Itaconic acid production is regulated by LaeA in *Aspergillus pseudoterreus*

Kyle R. Pomraning, Ziyu Dai, Nathalie Munoz, Young-Mo Kim, Yuqian Gao, Shuang Deng, Teresa Lemmon, Marie S. Swita, Jeremy D. Zucker, Joonhoo Kim, Stephen J. Mondo, Ellen Panisko, Meagan C. Burnet, Bobbie-Jo M. Webb-Robertson, Beth Hofstad, Scott E. Baker, Kristin E. Burnum-Johnson and Jon K. Magnuson

**ABSTRACT**

The global regulator LaeA controls secondary metabolism in diverse *Aspergillus* species. Here we explored its role in regulation of itaconic acid production in *Aspergillus pseudoterreus*. To understand its role in regulating metabolism, we deleted and overexpressed *laeA*, and assessed the transcriptome, proteome, and secreted metabolome prior to and during initiation of phosphate limitation induced itaconic acid production. We found that secondary metabolite clusters, including the itaconic acid biosynthetic gene cluster, are regulated by *laeA* and that *laeA* is required for high yield production of itaconic acid. Overexpression of LaeA improves itaconic acid yield at the expense of biomass by increasing the expression of key biosynthetic pathway enzymes and attenuating the expression of genes involved in phosphate acquisition and scavenging. Increased yield was observed in optimized conditions as well as conditions containing excess nutrients that may be present in inexpensive sugar containing feedstocks such as excess phosphate or complex nutrient sources. This suggests that global regulators of metabolism may be useful targets for engineering metabolic flux that is robust to environmental heterogeneity.

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**1. Introduction**

Itaconic acid is a five-carbon dicarboxylic acid that has been recognized as a platform chemical with broad applications in the production of commodity and specialty chemicals such as polymers, coatings and solvents (W et al., 2004). Iaconic acid is produced naturally from sugars by the filamentous fungus *Aspergillus terreus* during submerged fermentation culture at high titer and low pH (Larsen and Eimhjellen, 1955; Eimhjellen and Larsen, 1955). Significant market opportunities are available for the development of itaconic acid as a biorenewable product, however to be competitive with petrochemical-derived products, it was estimated in 2004 that the fermentation cost needed to be at or below $0.25/pound (W et al., 2004). The primary pathways to achieve this were identified as increased fermentation rate, improvement of final titer, and production from inexpensive C5 sugars from lignocellulosic feedstocks. Since then, demand has remained low.

Production of itaconic acid by *Aspergillus terreus* on glucose occurs via glycolysis and the tri-carboxylic acid cycle (Willke and Vorlop, 2001). Cis-aconitate is released by *aconitase* during isomerization of citrate to isocitrate and transported out of the mitochondrion by the mitochondrial carrier protein *mtdA* (Steiger et al., 2016). Cis-aconitate is then decarboxylated by the cis-aconitate decarboxylase (*cadA*) to itaconate in the cytosol. Deletion of the major facilitator superfamily protein from the itaconic acid biosynthetic cluster (*mfsA*) reduces, but does not eliminate, itaconic acid production (Deng et al., 2020); while over-expression improves productivity (Li et al., 2011) suggesting it plays a
role as the major, but not sole, transporter for itaconate across the plasma membrane (Shin et al., 2017). The genes encoding this metabolic pathway are colocalized on the chromosome suggesting their regulation may be coordinated as is the case for many secondary metabolite pathways in fungi (Li et al., 2011).

Overall titer, yield, and production rate have been improved by systematic alteration of culture conditions for high productivity from glucose (Hevekerl et al., 2014a, 2014b; Karaffa et al., 2015; Krull et al., 2017; Kuenz and Krull, 2018) while utilization of pentose sugars as well as the impact on productivity of nutrients and growth inhibitors present in lignocellulosic feedstocks (Saha and Kennedy, 2017, 2018, 2020; Saha et al., 2017, 2019; Kollath et al., 2019; Sandor et al., 2021) has been investigated. Limited efforts to produce itaconic acid from lignocellulosic feedstocks such as enzymatically saccharified wheat straw hydrolysate have generally yielded poor results due to the sensitivity of the process to impurities in the culture medium such as acetic acid and furfural (Kuenz and Krull, 2018; Saha et al., 2019) and the need to maintain phosphate (Willke and Vorlop, 2001) and manganese (Karaffa et al., 2015; Saha et al., 2019) limited conditions for high productivity.

High yields of itaconic acid production have been achieved using fungi (Xie et al., 2020; Sun et al., 2020; Becker et al., 2020; Zhao et al., 2018, 2019; Hosseinpour Tehrani et al., 2019) which are capable of producing itaconic acid from purified hexose and pentose sugars characteristic of the sugars present in lignocellulosic feedstocks at pH well below the pKa of itaconic acid (pKa = 3.58) allowing for production of the free acid (Bafana and Pandey, 2018). However, strategies are needed to improve the robustness of itaconic acid production to compositional variance present in lignocellulosic feedstocks since utilization of pure sugars, purified molasses, or starch hydrolysates are not economically competitive when compared to raw materials from the petrochemical industry (Kuenz and Krull, 2018).

