupling of both markers even though the markers are located on two different sides of the centromer of chromosome III.
Table 5. Pairwise marker analysis of the diploid strain JN20 (MA273.1 x JN3.2)

<table>
<thead>
<tr>
<th></th>
<th>fwnA</th>
<th>pyrG</th>
<th>+</th>
<th>nac'</th>
<th>prtT</th>
</tr>
</thead>
<tbody>
<tr>
<td>MA273.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JN3.2</td>
<td>olvA</td>
<td>+</td>
<td></td>
<td>argB</td>
<td>+</td>
</tr>
</tbody>
</table>

The frequencies of pairwise gene combination are shown in the lower left half of the table. For each gene combination the number of parental segregants (PS) or non-parental segregants (NPS) are indicated in the top left/bottom right (PS) or top right/bottom left (NPS), respectively. In the upper right half the recombination frequencies are given. Recombination frequencies are calculated as the number or non-parental segregants/total number of segregants x 100%; 140 segregants were analyzed.
Table 6. Identified secondary metabolites under different growth condition in *A. niger* and the effect of *laeA* inactivation on their production.

<table>
<thead>
<tr>
<th>Secondary metabolite</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aurasperone B</td>
<td>Production not affected by LaeA</td>
</tr>
<tr>
<td>Funalenone</td>
<td>Production not affected by LaeA</td>
</tr>
<tr>
<td>Kotanin*</td>
<td>Production of end product (Kotanin) not affected</td>
</tr>
<tr>
<td>Demethylkotanin*</td>
<td>Not present in ΔlaeA</td>
</tr>
<tr>
<td>Orlandin*</td>
<td>Not present in ΔlaeA</td>
</tr>
<tr>
<td>Asperrubrol</td>
<td>Production in subMM and CYA requires LaeA</td>
</tr>
<tr>
<td>Fumonisins B2/B4*</td>
<td>Production not affected by LaeA</td>
</tr>
<tr>
<td>Pyranopyrrol A</td>
<td>Production not affected by LaeA</td>
</tr>
<tr>
<td>Tensidol B</td>
<td>Production on CYA requires LaeA; production not affected in D15</td>
</tr>
<tr>
<td>Nigerazine</td>
<td>Production not affected by LaeA</td>
</tr>
<tr>
<td>Fungisporin A</td>
<td>Production not affected by LaeA</td>
</tr>
</tbody>
</table>

**Expressed only on agar conditions**

<table>
<thead>
<tr>
<th>Secondary metabolite</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atromentin</td>
<td>Production on CYA requires LaeA</td>
</tr>
<tr>
<td>Pyranonigrin S</td>
<td>Production not affected by LaeA</td>
</tr>
<tr>
<td>Pestalamide C</td>
<td>Production not affected by LaeA</td>
</tr>
<tr>
<td>JBIR86</td>
<td>Production on CYA requires LaeA</td>
</tr>
<tr>
<td>Nigragilin</td>
<td>Production not affected by LaeA</td>
</tr>
</tbody>
</table>

**Expressed only on YES**

<table>
<thead>
<tr>
<th>Secondary metabolite</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrophen</td>
<td>Production not affected by LaeA</td>
</tr>
<tr>
<td>Aspernigrin B</td>
<td>Production on CYA detected in <em>ΔlaeA</em></td>
</tr>
</tbody>
</table>

**Expressed only in subMM**

<table>
<thead>
<tr>
<th>Secondary metabolite</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMS-192548*</td>
<td>Production 1000x increased in SubMM in <em>ΔlaeA</em> and detected in CYA</td>
</tr>
</tbody>
</table>

**Expressed only in *ΔlaeA***

<table>
<thead>
<tr>
<th>Secondary metabolite</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspernigrin A</td>
<td>Production on CYA detected in <em>ΔlaeA</em></td>
</tr>
</tbody>
</table>

1 Considered as one group of secondary metabolites; 2 Fumonisins not detected in AB1.13, possibly because of mutation in the Fum gene cluster; 3 Minor amount detected in N402 on YES agar, not detected in AB1.13
Legends to the figures

Figure 1. Schematic overview of the lineage of the D15 mutant and its derivatives. The genotypes of the strains are given in Table 1.

