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Degradation of arginine by *Slackia exigua* ATCC 700122 and *Cryptobacterium curtum* ATCC 700683

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Uematsu H, Sato N, Djais A, Hoshino E. Degradation of arginine by Slackia exigua *ATCC 700122 and* Cryptobacterium curtum *ATCC 700683.*
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Slackia exigua ATCC 700122^T and *Cryptobacterium curtum* ATCC 700683^T were our isolates from infected root canal and human periodontal pocket, respectively; they are asaccharolytic anaerobic gram-positive rods, which are predominant in the oral cavity. They utilize arginine, so our aim was to investigate the pathway of arginine degradation. Metabolic end products were determined with high-performance liquid chromatography. The related enzymatic activities in cell-free extract were also assayed. Both *S. exigua* and *C. curtum* degraded arginine and produced substantial amounts of citrulline, ornithine and ammonia. Arginine and citrulline supported the growth of both strains. As the related enzymatic activities, arginine deiminase, ornithine carbamoyltransferase and carbamate kinase activities were detected in the cell-free extract of *S. exigua* and *C. curtum*. Arginase and urease activities were not detected in either organism. These results suggest that arginine was metabolized by the arginine deiminase pathway. Both *S. exigua* and *C. curtum* degrade arginine via the arginine deiminase pathway.

Key words: arginine; arginine deiminase pathway; *Cryptobacterium curtum*; *Slackia exigua*

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Slackia exigua [formerly *Eubacterium exiguum*, reclassified by Wade, (25)] has been isolated from infected root canals (15), and also detected from infected root canals (6) or periodontal pockets (4) by polymerase chain reaction (PCR). Immunoglobulin G (IgG) titers against this bacterial species are rather high in patients with periodontitis (19). *Cryptobacterium curtum* has also been isolated from human periodontal pockets and infected root canals (14). It is also frequently detected by PCR from periodontal pockets (9). Both *S. exigua* and *C. curtum* were found to be asaccharolytic anaerobic gram-positive rods (AAGPRs; 14, 15).

AAGPR has been named as one of the predominant groups of oral bacteria (13, 14, 15, 22), but they are still unfamiliar because they are difficult to culture. They do not utilize carbohydrates, so amino acids may be important metabolic substrates for growth, especially arginine and lysine (23, 24). Arginine may be provided by the enzymatic degradation of peptides, especially in inflamed lesions where trypsin-like enzymes produce peptides containing arginine at the C-terminal.

Therefore, the aim of this study was to investigate the degradation of arginine, using high-performance liquid chromatography (HPLC), and to determine the metabolic pathway.

Material and methods

Chemicals

Amino acids (alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine) and carbamoyl phosphate were purchased from Wako Pure Chemicals (Osaka, Japan). Citrulline and ornithine were from Sigma Chemical Co. (St Louis, MO). Nicotinamide adenine

dinucleotide (NAD), yeast hexokinase and glucose-6-phosphate dehydrogenase were obtained from Roche Diagnostics (Basel, Switzerland).

Bacteria

S. exigua ATCC 700122^T and *C. curtum* ATCC 700683^T were from our original stocks. They were maintained on brain–heart infusion (BHI; Difco Laboratories, Detroit, MI), –blood (sheep) agar plates (8) in an anaerobic glove box (Model AZ-Hard; Hirasawa, Tokyo, Japan) in an atmosphere of 10% H_2 and 10% CO_2 in nitrogen.

The growth of *C. curtum* was monitored by measuring the optical densities of cultures with a spectrophotometer (U-3200; Hitachi, Tokyo, Japan) at 660 nm for 7 days in BHI broth media (8), with or without the addition of arginine or citrulline or another amino acid at concentrations of 0, 10, 20, 30, 40 and 50 mMol. The bacterial growth of *S. exigua* was also monitored at 660 nm in tryptone broth, comprising tryptone 2%, yeast extract 1% and NaCl 0.5%, because the growth of *S. exigua* in BHI liquid media was rather poor ($OD_{660} = 0.021$).

