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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 E. Baker,‡ Jens C. Frisvad,§ Kristian F. Nielsen,§ Scott E. Baker,** and Arthur F.J. Ram*,1

*Molecular Microbiology and Biotechnology, Institute of Biology Leiden, Leiden University, 2333 BE, Leiden, The Netherlands, †Chemical and Biological Process Development Group, and ‡Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, Washington 99352, §Department of Systems Biology, Technical University of Denmark, 2800 Kgs Lyngby, Denmark, and **Dutch DNA Biotech, 3700 AJ Zeist, The Netherlands

**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 nonacidifying 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 nonacidifying 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 nonacidifying 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 nonacidifying 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 other 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.

**KEYWORDS** organic acids filamentous fungi bulk segregant analysis parasexual cycle genome sequencing

*Aspergillus niger* is a 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. 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 has revealed that the...
production of specific organic acids is clearly pH-dependent. Citric acid production is optimal at low pH (2.0) (Karaffa and Kubicek 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 pH 6.0, 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. 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. Oxalic acid production is most efficient between pH 5.0 and 8.0, and absent at pH 3.0 (de Ruijter et al. 1999; van den Hombergh et al. 2000; Andersen et al. 2009). Oxalic acid accumulation is the main cause of acidification of the medium during the late growth phases of batch cultures. Indeed, an A. niger mutant in which oxalic acid synthesis was abolished through inactivation of the oxaloacetate hydrolase (oahA) gene behaves as a nonacidifying mutant (Pedersen et al. 2000; Andersen et al. 2009; Li et al. 2013).

| Strain | Description | Reference |
|--------|-------------|-----------|
| N402   | cspA1 derivative of ATCC9029 | Bos et al. 1988 |
| N879   | fwnA1, argH12, pyrA5, leuA1, pheA1, lysD25, oilC2, cmrB12 | Bos et al. 1993 |
| AB4.1  | pyrG378 in N402 | van Hartingsveldt et al. 1987 |
| AB1.13 | pyrG378, nonacidifying | Punt et al. 2008 |
| AB1.13-pyrG* | MA169.4 | Carvalho et al. 2010 |
| D15#26 | pyrG378, nonacidifying | This study |
| D15#26-pyrG* | MA273.1 | This study |
| JN26.1 | pyrG378, nonacidifying | This study |
| JN26.1ΔoahA#76 | ΔoahA::pyrG76 in AB1.13 | Li et al. 2013 |
| AW8.4  | olvA::pyrG in MA169.4 | Jørgensen et al. 2011 |
| JN3.2  | argB::hygB in AW8.4 | Jing, unpublished |
| JN20   | Diploid | This study |
| JN21.1 | D15#26, pAO4-13-LaeA | This study |
| JN22.7 | D15#26, pAO4-13-LaeA | This study |
| JN24.6 | ΔlaeA in AB4.1, kusA::AfpyrG | This study |
| KB1001 | kusA::pyrG | Chiang et al. 2011 |
| KB1001ΔlaeA | ΔlaeA::hygB in KB1001 | This study |

This table lists the Aspergillus niger strains used in this study.
**MATERIALS AND METHODS**

**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 Figure 1. Strains were grown on minimal medium (MM) (Bennett and Lasure 1991) containing 1% (w/v) glucose, or on complete medium (CM) containing 2% (w/v) glucose, 0.5% (w/v) yeast extract, and 0.1% (w/v) 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°C. Skimmed milk, MacConkey agar plates to assay acidification contained MM + glucose medium without nitrate (ASP-N) (Arentshorst et al. 2012) supplemented with 1% skimmed milk (DFco) and 2% MacConkey agar. Preacidified (pH 3.0) skimmed 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 XL-1-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 (L-1412, Sigma-Aldrich, St. Louis) per g wet weight of mycelium. Genomic DNA was isolated as described (Arentshorst et al. 2012), using 40 mg lysing enzyme (L-1412, Sigma-Aldrich, St. Louis) per g wet weight of mycelium. Genomic DNA was isolated as described (Arentshorst et al. 2012), using 40 mg lysing enzyme (L-1412, Sigma-Aldrich, St. Louis) per g wet weight of mycelium. Genomic DNA was isolated as described (Arentshorst et al. 2012), using 40 mg lysing enzyme (L-1412, Sigma-Aldrich, St. Louis) per g wet weight of mycelium. Genomic DNA was isolated as described (Arentshorst et al. 2012), using 40 mg lysing enzyme (L-1412, Sigma-Aldrich, St. Louis) per g wet weight of mycelium. Genomic DNA was isolated as described (Arentshorst et al. 2012), using 40 mg lysing enzyme (L-1412, Sigma-Aldrich, St. Louis) per g wet weight of mycelium.

