## Title

Identification of a gene encoding the last step of the L-rhamnose catabolic pathway in Aspergillus niger revealed the inducer of the pathway regulator



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**Abstract**

In fungi, L-rhamnose (Rha) is converted via four enzymatic steps into pyruvate and L-lactaldehyde, which enter central carbon metabolism. In Aspergillus niger, only the genes involved in the first three steps of the Rha catabolic pathway have been identified and characterized, and the inducer of the pathway regulator RhaR remained unknown. In this study, we identified the gene (lkaA) involved in the conversion of L-2-keto-3-deoxyrhamnonate (L-KDR) into pyruvate and L-lactaldehyde, which is the last step of the Rha pathway. Deletion of lkaA resulted in impaired growth on L-rhamnose, and potentially in accumulation of L-KDR. Contrary to ΔlraA, ΔlrlA and ΔlrdA, the expression of the Rha-responsive genes that are under control of RhaR, were at the same levels in ΔlkaA and the reference strain, indicating the role of L-KDR as the inducer of the Rha pathway regulator.



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Table A.1. Plasmids used in this study.pdf [e-Component]

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**Data set** https://www.ncbi.nlm.nih.gov/sra/SRX7032121[accn]

Aspergillus niger DeltalraD Gene Expression Profiling

Transcriptome data on L-rhamnose

Dear Editor,

With this letter we would like to submit a revised version of our paper, entitled ‘Identification of a gene encoding the last step of the L-rhamnose catabolic pathway in *Aspergillus niger* revealed the inducer of the pathway regulator’. We have modified the paper according to most of the comments from the reviewers, but provided a rebuttal for some of the comments.

We hope you find this version of the manuscript suitable for your journal and look forward to a positive response.

Best wishes,

Ronald de Vries

**Response to comments from the editors and reviewers:**

## Reviewer 1

The manuscript is well written and interesting. To identify the aldolase in the pathway, which is from a completely different protein family is surprising. It would have been nice to have a brief characterization of the protein but is probably out of the scope of this paper.

- We agree with the reviewer, but unfortunately this is indeed outside the scope of this study.

General remarks:

The genes of the L-rhamnose pathway were first identified in Schefferomyces stipitis and called RHA1, LRA2, LRA3, LRA4, and LADH (for the L-lactaldehyde dehydrogenase). Is there a LADH in A. niger?

- A. niger does contain a LADH, but as lactaldehyde and pyruvate are also intermediates of other pathways, we did not include them in this study as we focused on the L-rhamnose specific steps and intermediates.

As a reason for renaming it was argued that in a different publication the deletion of the first three did not result in a phenotype. Are there indications based on the RNA seq data that there are homologs of the first three?

- Our RNAseq data revealed several genes without known function that are induced by L-rhamnose, but at this time we have not been able to identify the real 'backup-gene' for these steps. However, the fact that our deletion strains still have (some) growth on L-rhamnose indicates that other enzymes are involved in the pathway. This together with the gene/protein naming convention of Aspergillus, which consists of a three letter code that reflects the enzymatic function followed by a capital letter indicating the isogenes/enzymes, we feel that it is better to alter these names. On hindside we should already have done this in the previous paper (as several people from the field remarked upon since then), but we would rather correct this now than maintain an inaccurate stituation.

-**Reviewer 2**

- MS entitled "Identification of the last step of the L-rhamnose catabolic pathway in A. niger revealed the inducer of the pathway regulator"

The title is not correct - the last step (step 4) of the pathway is known for some time - it is the conversion for L-2-keto-3-deoxy-rhamnoate (LKDR) to pyruvate. What is unclear which gene encodes the encodes the enzyme responsible for this conversion.

- The reviewer is correct and we therefore changed the title to: Identification of a gene encoding the last step of the L-rhamnose catabolic pathway in Aspergillus niger revealed the inducer of the pathway regulator

The second part is also not correct - the inducer is not revealed but suggested from rather weak cicumstancial evidence. Because a KO of gene does not lead to downregulation of the pathway under otherwise inducing conditions which is the case for genes involved in step 1 to 3 it is

inferred that L-KDR must be the inducer. From previous reports cited by the authors we already could learn that L-KDR is a likely candidate because as already reported a KO of step 1-3 led to down-regulation of the pathway.

- We respectfully disagree with this argument. In the previous study we demonstrated that the neither L-rhamnose itself, nor its first two metabolic conversion products were the actual inducer, as blocking the pathway behind these steps abolished induction of the RhaR-regulated genes. At that time we were not able to evaluate the final L-rhamnose specific intermediate as we had not yet identified the gene that converted this to L-lactaldehyde and pyruvate. As the deletion of this gene does not result in absence of expression of RhaR-regulated genes, this clearly shows that the previous compound is sufficient for induction of RhaR. Therefore this study brings that from suggestion to evidence.

The most likely candidate for step 4 is NRRL3-08779 (Gruben et al 2014). This gene is rejected by the authors because it was not induced on Rha ( first argument) and (second argument) its expression is not controlled by RhaR which is the actually the same argument. The fact that 08779 is not induced on Rha is a weak argument for not to assess this gene by a KO. A number of others were tested by Khosravi et al 2017 sharing domain similarity but without succes and in the report lkaA was pointed as a gene involved in the fourth step.

- The text in the manuscript should have said 'not expressed on L-rhamnose', rather than 'not induced on L-rhamnose', which is now changed. Absence of expression on L-rhamnose is to our opinion a clear indication that this gene is not required for L-rhamnose conversion. Therefore this gene was not further investigated. The other genes tested in the previous study all had some level of expression on L-rhamnose.

From the manuscript it is unclear how the gene was discovered . Blastp yes - surrounding genes and more - no clue what that means - probability cutoff question mark - no explanation in the methods section either.
In short the authors need to add more detail

- This has been added as a separate section in Materials and Methods.

Next lkaA was knocked out and we do see a reduced growth on Rha but not no growth as was the case for step 1 -3 so the authors concluded this was not the only gene for step 4.

- In fact, our current and previous study shows that also for two of the other deletions there is still residual growth (although less), indicating that also there, possibly other genes are involved. However, the expression pattern with respect to induction was clearly different from the expression pattern in the lkaA deletion strain.

So what evidence do we have that lkaA is responsible for step 4: 1) A KO leads to reduced growth 2) lkaA is upregulated on Rha
What is against: Only reduced growth. No biochemical characterisation only PF03328 similarity

- In our opinion, a physiological effect (e.g. clearly reduced growth) is a stronger proof of function than in vitro biochemical analysis, as these enzymes can be active on compounds that are not physiologically relevant, which may result in wrong function assignment. We are planning to characterize this enzyme in a future project (as well as several other metabolic enzymes that have not yet been characterized) to match this with the genetic evidence, but that is unfortunately not possible in the current study.

RNAseq: A KO of lkaA basically has no effect. I would have expected that an increase of the L-KDR concentration as a result of the KO would lead to either superinduction or what could be tested a prolonged expression of the pathway of upon a shift from Rha to glucose

Note that a the effect of a KO of lkaA is indistinguishable from any other gene not involved in the Rha pathway

- The fact that a KO does not lead to higher expression of the genes is not an indication that this is not the inducer, as this would assume that a high level of the inducer is needed to lead to higher expression. Previously we already demonstrated that for the xylanolytic regulator XlnR the concentration of xylose does not affect the induction of xylanolytic genes through XlnR, but does affect their repression through CreA (at higher xylose levels), suggesting that the presence of a small amount of the inducer can already provide the maximal response (see: de Vries RP, Visser J, de Graaff LH. 1999. CreA modulates the XlnR induced expression on xylose of *Aspergillus niger* genes involved in xylan degradation. Res Microbiol 150: 281-285.). Our results indicate that this is likely also the case for RhaR.

In short the evidence presented is weak- lkaA is likely involved - but not the only factor

- We disagree that the evidence of the involvement of lkaA is weak as there is a clear phenotype of the deletion strain, specifically on L-rhamnose. We do agree that there must be other gene(s) involved, as is also indicated in our manuscript, but we currently have not yet identified this gene or genes.

