JOURNAL OF BACTERIOLOGY, Sept. 1971, p. 780-786

Vol. 107, No. 3
Printed in U.S.A.

Studies on the Physiology of *Bacillus fastidiosus*

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Received for publication 22 March 1971

Bacillus fastidiosus was grown in a minimal medium that contained uric acid or allantoin, aerated by vigorous stirring. A constant, optimum *p*H of 7.4 was maintained by controlled addition of sulfuric acid. Washed cells converted both urate and allantoin into carbon dioxide and ammonia, simultaneously assimilating part of the available carbon and nitrogen. Urate oxidase (formerly called uricase) was present in extracts from urate-grown but not allantoin-grown cells. The formation of urate oxidase was apparently induced by urate. Urea was detected as an intermediate in some but not all of these experiments. However, the high urease activity observed in cell-free extracts may have prevented accumulation of urea in many of the experiments. The presence of glyoxylate carboligase and tartronic semialdehyde reductase activities indicates that the glycerate pathway may be involved in urate and allantoin catabolism in this organism.

Bacillus fastidiosus was first isolated in 1929 by den Dooren de Jong (3) and described as an aerobic, rod-shaped organism, motile with peritrichous flagella, and able to form ovoid spores which are mostly located terminally. The organism appeared to be widely distributed in soil and was named for its fastidious demand for uric acid or allantoin as carbon and energy sources. As a result of the aerobic degradation of purine derivatives and of the nitrogen excretion in uricotelic animals, these compounds presumably provide support for *B. fastidiosus* in nature. Other bacterial species have been reported to metabolize uric acid and allantoin aerobically, but none of them seemed to be restricted to these compounds as was *B. fastidiosus*.

Despite this peculiarity, *B. fastidiosus* has attracted little attention during the past four decades. Only very recently it was reisolated independently by three research groups. After isolation by Butler (4), the fine structure of vegetative cells and spores was extensively studied by Leadbetter and Holt (4, 13). Simultaneously, several strains were reisolated at the Institut für Mikrobiologie in Göttingen, Germany, by D. Claus (*unpublished data*). He kindly supplied strain SMG 83 which was used during the present metabolic study. More recently, Mahler (15) employed urate oxidase for analytical application, using strains of *B. fastidiosus* isolated by soil enrichment with uric acid.

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MATERIALS AND METHODS

Media and growth of the organism. Lyophylized cells of *B. fastidiosus* SMG 83 (Culture collection, Institut für Mikrobiologie, Göttingen, Germany) were transferred to slants of a medium (*p*H 7.2) containing per liter: $Na_2HPO_4 \cdot 12H_2O$, 9 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; ferric ammonium citrate, 1.2 mg; $CaCl_2$, 20 mg; $MnCl_2 \cdot 4H_2O$, 1 mg; allantoin or uric acid, up to 4 g; and Bacto-Agar (Difco), 20 g. The slants were incubated at 30 C for 2 days and kept for several weeks at 4 C. For mass culturing, a suspension of 5 ml of this medium was spread on a 100-ml layer of solidified medium in a screw cap 500-ml prescription bottle lying flat. A heavy suspension of cells developed during a 48-hr incubation period and was transferred into 3 liters of the medium containing no agar. The culture was aerated with air slowly passed through the vessel and distributed by a magnetic stirring bar revolving at 500 to 600 rev/min. The doubling time under these conditions was 1.5 hr determined by turbidity readings. Growth ceased after 9 hr, when the *p*H of the culture medium had reached about 8.5. Up to 10 times the amount of phosphate buffer was tolerated by the bacteria, permitting higher cell yields at a doubling time of approximately 2.5 hr.

To avoid changes in *p*H while obtaining cells for physiological studies, 3-liter cultures were maintained at a constant *p*H of 7.4 during growth by using a combined glass electrode (GK2302C Radiometer) connected with a titrator (TTT 1, Radiometer). The addition of heat-sterilized 0.5 M sulfuric acid was controlled either by an automatic burette (ABU 12 Radiometer) in connection with a recorder (SBR 2C Titrigraph Radiometer) or by a magnetic valve.