**Construction of plasmids and strains**

D15#26 was transformed with the fwaA:hygB disruption plasmid (Jørgensen et al. 2011) to generate MA273.1 (pyrT-13, pyrG378, fwaA::hygB, pyrG, pyrT, Nac). 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. 2011). 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 Supporting Information, Table S1. These PCR fragments were used in a fusion PCR with the A. oryzae pyrG gene (pAO4-13) (de Ruiter-Jaacks et al. 1989) to generate the split-marker fragments. After amplification, the 5′-flank-pyrG and 3′-flank-pyrG fragments were purified from the agarose gel and subsequently transformed to the recipient A. niger strain AB4.1. Putative laeA disruption 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 A. 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 Top10 Escherichia coli competent cells by lithium acetate-mediated transformation (Life Technologies). The transformed bacterial colonies were screened for DNA fragment insertion by restriction endonuclease digestion with PvuII and XhoI. The ΔlaeA 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 nonacidifying phenotype of the D15 mutant (pJN33) was made by amplifying the laeA gene, including promoter and terminator sequences, with primers laeA(EcoR)5f and laeA(EcoR)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 cotransformation with the hygromycin resistance gene-containing plasmid pAN7.1.

To sequence the *olaA* 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.

### 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, pyrG378, Δfwna::hygB*, nonacidifying) and JN3.2 (*olvA::pyrG, argB::nicB*) was performed on MM after pregrowth of both strains for 36 hr in 0.5 ml CM containing uridine and arginine. The mycelial mat was fragmented using toothpicks and incubated for 7 d on MM. Spores from heterokaryotic mycelium were harvested and genotypically scored. A color marker-containing, haploid strains, we could identify diploids 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. Nonacidifying segregants were collected and, in total, 140 nonacidifying segregants were obtained. Seventy-eight segregants were individually grown in complete medium and, from each strain, 200 mg fresh weight mycelia were collected for genomic DNA isolation. Mycelia of ~20 strains (4 g of mycelia) was mixed and ground, and genomic DNA was isolated. Equal amounts of DNA of each of the four pools were 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.

### 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 HiSeq3000 were performed according to manufacturer’s protocols. Two sequencing reads of 150 cycles each using Read1 and Read2 sequencing primers were performed with the flow cell. For strains MA273.1 and JN3.2, 4.0 Gb of DNA sequence were 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. Based on the mapped reads, variants in the sample data were detected by comparison with the genome of *A. niger* MA273.1 (accession AY009652) 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.

### 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 × 10^7 spores and incubated at 30°C 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 supplier’s instructions (Jena BioScience). Broth samples of N402, AB1.13, D15 and ΔlaeA taken at 96 hr 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 ultrasonic bath for 20 min, and centrifuged at 4000 × 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 7 d, 3 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 UPLC (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 2009).

Results

Isolation of a nonacidifying A. niger strain D15#26

In a gene-expression study aimed at overproduction of bacterial levan-sucrase using cotransformation of low-protease A. niger mutant AB1.13 (Mattern et al. 1992; Punt et al. 2008) with the A. niger pyrG gene, a nonacidifying A. niger transformant showing increased growth on medium with inulin as a sole carbon source was isolated (E. Wanker and P. Punt, unpublished results). Acidification of the medium by A. niger can be easily visualized 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 no precipitate is formed in the D15 mutant (Figure 2).

The mutant, displaying a nonacidifying phenotype, was crossed to A. niger strain N879 and a nonacidifying 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 transformation experiment that gave rise to strain D15 (data not shown). Another effect of the reduced acidification of this strain was that 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 remained around 6.5, whereas the N402 and the AB1.13 strains showed 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).

The nonacidifying phenotype in D15 is not caused by a mutation in the oahA gene

A low-protease, nonacidifying A. niger mutant was previously isolated by van den Hombergh and coworkers (van den Hombergh et al. 1995). This mutant, named prtf, lacks oxaloacetate acetylhydrolase 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 nonacidifying 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 1000 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.

