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Published in:
Journal of Bacteriology

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

Publication date:
1987

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Regulation of Arginine-Ornithine Exchange and the Arginine Deiminase Pathway in *Streptococcus lactis*

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Streptococcus lactis metabolizes arginine by the arginine deiminase (ADI) pathway. Resting cells of *S. lactis* grown in the presence of galactose and arginine maintain a high intracellular ornithine pool in the absence of arginine and other exogenous energy sources. Addition of arginine results in a rapid release of ornithine concomitant with the uptake of arginine. Subsequent arginine metabolism results intracellularly in high citrulline and low ornithine pools. Arginine-ornithine exchange was shown to occur in a 1-to-1 ratio and to be independent of a proton motive force. The driving force for arginine uptake in intact cells is supplied by the ornithine and arginine concentration gradients formed during arginine metabolism. These results confirm studies of arginine and ornithine transport in membrane vesicles of *S. lactis* (A. J. M. Driessen, B. Poolman, R. Kiewiet, and W. N. Konings, Proc. Natl. Acad. Sci. USA, 84:6093–6097). The activity of the ADI pathway appears to be affected by the internal concentration of (adenine) nucleotides. Conditions which lower ATP consumption (dicyclohexylcarbodiimide, high pH) decrease the ADI pathway activity, whereas uncouplers and ionophores which stimulate ATP consumption increase the activity. The arginine-ornithine exchange activity matches the ADI pathway most probably by adjusting the intracellular levels of ornithine and arginine. Regulation of the ADI pathway and the arginine-ornithine exchanger at the level of enzyme synthesis is exerted by glucose (repressor, antagonized by cyclic AMP) and arginine (inducer). An arginine/ornithine antiport was also found in *Streptococcus faecalis* DS5, *Streptococcus sanguis* 12, and *Streptococcus milleri* RH1 type 2.

The arginine deiminase (ADI) pathway is widely distributed among bacteria and serves as the sole or an additional source of energy, carbon, and/or nitrogen in these organisms (2, 24). The enzymatic properties of the ADI pathway have been analyzed in variety of bacteria (1, 2, 4, 23, 25). The ADI pathway includes (i) ADI, which catalyzes the conversion of arginine into citrulline and ammonia in an essentially irreversible reaction; (ii) ornithine carbamoyltransferase, which catalyzes the phosphorolysis of citrulline, yielding ornithine and carbamoylphosphate (this step is thermodynamically limiting since the equilibrium of the reaction strongly favors the formation of citrulline [$K \sim 10^5$] [20, 23]; (iii) carbamate kinase, which catalyzes the reversible conversion of carbamoylphosphate and ADP into ATP, carbon dioxide, and ammonia. The equilibrium of this reaction is in favor of ATP synthesis (26).

Control of enzyme synthesis of the ADI pathway has been studied for the three cytoplasmic enzymes (1, 2, 11, 22). For *Streptococcus lactis*, ADI and ornithine carbamoyltransferase are inducible, whereas carbamate kinase is present constitutively (1). The enzymes are coordinately synthesized in *Streptococcus faecalis* and *Pseudomonas aeruginosa* (11, 22), and there is evidence for a four-gene cluster in *P. aeruginosa* encoding ADI, ornithine carbamoyltransferase, carbamate kinase, and a gene product not yet identified (27). For these and other organisms, arginine has been shown to give a inductive response, whereas conditions which favor the energy status of the cells repress enzyme formation (2).

In many bacteria possessing the ADI pathway, 1 mol of ornithine is excreted per mol of arginine metabolized (1, 27). Some mutant strains have also been shown to excrete citrulline instead of ornithine (4, 27, 29). Arginine catabolism by the ADI pathway yields only one ATP per arginine. The net gain of metabolic energy, however, will depend on the energetic costs of arginine uptake and ornithine excretion. Studies of the molar growth yields on arginine in *S. lactis*, *S. faecalis*, and other bacteria indicated the net formation of one ATP per arginine (1, 12), implying that no metabolic energy is needed for the transport processes. This indication is confirmed by transport studies in membrane vesicles of *S. lactis*, which revealed the presence of an arginine/ornithine antiporter catalyzing the stoichiometric exchange of arginine for ornithine (3a). Arginine-ornithine exchange activity exhibited saturation kinetics with respect to the external arginine and the internal ornithine concentration and appeared to be independent of the magnitude of the proton motive force. In addition to heterologous exchange, the antiport system catalyzes homologous exchange of arginine and ornithine (3a).

In this study, we determined the (in vivo) role of the arginine/ornithine antiporter in the ADI pathway. Information is presented on the short-term regulation of arginine-ornithine exchange and ADI pathway activity by pathway intermediates as well as on the long-term regulation of the enzymes by protein synthesis.

MATERIALS AND METHODS

Organisms and culture conditions. The following strains were kindly provided by A. H. Weerkamp (Dental School, University of Groningen): *Streptococcus salivarius* HB, *Streptococcus sanguis* 12, *Streptococcus milleri* RH1 type 2, *Streptococcus mutans* NCTC 10449 and C67. *S. faecalis* DS5 was obtained from the Department of Genetics, University of Groningen. Other strains used were *Streptococcus cremoris* Wg2, *S. lactis* ML3, *Clostridium acetobutylicum* ATCC 824, and *P. aeruginosa* LMD80.53. Strains were grown anaerobically on MRS broth (3) in the presence of 50 mM galactose and 25 mM arginine unless indicated otherwise. For some experiments, galactose was replaced by glucose (50 mM) and/or the arginine supplement to the

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medium was omitted. *S. lactis* ML3, *S. cremoris* Wg2, and *C. acetobutylicum* were grown at 30°C; all other strains were grown at 37°C. After growth overnight, the bacteria were harvested by centrifugation, washed twice, and resuspended in buffer to a final protein concentration of 40 to 80 mg/ml. These concentrated cell suspensions were used immediately or frozen (and stored) in liquid nitrogen until use.