Treatment of cells. The cells were harvested after approximately 250 ml of the solution of sulfuric acid had been added to a 3-liter culture. The suspension was

brought to *p*H 6.8, cooled to 4 C, and harvested by centrifugation for 15 min in a GS-3 rotor of a Sorvall RC2-B centrifuge at 3,000 × *g*. The yellow mass was washed several times with phosphate buffer (*p*H 7.0) with the same molarity as the culture medium. Usually about 1 g (wet weight) of cells was obtained per liter under these conditions.

Sonic treatment. Cell-free extracts were prepared by sonic treatment at 0 to 8 C, by using a model W140D Branson Sonifier (Heat Systems, Ultrasonics). At the full output possible when using the microtip, each milliliter of suspension was sonically treated twice for 10 sec each time. Unbroken cells and crude particles were removed by centrifugation for 20 min at 10,000 × *g* at 4 C. In some experiments, the extract was centrifuged at 100,000 × *g* in a fixed-angle rotor type 65 in a Beckmann model L2-65B centrifuge.

Analytical procedures. Protein content of extracts, culture turbidity, and uric acid concentrations were determined as previously described (7, 19). A Hitachi Perkin-Elmer spectrophotometer, model 139, in connection with a Photovolt linear/log recorder (Varicord, model 43) was used for all spectrophotometric determinations. Urease (urea amidohydrolase, EC 3.5.1.5) activity was measured by the method of Kaltwasser and Schlegel (8), using a coupled enzyme assay to determine the rate of ammonia production. This system was also employed to measure ammonia concentrations and, by treatment of reaction mixtures with urease, to determine urea in the supernatant fluid. Glyoxylate carboligase (glyoxylate carboxy-lyase, EC 4.1.1.b) activity (2) was determined manometrically at *p*H 6.4 under nitrogen atmosphere by the method of Krakow and Barkulis (12). Urate oxidase (urate: oxygen oxidoreductase, EC 1.7.3.3) was determined spectrophotometrically at 30 C in quartz cuvettes containing: lithium-urate, 0.25 μmole; Na_2HPO_4, 50.4 μmoles; KH_2PO_4, 22 μmoles; $MgSO_4$, 1.63 μmoles; and varying amounts of protein in a 3-ml total volume at *p*H 7.2. Decreases in optical density (OD) at 293 nm in a 1-cm light path were recorded, and the rate was determined at OD 0.9 (78 μM urate). The manometric urate oxidase assay was performed as previously described (7). To measure tartronic semialdehyde reductase [D-glycerate: NAD(P) oxidoreductase, EC 1.1.1.60], 10 μmoles of glyoxylate was incubated with dialyzed extract in a mixture containing varying amounts of protein, 115 μmoles of tris(hydroxymethyl) aminomethane (*p*H 7.2), 10 μmoles of $MgCl_2$, and 1.0 μmole of thiaminepyrophosphate for 10 min at 30 C prior to the addition of 0.6 μmole of reduced pyridine nucleotides; final volume, 3 ml. The OD change at 366 nm occurring after addition of the electron donors was recorded. A 10-min preincubation in the presence of glyoxylate was required to obtain maximum rates of initial reduced nicotinamide adenine dinucleotide (phosphate) [NAD(P)H] oxidation under conditions described in Table 2. Slow and increasing rates were observed when glyoxylate and NAD(P)H were added simultaneously. These observations indicate that the oxidation of NAD(P)H was due to a two-step enzymatic reaction rather than to glyoxylate reductase (EC 1.1.1.26). All specific enzyme activities reported were corrected for the rates observed in substrate-free controls.

All biochemicals and enzymes were obtained from Sigma Chemical Co., except urease, which was purchased also from Boehringer Mannheim Corp.

RESULTS

When varying amounts of substrate were added to washed allantoin-grown cells, about twice as much oxygen was consumed per mole of urice acid than per mole of allantoin. The rate of oxygen uptake during oxidation of uric acid increased with time, which was not the case during oxidation of allantoin. These observations may be interpreted as indicating that the enzyme urate oxidase catalyzed uptake of the additional quantity of oxygen and that this enzyme was not present initially in allantoin-grown cells.

