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*a tale of two fungi*

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*Published in:*
Current Opinion in Biotechnology

*DOI (link to publication from Publisher):*
10.1016/j.copbio.2019.09.014



*Publication date:*
2020

*Document Version*
Publisher's PDF, also known as Version of record

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Current Opinion in Biotechnology

# Metabolic specialization in itaconic acid production: a tale of two fungi

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Some of the oldest and most established industrial biotechnology processes involve the fungal production of organic acids. In these fungi, the transport of metabolites between cellular compartments, and their secretion, is a major factor. In this review we exemplify the importance of both mitochondrial and plasma membrane transporters in the case of itaconic acid production in two very different fungal systems, *Aspergillus* and *Ustilago.* Homologous and heterologous overexpression of both types of transporters, and biochemical analysis of mitochondrial transporter function, show that these two fungi produce the same compound through very different pathways. The way these fungi respond to itaconate stress, especially at low pH, also differs, although this is still an open field which clearly needs additional research.

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**Current Opinion in Biotechnology** 2020, **62**:153–159

This review comes from a themed issue on **Environmental biotechnology**

Edited by **David R Johnson** and **Stephan Noack**

For a complete overview see the Issue and the Editorial

Available online 2nd November 2019

**https://doi.org/10.1016/j.copbio.2019.09.014**



## Introduction

Transport reactions are essential for living cells in order to shuttle metabolites between cellular compartments and to interact with their surrounding environment. Organic acids like itaconate, succinate, pyruvate, malate, citrate and *cis*-aconitate require a transport system to efficiently cross cellular membranes when they are present in the dissociated form, as is usually the case at physiological pH levels. In biotechnology, the importance of the transport of organic acids has gained increasing recognition in the last decade, as they can contribute significantly to the performance of production hosts [1]. This mini-review aims to highlight the importance of transporters for metabolic specialization in biotechnology, with main focus on fungal itaconic acid production.

## Metabolic specialization in two itaconate producing fungi

The Ascomycete *Aspergillus terreus* and the Basidiomycete *Ustilago maydis* both naturally produce itaconic acid. These two fungi can be considered as model organisms for this trait, although other members of their families have also been shown to produce itaconate, especially in the Ustilaginaceae family [2,3]. Itaconic acid has been a commercial industrial biochemical since the 1950s, mainly due to its versatility as polymer building block with both dicarboxylate and ethylene side chain functionality [4,5]. Its role in the immune response of higher Eukaryotes is also gaining increasing recognition (Box 1). *A. terreus* has classically been the organism of choice for industrial itaconic acid production, mainly due to its outstanding tolerance to low pH stress along with very high product titers and yields [4,6••,7]. In contrast, itaconate production by *Ustilago* has only been intensively studied in the last decade, mainly due to its yeast-like morphology, which is beneficial for large-scale process development in comparison to the filamentous aspergilli [8]. Initially, wild type *U. maydis* had a much lower tolerance to low pH and poor itaconate yield, titer and rate, but these drawbacks have been addressed recently by strain selection and metabolic engineering [9•,10]. In both fungi, the metabolic pathway for itaconate has been characterized, and the associated gene clusters have recently been identified [9•,11,12]. Interestingly, the metabolic steps involved in these pathways differ in the two fungi (Figure 1). *A. terreus* expresses a *cis*-aconitate decarboxylase, while *U. maydis* utilizes an aconitate-delta-isomerase in conjunction with a specific *trans*-aconitate decarboxylase. In contrast to this difference, the itaconate production pathways of both fungi contain similar transport steps, which will be discussed in more detail. The availability of target genes from evolutionary distinct organisms makes the itaconate gene cluster an interesting research subject to investigate the impact of metabolite transport on the production of organic acids.