# Highlights

- *lkaA* is involved in the last step of the L-rhamnose catabolic pathway in *A. niger*
- L-2-keto-3-deoxyrhamnonate is the inducer of the pathway regulator RhaR

# Identification of a gene encoding the last step of the L-rhamnose catabolic pathway in *Aspergillus niger* revealed the inducer of the pathway regulator

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# Abstract

In fungi, L-rhamnose (Rha) is converted via four enzymatic steps into pyruvate and L-lactaldehyde, which enter central carbon metabolism. In *Aspergillus niger*, only the genes involved in the first three steps of the Rha catabolic pathway have been identified and characterized, and the inducer of the pathway regulator RhaR remained unknown. In this study, we identified the gene (*lkaA*) involved in the conversion of L-2-keto-3-deoxyrhamnonate (L-KDR) into pyruvate and L-lactaldehyde, which is the last step of the Rha pathway. Deletion of *lkaA* resulted in impaired growth on L-rhamnose, and potentially in accumulation of L-KDR. Contrary to Δ*lraA,* Δ*lrlA* and Δ*lrdA*, the expression of the Rha-responsive genes that are under control of RhaR, were at the same levels in Δ*lkaA* and the reference strain, indicating the role of L-KDR as the inducer of the Rha pathway regulator.

**Keywords:** L-rhamnose catabolic pathway; RhaR; inducer; pectinolytic enzymes; gene regulation

# Abbreviations

**CM**, complete medium; **5-FOA**, 5-fluoroorotic acid; **GalUA**, D-galacturonic acid; **HGA**, homogalacturonan; **L-KDR**, L-2-keto-3-deoxyrhamnonate; **LraA**, L-rhamnose-1-dehydrogenase, **LrlA**, L-rhamnono-γ-lactonase, **LrdA**, L-rhamnonate dehydratase,

**LkaA**, L-2-keto-3-deoxyrhamnonate aldolase; **NHEJ**, non-homologous end-joining; **RG-I**, rhamnogalacturonan I; **RG-II**, rhamnogalacturonan II; **Rha**, L-rhamnose; **RhaR**, L-rhamnose responsive transcription factor; **RhtA**, L-rhamnose transporter; **MM**, minimal medium; **XGA**, xylogalacturonan

# Introduction

Pectin is one of the major components of plant cell walls. It represents a group of complex heteropolysaccharides with high diversity in their structure, which are composed of four structural elements: homogalacturonan (HGA), xylogalacturonan (XGA), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Voragen *et al.*, 2009). Due to the rather intricate composition of pectin, many fungi, including *Aspergillus niger,* produce a broad range of pectinolytic enzymes to efficiently degrade these polysaccharides (de Vries and Visser, 2001; Martens-Uzunova and Schaap, 2009; Benoit *et al.*, 2012). These enzymes promote the decomposition of pectin into monosaccharides that the fungus can uptake and use as carbon source.

Although galacturonic acid (GalUA) is the predominant component of pectin, several other sugars are also part of its structure (Voragen *et al.*, 2009). One of these is L-rhamnose (Rha), which is a hexose sugar abundantly present in both RG-I and RG-II. The backbone of RG-I is composed of alternating GalUA and Rha residues, while in RG-II, Rha is part of its side chains. Pectinolytic enzymes specifically involved in the release of Rha from pectin include exo- and endo-rhamnogalacturonase (EC 3.2.1.67), and α-L-rhamnosidase (EC 3.2.1.40), aided by rhamnogalacturonan galacturonohydrolase (exo-rhamnogalacturonase, EC 3.2.1.-), rhamnogalacturonan lyase (EC 4.2.2.-) and rhamnogalacturonan acetylesterase (EC 3.1.1.-) (de Vries *et al.*, 2000; de Vries and Visser, 2001; Voragen *et al.*, 2009).

After Rha is released, it is taken up in the fungal cell and converted through the fungal Rha catabolic pathway into pyruvate and L-lactaldehyde in four enzymatic steps (Figure 1), which are sequentially catalyzed by L-rhamnose-1-dehydrogenase (LraA; EC 1.1.1.173), L-rhamnono-γ-lactonase (LrlA, formerly LraB; EC 3.1.1.65), L-rhamnonate dehydratase (LrdA, formerly LraC; EC 4.2.1.65) and L-2-keto-3-deoxyrhamnonate (L-KDR) aldolase (LkaA; EC 4.1.2.53) (Watanabe *et al.*, 2008). In *A. niger*, the genes involved in the first three enzymatic steps of this pathway have been identified and characterized (Khosravi *et al.*, 2017). However, the gene(s) involved in the last conversion step of the pathway remains unknown. Previously, a bidirectional BlastP analysis against the *Aspergillus* genome database (www.aspgd.org/) showed that NRRL3_08779 is the closest *A. niger* homolog of *Schefferomyces stipitis* Lra4 (Gruben *et al.*, 2014). However, this gene was not expressed on Rha and its expression was not controlled by RhaR (Khosravi *et al.*, 2017). Therefore, the involvement of three other putative genes,

NRRL3_03899, NRRL3_05649 and NNRL3_06731, identified based on their InterPro and PFAM domain similarity to those found in Lra4 of *S. stipitis*, was assessed (Khosravi *et al.*, 2017). In that case, all three genes were shown to be specifically upregulated in Rha, nevertheless, their deletion did not affect growth when Rha was used as sole carbon source, showing that they are not involved in Rha metabolism in *A. niger*.

We have renamed the genes of the Rha catabolic pathway, as our recent results clearly indicate that multiple genes/enzymes may be involved in several of the steps. According to common practice in *Aspergillus* gene/enzyme naming, genes are commonly referred to by a 3-letter code, reflecting their function, followed by a letter, indicating the iso-genes. The previously used names for the Rha pathway genes (*lraA*, *lraB* and *lraC*) do not follow this structure, as they encode diverse enzymatic functions, but have the same three-letter code. The use of the same code for different enzymes prevents the use of this code for iso-genes encoding enzymes with similar activity. The new names suggested in this paper, provide a different three-letter code for each enzyme activity, as well as the option for referring to their corresponding iso-genes by the capital letter behind it.

Induction of the genes required for the degradation of pectin and release of Rha, transport of Rha into the cell and Rha catabolism, have been previously shown to be under control of the transcriptional regulator RhaR in *A. niger* (Gruben *et al.*, 2014; Sloothaak *et al.*, 2016; Gruben *et al.*, 2017; Khosravi *et al.*, 2017). The deletion of *rhaR* resulted in strong reduction in expression of the catabolic pathway genes *lraA*, *lrlA* and *lrdA*, the Rha transporter gene *rhtA* (Sloothaak *et al.*, 2016), as well as several Rha-releasing enzymes, during growth on both Rha and L-rhamnonate. The presence of Rha, even in low concentrations, has been shown to specifically induce the expression of *rhaR* (Sloothaak *et al.*, 2016). Khosravi *et al.* (2017) showed that single gene deletions of *lraA*, *lrlA* and *lrdA* abolished induced expression of the RhaR-related genes, suggesting that the inducer of this regulator is further downstream in the pathway.

In this study, identification of the *lkaA* gene that is involved in the last step of Rha catabolism in *A. niger* showed that L-2-keto-3-deoxy-rhamnonate is the inducer of the RhaR regulator. In particular, deletion of *lkaA* and transcriptomic analysis showed that the induction of the Rha pathway genes, the RhaR-regulated pectinolytic genes and *rhtA* in the Δ*lkaA* strain was maintained at similar levels as the reference strain.

# Material and methods

## Strains, media and growth conditions

The uridine auxotrophic and non-homologous end-joining (NHEJ) deficient *A. niger strain* N593Δ*kusA* (reference strain) was used as parental strain for the construction of the Δ*lraA*, Δ*lrlA*, Δ*lrdA*, Δ*lkaA* and Δ*03333* mutants. For the double Δ*lkaA*Δ*03333* mutant, the Δ*lkaA* strain was used as parental strain. All strains described in this study were deposited

in the CBS strain collection of the Westerdijk Fungal Biodiversity Institute under accession numbers listed in Table 1. All strains were grown at 30°C using Minimal Medium (MM, pH 6) or Complete Medium (CM, pH 6) with the appropriate carbon source (de Vries *et al.*, 2004). For solid cultivation, 1.5% (w/v) agar was added in the medium and, unless stated otherwise, all agar plates contained 1% D-glucose as carbon source. As required, media of auxotrophic strains were supplemented with 1.22 g/L uridine, while a final concentration of 1.3 mg/mL of 5-fluoroorotic acid (5-FOA) was used for counterselection of strains carrying the *pyrG* marker gene on the self-replicating plasmid.

