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Activities of Arginine Dihydrolase and Phosphatase in *Streptococcus faecalis* and *Streptococcus faecium*

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Strains of *Streptococcus faecalis* and *S. faecium* are known to produce ammonia from arginine, but only *S. faecalis* couples the adenosine triphosphate (ATP) produced through the arginine dihydrolase pathway to growth processes. The specific activities of the arginine dihydrolase enzymes were found to be much lower in *S. faecium* (0.01 to 0.10) than in *S. faecalis* (0.24 to 1.60). Phosphatase activities in both strains were similar (up to 0.11), but equaled or exceeded the activities of the arginine dihydrolase enzymes in *S. faecium*. The failure of *S. faecium* to show increased growth in arginine media is explained on the basis of low activities of the arginine dihydrolase enzymes coupled with sufficient phosphatase activity to negate any benefit from ATP formed.

The arginine dihydrolase pathway catalyzes the hydrolysis of arginine to ornithine, carbon dioxide, and ammonia with the formation of 1 mole of adenosine triphosphate (ATP) per mole of arginine utilized (14, 17, 19). The pathway consists of three enzymes (12, 17): arginine deiminase (EC 3.5.3.6), ornithine transcarbamylase (EC 2.1.3.3.), and carbamate kinase (EC 2.7.2.2). Strains of *Streptococcus faecalis* hydrolyze arginine by this route, with the ATP formed contributing to increased growth, as shown by the determination of molar growth yields (2). The closely related species *S. faecium* produces ammonia from arginine, but it does not show increased cell crops in media containing added arginine (5, 6). This observation has seemed anomalous since Jones, Spector, and Lipmann (13) used a culture of *S. faecium* (at that time designated as *S. faecalis* R) in their demonstration of carbamate kinase activity. Thus, the failure of arginine to stimulate growth of *S. faecium* has been puzzling. Deibel (5) has suggested that *S. faecium* either may have a less efficient energy-coupling mechanism or that a different means of degrading citrulline exists in this species. An evaluation of the utilization of arginine and the specific activities of both the arginine dihydrolase enzymes and phosphatase in strains of *S. faecium* and *S. faecalis* is reported in this paper.

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MATERIALS AND METHODS

Cultures. The organisms used in this study were *S. faecalis* var. *liquefaciens* R64A (20), *S. faecalis* var. *liquefaciens*, ATCC 13398, *S. faecalis*, ATCC 11420, *S. faecium* A-2, *S. faecium* A-11, and *S. faecium* A-36. All strains of *S. faecium* were obtained from J. O. Mundt, Department of Microbiology, University of Tennessee. Stock cultures were stored at −18 C in Brain Heart Infusion (BHI) broth (Difco) containing 16% glycerol.

Media and cultivation. A Casamino Acids-yeast extract medium was used for growth experiments. The medium, containing 1.0% Casamino Acids (Difco), 0.1% yeast extract (Difco), 0.65% K_2HPO_4, 0.51% KH_2PO_4, 0.72% glucose, and 0.21% L-arginine hydrochloride, was sterilized as four separate solutions (Casamino Acids and yeast extract, glucose, arginine, and mono- and dipotassium phosphates) and combined aseptically. The finished medium contained about 40 μmoles of glucose and about 12 μmoles of arginine hydrochloride per ml. Growth experiments were performed both with and without the addition of arginine. Tubes containing 50 ml of Casamino Acids-yeast extract broth were inoculated with 1.0 ml of a washed cell suspension (absorbance, 0.1). Incubation was at 37 C in stationary culture. Samples were withdrawn periodically and assayed for cell density, glucose, and arginine.

Cells for enzyme preparations were grown in BHI broth supplemented with 40 μmoles of glucose and either 10 or 50 μmoles of L-arginine hydrochloride per ml.

Production of ammonia. A 50-ml amount of tryptone-yeast extract medium (7), supplemented with 50 μmoles of arginine per ml, was inoculated with

washed cells as described above. Cultures were incubated at 37 C and portions were analyzed for ammonia after 24 hr.

