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Arginine Metabolism by *Spiroplasma citri*

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INTRODUCTION

The newly identified organism *Spiroplasma citri* (Saglio *et al.*, 1973), the leafhopper-transmitted agent of stubborn and little-leaf diseases of citrus (Markham *et al.*, 1974), exhibits many properties characteristic of fermentative mycoplasmas, including the ability to use glucose and fructose as energy substrates. It has also been reported that it does not hydrolyse arginine (Bové *et al.*, 1973; Saglio *et al.*, 1973, 1974). However, the pH fluctuations observed in spiroplasma cultures supplemented with arginine suggested that *S. citri* might be able to metabolize this amino acid.

METHODS

Culture. Spiroplasma citri, little-leaf strain (SP-A), was grown in a basal spiroplasma medium (BSM) containing: 2·1 % (w/v) PPLO broth (Difco), 0·5 % (w/v) yeast extract (Oxoid), 1 % (w/v) sodium chloride, 15 % (v/v) horse serum no. 6 (Wellcome Reagents Ltd, Beckenham, Kent, heated to 60 °C for 30 min before use), phenol red (10 μg ml^{-1}), 0·025 % thallous acetate; and supplemented as indicated with 0·1 % glucose, 0·1 % fructose, or 0·42 % L-arginine monohydrochloride. When necessary 1 % (w/v) agar was used to solidify this medium. The pH of the medium, normally about 7·6, was adjusted, when required, with 1 M-hydrochloric acid or 1 M-sodium hydroxide. Cultures were incubated at 32 °C.

Measurement of pH and assay of ammonia. Daily measurements of pH and ammonia concentration were made on samples (0·25 ml) from 10 ml cultures. The pH was measured with a Pye Ingold micro-electrode (Pye Unicam, Cambridge). Ammonia was assayed by the Nessler method (Vogel, 1961), following removal of the acid-insoluble material by adding an equal volume of 10 % (w/v) trichloroacetic acid (TCA), and 20-fold dilution in distilled water. Extinction was measured at 412 nm with reference samples prepared from uninoculated medium.

Electrophoresis. One-dimensional electrophoresis of the amino acids in the cultures was carried out by the flat-plate technique of Trim & Dickerson (1974) using Whatman 3MM paper and a buffer solution, pH 6.5, containing 10 % (v/v) pyridine and 0·4 % acetic acid. Samples (0·1 ml) of 2 ml cultures grown in BSM supplemented with combinations of sugars, 0·42 % (20 mM) L-arginine monohydrochloride and L-[U-^{14}C]arginine monohydrochloride (318 mCi mmol^{-1}, about 5 μCi ml^{-1}), were added to an equal volume of 10 % TCA, centrifuged, and 10 μl portions of the supernatant solution spotted at the anode end of the paper. A potential gradient of 33 V cm^{-1} was applied for 3 h. Spots were located by spraying with ninhydrin (0·2 % in acetone) and identified by comparison with amino acid standards and samples of uninoculated media run under the same conditions. Subsequently the amino acid spots on the electrophoresis papers were cut out, placed in scintillation vials with 5 ml of scintillation mixture [containing 4 g 2,5-diphenyloxazole (PPO) and 50 mg 1,4-di-2-

(5-phenyloxazolyl) benzene (POPOP) in 1 l toluene] and counted for 5 min in a liquid scintillation counter.

Measurement of growth. Growth of *S. citri* was assessed by colony counts made on 10 μl samples of serial 10-fold dilutions of the cultures. Plates were incubated for 7 days and counted after staining with Dienes' stain (Scriba, 1968).

RESULTS

Ammonia production

Spiroplasmas grew well in BSM supplemented with glucose and fructose: the acid produced caused a fall in pH from 7·6 to below 5·4 in 4 days without any detectable liberation of ammonia. If 20 mM-arginine was also added, up to 30 mM-ammonia was released, which completely neutralized the acid, producing a characteristic V-shaped pattern of pH fluctuation (Fig. 1). Supplementation of BSM with arginine alone resulted in the release of less than 6 mM-ammonia accompanied by a small rise in pH. Added urea was not metabolized.

Strains of *S. citri* from Morocco (R8-A2) and California (C189) produced pH changes similar to those shown in Fig. 1 and consistently gave rise to more ammonia when sugars were present in the medium.

Electrophoresis

The amino-acid pattern of cultures containing labelled arginine with excess unlabelled amino acid and sugars showed that, after 2 to 3 days' incubation, the concentration of arginine began to fall steadily accompanied by the appearance of increasing amounts of ornithine. It was not possible to determine visually if there was also an increase in citrulline concentration, because the acid-soluble fraction of BSM contained material with the same mobility as citrulline.