Physiological analysis of the D15 mutant

The nonacidifying 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 strains acidified quickly to reach a pH value of 3.5. At later time points (72 hr 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

Table 2 Culture pH and relative protease activity during batch growth

| Strain   | Phenotype | pH | 24 hr Relative Protease Activity | pH | 48 hr Relative Protease Activity | pH | 72 hr Relative Protease Activity | pH | 96 hr Relative Protease Activity | pH | 120 hr Relative Protease Activity |
|----------|-----------|----|---------------------------------|----|---------------------------------|----|---------------------------------|----|---------------------------------|----|---------------------------------|
| N402     | —         | 3.7| 39%                            | 4.4| 57%                             | 4.6| 89%                             | 5.2| 79%                             | 5.3| 100%                            |
| AB1.13   | prtT      | 3.5| 23%                            | 4.1| 25%                             | 4.0| 38%                             | 4.3| 38%                             | 4.8| 48%                             |
| D15#26   | prtT, nac | 6.3| 5%                             | 6.6| 5%                             | 6.3| 9%                             | 6.6| 16%                             | 6.8| 10%                             |

* Relative protease activity expressed as percentage of the protease activity in the culture fluid of wild-type (N402) after 120 hr of growth. Protease acidity was determined using the P-check assay. For the growth experiments, pyrG+ (uridine-prototrophic strains) were cultivated. nac = nonacidifying.
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 the 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 was 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. 2004). 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 was severely reduced at pH 5.0 (2.8 g biomass/liter) 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 was severely reduced at pH 5.0 (2.8 g biomass/liter) 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.

**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)**

| Strain | Cultivation pH | pH Control | EFT hr | dwt g/L | Gluconic Acid g/L | Oxalic Acid g/L | Citric Acid g/L |
|--------|----------------|------------|--------|---------|------------------|----------------|----------------|
| D15    | 3              | Acid       | 45     | 15      | 1.3              | n.d            | n.d            |
| N402   | 3              | Acid       | 42     | 10.9    | 3.1              | n.d            | 0.16           |
| D15    | 4              | Acid       | 49     | 17      | 1.0              | n.d            | n.d            |
| N402   | 4              | Acid       | 79     | 15      | 0.9              | 1.9            | 0.7            |
| D15    | 5              | Acid       | 42     | 13      | 12.6             | 0.5            | n.d            |
| N402   | 5              | Base       | 49     | 2.8     | 30.5             | 4.3            | 1.6            |
| D15    | 6              | Base       | 42     | 2.4     | 36.4             | 0.8            | n.d            |
| N402   | 6              | Base       | 48     | 1.8     | 32.2             | 4.4            | 0.16           |
| D15    | 7              | Base       | 48     | 2      | 39.0             | 1.6            | n.d            |
| N402   | 7              | Base       | 63     | 1.7     | 43.2             | 3.4            | 1.2            |

EFT, elapsed fermentation time; dwt, dry weight; n.d, not detected.

Using the complementary color markers (fwnA and olvA) and the complementary auxotrophies (pyrG and argB), a diploid was isolated from heterokaryotic mycelium. The resulting black-condiating, prototrophic, diploid strain (JN20) acidified the medium, showing that the nonacidifying trait in D15 was recessive (Figure 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 A. niger diploid strain in the presence of sublethal 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. 1988). 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, pyrG, and argB auxotrophies, acidification phenotype, and their pro tease production phenotype (Table S2). The possible genotypes of segregants and the number of segregants with the same genotype are presented in Table S3. 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 fwnA and olvA were localized on different arms of linkage group I, and no haploid recombinants producing black spores were isolated in our segregants. Two possible fwnA/olvA double mutants were detected in the segregants since such fwnA mutants are pyrG+, indicating that they might also harbor the olvA:pyrG disruption (Table S2). Table 5 presents the results from the marker linkage analysis. Because the olvA gene is disrupted by the pyrG gene, all olvA strains are pyrG+. The argB gene is on the same linkage group as the olvA marker (linkage group I), explaining the observed linkage of olvA and argB. We also noticed the strong coupling of the fwnA::hygB disruption with the pyrG gene. The pyrG is reported to be localized on the left arm of linkage group III, but our data show strong linkage between the pyrG marker and the fwnA marker (Table 5).

**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 fwnA::hygB deletion cassette (Jørgensen et al. 2011). The 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 pyrG auxotrophic marker. MA273.1 was crossed with JN3.2 (olvA:pyrG, argB::pyrG).