For growth profiling, 6 cm petri dishes with vents containing MM agar supplemented with 25 mM D-glucose (Sigma, G8270) or Rha (Sigma, 83650) were used. Spores were harvested from CM agar plates in ACES buffer, after five days of growth, and counted using a haemocytometer. Growth profiling plates were inoculated with 1000 spores in 2 µl, and incubated at 30°C for 5 days. All liquid cultures were incubated in an orbital shaker at 250 rpm and 30°C. For transfer experiments, the pre-cultures containing 250 ml CM with 2% D-fructose in 1 L Erlenmeyer flasks were inoculated with $10^6$ spores/ml and incubated for 16 h. Thereafter, the mycelia were harvested by filtration on sterile cheesecloth, washed with MM and ~0.5 g (dry weight) was transferred to 50 ml Erlenmeyer flasks containing 10 ml MM supplemented with 25 mM Rha. All cultures were performed in triplicate. After 2 h of incubation, the mycelia were harvested by vacuum filtration, dried between tissue paper and frozen in liquid nitrogen. Samples were stored at -80°C.

## Identification of candidate genes

Pathway hole filler (Green and Karp, 2004) from Pathway Tools software (Karp *et al.*, 2016) was used to identify missing enzymes in the manually curated *A. niger* carbon metabolic network (Aguilar-Pontes *et al.*, 2018) based on *A. niger* NRRL 3 genome (Vesth *et al.*, 2018; Aguilar-Pontes *et al.*, 2018). Sequences for enzymes catalyzing the last step of the Rha pathway associated to EC 4.1.2.53 (2-keto-3-deoxy-L-rhamnonate aldolase) function were retrieved from Swiss-Prot (Boutet *et al.*, 2016), MetaCyc PGDB (Caspi *et al.*, 2016), including *Candida albicans* SC5314 and *Saccharomyces cerevisiae* PGDB YeastCyc (Karp *et al.*, 2019). Their amino acid sequences were then used as queries in a BLASTP search against *A. niger* NRRL 3 full proteome with the default E-value cutoff of 10. Each of the candidate hits in the BLASTP results are evaluated by calculating the probability that the sequence encodes the desired function based on operon-, homology- and pathway-based data using the Bayesian network described in (Green and Karp, 2004). No hits were identified using the default probability-score of 0.9, however, 5 candidate hits were identified with a probability-score cutoff of 0.75 (Table 2).

## Protoplast-mediated transformation, mutant purification and screening

For creation of all the mutants described in this study, the CRISPR/Cas9 system, as designed by (Song *et al.*, 2018), was used. The Geneious R11 software (Kearse *et al.*, 2012) was used for the identification of 20 bp guide sequences for our target genes against the *A. niger* NRRL 3 genome. The guide sequences and plasmids used in this study are listed in Table A.1.

To construct linear deletion DNA cassettes, the upstream and downstream flanking regions of the genes *lraA*, *lrlA*, *lrdA*, *lkaA* and *03333* were amplified by PCR using gene specific primers (see Table A.2). PCR amplification was performed using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific), following manufacturer's instructions. Genomic DNA from reference strain was used as a template. The upstream reverse and the downstream forward primers were designed to harbor a barcode sequence [actgctaggattcgctatcg]. This sequence was used as the homologous region for the fusion of these two fragments in a PCR reaction, to generate the linear deletion DNA cassette. The amplified deletion cassettes were purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences).

*A. niger* protoplasting was performed as described by (Kusters-van Someren *et al.*, 1991) with some modifications. In particular, young mycelia from overnight culture were harvested by vacuum filtration, washed with 0.6 M $MgSO_4$ and dried between two tissue paper sheets. Mycelia were then incubated with VinoTaste® Pro lysing enzyme (0.75 g/gDW mycelia), dissolved in PS buffer (0.8 M sorbitol, 0.2 M sodium phosphate buffer pH 7.5), in an orbital shaker at 100 rpm and 34°C. When free protoplasts were abundantly present (after ~2.5h), mycelial debris was removed by filtration through Miracloth and protoplasts were collected by centrifugation (10 min, 3000 rpm, 4°C). Protoplasts were then washed twice with ice-cold SC solution (1 M sorbitol, 50 mM $CaCl_2*2H_2O$) and resuspended in that buffer at an approximate concentration of $2*10^7$ protoplasts/ml. Transformation of *A. niger* protoplasts was performed as described in detail by (Kowalczyk *et al.*, 2017).

All transformations were carried out using 0.8 µg of ANEp8-Cas9-gRNA plasmid DNA together with 4-6 µg of purified linear deletion DNA cassette. Since the reference strain is NHEJ-deficient, construction of mutants using a rescue cassette resulted in clean deletions. Transformants were plated on MM plates with 0.95 M sucrose. Five colonies per mutant were randomly selected from the transformation plates and streak-purified twice on MM plates. For *A. niger* colony PCR, genomic template DNA was isolated from mycelia of putative deletion strains using the Wizard® Genomic DNA Purification Kit (Promega). Correct mutants were identified by PCR amplification of the sequences flanking the CRISPR/Cas9 cut site, using primers listed in Table 4. Prior to storage,

mutants were re-inoculated twice on MM plates supplemented with 1% D-glucose and uridine, and subsequently on plates with 5-FOA aiming on counterselection against the ANEp8-Cas9-gRNA plasmids.

## Transcriptome sequencing and analysis

The transcriptomic response of Δ*lkaA* induced after 2 h on Rha was analyzed using RNA-seq analysis. Total RNA was extracted from grinded mycelial samples using TRIzol® reagent (Invitrogen, Breda, The Netherlands) and purified with the NucleoSpin® RNA Clean-up Kit (Macherey-Nagel), while contaminant gDNA was removed by rDNase treatment directly on the silica membrane. The RNA quality and quantity were analyzed with a RNA6000 Nano Assay using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Purification of mRNA, synthesis of cDNA library and sequencing were conducted at DOE Joint Genome Institute (JGI).

RNA sample preparation was performed on the PerkinElmer Sciclone NGS robotic liquid handling system using the Illumina TruSeq Stranded mRNA HT sample prep kit, utilizing poly-A selection of mRNA following the protocol outlined by Illumina: https://support.illumina.com/sequencing/sequencing_kits/truseq-stranded-mrna.html, and with the following conditions: total RNA starting material was 1 µg per sample and eight cycles of PCR was used for library amplification. The prepared libraries were quantified using KAPA Biosystem's next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. The quantified libraries were then multiplexed with other libraries, and the pool of libraries was then prepared for sequencing on the Illumina NovaSeq sequencer using NovaSeq XP V1 reagent kits, S4 flow cell, and following a 2x150 indexed run recipe.

Using BBDuk (https://sourceforge.net/projects/bbmap), raw reads were evaluated for artifact sequence by kmer matching (kmer = 25), allowing one mismatch and detected artifact was trimmed from the 3' end of the reads. RNA spike-in reads, PhiX reads and reads containing any Ns were removed. Quality trimming was performed using the phred trimming method set at Q6. Finally, following trimming, reads under the length threshold were removed (minimum length 25 bases or one third of the original read length – whichever was longer). Filtered reads from each library were aligned to the *A. niger* NRRL 3 (http://genome.jgi.doe.gov/Aspni_NRRL3_1) genome assembly using HISAT2 version 2.1.0 (Kim *et al.*, 2015). FeatureCounts (Liao *et al.*, 2014) was used to generate the raw gene counts using gff3 annotations. Only primary hits assigned to the reverse strand were included in the raw gene counts (-s 2 -p --primary options). The reads from each of the RNAseq samples were deposited with the Sequence Read Archive at NCBI with individual sample accession numbers (SRP225871, SRP225872, SRP226530, SRP226531 and SRP226532)

Statistical analysis was performed using DESeq2 (Love *et al.*, 2014). Transcripts were considered differentially expressed if the DESeq2 fold change was > 1.5 or < 0.67 and

Padj < 0.05 as well as the FPKM > 50 in at least one of the two conditions being compared. Transcripts with FPKM ≤ 50 were considered lowly (i.e. not substantially) expressed.

