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ORIGINAL ARTICLE

Weissella halotolerans W22 combines arginine deiminase and ornithine decarboxylation pathways and converts arginine to putrescine

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Keywords

amino acid decarboxylation, arginine, ornithine, putrescine, *Weissella*.

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Abstract

Aims: To demonstrate that the meat food strain *Weissella halotolerans* combines an ornithine decarboxylation pathway and an arginine deiminase (ADI) pathway and is able to produce putrescine, a biogenic amine. Evidence is shown that these two pathways produce a proton motive force (PMF).

Methods and Results: Internal pH in *W. halotolerans* was measured with the sensitive probe 2′,7′–bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein. Membrane potential was measured with the fluorescent probe 3,3′-dipropylthiocarbocyanine iodine. Arginine and ornithine transport studies were made under several conditions, using cells loaded or not loaded with the biogenic amine putrescine. ADI pathway caused an increase in ΔpH dependent on the activity of F_0F_1ATPase. Ornithine decarboxylation pathway generates both a ΔpH and a ΔΨ. Both these pathways lead to the generation of a PMF.

Conclusions: *Weissella halotolerans* W22 combines an ADI pathway and an ornithine decarboxylation pathway, conducing to the production of the biogenic amine putrescine and of a PMF. Transport studies suggest the existence of a unique antiporter arginine/putrescine in this lactic acid bacteria strain.

Significance and Impact of the Study: The coexistence of two different types of amino acid catabolic pathways, leading to the formation of a PMF, is shown for a *Weissella* strain for the first time. Moreover, a unique antiport arginine/putrescine is hypothesized to be present in this food strain.

Introduction

The catabolism of amino acids by lactic acid bacteria (LAB) has implications for the quality and safety of fermented foods (Silla Santos 1996; Vergés *et al.* 1999). Amino acids are the precursors of compounds that significantly contribute to the characteristic flavour of some foodstuffs (Fernandez and Zúñiga 2006). But amino acids can also be converted to biogenic amines, by deiminase and decarboxylation pathways present in LAB that colonize the food product (Pereira *et al.* 2001). Biogenic amines can compromise the health of the consumer being responsible for several food-poisoning incidents causing headaches, palpitations, flushing, hypertension or vomiting (van de Vossenberg *et al.* 1998; Lonvaud-Funel 2006; Alberto *et al.* 2007; Lucas *et al.* 2007).

Biogenic amine production is a beneficial physiological process for those strains that have acquired the capacity to produce them and amino acid decarboxylation pathways can lead to the production of a proton motive force (PMF) by a secondary mechanism (Konings 2002) in which protons are consumed in the cytoplasmic decarboxylation reaction and membrane potential by coupling uptake of amino acid with the extrusion of biogenic amine.

Arginine is one of the amino acids most commonly found in fermented meat products (Vergés *et al.* 1999). The main arginine catabolic pathway performed by LAB is the arginine deiminase (ADI) pathway, which has been

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described in strains belonging to genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Streptococcus* and *Weissella* (Ammor and Mayo 2007). The ADI pathway comprises three sequential reactions catalysed by ADI (EC 3.5.3.6), ornithine carbamoyl-transferase (OTC; EC 2.1.3.3) and carbamate kinase (CK; EC 2.7.2.2) and leads to the conversion of arginine into ornithine, ammonia and CO_2, with the concomitant production of 1 mol of ATP per mol of arginine consumed (Fig. 1). In the first step, ADI converts arginine to citrulline and ammonia. The product citrulline is cleaved by OTC into ornithine and carbamoyl-phosphate in the second step, after which the latter is used by CK to convert ADP to ATP followed by the spontaneous decomposition of carbamate into ammonia and CO_2. Importantly, a secondary transporter catalysing electroneutral arginine/ornithine exchange is responsible for the coupled uptake of the precursor and the excretion of the product of the pathway (Christensen *et al.* 1999). Because the ADI pathway provides energy (ATP), it is believed to have an important role in obtaining energy in nutrient-limited environments. This is confirmed by the fact that in most bacteria, energy depletion seems to be a key triggering factor for the induction of the ADI pathway. Additionally, the ADI pathway is also believed to play a role in the protection against an acidic environment, through the production of ammonia. Deiminase pathways have been described only for arginine and agmatine (Driessen *et al.* 1988; Lucas *et al.* 2007). Many amino acids can be decarboxylated in a single step by a cytoplasmic decarboxylase, which together with a transporter is responsible for the coupled uptake of the amino acid and excretion of the produced biogenic amine, and form an autonomous metabolic pathway, as is exemplified for the pathway in Fig. 1.