For the continuous culture experiments, *S. lactis* ML3 was grown on a chemically defined medium as described previously (19) with the following modifications. (i) Glucose (30 mM) was added instead of lactose as the growth-limiting substrate. (ii) The arginine concentration was raised to 4 mM. (iii) Glutamate and aspartate were replaced by glutamine (2.67 mM) and asparagine (2.63 mM), respectively. (iv) The final tyrosine concentration was 1.1 mM. Chemostat cultures were grown anaerobically under a N_2 atmosphere in glass fermentors with a working volume of 185 ml at 30°C and controlled at pH 6.4. For the determination of ADI pathway activity and the transport measurements, 100-ml samples were withdrawn from the chemostat, centrifuged, washed twice with 50 mM potassium PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] (pH 6.0) supplemented with 5 mM $MgSO_4$, and stored in liquid nitrogen until use.

Transport assay. Cells were resuspended to a final protein concentration of 2 to 50 mg/ml in 50 mM potassium PIPES (pH 6.0) supplemented with 5 mM $MgSO_4$. [^{3}H]ornithine or unlabeled ornithine was added to a final concentration of 1 to 5 mM, and the cells were incubated for 1 h at 20 to 22°C. Subsequently, the cells were centrifuged and suspended to a final protein concentration of about 50 mg/ml in the same buffer without ornithine. For transport measurements, 2 μl of the cell suspension was diluted into 200 μl of 50 mM potassium PIPES (pH 6.0) containing 5 mM $MgSO_4$ and [^{14}C]arginine (100 to 1,000 μM, final concentration), [^{14}C] citrulline (50 μM, final concentration), or no added amino acids. Transport measurements were performed at 15°C unless indicated otherwise. At the time intervals indicated, samples were filtered (cellulose-acetate filters, 0.45-μm pore size; Millipore Corp., Bedford, Mass.) and washed twice with ice-cold 0.1 M LiCl as described previously (15).

Transport studies in membrane vesicles were performed essentially as described previously (3a). Membrane vesicles were incubated for 2 h at 20 to 22°C with 500 μM ornithine and subsequently pelleted by centrifugation (48,000 × *g* for 30 min at 4°C). After resuspension in the same buffer (20 to 25 mg of protein per ml), samples (4 μl) were diluted 100-fold into buffer containing various concentrations of [^{14}C]arginine. Subsequent actions were as described above for experiments with intact cells.

ADI pathway activity. Cells were diluted to a final protein concentration of 0.2 to 0.3 mg/ml in 4 ml of 1.5 mM potassium MES (morpholineethanesulfonic acid)–1.5 mM potassium PIPES–1.5 mM potassium HEPES-(*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–1.5 mM potassium Tricine–100 mM KCl–5 mM $MgSO_4$ (pH 6.6, unless indicated otherwise). The reaction was started upon the addition of 2.0 mM arginine. The alkalinization of the medium was recorded in a range in which the change in external pH was less than 0.02 pH units and linear in time. Changes in pH were converted into nanomoles of OH^- or ammonia by calibration of the cell suspension with 2- to 4-μl portion of 100 mM KOH or 100 mM ammonia. The measurements were performed at 20°C.

Intracellular amino acid concentrations. For experiments with resting cell suspensions, cells were suspended to a final

protein concentration of 0.8 to 1.5 mg/ml in buffer as described for the ADI pathway activity measurements. Samples (1.0 ml) were taken before or after the addition of arginine and transferred to microcentrifuge tubes containing 0.8 ml of silicon oil (density, 1.03 mg/ml) on top of 0.2 ml of 7% (wt/vol) perchloric acid and 4.5 mM EDTA. The mixture was centrifuged for 4 min at 12,000 × *g*. Fractions (0.5 ml) were taken from the supernatant and, after removal of the remaining water and oil layers, from the perchloric extract (0.15 ml). The perchloric acid extract was mixed with 0.2 ml of 1 N $KOH-KHCO_3$ to give a final pH of 9.5. Supernatant fractions were treated similarly with perchloric acid followed by neutralization with 1 N $KOH-KHCO_3$. The samples were stored at -20°C. The same procedure was used for samples (1.0 ml) which were drawn from chemostat cultures. Amino acids were analyzed after derivation with dansyl chloride and separated by reversed-phase high-performance liquid chromatography on a C18 column (μBondapak C18, 3.9 mm by 30 cm; Water Associates, Inc., Milford, Mass.) as described previously (16). Intracellular amino acid concentrations were calculated from the amount present in the cell extracts after correction for medium adhering to the cellular surface during silicon oil centrifugation (16).

Other analytical procedures. Membrane vesicles of *S. lactis* ML3 were prepared by osmotic lysis as described previously (18) and stored in liquid nitrogen until use. ATP concentrations were determined by the firefly luciferase assay as described previously (17). Protein was measured by the method of Lowry et al. (8), using bovine serum albumin as the standard.

Materials. L-(2,3-^{3}H)ornithine (1.1 TBq/mmol) was obtained from New England Nuclear Corp. (Boston, Mass.). L-[U-^{14}C]arginine (11 TBq/mol) and L-[U-^{14}carbamoyl] citrulline (1.85 TBq/mol) were purchased from the Radiochemical Centre (Amersham, England). All other materials were obtained from commercial sources with the highest grade of purity available.