To acquire further research to elucidate the genetic background of the mutant strain.

**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 fwnA::hygB deletion cassette (Jørgensen et al. 2011). The 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 pyrG auxotrophic marker. MA273.1 was crossed with JN3.2 (olvA:pyrG, argB::pyrG).
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 from each of the four pools was combined to obtain the genomic DNA pool for sequencing.

**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 again found the SNP in the D15 mutant that is responsible for the *prtT* phenotype. Subsequently, we looked for homologous 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 group II. Three other SNPs in linkage group II showed a high (98%), but not absolute conservation. Apparently, these SNPs are linked to our

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**Table 4 Distribution of marker alleles among the 140 segregants**

| Marker       | # of Segregants | # of Segregants |
|--------------|-----------------|-----------------|
| fnvA/olvA    | 64 fnvA         | 76 olvA         |
| pyrG         | 78 pyrG         | 62 pyrG         |
| argB         | 64 argB         | 76 argB         |
| Nonacidifying| 62 Acidifying   | 78 Nonacidifying|
| prtT         | 68 prtT         | 72 prtT         |

Segments are either *fnvA* or *olvA* due to the tight coupling of both markers even though the markers are located on two different sides of the centromere III.

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**Table 5 Pairwise marker analysis of the diploid strain JN20 (MA273.1 (fnvA, pyrG, argB+, nac, prtT) x JN3.2 (olvA, pyrG+, argB, nac+, prtT))**

| markers       | fnvA | olvA | pyrG+ | argB+ | nac+ | prtT+ |
|---------------|------|------|-------|-------|------|-------|
| fnvA          | 64   | 0    | 1%    | 38%   | 47%  |
| olv           | 0    | 76   | 0%    | 0%    | 0%   |
| pyrG+        | 62   | 0    | 2%    | 39%   | 48%  |
| +            | 2    | 76   |       |       |      |
| +            | 63   | 1    | 61%   | 37%   | 47%  |
| argB+        | 1    | 75   | 1%    | 75%   |      |
| +            | 72   | 35   | 27%   | 35%   | 26%  |
| nac+         | 37   | 41   | 35%   | 43%   | 38%  |
| +            | 27   | 35   | 27%   | 35%   | 26%  |
| prtT+        | 26   | 46   | 24%   | 48%   | 26%  |
| +            | 30   | 46   | 24%   | 48%   | 26%  |

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 nonparental 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 nonparental segregants / total number of segregants × 100%. 140 segregants were analyzed.

The *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 *laeA* gene by Southern blot (Figure S1). The Δ*laeA* mutant was also cultivated in shake flasks cultures as described for the D15 and the N402 strains (see above). Similar to the D15 mutant, the pH of the Δ*laeA* culture remained between 5.5 and 6.5 during the entire cultivation period. Organic acid analysis of the medium samples of the Δ*laeA* mutant also confirmed that the levels of citric and oxalic acid were reduced (data not shown). Both the complementation experiment and the targeted deletion of *laeA* show that the mutation in *laeA* in the D15 mutant is responsible for the acidification defect in the D15 mutant.

In order to assess the effect of *laeA* deletion under classical citric acid production conditions, we used strain ATCC11414, which is a spontaneous derivative of ATCC11015 (Dai et al. 2004; Baker 2006). Proper deletion of *laeA* in the ATCC11414 background was verified via diagnostic PCR (data not shown). Under low-manganese, high-glucose conditions, the parent strain can produce significant amounts of citrate. Deletion of *laeA* in this background resulted in a complete absence of citrate production in comparison to the parental strain, which made 30 g/l citric acid, indicating that LaeA is also required under high-citrate production conditions (Figure 3A). Deletion of *laeA* in the ATCC11414 background 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 Δ*laeA* strain were smaller and the mycelium was much more dispersed (Figure 3B).

**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 (Bok and Keller 2004; Sugui et al. 2007; Perrin...
et al. 2007; Bok et al. 2009; Butchko et al. 2012; Karimi-Aghcheh et al. 2013). We observed, when working with the D15 or ΔlaeA strains, that plate-grown mycelium was yellowish and not greyish as seen in the wild-type, and that in submerged cultures of the ΔlaeA mutant the medium turned purple (Figure 4). It is also apparent from Figure 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 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 ΔlaeA 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 ΔlaeA 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, were produced in much higher amounts in the ΔlaeA 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 JBIIR86, 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 results). The production of tensidol B on CYA was absent in the ΔlaeA 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.