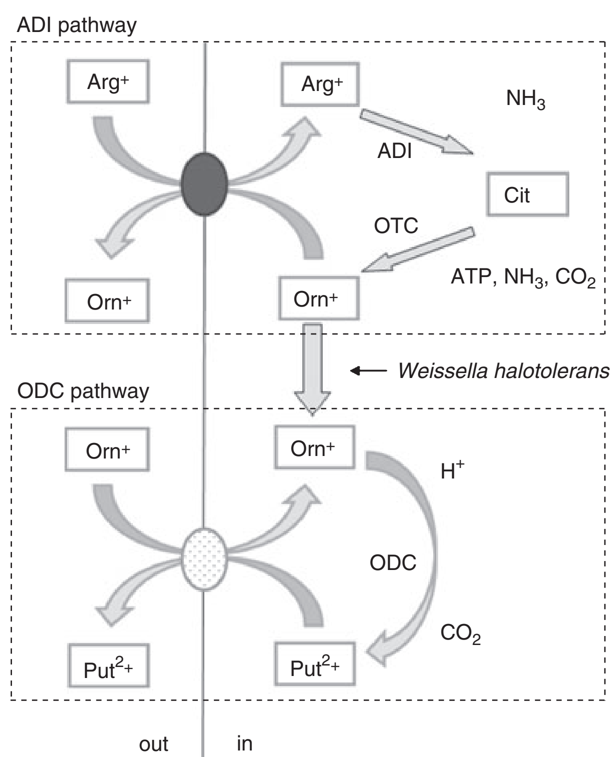

Figure 1 Proposed model for combined arginine deiminase (ADI) pathway and ornithine decarboxylation pathway in strain *Weissella halotolerans* W22. The ADI pathway comprises three sequential reactions: in the first step, ADI converts arginine to citrulline, ammonia and carbon dioxide. Then, the product citrulline is cleaved by OTC into ornithine and carbamoyl-phosphate in the second step, after which the latter is used by carbamate kinase (CK) to convert ADP to ATP followed by the spontaneous decomposition of carbamate into ammonia and CO_2. Importantly, a secondary transporter catalyzing electroneutral arginine/ornithine exchange is responsible for the couple uptake of the precursor and the excretion of the product of the pathway. ADI, ADI pathway; ATP, adenosine triphosphate; Arg, arginine; Cit, citruline; CO_2, carbon dioxide; H^+, hydrogen ion; NH_3, ammonium; ODC, ornithine decarboxylase; Orn, ornithine; OTC, ornithine transcarbamilase; Put, putrescine.

Examples would be the conversion of histidine into histamine, tyrosine into tyramine, lysine into cadaverine and, also, ornithine into putrescine (Kashigwagi *et al.* 1992; Konings *et al.* 1997; Marcobal *et al.* 2004). The decarboxylation reaction consumes a proton, resulting in cytoplasm alkalinization. Additionally, it introduces a charge difference across the membrane between the precursor and the product, i.e. monovalent ornithine is converted to divalent putrescine, which results in the generation of a membrane potential of physiological polarity when the two are exchanged by the (ornithine/putrescine) transporter. Taken together, alkalinization of the cytoplasm and membrane potential generation are equivalent to pumping protons outside the cell resulting in PMF formation (Wolken *et al.* 2006; Lucas *et al.* 2007).

Previous studies identified the genes coding for the enzymes of ADI pathway in *Weissella halotolerans* W22 (C.I. Pereira, A.T. Pires, H. Silva, C. Leitao, M.V. San Romao and M.T.B. Crespo, unpublished data). Interestingly, in the same study, the strain W22 was also found to be able to decarboxylate ornithine (Poolman *et al.* 1987) into the corresponding biogenic amine putrescine, and the gene coding for ornithine decarboxylase (OTC) was also identified. This is surprising because ornithine is a metabolite common to both pathways, and the activity of the OTC in the cytoplasm would interfere with the completion of the ADI pathway (Fig. 1). In this study, we demonstrate that in the same cells of *W. halotolerans* W22, both the ADI and ornithine decarboxylation pathways can operate independently and produce ATP and PMF respectively. Transport studies suggest the presence of an arginine/putrescine exchanger in the membrane of *W. halotolerans.*

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Materials and methods

Materials

L-[U-^{14}C]Arginine and L-[U-^{14}C]Ornithine were purchased from GE Healthcare Europe GmbH (Munich, Germany). The pH-sensitive fluorescent probe 2′,7′–bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein (BCECF) and the fluorescent probe 3,3′-dipropylthiocarbocyanine iodine [DiSC$_3$(5)] were obtained from Molecular Probes (Eugene, OR, USA). The ATPase inhibitor *N*,*N*′-dicyclohexylcarbodiimide (DCCD), putrescine, valinomycin and nigericin were all purchased from Sigma-Aldrich. De Man, Rogosa and Sharp broth (MRS) medium was obtained from Merck KGaA (Darmstadt, Germany). BCA Protein Assay kit was purchased from Pierce (Rockford, IL, USA). All other chemicals were of reagent grade.