RESULTS

Arginine-ornithine exchange in *S. lactis*. Galactose-arginine-grown cells of *S. lactis* ML3 maintained a high intracellular concentration of ornithine (10 to 15 mM) for several hours when suspended in buffer without an exogenous energy source. Even after methyl-1-thio-β-D-galactopyranoside treatment to deplete the cells of endogenous energy sources (15, 16), high ornithine concentration gradients were maintained. Addition of [^{3}H]ornithine resulted in a rapid exchange of intracellular and extracellular ornithine (data not shown).

To monitor the exchange of arginine and ornithine, we diluted [^{3}H]ornithine-loaded cells 100-fold into medium with or without [^{14}C]arginine (Fig. 1). A rapid uptake of [^{14}C]arginine was observed concomitant with the efflux of [^{3}H]ornithine. Efflux of [^{3}H]ornithine was not observed in the absence of external arginine, indicating that arginine uptake and ornithine exit are coupled directly. The stoichiometry of arginine-ornithine exchange varied between 1.0 and 1.3 during the first 5 s after dilution of the cells. The initial exchange activity appeared not to be affected by uncouplers or a combination of ionophores which dissipate the proton motive force (Δp) (Fig. 2). The Δp maintained by resting cells of *S. lactis* was about -100 mV, whereas the intracellular ATP levels were 0.1 mM or less in the absence of exogenous energy sources (7; data not shown). Importantly, neither exchange between citrulline and ornithine nor
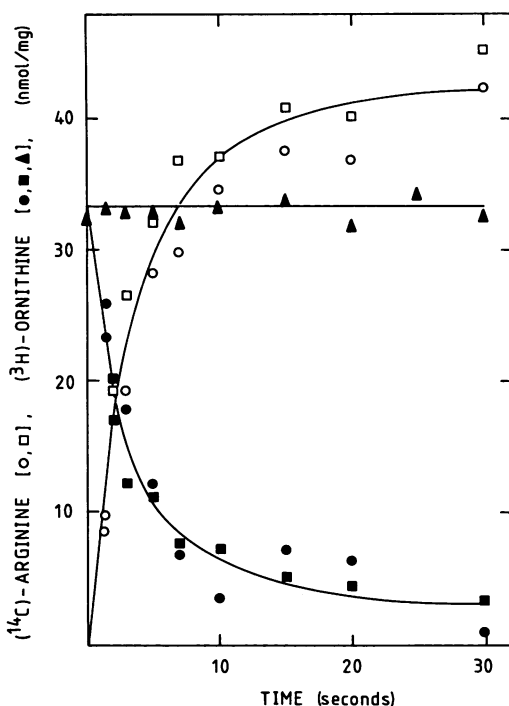
FIG. 1. Arginine-ornithine exchange in *S. lactis* ML3. Concentrated cell suspensions of galactose-arginine-grown cells loaded with [³H]ornithine were diluted 100-fold into 50 mM potassium PIPES (pH 6.0)–5 mM MgSO₄ containing no (▲), 100 µM (○, ●), or 1 mM (□, ■) [¹⁴C]arginine. The final protein concentration was 0.91 mg/ml. The intracellular ornithine concentrations were determined in parallel experiments after separation of the cells from the medium by silicon oil centrifugation as described in the text.
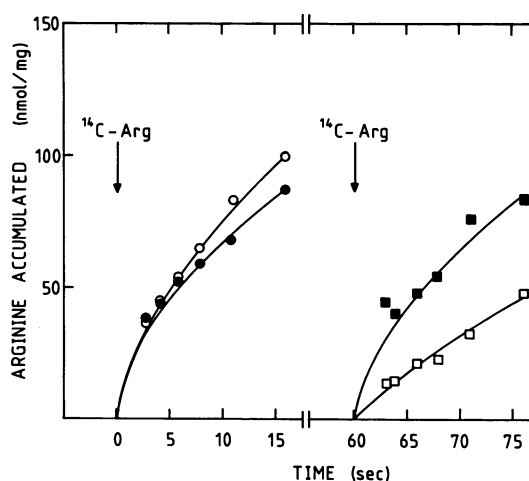

FIG. 2. Unidirectional rates of arginine uptake after various periods of arginine metabolism in the presence and absence of valinomycin plus nigericin. At time zero, galactose-arginine-grown cells of *S. lactis* ML3 were diluted 100-fold into 50 mM potassium PIPES (pH 6.6)–5 mM MgSO₄ containing 200 µM arginine and in the absence (○, □) or presence (●, ■) of 3 µM valinomycin plus 1.5 µM nigericin. [¹⁴C]arginine (3.6 µM; 11 TBq/mmol) was added at time zero (○, ●) and after 60 s of arginine metabolism (□, ■). Corrections were made for differences in specific activity of [¹⁴C]arginine between samples to which [¹⁴C]arginine was added after 0 and 60 s of metabolism, respectively. The final protein concentration was 0.64 mg/ml.

citrulline uptake driven by glycolysis was observed in these cells (data not shown). Since the initial arginine-ornithine exchange rates were high, most of the experiments were performed at 15°C. Estimation of the exchange activity in galactose-arginine-grown cells at 30°C indicated initial rates of 0.5 to 1.0 µmol/min × (mg of protein).

Regulation of arginine-ornithine exchange activity. The unidirectional rates of arginine uptake 0 and 60 s after addition of arginine are shown in Fig. 2. At zero time, uptake of [¹⁴C]arginine was rapid and exchange activity appeared not to be affected by the addition of valinomycin plus nigericin. After 60 s of arginine metabolism, the unidirectional rate of [¹⁴C]arginine uptake appeared to be reduced about threefold compared with the uptake at zero time. At 60 s, the exchange activity was stimulated significantly by valinomycin plus nigericin. This stimulation can be related to changes in the intracellular ornithine concentration, one of the substrates of the exchange reaction (see below).