Bioconversion processes often fail when moved to non-ideal conditions in part because the organism responds to the new environment by altering the expression of genes which subsequently changes flux through the organism’s metabolic network. We hypothesized that altering the organism’s ability to respond to environmental perturbation, by modification of global regulatory mechanisms, would improve robustness of the bioprocess to environmental change. In many fungi the putative methyltransferase laeA is a global regulator of genes present in secondary metabolite clusters and the response to environmental cues (Bayram and Braus, 2012; Amare and Keller, 2014; Gerke and Braus, 2014). In Trichoderma reesei, laeA regulates expression of cellulases and polysaccharide hydrolases (Karimi Aghcheh et al., 2013) as well as a
Fig. 2. Regulation of itaconic acid production by LaeA in *Aspergillus pseudotereus*. Strains were cultivated in shake flasks for 8 days until all of the glucose and xylose present in the medium had been consumed from an initial concentration of 50 g/L total sugar. A) The major products of the cultivation are biomass and itaconic acid. Both are affected by *laeA* deletion and overexpression. B) Production of other tricarboxylic acid cycle derived organic acids at minor levels. Citric acid was not detected at > 5 mg/L in any sample. C) Correlation between itaconic acid production and final biomass or other acids. GX (glucose/xylose medium), P (phosphate), N (yeast extract). Asterisks indicate significant differences between either the *laeA* + or Δ*laeA* strain and the wild-type in each condition.
variety of secondary metabolite clusters (Karimi-Aghcheh et al., 2013), while in *Aspergillus nidulans*, LaeA forms a complex with VeA and VelB that affects the production of secondary metabolites and participates in light-responsive developmental regulation (Bayram et al., 2008). LaeA has also been implicated in regulation of central carbon metabolism and citric acid production in *Aspergillus carbonarius* (Linde et al., 2016) and *Aspergillus luchuensis* (Kadooka et al., 2020). Here we examined the impact of laeA on production of itaconic acid, characterized its impact as a global regulator of gene expression, and examined its utility for bioconversion of lignocellulosic feedstocks by *Aspergillus pseudotereus*.

2. Methods

2.1. Cultivation conditions

*A. pseudotereus* strain ATCC®32359™ was obtained from American Type Culture Collection (Manassas, Virginia). Overexpression and deletion strains of *laeA* were described previously (Dai and Baker, 2016). All strains were maintained on potato dextrose agar (BD, USA). Spore inoculum was grown on potato dextrose agar at 30°C and harvested after 5 days. Transformants were selected on minimum medium agar (10 g/L glucose, 6 g/L Na₂NO₃, 0.52 g/L KCL, 0.52 g/L MgSO₄.7H₂O, 1.7 g/L KH₂PO₄, 0.1 mg/L Bi, 0.1 mg/L pyridoxine·HCl, 0.1 mg/L thiamine·HCl, 0.1 mg/L riboflavin, 0.1 mg/L para-aminobenzoic acid, 0.1 mg/L nicotinic acid, 22 mg/L ZnSO₄.7H₂O, 11 mg/L H₂BO₃, 5 mg/L MnCl₂.4H₂O, 5 mg/L FeSO₄.7H₂O, 1.7 mg/L CoCl₂.6H₂O, 1.6 mg/L CuSO₄.5H₂O, 1.5 mg/L Na₂MoO₄.2H₂O, 50 mg/L Na₂EDTA, and 15 g/L agar) supplemented with 100 µg/ml hygromycin or 250 µg/ml bleomycin as appropriate. For shake-flask experiments, *A. pseudotereus* strains were cultivated in minimal glucose/x-ylose medium (GX) (32.4 g/L glucose, 17.6 g/L xyllose, 2.36 g/L (NH₄)₂SO₄, 0.11 g/L KH₂PO₄, 2.08 g/L MgSO₄.7H₂O, 0.13 g/L CaCl₂.2H₂O, 74 mg/L NaCl, 1.3 mg/L ZnSO₄.7H₂O, 0.7 mg/L MnCl₂.4H₂O, 5.5 mg/L FeSO₄.7H₂O, 0.2 mg/L CuSO₄.5H₂O, pH 3.4) (Riscaldati et al., 2000) at 30°C and 200 rpm unless otherwise noted. To the GX medium 0.14 g/L KH₂PO₄ was added to make GX-P and GX-NP and 2 g/L yeast extract was added to make GX-N and GX-NP. Spore suspensions are maintained in 15% glycerol at ~80°C. For multi-omic analyses spores from wild-type (ATCC®32,359™) and *laeA* overexpressing *A. pseudotereus* were inoculated at 10³/0.1 mL into 50 mL production medium (PM) (100 g/L glucose, 2.36 g/L (NH₄)₂SO₄, 0.11 g/L KH₂PO₄, 2.08 g/L MgSO₄.7H₂O, 0.13 g/L CaCl₂.2H₂O, 74 mg/L NaCl, 1.3 mg/L ZnSO₄.7H₂O, 0.7 mg/L MnCl₂.4H₂O, 5.5 mg/L FeSO₄.7H₂O, 0.2 mg/L CuSO₄.5H₂O, pH 3.4) (Riscaldati et al., 2000) in 250 ml smooth-walled Erlenmeyer flasks at 30°C and 200 rpm in an orbital shaker.

