Thermodynamic boundary conditions suggest that a passive transport step suffices for citrate excretion in *Aspergillus* and *Penicillium*

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Excretion of organic acids, e.g. citrate, by anamorphic fungi is a frequent phenomenon in natural habitats and in laboratory cultures. In biotechnological processes for citrate production with *Aspergillus niger* extracellular citrate concentrations up to 1 mol l⁻¹ are achieved. Intracellular citrate concentrations are in the millimolar range. Therefore the question arises whether citrate excretion depends on active transport. In this article thermodynamic calculations are presented for citrate excretion by *A. niger* at an extracellular pH of 3 and by *Penicillium simplicissimum* at an extracellular pH of 7. From the results of these calculations it is concluded that in both cases a passive transport step suffices for citrate excretion.

INTRODUCTION

If microbial metabolites are excreted, they must pass the plasma membrane. Most metabolites are charged and cannot cross this barrier by simple diffusion through the lipid bilayer – a transport protein must be involved. This transport step may influence the yield of a biotechnological production process, as exemplified, for instance, in amino acid production by *Corynebacterium glutamicum* (Burkovski & Krämer, 2002) and lactic acid production by *Lactobacillus* spp. (Maris *et al.*, 2004). One important point in this respect is whether a transport step is active or passive. Active transport means that one and the same transport protein couples the flux of a substrate to the consumption of energy (in the form either of an ion gradient or the hydrolysis of ATP) to enable transport of the substrate against its electrochemical potential gradient. Whether or not there is a need for an active transport step is determined by the thermodynamic boundary conditions – and so also is the final extracellular concentration of a metabolite that can be achieved by an excretion process.

In this article I present the results of thermodynamic calculations to evaluate whether the excretion of citrate by *Aspergillus niger* and *Penicillium simplicissimum* needs an active transport step or may proceed passively. Another aim is to present in detail the thermodynamic boundary conditions for the excretion of a model anionic metabolite

under the assumption that the plasma membrane potential is inside negative – as usual in the filamentous fungi studied up to now.

The excretion of intermediates of the TCA cycle (organic acids; for instance citrate, oxalate or succinate) is a characteristic feature of many anamorphic fungal species, such as *Aspergillus* spp. and *Penicillium* spp. Excretion of organic acids is observed in natural habitats (Gadd, 1999) and during growth on solid/liquid media in the laboratory (Foster, 1949). Excretion of citrate by *P. simplicissimum*, for instance, may be used for the leaching of metals from industrial wastes and low-grade ores (Burgstaller & Schinner, 1993). Excretion of citrate by *A. niger* is exploited in biotechnological processes for commercial citric acid production (Roehr *et al.*, 1996). It is in the latter biotechnological process that extracellular citric acid concentrations up to 1 mol l⁻¹ are achieved. Because the intracellular citrate concentration is about 100-fold lower, it is of interest to study the transport process(es), which is (are) responsible for excreting citrate from the cytosol to the medium (Burgstaller, 1997; Ruijter *et al.*, 2002). The kinetics and energetics of this transport protein have not been investigated in detail in filamentous fungi up to now. An active mode of transport could have consequences for the overall energy balance of hyphae, especially if the conditions demand the input of a considerable amount of energy (for instance at low pH and high product concentration). Exactly this was postulated to be the case in the excretion of lactic acid by *Lactobacillus* spp. (Maris *et al.*, 2004).

I examined the thermodynamic constraints for citrate excretion in two different situations: citrate production by *A. niger* at pH 3 and high extracellular citrate concentration (0.5 M), and citrate excretion by *P. simplicissimum* at pH 7

Abbreviations: $\Delta\Psi$, electrical potential difference between the inside and the outside of the cell (membrane potential at the plasma membrane); pK_a , negative decadic logarithm of the acid dissociation constant; pK_c , negative decadic logarithm of the complex formation constant; pH_e , extracellular pH; pH_i , intracellular pH; pH_{cyt} , cytoplasmic pH; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; 2,4-DNP, 2,4-dinitrophenol.

and low extracellular citrate concentration (0.006 M). Whenever possible, I used measured data reported in the literature for the intracellular pH, the intracellular citrate concentration and the membrane potential across the plasma membrane. Additionally, I emphasized the differences that arise from citrate being a polyprotic acid, compared to the monoprotic lactic acid examined by Maris *et al.* (2004). The calculations and their results are presented explicitly to enable the reader to follow the arguments in detail.