# Results & Discussion

## Deletion of *lkaA* results in reduced growth on Rha

Candidate genes for the last step of the *A. niger* Rha catabolic pathway were identified as indicated in Materials and Methods. All of the candidates contained an HpcH/HpaI aldolase/citrate lyase domain (IPR005000, PF03328) according to InterPro (Mitchell *et al.*, 2019) and PFAM (El-Gebali *et al.*, 2019) database. This domain is also found in a number of proteins, including 2-keto-3-deoxy-L-rhamnonate aldolase (EC:4.1.2.53), 5-keto-4-deoxy-D-glucarate aldolase (EC:4.1.2.20) and citrate lyase subunit beta (EC:4.1.3.6).

Comparison of the expression levels of these five candidate genes on Rha and D-glucose (Table 2) showed that only the expression of NRRL3_08604, referred to from now on as *lkaA*, was significantly upregulated (40-fold) on Rha compared to D-glucose. However, analysis of the microarray data generated by Gruben *et al.* (2017) revealed that the expression of NRRL3_03333 was also induced (2.4-fold upregulated) on Rha compared to D-glucose. Therefore, both of these genes were selected for further analysis. The other three putative genes were either not induced by Rha or not expressed on either sugar in both datasets, and were therefore excluded as candidates. Neither *lkaA* nor NRRL3_03333 were homologues of *S. stipitis* Lra4, nor did they belong to the same aldolase families as the previously described putative *lkaA* genes (see Table A.3).

The *lkaA* gene was the only candidate that was strongly upregulated on Rha compared to D-glucose, which was regulated by RhaR (Table 2). Additionally, expression of *lkaA* was reduced in all three Δ*lraA*, Δ*lrlA* and Δ*lrdA* Rha metabolic mutants compared to the reference strain (Table 3). Deletion of *lkaA* resulted in reduced growth and sporulation on Rha as sole carbon source, which clearly showed that this gene is involved in the Rha catabolic pathway (Figure 2). However, the residual growth on Rha suggests that the Δ*lkaA* mutant is still able to metabolize this sugar. Deletion of the NRRL3_03333 alone or in combination with Δ*lkaA* did not affect growth on Rha, indicating that this gene is not a paralog of *lkaA*.

## Deletion of *lkaA* does not affect induction of RhaR regulated genes

As mentioned earlier, RhaR is activated in the presence of Rha. However, deletion of *lraA* resulted in inactivation of RhaR-mediated expression (Khosravi *et al.*, 2017), which

showed that Rha is not the actual inducer. Similarly, deletion of *lrlA* and *lrdA* also inactivated RhaR-mediated expression, demonstrating that neither L-rhamnono-γ-lactonase nor L-rhamnonate are inducers of RhaR.

This suggested that the inducer is located further down in the Rha catabolic pathway, and therefore the expression of the Rha-responsive genes that are under control of RhaR was also examined in the *lkaA* deletion mutant. The reference and the Δ*lkaA* strains were transferred to MM medium with 25 mM Rha, followed by RNA-seq analysis. The expression of *lkaA* in the Δ*lkaA* strain compared to reference strain was abolished, confirming the deletion of this gene. However, the expression levels of *lraA, lrlA*, *lrdA* and of the pathway regulator *rhaR* were the same as for the reference strain (Figure 3; Table 3a), demonstrating that deletion of *lkaA* did not abolish activation of RhaR. This result was confirmed by qPCR analysis (data not shown).

The expression level of the *rhtA* Rha transporter followed the same pattern (Table 3b). Interestingly, two other putative transporter genes (NRRL3_09860 and NRRL3_02828) showed a similar expression profile, suggesting their involvement in Rha transport. While both genes were significantly downregulated in the Δ*lraA*, Δ*lrlA*, Δ*lrdA and* Δ*rhaR* mutants (Khosravi *et al.*, 2017), their expression in the Δ*lkaA* mutant was similar to the reference strain. The same was observed for a third putative transporter gene (NRRL3_06137), but its expression levels were very low compared to *rhtA* and the other two candidate Rha transporter genes.

Finally, the expression of CAZy genes, previously shown to be regulated by RhaR (Gruben *et al.*, 2017), was also compared between the *lkaA* deletion mutant and the reference strain. Several pectinolytic genes involved in the degradation of the RG-I backbone had similar expression levels in Δ*lkaA* and the reference strain. These included two GH28 exo-rhamnogalacturonases (*rgxA* and *rgxB*), five putative GH78 a-L-rhamnosidases (NRRL3_02162, NRRL3_06304, NRRL3_03279, NRRL3_04245 and NRRL3_07520), one GH105 unsaturated rhamnogalacturonan hydrolase (*urhgA*), one PL4 rhamnogalacturonan lyase (*rglB*) and two CE12 rhamnogalacturonan acetyl esterase (*rgaeA* and *rgaeB*) (Table 3c). A similar pattern was also observed for a gene (*lacC*) encoding a GH35 β-1, 4-galactosidase acting on the pectic side chains. Previously, these genes were reported to be significantly (>1.5 fold) down-regulated in all Δ*lraA*, Δ*lrlA*, Δ*lrdA and* Δ*rhaR* mutants compared to the reference strain on Rha (Khosravi *et al.* 2017).

Similar situation was also described for the GalUA catabolic pathway of *A. niger* (Alazi *et al.*, 2017). In this pathway, which actually shares similarities regarding the conversion reactions of the pathway intermediates with the Rha catabolic pathway, deletion of *gaaC* led to the identification of 2-keto-3-deoxy-L-galactonate as the inducer of the transcriptional regulator GaaR. In particular, accumulation of 2-keto-3-deoxy-L-

296 galactonate caused induction of the genes involved in pectin degradation, GalUA
297 transport and GalUA catabolism.

298 ## Conclusions

299 Our results clearly demonstrate that in the Δ*lkaA* mutant the actual inducer of the RhaR
300 regulator is still present. Since LkaA catalyzes the conversion of L-KDR into pyruvate and
301 L-lactaldehyde, we conclude that L-KDR is the responsible metabolite for the induction of
302 the Rha-responsive genes in *A. niger*. As the products of L-KDR conversion, pyruvate
303 and L-lactaldehyde, are part of central metabolism, L-KDR is also the last Rha-specific
304 metabolite of this pathway.

305

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## Figure Legends

**Figure 1:** Graphical representation of the L-rhamnose (Rha) catabolism and transcriptional regulation of the Rha-responsive genes in *A. niger*. The pathway regulator RhaR, which is activated by L-2-keto-3-deoxy-rhamnonate (red arrow), induces the genes required for the degradation of pectin and release of Rha, transport of Rha intracellularly and Rha catabolism (green arrows). LraA = L-rhamnose-1-dehydrogenase, LrlA = L-rhamnono-γ-lactonase, LrdA = L-rhamnonate dehydratase, LkaA = L-2-keto-3-deoxyrhamnonate (L-KDR) aldolase.

**Figure 2:** Growth profiles of the reference strain (N593 Δ*kusA*) and the deletion mutants, Δ*lraA*, Δ*lrlA*, Δ*lrdA*, Δ*lkaA*, Δ03333 and Δ*lkaA*Δ03333, on solid MM without any carbon source, with 25 mM L-rhamnose or with 25 mM D-glucose. Strains were grown for 5 days at 30°C.

**Figure 3:** Comparison of *A. niger lraA*, *lrlA*, *lrdA*, *lkaA*, *rhaR* and *rhtA* expression levels (FPKM) between the reference and the Δ*lkaA* strains. The expression was measured after transferring both strains for 2 h on 25 mM Rha. The expression levels represent mean values of triplicate samples. The cut-off for differential expression is DESeq2 fold change >1.5 or <0.67 and padj_value <0.05. Significant differences in gene expression between these two strains are highlighted with an asterisk (*).

