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

Nick Wierckx[1], Gennaro Agrimi[2], Peter Stephensen Lübeck[3], Matthias G Steiger[4], Nuno Pereira Mira[5] and Peter J Punt[6]

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.

Addresses
[1] Forschungszentrum Jülich, Institute of Bio- and Geosciences (IBG-1) and Bioeconomy Science Center (BioSC), 52425 Jülich, Germany
[2] University of Bari "Aldo Moro", Department of Biosciences, Biotechnologies and Biopharmaceutics, via Orabona 4, 70125 Bari, Italy
[3] Aalborg University, Department of Chemistry and Bioscience, Section for Sustainable Biotechnology, A.C. Meyers Vaenge 15, DK-2450 Copenhagen SV, Denmark
[4] Austrian Centre of Industrial Biotechnology, Institute of Chemical, Environmental and Bioscience Engineering, TU Wien, Getreidemarkt 1a, 1060 Vienna, Austria
[5] Instituto Superior Técnico, Universidade de Lisboa, iBB - Institute for Bioengineering and Biosciences, Department of Bioengineering, Av. Rovisco Pais, 1049-001, Lisboa, Portugal
[6] Dutch DNA Biotech BV Padualaan 8, 3584CH Utrecht, the Netherlands

Corresponding author: Wierckx, Nick (n.wierckx@fz-juelich.de)

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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

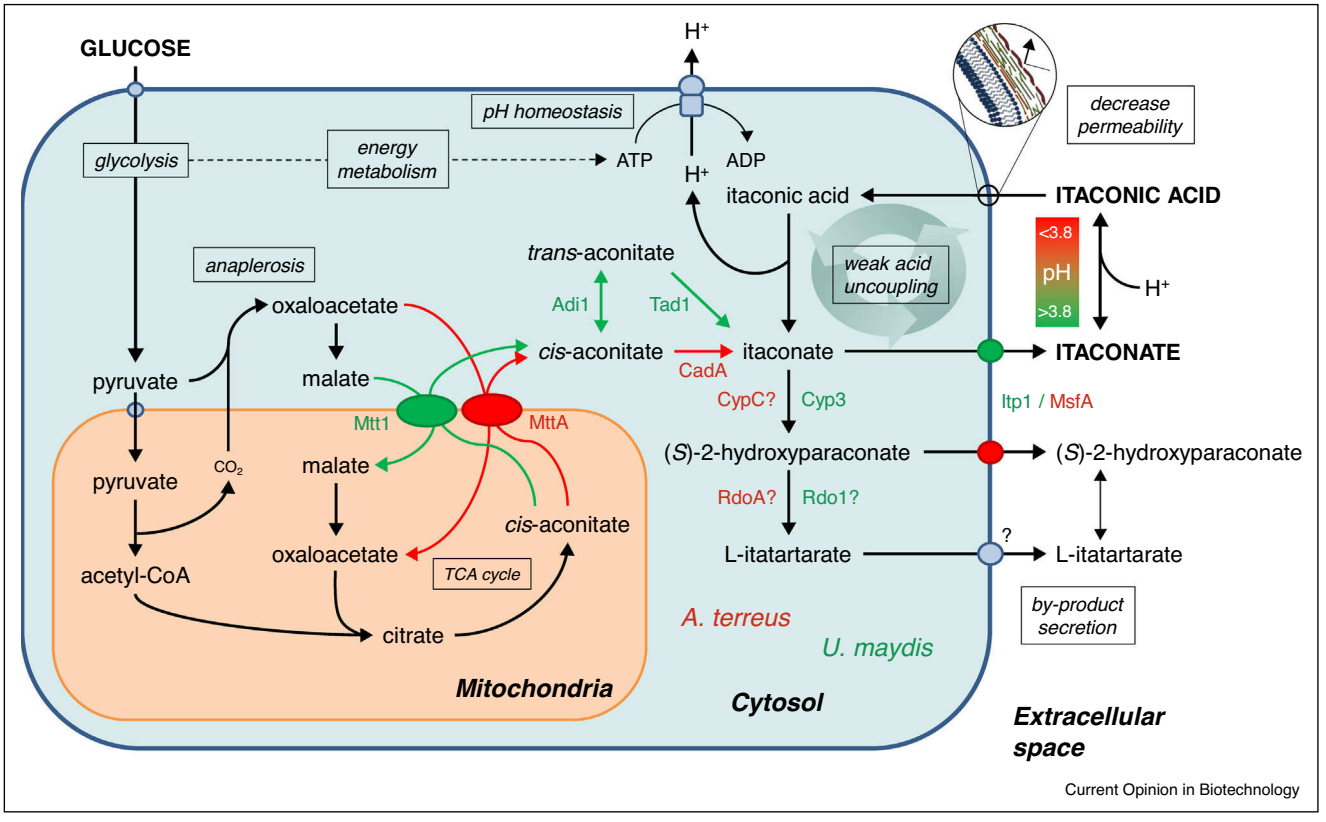
Itaconic acid is a highly versatile secondary metabolite and recent discoveries have shown that it is involved in a multitude of cellular functions. It is produced in mammalian macrophages [13] through the strong induction of Immune Responsive Gene 1 which encodes a *cis*-aconitate decarboxylase (Cad) [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 metabolic pathways localized totally or