The results of the thermodynamic calculations presented indicate that in almost all considered cases a passive transport step would suffice to explain measured extracellular citrate concentrations. However, the experimental testing of this hypothesis must await results from an ongoing study of the transport of citrate in *P. simplicissimum* using plasma membrane vesicles. Such detailed studies of organic acid transporters are useful, as stated by Maris *et al.* (2004): ‘...research into the mechanism and substrate-specificity of organic-acid transporters is highly relevant for successful engineering of micro-organisms for organic-acid production’.

THEORETICAL ANALYSES

Speciation of citric acid. Citric acid is a polyprotic (or polyvalent) acid forming the four species H_3Cit^0 , H_2Cit^{1-} , $HCit^{2-}$ and Cit^{3-} . The anion Cit^{3-} forms complexes with magnesium ions ($MgCit^{1-}$; the complex $MgHCit^0$ has a stability constant that is two orders of magnitudes lower, and is neglected here). To calculate the electrochemical potential gradient for the different citrate species, their intracellular and extracellular concentrations must be known. For this, data are needed about the intracellular (better: the cytoplasmic) concentrations of citrate and magnesium, the intracellular (better: the cytoplasmic) pH, and the membrane potential across the plasma membrane.

Assuming an intracellular ionic strength of 0.25, the pK_a values of citric acid are 2.9, 4.3 and 5.6. The pK_c (negative decadic logarithm of the complex formation constant) values for the two relevant magnesium-citrate complexes are -3.2 (for $MgCit^{1-}$) and -1.3 (for $MgHCit^0$) (Kwack & Veech, 1992). For extracellular citrate, the species distribution was calculated at an ionic strength of zero using the pK_a values 3.1, 4.8 and 6.4. The pK_c for $MgCit^{1-}$ at zero ionic strength is -4.8, and for $MgHCit^0$ it is -1.6 (Sillen & Martell, 1964).

The concentrations of the different citrate species, including magnesium complexes, at an intracellular pH of 7.6 and 6, as well as at an extracellular pH of 3 and 7, were calculated with the program EQCAL (by L. Backman, BIOSOFT, Cambridge, UK, 1988).

Intracellular citrate. Total intracellular citrate in *A. niger* was reported to be between 2 mM and 30 mM (Prömpfer *et al.*, 1993; Netik *et al.*, 1997). Cytoplasmic citrate was suggested to be 6 mM and mitochondrial citrate 31 mM (Alvarez-Vasquez *et al.*, 2000). These values correspond to a total citrate concentration of 10 mM, if the mitochondrial volume is assumed to be 15% of the total cell volume (Alvarez-Vasquez *et al.*, 2000). I used a cytoplasmic citrate concentration of 6 mM for the calculations.

Total intracellular citrate in *P. simplicissimum* during growth is between 10 mM and 50 mM in batch cultures (Gallmetzer *et al.*, 1998), and between 20 mM and 60 mM in chemostat cultures (Gallmetzer &

Burgstaller, 2001). In non-growing hyphae total intracellular citrate is between 2 mM and 20 mM (Gallmetzer *et al.*, 1998). These values are somewhat higher than the values reported for *A. niger*. To facilitate the comparison between citrate excretion by *A. niger* and *P. simplicissimum* I used also 6 mM for cytoplasmic citrate in *P. simplicissimum* for the calculations.

Intracellular magnesium. Reported values for total intracellular magnesium in *Aspergillus nidulans* and *Trichoderma aureoviride* are between 6 mM and 37 mM (Bushell & Bull, 1974; Pitt & Bull, 1982), and between 3 mM and 12 mM in *Penicillium chrysogenum* (Okorokov *et al.*, 1975). Cytoplasmic magnesium in *Neurospora crassa* (Levina *et al.*, 1995), *Saccharomyces cerevisiae* (Beeler *et al.*, 1997) and mammalian cells (Reich & Sel'kov, 1981) was reported to be between 0.1 mM and 1 mM. In *N. crassa* the percentage of magnesium storage in vacuoles can vary between 10% (Cramer & Davis, 1984) and 80% (Keenan *et al.*, 1997) and the percentage of vacuoles can vary between <5% and >50% of the cytoplasmic volume (Slayman *et al.*, 1995). Because of these wide ranges I assumed a cytoplasmic magnesium concentration equimolar to the cytoplasmic citrate concentration (6 mM). The consequence of this assumption is that citrate in the cytoplasm exists mainly as a magnesium complex (Table 1) – just as is assumed for ATP (O'Sullivan & Smithers, 1979). Complexation of magnesium with ATP was neglected, because a lower available magnesium concentration would only increase the concentration of Cit^{3-} and thus strengthen the hypothesis of a passive transport step for citrate excretion.