**Box 1 Relevance of itaconic acid in higher Eukaryotes**

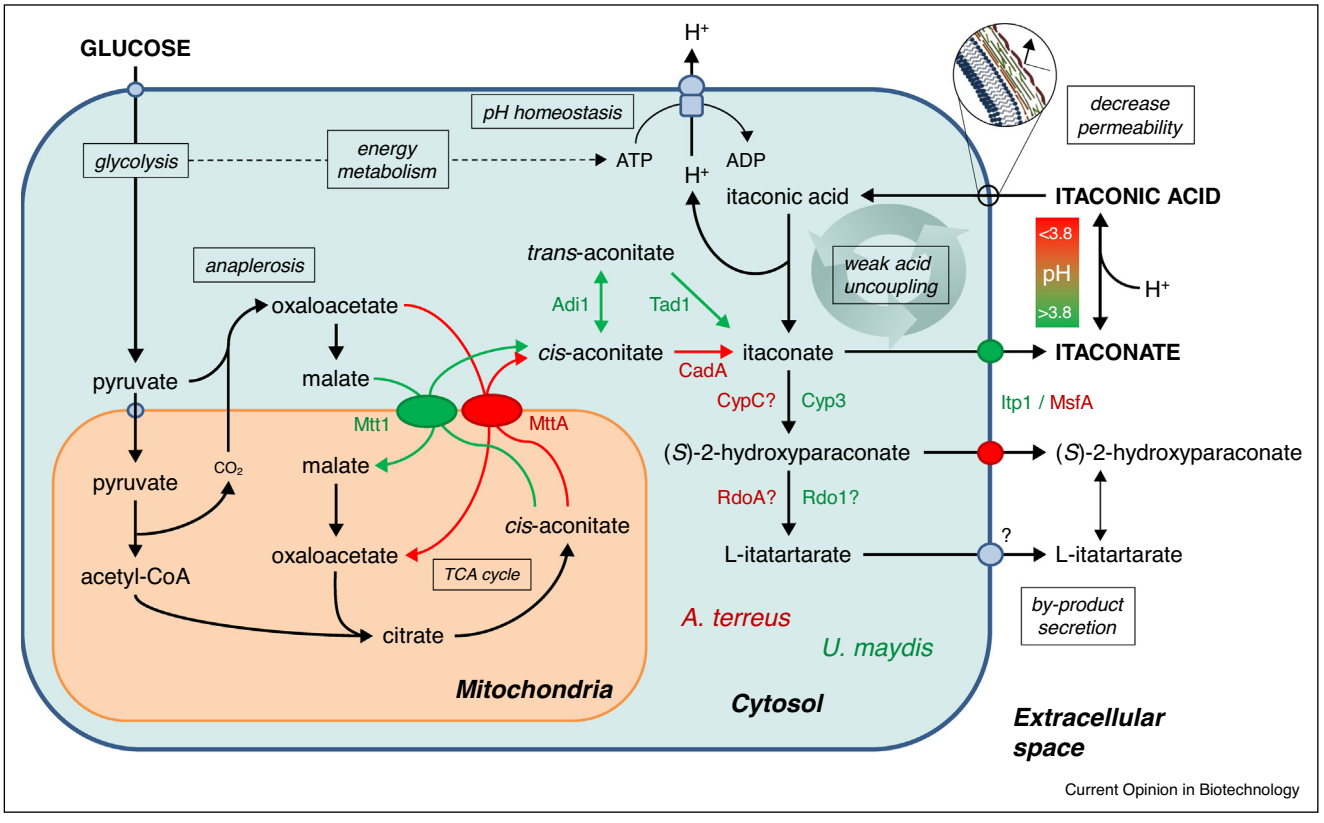
Itaconic acid is a highly versatile secondary metabolite and recent discoveries have shown that it is involved in a multitude of cellular functions. It is produced in mammalian macrophages [13] through the strong induction of Immune Responsive Gene 1 which encodes a *cis*-aconitate decarboxylase (Cad) [14]. There, it inhibits the metabolism of pathogenic bacteria as part of the immune response. In addition, it acts as an immunomodulatory signaling molecule [15,16] and its production affects diverse metabolic and cellular systems such as the glyoxylate shunt [13] and the TCA cycle [17] and it is linked to vitamin B12 deficiency [18]. The defensive production of itaconate in mammals has given rise to bacteria that can degrade this compound. Interestingly, this trait is rather specific for pathogens, indicating an evolutionary link between itaconate production in mammals and degradation in their pathogens [19]. In contrast to this detailed knowledge on the biological role of itaconate production in higher eukaryotes, little is known about the ecological reason why *Ustilago* and *Aspergillus*, two fungi with very different lifestyles, produce itaconate. This should be further investigated.

## Transporters, engineering and product specificity

In both *A. terreus* and *U. maydis*, a mitochondrial carrier family (MCF) protein (MttA and Mtt1) and a major facilitator superfamily (MFS) protein (MfsA and Itp1) are encoded in the respective itaconate gene clusters (Transporter Classification Database 2.A.29 and 2.A.1, respectively). These two transporter classes also play important roles in the production of other organic acids (Box 2). Regarding the MFS transporters, it is interesting to note that Itp1 and MfsA are not reciprocal best hits in terms of sequence identity. It is therefore likely that, although putative organic acid transporters can be uncovered by sequence comparison to Itp1 and MfsA, sequence identity will not predict substrate specificity (Table 1). The MFS proteins enable secretion of itaconate, while the MCF proteins transport *cis*-aconitate from the mitochondria to the cytosol. In *U. maydis* it was shown that deletion of either of the transporter genes causes a significant reduction in secreted itaconate [9•], while the deletion of *mtt1* in the related species *Ustilago cynodontis* completely abolishes itaconate production [10]. This highlights the metabolic importance of these transporters. Interestingly, no equivalent knockout studies have been performed in *A. terreus*, although the relevance of MfsA and MttA for itaconate production is evident from heterologous overexpression studies.

Overexpression of MttA in *A. terreus* yielded no significant improvement over the wild type levels [32]. However, during itaconate production *mttA* transcript levels are

**Figure 1**




Overview of metabolic and (sub)cellular aspects of fungal itaconic acid production. Enzymes specific to *A. terreus* are indicated in red, *U. maydis* in green.

**Box 2 Transport systems for the secretion of malate, citrate, succinate**

The impact of transport for the secretion of organic acids was also demonstrated for other acids. In *A. niger*, CexA, a homologue of Itp1, was identified as the main citrate export gene of the plasma membrane [20••]. In addition, the mitochondrial carrier Yhm2 was also shown to be critical, as deletion of its gene decreased citrate production by 45% in *A. niger* [21] and completely abolished it in *Yarrowia lipolytica* [22•]. CtpA has been demonstrated to provide a minor but significant contribution to citrate production in *Aspergillus kawachii* [23•]. In *S. cerevisiae* it has been shown that malic acid production was increased 100-fold if a malate transporter from *Schizosaccharomyces pombe* was overexpressed in combination with an overexpressing of pyruvate carboxylase and retargeting a malate dehydrogenase to the cytosol [24]. In *A. carbonarius* the overexpression of a C4-dicarboxylate transporter gene increased malate and succinate production and in combination with the overexpression of a fumarate reductase shifted the product pattern towards succinate [25]. Similar experiments were performed in *A. oryzae* and *Ustilago trichophora* to improve malate production [26,27]. More recently also a MFS specific for citramalate production was identified (Table 1).