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

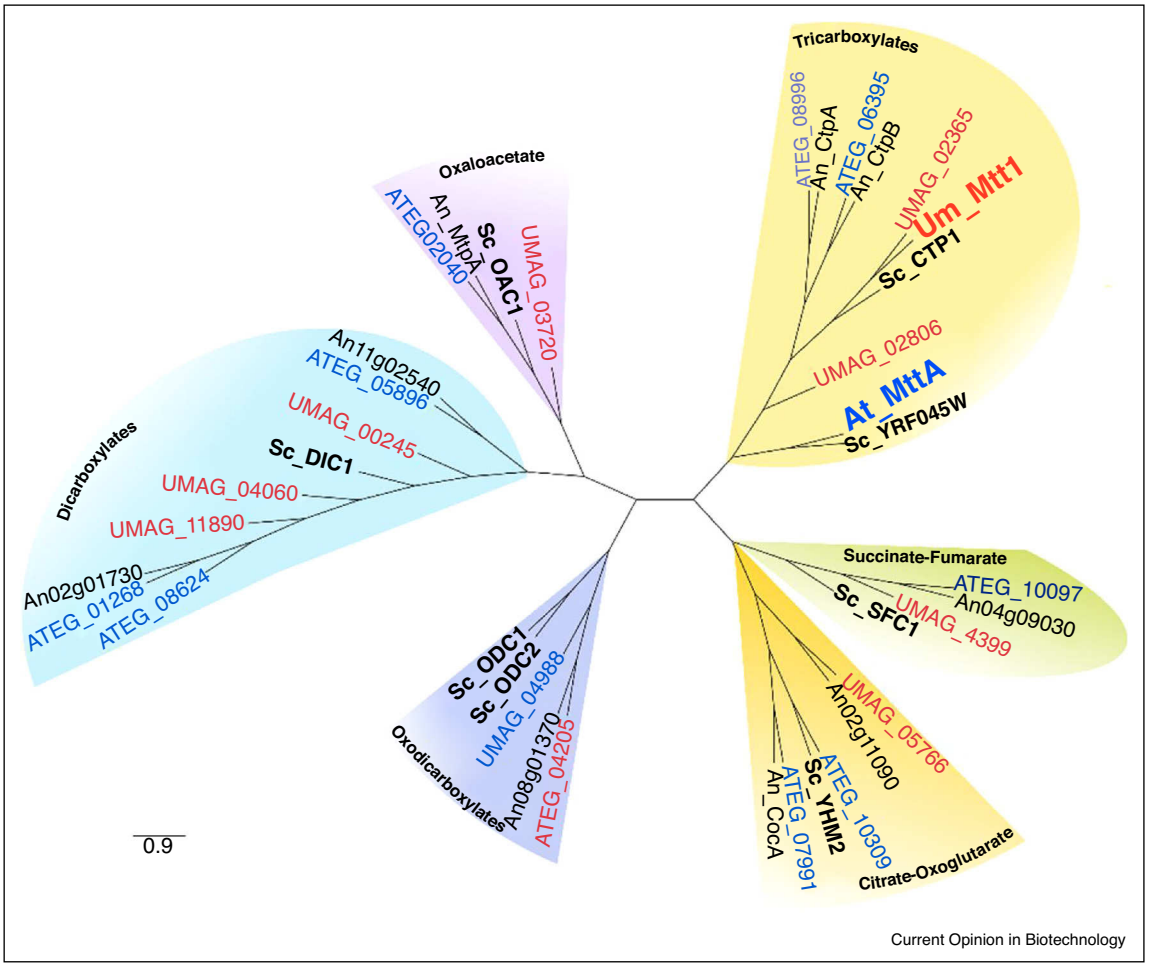
Name	Accession number	Identity % versus Itp1	Identity% versus MfsA	Organism	Function
Um_Itp1 homologs					
At_ATEG_03972	XP_001213150	**37**	24	*A. terreus*	Unknown
At_ATEG_04174	XP_001213352	**36**	24	*A. terreus*	Unknown
An_CexA	XP_001398400	**36**	19	*A. niger*	Citrate transporter [20••]
An_MfsB	XP_001393344	**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]

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.

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