Micro-organism and growth conditions

The strain *W. halotolerans* W22 was isolated from a traditional Portuguese fermented sausage. The strain was grown at 30°C without agitation in modified MRS medium containing (g l^{-1}): 5, glucose (Merck); 4, yeast extract (Oxoid, UK); 10, peptone (Merck); 2, potassium dihydrogenphosphate (Panreac Quimica, Lyon, France); 0·2, magnesium sulfate heptahydrated; 0·1, manganesium sulfate hydrated and 1 ml, Tween 80. When stated, the growth medium was supplemented with 20 mmol l^{-1} arginine (Sigma Aldrich). The pH was adjusted to 6·3–6·5. Growth was estimated by measuring optical density at 600 nm (OD$_{600}$) in a UV/visible spectrophotometer (UltroSpec 2100 Pro; GE Healthcare Europe).

Acid shock experiments

Acid shock experiments were performed as described by Iyer *et al.* (2003) with some modifications. Briefly, cells were grown overnight in modified MRS medium as previously mentioned. Stationary-phase cultures were diluted 1 : 1000 into prewarmed MRS medium at pH 2·5, without amino acid supplementation (control) or supplemented with 10 mmol l^{-1} of arginine or 10 mmol l^{-1} of ornithine. The cultures were incubated at 30°C, and after 3 h appropriate serial dilutions were plated on MRS agar plates, in triplicate. Colony forming units (CFUs) were counted after 24 h incubation at 30°C. Survival efficiency was defined as the percentage of survivor colonies relative to the number of colonies plated directly from the stationary-phase culture without the acid shock step. Results are the mean of three independent CFU plate countings.

Measurement of internal pH and membrane potential

An overnight culture of *W. halotolerans* W22 was diluted into fresh modified MRS medium to an initial OD$_{600}$ of 0·1. When the growth culture reached an OD$_{600}$ of 0·6–0·8, cells were harvested by centrifugation (10 000 ***g***, 10 min, 4°C), washed with 50 mmol l^{-1} potassium phosphate buffer (pH 5·5) (KPi buffer) and resuspended in 1 ml of the same buffer.

The internal pH was measured with the sensitive probe BCECF as described by Magni *et al.* (1999). Briefly, 1 μl of a 10 mmol l^{-1} BCECF solution was added to 20 μl of a cell suspension typically containing 50 mg ml^{-1} of protein, followed by 2·5 μl of 0·5 mol l^{-1} HCl to shock the probe into the cells. The suspension was left for 5 min. at room temperature, after which 1 ml of KPi buffer (pH 5·5) was added. The cells were spun down, resuspended in 200 μl of KPi buffer and kept on ice until use. Reactions were carried out in 2 ml buffer containing 10 μl of the BCECF-loaded cells at 30°C, under constant stirring. At time intervals, glucose, arginine and ornithine were added (10 mmol l^{-1} each in reaction). The fluorescent signal was sampled every second. Fluorescence was directly correlated with the intracellular pH of cells suspended in the same buffer containing Triton X and the ionophores valinomycin and nigericin, to completely abolish PMF.

The membrane potential was measured qualitatively with the fluorescent probe DiSC$_3$(5). An increase in electrical potential across the membrane correlates with a decrease in fluorescence intensity. For each experiment, 10 μl of a cell suspension prepared as described was added to 2 ml of KPi buffer at 30°C, and 4 μl of a 1 mmol l^{-1} solution of DiSC$_3$(5) was subsequently added. At time intervals, glucose, arginine and ornithine were added (10 mmol l^{-1} each in reaction). The fluorescence signal was recorded every second. In both assays, F$_0$F$_1$-ATPase activity was inhibited by preincubation of the cells with 30 mmol l^{-1} DCCD for 30 min at room temperature. Valinomycin (a K$^+$ ionophore) and nigericin (a K$^+$/H$^+$ exchanger) were used in sample reactions at final concentrations of 0·75 μmol l^{-1}.

Transport assays using whole cells

Strain *W. halotolerans* W22 was grown in as described before in modified MRS medium supplemented with 20 mmol l^{-1} arginine. Cells from a culture grown to an OD$_{600}$ of 0·6–0·8 were harvested by centrifugation, washed with KPi buffer and resuspended in 1 ml of the same buffer. Three microlitres of this cell suspension were diluted into 93 μl of the same buffer. At time 0, L-[U-^{14}C]arginine or L-[U-^{14}C]ornithine was added to achieve final concentrations of 0·3 μmol l^{-1} or 0·4 μmol l^{-1}

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respectively. Uptake was stopped at indicated times by the addition of 2 ml of ice-cold 0·1 mol l^{-1} LiCl solution immediately followed by filtering through a 0·45 μmol l^{-1} pore-sized nitrocellulose filter (BA85; Schleifer & Schuell GmbH, Dassel, Germany). The filter was washed once with 2 ml of ice-cold 0·1 mol l^{-1} LiCl and submerged in Emulsifier Scintillation Plus scintillation fluid (Hewlett Packard, CA), and the retained radioactivity was counted in Tri-Carb 2000CA liquid scintillation counter (Packard Instrumentation). The background was estimated by adding 2 μl of the radiolabelled substrate to the cell suspension immediately after the addition of 2 ml of ice-cold LiCl, followed by filtering. Cells were energized by incubating the cell suspension with KPi buffer containing 20 mmol l^{-1} glucose, at room temperature for 5 min, under conditions of constant stirring.