already high and any additional increase does not lead to a further improvement [12]. In heterologous systems like *Aspergillus niger* the expression of the mitochondrial carrier MttA improved the production of itaconate [33–35]. Furthermore, it was shown that overexpression of *mttA* in *A. niger* leads to the secretion of significant amounts of *cis*-aconitate, even to the extracellular space [36]. The overexpression of MfsA or Itp1 leads to different results. Although the importance of these genes for itaconate production was demonstrated [9•,10] a mere overexpression does not lead to a significant improvement of itaconate in *U. maydis*. Nevertheless, in a heterologous system like *A. niger*, *mfsA* expression can have a positive impact on itaconate levels [34,37]. Recent research on heterologous itaconate production in *A. niger* has shown that relocation of specific steps of the biosynthetic pathway, either by modified subcellular targeting of aconitase and CadA [33], or by introducing a cytosolic citrate synthase pathway [38] may lead to improved organic acid levels, possibly by circumventing rate-limiting transport steps.

In *U. maydis* deletion mutants, itaconate production can be complemented by expression of the equivalent *A. terreus* genes, showing that they have a similar function in both species. However, the expression of the transporters from the two species yields quantitative differences [30••]. Expression of MttA from *A. terreus* enables more efficient itaconate production than the native Mtt1. On the other hand, MfsA seems to have a higher affinity for the downstream oxidation product (*S*)-2-hydroxyparaconate, which is produced from itaconate in the cytosol by a P450 monooxygenase by both *U. maydis* and *A. terreus* [9•,39]. These quantitative differences are likely related to different substrate specificities of both types of transporters ([30••,52].

## Biochemistry of mitochondrial transporters, specificity, interaction

In the itaconate production pathway, *cis*-aconitate synthetized in the mitochondrial matrix must be transported into the cytosol where it can be converted into itaconate. For this task Mtt1 in *U. maydis* [9•] and MttA in *A. terreus* [36] has been demonstrated to be a critical factor.

MCF members are responsible for the shuttling of metabolites such as dicarboxylates, tricarboxylates, amino acids, keto acids, as well as nucleotides and coenzymes across the inner mitochondrial membrane. They display a characteristic tripartite structure consisting of three domains of about 100 amino acids. Each domain contains two hydrophobic stretches that span the membrane as α-helices showing a characteristic sequence motif [40]. Most of these proteins are antiporters and display a strict substrate specificity which should be carefully considered when dealing with

**Table 1**

**Closest homologs (indicated in bold) of Itp1 and MfsA in *U. maydis* (Um), *A. terreus* (At), *A. niger* (An) and *S. cerevisiae* (Sc). Um_Itp1 appears to be more closely related to An_CexA, An_MfsB and At_ATEG_03972 than to At_MfsA**