Preparation of washed microorganisms

The *S. exigua* and *C. curtum*, grown at a late log-phase in the presence of 30 mM arginine, were harvested by centrifugation (8000 **g**, for 10 min at 4°C) using tightly capped centrifuge tubes, washed three times with 40 mMol potassium phosphate buffer (pH 7.0) and resuspended in the same buffer. All the procedures were carried out under strict anaerobic conditions in the anaerobic glove box. The tightness of the centrifuge tubes was carefully checked by use of methylviologen as an indicator before and after use.

Analyses of metabolic products

The reaction mixture contained in 1 ml: 0.5 ml washed cell suspension at various cell concentrations (indicated elsewhere), 100 μmol potassium phosphate (pH 7.0), 10 μmol $MgCl_2$ and 10 μmol arginine, citrulline or one of the other 19 amino acids. The reaction was carried out in the anaerobic glove box at 37°C for 30 min and was stopped by the addition of 0.1 ml of 40% (w/v) metaphosphoric acid or 60% perchloric acid.

Ion exclusion HPLC with an electroconductivity detector (CDD-6A L-3720, Hitachi, Tokyo, Japan) was used to identify organic acids (nC_1–nC_6, iC_4–iC_6 and α-keto-C_3–C_6) separated on a Shim-pack SCR-102H column (8 mm internal diameter by 300 mm; Shimadzu, Kyoto, Japan), after passing through a guard column of SCR-102H (6 mm internal diameter by 50 mm; Shimadzu), and eluted with 5 mmol *p*-toluenesulphonic aqueous solution as solvent at a flow rate of 0.8 ml/min at 40°C. To detect, the acidic eluent was neutralized with 20 mMol bis(2-hydroxyethyl)-iminotris(hydroxymethyl)methane aqueous solution containing 5 mMol *p*-toluenesulphonic acid and 100 μMol EDTA as post column procedures. The detection limit was 0.05 mMol.

HPLC with a fluorescence spectrophotometer (F-1000; Hitachi; emission at 440 nm and excitation at 360 nm) was used to identify *o*-phthalaldehyde (OPA) derivatives of arginine, citrulline, ornithine and other amino acids (7, 16) after separation on a Hitachi 2619-F column (4 mm internal diameter by 150 mm; Hitachi) at 60°C. The detection limit was 0.05 mMol. Ammonia was determined enzymatically using glutamate dehydrogenase (2).

Enzymatic assays

For the preparation of cell-free extract, washed organisms were disintegrated with a sonic oscillator (Handy Sonic model UR-20P, Tomy Seiko, Tokyo, Japan) at power setting 10, for 10 min, at 4°C. The cell debris was removed by centrifugation (10,000 **g**, for 10 min at 4°C). All procedures were carried out under anaerobic conditions. Protein concentration was determined using either the Biuret method (10) or the Lowry method (11).

Activities of arginine deiminase (EC 3.5.3.6), ornithine carbamoyltransferase (EC 2.1.3.3) and carbamate kinase (EC 2.7.2.2) were assayed as described by Archibald (1), Stalon et al. (21) and Mercenier et al. (12), respectively. For arginase activity (EC 3.5.3.1), the rate of urea formation from arginine was determined as described by Schmike (17). Urease (EC 3.5.1.5) was assayed as described by Schlegel and Kaltwasser (18). All enzymatic reactions were carried out in the anaerobic glove box.

Results

Bacterial growth

Bacterial growth of *S. exigua* and *C. curtum* was enhanced by only arginine or citrulline among the 21 amino acids tested (Fig. 1). The doubling time of *S. exigua* in tryptone media enriched with 30 mMol arginine was 7.8 h, and that of *C. curtum* in BHI media enriched with 30 mMol arginine was 12.0 h. The growing *S. exigua* consumed 28 mMol arginine and produced 17 mMol citrulline and 10 mMol ornithine after 7 days ; *C. curtum* consumed 15 mMol arginine and produced 1 mMol citrulline and 13 mMol ornithine after 7 days. No other detectable end products from arginine were found by HPLC.