### Table 2

| GO term                                      | Fold enrichment | Corrected p-value |
|----------------------------------------------|-----------------|-------------------|
| Monooxygenase activity                       | 2.09            | 1.10E-08          |
| Metabolic process                            | 1.29            | 5.50E-07          |
| Electron transport                           | 1.52            | 7.11E-07          |
| Integral to membrane                        | 1.44            | 1.31E-06          |
| Oxidoreductase activity                      | 1.41            | 2.21E-06          |
| Catalytic activity                           | 1.28            | 2.11E-05          |
| Carbohydrate metabolic process               | 1.55            | 1.14E-03          |
| Heme binding                                 | 1.66            | 2.25E-03          |
| Iron ion binding                             | 1.66            | 7.17E-03          |
| Hydrolyzing O-glycosyl compounds             | 1.68            | 1.12E-02          |

### Table 3

| Pathway                              | Fold-change (log) | Up-regulated | Down-regulated | Quantified |
|--------------------------------------|-------------------|--------------|----------------|------------|
| Styrene degradation                  | 3.83              | 2 (100%)     | 0 (0%)         | 2          |
| CS-branched dibasic acid metabolism  | 2.64              | 1 (50%)      | 0 (0%)         | 2          |
| Cysteine degradation                 | 2.06              | 2 (67%)      | 0 (0%)         | 3          |
| Glyoxylate and dicarboxylate metabolism| 1.49             | 3 (60%)      | 1 (20%)        | 5          |
| Purine metabolism                    | 1.31              | 8 (67%)      | 0 (0%)         | 12         |
| Beta-alanine metabolism              | 0.97              | 7 (78%)      | 1 (11%)        | 9          |
| Starch and sucrose metabolism        | 0.84              | 2 (50%)      | 0 (0%)         | 4          |
| Mevalonate pathway                   | 0.80              | 1 (33%)      | 0 (0%)         | 3          |
| Galactose metabolism (melibiase)      | 0.75              | 5 (83%)      | 0 (0%)         | 6          |
| Glycolysis/glucogenesis               | 0.66              | 2 (67%)      | 0 (0%)         | 3          |
| Xenobiotics/biodegradation and metabolism| 0.59             | 3 (43%)      | 0 (0%)         | 7          |
| Stachyose, raffinose, sucrose degradation| 0.58             | 5 (71%)      | 0 (0%)         | 7          |
| Metabolism of terpenoids and polyketides| 0.55             | 3 (75%)      | 1 (25%)        | 4          |
| Phenylalanine metabolism             | 0.54              | 3 (50%)      | 1 (17%)        | 6          |
| Starch and sucrose metabolism        | 0.51              | 21 (53%)     | 7 (18%)        | 40         |

### Table 3 (continued)

| Pathway                              | Fold-change (log) | Up-regulated | Down-regulated | Quantified |
|--------------------------------------|-------------------|--------------|----------------|------------|
| Amino acid metabolism                | −0.51             | 0 (0%)       | 2 (100%)       | 2          |
| Vitamin B6 metabolism                | −0.53             | 0 (0%)       | 4 (67%)        | 6          |
| One carbon pool by folate            | −0.62             | 0 (0%)       | 4 (100%)       | 4          |
| Valine, leucine and isoleucine biosynthesis| −0.68          | 0 (0%)       | 3 (100%)       | 3          |
| Formation of unsaturated cystolic fatty acids| −0.74         | 0 (0%)       | 3 (75%)        | 4          |
| Purine metabolism (salvage pathways) | −0.75             | 0 (0%)       | 2 (100%)       | 2          |
| Fructose and mannose metabolism      | −0.77             | 0 (0%)       | 2 (100%)       | 2          |
| Biosynthesis of unsaturated fatty acids| −0.77             | 0 (0%)       | 1 (50%)        | 2          |
| Vanillate degradation                | −0.78             | 0 (0%)       | 1 (50%)        | 2          |
| Amino sugar and nucleotide sugar metabolism| −0.86             | 0 (0%)       | 2 (67%)        | 3          |
2.2. Strain construction

Over-expression and deletion cassettes for *A. pseudoterreus* laeA were described previously (Dai and Baker, 2016). Briefly, the double-joint PCR method (Yu et al., 2004) was applied to prepare the laeA deletion construct with oligos P1 to P8. Oligo pair P15/P16 and P17/P18 were used to isolate the *gpdA* (M33539.1) promoter and the *laeA* (AY394722.1) coding region of *Aspergillus nidulans*, which were fused together by PCR. The 5′-upstream fragment of the *A. niger* pyrG gene (P19/P20), the gpdAp-laeA fragment (P21/P22), and *A. nidulans* TrpC gene models

| Family                  | Up-regulated | Down-regulated | Quantified |
|-------------------------|--------------|----------------|------------|
| Glycoside hydrolase     | 75 (43%)     | 23 (13%)       | 173        |
| Glycosyl transferase    | 13 (20%)     | 13 (20%)       | 65         |
| Auxiliary activity      | 14 (37%)     | 8 (21%)        | 38         |
| Carbohydrate binding    | 11 (37%)     | 4 (13%)        | 30         |
| Carbohydrate esterase   | 9 (39%)      | 3 (13%)        | 23         |
| Polysaccharide lyase    | 3 (25%)      | 2 (17%)        | 12         |
transcriptional terminator (P23/P24), Aspergillus oryzae pyrithiamine resistance (ptrA) marker gene (P25/P26), and the 3′-downstream fragment of A. niger pyrG gene (P27/P28) were prepared by PCR and fused together by yeast gap-repair cloning method (Dai et al., 2013) to form the transgene expression construct. Finally, the laeA over-expression construct for A. pseudotereus was prepared by the yeast gap-repair cloning method. The laeA over-expression cassette was targeted to the 5′-upstream region of laeA (between −1.7 kb and −1.57 kb). Primers P29/P30 were used to amplify the laeA upstream fragment between 2716 bp and 1698 bp of A. pseudotereus, P31/P32 for the entire fragment of gpdA-laeA-trpC- ptrA, and P33/P34 for the laeA upstream fragment between 1571 bp and 493 bp of A. pseudotereus prior to assembly and transformation. Deletion of laeA in transgenic strains of A. pseudotereus was confirmed by PCR with oligo pair P1/P107 and P108/P8. Integration of the laeA over-expression cassette was confirmed by PCR with oligo pairs P23/P32 and P31/P32. Oligos are presented in Table 1.