To load cells with putrescine, these were obtained by incubation of the cell suspension for 1 h at 30°C with 5 mmol l^{-1} of putrescine and 4 μmol l^{-1} final concentrations of valinomycin and nigericin. Reactions were initiated by diluting 3 μl of this cell suspension in 97 μl of buffer containing labelled arginine or labelled ornithine. Valinomycin and nigericin were added to give a final concentration of 2% (v/v). When stated, in the Results section, not labelled putrescine was added to the reaction buffer. Chase experiments were performed by allowing cells to uptake labelled arginine or labelled ornithine. After 1 min of arginine or ornithine uptake, 1 mmol l^{-1} cold (not labelled) ornithine or arginine respectively, or cold putrescine, were added to the reactions mixture. Retained radioactivity was measured as before.

Protein quantification

Total protein was quantified using the BCA Protein Assay kit from Pierce, using bovine serum albumin as the standard for quantification. Five millilitres of a cell culture was centrifuged (13 200 ***g***, 8 min), and the cells were washed with 50 mmol l^{-1} KPi buffer and diluted into 1 ml of the same buffer. Cells were disrupted by sonication performed on ice, at 50% potency for 30 s followed by 30 s pause, for a total of 3 min. The total protein of this cell suspension was then quantified.

Results

Arginine and ornithine requirement in acid shock survival

Weissella halotolerans W22 cells were challenged with acid shock in the presence of 10 mmol l^{-1} concentration of arginine or ornithine, and survival efficiency was assessed. Control cells (with no added amino acid) showed high susceptibility to acid shock (35% survival), but addition of 10 mmol l^{-1} arginine to the shock medium raised the survival efficiency to 55%.

Remarkably, when 10 mmol l^{-1} ornithine was used to supplement the medium instead of arginine, the survival efficiency was even higher (95%). These results clearly show the major role of arginine and even more of ornithine in the response of *W. halotolerans* W22, when imposed to an acid stress, and prompted us to investigate the role of arginine and ornithine in the energetic state of the strain W22.

ΔpH generation by arginine and ornithine catabolism in *W. halotolerans* W22

The bioenergetic consequences of arginine and ornithine catabolism pathways in resting cells of *W. halotolerans* W22 were investigated by monitoring the two components of the PMF, i.e. the pH gradient (ΔpH) and the membrane potential (ΔΨ) across the cytoplasmic membrane. Resting cells of strain W22, when suspended in 50 mmol l^{-1} potassium phosphate buffer pH 5·5, were able to maintain an intracellular pH (pH_{in}) of about 6·5, which corresponds to a pH gradient of 1·0 unit, inside alkaline (Fig. 2). Addition of arginine (Fig. 2a) to these cells resulted in a net alkalinization of the cytoplasm. After a transient acidification, the pH reached a value close to 7·4, corresponding to a ΔpH of 1·9. Under these conditions, addition of valinomycin, a K^+ ionophore that converts membrane potential in ΔpH, resulted in a further increase of the internal pH up to a final ΔpH of 2·1. Addition of nigericine, which catalyses electroneutral exchange between K^+ and H^+, resulted in a complete abolishment of ΔpH (data not shown).

Weissella halotolerans W22 cells metabolizing arginine through ADI pathway produce ammonia that alkalinizes the medium. In the ADI pathway, ATP is formed, which is used to pump protons across the cytoplasmic membrane by F_0F_1ATPase to generate PMF. This was confirmed by preincubating the cells with the F_0F_1ATPase inhibitor DCCD. Then, addition of arginine did not result in the alkalinization of the cytoplasm (Fig. 2a). Instead, an acidification of the cytoplasm by half a pH unit was observed, suggesting that the initial acidification in the untreated cells is related to the metabolism of arginine in the cells. The results are consistent with the function of the ADI pathway in providing the cell with ATP, which can be hydrolysed by F_0F_1ATPase to extrude protons from the cell cytoplasm.

The same resting cells of strain W22 were also assayed for the cytoplasmic pH in response to the addition of ornithine (Fig. 2b). Addition of ornithine caused an immediate increase of internal pH to a value close to 7·4,

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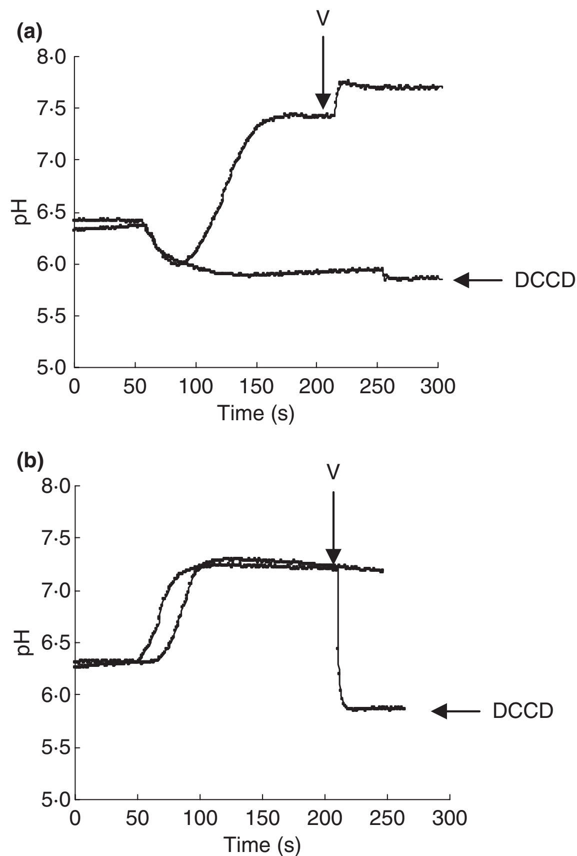