Cytoplasmic pH. The cytoplasmic pH in *A. niger* at an extracellular pH (pH_e) of 2 (that is the extracellular pH during the citric acid production process) was reported as 7.6 (Hesse *et al.*, 2002); the mean intracellular pH at $pH_e=2$ was 7.0 (Jernejc & Legisa, 2004). In *P. simplicissimum* the mean intracellular pH of non-growing hyphae at an extracellular pH of 6 was between 6.5 and 7.2 (Firler *et al.*, 1998). For the calculations I assumed a cytoplasmic pH of 7.6.

Membrane potential across the plasma membrane. The most reliable – i. e. electrophysiological – data for the electrical potential gradient across the plasma membrane of a fungus were reported for *N. crassa* (Slayman, 1965a). Therefore it seems reasonable to use these data as a guideline for thermodynamic calculations. In *N. crassa* the plasma membrane potential at a $pH_e=3$ is still negative (Fig. 1). However, a slightly positive membrane potential may be supposed to develop in fungi living in more acidic environments than *N. crassa* (Roos & Slavik, 1987).

Table 1. Cytoplasmic concentrations of the main citrate species at a cytoplasmic pH of 7.6 and 6.0

Total cytoplasmic citrate is 6 mM; total cytoplasmic magnesium is 6 mM; ionic strength $\mu=0.25$. Values in parentheses were calculated in the absence of magnesium.

Citrate species	Concn (mM)	Percentage of total citrate
pH_{cyt} = 7.6		
MgCit ¹⁻	4.3 (0.0)	72 (0)
Cit ³⁻	1.7 (5.9)	28 (98)
HCit ²⁻	– (0.1)	– (2)
pH_{cyt} = 6.0		
MgCit ¹⁻	4.1 (0.0)	68 (0)
Cit ³⁻	1.4 (4.3)	23 (72)
HCit ²⁻	0.5 (1.7)	8 (28)

Fig. 1. Dependence of the plasma membrane potential on the extracellular pH in *N. crassa* measured with capillary electrodes (modified after Slayman, 1965a).

Calculation of extracellular total citrate concentration at equilibrium. In general, transport systems for citrate do not accept all citrate species equally (Krom *et al.*, 2003). Therefore, I calculated the electrochemical potential gradient for each citrate species separately. For this I used the Nernst equation (see, for instance, Nicholls & Ferguson, 2002). With this equation it is possible to calculate the equilibrium concentration of an ion on one side of a membrane, if the membrane potential and the concentration of the ion on the other side of the membrane are known:

$$\Delta\Psi = 2.3 \frac{RT}{mF} \log \left(\frac{C_o}{C_i} \right) \quad (1)$$

or (for 30 °C):

$$\Delta\Psi = \frac{60}{m} \log \left(\frac{C_o}{C_i} \right) \quad (2)$$

(*R*, gas constant; *T*, temperature; *m*, charge of an ion; *F*, Faraday constant; *C_i*, intracellular concentration of an ion; *C_o*, extracellular concentration of an ion).

In the following I describe the single steps of this calculation for one citrate species.

1. The cytoplasmic concentration of a citrate species was calculated (using the selected values for cytoplasmic citrate, cytoplasmic magnesium and cytoplasmic pH, as well as *pK_a* values of citric acid, and the stability constants of magnesium–citrate complexes at an ionic strength of 0.25).

2. The Nernst equation and selected membrane potentials (+50 mV, −50 mV, −200 mV) were used to calculate the theoretical extracellular concentration of the citrate species at equilibrium, i.e. at a purely passive distribution of a citrate species between the cytoplasm and the medium. No variation of parameters other than the membrane potential was used for the calculations, because the results also hold for variations of these parameters within the range of their experimentally measured values.

3. Then the theoretical total extracellular citrate concentration under equilibrium conditions was calculated. For this the calculated extracellular equilibrium concentration of the citrate species and the calculated extracellular distribution of all citrate species at the respective extracellular pH were used.