2.3. Reference genome sequencing, assembly, and annotation

Genomic DNA and RNA was isolated from A. pseudotereus (ATCC®32359™) using a yeast genomic DNA purification kit (AMRESCO, Solon, OH) and Maxwell 16 LEV Plant RNA kit (Promega, Madison, WI) respectively. Genomic DNA was sequenced by paired-end 250 base pair sequencing on an Illumina MiSeq platform (San Diego, CA) and assembled into contigs with the CLC Genomics Workbench (Qiagen, Hilden, Germany). Stranded RNA from A. pseudotereus (ATCC®32359™) grown in YPD (10 g/L peptone, 10 g/L yeast extract, 20 g/L glucose), YES (150 g/L sucrose, 20 g/L yeast extract, 50 mg/L MgSO₄·7H₂O, 10 mg/L ZnSO₄·7H₂O, 5 mg/L CuSO₄·5H₂O), MM, and MM-W (20 g/L wheat instead of 20 g/L glucose) medium was sequenced by paired-end 100 base pair sequencing on an Illumina HiSeq2500 platform. RNA sequences were used to produce a high-quality genome annotation with the JGI genome annotation pipeline. The assembled genome has been deposited at Genbank (Accession: PRJNA420104) and the annotated version has been made available through the MycoCosm portal (https://mycocosm.jgi.doe.gov/Asppseute1/Asppseute1.home).
Significantly up- and down-regulated proteins in the LaeA overexpression strain versus wild-type during growth (36h) and the transition to production phase (60h and 84h) were assessed for enrichment of gene ontology terms. Hyper-geometric test p-values are shown.

### Table 5

| GO term | Hour | Proteins (sig./ background) | Fold enrichment | p-value |
|---------|------|-----------------------------|-----------------|---------|
| Oxidoreductase activity | 36   | 14/203                       | 2.62            | 9.14E-04|
| One-carbon compound metabolic process | 36   | 2/2                          | 28.13           | 1.19E-03|
| Peroxidase activity | 36   | 3/9                          | 14.56           | 1.53E-03|
| Phospholipase D activity | 36   | 2/3                          | 26.85           | 2.25E-03|
| Protein transporter activity | 36   | 4/21                         | 8.29            | 2.28E-03|
| Metallopeptidase activity | 36   | 3/12                         | 11.26           | 3.78E-03|
| Response to oxidative stress | 36   | 3/12                         | 9.02            | 7.00E-03|
| O-glycosyl compound hydrolase activity | 60   | 4/35                         | 8.26            | 2.47E-03|
| Carbohydrate metabolic process | 60   | 5/67                         | 4.71            | 6.82E-03|
| Serine carboxypeptidase activity | 60   | 2/8                          | 20.04           | 7.31E-03|
| Ubiquitin-cytoschrome-c reductase activity | 84   | 3/6                          | 20.58           | 3.87E-04|
| Mitochondrial electron transport | 84   | 2/2                          | 34.35           | 7.45E-04|
| Metabolism of terpenoids and polyketides | 84   | 2/3                          | 25.28           | 2.19E-03|
| Metabolism of other amino acids | 84   | 3/13                         | 9.86            | 4.41E-03|

### Down-regulated proteins

| GO term | Hour | Proteins (sig./ background) | Fold enrichment | p-value |
|---------|------|-----------------------------|-----------------|---------|
| Small subunit processome | 36   | 3/4                          | 12.57           | 8.92E-04|
| Protein serine/threonine kinase activity | 60   | 8/38                         | 4.38            | 6.39E-04|
| Hydrolase activity, acting on ester bonds | 60   | 4/9                          | 9.51            | 7.79E-04|
| Metal ion binding | 60   | 6/24                         | 5.33            | 1.22E-03|
| Protein kinase activity | 60   | 8/42                         | 3.97            | 1.29E-03|
| Protein amino acid phosphorylation | 60   | 8/45                         | 3.73            | 1.87E-03|
| Protein-tyrosine kinase activity | 60   | 7/37                         | 4.01            | 2.73E-03|
| Hydrolase activity | 60   | 10/73                        | 2.81            | 4.41E-03|
| Myosin complex | 60   | 2/4                          | 15.33           | 8.30E-03|
| Methyltransferase activity | 60   | 4/16                         | 5.65            | 8.42E-03|
| Riboflavin metabolism | 84   | 6/10                         | 7.78            | 3.33E-05|
| Folate biosynthesis | 84   | 7/15                         | 6.13            | 5.65E-05|
| Metabolism of cofactors and Vitamins | 84   | 13/63                        | 2.68            | 6.19E-04|
| Hydrolase activity, acting on ester bonds | 84   | 4/9                          | 8.35            | 1.29E-03|
| Acid phosphatase activity | 84   | 4/10                         | 7.61            | 2.04E-03|
| Cellular metabolic process | 84   | 5/16                         | 5.79            | 2.06E-03|
| Fatty-acid ligase activity | 84   | 2/2                          | 16.31           | 3.66E-03|
| Double-strand break repair | 84   | 2/2                          | 15.98           | 3.78E-03|