The amounts of oxygen consumed and of carbon dioxide produced during the degradation of urate and allantoin were determined after preincubation of allantoin-grown cells with uric acid (Table 1). With uric acid provided, approximately 0.5 mole of oxygen was consumed and 1 mole of carbon dioxide was produced in excess of the quantities consumed and produced during degradation of allantoin. This result is in good agreement with the stoichiometry of the urate oxidase reaction. From allantoin, four additional moles of carbon dioxide might be expected to be released during complete degradation. However, only a fraction of this amount was recovered, indicating that a considerable portion of the available carbon was very likely incorporated into the growing cells. If it is assumed that only the glyoxylate moiety is utilized as an energy source, the data in Table 1 would indicate that more than 60% of the glyoxylate carbon was assimilated. The data are average numbers and are based on the manometric data only. They are not corrected for the carbon present in the small amounts of urea detected in some of these experiments.

pH requirement. Since the growth of *B. fastidiosus* is self inhibitory and ceases at about *p*H 8.5, acid was added to neutralize the ammonia accumulating during growth. Although a rather wide *p*H range was tolerated when the oxygen uptake by washed cells was determined in the presence of allantoin, the *p*H range tolerated by germinating spores was narrower. An optimum at about 7.5 was observed when the respiration rate was measured in germinating spore suspensions 3 hr after heat treatment.

Heat treatment. A suspension containing both sporulated and nonsporulated cells was heated for 3 min before determination of the respiration rate. Temperatures higher than 55 C destroyed initial respiratory activity that was presumably

TABLE 1. *Uptake of oxygen and the release of carbon dioxide, ammonia, and urea during the degradation of urate and allantoin by whole cells*[a]

Organic substrate	Oxygen	Carbon			Nitrogen		
	O_2 consumed (moles per mole of substrate)	CO_2 released (moles per mole of substrate)	CO_2 assimilated (moles per mole of substrate)	2 C-moiety assimilated (%)	N released (equivalents per mole of substrate)		
					Urea	Ammonia	Sum
Uric acid	0.881	3.67	1.33	66.5	0.92 0.0	2.90 3.74	3.78
Allantoin	0.405	2.78	1.22	62.0	0.67 0.00	3.18 3.80	3.83

[a] A 100-mg amount of wet cells, grown with allantoin and incubated for 3 hr with uric acid, was washed twice and suspended in a manometer vessel containing: Na_2HPO_4, 37.8 μmoles; KH_2PO_4, 16.5 μmoles; and $MgSO_4$, 1.24 μmoles, in 2.6 ml at *p*H 7.2 and 30 C. Uric acid (2 or 3 μmoles) or allantoin (3 or 4 μmoles) was added from the side arm at zero time. Oxygen uptake was determined with KOH in the center well. Carbon dioxide was measured by Warburg's direct manometric method with 0.1 ml of 1 M H_2SO_4 tipped from the side arm at zero time and after 2 or 3 hr, at which time the entire substrate had been utilized. The values are corrected for endogenous metabolism.

due to nonsporulated cells. During the following 3 hr of incubation, oxygen uptake increased as a consequence of spore germination. The respiration rates determined after 160 min indicated that spore germination was stimulated by heat. The optimum temperature was 65 C under these conditions. No oxygen uptake was observed in the sample that had been heated for 3 min at 98 C, indicating that spores treated at this temperature did not germinate.

Urate degradation. Addition of uric acid to a light suspension of uric acid-grown cells resulted in immediate degradation of the substrate (Fig. 1). Almost 4 moles of ammonia was finally released per mole of uric acid consumed. Although urea was not detected during the course of this experiment, it did appear in the supernatant fluid when allantoin-grown cells were provided at a much higher cell density (Fig. 2).

Enzymes. In the presence of cell-free extracts from uric acid-grown cells, uric acid disappeared rapidly, as indicated by the loss in absorbancy at 293 nm (Fig. 3). The specific urate oxidase activity in the extracts was not diminished after centrifugation for 3 hr at 100,000 × *g*. It thus appears that urate oxidase is not bound to subcellular particles in *B. fastidiosus*. In accordance with the stoichiometry of the urate oxidase and catalase reaction, 0.485 mole of oxygen was consumed per mole of urate incubated with the extracts, as expected from work with *Hydrogenomonas* H 16 (5). In the latter case, 0.45 mole of oxygen was consumed under similar experimental conditions.