Figure 2 ΔpH generation by arginine (panel a) and by ornithine (panel b). At the times indicated by the arrows, valinomycin was added to the reaction. The same experiment was performed with cells previously incubated with *N*,*N'*-dicyclohexylcarbodiimide.

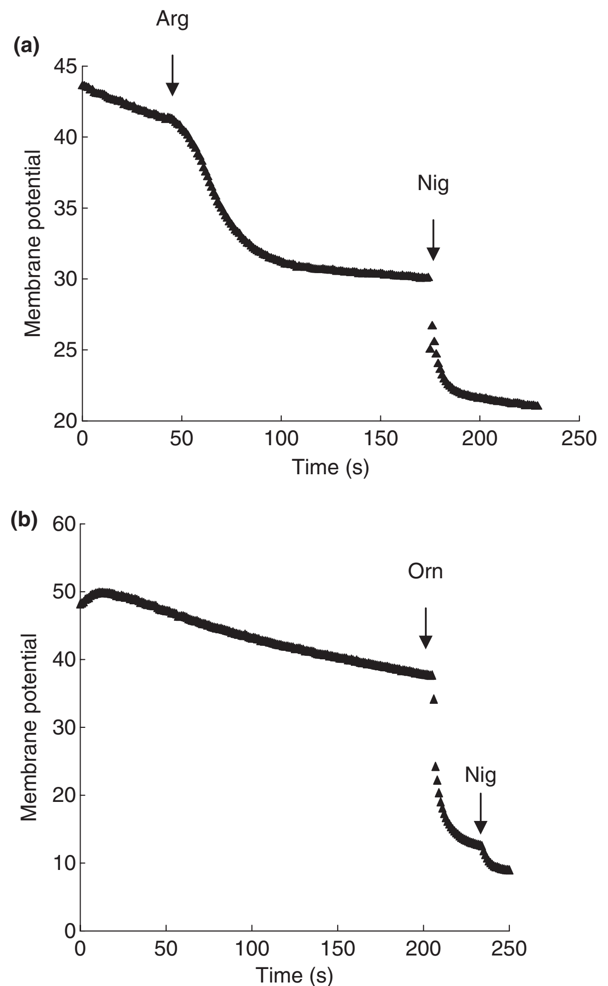

Figure 3 Membrane potential variation upon arginine (panel a) and ornithine (panel b) addition to resting cells of *Weissella halotolerans* W22. Arginine (Arg) or ornithine (Orn), were added to the reactions, as well as nigericin, at the times indicates by the arrows.

corresponding to a ΔpH of 1·9. Nigericin addition completely dissipated the pH gradient (not shown). Addition of valinomycin did not further increase the pH gradient, suggesting that in this case the PMF is solely composed of a ΔpH.

Contrary to the effect of arginine addition to DCCD-treated cells, addition of ornithine resulted in an equal increase of internal pH, as observed for the untreated cells, indicating that F_0F_1ATPase does not play a role in the alkalinization of cytoplasm during ornithine catabolism in cells W22, which is consistent with an ornithine decarboxylation pathway.

Membrane potential measurements in whole cells of *Weissella* W22

The formation of a membrane potential (ΔΨ) by *Weissella* W22, as a result of arginine metabolism by ADI pathway, was also studied (Fig. 3). Addition of arginine (Fig. 3a) to these resting cells caused an immediate increase in membrane potential (about 10% increase), after which a steady state was achieved.

Addition of nigericin augmented the ΔΨ to a maximal value, as a result of conversion of ΔpH into ΔΨ. At this point, PMF was constituted solely by ΔΨ. Valinomycin also increased ΔΨ of these cells, but at a much lower rate than nigericin. These results suggest that both ionophores, valinomycin and nigericin, stimulate the ADI pathway, resulting in an increase in PMF.

When the same experiments were performed with DCCD-treated cells, they did not show any variation in membrane potential after arginine addition. This observation indicates that ADI pathway in W22 cells is directly coupled to F_0F_1ATPase activity. In parallel, when ornithine was added – instead of arginine – to resting cells of strain *Weissella* W22 (Fig. 3b), an immediate increase in membrane potential was observed (about 35% increase).

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However, in this case, addition of valinomycin decreased $\Delta\Psi$ by about 20%. Because valinomycin abolishes the $\Delta\Psi$, PMF in this case is composed only of ΔpH. Again this is consistent with the ornithine decarboxylation pathway where a ΔpH is expected to be formed as a result of cytoplasmic protons' consumption during the decarboxylation reaction.