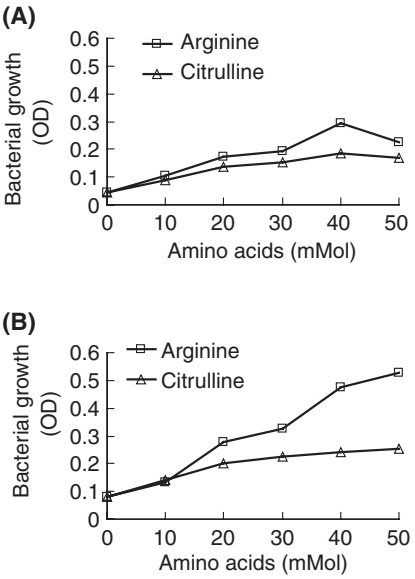

Fig. 1. Bacterial growth (OD) of *Sackia exigua* (A) and *Cryptobacterium curtum* (B) after 7 days with the addition of arginine or citrulline in tryptone broth or BHI broth respectively.

Arginine and citrulline metabolism in washed microorganisms and enzymatic activities

Washed *S. exigua* metabolized arginine mainly to citrulline, and small amounts of ornithine were also produced. They also metabolized citrulline to ornithine (Table 1) and the other citrulline was not consumed. No other organic acids and amino acids were detected.

Washed microorganisms of *C. curtum* metabolized arginine to small amounts of citrulline and large amounts of ornithine. They also metabolized citrulline to ornithine (Table 1) and the other citrulline was not consumed. No other organic acids and amino acids were detected.

Activities of arginine deiminase, ornithine carbamoyltranseferase and carbamate kinase were found in the cell-free extracts of both organisms (Table 2). Arginase may be another possible method for enzymatic conversion of arginine to ornithine. Arginase activity, which converts arginine to

Table 1. Arginine and citrulline utilization[1] by washed microorganisms of *Slackia exigua* and *Cryptobacterium curtum*

	S. exigua[2]	*C. curtum*[2]	*S. exigua*[2]	*C. curtum*[2]
	From Arginine (mMol)		From Citrulline (mMol)	
	(10.0 ± 0.0)	(8.7 ± 0.2)	(1.1 ± 0.2)	(4.1 ± 0.5)
Products(mMol)				
Citrulline	8.2 ± 03[3]	1.1 ± 0.5		
Ornithine	1.4 ± 0.1	7.4 ± 0.8	1.2 ± 0.2	4.9 ± 0.7
Ammonia	9.4 ± 0.3	8.4 ± 0.4	1.5 ± 0.4	3.6 ± 0.1

[1]No metabolic end products were detected from the other 19 amino acids (see Material and methods).
[2]Cell concentration: 6.27 mg as protein/ml reaction mixture for *S. exigua* and 10.25 mg as protein/ml reaction mixture for *C. curtum*.
[3]Mean ± SD of three independent experiments.

Table 2. Enzymatic activities of *Slackia exigua* and *Cryptobacterium curtum*

	Specific activity[1] (μMol/min/mg protein)		
	Arginine deiminase	Ornithine carbamoyl transferase	Carbamate kinase
S. exigua	3.34 ± 0.31	26.5 ± 2.3	4.14 ± 0.16
C. curtum	0.245 ± 0.017	29.5 ± 4.5	1.40 ± 0.39

[1]Mean ± SD of three independent experiments.