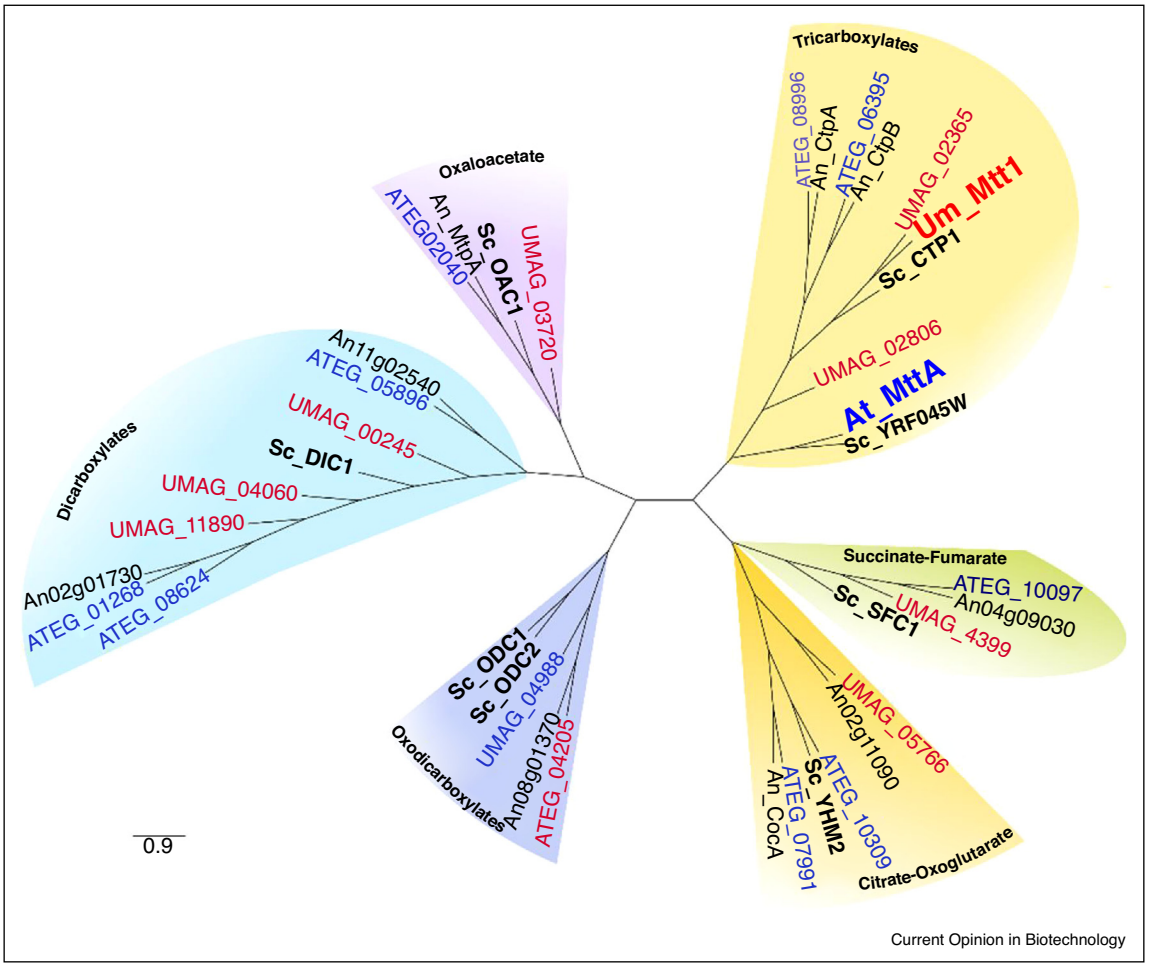
| Name | Accession number | Identity % versus Itp1 | Identity% versus MfsA | Organism | Function |
|---|---|---|---|---|---|
| Um_Itp1 homologs | | | | | |
| At_ATEG_03972 | XP_001213150 | **37** | 24 | *A. terreus* | Unknown |
| At_ATEG_04174 | XP_001213352 | **36** | 24 | *A. terreus* | Unknown |
| An_CexA | XP_001398400 | **36** | 19 | *A. niger* | Citrate transporter [20••] |
| An_MfsB | XP_001393344 | **33** | 24 | *A. niger* | Citramalate transporter (Hossain and Punt, in preparation) |
| Sc_Qdr1p | NP_012146 | **31** | 19 | *S. cerevisiae* | Multidrug transporter [28] |
| Sc_Qdr3p | NP_009599 | **25** | 24 | *S. cerevisiae* | Putative itaconate importer [29] |
| At_MfsA homologs | | | | | |
| An_ANI_1_2702024 | XP_001399891 | 21 | **33** | *A. niger* | Unknown |
| Um_Itp1 | XP_011388398 | **100** | 21 | *U. maydis* | Itaconate/p-OH-paraconate transporter [30••] |
| Sc_Dtr1p | NP_009739 | **25** | 22 | *S. cerevisiae* | Dityrosine transporter [31] |

metabolic pathways localized totally or partially in the mitochondrial matrix. The characterization of MCF members requires a biochemical and a physiological complementary approach. The biochemical characterization can be carried out by the recombinant expression, purification and reconstitution of these proteins into liposomes followed by transport assays using radiolabeled molecules. These studies should be complemented by a physiological approach in which the role of the proteins in the cellular metabolism can be determined *in vivo* for example studying knock-out or overexpression strains as described above [30••].

The extensive and systematic characterization of MCF members carried out in *Saccharomyces cerevisiae* using this experimental approach [40], has allowed their classification into several subfamilies. A phylogenetic tree showing the classification of the MCF members based on their sequence features, involved in the transport of di-carboxylic or tri-carboxylic acids from *S. cerevisiae*, *U. maydis*, *A. terreus* and *A. niger* is shown in Figure 2.

Mtt1 and MttA cluster with the tricarboxylate MCF subfamily. The main biochemically characterized protein in this cluster is *S. cerevisiae* Ctp1, whose specificity is restricted mainly to tricarboxylates including citrate and isocitrate [41]. Mtt1 and MttA can only be found in itaconate producing organisms and are not highly related in terms of sequence; Mtt1 clusters with two other *U. maydis* putative tricarboxylate transporters encoded by UMAG_02365 and UMAG_02806. The latter gene lies in a cluster that contains a second aconitate-delta-isomerase [42] and an MFS transporter that together may constitute a *trans*-aconitate metabolic pathway. MttA clusters with the uncharacterized *S. cerevisiae* protein YRF045w.

**Figure 2**




Phylogenetic tree of carboxylic acid mitochondrial carriers. The tree was constructed using the sequences of known and putative di-carboxylic and tri-carboxylic acid carriers from *U. maydis* (in red), *A. terreus* (in blue), *S. cerevisiae* (in black, bold) and *A. niger* (in black). The names of the mitochondrial carriers and/or their coding ORF are indicated on the terminal nodes. The phylogenetic tree was constructed using PhyML v3.1 in seaview4 from a Muscle multiple-sequence alignment and drawn in FigTree v1.4.2.

Previous studies give strong indications that both Mtt1 and MttA proteins export *cis*-aconitate from the mitochondria, with indirect evidence pointing to malate as the antiport substrate [36,43]. This tricarboxylate/dicarboxylate antiport activity makes sense given the metabolic context (Figure 1), as anaplerotic reactions are essential for reaching high itaconate yields. However, recent experiments carried out with recombinant Mtt1 and MttA reconstituted into liposomes challenge the assumed counter-substrate (Figure 1; [52]). The differences in Mtt1 and MttA substrate specificity likely reflect a different metabolic flux distribution in the two organisms which should be further investigated in light of these recent results.