# Tables

**Table 1:** *A. niger* strains used in this study.

| Strains | Formerly known as | Gene ID | Description | CBS number | Genotype | Reference |
|---|---|---|---|---|---|---|
| *Reference strain* (N593 Δ*kusA*) | - | - | - | CBS 138852 | *A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$ | (Meyer *et al.*, 2007) |
| Δ*lraA* | Δ*lraA* | NRRL3_01494 | L-rhamnose-1-dehydrogenase | CBS 144623 | *A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$, ***lraA***$^{-}$ | This study |
| Δ*lrlA* | Δ*lraB* | NRRL3_01493 | L-rhamnono-γ-lactonase | CBS 144300 | *A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$, ***lrlA***$^{-}$ | This study |
| Δ*lrdA* | Δ*lraC* | NRRL3_01495 | L-rhamnonate dehydratase | CBS 144313 | *A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$, ***lrdA***$^{-}$ | This study |
| Δ*lkaA* | Δ*lraD* | NRRL3_08604 | L-2-keto-3-deoxyrhamnonate aldolase | CBS 144626 | *A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$, ***lkaA***$^{-}$ | This study |
| Δ*03333* | - | NRRL3_03333 | putative L-2-keto-3-deoxyrhamnonate aldolase | CBS 145852 | *A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$, ***03333***$^{-}$ | This study |
| Δ*lkaA*Δ*03333* | - | NRRL3_08604 NRRL3_03333 | - | CBS 145938 | *A. niger* N593, *cspA1*, *kusA*::*amdS*, *pyrG*$^{-}$, ***lkaA*$^{-}$, *03333*$^{-}$** | This study |

**Table 2:** Expression levels (FPKM) of putative *lkaA* genes in *A. niger* reference (Ref) and *ΔrhaR* strains, after their transfer for 2 h in liquid MM with 25 mM Rha or 25 mM D-glucose. The values are averages of duplicates. Fold changes >1.5 and <0.67 are highlighted in green and yellow, respectively, and padj_values <0.05 are indicated with an asterisk (*). Genes selected for further analysis are underlined.

| | | | RNA-sequencing This study | | | Microarray [a] Gruben *et al.* (2017) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| ***A. niger*** **NRRL3 model ID** | ***A. niger*** **CBS 513.88 model ID** | **Gene** | **Mean Ref Glc** | **Mean Ref Rha** | **Fold change Ref Rha / Ref Glc** | **Mean Ref Glc** | **Mean Ref Rha** | **Mean Δ*rhaR* Rha** | **Fold change Ref Rha / Ref Glc** | **Fold change Δ*rhaR* Rha / Ref Rha** |
| NRRL3_08604 | An03g02490 | *lkaA* | 39.7 | 1591.9 | 40.1 * | 306.7 | 8462.1 | 849.8 | 27.6 * | 0.1 * |
| NRRL3_03333 | An12g05070 | | 16.5 | 16.5 | 1.0 | 44.2 | 103.8 | 79.3 | 2.4 * | 0.8 * |
| NRRL3_00191 | An09g02440 | | 0.1 | 0.5 | 8.7 * | 21.8 | 26.7 | 19.0 | 1.2 | 0.7 * |
| NRRL3_09072 | An12g01610 | | 0,0 | 0.1 | 5.4 | 26.7 | 28.6 | 29.7 | 1.1 | 1.0 |
| NRRL3_06259 [b] | - | | 1.0 | 0.9 | 0.9 | - | - | - | - | - |

[a] Based on the microarray dataset generated by Gruben *et al.* (2017).

[b] Expression data for NRRL3_06259 were not available in the microarray dataset generated by Gruben *et al.* (2017). Since, this gene was lowly expressed (FPKM < 50) in our RNA-seq data, it was excluded for further analysis.

**Table 3:** RNA-seq analysis of Rha-responsive genes, involved in (a) Rha catabolism, (b) transport of Rha intracellularly and (c) degradation of pectin and release of Rha, in *A. niger* Δ*lkaA* and the reference strains. For both strains, expression levels (FPKM) were measured after their transfer for 2 h in MM with 25 mM Rha. Genes with FPKM values <50 are considered lowly expressed and marked in red font. The values are averages of duplicates. The fold change is the difference between the deletion mutants and the reference strain. Fold changes >1.5 and <0.67 are highlighted in green and red, respectively, and padj_values <0.05 are indicated with an asterisk (*). [a] based on Khosravi *et al.* (2017)

| | | | | | | RNA-sequencing | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | This study | [a](Khosravi *et al.*, 2017) | | | |
| | | ***A. niger*** **NRRL 3 model ID** | **Gene** | **mean Δ*lkaA*_Rha** | **mean Ref_Rha** | **fold change Δ*lkaA*/Ref** | **fold change Δ*lraA*/ Ref** | **fold change Δ*lrlA*/ Ref** | **fold change Δ*lrdA*/ Ref** | **fold change Δ*rhaR*/ Ref** |
| a | **Regulator** | NRRL3_01496 | *rhaR* | 148,08 | 178,20 | 0,83 * | 0,51 * | 0,64 * | 0,02 * | 0,01 * |
| | **L-rhamnose catabolic pathway genes** | NRRL3_01494 | *lraA* | 2346,96 | 1813,98 | 1,29 * | 0,00 * | 0,13 * | 0,17 * | 0,04 * |
| | | NRRL3_01493 | *lrlA* | 753,78 | 571,30 | 1,32 * | 0,5 * | 0,01 * | 0,54 * | 0,3 * |
| | | NRRL3_01495 | *lrdA* | 13031,16 | 11841,31 | 1,10 * | 0,09 * | 0,13 * | 0,00 * | 0,00 * |
| | | NRRL3_08604 | *lkaA* | 27,23 | 1591,85 | 0,02 * | 0,08 * | 0,1 * | 0,27 * | 0,16 * |
| b | **L-rhamnose transporter genes [c](Sloothaak *et al.*, 2016)** | NRRL3_03278 | *rhtA* | 1275,54 | 936,98 | 1,36 * | 0,19 * | 0,30 * | 0,00 * | 0,01 * |
| | | NRRL3_09860 | | 1522,69 | 1337,17 | 1,14 * | 0,06 * | 0,08 * | 0,05 * | 0,05 * |
| | | NRRL3_02828 | | 1286,80 | 1438,45 | 0,89 | 0,14 * | 0,19 * | 0,01 * | 0,01 * |
| | | NRRL3_03147 | | 1097,04 | 1306,42 | 0,84 * | 1,40 | 1,94 * | 1,61 * | 1,08 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | NRRL3_10300 | | 262,18 | 307,30 | 0,85 * | 1,44 | 1,69 * | 2,13 * | 3,27 * |
| | NRRL3_01652 | | 190,52 | 73,62 | 2,59 * | 2,34 * | 1,56 | 1,23 | 0,77 * |
| | NRRL3_06137 | | 5,96 | 4,84 | 1,23 | 0,63 | 0,37 | 0,39 | 3,46 |
| | NRRL3_00235 | | 7,93 | 4,59 | 1,73 * | 1,97 * | 1,16 | 1,52 | 6,81 * |