Radioactivity was only associated with the arginine, ornithine and citrulline spots. There was good correlation between the decrease in counts per minute (c.p.m.) in the arginine spots and the increase in c.p.m. in ornithine and citrulline spots, after allowing for the calculated loss in radioactivity due to the formation of carbamyl phosphate and the presence of 5 to 10 % of the initial radioactivity in the acid-insoluble fraction. In all experiments, after 8 days, at least 20 % of the total radioactivity was in the form of [^{14}C]citrulline indicating that the breakdown of arginine to ornithine was incomplete. Even when the incubation period was extended to 14 days, there was no decline in the concentration of [^{14}C]citrulline. In cultures supplemented with sugars and [^{14}C]arginine (5 μCi ml^{-1}) but no unlabelled amino acid, the degradation of arginine was much faster, complete breakdown taking only 48 h. However, up to 25 % of the total radioactivity still remained in the form of [^{14}C]citrulline. When the medium was supplemented with arginine alone, the organisms failed to use more than 20 % of the labelled amino acid, despite extended incubation, but still produced the same pattern of incomplete degradation.

Effect of pH on ammonia production

Arginine hydrolysis was not stimulated at any particular pH, but in media supplemented only with arginine it was slightly enhanced at initial values between pH 7·2 and pH 6·6, when up to 10 mM-ammonia was produced. Adjusting the starting pH of medium supplemented with sugars and arginine to pH 7 resulted in the release of nearly 32 mM-ammonia. Below this pH, progressively less ammonia was produced although the characteristic V-shaped pattern of pH fluctuation was maintained. At initial values below pH 5·5, the organism did not grow.

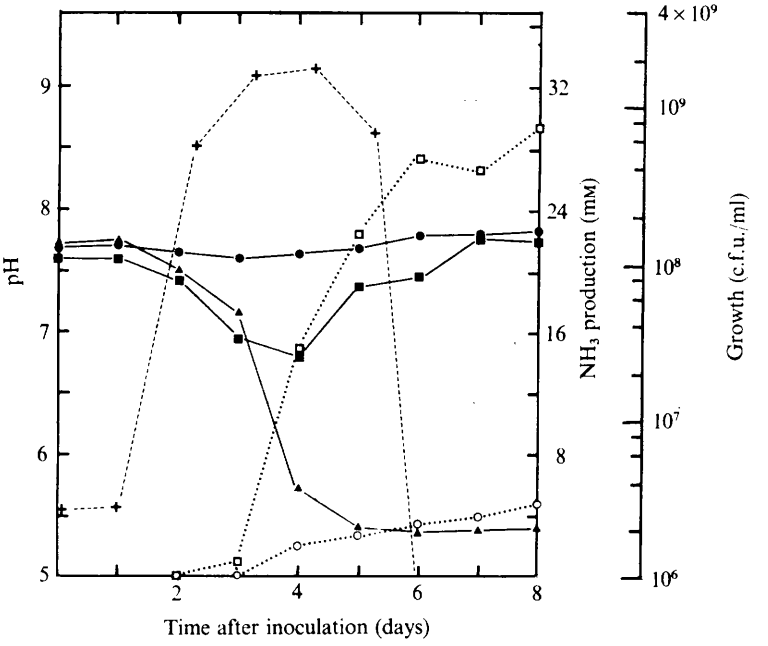

Fig. 1. Changes in pH (solid symbols) and ammonia production (open symbols) in cultures of *S. citri* (SP-A) grown in BSM supplemented with arginine (●), arginine and sugars (■), and sugars alone (▲); and growth of SP-A in BSM with sugars as measured by colony forming units (c.f.u) (+).

DISCUSSION

The increase in concentration of ornithine and citrulline as the degradation of arginine proceeded, and the absence of urease activity, indicated that the arginine dihydrolase pathway was operative in *S. citri*. In non-fermentative mycoplasmas, the course of this pathway varies according to the metabolic activity of the organisms. In actively growing mycoplasma cells, the constant requirement for ATP biases the combined reactions towards carbamyl phosphate formation and ensures the complete breakdown of citrulline to ornithine. When the cells enter the stationary phase, the greatly decreased requirement for ATP causes citrulline breakdown to stop but arginine degradation continues and citrulline accumulates (Schimke & Barile, 1963; Smith, 1971). In cultures of *S. citri*, citrulline accumulated in both growing and stationary-phase cultures and this decreased the formation of ATP and liberation of NH_3 by the arginine dihydrolase pathway. Complete metabolism of 0·42 % (20 mM-arginine) hydrochloride by this pathway would liberate nearly 40 mM-ammonia in the culture. It appeared that the incomplete degradation of citrulline to ornithine, which occurred even under optimum growth conditions, reflected the limited contribution of this pathway to the overall energy requirements of *S. citri*.

Complete utilization of arginine only occurred when an alternative energy source in the form of glucose or fructose was present to encourage growth. This may explain why previous workers failed to detect arginine hydrolysis in *S. citri* by the standard test of Aluotto *et al.* (1970) in which arginine was the only energy source. The poor growth of spiroplasmas in the medium used for this test resulted in insufficient ammonia being released to cause a detectable change in the colour of the phenol red indicator. P. Saglio has since repeated the

arginine hydrolysis tests on *S. citri* and has confirmed that this organism hydrolyses arginine (unpublished results).

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