Cell-free extracts. A 2-liter amount of the supplemented BHI broth was inoculated with 500 ml of a 12-hr culture grown in the same medium. After 6 hr at 37 C the cells were removed by centrifugation, washed once in water, and resuspended to 15 ml. Dry weight of the cell mass was determined by optical density measurements at 500 nm. The cells were broken either by treatment with an ultrasonic probe (Branson Sonifier, Branson Instruments, Inc., Stamford, Conn.) at 20,000 cycles per sec for 30 min or with a French pressure cell (Yellow Springs Instrument Company, Inc., Yellow Springs, Ohio) at 10,000 psi. Care was taken to keep the suspension cold during both operations. Cellular debris was removed by centrifugation in the cold at 15,000 × *g*. The extracts were stored at −18 C and discarded after 10 days.

Enzyme assays. Arginase (EC 3.5.3.1) was determined by measuring the amount of urea produced (10). A 1-ml amount of suitably diluted cell-free extract, which had been activated by incubation with manganous ions and 0.5 ml of 0.85 M arginine (*p*H 9.5), was mixed and incubated at 25 C for 10 min; the reaction was stopped by the addition of 0.2 ml of 87% acetic acid. Urea was determined by the method of Engel and Engel (8).

Urease (EC 3.5.1.5) activity was measured by determining the amount of ammonia released from urea. A 1-ml amount of cell-free extract, 0.5 ml of 3% urea, and 0.5 ml of 9.6% phosphate buffer were mixed and incubated at 30 C for 20 min; 1.0 ml of 1 N HCl was added to stop the reaction. Ammonia was determined by a modification of the method of Ternberg and Hershey (21). To 1.0 ml of diluted ammonia solution were added 1.0 ml of 1 N NaOH, 1.0 ml of alkaline-hypochlorite reagent (2.5 g of NaOH and 5.0 ml of commercial hypochlorite diluted to 100 ml), and 1.0 ml of phenol reagent (5.0 g of phenol and 25 mg of sodium nitroprusside in 100 ml of water). The reagents were mixed, diluted to 10 ml with water, and incubated at 37 C for 30 min. The optical density was determined at 590 nm.

Arginine deiminase activity was estimated by measuring the amount of citrulline released (17). A 1-ml amount of 0.2 M phosphate buffer (*p*H 6.5), 0.4 ml of 0.1 M L-arginine (*p*H 6.5), 1.4 ml of water, and 0.2 ml of cell-free extract were mixed and incubated at 37 C for 30 min; the reaction was stopped by the addition of 0.2 ml of 70% perchloric acid. Citrulline was determined by its reaction with diacetyl monoxime (18).

Ornithine transcarbamylase activity was measured by determining the amount of ornithine produced in the reaction (17). A 1-ml amount of acetate buffer (*p*H 5.8), 0.5 ml of 0.01 M adenosine 5′-monophosphate, 0.1 ml 0.1 M $MgCl_2$, 0.7 ml of 0.1 M phosphate buffer (*p*H 5.8), 0.2 ml of 0.2 M DL-citrulline, and 0.5 ml of cell-free extract were mixed and incubated at 37 C for 30 min; the reaction was stopped by the addition of 0.2 ml of 2 M sulfuric acid. Ornithine was determined colorimetrically by a modification of the Litwack procedure (4).

Carbamate kinase activity was estimated by measuring the amount of ATP released in the reaction (11). To 0.3 ml of cell-free extract was added 0.2 ml of an assay mixture containing 1 part of 1 M acetate buffer (*p*H 5.5), 0.4 part of 0.1 M adenosine diphosphate, 0.4 part of 0.1 M dilithium carbamyl phosphate, and 0.1 part of 0.4 M $MgCl_2$. The reagents were mixed, incubated at 37 C for 10 min, and cooled to room temperature; the remaining carbamyl phosphate was hydrolyzed by the addition of 1 ml of 1 N KOH. Since ATP was not hydrolyzed under these conditions, the amount of ATP produced was determined as the difference in the phosphate concentrations of the samples and controls containing no enzyme.

Phosphate determinations were made by a modification of the procedure of Allen (1). Water (3 ml), 1.0 ml of phosphate solution, 0.4 ml of amidol reagent (1% amidol in 20% sodium bisulfite), 0.4 ml of 70% perchloric acid, and 0.2 ml of 8.3% ammonium molybdate were mixed and allowed to stand for 30 min; the optical density was determined at 600 nm.

Phosphatase activity was assayed with three substrates, *p*-nitrophenyl phosphate, carbamyl phosphate, and ATP. A citrate buffer was employed in the range from *p*H 3.0 to 6.0, tris(hydroxymethyl)aminomethane-maleate from *p*H 6.0 to 9.0, and glycine-NaOH above *p*H 9.0.