Arginine and ornithine transport in whole cells

Uptake of the basic amino acids arginine and ornithine was studied using metabolizing cells of *W. halotolerans* W22, at pH 5·5 and 30°C.

Cells of *W. halotolerans* W22 energized with glucose were studied for the uptake of arginine and ornithine (Fig. 4). In both cases, uptake was higher in nonenergized cells. Ornithine was accumulated in the cells, whereas no uptake was detected for arginine in the presence of glucose. This observation suggests that uptake of arginine is inhibited in cells with available energy. Moreover, ornithine uptake, although reduced, occurs even under high cellular energy levels.

Because, in strain W22, putrescine is produced from decarboxylation of ornithine, the existence of a transporter mediating the extrusion of putrescine was anticipated. Therefore, uptakes of arginine and ornithine, in cells loaded with putrescine or not loaded but assayed in buffer with 5 mmol l^{-1} putrescine, were measured. Figure 5 shows that the uptake of arginine was higher in cells loaded with putrescine, whereas uptake of ornithine was not dependent on the presence of putrescine inside the cells (compared to control cells not loaded with putrescine). However, when uptakes were measured in nonloaded cells,

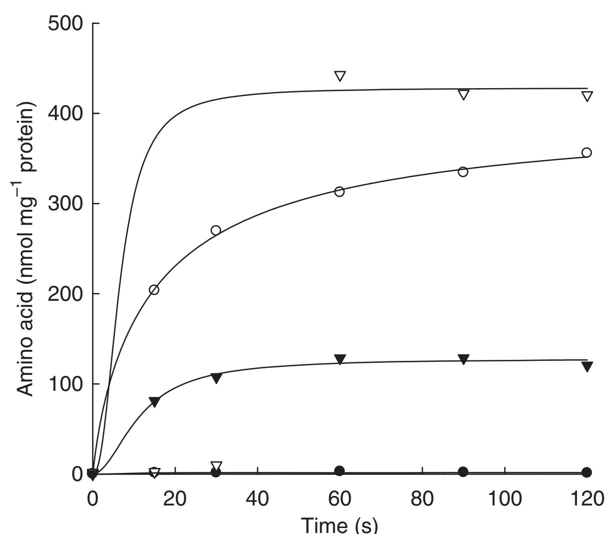

Figure 4 Arginine (○, ●) and ornithine (△, ▲) uptake in control cells (○, △) and in cells energized with glucose (●, ▲).

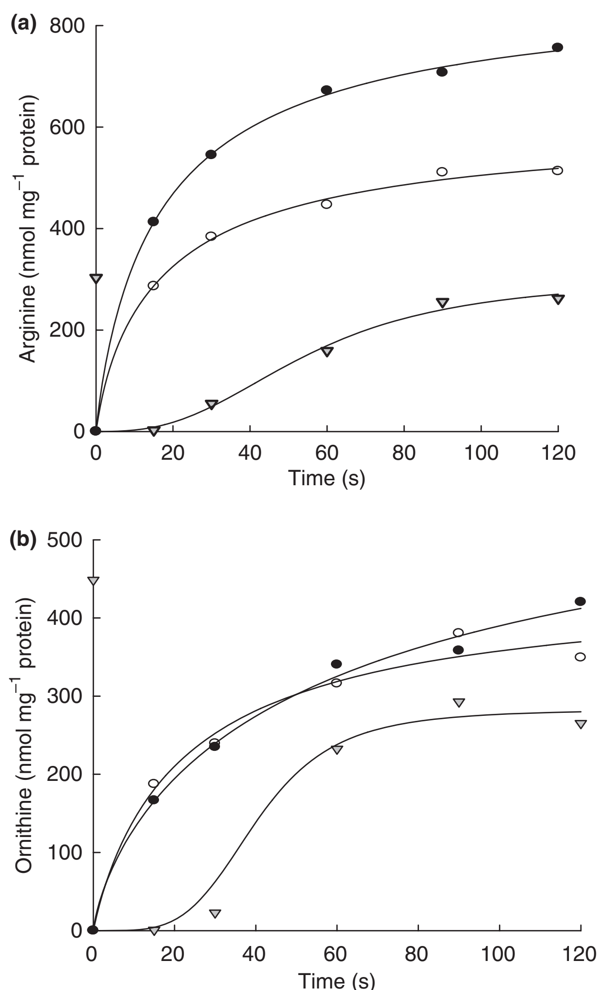

Figure 5 Uptake of arginine (panel a) and of ornithine (panel b). Uptake in cells loaded (●) and not loaded (○) with putrescine. The same assays were performed diluting non loaded cells in buffer with 5 mmol l^{-1} putrescine (▼).

but with 5 mmol l^{-1} putrescine in the buffer reaction, both arginine and ornithine uptakes decreased.