4. This calculated total extracellular citrate concentration was then compared with observed total extracellular citrate concentrations in cultures of *A. niger* and *P. simplicissimum* (500 mM was used for *A. niger* and 6 mM for *P. simplicissimum*).

5. If the selected value for total extracellular citrate concentration (500 mM for *A. niger* and 6 mM for *P. simplicissimum*) was lower than the calculated total extracellular citrate concentration at equilibrium, passive transport was taken to be sufficient for citrate excretion.

RESULTS AND DISCUSSION

Excretion of uncharged citric acid via simple or facilitated diffusion

The cytoplasmic concentration of undissociated citric acid is very low: 2×10^{-10} mM at 6 mM total cytoplasmic citrate concentration (see Table 3). The permeability coefficient (basal permeability for the lipid bilayer) for undissociated citric acid was estimated as about 10^{-7} cm s^{−1} (Gallmetzer *et al.*, 1998). Thus the excretion rate that is possible for undissociated citric acid by simple diffusion is several orders of magnitude lower than measured citrate excretion rates (Gallmetzer *et al.*, 1998). Even if diffusion of undissociated citric acid were facilitated by a transport protein, the low intracellular concentration of this species would not result in a considerable extracellular citrate accumulation, at least at low extracellular pH, when the extracellular concentration of undissociated citric acid is high. Therefore excretion of undissociated citric acid could be neglected.

Excretion of charged citrate species

Of all charged citrate species only HCit^{2−}, Cit^{3−} and MgCit^{1−} occur at relevant concentrations in the cytoplasm (Table 1). The consequence is that citrate excretion always means the excretion of negative charges. Because the principle of overall electroneutrality must be obeyed, excretion of a negatively charged citrate species must be accompanied by either the uptake of negative charges or excretion of positive charges (see Conclusions).

The distribution of extracellular citrate species at *pH_e* = 3 (*A. niger*; 500 mM extracellular citrate) and at *pH_e* = 7 (*P. simplicissimum*; 6 mM extracellular citrate) is shown in Table 2. In Tables 3, 4 and 5 the equilibrium concentrations of total extracellular citrate, calculated for the main intracellular citrate species, are given for the membrane potentials +50 mV (Table 3), −50 mV (Table 4) and −200 mV (Table 5). These values illustrate that even under the most unfavourable conditions (*pH_e* 3 and +50 mV) no active transport is needed for citrate excretion, if either Cit^{3−} or MgCit^{1−} is the transported species (Table 3). This applies all the more if the plasma membrane potential is inside negative (Tables 4 and 5). And even considering all the uncertainties concerning cytoplasmic citrate and magnesium concentrations, passive excretion should suffice for extracellular citrate accumulation if either Cit^{3−} or MgCit^{1−} is the transported species.

Table 2. Extracellular concentrations of the main citrate species at an extracellular pH of 3 and 7

pH_e=3: total extracellular citrate concentration is 500 mM; total extracellular magnesium concentration is 6 mM; ionic strength μ=0. pH_e=7: total extracellular citrate concentration is 6 mM; total extracellular magnesium concentration is 6 mM; ionic strength μ=0. Values in parentheses were calculated in the absence of magnesium.

Citrate species	Concn (mM)	Percentage of total citrate
pH_e=3		
HCit ²⁻	3	1
H ₂ Cit ¹⁻	220	44
H ₃ Cit ⁰	277	55
MgCit ¹⁻	0.5	0.1
pH_e=7		
HCit ²⁻	0.07 (1.2)	1 (20)
Cit ³⁻	0.3 (4.8)	5 (80)
MgCit ¹⁻	5.7 (0)	95 (0)

Excretion of citrate coupled to the proton-motive force or to hydrolysis of ATP

The proton-motive force in growing hyphae is composed of an inside negative membrane potential and an inward-directed concentration gradient of protons. If excretion of citrate were to proceed via a citrate/proton symport, then protons would have to move against their electrochemical potential gradient. This would only be possible if citrate moves down its electrochemical potential gradient – and then there would be no reason for coupling citrate excretion to an excretion of protons.