### Table 5 (continued)

| GO term | Hour | Proteins (sig./ background) | Fold enrichment | p-value |
|---------|------|-----------------------------|-----------------|---------|
| Fatty acid metabolism | 84   | 2/2                         | 12.07           | 6.40E-03|
| G-protein coupled receptor protein signaling | 84   | 3/7                          | 8.23            | 6.68E-03|

2.4. Multi-omic analyses

Tissue samples were flash frozen in liquid nitrogen after collection. RNA was isolated using a Maxwell 16 LEV Plant RNA kit and was sequenced by stranded single-end 50 base pair sequencing on an Illumina HiSeq2500 platform. Sequencing reads were mapped to the *A. pseudoterreus* coding sequence models, using Bowtie2 v2.2.8 (Langmead and Salzberg, 2012). The alignments were sorted, converted to bam format, and quantified with Samtools v1.3.1 (Li, 2011). For global extracellular metabolomics analysis, supernatants were dried, chemically derivatized, acquired and analyzed as previously reported (Pomraning et al., 2021). Specific metabolites were also quantified using a GC-MS with external calibration curves corresponding to authentic chemical standards as described previously (Pomraning et al., 2021). Absolute quantification of some metabolites was performed by HPLC. Samples were filtered with a 0.2 μm syringe filter and analyzed for 45 min using an Aminex HPX-87H ion exclusion column with a 1 mM H2SO4 flow of 0.6 ml/ml. The temperature of the column was 60°C. The refractive index at 45°C and the UV absorption at 210 nm were measured. Targeted and global proteomics was performed as previously described (Pomraning et al., 2021).

2.5. Data analysis

For protein samples, sample level quality was ensured by a robust Principal Component Analysis to compute a robust Mahalanobis distance based on sample-level parameters (Matzke et al., 2011). The default for normalization is standard global median centering to account for total abundance differences between samples. A test was performed to assure that these factors are not biased (Webb-Robertson et al., 2010). For this dataset there was no bias detected and we utilized global median centering (Callister et al., 2006). Protein quantification was performed with standard reference-based median averages (Polpitiya et al., 2008; Matzke et al., 2013). Statistics were performed with established standard methods (Webb-Robertson et al., 2017). For RNA samples, raw read alignment counts were assessed statistically in R v3.5 using the DESeq2 method as implemented in Bioconductor v3.8 (Love et al., 2014). For visualization purposes the aligned reads were examined in the Integrative Genomics Viewer (Robinson et al., 2011). Enrichment of gene ontology terms used FunRich (Pathan et al., 2015).

3. Results

3.1. Genome assembly and annotation of Aspergillus pseudoterreus

We sequenced the genome of *A. terreus* strain ATCC®32359™ (derived from NRRL 1960 (Nakagawa et al., 1975)) as a resource for development as a bioproduction platform organism to an average depth of 109x and assembled into 272 contigs. The resulting assembled genome sequence is 29.5 Mb (N_{50} 678,262 bp; N_{max} 1,693,594 bp) with...
G + C content of 52.3%. Stranded RNA isolated from four growth conditions (YPD, YES, MM, and MM-W) was sequenced and used to annotate the genome with the JGI annotation pipeline. Comparison of randomly selected 1 kilobase sections of the genome with other Aspergillus species by Blastn identified the recently described A. pseudoterreus as the most similar species to strain ATCC®32359™ (Samson et al., 2011). Phylogenetic analysis using the β-tubulin genes with those from other Aspergillus species confirmed this (Fig. 1). We therefore propose designation of strain ATCC®32359™ as A. pseudoterreus and have named it as such in the genome sequence assembly deposited at Genbank (Accession: PRJNA420104) and the annotated sequence available through the JGI’s MycoCosm portal (https://mycocosm.jgi.doe.gov/Asppseute1/Appseute1.home.html).

3.2. LaeA regulates itaconic acid production

The global regulator LaeA controls secondary metabolism in a wide variety of fungi (Bayram and Braus, 2012; Amare and Keller, 2014; Gerke and Braus, 2014). To assess the impact of LaeA on metabolism in A. pseudoterreus we replaced the endogenous laeA gene with hph by targeted homologous recombination to create a deletion strain and overexpressed the laeA homolog from A. nidulans to create a laeA overexpression strain. Overexpression, deletion, and the parental wild-type strain were cultivated for eight days in shake flasks in minimal medium with glucose and xylose present at a ratio typical of a lignocellulosic feedstock (GX) to examine the effect of laeA on itaconic acid production (Fig. 2a). Deletion of laeA nearly eliminated itaconic acid production (decrease of 94%) while overexpression increased itaconic acid production (decrease of 13%) on GX medium. This suggests that LaeA is negatively correlated with cis-aconitic acid production (R² = 0.81) and to a lesser extent succinic acid production (R² = 0.61) (Fig. 2c). Overall, this suggests that the processes regulated by LaeA in A. pseudoterreus promote flux away from growth and toward production of itaconic acid.

3.3. Transcriptome analysis of LaeA overexpression

We quantified transcripts by RNA-sequencing of wild-type and the LaeA overexpression strains of A. pseudoterreus during growth phase at 36 hours in shake flasks to assess global effects on gene expression. Global profiling quantified 10,228 transcripts of which 1089 are negatively correlated with biomass production across the different mutant and medium conditions examined (R² = 0.94) and is positively correlated with cis-aconitic acid production (R² = 0.81) and to a lesser extent succinic acid production (R² = 0.61) (Fig. 2d). Overall, this suggests that the processes regulated by LaeA in A. pseudoterreus promote flux away from growth and toward production of itaconic acid.
The phosphate level was increased (from 0.11 to 0.25 g/L KH\textsubscript{2}PO\textsubscript{4}) and the uptake of phosphate is dependent on limitation of phosphate (Riscaldati et al., 2000). When LaeA overexpression strain was grown, the transcription factor, regulates phosphate acquisition. This indicates that, in addition to CO\textsubscript{2} assimilation and leaves, well over half the carbon unaccounted for in the \textit{Δ}laeA strain.