The activity of a nonspecific phosphatase was determined by its action on *p*-nitrophenyl phosphate. The *p*-nitrophenol formed was determined colorimetrically at 430 nm. A 1-ml amount of cell-free extract, 1.5 ml of buffer, and 1.5 ml of substrate (0.4%) were mixed and incubated at 37 C for 30 min; reaction was stopped by the addition of 1.0 ml of 10% NaOH.

Phosphatase activity was also measured by determining the amount of phosphate released from carbamyl phosphate. To 0.1 ml of 0.1 M dilithium carbamyl phosphate were added 0.2 ml of buffer and 0.7 ml of cell extract. The mixture was incubated at 37 C for 10 min, and the amount of phosphate was determined as above.

Adenosine triphosphatase activity was determined by a modification of the procedure of Lowry et al. (16). To 0.1 ml of 0.005 M ATP were added 0.3 ml of buffer, 0.5 ml of water, and 0.1 ml of enzyme. The mixture was incubated at 37 C for 30 min, and the amount of phosphate released was determined as above.

Chemical analyses. Glucose was determined with anthrone reagent (9), and arginine was measured by a modified Sakaguchi procedure (15). Protein was determined by measuring the absorption at 210 nm and by calculating the protein concentration using an extinction coefficient ($E_{1cm}^{1\%}$) of 205 (22).

Physiological tests. All organisms were classified with the scheme of Deibel (6). Physiological tests were conducted according to the procedures of Deibel, Lake, and Niven (7).

RESULTS AND DISCUSSION

The results of the physiological tests showed that all organisms employed were typical of their respective species according to established criteria

TABLE 1. *Growth yields of S. faecalis and S. faecium in arginine media*

Organism	Medium	Glucose used	Arginine used	Cell yield[a]
		μmoles/ml	*μmoles/ml*	*μg/ml*
S. faecalis strains				
R64A	Basal	26.6	2.4	580
	Arginine	29.9	12.4	870
13398	Basal	25.9	2.5	660
	Arginine	27.5	13.0	800
11420	Basal	22.5	2.6	490
	Arginine	26.7	13.5	800
S. faecium strains				
A-2	Basal	10.1	0.9	120
	Arginine	10.4	1.9	130
A-11	Basal	10.7	0.9	120
	Arginine	8.5	1.3	120
A-36	Basal	9.1	0.8	120
	Arginine	13.0	1.6	140

[a] Cell yield after 12 hr of growth at 37 C (early stationary phase).

TABLE 2. *Specific activities of enzymes of the arginine dihydrolase system in S. faecalis and S. faecium*[a]

Organism	Arginine concentration in growth medium[b]	Arginine deiminase	Ornithine transcarbamylase	Carbamate kinase
	μmoles/ml			
S. faecalis strains				
R64A	10	0.68	0.24	0.31
	50	1.43	0.93	1.20
13398	10	0.81	0.27	0.49
	50	1.60	1.07	1.29
11420	10	0.83	0.28	0.33
	50	1.52	0.90	1.15
S. faecium strains				
A-2	10	0.01	0.003	0.02
	50	0.11	0.03	0.05
A-11	10	0.01	0.003	0.01
	50	0.09	0.04	0.03
A-36	10	0.02	0.003	0.01
	50	0.08	0.03	0.04

[a] Specific activities expressed as μmole of product released per milligram of protein.

[b] Cells grown in 10 μmoles of arginine per ml were broken by sonic treatment, whereas those grown in 50 μmoles of arginine per ml were broken with the French pressure cell.

(6). Strains of *S. faecalis* had increased growth yields in media containing added arginine, whereas strains of *S. faecium* did not (Table 1). The high growth yields observed in cultures of *S. faecalis* grown in glucose media have been reported previously (3).