Substrate/proton antiports or ATP-dependent excretion pumps mediate the excretion of antifungal drugs in fungi (White *et al.*, 1998). For these transport mechanisms an energy source – ultimately ATP – is used. Theoretically, both transport mechanisms could also be used for citrate excretion. Because ‘uphill’ citrate excretion is not necessary under the relevant conditions (see Tables 3, 4 and 5) the question arises why a cell should spend energy for citrate excretion. However, excretion of antifungal drugs is probably also a ‘downhill’ transport process and nevertheless their excretion seems to be energized (White *et al.*, 1998).

Conclusions

The main cytoplasmic citrate species are Cit³⁻ or MgCit¹⁻ (Table 1), the fraction of each species depending on the actual cytoplasmic magnesium concentration. The plasma membrane potential is most probably inside negative (Ballarin-Denti *et al.*, 1994). If either Cit³⁻ or MgCit¹⁻ is transported, then under the considered conditions there is no thermodynamic necessity for an active excretion of citrate. This is true for citrate excretion at low pH and high extracellular citrate concentration (at least up to 500 mM extracellular citric acid), as well as at neutral pH and low extracellular citrate concentration. A passive transport step for citrate excretion is thus the simplest hypothesis explaining the driving force for citrate excretion, and this hypothesis should be assumed to be valid as long as it is not disproved by valid experimental data. In other words, in the case of *A. niger* the dominant driving force is the low extracellular pH: citrate is transported to the medium as Cit³⁻ (or MgCit¹⁻) and immediately protonated to undissociated citric acid. This removal of Cit³⁻ allows for a continued

Table 3. Calculated total extracellular citrate concentration at equilibrium for a ΔΨ of +50 mV and a pH_e of 3

Values were calculated for six different citrate species. It was assumed that transport was in each case only by one specific citrate species and that the specific citrate species was distributed between inside and outside purely passively. Active transport was assumed to be needed if the calculated total extracellular citrate concentration at equilibrium was lower than 500 mM (a usual concentration of extracellular citrate in citric acid production by *A. niger*). For greater clarity the values were rounded off to whole numbers. The extracellular pH is 3, cytoplasmic pH is 7.6, cytoplasmic magnesium concentration is 6 mM, extracellular magnesium concentration is 6 mM. All concentrations are in mM.

Excreted citrate species	Cytoplasmic concn	Extracellular equilibrium concn	Calculated total extracellular citrate	Active transport needed
H ₃ Cit ⁰	2 × 10 ⁻¹⁰	2 × 10 ⁻¹⁰	3 × 10 ⁻¹⁰	Yes
H ₂ Cit ¹⁻	8 × 10 ⁻⁶	1 × 10 ⁻⁶	3 × 10 ⁻⁶	Yes
HCit ²⁻	2 × 10 ⁻²	4 × 10 ⁻⁴	5 × 10 ⁻²	Yes
Cit ³⁻	2 × 10 ⁰	5 × 10 ⁻³	2 × 10 ³	No
MgCit ¹⁻	4 × 10 ⁰	6 × 10 ⁻¹	7 × 10 ²	No
MgHCit ⁰	1 × 10 ⁻⁹	1 × 10 ⁻⁹	2 × 10 ⁰	Yes

Table 4. Calculated total extracellular citrate concentration at equilibrium for a $\Delta\Psi$ of -50 mV and a pH_e of 3

See the legend of Table 3 for details.

Excreted citrate species	Cytoplasmic concn	Extracellular equilibrium concn	Calculated total extracellular citrate	Active transport needed
H_3Cit^0	2×10^{-10}	2×10^{-10}	3×10^{-10}	Yes
$\text{H}_2\text{Cit}^{1-}$	8×10^{-6}	6×10^{-5}	1×10^{-4}	Yes
HCit^{2-}	2×10^{-2}	8×10^{-1}	1×10^2	Yes
Cit^{3-}	2×10^0	5×10^2	2×10^8	No
MgCit^{1-}	4×10^0	3×10^1	3×10^4	No
MgHCit^0	1×10^{-9}	1×10^{-9}	2×10^0	Yes

diffusion of Cit^{3-} down an electrical and concentration gradient. The process is similar to the accumulation of citrate in tonoplasts of acid lime juice cells (Brune *et al.*, 1998). In the case of *P. simplicissimum* – i. e. at an extracellular pH of 7 – the much higher membrane potential is the dominant driving force.