With ornithine-loaded membrane vesicles of *S. lactis*, the maximal rate of arginine uptake has been shown to increase with increased internal ornithine concentration (3a). To determine whether arginine/ornithine antiport activity in intact cells is affected by changes in the intracellular levels of ornithine (and other possible effectors), we measured the intracellular concentrations of ADI pathway intermediates. The intracellular citrulline and ornithine concentrations in resting galactose-arginine-grown cells were 0.7 and 11.9 mM, respectively (Table 1). Upon addition of arginine, the intracellular ornithine pool fell rapidly to about 2.5 mM, whereas the citrulline pool rose to concentrations exceeding

20 mM. For technical reasons (the arginine peak was not sufficiently separated from the glycine peak in reversed-phase high-performance liquid chromatography assays), the precise arginine concentrations could not be determined. Estimation of the intracellular arginine pool after 60 s of metabolism indicated a concentration of less than 1 mM.

In contrast to cells preloaded with ornithine (see Materials and Methods), unloaded cells contained high concentrations of lysine (up to 10 mM) in addition to ornithine. The internal lysine pool fell to below 1 mM within 60 s of arginine metabolism (data not shown). Exchange between arginine and lysine (3a) (along with arginine-ornithine exchange) most probably explains why the increase in citrulline exceeded the decrease in ornithine in these unloaded cells (Table 1). Upon addition of arginine to ornithine-preloaded cells (containing

TABLE 1. Changes in the intracellular concentrations of citrulline and ornithine upon addition of arginine to resting cells of *S. lactis* ML3[a]

Time (s)	Without further additions		In the presence of valinomycin and nigericin	
	Citrulline (mM)	Ornithine (mM)	Citrulline (mM)	Ornithine (mM)
0	0.7	11.9	0.8	11.2
15	16.7	2.6	15.2	5.9
60	22.9	2.6	18.6	7.3
75	22.7	2.4	18.0	6.9

[a] Cells were suspended to a final protein concentration of 1.47 mg/ml into 5 mM potassium PIPES–50 mM KCl–5 mM MgSO₄ buffer (pH 6.7). At time zero, arginine was added to a final concentration of 4.0 mM. At the times indicated, the cells were separated from the medium by silicon oil centrifugation as described in the text. Measurements were performed in duplicate.
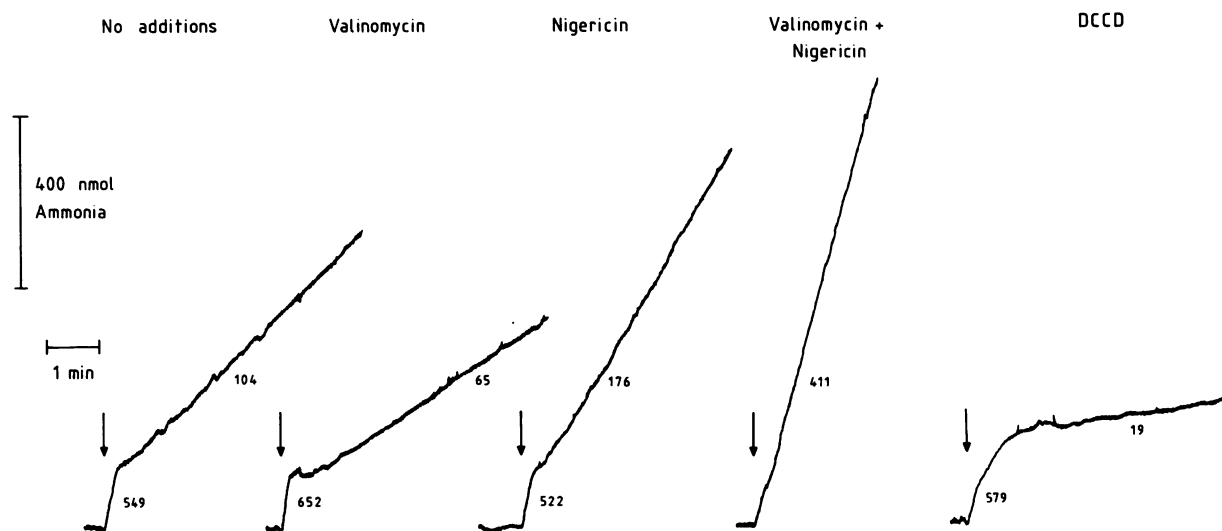
FIG. 3. Effect of ionophores and DCCD on the ADI pathway activity in S. lactis ML3. Cells were suspended to a final protein concentration of 0.3 mg/ml into 1.5 mM potassium MES–1.5 mM potassium PIPES–1.5 mM potassium HEPES–1.5 mM potassium Tricine–100 mM KCl–5 mM MgSO$_4$ (pH 6.6). The arrows indicate the addition of arginine to a final concentration of 2 mM. Valinomycin and nigericin were added (where indicated) to final concentrations of 1.5 and 0.75 μM, respectively. For DCCD treatment, the cells were preincubated with 200 μM DCCD for 30 min at 30°C. The numbers indicate the ADI pathway activity in nanomoles of ammonia produced per minute per milligram of protein.

virtually no lysine), the increase in citrulline was stoichiometric with the decrease in ornithine (data not shown).