In a chase experiment where arginine uptake was chased with ornithine and putrescine (Fig. 6a), it was evident that the arginine transporter also had affinity for ornithine and putrescine. The ornithine transporter was also studied in a chase experiment. Figure 6(b) shows that putrescine is also a substrate of the ornithine transporter. In fact, when putrescine was added to the reaction buffer, the rate of ornithine uptake was inhibited, suggesting that putrescine is co-transported by the transporter that mediates uptake of ornithine.

Discussion

In a previous work (C.I. Pereira, A.T. Pires, H. Silva, C. Leitao, M.V. San Romao, M.T.B. Crespo, unpublished

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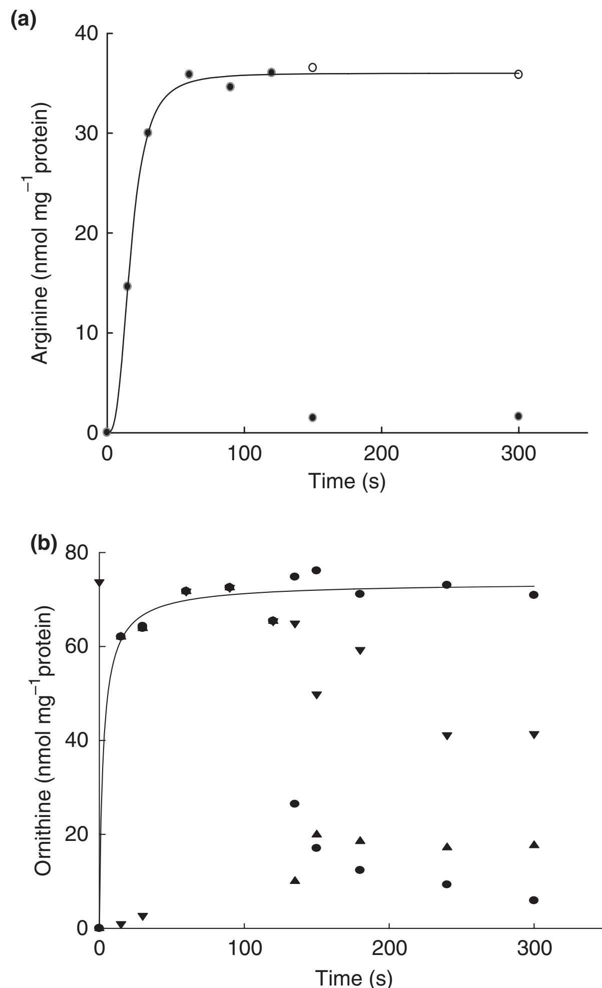

Figure 6 (a) Arginine chase experiment. Cells were allowed to take arginine (open circles). At 120 s putrescine was added to the reaction (closed symbols). (b) Ornithine chase assay. Cells were allowed to take ornithine in the control experiment (open circles). Then, at 120 s either ornithine was added (closed circles) or arginine (lower traingles) or putrescine (upper triangles) were added, and internalized ornithine was measured.

data), we have reported that *W. halotolerans* W22 possesses an active and functional ADI system. In addition, PCR screening revealed that an ornithine decarboxylase (*odc*) gene was also present in this strain. Further studies showed that this strain produced putrescine by decarboxylation of ornithine. Therefore, we firmly believe that both ADI pathway and ornithine decarboxylation pathway are active, simultaneously functional and sharing ornithine as a common metabolite, in the strain *W. halotolerans* W22. A proposed mechanism is illustrated in Fig. 1 for the interaction between the two pathways.

The role of amino acid decarboxylations in ameliorating internal cellular pH when cells are exposed to an acid stress has been described by several authors (Iyer *et al.* 2003; Richard and Foster 2004; van de Vossenberg *et al.* 1998). Because the cells of *W. halotolerans* W22 were able to maintain high cell viability when subjected to an acid stress when exposed to pH 2·5 in the presence of arginine or ornithine, the function of both amino acids was studied deeply. Two amino acid–dependent systems were addressed: ADI pathway depending on arginine and ornithine decarboxylation pathway depending on ornithine. It was demonstrated by the bioenergetic measurements that both ADI and OTC pathways generate a PMF in W22 cells. ADI pathway increases ΔpH (higher inside) which was confirmed by ΔpH abolishment upon addition of the ionophore nigericin. The ΔpH was maximal after valinomycin addition, which by converting $\Delta\Psi$ (Inside negative) to ΔpH (inside alkaline) indicates that PMF was solely composed of the pH gradient component. The increase in ΔpH caused by ADI pathway was shown to be dependent on the activity of F_0F_1ATPase, suggesting that intracellular decrease of protons during ADI metabolism is directly coupled to proton extrusion by F_0F_1ATPase.

As reported (Arena *et al.* 1999), arginine catabolism by ADI pathway produces 1 mol of ATP that can be hydrolysed by F_0F_1ATPase to extrude protons out of the cell. The ADI pathway requires two transport steps across the cytoplasmic membrane: the uptake of arginine and the excretion of the end-product ornithine. When each of these transport steps would require energy in the form of a PMF or ATP, the contribution to metabolic energy by arginine metabolism would not be significant. LAB and other bacteria (Poolman *et al.* 1987; Driessen et al. 1988) have cleverly solved this problem by coupling the uptake of arginine with the excretion of ornithine.