As already mentioned, electroneutrality considerations force the outward transport of positive charges or the inward transport of negative charges simultaneously with the excretion of negatively charged citrate. The two most obvious possibilities for a charge-balancing ion flow are an efflux of potassium or an efflux of protons. An efflux of potassium was postulated to be the main charge-balancing ion flow during citrate excretion in roots from white lupin (Zhang *et al.*, 2004). An efflux of protons was postulated as the main charge-balancing ion flow in *Penicillium cyclopium* (Roos & Slavik, 1987) and in *N. crassa* (Slayman *et al.*, 1990; but only

at high extracellular pH). Potassium efflux would be a passive charge-balancing ion flow, whereas proton efflux would have to be active. A proton efflux could either be coupled directly to citrate excretion (via a citrate/proton symport similar to the excretion of lactate together with protons in *Escherichia coli* and *Lactobacillus lactis*; Konings *et al.*, 1992) or take place via the plasma membrane H^+ -ATPase. In the latter case this would be an active charge-balancing ion flow, because the H^+ -ATPase needs ATP. In this case the overall transport process (the citrate transport step plus the charge-balancing proton excretion) might be called active and one could say that in terms of overall cell physiology, free energy expenditure is necessary for citrate excretion. However, direct experimental evidence for an involvement of the H^+ -ATPase is not easy to achieve, because the membrane potential would have to be clamped to avoid simultaneous depolarization of the plasma membrane if the H^+ -ATPase is inhibited.

Table 5. Calculated total extracellular citrate concentration at equilibrium for a $\Delta\Psi$ of -200 mV and a pH_e of 7

Values were calculated for six different citrate species. It was assumed that transport was in each case only by one specific citrate species and that the specific citrate species was distributed between inside and outside purely passively. Active transport was assumed to be needed if the calculated total extracellular citrate concentration at equilibrium was lower than 6 mM (a usual concentration of extracellular citrate in cultures of *P. simplicissimum*). For greater clarity the values were rounded off to whole numbers. The extracellular pH is 7, cytoplasmic pH is 7.6, cytoplasmic magnesium concentration is 6 mM, extracellular magnesium concentration is 6 mM. All concentrations are in mM.

Excreted citrate species	Cytoplasmic concn	Extracellular equilibrium concn	Calculated total extracellular citrate	Active transport needed
H_3Cit^0	2×10^{-10}	2×10^{-10}	2×10^{-2}	Yes
$\text{H}_2\text{Cit}^{1-}$	8×10^{-6}	2×10^{-2}	2×10^2	No
HCit^{2-}	2×10^{-2}	8×10^4	7×10^6	No
Cit^{3-}	2×10^0	2×10^{10}	4×10^{11}	No
MgCit^{1-}	4×10^0	9×10^3	1×10^4	No
MgHCit^0	1×10^{-9}	1×10^{-9}	3×10^0	Yes

If the actual (free) cytoplasmic magnesium concentration were lower than the assumed 6 mM (Lichko *et al.*, 1982), then the conclusion of a passive transport step for citrate excretion would even be strengthened, because the concentration of Cit^{3-} would increase. Only if HCit^{2-} were the transported species, then – depending on the actual value of the membrane potential – would active transport probably be needed for citrate excretion.

Following this line of argument, reports of an active transport step for citrate excretion in *A. niger* should be regarded with caution. The sometimes mentioned evidence for an active transport – inhibition of citrate excretion by metabolic inhibitors (CCCP, 2,4-DNP, NaN_3) – could also be due to a secondary effect, because metabolic inhibitors may depolarize the plasma membrane (Slayman, 1965b) and could thus reduce citrate excretion, either via closing of channels (Kollmeier *et al.*, 2001) or via decreasing the electrochemical potential gradient for the transported citrate species.

Albeit passive, citrate excretion must always be mediated by a transport protein, because charged molecules are transported. The transport mechanism mediating citrate excretion in filamentous fungi is still unknown. Excretion of citrate across plant plasma membranes is most probably mediated by channel-like transport proteins (Kollmeier *et al.*, 2001; Zhang *et al.*, 2004). To investigate this hypothesis in fungi, experiments with plasma membrane vesicles and/or electrophysiological studies are necessary.

If the transported citrate species turns out to be actually MgCit^{1-} , then magnesium ions should appear in the extracellular medium. This magnesium would either accumulate extracellularly or be taken up again. A careful examination of cytoplasmic and extracellular magnesium concentrations during citrate excretion could therefore help to identify the citrate species that is actually transported.

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