The detailed knowledge of the biochemical features of these transporters can help the metabolic engineering of microorganisms. For example, it can be envisaged that the deletion of the putative homologs of the citrate/oxoglutarate transporter can push the TCA cycle flux towards *cis*-aconitate and hence itaconate, especially in heterologous systems such as *A. niger*. Deletion of a putative mitochondrial oxaloacetate transporter in *Aspergillus carbonarius* results in less citric acid production and an increase of malic acid in the growth medium (L. Yang *et al.*, unpublished).

## Toxicity: beyond export

Although production of itaconic acid at a low pH is a desired trait to reduce costs in the downstream stage, this can be hampered by its accumulation at high concentrations in the acidic fermentation broth, especially at pHs below the second pKa of itaconic acid (3.84) where the undissociated form will prevail (Figure 1, [44]). Due to its lipophilic character, undissociated itaconic acid can permeate the cellular envelope simply by passive diffusion exerting a toxic effect for producing cells, compromising yield and/or productivity [45]. Consistent with this idea, a pulse adjustment in pH from 1.8 to 5 after two days of fermentation led to an increase of about 50% in the titer of itaconate produced by *A. terreus* [46]. Adjustment of the pH to a controlled level of 3.4 after 2.1 days was found to enable the most efficient itaconic acid production up to 160 g $L^{-1}$, with higher pH levels affecting morphology. Lower pH values yielded lower overall titers, while the concentration of the protonated form stayed relatively constant [47••]. Due to its charge itaconate accumulates intracellularly and its export requires the activity of a specific exporter like MfsA or Itp1. However, at low extracellular pH further adaptive responses are required as otherwise a futile cycle is created where itaconate is extruded to the medium, and returns to its undissociated form which can re-enter the cell by passive diffusion, thereby acidifying the cytosol (Figure 1). This cycle, also known as weak acid uncoupling, must be counteracted by pH-homeostasis mechanisms of the cell, primarily the plasma membrane $H^+$-ATPase [48]. These energy-demanding mechanisms can be powered by the fact that the conversion from glucose to itaconate yields an NADH surplus [49]. In *S. cerevisae*, this futile cycle can be aggravated by the disruption of the putative itaconic acid exporter Qdr3 (N.P. Mira *et al.*, unpublished data). This MFS transporter shows sequence similarity to Itp1 (Table 1), but its heterologous expression in an *U. maydis itp1* knockout could not complement the itaconate production phenotype [29]. Recent insights on adaptive responses to stress induced by organic acids at a low pH in *S. cerevisiae* have further uncovered that modifications in the structure of the cell wall and/or of the plasma membrane result in reduced porosity to the undissociated form of organic acids [45]. More recently, a similar mechanism was observed in other acid-tolerant yeast species [50]. Such modifications open the door to new interventions aiming to improve robustness of producing cells by 'cell envelope engineering', a strategy that has been used with success to improve production of short-chain fatty acids and other membrane-damaging compounds by *Escherichia coli* [51]. While in bacterial cells it is known that itaconate exerts a toxic effect by inhibiting the activity of isocitrate lyase, in fungal cells little is known on the topic. The fact that *A. terreus* is able to produce itaconic acid at high concentrations at low pH suggests that this species is equipped with efficient tolerance mechanisms and the identification of the biochemical factors underlying those traits could be of interest to guide the engineering of more robust strains. Although this is a promising approach, the pursuit of this road will likely need to involve detailed investigations in more accessible systems.

## Conclusions

In this review we have addressed the role of transport phenomena in fungal organic acid production, with a focus on a system where both mitochondrial and plasma membrane transporters from two distinct industrial production hosts have been compared experimentally, that is, itaconic acid production. In our opinion, the role of organic acid transport has long been underestimated. Detailed multidisciplinary research has resulted, and will further result, in improved organic acid production strains. Such attempts to improve organic acid production can be accompanied by significant rewiring of biosynthetic pathways. This may lead to increased levels of the desired organic acid, but also the production of new chemicals of potential industrial interest such as *cis*-aconitate, (*S*)-2-hydroxyparaconate and citramalate. In our opinion, the balanced interplay between mitochondrial and plasma membrane transporters, and their substrate specificity, significantly shapes the overall organic acid secretion capability of fungi. Given the intricate interconnection of virtually all industrially relevant organic acids, it is clearly to be expected that the knowledge on their transport will also contribute to future strain

improvement, thereby contributing to the advancement of sustainable chemicals production.

## Conflict of interest statement

N. Wierckx is co-inventor of several patent applications on organic acid production with *Ustilago* and related fungi. M. G. Steiger is co-inventor of patents on organic acid production with *Aspergillus niger* and related fungi. P.J. Punt, as CTO of Dutch DNA Biotech, is co-inventor of several patent applications on organic acid production in *Aspergillus niger* and other fungal and yeast species. P.S. Lübeck, N.P. Mira, and G. Agrimi have no potential conflict of interest to declare.