c

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **CAZy under RhaR regulation** | NRRL3_02832 | *rgxA* | 694,08 | 547,63 | 0,79 * | 0,03 * | 0,07 * | 0,02 * | 0,02 * |
| | NRRL3_08631 | *rgxB* | 109,66 | 139,27 | 1,27 * | 0,01 * | 0,04 * | 0,00 * | 0,00 * |
| | NRRL3_10559 | *rgxC* | 8,34 | 15,53 | 1,86 * | 0,06 * | 0,13 * | 0,06 * | 0,08 * |
| | NRRL3_02162 | | 5437,00 | 3717,68 | 0,68 * | 0,04 * | 0,09 * | 0,02 * | 0,01 * |
| | NRRL3_06304 | | 104,70 | 119,24 | 1,14 * | 0,03 * | 0,05 * | 0,02 * | 0,02 * |
| | NRRL3_03279 | | 1009,92 | 1098,73 | 1,09 | 0,19 * | 0,26 * | 0,01 * | 0,01 * |
| | NRRL3_04245 | | 50,89 | 43,74 | 0,86 * | 0,01 * | 0,01 * | 0,01 * | 0,01 * |
| | NRRL3_07520 | | 429,93 | 485,94 | 1,13 * | 0,1 * | 0,22 * | 0,04 * | 0,04 * |
| | NRRL3_00839 | *urhgA* | 4032,51 | 4531,18 | 1,12 * | 0,08 * | 0,12 * | 0,02 * | 0,02 * |
| | NRRL3_10115 | *rglB* | 2632,37 | 3144,23 | 1,19 * | 0,03 * | 0,07 * | 0,00 * | 0,01 * |
| | NRRL3_00169 | *rgaeA* | 207,88 | 233,14 | 1,12 | 0,27 * | 0,26 * | 0,08 * | 0,06 * |
| | NRRL3_07501 | *rgaeB* | 569,31 | 535,23 | 0,94 | 0,19 * | 0,21 * | 0,06 * | 0,05 * |
| | NRRL3_11738 | *lacC* | 178,79 | 228,08 | 1,28 * | 0,07 * | 0,16 * | 0,03 * | 0,05 * |
| | NRRL3_01071 | *lacE* | 9,76 | 17,88 | 1,83 * | 0,12 * | 0,10 * | 0,21 * | 0,18 * |
| | NRRL3_02931 | *faeB* | 28,23 | 19,30 | 0,68 * | 0,33 * | 0,27 * | 0,28 * | 0,25 * |
| | NRRL3_02827 | | 45,97 | 35,42 | 0,77 * | 0,07 * | 0,06 * | 0,02 * | 0,03 * |

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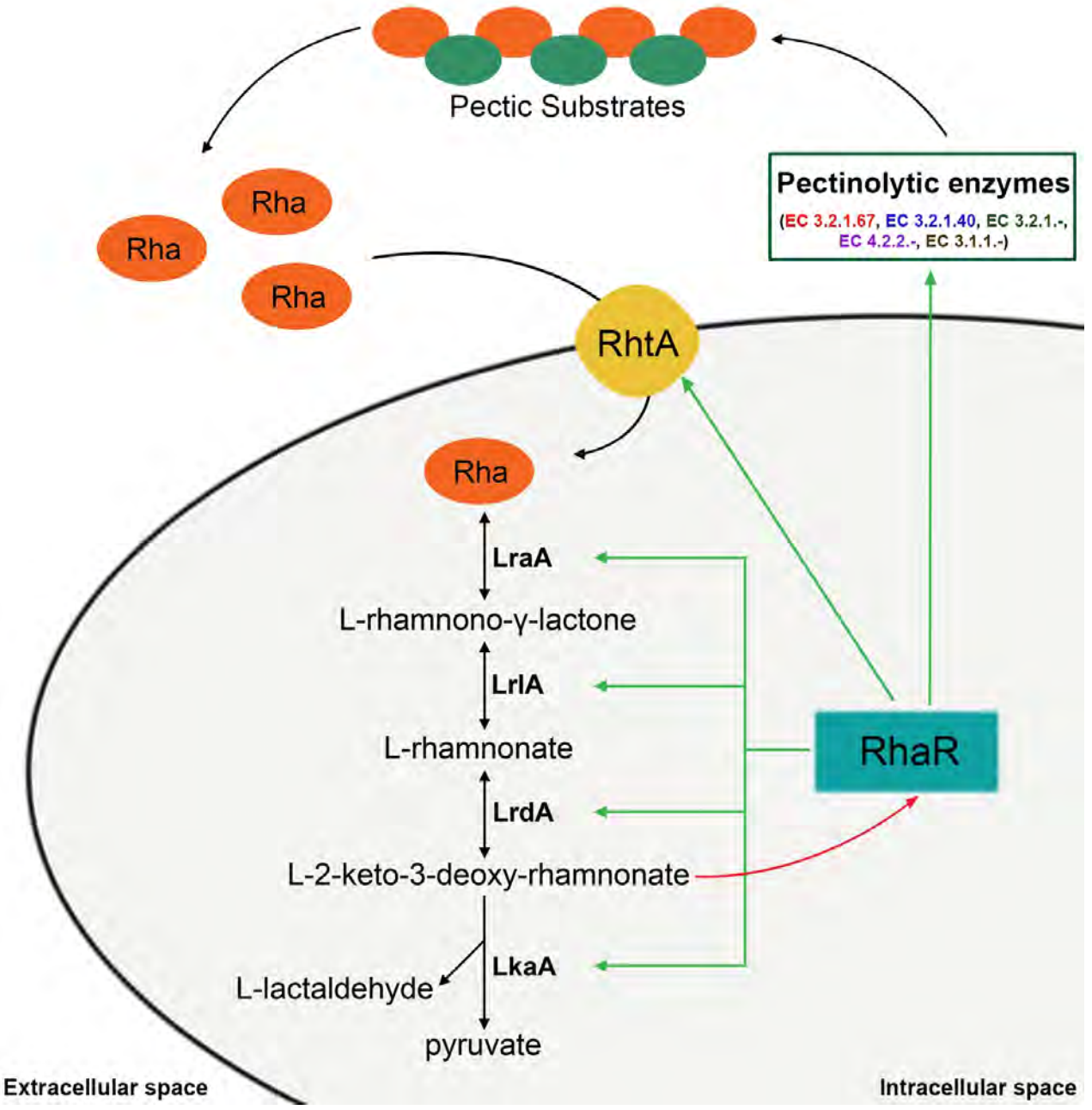
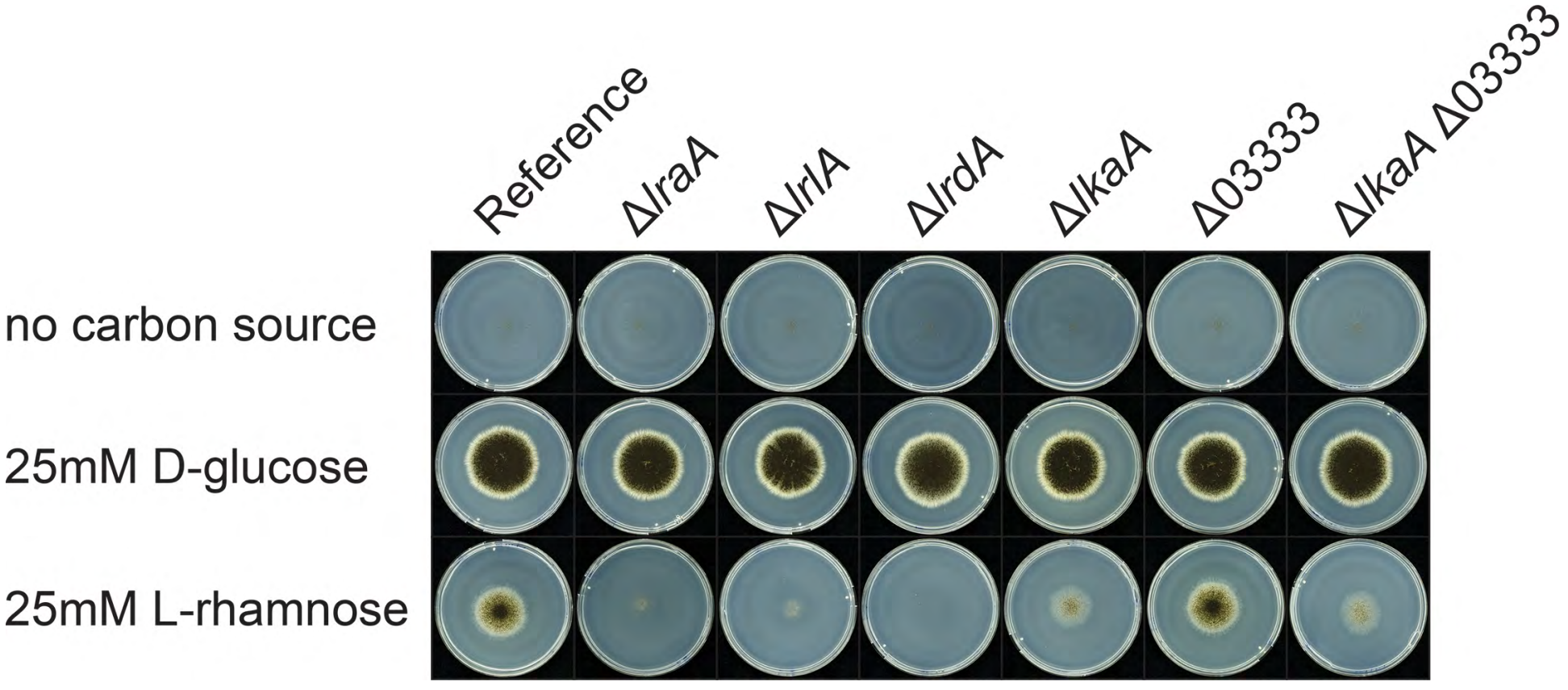
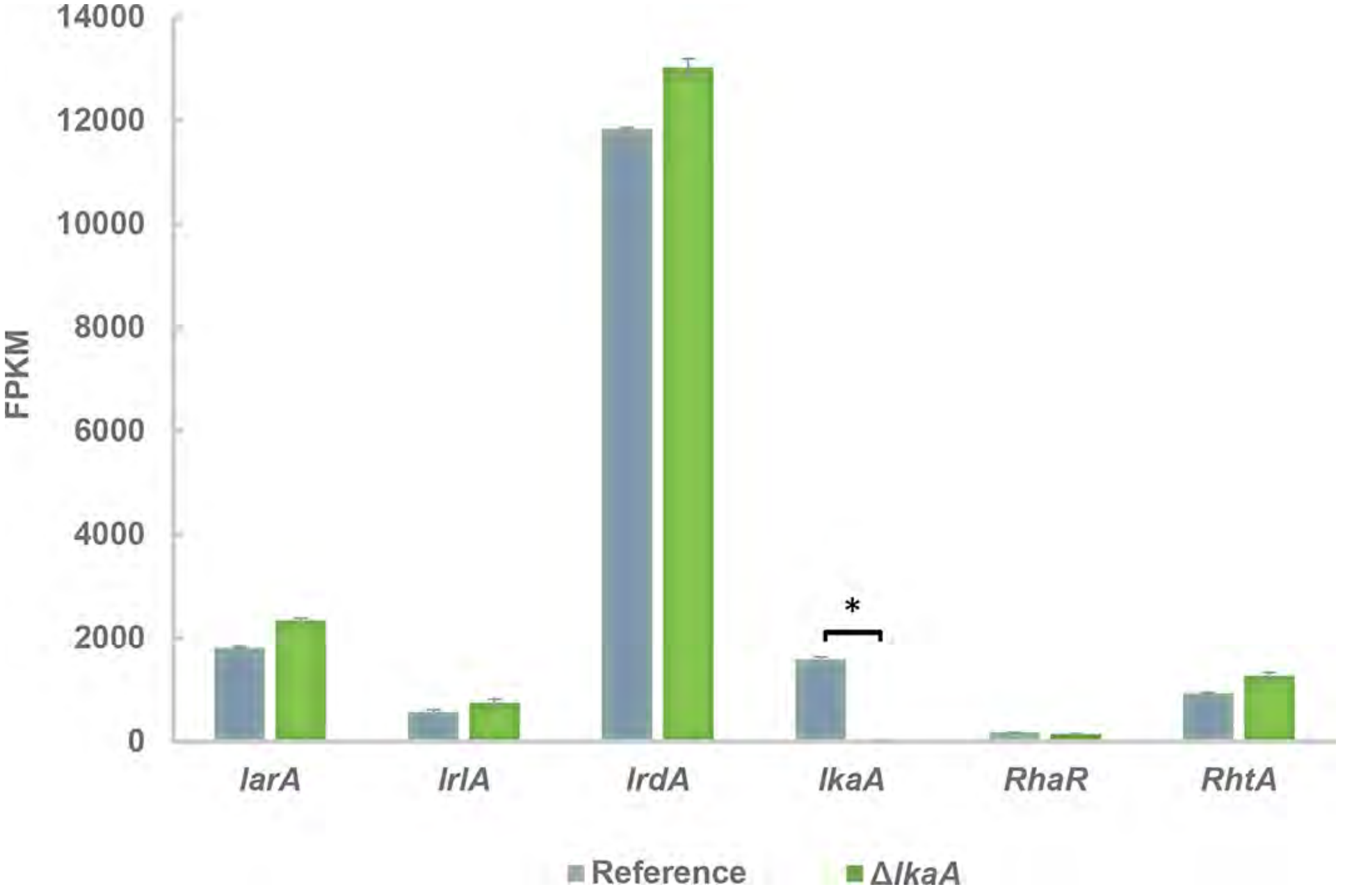