The changes in the intracellular concentrations of ornithine and citrulline in the presence of valinomycin plus nigericin were smaller than those in the absence of these ionophores (Table 1). Consequently, the citrulline/ornithine ratio during arginine metabolism was significantly decreased under these conditions. The decrease in internal lysine concentration (upon addition of arginine) was not affected by the ionophores (data not shown). Therefore, the increased rate of arginine-ornithine exchange after 60 s of arginine metabolism in the presence of valinomycin plus nigericin (Fig. 2) is caused most probably by the increased ornithine concentration.

Regulation of ADI pathway. The addition of arginine to a resting cell suspension of S. lactis resulted in a rapid alkalinization of the medium for about 15 s, followed by a much lower rate of alkalinization owing to steady-state activity of the ADI pathway, until all the arginine was consumed (Fig. 3). A lower steady-state activity was found in the presence of the ionophore valinomycin, which collapses the membrane potential (Δψ), whereas the ionophore nigericin, which collapses the pH gradient (ΔpH) across the cytoplasmic membrane, resulted in higher steady-state activity. The combination valinomycin plus nigericin or the protonophore SF6847 (data not shown), which dissipate the total proton motive force (Δp), gave the highest pathway activities. The rapid phase of alkalinization of the medium was not observed under the last conditions (Fig. 3). This is in agreement with the observations of arginine uptake in the presence of valinomycin plus nigericin; the rates were similar after 0 and 60 s of metabolism (Fig. 2). Since arginine-ornithine exchange activity is not directly affected by the Δp (see also reference 3a) the ionophores must influence the ADI pathway activity in other ways, possibly by influencing the (adenine) nucleotide concentration or the internal pH or both.

Role of adenine nucleotides. The activity of the F$_0$F$_1$-

ATPase could influence the activity of the ADI pathway most likely by changes in the intracellular concentrations of (adenine) nucleotides. The intracellular ATP concentrations measured after 60 to 90 s of arginine metabolism were reduced from 1.5 to 0.6 mM by valinomycin plus nigericin. Valinomycin alone had no significant effect, whereas the addition of nigericin alone resulted in a decrease of the ATP pool to about 1.0 mM (data not shown). These results indicate that the changes in ADI pathway activity correlate with change in the internal ATP pools. Further evidence in favor of a role of ATP (or adenine nucleotides) in regulating the ADI pathway activity is given by experiments with N,N'-dicyclohexylcarbodiimide (DCCD), a specific inhibitor of F$_0$F$_1$-ATPase. In DCCD-treated cells, the steady-state activity of the ADI pathway was decreased significantly, whereas the initial phase of medium alkalinization (i.e., when the internal ATP concentrations were still low) was similar to that of control cells (Fig. 3). The ADI pathway activity in DCCD-treated cells could not be stimulated by the addition of valinomycin plus nigericin (data not shown). In these studies, exponentially growing cells were used since cells from later stages are poorly sensitive to DCCD (unpublished data).

pH effects. The effect of external pH on the ADI pathway activity was studied in the absence or presence of valinomycin plus nigericin (Fig. 4). In the presence of these ionophores, the Δp is dissipated and the intracellular pH is set at pH values 0.7 to 0.8 pH units more acidic than the outside medium (14). Without ionophores, the ADI pathway was most active at about pH 6; the activity decreased with increasing external pH. In the presence of valinomycin plus nigericin, the activity was significantly increased by the ionophores at pH 5.5 or higher, indicating that these ionophores do not stimulate only by affecting the internal pH. The ADI pathway activity was maximal between pH 6 and 7. Between pH 5.1 to 5.5 the ADI pathway activity was not stimulated by valinomycin plus nigericin, although the
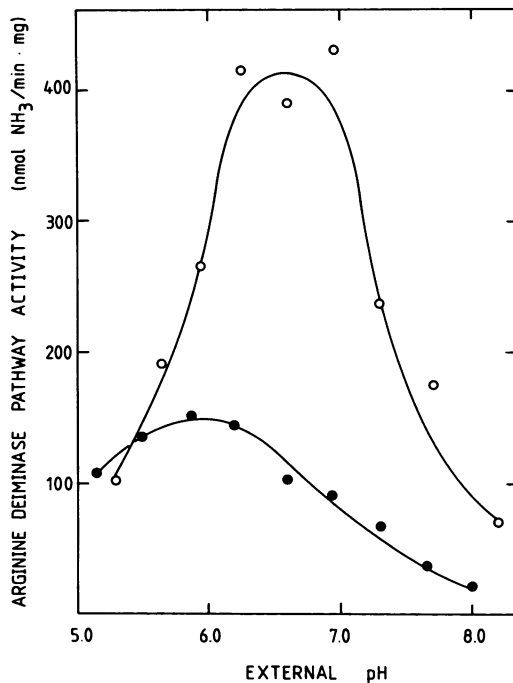
FIG. 4. pH dependence of ADI pathway activity in *S. lactis* ML3. Cells were suspended in buffer with the pH indicated as described in the legend to Fig. 3 in the absence (●) or presence (○) of valinomycin plus nigericin.

pathway was still rather active despite the low internal pH, approximately 5 (14).

The pH dependence of the kinetic parameters of arginine-ornithine exchange was studied in membrane vesicles of *S. lactis* under conditions in which internal and external pH were equal. To achieve this, ornithine-loaded membrane vesicles were diluted 100-fold into a buffer containing various concentrations of [^{14}C]arginine. The affinity constant (K_t) for arginine uptake appeared to be pH independent, whereas the maximal activity (V_{max}) was optimal at pH 6.0 and 7.0 (Table 2).