## Acknowledgements

This review was conceived as part of the ERA-IB consortium TTRAFFIC – Toxicity and Transport For Fungal production of Industrial Compounds. We gratefully acknowledge Dr Gertien Smits for her efforts during the conception phase of this consortium. Dr Hamed Hosseinpour-Tehrani is acknowledged for his help with manuscript formatting and proofing. The work of NW and GA was funded by the German Federal Ministry of Food and Agriculture (BMEL), through the Specialist agency renewable raw materials e. V. (FNR) (FKZ 22030515). The scientific activities of the Bioeconomy Science Center were financially supported by the Ministry of Culture and Science within the framework of the NRW Strategieprojekt BioSC (No. 313/323-400-002 13). PSL acknowledges the Danish Strategic Research Program for support of MycoFuelChem Grant no. 11-116803. NPM thanks the financial support from Fundação Ciência e Tecnologia through contract ERA-IB-2-6/0003/2014, and contract UID/BIO/04565/2013 and UID/BIO/04565/2019, which supports the research activities at iBB. Programa Operacional Regional de Lisboa 2020 is also acknowledged for its financial support to iBB (project no. 007317). The work of MGS has been supported by the Austrian Federal Ministry for Digital and Economic Affairs (bmwd), the Federal Ministry for Transport, Innovation and Technology (bmvit), the Styrian Business Promotion Agency SFG, the Standortagentur Tirol, Government of Lower Austria and ZIT – Technology Agency of the City of Vienna through the COMET-Funding Program managed by the Austrian Research Promotion Agency FFG.

## References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. van der Straat L, de Graaff LH: **Pathway transfer in fungi**. *Bioengineered* 2014, **5**:335-339.

2. Geiser E, Hosseinpour Tehrani H, Meyer S, Blank LM, Wierckx N: **Evolutionary freedom in the regulation of the conserved itaconate cluster by Ria1 in related Ustilaginaceae**. *Fungal Biol Biotechnol* 2018, **5**:14.

3. Geiser E, Wiebach V, Wierckx N, Blank LM: **Prospecting the biodiversity of the fungal family Ustilaginaceae for the production of value-added chemicals**. *Fungal Biol Biotechnol* 2014, **1**:2.

4. Okabe M, Lies D, Kanamasa S, Park EY: **Biotechnological production of itaconic acid and its biosynthesis in *Aspergillus terreus***. *Appl Microbiol Biotechnol* 2009, **84**:597-606.

5. Robert T, Friebel S: **Itaconic acid – a versatile building block for renewable polyesters with enhanced functionality**. *Green Chem* 2016, **18**:2922-2934.

6. •• Kuenz A, Krull S: **Biotechnological production of itaconic acid-things you have to know**. *Appl Microbiol Biotechnol* 2018, **102**:3901-3914.
This review on itaconic acid production provides an especially comprehensive overview on process development.

7. Karaffa L, Kubicek CP: **Citric acid and itaconic acid accumulation: variations of the same story?** *Appl Microbiol Biotechnol* 2019, **103**:2889-2902.

8. Regestein L, Klement T, Grande P, Kreyenschulte D, Heyman B, Maßmann T, Eggert A, Sengpiel R, Wang Y, Wierckx N *et al.*: **From beech wood to itaconic acid: case study on biorefinery process integration**. *Biotechnol Biofuels* 2018, **11**:279.

9. • Geiser E, Przybilla SK, Engel M, Kleineberg W, Buttner L, Sarikaya E, Den Hartog T, Klankermayer J, Leitner W, Bölker M *et al.*: **Genetic and biochemical insights into the itaconate pathway of *Ustilago maydis* enable enhanced production**. *Metab Eng* 2016, **38**:427-435.
The oxidation of itaconate to 2-hydroxyparaconate by Cyp3 is elucidated, enabling enhanced production of itaconate.

10. Hosseinpour Tehrani H, Tharmasothirajan A, Track E, Blank LM, Wierckx N: **Engineering the morphology and metabolism of pH tolerant *Ustilago cynodontis* for efficient itaconic acid production**. *Metab Eng* 2019, **54**:293-300.

11. Geiser E, Przybilla SK, Friedrich A, Buckel W, Wierckx N, Blank LM, Bölker M: ***Ustilago maydis* produces itaconic acid via the unusual intermediate *trans*-aconitate**. *Microb Biotechnol* 2015, **9**:116-126.

12. Li A, van Luijk N, ter Beek M, Caspers M, Punt P, van der Werf M: **A clone-based transcriptomics approach for the identification of genes relevant for itaconic acid production in *Aspergillus***. *Fungal Genet Biol* 2011, **48**:602-611.

13. Strelko CL, Lu W, Dufort FJ, Seyfried TN, Chiles TC, Rabinowitz JD, Roberts MF: **Itaconic acid is a mammalian metabolite induced during macrophage activation**. *J Am Chem Soc* 2011, **133**:16386-16389.

14. Michelucci A, Cordes T, Ghelfi J, Pailot A, Reiling N, Goldmann O, Binz T, Wegner A, Tallam A, Rausell A *et al.*: **Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production**. *Proc Natl Acad Sci U S A* 2013, **110**:7820-7825.

15. Mills EL, Ryan DG, Prag HA, Dikovskaya D, Menon D, Zaslona Z, Jedrychowski MP, Costa ASH, Higgins M, Hams E *et al.*: **Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1**. *Nature* 2018, **556**:113-117.

16. Bambouskova M, Gorvel L, Lampropoulou V, Sergushichev A, Loginicheva E, Johnson K, Korenfeld D, Mathyer ME, Kim H, Huang LH *et al.*: **Electrophilic properties of itaconate and derivatives regulate the IκBζ-ATF3 inflammatory axis**. *Nature* 2018, **556**:501-504.

17. Cordes T, Wallace M, Michelucci A, Divakaruni AS, Sapcariu SC, Sousa C, Koseki H, Cabrales P, Murphy AN, Hiller K *et al.*: **Immunoresponsive gene 1 and itaconate inhibit succinate dehydrogenase to modulate intracellular succinate levels**. *J Biol Chem* 2016, **291**:14274-14284.