During anaerobic incubation of dialyzed extracts and glyoxylate, 0.507 mole of carbon dioxide was released per mole of substrate provided. Thiaminepyrophosphate and Mg^{2+} were required for this reaction. In the absence of both these substances, carbon dioxide was evolved at only 11% of the rate observed in the complete reaction mixture. In the absence of thiaminepyrophosphate, the rate of release was 36% of that of the complete reaction mixture, whereas omission of Mg^{2+} resulted in evolution at 65% of the rate observed for the complete mixture. In a qualitative test, the reaction product formed an orange precipitate with acid 2,4-dinitrophenylhydrazine under conditions previously described (6). Dissolved in dilute alkali, the precipitate was deep purple with maximum light absorbancy around 565 nm, which is characteristic for tartronic semialdehyde (9). According to these criteria, it can be assumed that glyoxylate carboligase was present in the cell-free extracts.

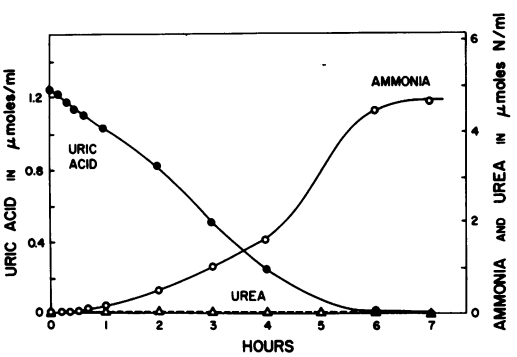

FIG. 1. *Degradation of uric acid by urate-grown cells. Approximately 300 mg of cells was harvested from 100 ml of medium, washed, and suspended in 200 ml of growth medium containing 1.25 μmoles of lithium-urate per ml and incubated at 30 C.*

However, the specific activity for this enzyme (Table 2) appeared to be rather low in comparison with the activity of other uric acid, allantoin, or glycollate metabolizing bacteria (6, 10). Since tartronic semialdehyde reductase (or D-glycerate-3-dehydrogenase) was detected only in minor amounts (Table 2), the glycerate pathway may not be one of the major routes by which allantoin is metabolized in this organism.

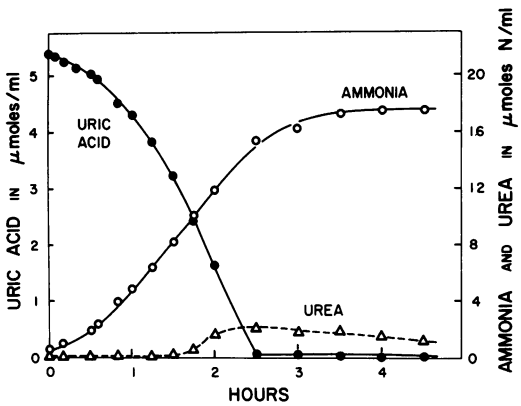

FIG. 2. *Degradation of uric acid by allantoin-grown cells. A 1-g amount of washed cells was incubated at 30 C in 200 ml of growth medium containing 5.4 μmoles of lithium-urate per ml.*

Rather high amounts of specific urease activity were observed in extracts of *B. fastidiosus* (Table 2). This probably explains why urea was detected in some but not all of these experiments. In uric acid-grown *Hydrogenomonas* H 16, the urease activity was lower by three orders of magnitude. These cells released considerable amounts of urea into the medium under similar experimental conditions (7).

Control of urate oxidase formation. Almost no urate oxidase activity was detected spectrophotometrically in extracts of cells grown on allantoin (Table 2). In contrast to the action of uric acid-grown cells (Fig. 1), there was a lag period in utilization of urate by allantoin-grown cells (Fig. 4). As expected, the increase in specific activity did not occur in cells incubated in the presence of allantoin or glyoxylate or in the absence of a substrate (Fig. 5). It thus appears that urate oxidase is an inducible enzyme and that uric acid acts as the inducing substrate in *B. fastidiosus*.

With both uric acid and allantoin present, an increase in urate oxidase activity was followed by a decrease, suggesting the possibility that the presence of allantoin suppressed further urate oxidase formation. Data consistent with this supposition were then obtained (Fig. 6). As be-