DISCUSSION
Owing to its low production of proteases, the A. niger D15 mutant has been used in various studies of the production of heterologous proteins (Gordon et al. 2002; Rose and van Zyl 2002; Record et al. 2003; Benoit

Figure 3 Citric acid levels of ATCC11414 and the ATCC11414ΔlaeA strain. (A) Bar graph showing the results of citric acid production after 5 d in citric acid-production culture medium of the parental strain (ATCC11414-kusA), and the laeA 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 (1 × 10^6 conidia/ml) were inoculated into 75 ml of citric acid-production medium in 250 ml siliconized baffled flasks and shaken at 200 rpm at 30°C for 5 d. Pellet formation from each culture was determined microscopically after 5 d of growth.

Figure 4 Secretion of secondary metabolites by the A. niger laeA mutant on minimal medium (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°C for 5 d. For batch cultures, spores were inoculated at a density of 1 × 10^6 spores/ml and grown at 30°C for 5 d.
**Table 6** Identified secondary metabolites under different growth condition in *A. niger* and the effect of laeA inactivation on their production

| Secondary metabolite               | Remark                                      |
|------------------------------------|---------------------------------------------|
| Aurasperone B                      | Production not affected by LaeA             |
| Funalenone                         | Production not affected by LaeA             |
| Kotanin*                           | Production of end product (Kotanin) not affected |
| Demethylkotanin*                   | Not present in ΔlaeA                       |
| Orilandin*                         | Not present in ΔlaeA                       |
| Aspernubrol                        | Production in subMM and CYA requires LaeA   |
| Fumonisin B2/B4                     | Production not affected by LaeA             |
| Pyranopyrrol A                     | Production not affected by LaeA             |
| Tensidol B                         | Production on CYA requires LaeA; production not affected in D15 |
| Nigerazine                          | Production not affected by LaeA             |
| Fungispiorin A                     | Production not affected by LaeA             |
| **Expressed only on agar conditions** |                                             |
| Atromentin                         | Production on CYA requires LaeA             |
| Pyrononigrin S                     | Production not affected by LaeA             |
| Pestalamide C                      | Production not affected by LaeA             |
| JBR186                             | Production on CYA requires LaeA             |
| Nigraglin                          | Production not affected by LaeA             |
| **Expressed only on YES**           |                                             |
| Pyrophen                           | Production not affected by LaeA             |
| Aspermigrin B                      | Production on CYA detected in ΔlaeA         |
| **Expressed only in subMM**        |                                             |
| BMS-192548c                        | Production 1000x increased in SubMM in ΔlaeA and detected in CYA |
| **Expressed only in ΔlaeA**        |                                             |
| Aspermigrin A                      | Production on CYA detected in ΔlaeA         |

* Considered as one group of secondary metabolites.
* Fumonisin not detected in AB1.13, possibly because of mutation in the Fum gene cluster.
* Minor amount detected in N402 on YES agar, not detected in AB1.13.

...et al. 2007; Chimphango et al. 2012; Turbe-Doan et al. 2013; Benghazi et al. 2014; Piumi et al. 2014; Zwane et al. 2014). The D15 strain does not only contain a mutation in the protease regulator gene (*prtT*) (Punt et al. 2008) but also a mutation leading to a nonacidifying 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 *A. niger* genomic cosmId library that has been successfully used before (Punt et al. 2008; Damveld et al. 2009; Meyer et al. 2009). However, complementation of the D15 nonacidifying 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 (Figure 1), 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 *Neurospora crassa* (Pomranging et al. 2011), *Sordaria macrospora* (Nowroussian et al. 2012), and *A. nidulans* (Bok et al. 2014), either via a pooled-segregant approach (Pomranging et al. 2011; Nowroussian et al. 2012), or via successive backcrossings (Bok et al. 2014) using the sexual cycle. Since *A. niger* does not have a sexual cycle, which is normally used to obtain segregants, we employed the parasexual cycle of *A. niger* to generate segregants (Pontecorvo et al. 1953). For bulk segregant analysis, a pool of 78 nonacidifying 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 (Table S4). 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)Cys₆ 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 an 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 results 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; Sugui et al. 2007), *Penicillium chrysogenum* (Kosalková et al. 2010), *P. citrinum* (Xing et al. 2010), *F. fujikuroi* (Wiemann et al. 2007), *Fusarium 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 asexual 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 (Kamererwerd et al. 2011). Whether and to what 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 what 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 [BIR86], from three very different pathways (aperrubrol is from the mixed polyketide-terpene pathway, atromentin from the shikimic acid pathway, whereas [BIR86] 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, 2009). Interestingly, deletion of laeA also leads to increased production of two secondary metabolites (BMS-192548 and aspergigin 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 resulted in increased melanin and bikaverin production, respectively, (Wu et al. 2012; Wiemann et al. 2010).