18. Shen H, Campanello GC, Flicker D, Grabarek Z, Hu J, Luo C, Banerjee R, Mootha VK: **The human knockout gene CLYBL connects itaconate to vitamin B12**. *Cell* 2017, **171**:771-782.e711.

19. Sasikaran J, Ziemski M, Zadora PK, Fleig A, Berg IA: **Bacterial itaconate degradation promotes pathogenicity**. *Nat Chem Biol* 2014, **10**:371-377.

20. •• Steiger MG, Rassinger A, Mattanovich D, Sauer M: **Engineering of the citrate exporter protein enables high citric acid production in *Aspergillus niger***. *Metab Eng* 2019, **52**:224-231.
Citrate export is mediated by cexA encoding a major facilitator superfamily transporter in *A. niger*. Engineering this gene has a significant impact on citric acid secretion and demonstrates the impact of transport reactions on cellular productivity.

21. Kirimura K, Kobayashi K, Yoshioka I: **Decrease of citric acid produced by *Aspergillus niger* through disruption of the gene encoding a putative mitochondrial citrate-oxoglutarate shuttle protein**. *Biosci Biotechnol Biochem* 2019, **83**:1538-1546.

22. • Yuzbasheva EY, Agrimi G, Yuzbashev TV, Scarcia P, Vinogradova EB, Palmieri L, Shutov AV, Kosikhina IM, Palmieri F, Sineoky SP: **The mitochondrial citrate carrier in *Yarrowia lipolytica*: its identification, characterization and functional**

**significance for the production of citric acid**. *Metab Eng* 2019, **54**:264-274.
This work reveals a mitochondrial carrier responsible for driving fungal citrate production.

23. • Kadooka C, Izumitsu K, Onoue M, Okutsu K, Yoshizaki Y, Takamine K, Goto M, Tamaki H, Futagami T: **Mitochondrial citrate transporters CtpA and YhmA are required for extracellular citric acid accumulation and contribute to cytosolic acetyl coenzyme a generation in *Aspergillus luchuensis* mut. Kawachii**. *Appl Environ Microb* 2019, **85** e03136-18.
This work reveals that not only CtpA but also YhmA is necessary to transport citrate to the cytosol which is also the major source for cytocsolic acetyl-CoA.

24. Zelle RM, de Hulster E, van Winden WA, de Waard P, Dijkema C, Winkler AA, Geertman J-MA, van Dijken JP, Pronk JT, van Maris AJA: **Malic acid production by *Saccharomyces cerevisiae*: engineering of pyruvate carboxylation, oxaloacetate reduction, and malate export**. *Appl Environ Microbiol* 2008, **74**:2766-2777.

25. Yang L, Christakou E, Vang J, Lübeck M, Lübeck PS: **Overexpression of a C4-dicarboxylate transporter is the key for rerouting citric acid to C4-dicarboxylic acid production in *Aspergillus carbonarius***. *Microb Cell Fact* 2017, **16**:43.

26. Brown SH, Bashkirova L, Berka R, Chandler T, Doty T, McCall K, McCulloch M, McFarland S, Thompson S, Yaver D *et al.*: **Metabolic engineering of *Aspergillus oryzae* NRRL 3488 for increased production of L-malic acid**. *Appl Microbiol Biotechnol* 2013, **97**:8903-8912.

27. Zambanini T, Hosseinpour Tehrani H, Geiser E, Sonntag CK, Buescher JM, Meurer G, Wierckx N, Blank LM: **Metabolic engineering of *Ustilago trichophora* TZ1 for improved malic acid production**. *Metab Eng Commun* 2017, **4**:12-21.

28. Nunes PA, Tenreiro S, Sa-Correia I: **Resistance and adaptation to quinidine in *Saccharomyces cerevisiae:* role of QDR1 (YIL120w), encoding a plasma membrane transporter of the major facilitator superfamily required for multidrug resistance**. *Antimicrob Agents Chemother* 2001, **45**:1528-1534.

29. Lóia ACR: *Metabolic Engineering for Enhancement of Itaconic Acid Production in **Ustilago** including Introduction of the QDR3 gene from **Saccharomyces cerevisiae**. Master Thesis*. RWTH Aachen University; 2017. Accessible at: https://fenix.tecnico.ulisboa.pt/downloadFile/1126295043835552/Thesis-Ana_Submit.pdf.

30. •• Hosseinpour Tehrani H, Geiser E, Engel M, Hartmann SK, Hossain AH, Punt PJ, Blank LM, Wierckx N: **The interplay between transport and metabolism in fungal itaconic acid production**. *Fungal Genet Biol* 2019, **125**:45-52.
Transporters involved in itaconate production in *Ustilago* and *Aspergillus* yield different results with respect to production of itaconate and 2-hydroxyparaconate when expressed in the same biological background.

31. Morishita M, Engebrecht J: **Sorting signals within the *Saccharomyces cerevisiae* sporulation-specific dityrosine transporter, Dtr1p, C terminus promote golgi-to-prospore membrane transport**. *Eukaryotic Cell* 2008, **7**:1674-1684.

32. Huang XN, Lu XF, Li YM, Li X, Li JJ: **Improving itaconic acid production through genetic engineering of an industrial *Aspergillus terreus* strain**. *Microb Cell Fact* 2014, **13**:119.