Figure 2. MacConkey agar milk plates for assaying medium acidification. The milk powder in MacConkey milk agar plates remains soluble at pH >5.0. Acidification around the colony results in precipitation of the skim milk.

Figure 3. Citric acid levels of ATCC11414 and the ATCC11414ΔlaeA strain. A) Bar graph showing the results of citric acid production after 5 days in citric acid-production culture medium of the parent strain (ATCC11414-kusA), and the laeAΔ mutant. The data for each strain are the average of at least three biological replicates. B) and C) The effects of laeA deletion on A. niger morphology. The conidia (1x10⁶ conidia/ml) were inoculated into 75 ml of citric acid-production medium in 250-ml silanized baffled flasks and shaken at 200 rpm and 30° for 5 days. Pellet formation from each culture was determined microscopically after 5 days of growth.

Figure 4. Secretion of secondary metabolites by the A. niger laeA mutant on MM agar plates and MM-shake flask cultures. Spores of the wild type and mutant were streaked to single colonies on MM agar plates and incubated at 30° for five days. For batch cultures, spores were inoculated at a density of 1x10⁶ spores/ml and grown at 30° for five days.

S1 Table: Primers used in this study
S2 Table: Phenotype of 140 segregants
S3 Table: Genotypes of segregants

S4 Table: Overview of SNP analysis in the parental strains and the pool of segregants.

S5 Table: Secondary metabolites. Numbers indicate peak areas of given metabolites, which represent a relative amount of the particular secondary metabolite.

S1 Figure. Verification of the laeA deletion strain in the N402 background. A) Strategy and primer design for disruption of the laeA gene using the split-marker method (Arensthorst et al. 2015). B) Schematic representation of the laeA locus of the wild type and after laeA deletion. Predicted sizes of the DNA fragments hybridizing with the indicated probes are shown. C) Southern blot analysis of genomic DNA of AB4.1 (lane 1), JN23.1 (lane 2) and JN24.6 (lane 3). Left panel: agarose gel stained with ethidium bromide. MW = molecular weight marker size (in kb). Right panel: Southern blot after hybridization.
Figure 1

UV irradiation (van Hartingsveldt et al., 1987)

UV irradiation (Mattern et al., 1992)

Transformation pAB4.1

AB4.1

Targeted deletion ku70 (Carvalho et al., 2010)

AB1.13

pyrG, prtT

MA169.4

pyrG, ku70::amdS

Targeted deletion olvA (Jorgensen et al., 2011)

D15

prrt, nacA

N879

N879/D15

Targeted deletion fwnA (this study)

D15#26

pyrG, prtT, nacA

MA273.1

fwnA::hygB, pyrG, prtT, nacA

JN3.2

olvA::pyrG, argB::hygB, ku70::amdS

JN20 (diploid)

fwnA'/fwnA'; olvA'/olvA', argB'/argB', pyrG'/pyrG'; ku70'/ku70'; prtT'/prtT'; nacA'/nacA

Haploidisation

JN20 segregants
Figure 2

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>AB4.1</td>
<td>D15#26</td>
<td>JN3.2</td>
<td>MA273.1</td>
<td></td>
</tr>
<tr>
<td>pyrG-</td>
<td>prtT, nacA, pyrG-</td>
<td>olvA, argB</td>
<td>fwnA, prtT, nacA, pyrG-</td>
<td></td>
</tr>
</tbody>
</table>

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>JN20</td>
<td>JN24.6</td>
<td>JN21.1</td>
</tr>
<tr>
<td>diploid</td>
<td>ΔlaeA</td>
<td>D15#26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pAOpyrG</td>
</tr>
</tbody>
</table>

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>JN22.7</td>
<td>D15#26</td>
</tr>
<tr>
<td></td>
<td>pAOpyrG-laeA</td>
</tr>
</tbody>
</table>
Figure 3
Figure 4

Top view          Bottom view

N402

ΔlaeA