The link between LaeA and the production of citric acid or secondary metabolites changes our view of citric acid production in A. niger as a process belonging to primary metabolism. Both citrate and the 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 a need to consider the production of citric acid in A. niger as a process belonging to secondary metabolism. Oxalic acid production from oxaloacetate, without involvement of the tricarboxylic acid cycle, has also been previously reported (Kubicek 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, gene clusters are frequently involved in the production of secondary metabolites. Interestingly, 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.

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LITERATURE CITED
Andersen, M. R., L. Lehmann, and J. Nielsen, 2009 Systemic analysis of the response of Aspergillus niger to ambient pH. Genome Biol. 10: R47.

Andersen, M. R., M. P. Salazar, P. J. Schaap, P. J. I. van de Vondervoorst, D. Culley et al., 2011 Comparative genomics of citric-acid-producing Aspergillus niger ATCC 1015 vs. enzyme-producing CBS 513.88. Genome Res. 21: 885–897.

Andersen, M. R., J. B. Nielsen, A. Kligtgaard, L. M. Petersen, M. Zachariasen et al., 2013 Accurate prediction of secondary metabolite gene clusters in filamentous fungi. Proc. Natl. Acad. Sci. USA 110: E99–E107.

Arentshorst, M., A. F. J. Ram, and V. Meyer, 2012 Using non-homologous end-joining-deficient strains for functional gene analyses in filamentous fungi. Methods Mol. Biol. 835: 133–150.

Arentshorst, M., J. Niu, and A. F. Ram, 2015 Efficient generation of Aspergillus niger knock out strains by combining NHEJ mutants and a split marker approach, pp. 263–272 in Genetic Transformation Systems in Fungi, Vol. 1, edited by van den Berg, M. A., and K. Maruthachalam. Springer International Publishing, Switzerland

Baker, S. E., 2006 Aspergillus niger genomics: past present and into the future. Med. Mycol. 44(Suppl 1): S17–S21.

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

Benghazi, L., E. Record, A. Suárez, J. A. Gomez-Vidal, J. Martinez et al., 2014 Production of the Phanerochaete flavido-alba laccase in Aspergillus niger for synthetic dyes decolorization and biotransformation. World J. Microbiol. Biotechnol. 30: 201–211.

Benoit, I., M. Asther, Y. Bourne, D. Navarro, S. Canaan et al., 2007 Gene overexpression and biochemical characterization of the biotechnologically relevant chlorogenic acid hydrolase from Aspergillus niger. Appl. Environ. Microbiol. 73: 5624–5632.

Bennett, J. W., and L. L. Lasure, 1991 Growth media, pp. 441–458 in More Gene Manipulations in Fungi, edited by Bennett, J. W., and L. L. Lasure. Academic Press, San Diego.

Bergmeyer, H. U., 1985 Metabolites 2: tri- and dicarboxylic acids, purines, pyrimidines and derivatives, coenzymes, inorganic compounds, pp. 5–10 in Citric Acid, edited by Bergmeyer, H. U., and J. Bergmeyer, and Grassl, M. VCH.

Bok, J. W., and N. P. Keller, 2004 2006, LaeA, a regulator of secondary metabolism in Aspergillus spp. Eukaryot. Cell 3: 527–535.

Bok, J. W., D. Noordermeer, S. P. Kale, and N. P. Keller, 2006 Secondary metabolic gene cluster silencing in Aspergillus nidulans. Mol. Microbiol. 61: 1636–1645.

Bok, J. W., Y.-M. Chiang, E. Szewczyk, Y. Reyes-Dominguez, A. D. Davidson et al., 2009 Chromatin-level regulation of biosynthetic gene clusters. Nat. Chem. Biol. 5: 462–464.