Induction of arginine-ornithine exchange and ADI pathway activity. Exchange between arginine and ornithine was observed not only in galactose-arginine-grown cells, but also in glucose-grown cells (Table 3). The intracellular ornithine pool in various experiments appeared to be two to three

TABLE 2. pH dependence of the kinetic parameters of arginine-ornithine exchange in membrane vesicles of *S. lactis* ML3[a]

pH	K_t (μM)	V_{max} [nmol of arginine/min × (mg of protein)]
5.0	0.9	29
6.0	0.9	77
7.0	0.8	72
8.0	0.9	40

[a] Membrane vesicles incubated in 30 mM potassium MES–30 mM potassium PIPES–30 mM potassium HEPES buffer at the indicated pH and supplemented with 500 μM ornithine were diluted 100-fold into the same buffer lacking ornithine but containing various concentrations of [^{14}C]arginine (0.3 to 10 μM). The initial arginine uptake rates were estimated in triplicate after 4 s of incubation. A final protein concentration of 0.10 to 0.15 mg/ml was used. The transport experiments were performed at 20°C.

TABLE 3. Effect of growth medium composition on arginine-ornithine exchange and ADI pathway activity in *S. lactis* ML3[a]

Sugar	Arginine (mM)	Cyclic AMP (mM)	Arginine-ornithine exchange activity [nmol of arginine/min × (mg of protein)]	ADI pathway activity [nmol of NH₃/min × (mg of protein)]
Galactose	0	0	50	100
Galactose	25	0	155	434
Galactose	0	2.0	53	89
Glucose	0	0	21	64
Glucose	25	0	93	255
Glucose	0	2.0	40	91
Glucose	25	2.0	114	341

[a] Cells were grown in MRS broth (3) containing the supplements listed above. For arginine-ornithine exchange measurements, the cells were diluted 100-fold to final protein concentrations of 0.4 to 0.6 mg/ml with 50 mM potassium PIPES (pH 6.6)–5 mM MgSO₄ containing 2.0 μM valinomycin and 1.0 μM nigericin. The final [^{14}C]arginine concentration in the transport assays was 100 μM. The initial rates of arginine uptake were estimated (in duplicate) after 2.5 s of incubation at 15°C. For ADI pathway measurements, the cells were suspended to protein concentrations of 0.23 to 0.34 mg/ml into 2.5 mM potassium PIPES (pH 6.6)–100 mM KCl–5 mM MgSO₄ containing 2.0 μM valinomycin and 1.0 μM nigericin. The final arginine concentration was 2 mM. The measurments were performed at 20°C.

times lower in glucose-grown cells than in galactose-arginine-grown cells. The ornithine pools could be raised in these cells by incubating diluted cell suspensions (1 to 2 mg of protein per ml) with relatively high concentrations of ornithine. Net uptake of ornithine occurred most probably via the H$^+$-symporter, which has a specificity for lysine and ornithine (unpublished data), and by ornithine-lysine exchange (see above) (3a).

To measure the arginine and ornithine concentration gradients and the effect of glucose on the arginine-ornithine exchange and ADI pathway activity, we grew *S. lactis* cells on a chemically defined medium with glucose as the growth-limiting substrate. Arginine was converted almost stoichiometrically into ornithine at low dilution rates, whereas above a dilution rate of 0.5 h^{-1} only a small fraction was converted into ornithine (Fig. 5A). The ornithine concentration gradients varied between 2.0 and 2.5 at dilution rates below 0.6 h^{-1}. The intracellular arginine concentrations were too low

TABLE 4. Arginine-ornithine exchange in bacteria[a]

Organism	Initial rate of arginine uptake [nmol of arginine/min × (mg of protein)]
S. cremoris Wg2	0
S. faecalis DS5	221[b]
S. sanguis 12	321[b]
S. salaivarius HB	0
S. mutans NCTC 10449 and C67-1	0
S. milleri RH1 type 2	72[b]
S. mitis BMS	0
P. aeruginosa LMD80.53	114[c]
C. acetobutylicum ATCC 824	0

[a] [^{3}H]ornithine-loaded cells were diluted 100-fold into 50 mM potassium PIPES (pH 6.0)–5 mM MgSO₄ in the presence or absence of 100 μM [^{14}C]arginine. The final protein concentrations varied between 0.5 and 0.9 mg/ml. Initial rates of arginine uptake were estimated (in duplicate) after 2.5 s of incubation. In none of the organisms was [^{3}H]ornithine efflux observed in the absence of external arginine. All organisms were grown in the presence of galactose and arginine, as described in the text.
[b] [^{14}C]arginine uptake concomitant with [^{3}H]ornithine efflux.
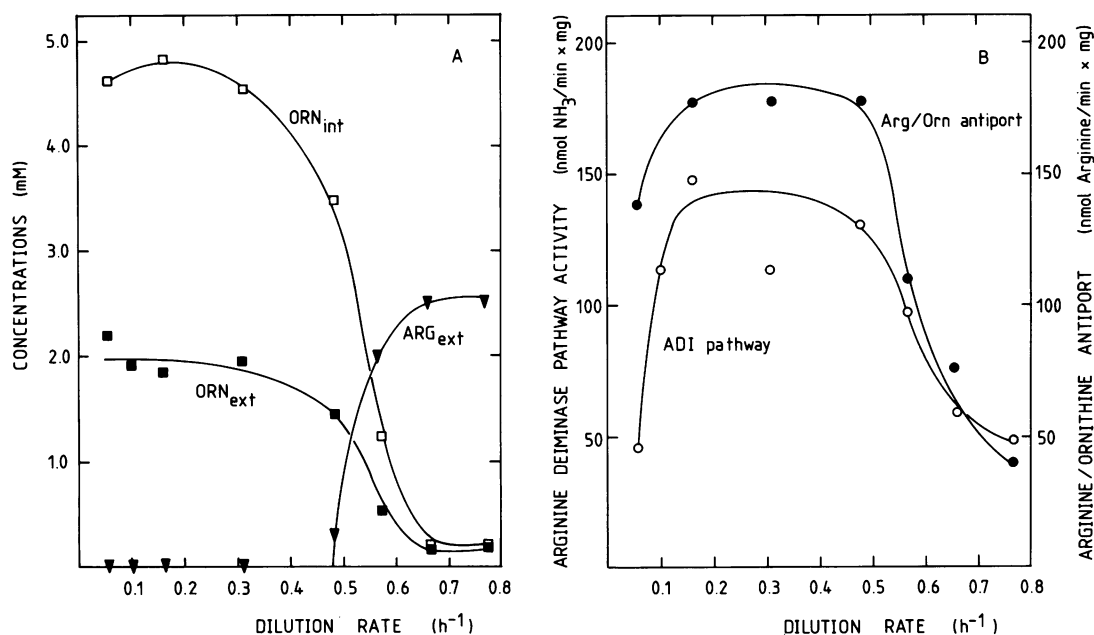[c] [^{14}C]arginine uptake without [^{3}H]ornithine efflux.