Pectic Substrates
Pectinolytic enzymes
(EC 3.2.1.67, EC 3.2.1.40, EC 3.2.1.-, EC 4.2.2.-, EC 3.1.1.-)
Rha
Rha
Rha
RhtA
Rha
LraA
L-rhamnono-γ-lactone
LrlA
L-rhamnonate
LrdA
L-2-keto-3-deoxy-rhamnonate
LkaA
L-lactaldehyde
pyruvate
RhaR
Extracellular space
Intracellular space


Reference
ΔlraA
ΔlrlA
ΔlrdA
ΔlkaA
Δ03333
ΔlkaA Δ03333
no carbon source
25mM D-glucose
25mM L-rhamnose


14000
12000
10000
8000
6000
4000
2000
0
FPKM
*
larA
lrlA
lrdA
lkaA
RhaR
RhtA
Reference
ΔlkaA

Author statement

**Tania Chroumpi:** Investigation, Writing – Original Draft. **Maria Victoria Aguilar-Pontes**: Formal Analysis, Data Curation, Writing – Original Draft. **Mao Peng**: Formal Analysis, Data Curation, Writing – Review & Editing. **Mei Wang**: Investigation. **Anna Lipzen**: Formal Analysis. **Vivian Ng**: Project administration. **Igor V. Grigoriev**: Supervision, Writing – Review & Editing. **Miia R. Mäkelä**: Writing – Review & Editing. **Ronald P. de Vries:** Conceptualization, Supervision, Writing – Review & Editing, Funding acquisition.

**Table A.1:** Plasmids used in this study.

| Plasmid | Description | CRISPR guide sequence | Reference |
|---|---|---|---|
| ANEp8-Cas9 | Extra-chromosomal *cas9* expressing plasmid | - | Song *et al.* (2018) |
| ANEp8-Cas9-gRNA (*lraA*) | ANEp8-Cas9 with gRNA targeting *lraA* | GTAGCCGTAAACCACCTCGG | This study |
| ANEp8-Cas9-gRNA (*lrlA*) | ANEp8-Cas9 with gRNA targeting *lrlA* | CATCTTTGTGGCCTTCCCCG | This study |
| ANEp8-Cas9-gRNA (*lrdA*) | ANEp8-Cas9 with gRNA targeting *lrdA* | ATGACCGTCGAAGTCATCCG | This study |
| ANEp8-Cas9-gRNA (*lkaA*) | ANEp8-Cas9 with gRNA targeting *lkaA* | GTCCTAGTAGACGCCGAGCA | This study |
| ANEp8-Cas9-gRNA (03333) | ANEp8-Cas9 with gRNA targeting NRRL3_03333 | ACGCAATCTGTGCAGTACCA | This study |

**Table A.2:** Primers used in this study. The 20 bp guide RNA (gRNA) sequences designed for the deletion of our target genes are indicated in red font. The linker sequence is depicted in lowercase/ bold font.