### 3.4. Metabolic analysis of LaeA overexpression

Production of itaconic acid in the medium used in these experiments is dependent on limitation of phosphate (Riscaldati et al., 2000). When the phosphate level was increased (from 0.11 to 0.25 g/L KH\textsubscript{2}PO\textsubscript{4}) the wild-type strain produced more biomass and less itaconic acid. In the LaeA overexpression strain the impact of additional phosphate was attenuated while in the deletion strain addition of phosphate decreased itaconic acid production to below the limit of detection (Fig. 2A). We performed global extracellular metabolomics analysis to identify products secreted by \textit{A. pseudoterrae} during itaconic acid production that are specifically enriched in the \textit{Δ}laeA strain suggesting the unaccounted carbon is primarily in the form of biomass and gaseous products.

![Fig. 3a](image)

**Table 6**

| Regulators of phosphate acquisition | Homologs | RNA (36h) | Protein (60h) |
|------------------------------------|----------|-----------|--------------|
| Protein ID | Annotation | A. nidulans | N. crassa | S. cerevisiae | Δ g-value | Δ p-value | Δ g-value | Δ p-value | Δ p-value |
| 520908 | Ankyrin repeat protein; inhibits Pho80/Pho85 complex | AN4310 | nuc-2 | PHO81 | –0.19 | 6.8E-02 | – | – | – |
| 508770 | Cyclin involved in phosphate homeostasis; interacts with Pho85 | AN5156 | preg | PHO80 | –0.41 | 5.0E-04 | – | – | – |
| 187867 | Cyclin-dependent protein kinase; interacts with cyclin Pho80 | AN8261 | mdk-1 | PHO85 | 0.16 | 1.3E-01 | – | – | – |
| 414825 | Transcription factor, regulates phosphate acquisition | AN8271 | nuc-1 | PHO4 | –0.13 | 2.3E-01 | – | – | – |

### Enzymes involved in phosphate acquisition

| Homologs | RNA (36h) | Protein (60h) |
|----------|-----------|--------------|
| Protein ID | Annotation | A. nidulans | N. crassa | S. cerevisiae | Δ g-value | Δ p-value | Δ g-value | Δ p-value | Δ p-value |
| 482564 | Acid phosphatase | AN8063 | pho-13 | – | 0.25 | 1.5E-02 | 1.03 | 1.2E-01 | –0.80 | 5.2E-01 | 1.14 | 8.9E-01 |
| 456862 | Acid phosphatase | AN2360 | phe-3 | – | – | –1.02 | 1.3E-04 | – | – | –3.68 | 9.2E-05 | – | 1.90 | 2.8E-05 |
| 7506 | Acid phosphatase | AN4055 | pho-9 | – | – | –0.16 | 2.2E-01 | – | – | –0.48 | 2.1E-02 | 0.25 | 6.5E-01 |
| 526208 | Acid phosphatase | AN0952 | pho-8 | – | – | –0.52 | 1.3E-04 | – | – | –0.82 | 1.9E-02 | – | 2.4E-04 |
| 430438 | Acid phosphatase | AN7142 | – | – | –1.24 | 1.3E-04 | – | – | –2.02 | 1.8E-04 | – | 1.58 | 1.2E-04 |
| 472595 | Alkaline phosphatase | AN11069 | pho-11 | – | 0.10 | 3.6E-01 | – | – | – | – | – | – |
| 58394 | Alkaline phosphatase | AN2493 | pho-2 | – | – | –0.39 | 1.2E-02 | – | – | – | – | – | – |
| 456715 | Alkaline phosphatase | AN8622 | pho-12 | – | – | –0.94 | – | – | – | – | – | – |
| 508770 | Vacular alkaline phosphatase | AN10563 | pho-10 | PHO8 | – | –0.40 | 1.3E-04 | – | – | – | – | – |
| 523629 | High-affinity phosphate permease | AN0217 | phe-5 | PHO84 | – | –1.78 | 1.3E-04 | – | – | – | – | – |
| 455719 | Low-affinity phosphate permease | AN0469 | pho-6 | PHO91 | – | –0.39 | 1.3E-04 | – | – | – | – | – |
| 203804 | Low-affinity phosphate transporter | AN5935 | pho-7 | PHO84 | – | –0.55 | 1.3E-04 | – | – | – | – | – |
| 456717 | Phosphate permease | AN10343 | pho-4 | PHO89 | – | –0.79 | 1.9E-02 | – | – | – | – | – |
| 499196 | Phosphate transporter | AN8040 | pho-15 | PHO88 | – | –0.15 | 1.4E-01 | – | – | – | – | – |

**K.R. Pomraning et al.**

Metabolic Engineering Communications 15 (2022) e00203
Deletion of laeA resulted in loss of many extracellular metabolites including aromatic compounds such as 3-methoxyanthranilic acid, 3-dehydroxyshikimic acid, and protocatechuic acid as well as a wide variety of unidentified metabolites that may be byproducts of secondary metabolism. Expression of many of these metabolites was increased by laeA overexpression, consistent with control of their production as secondary metabolites. In general, the level of secreted metabolites in the laeA deletion strain was lower, however, addition of phosphate lead to a profound impact on the secreted metabolome (Fig. 3B) that includes higher extracellular concentration of sugars and their derivatives (melibiose, cellobiose, sophorose, lactose, lactulose, lactobionic acid, 1,5-anhydrohexitol, and trehalose), sugar alcohols (xylitol, galactitol, erythropentitol, and palatinitol), and 2,3-butanediol suggesting laeA may be required for sensing or appropriately responding to phosphate availability.