FIG. 5. Effect of dilution on the internal and external arginine and ornithine concentrations (A) and the arginine-ornithine exchange and ADI pathway activity (B) of chemostat-grown cells of *S. lactis* ML3. For the determination of the ADI pathway activity (O), the cells were suspended to a final protein concentration of 0.6 to 0.8 mg/ml into 5 mM potassium PIPES (pH 6.7)–50 mM KCl–5 mM MgSO$_4$. Arginine was added to a final concentration of 2 mM. Pathway activity was determined under steady-state conditions, i.e., after the rapid initial alkalinization of the medium (Fig. 3). For the determination of the arginine-ornithine exchange activity (●), ornithine-loaded cells were diluted 50-fold into 50 mM potassium PIPES (pH 6.0)–5 mM MgSO$_4$ containing 100 μM [^{14}C]arginine. The final protein concentrations varied between 0.6 and 0.9 mg/ml. Arginine-ornithine exchange rates were determined (in triplicate) from the amount of [^{14}C]arginine taken up after 2.5 s. The arginine-ornithine exchange and ADI pathway activity were both measured at 20°C. ext, External; int, internal.

to measure accurately. The arginine-ornithine exchange and the ADI pathway activity (both measured at 20°C) were highest at dilution rates between 0.15 to 0.5 h^{-1}. At higher dilution rates, these activities decreased in parallel (Fig. 5B) concomitant with the appearance of glucose in the culture fluid (data not shown). The arginine-ornithine exchange rates, which were estimated from the uptake of arginine after 2 to 3 s, are most probably underestimates owing to the nonlinearity of transport during this time interval (Fig. 1 and 2). Surprisingly, at a dilution rate of 0.055 h^{-1} the ADI pathway activity was also reduced. Glucose was not detectable in the medium under these conditions.

The regulation of enzyme synthesis of the ADI pathway and the arginine/ornithine antiporter was also studied in cells grown on complex broth (3) containing various supplements (Table 3). Low activities were found in cells grown on glucose or galactose without further additions. Pathway and antiporter activity were stimulated three- to fivefold in the presence of (25 mM) arginine (Table 3). The activity of the ADI pathway and arginine-ornithine exchange in cells grown in the presence of glucose was always lower than in cells grown with galactose. The weak repressing effect of glucose was largely abolished by cyclic AMP (Table 3).

Arginine-ornithine exchange in other bacteria. Experiments similar to that shown in Fig. 1 were performed in other bacteria to measure [^{14}C]arginine uptake and arginine-stimulated [^{3}H]ornithine efflux (Fig. 4). The organisms were grown in complex medium in the presence of galactose and arginine. The exchange rates were determined from the uptake of [^{14}C]arginine after 2 s. Arginine-ornithine exchange activity was present in *S. faecalis* DS5 (group D) and some viridans streptococci (*S. sanguis* 12 and *S. milleri* RH1 type 2). Efflux of [^{3}H]ornithine was observed only concom-

itant with uptake of [^{14}C]arginine in these bacteria. In contrast, high rates of arginine uptake without ornithine efflux occurred in *P. aeruginosa* LMD80.53.

DISCUSSION

This paper describes some physiological properties of a novel transport system which catalyzes the stoichiometric exchange of arginine for ornithine (3a). The driving force for arginine uptake is supplied by the ornithine and arginine gradients, which are replenished continuously by arginine metabolism. Consequently, the net gain of metabolic energy by the ADI pathway is one ATP per arginine metabolized, since no metabolic energy is spent for the uptake of arginine or the excretion of ornithine. The efficiency of the antiport system, as inferred from the V_{max}/K_t ratio, is extremely high in comparison to the efficiency of other secondary transport systems. Although it is difficult to make exact estimates, arginine uptake rates of 0.5 to 1.0 μmol/min × (mg of protein) were calculated for intact cells. The affinity constant (K_t) for arginine uptake is approximately 1 μM (Table 2).