Bok, J. W., P. Wiemann, G. S. Garvey, F. Y. Lim, B. Haas et al., 2014 Illumina identification of RsrA, a conserved C2H2 transcription factor coordinating the NapA mediated oxidative stress signaling pathway in Aspergillus. BMC Genomics 15: 1011.

Bos, C. J., A. J. Debets, K. Swart, A. Huybers, G. Kobus et al., 1988 Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in Aspergillus niger. Curr. Genet. 14: 437–443.
Nowrousian, M., J. Teichtert, S. Masloff, and U. Kück, 2012 Whole-genome sequencing of Sordaria macrospora mutants identifies developmental genes. G3 (Bethesda) 2: 261–270.

Oda, K., A. Kobayashi, S. Ohashi, and M. Sano, 2011 Aspergillus oryzae laccase regulates kojic acid synthesis genes. Biosci. Biotechnol. Biochem. 75: 1832–1834.

Park, Y., R. M. Gonzalez-Martinez, G. Navarro-Cerrillo, M. Chakroun, Y. Kim et al., 2014 ABC transporter mediate insect resistance to multiple Bt toxins revealed by bulk segregant analysis. BMC Biol. 12: 46.

Pedersen, H., B. Christensen, C. Hjort, and J. Nielsen, 2000 Construction and characterization of an oxalic acid nonproducing strain of Aspergillus niger. Metab. Eng. 2: 34–41.

Pel, H., J. H. de Winde, D. B. Archer, P. S. Dyer, G. Hofmann et al., 2007 Genome sequencing and analysis of the versatile cell factory Aspergillus niger CBS 513.88. Nat. Biotechnol. 25: 221–231.

Pétalva, M. A., and H. N. Arst, 2002 Regulation of gene expression by ambient pH in filamentous fungi and yeasts. Microbiol. Mol. Biol. Rev. 66: 426–446.

Perlmutter, D., D. A., Kita, and W. H. Peterson, 1946 Production of citric acid from cane molasses. Arch. Biochem. 11: 123–129.

Perrin, M. N., D. N. Fedorova, J. W. Bok, R. A. Cramer, J. R. Wortman et al., 2007 Transcriptional regulation of chemical diversity in Aspergillus fumigatus by LAAe. PLoS Pathog. 3: e50.

Piuni, F., A. Levassieur, D. Navarro, S. Zhou, Y. Mathieu et al., 2014 A novel glucose dehydrogenase from the white-rot fungus Pycnoporus cinnabarinus: production in Aspergillus niger and physicochemical characterization of the recombinant enzyme. Appl. Microbiol. Biotechnol. 98: 10105–10118.

Pomranning, K. R., K. M. Smith, and M. Freitag, 2011 Bulk segregant analysis followed by high-throughput sequencing reveals the Neurospora cell cycle gene, ndc-1, to be allelic with the gene for ornithine decarboxylase, spe-1. Eukaryot. Cell 10: 724–733.

Pontecorvo, G., J. A. Roper, and E. Forbes, 1953 Genetic recombination without sexual reproduction in Aspergillus niger. J. Gen. Microbiol. 8: 198–210.

Poulson, L., M. R. Andersen, A. E. Lantz, and J. Thykaer, 2012 Identification of a transcription factor controlling pH-dependent organic acid response in Aspergillus niger. PLoS One 7: e50596.

Punt, P. J., F. H. Schuren, J. Lehmebeck, T. Christensen, C. Hjort et al., 2008 Characterization of extracellular protease encoding genes. Fungal Genet. Biol. 45: 1591–1599.

Record, E., M. Asther, C. Sigoillot, S. Pagès, P. J. Punt et al., 2003 Overproduction of the Aspergillus niger terufol enzyme for pulp bleaching application. Appl. Microbiol. Biotechnol. 62: 349–355.

Rose, S. H., and W. H. van Zyl, 2002 Constitutive expression of the Trichoderma reesei beta-1,4-xylanase gene (xyn2) and the beta-1,4-endoglucanase gene (egl) in Aspergillus niger in molasses and defined glucose media. Appl. Microbiol. Biotechnol. 58: 461–468.

Ruijter, G. J., P. J. van de Vondervoort, and J. Visser, 1999 Oxalic acid production by Aspergillus niger: an oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese. Microbiology 145: 2569–2576.