3.5. Proteome analysis of LaeA overexpression

We profiled the proteome of the wild-type and LaeA overexpression strains at three time-points during growth (36h) and transition to phosphate depletion induced organic acid production phase (60h and 84h) to better understand how LaeA regulates the physiology of A. pseudotereus. Global profiling of wild-type and LaeA overexpression strains of A. pseudotereus quantified 27,203 peptides (corresponding to 3193 proteins), and 188 targeted peptides were used to quantify 74 proteins involved in central carbon metabolism. Of the quantified proteins, 890 are expressed at a significantly different level between the wild-type and LaeA overexpression strain during at least one time-point (p < 0.05).

To examine the function of the proteins regulated by LaeA, functional enrichment analysis for metabolic pathways, biological processes, molecular functions, and cellular compartments was performed for up- and down-regulated genes at 36, 60, and 84 hours (Table S5). The most significantly enriched (p < 0.05 after Bonferroni correction) gene ontology terms are associated with proteins up- and down-regulated at 84 hours and include electron transport, terpene and polyketide metabolism (up-regulated) and riboflavin, folate, and metabolism of other cofactors and vitamins (down-regulated).

We identified regulatory genes to better understand how LaeA
impacts gene regulation in A. pseudotereus. Kinases, phosphatases, and transcription factors were predicted from the genome of A. pseudotereus. InterProScan (Jones et al., 2014) was used to identify proteins with kinase or phosphatase domains while transcription factors were identified by homology with known fungal transcription factors present in the Fungal Transcription Factor Database (Park et al., 2008) and the Transcription factor prediction database (Wilson et al., 2008). From this we identified 326 kinases, 142 phosphatases, and 550 transcription factor candidates from the A. pseudotereus genome, many of which are differentially regulated. Of these proteins the acid phosphatase Pho-3 is the most significantly up-regulated in response to phosphate limitation in the wild-type strain (30.2x from 36h to 84h) and is significantly up-regulated compared with the LaeA overexpression strain. Analysis of all differently expressed phosphatases suggests a much more limited response to phosphate limitation when LaeA is overexpressed (Fig. 5).

4. Discussion and conclusions

Many bioprocesses are optimized around limitation of specific nutrients. Bioreactor conditions to cultivate strains of A. terreus and A. pseudotereus have been optimized to produce high titers of itaconic acid but often exhibit trade-offs between production rate and overall yield in response to phosphate level (Hevekerl et al., 2014a; Krull et al., 2017; Riscaldati et al., 2000). A low phosphate level is used to limit growth and increase yield and may also limit the impact of excess manganese (Saha and Kennedy, 2020). However, with excess phosphate more biomass is produced allowing a higher overall conversion rate (Hevekerl et al., 2014b). Process control by nutrient limitation is possible under tightly controlled conditions, however heterogeneous feedstocks such as lignocellulosic sugar streams can dramatically impact productivity. In A. pseudotereus we found that production of organic acids is reduced when using sugars from lignocellulosic feedstocks which contain a mixture of chemicals that act as nutrients and growth inhibitors (Chen et al., 2016). We therefore sought a means to increase the reliability of bioproduction in the absence of specific nutrient limitations. In many fungi the global regulator LaeA controls secondary metabolism and we hypothesized that the itaconic acid gene cluster in A. pseudotereus (Deng et al., 2020) is controlled by LaeA.

We found that laeA is required for high production of itaconic acid and that overexpression makes itaconic acid production from a mixed glucose/xyllose sugar stream more robust to the presence of excess phosphate or mixed nutrients from yeast extract (Fig. 2). Systematic examination of the effects of LaeA overexpression in phosphate limited medium found that proteins with acid phosphatase activity tend to be down-regulated and that genes involved in purine metabolism as well as amino sugar and nucleotide metabolism pathways are significantly down-regulated at the transcript level (Tables 3 and 5) suggesting the response to phosphate limitation and initiation of phosphate scavenging may be delayed or suppressed by overexpression of LaeA.

Metabolic pathways involved in phosphate acquisition are normally repressed but become active in phosphate limited conditions. For example, genetic screens in the filamentous fungus Neurospora crassa and the yeast Saccharomyces cerevisiae have identified a variety of acid and alkaline phosphatases, as well as phosphate transport systems that are expressed specifically under conditions of phosphate limitation (Mann et al., 1989; Nelson et al., 1976; Gleason and Metzenberg, 1974). This is controlled by the Pho80/Pho85 cyclin/cyclin dependent kinase complex which phosphorylates the basic helix-loop-helix transcription factor Pho4 (Kaffman et al., 1994). Pho4 then binds upstream of phosphate acquisition genes to regulate their transcription (Peleg and Metzenberg, 1994) while the ankyrin repeat protein Pho81 inhibits phosphorylation by Pho80/Pho85 (Gras et al., 2009; Schneider et al., 1994; Waters et al., 2004). In A. pseudotereus, when LaeA is overexpressed, 9/13 quantified genes involved in phosphate acquisition are repressed at 36 hours (g < 0.05) and for genes where protein was quantified, such as homologs of the acid phosphatases pho-3 (456,862) and pacA (430,438), this repression is maintained later in the culture at 60 and 84 hours (Table 6), suggesting LaeA overexpression may inhibit activation of these genes in phosphate limited conditions. Of the proteins regulating phosphate acquisition, only the PHO80 cyclin homolog is significantly down-regulated while homologs of PHO4, PHO81, and PHO85 are not affected at 36h.