Some of the properties of this antiport system and its role in the ADI pathway deserve further attention. Heterologous exchange between arginine and ornithine is electroneutral. The stimulation of the unidirectional rate of arginine uptake after 60 s of arginine metabolism by valinomycin plus nigericin (Fig. 2) is most probably due to the stimulation of arginine metabolism by the increased ATP consumption, which in turn alters the steady-state levels of citrulline and ornithine. In the presence of valinomycin plus nigericin, the citrulline pool is reduced slightly, but more importantly, the ornithine pool is increased about threefold (Table 1). The dependence of arginine uptake on the internal ornithine

concentration has been demonstrated in membrane vesicles of S. lactis ML3 (3a). The apparent K_t(exit) for ornithine was estimated to be 0.1 mM. This K_t value is lower than the lowest concentration of ornithine found in intact cells metabolizing arginine (Table 1). It should be emphasized that besides ornithine, the small but significant concentration of intracellular arginine may also influence net antiport activity by competition of arginine with ornithine for the exchange reaction. Homologous exchange of arginine (and also ornithine) has been observed in membrane vesicles of S. lactis (3a). Depending on the relative K_t values and the intracellular concentrations of arginine and ornithine, the transport system catalyzes either arginine-ornithine or arginine-arginine exchange. Ornithine-ornithine exchange will be of minor importance, since the K_t(entry) for ornithine is much higher than that for arginine (3a), whereas initially, the extracellular ornithine concentration is much lower than the arginine concentration. The net rate of arginine uptake in cells metabolizing arginine could therefore be modulated by changes in intracellular ornithine concentrations in the millimolar range. Finally, under some conditions exchange between arginine and lysine (when present, see Results) can also influence net arginine uptake (3a).

No exchange between ornithine (or arginine) and citrulline was found in membrane vesicles or in intact cells. This enables the cells to maintain the high intracellular concentrations of citrulline required to drive the thermodynamically unfavorable reaction, catalyzed by ornithine carbamoyltransferase, toward ornithine and carbamoylphosphate (20, 23).

The ADI pathway is regulated indirectly by the proton motive force, which influences the consumption and consequently the levels of (adenine) nucleotides as indicated by the following observations. At first, uncouplers and ionophores, which stimulate the F_0F_1-ATPase by abolishing the back-pressure effect of the proton motive force on proton translocation, stimulate the activity of the ADI pathway. The intracellular levels of ATP are decreased in these cells. The inhibitory effect of valinomycin alone could be related to the increase in the intracellular pH which in turn inhibits the ATPase (13). Second, DCCD, a specific inhibitor of the F_0F_1-ATPase, inhibits the ADI pathway. Third, the initial phase of arginine metabolism, i.e., when the intracellular ATP levels are still low, is always rapid independent of the presence of ionophores or DCCD. Fourth, increasing pH inhibits both F_0F_1-ATPase and ADI pathway activity. Measurement of ATP hydrolysis by membrane vesicles of S. lactis shows a sharp decline in ATPase activity upon an increase of the pH from 7.2 to 8.0 [unpublished data].) Based on a diminished rate of proton extrusion and the pH dependence of the F_0F_1-ATPase in S. faecalis, it has also been suggested that ATPase activity is highly decreased at alkaline pH values (5, 6). It should be noted that, in general, ADI is also inhibited at alkaline pH values (28).

The regulation of the ADI pathway by ATP (and possible other [adenine] nucleotides) could be exerted on carbamate kinase, for which ADP is a substrate and ATP a product, but possibly also on ornithine carbamoyltransferase. In Pseudomonas fluorescens, ornithine carbamoyltransferase is inhibited by pyrimidine and purine triphosphonucleotides which increase the threshold substrate concentration required to activate the enzyme, whereas nucleotide monophosphates restore the normal kinetic behavior (2, 25). Whether ornithine carbamoyltransferase in S. lactis is regulated allosterically by (adenine) nucleotides is unknown at present. Although the intracellular pools of adenine nucleotides (ex-

cept for ATP) have not been measured under conditions in which the activity of the ADI pathway is modulated by ionophores, the increase in ADI pathway activity by nigericin (with or without valinomycin) is consistent with the decrease in ATP concentration. A decrease in ATP concentration will stimulate the activity of carbamate kinase and (possibly) ornithine carbamoyltransferase, and depending on which enzyme(s) control(s) the pathway flux, this may stimulate the pathway activity.

The inhibition of ADI pathway activity by valinomycin plus nigericin below pH 6 is most probably due to the acidic cytoplasm under these conditions. Arginine-ornithine exchange (Table 2), ADI (28), and F_0F_1-ATPase (and perhaps other pathway enzymes) are inactivated under these conditions. The ADI pathway of S. lactis, however, appears to be much more acid tolerant than the Embden-Meyerhof pathway (9), as had already been noted (indirectly) for other streptococci (10). In agreement with the different (internal) pH dependencies of these pathways are the high ATP pools obtained by arginine metabolism at acid pH values, at which the ATP pools in glycolyzing cells have already fallen to zero (14).

The activity of arginine-ornithine exchange and the ADI pathway are also (long-term) regulated at the level of enzyme synthesis. Like the regulation of ADI and ornithine carbamoyltransferase (1), arginine-ornithine exchange activity is highest in cells grown in the presence of arginine and in the absence of glucose (Fig. 5; Table 3). The weak repressing effect of glucose is antagonized by the addition of cyclic AMP to the growth medium, suggesting that glucose acts by catabolite repression.

Besides its role in the ADI pathway, the arginine/ornithine antiporter may also play a role in other pathways of arginine degradation. For instance, the hydrolytic cleavage of arginine to ornithine and urea has been demonstrated in various organisms (2). The fate of ornithine and urea may differ among organisms since in some bacteria ornithine is not metabolized further (2).

Some lactic acid bacteria can use agmatine as an energy source by metabolizing it in the agmatine deiminase pathway (21). In analogy to arginine/ornithine antiport as the initial step of the ADI pathway, agmatine uptake may occur in exchange for putrescine.

ACKNOWLEDGMENTS

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