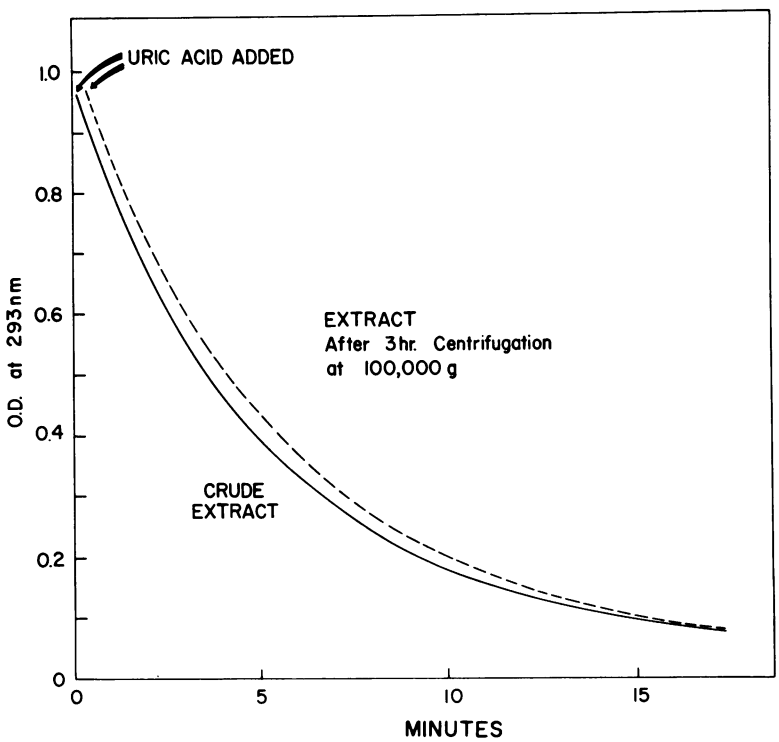

FIG. 3. *Disappearance of urate during the incubation with cell-free extracts from uric acid-grown cells. Each cuvette contained 0.2 ml of extract (0.12 mg of protein before centrifugation).*

TABLE 2. *Specific enzyme activities determined in extracts of Bacillus fastidiosus*

Enzyme	Cells grown with	Specific enzyme activity
Urate oxidase	Allantoin	Uric acid (0.33 μmole/min/g)
	Uric acid	Uric acid (146.0 μmoles/min/g)
Glyoxylate carboligase	Allantoin	CO_2 (96.9 μmoles/min/g)
Urease	Allantoin	NH_3 (1,977.0 μmoles/min/g)
Tartonic semialdehyde reductase	Allantoin	NADH[a] (11.4 μmoles/min/g)
		NADPH[b] (9.4 μmoles/min/g)

[a] Reduced nicotinamide adenine dinucleotide.
[b] Reduced nicotinamide adenine dinucleotide phosphate.

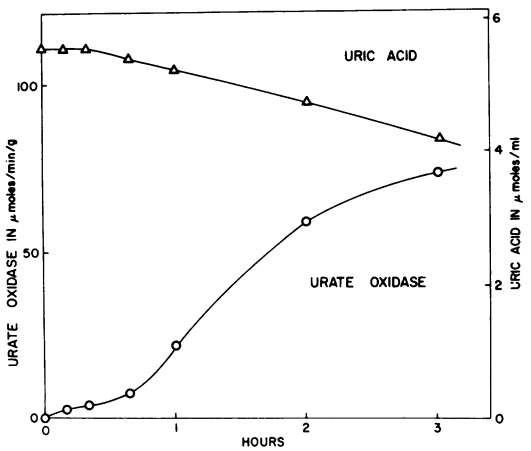

FIG. 4. *Inductive formation of urate oxidase in allantoin-grown cells. Cells grown in 200 ml of allantoin medium were washed twice and incubated at pH 7.2 at 30 C in 80 ml of basal medium containing 5.55 μmoles of uric acid per ml. Uric acid content of 10-ml samples of the supernatant fluid was determined. Urate oxidase in the washed and sonically treated cells was measured spectrophotometrically with 80 to 150 μg of protein per assay.*

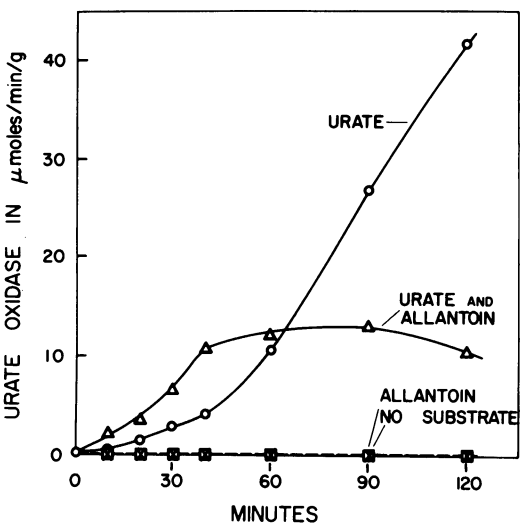

FIG. 5. *Formation of urate oxidase in washed, allantoin-grown cells. A 500-mg amount of wet cells was incubated in 100 ml of growth medium containing 2 mmoles of the substrates indicated. The cells were treated as in Fig. 6.*