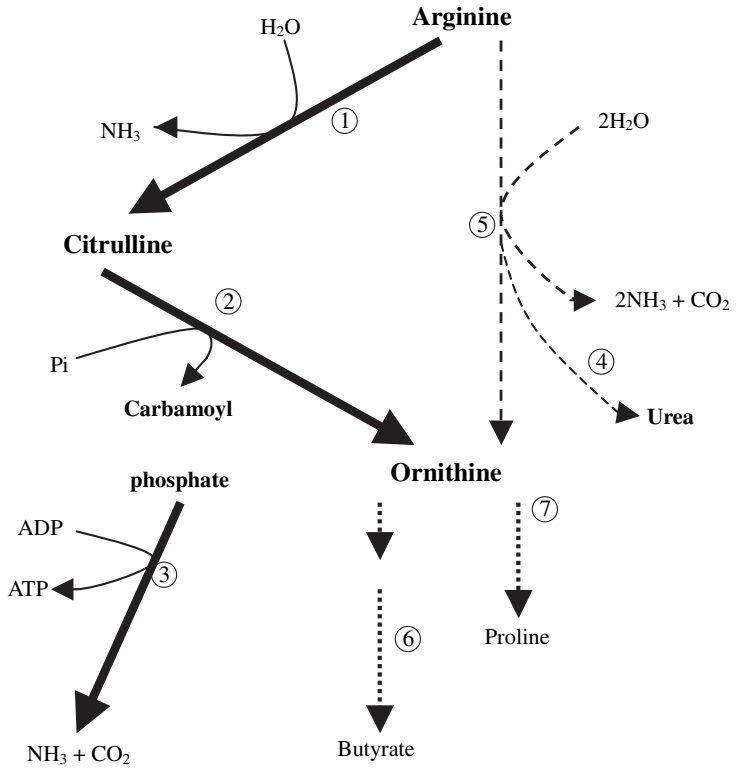

Fig. 2. Reported pathway of arginine degradation (3, 5, 23, 24). (1), arginine deiminase; (2), ornithine carbamoyltransferase; (3), carbamate kinase; (4), arginase; (5), enzymatic conversion of arginine to ornithine and ammonia. Arginine deaminase pathway: (1) + (2) + (3); in *Eubacterium nodatum* (23), *Eubacterium minutum* (24) and *Treponema denticola* (3) as well as in *S. exigua* and *C. curtum*. (5) + (6); in *Filifactor alocis* (24). Butyrate (6) is produced by *E. nodatum* (22) and *E. minutum* (24) and proline (7) is produced by *T. denticola*.

ornithine and urea, was not detected. Nor was any urease activity detected. These results suggested that arginine was degraded through the arginine deiminase pathway and that ATP was formed in this pathway. Arginine deiminase activity was higher in *S. exigua* than in *C. curtum*. Other enzyme activities involving the arginine deiminase pathway, i.e. ornithine carbamoyltranseferase and carbamate kinase, were of a similar order in both microorganisms.

Discussion

Arginine and/or oligopeptides containing arginine are products of trypsin-like proteinases and peptidases. Such enzymatic activities are often detected at the site of the inflamed connective tissue, such as periodontal and endodontic lesions, where asaccharolytic anaerobic gram-positive rods, such as *C. curtum*, *Eggerthella lenta*, *Eubacterium brachy*, *Eubacterium infirmum*, *Eubacterium minutum*, *Eubacterium nodatum*, *Eubacterium saphenum*, *Eubacterium sulci*, *Filifactor alocis*, *Mogibacterium timidum* and *S. exigua* are isolated as the predominant bacteria (13–15, 22). Thus, some asaccharolytic anaerobic gram-positive rods might live on arginine (23, 24). In fact, the growth of *S. exigua* and *C. curtum* was supported by arginine.

There are several reports on arginine metabolism in oral anaerobic bacteria. *E. nodatum* and *E. minutum* degrade arginine to butyrate through the arginine deiminase pathway, and *F. alocis* did it by an unnamed pathway, respectively (23, 24). The arginine deiminase pathway was also found in *S. exigua* and *C. curtum*.

Arginine deiminase, ornithine carbamoyltransferase and carbamate kinase constitute the deiminase pathway which catalyses the conversion of arginine into ornithine, ammonia and carbon dioxide with the formation of 1 mol ATP/mol arginine consumed (Fig. 2). The pathway is widely distributed among prokaryotic organisms (5). Arginine utilization by the arginine deiminase pathway is characterized by abundant ornithine excretion, indicating that only the guanidino group of arginine is used (5). Both *S. exigua* and *C. curtum* also produced ornithine as an end product. This result resembled that of *E. lenta* (20). However, *Treponema denticola* and some *Clostridium* spp. are able to dissimilate ornithine by either the action of an ornithine cyclase yielding proline or by a Stickland reaction producing 5-aminovalerate (3, 5). Some butyrate-producing AAGPRs also metabolize ornithine further to butyrate (23, 24). *Slackia exigua* and *C. curtum* did not produce butyrate or other products from ornithine, which might be one of the reasons for their poor growth (Fig. 2). *S. exigua* produced much citrulline in experiments with both culture and washed organisms. This might be a character of *S. exigua* and this bacterium could produce only small amounts of ATP, because the arginine deiminase pathway was stopped at citrulline.

The growth of both strains was not enhanced by any of the other 19 amino acids and washed organisms could not use each of the other 19 amino acids. Therefore, *S. exigua* and *C. curtum* could utilize limited types of substrate for their metabolic activity, which might also be the reason for their poor growth.

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