Sambrook, J., T. Maniatis, and E. F. Fritsch, 1989 Molecular Cloning: A Laboratory Manual, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schachtschabel, D., M. Arentshorst, B. M. Nitsche, S. Morris, K. F. Nielsen et al., 2013 The transcriptional repressor TupA in Aspergillus niger is involved in controlling gene expression related to cell wall biosynthesis, development, and nitrogen source availability. PLoS One 8: e78102.

Seiboth, B., R. A. Karimi, P. A. Phatale, R. Linke, L. Hartial et al., 2012 The putative protein methyltransferase LAE1 controls cellulase gene expression in Trichoderma reesei. Mol. Microbiol. 84: 1150–1164.

Sugui, J. A., J. Pardo, Y. C. Chang, A. Mullbacher, K. A. Zarembner et al., 2007 Role of laeA in the regulation of alb1, gliP, conidial morphology, and virulence in Aspergillus fumigatus. Eukaryot. Cell 6: 1552–1561.

Turbe-Doan, A., Y. Arfi, E. Record, J. Estrada-Alvarado, and A. Levasseur, 2013 Heterologous production of cellulbiose dehydrogenases from the basidiomycete Coprinopsis cinerea and the ascomycete Podospora anserina and their effect on saccharification of wheat straw. Appl. Microbiol. Biotechnol. 97: 4873–4885.

van den Hombergh, J. P., P. J. van de Vondervoort, N. C. van der Heijden, and J. Visser, 1995 New protease mutants in Aspergillus niger result in strongly reduced in vitro degradation of target proteins; genetical and biochemical characterization of seven complementation groups. Curr. Genet. 28: 299–308.

van den Hombergh, J. P., A. P. MacCabe, P. J. van de Vondervoort, and J. Visser, 1996 Regulation of acid phosphatases in an Aspergillus niger pacD disruption strain. Mol. Gen. Genet. 251: 542–550.

van den Hombergh, J. P., M. D. Sollewijn Gelpke, P. J. van de Vondervoort, F. P. Buxton, and J. Visser, 1997a Disruption of three acid proteases in Aspergillus niger: effects on protease spectrum, intracellular proteolysis, and degradation of target proteins. Eur. J. Biochem. 247: 603–613.

van den Hombergh, J. P., P. J. van de Vondervoort, L. Fraissinet-Tachet, and J. Visser, 1997b Aspergillus as a host for heterologous protein production: the problem of proteases. Trends Biotechnol. 15: 256–263.

van de Vondervoort, P. J. I., S. M. J. Langelveld, J. Visser, N. M. E. van Peij, H. J. P. et al., 2007 Identification of a mitotic recombination hotspot on chromosome III of the assexual fungus Aspergillus niger and its possible correlation with elevated basal transcription. Curr. Genet. 52: 107–114.

van Hartingsveldt, W., J. E. Mattern, C. M. van Zeijl, P. H. Pouwels, and C. A. van den Hondel, 1987 Development of a homologous transformation system for Aspergillus niger based on the pyrG gene. Mol. Gen. Genet. 206: 71–75.

Wenger, J. W., K. Schwartz, and G. Sherlock, 2010 Bulk segregant analysis by high-throughput sequencing reveals a novel xylose utilization gene from Saccharomyces cerevisiae. PLoS Genet. 6: e1000942.

Wiemann, P., D. W. Brown, K. Kleigreve, J. W. Bok, N. P. Keller et al., 2010 FvVen1 and FvLae1, components of a velvet-like complex in Fusarium fujikuroi, affect differentiation, secondary metabolism and virulence. Mol. Microbiol. 77: 972–994.

Wu, D., S. Oide, N. Zhang, M. Y. Choi, and B. G. Turgeon, 2012 ChLae1 and ChVen1 regulate T-toxin production, virulence, oxidative stress response, and development of the maize pathogen Cochliobolus heterostrophus. PLoS Pathog. 8: e1002542.

Xing, W., C. Deng, and C.-H. Hu, 2010 Molecular cloning and characterization of the global regulator LaeA in Penicillium citrinum. Biotechnol. Lett. 32: 1733.

Zwane, E. N., S. H. Rose, W. H. van Zyl, K. Rumbold, and M. Viljoen-Bloom, 2014 Overexpression of Aspergillus tubingensis facA in protease-deficient Aspergillus niger enables fullic acid production from plant material. J. Ind. Microbiol. Biotechnol. 41: 1027–1034.