Qualitative tests showed that arginase and urease were absent in both species; however, the enzymes of the arginine dihydrolase system could be detected in all cultures grown in media containing arginine. Consequently, the specific activities of the several enzymes involved were determined (Table 2). The cells from cultures containing 10 μmoles of arginine per ml were broken by sonic treatment, whereas those from cultures containing 50 μmoles of arginine per ml were broken with the French pressure cell. The higher levels of activity found in cultures grown in the greater concentration of arginine were due, in part, to more efficient cell breakage. Although significant levels of enzyme activity were found in the strains of *S. faecium*, the specific activities were far greater in the strains of *S. faecalis*. Even though both species had significant levels of phosphatase activity, the phosphatase activities in *S. faecium* exceeded the activities of the arginine

TABLE 3. *Phosphatase activity in S. faecalis and S. faecium*[a]

Organism	Arginine concentration in growth medium[b]	Phosphatase substrates tested		
		Carbamyl phosphate	*p*-Nitrophenyl phosphate	ATP
	μmoles/ml			
S. faecalis strains				
R64A	10	0.05	0.065	0.06
	50	0.12	0.10	0.10
13398	10	0.04	0.05	0.06
	50	0.09	0.12	0.10
11420	10	0.045	0.05	0.06
	50	0.10	0.10	0.11
S. faecium strains				
A-2	10	0.035	0.045	0.04
	50	0.09	0.09	0.10
A-11	10	0.04	0.035	0.04
	50	0.10	0.11	0.11
A-36	10	0.04	0.04	0.04
	50	0.08	0.10	0.10

[a] Specific activities expressed as μmole of product released per milligram of protein.

[b] Cells grown in 10 μmoles of arginine per ml were broken by sonic treatment, whereas cells grown in 50 μmoles of arginine per ml were broken with the French pressure cell.

dihydrolase enzymes (Table 3). Although *S. faecium* generated ATP from arginine, the phosphatase activity was great enough to overbalance this contribution to biosynthetic needs. It should be noted that since the phosphatases assayed are all phosphomonoesterases and had similar activities on all three substrates, it is possible that rather than three separate enzymes only a single nonspecific phosphatase was involved.

Diebel (5) reported that the ammonia released by cultures of *S. faecium* equaled that of *S. faecalis* after 20 to 24 hr of growth and that the production of ammonia by *S. faecium* began after a delay of approximately 12 hr. The cultures of *S. faecium* used in this study did not exhibit this pattern, as demonstrated by measurement of arginine utilization (Fig. 1). Although the utilization of arginine by *S. faecium* did not begin immediately after inoculation, it ceased after 8 hr with more than 70% of the arginine remaining in the medium. *S. faecalis*, by comparison, completely depleted the arginine after about 12 hr of incubation.

The *S. faecium* strains did not produce large quantities of ammonia, as shown by measuring ammonia production in a tryptone-yeast extract medium containing 50 μmoles of arginine per ml. After 24 hr of incubation, *S. faecium* produced approximately 9 μmoles of ammonia per ml, whereas *S. faecalis* produced about 64 μmoles.

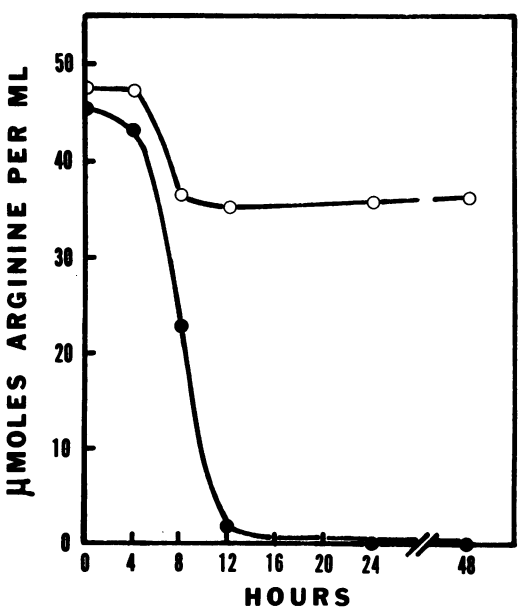

FIG. 1. *Utilization of arginine by S. faecalis* (●) *and S. faecium* (○). *Casamino Acids medium was inoculated with 1.0 ml of washed cells (absorbance, 0.1). Incubation was at 37 C.*

These data demonstrate that although both *S. faecium* and *S. faecalis* possess the enzymes of the arginine dihydrolase system, *S. faecium* is unable to produce sufficient ATP to bring about measurable increases in growth. Furthermore, the phosphatase activity in *S. faecium* is great enough to hydrolyze the ATP formed through the dihydrolase pathway, thus negating any contribution to the overall economy of the cell.

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