| Primer name | Sequence (5' to 3') | Used for |
|---|---|---|
| G119_01493_fw | CATCTTTGTGGCCTTCCCCGGTTTTAGAGCTAGAAATAGCAAG | construction of the gRNA (*lrlA*) |
| G119_01493_rev | CGGGGAAGGCCACAAAGATGGACGAGCTTACTCGTTTCG | construction of the gRNA (*lrlA*) |
| left_01493_fw | CCTGGCATGGTTCTGGTAATTGG | amplification of *lrlA* 5' flank |
| left_01493_rev | **cgatagcgaatcctagcagt**TGGCATTGTCGTGGGTGTAG | amplification of *lrlA* 5' flank |
| right_01493_fw | **actgctaggattcgctatcg**GTGTGTAATGTGGGTGGAGG | amplification of *lrlA* 3' flank |
| right_01493_rev | GGTAACAATCCCACGAGAAGC | amplification of *lrlA* 3' flank/ checking the presence or absence of the *lrlA* |
| 01493_NEST_fw | ACGAGTCAGGAGGGTGCTTG | fusion of *lrlA* 5' and 3' flanks |
| 01493_NEST_rev | CGACCATCAACCACAATCAAC | fusion of *lrlA* 5' and 3' flanks |

| | | |
|---|---|---|
| G12_01494_fw | GTAGCCGTAAACCACCTCGGGTTTTAGAGCTAGAAATAGCAAG | construction of the gRNA (*lraA*) |
| G12_01494_rev | CCGAGGTGGTTTACGGCTACGACGAGCTTACTCGTTTCG | construction of the gRNA (*lraA*) |
| left_01494_fw | GACGGGACTAAGGGATTTGC | amplification of *lraA* 5' flank |
| left_01494_rev | **cgatagcgaatcctagcagt**TGTGATGGGTTGATTGTGGTTG | amplification of *lraA* 5' flank |
| right_01494_fw | **actgctaggattcgctatcg**CTGGAAGAGGCTGCTAATGTG | amplification of *lraA* 3' flank |
| right_01494_rev | GACTCCCACATCCACCTCTTCC | amplification of *lraA* 3' flank/ checking the presence or absence of the *lraA* |
| 01494_NEST_fw | AGAGATACCAATGACCTGTTCG | fusion of *lraA* 5' and 3' flanks |
| 01494_NEST_rev | ACCACCTCCATTCCCACATC | fusion of *lraA* 5' and 3' flanks |
| G121_01495_fw | ATGACCGTCGAAGTCATCCGGTTTTAGAGCTAGAAATAGCAAG | construction of the gRNA (*lrdA*) |
| G121_01495_rev | CGGATGACTTCGACGGTCATGACGAGCTTACTCGTTTCG | construction of the gRNA (*lrdA*) |

| | | |
|---|---|---|
| left_01495_fw | CGAAGGACTGGTGATGGATGG | amplification of *lrdA* 5’ flank |
| left_01495_rev | **cgatagcgaatcctagcagt**ATGTTGGCAGTAGTTTAGCGGAG | amplification of *lrdA* 5’ flank |
| right _01495_fw | **actgctaggattcgctatcg**GCTTCAATTCTCACTCCTGC | amplification of *lrdA* 3’ flank |
| right _01495_rev | TCCACATCAGAGAGATCATCAC | amplification of *lrdA* 3’ flank/ checking the presence or absence of the *lrdA* |
| 01495_NEST_fw | AGCCGTCTCTGATGGTGAGC | fusion of *lrdA* 5’ and 3’ flanks |
| 01495_NEST_rev | CACATAACCACTCAACTCCTCAC | fusion of *lrdA* 5’ and 3’ flanks |
| G37_08604_fw | GTCCTAGTAGACGCCGAGCAGTTTTAGAGCTAGAAATAGCAAG | construction of the gRNA (*lkaA*) |
| G37_08604_rev | TGCTCGGCGTCTACTAGGACGACGAGCTTACTCGTTTCG | construction of the gRNA (*lkaA*) |
| left_08604_fw | CTTGCTACTATCGACAACACAGG | amplification of *lkaA* 5’ flank |
| left_08604_rev | **cgatagcgaatcctagcagt**GGAGATGATCCTGAGCGTGG | amplification of *lkaA* 5’ flank |

| | | |
|---|---|---|
| right_08604_fw | **actgctaggattcgctatcg**GTTGAAGAGCGTTACGGAGG | amplification of *lkaA* 3’ flank |
| right_08604_rev | TGATTCCGTTAGTCGTTCTTCC | amplification of *lkaA* 3’ flank/ checking the presence or absence of the *lkaA* |
| 08604_NEST_fw | AGTAGCACAGCCAACAAGAACG | fusion of *lkaA* 5’ and 3’ flanks |
| 08604_NEST _rev | TGAGCAAATCAAGCAGAGAGAGG | fusion of *lkaA* 5’ and 3’ flanks |
| G53_03333_fw | ACGCAATCTGTGCAGTACCAGTTTTAGAGCTAGAAATAGCAAG | construction of the gRNA (03333) |
| G53_03333_rev | TGGTACTGCACAGATTGCGTGACGAGCTTACTCGTTTCG | construction of the gRNA (03333) |
| left_03333_fw | GGAGCAGCAATGGAAATGAAAC | amplification of NRRL3_03333 5’ flank |
| left_03333_rev | **cgatagcgaatcctagcagt**CGATCATAGGCAGGGTAGATTG | amplification of NRRL3_03333 5’ flank |
| right_03333_fw | **actgctaggattcgctatcg**GTGTCGGTAGTGTCAGGAGG | amplification of NRRL3_03333 3’ flank |
| right_03333_rev | TGTTGGAAGAGAAGCCGAGG | amplification of NRRL3_03333 3’ flank/ checking the presence or absence of the NRRL3_03333 |

| | | |
|---|---|---|
| 03333_NEST_fw | ACCGAAGGTGGTTAGTTCATGC | fusion of NRRL3_03333 5’ and 3’ flanks |
| 03333_ NEST _rev | TTGATGAATCCGCGAAGGATAGG | fusion of NRRL3_03333 5’ and 3’ flanks |
| linker_fw | ACTGCTAGGATTCGCTATCG | checking the presence or absence of the target genes |

**Table A.3: Comparison of the domains in characterized L-2-keto-3-deoxyrhamnonate aldolase**

| Strains | Gene ID | Annotation | InterProScan | Pfam | References |
|---|---|---|---|---|---|
| *Schefferomyces stipitis* | PICST_64442 | L-KDR aldolase | IPR002220:DapA-like \| IPR013785:Aldolase-type TIM barrel | PF00701:Dihydrodipicolinate synthetase family | [a] (Koivistoinen *et al.*, 2012) |
| *Aspergillus niger* NRRL3 | NRRL3_03899 | N-acetylneuraminate lyase | IPR002220:DapA-like \| IPR013785:Aldolase-type TIM barrel | PF00701:Dihydrodipicolinate synthetase family | [b](Khosravi *et al.*, 2017) |
| | NRRL3_05649 | N-acetylneuraminate lyase | IPR013785:Aldolase-type TIM barrel \| IPR002220:DapA-like | PF00701:Dihydrodipicolinate synthetase family | [b](Khosravi *et al.*, 2017) |
| | NRRL3_06731 | DapA-like protein | IPR013785:Aldolase-type TIM barrel \| IPR002220:DapA-like | PF00701:Dihydrodipicolinate synthetase family | [b](Khosravi *et al.*, 2017) |
| | NRRL3_08604 | HpcH/ HpaI aldolase/citrate lyase domain-containing protein | IPR005000: HpcH/HpaI aldolase/citrate lyase domain | PF03328: HpcH/HpaI aldolase/citrate lyase family | This study |
| | NRRL3_03333 | HpcH/ HpaI aldolase/citrate lyase domain-containing protein | IPR005000: HpcH/HpaI aldolase/citrate lyase domain | PF03328: HpcH/HpaI aldolase/citrate lyase family | This study |

[a] Koivistoinen, O.M., Arvas, M., Headman, J.R., Andberg, M., Penttila, M., Jeffries, T.W., Richard, P., 2012. Characterisation of the gene cluster for L-rhamnose catabolism in the yeast *Scheffersomyces* (*Pichia*) *stipitis*. Gene 492, 177-85.

[b] Khosravi, C., Kun, R.S., Visser, J., Aguilar-Pontes, M.V., de Vries, R.P., Battaglia, E., 2017. *In vivo* functional analysis of L-rhamnose metabolic pathway in *Aspergillus niger*: A tool to identify the potential inducer of RhaR. BMC Microbiol. 17, 214.