The presence of growth promoting nutrients, such as those found in the complex micro- and macro-nutrient source yeast extract, also significantly decreases production of itaconic acid and pushes the carbon balance toward growth. Like with excess phosphate, overexpression of LaeA limited growth and improved the yield of itaconic acid from mixed sugars when challenged with excess nutrients that may be available in inexpensive lignocellulosic feedstocks or other carbohydrate containing waste-streams such as stillage produced by bioethanol refineries (Kim et al., 2008) or wastewater from potato processing (Laski et al., 2010).

Overexpression of LaeA results in global impacts on cellular functions and metabolism confirming its role as a master regulator in A. pseudotereus. Notably genes systematically categorized as involved in secondary metabolism were up-regulated in response to LaeA overexpression as has been described in a wide variety of Aspergilli and related fungi (Zhang et al., 2020; Lin et al., 2020; Deng et al., 2020; Grau et al., 2015; Wang et al., 2015; Liu et al., 2015; Jiang et al., 2015; Hong et al., 2015; Oda et al., 2011; Kale et al., 2008; Bok and Keller, 2004). Only more recently has the involvement of laeA in central metabolism been noted for its impact on citric acid production (Linde et al., 2016; Niu et al., 2015). Studies in Aspergillus luchuensis linked the impact of laeA on citric acid production to expression of the citric acid exporter caa1 (Kadooka et al., 2020). Likewise, here we found that laeA regulates all of the genes in the itaconic acid cluster including the biosynthetic gene caa1 and the mitochondrial and plasma membrane transporters mttA and mfaA (Deng et al., 2020). Within central metabolism, LaeA overexpression resulted in down-regulation of enzymes involved in glycolysis, the pentose phosphate pathway, and formation of acetyl-CoA via the pyruvate dehydrogenase complex but up-regulation of malic enzyme and citrate synthase (Fig. 6). The carbon-balance suggests the ΔlaeA strain produces substantially more CO2 than the strains that produce itaconic acid (Fig. 4), which is notable because CadA itself releases a mole of CO2 for every mole of itaconic acid produced. This suggests that interrupting export of cis-aconitate from the mitochondria promotes continued and potentially futile respiration via cycling of the tricarboxylic acid cycle which produces 2 mol of CO2.

The results presented herein suggest that the higher yield of itaconic acid observed in the LaeA overexpression strain is due not only to increased expression of enzymes in the itaconic acid gene cluster, but decreased growth due to phosphate limitation and increased expression of key enzymes supplying precursor metabolites. Utilization of a global gene regulator such as LaeA to control pathway expression has promise for bioconversion of feedstocks with heterogeneous composition. However, the functionality of LaeA, including its enzymatic activity and target(s) remains an enigma, and increased expression of other secondary metabolite clusters may contaminate the bioprocess with additional complex chemicals and add cost to the purification process.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Author statement

Kyle R Pomraning: Conceptualization, Methodology, Writing - Original Draft, Writing - Review & Editing, Visualization, Investigation Ziyu Dai: Conceptualization, Visualization, Writing - Review & Editing, Investigation, Nathalie Munoz: Investigation, Young-Mo Kim:
Investigation, Yuqian Gao: Investigation, Shuang Deng: Conceptualization, Investigation, Teresa Lemmon: Investigation, Marie S Swita: Investigation, Jeremy D Zucker: Investigation, Joonhoon Kim: Investigation, Stephen J Mundo: Investigation, Ellen Panisko: Investigation, Meagan C Burnet: Investigation, Bobbie-Jo M Webb-Robertson: Formal analysis, Beth Hofstad: Project administration, Investigation, Scott E Baker: Conceptualization, Investigation, Kristin E Burnum-Johnson: Conceptualization, Investigation, Jon K Magnuson: Conceptualization, Project administration, Funding acquisition.

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Additional Files

Additional file 1. xlsx
Excel file containing RNA-seq analysis as well as targeted and global proteomics data and extracellular metabolomics data.

Declaration of competing interest

The authors declare no competing interests.

List of abbreviations

MM – minimal medium
PM – production medium
GX – minimal glucose/xylose medium
GX + P – minimal glucose/xylose medium with added phosphate
GX + N – minimal glucose/xylose medium with added yeast extract
GX + NP – minimal glucose/xylose medium with added yeast extract and phosphate
GM – germination medium
DMR – medium with sugars from deacetylated and mechanically refined corn stover
atA – terreic acid cluster gene A
afB – terreic acid cluster gene B
atC – terreic acid cluster gene C
atD – terreic acid cluster gene D
atE – terreic acid cluster gene E
atF – terreic acid cluster gene F
TF – itaconic acid gene cluster zinc-finger transcription factor
mtA – itaconic acid gene cluster mitochondrial transporter
cad – itaconic acid gene cluster cis-aconitate decarboxylase
mfsA – itaconic acid gene cluster major facilitator superfamily transporter
p450 – itaconic acid gene cluster p450 domain containing protein

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2022.e00203.
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