33. Blumhoff ML, Steiger MG, Mattanovich D, Sauer M: **Targeting enzymes to the right compartment: metabolic engineering for itaconic acid production by *Aspergillus niger***. *Metab Eng* 2013, **19**:26-32.

34. Hossain AH, Li A, Brickwedde A, Wilms L, Caspers M, Overkamp K, Punt PJ: **Rewiring a secondary metabolite pathway towards itaconic acid production in *Aspergillus niger***. *Microb Cell Fact* 2016, **15**:130.

35. van der Straat L, Vernooij M, Lammers M, van den Berg W, Schonewille T, Cordewener J, van der Meer I, Koops A, de Graaff LH: **Expression of the *Aspergillus terreus* itaconic acid biosynthesis cluster in *Aspergillus niger***. *Microb Cell Fact* 2014, **13**:11.

36. Steiger MG, Punt PJ, Ram AFJ, Mattanovich D, Sauer M: **Characterizing MttA as a mitochondrial *cis*-aconitic acid transporter by metabolic engineering**. *Metab Eng* 2016, **35**:95-104.

37. Li A, Pfelzer N, Zuijderwijk R, Brickwedde A, van Zeijl C, Punt P: **Reduced by-product formation and modified oxygen availability improve itaconic acid production in *Aspergillus niger***. *Appl Microb Biotechnol* 2013, **97**:3901-3911.

38. Hossain AH, Ter Beek A, Punt PJ: **Itaconic acid degradation in *Aspergillus niger*: the role of unexpected bioconversion pathways**. *Fungal Biol Biotechnol* 2019, **6**:1.

39. Steiger MG, Wierckx N, Blank LM, Mattanovich D, Sauer M: In *Itaconic Acid - An Emerging Building Block*. Edited by Wittman C, Liao JC. Wiley-VCH Verlag GmbH & Co. KGaA; 2017. Chapter 15.

40. Palmieri F, Agrimi G, Blanco E, Castegna A, Di Noia MA, Iacobazzi V, Lasorsa FM, Marobbio CM, Palmieri L, Scarcia P *et al.*: **Identification of mitochondrial carriers in *Saccharomyces cerevisiae* by transport assay of reconstituted recombinant proteins**. *Biochim Biophys Acta (BBA) Bioenerget* 2006, **1757**:1249-1262.

41. Kaplan RS, Mayor JA, Gremse DA, Wood DO: **High level expression and characterization of the mitochondrial citrate transport protein from the yeast *Saccharomyces cerevisiae***. *J Biol Chem* 1995, **270**:4108-4114.

42. Geiser E: *Itaconic Acid Production by **Ustilago Maydis.** PhD Thesis*. Apprimus Verlag; 2015. Accessible at https://publications.rwth-aachen.de/record/478377/files/478377.pdf.

43. Jaklitsch WM, Kubicek CP, Scrutton MC: **The subcellular organization of itaconate biosynthesis in *Aspergillus terreus***. *J Gen Microbiol* 1991, **137**:533-539.

44. Willke T, Vorlop KD: **Biotechnological production of itaconic acid**. *Appl Microb Biotechnol* 2001, **56**:289-295.

45. Mira NP, Teixeira MC, Sa-Correia I: **Adaptive response and tolerance to weak acids in *Saccharomyces cerevisiae*: a genome-wide view**. *OMICS* 2010, **14**:525-540.

46. Hevekerl A, Kuenz A, Vorlop KD: **Influence of the pH on the itaconic acid production with *Aspergillus terreus***. *Appl Microbiol Biotechnol* 2014, **98**:10005-10012.

47. •• Krull S, Hevekerl A, Kuenz A, Prüße U: **Process development of itaconic acid production by a natural wild type strain of *Aspergillus terreus* to reach industrially relevant final titers**. *Appl Microbiol Biotechnol* 2017, **101**:4063-4072.
This work characterizes the relation of extracellular pH and itaconic acid production in *A. terreus* in detail, including novel insights into the efficient induction of itaconate production by low pH, and not phosphate limitation.

48. Kane PM: **Proton transport and pH control in fungi**. In *Yeast Membrane Transport, Advances in Experimental Medicine and Biology*, , vol 892. Edited by Ramos J, Sychrová H, Kschischo M. Springer International; 2016:33-68.

49. Hartmann SK, Stockdreher Y, Wandrey G, Hosseinpour Tehrani H, Zambanini T, Meyer AJ, Büchs J, Blank LM, Schwarzländer M, Wierckx N: **Online in vivo monitoring of cytosolic NAD redox dynamics in *Ustilago maydis***. *Biochim Biophys Acta (BBA) Bioenerget* 2018, **1859**:1015-1024.

50. Palma M, Guerreiro JF, Sa-Correia I: **Adaptive response and tolerance to acetic acid in *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*: a physiological genomics perspective**. *Front Microbiol* 2018, **9**:274.

51. Tan ZG, Yoon JM, Nielsen DR, Shanks JV, Jarboe LR: **Membrane engineering via *trans* unsaturated fatty acids production improves *Escherichia coli* robustness and production of biorenewables**. *Metab Eng* 2016, **35**:105-113.

52. Scarcia P, Gorgoglione R, Messina E, Fiermonte G, Blank LM, Wierckx N, Palmieri L, Agrimi G: **The mitochondrial *cis*-aconitate transporter of *U. maydis* and *A. terreus*: same physiological role but different biochemical function**. *FEBS Letters* 2019 http://dx.doi.org/10.1002/1873-3468.13645.