On the contrary, ornithine decarboxylation pathway generates both a ΔpH and a $\Delta\Psi$. The process of precursor/product exchange coupled to decarboxylation does not involve the net translocation of protons across the membrane as in the case with true proton pumps, like the F_0F_1ATPase. By compartmentalization of the decarboxylation pathway, the energy of the decarboxylation reaction can be converted into a PMF. In the ornithine decarboxylation reaction, protons are taken up from the cytoplasm, resulting in an increase of the cytoplasmic pH, and consequently generate a transmembrane pH gradient. In addition, in the physiological conditions studied in this work, putrescine bears a +2 net charge compared to ornithine (+1). As described, a precursor/product exchange in which a net positive charge is translocated from inside to outside can generate an electrical potential across the membrane.

The fact that ADI pathway was inhibited by DCCD, whereas in the same cells ornithine decarboxylation was not, suggest the existence of two transporters, one mediating arginine uptake (in antiport with ornithine

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extrusion) and another catalysing ornithine uptake (in antiport with putrescine extrusion). Moreover, when cells were energized with glucose, arginine uptake was inhibited, whereas ornithine uptake was not. Again, these results point towards the existence of two different transporters.

Moreover, ATP produced in the pathway can be used for other metabolic energy requiring processes in the cell (Poolman 1993; Konings *et al.* 1995, 1997). Under energy-limited conditions, arginine metabolism supplies additional metabolic energy and prevents a drop of the PMF below viable levels (Richard and Foster 2004). Because ATP is a product of ADI pathway, it is not surprising that an energy source, like glucose, would inhibit the ADI pathway. This evidences the role of ADI pathway as an alternative physiological pathway to produce ATP in energy-deficient conditions. Metabolism of arginine to ornithine, ammonia and CO_2 (in the ratio 1 : 2 : 1) via the ADI pathway, as referred previously, provides various LAB with an additional substrate-level phosphorylation process (Abdelal 1979).

Arginine in the cytoplasm is rapidly converted to ornithine, the internal arginine concentration is very low, and the arginine gradient remains directed from outside to inside. Ornithine, on the other hand, is produced internally which leads to high concentrations inside, while the concentration of ornithine in the medium will be low. Consequently, the ornithine concentration gradient is directed from in to out. Results obtained in this study show that in W22 cells, ornithine uptake is more efficient than arginine uptake, which is in accordance with the results reported (Driessen et al. 1988), evidencing that many LAB maintain high intracellular concentrations of ornithine. Therefore, lower uptake rates for ornithine compared to arginine, in cells grown in the presence of arginine, are not surprising. We believe that the arginine/ornithine antiporter expression is enhanced, and therefore extrusion of ornithine in exchange for arginine uptake results in lower net uptakes of ornithine. The presence of arginine in the growth medium also stimulates the activity of other enzymes of the ADI pathway.

Although it has always been described that arginine/ornithine antiport is electroneutral (Konings *et al.* 1997; Fernandez and Zúñiga 2006; Lucas *et al.* 2007) (because at the pH conditions of the assay, both arginine and ornithine bear a net positive charge of +1), ΔpH measurement assays showed an initial decrease in intracellular pH immediately after arginine addition. This effect can be attributed to the metabolism of arginine by W22 cells. Both gradients of arginine and ornithine therefore contribute to the driving force for the electroneutral arginine/ornithine exchange process, and additional metabolic energy is not needed.

Results obtained in this study during the arginine uptake experiments suggest the presence of an antiport arginine/putrescine. Several facts support this hypothesis: (i) arginine transport seems to be more active than the ornithine transporter; (ii) when cells were loaded with putrescine, arginine uptake was stimulated; and (iii) chase experiments showed that putrescine is also a subtract for the transporter that mediates arginine uptake.

However, these results need to be carefully interpreted. In fact, a question remains: Does arginine also exchange with putrescine as might be anticipated when both pathways are present at the same time? Figure 6 appears to confirm this, but what is really exchanging? Is it the pool inside still arginine, or is it converted to ornithine and we are looking at ornithine/putrescine exchange?

The best system for a detailed clarification of these results of transport processes would be an isolated membrane vesicles system. However, so far, preparing functional vesicles for these bacteria has not been possible, and the clarification of this hypothesis remains to be elucidated. Nevertheless, unlike reported previously for other species of LAB (Alberto *et al*. 2007; Konings 1994; Konings 2006; Marques *et al*. 2008; Salema *et al*. 1996), besides increasing intracellular pH and producing ATP, ADI pathway in *W. halotolerans* W22 provides ornithine, which is used as a substrate for another metabolic pathway: ornithine decarboxylation. For the first time, the coexistence of two different types of amino acid catabolic pathways in the same strain of LAB is shown. Also, up-to-date and as far as we are aware of, this is the first time such decarboxylation pathways are described for a strain of the *Weissella* genera.

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