fore, addition of uric acid to a suspension of allantoin-grown cells led to the formation of urate oxidase. When allantoin was added 30 min after the addition of urate, however, no further increase in specific urate oxidase activity was observed. Rather, the specific enzyme activity decreased slowly, indicating that synthesis of urate oxidase was apparently diminished if allantoin was present in addition to uric acid. Similar addition of glyoxylate did not have such an effect. Studies of the precise mechanism of allantoin action will be continued.

DISCUSSION

Den Dooren de Jong's observation that *B. fastidiosus* did not utilize common organic substrates other than uric acid and allantoin (3) was also substantiated by Leadbetter and Holt (4, 13) and by Claus (*unpublished data*). The latter recognized allantoic acid as the third utilizable compound, but our attempts to grow the organism at the expense of adenine, adenosine monophosphate, guanine, xanthine, hypoxanthine, glycine, serine, and methylated purine derivatives (theophylline, theobromine, and caffeine) were unsuccessful. There was no stimulation of endogenous respiration of carefully washed cells of *B. fastidiosus* by ammonium chloride (*unpublished data*) and thus no indication that ammonia might be required for respiration as in *B. pasteurii* (24). Since only nitrogenous organic substrates are known to be utilized, and these ultimately give rise to ammonia, the effect of this ion can be studied on endogenous respiration only.

The enzyme urate oxidase, which is bound to

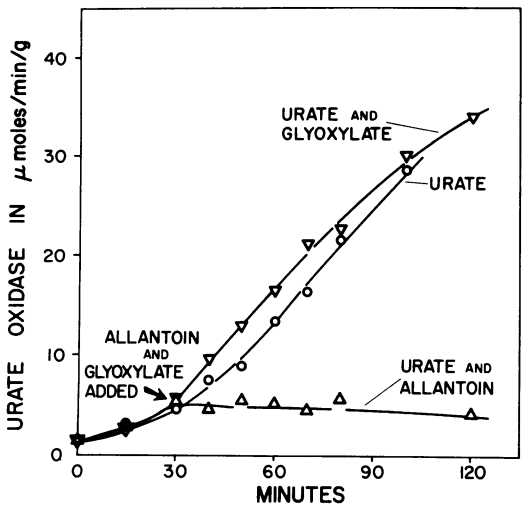

FIG. 6. *Suppression of urate-induced urate oxidase formation by allantoin. In each of three vessels, 1.23 g of wet cells was incubated in 70 ml of growth medium and 2.5 mmoles lithium-urate. After 30 min, 50 ml of 0.1 M allantoin, 0.1 M glyoxylate, or water was added. Cells from 8-ml and 12-ml samples were washed twice, frozen, sonically treated, and assayed.*

subcellular particles in *Hydrogenomonas*, *Pseudomonas aeruginosa*, and *Micrococcus denitrificans* (5), appeared to be soluble in *B. fastidiosus*, which is similar in this respect to the situation in *Arthrobacter pascens* (1, 16). During the complex reaction catalyzed by urate oxidase, two electrons are transferred from the substrate to oxygen, leading to the formation of hydrogen peroxide, carbon dioxide, and allantoin according to equation 1. The hydrogen peroxide is subsequently destroyed by catalase (equation 2). No utilizable energy becomes available during urate oxidation.

$$\text{Urate} + O_2 \rightarrow \text{Allantoin} + CO_2 + H_2O_2 \quad (1)$$

$$H_2O_2 \rightarrow \text{Water} + 0.5\ O_2 \quad (2)$$

$$\text{Allantoin} \rightarrow \text{Glyoxylate} + 2\ CO_2 + 4\ NH_3 \quad (3)$$

$$\text{Glyoxylate} + O_2 \rightarrow 2\ CO_2 + \text{water} \quad (4)$$

We have seen that the formation of urate oxidase is apparently induced by uric acid in *B. fastidiosus* (Fig. 5 and 6). Allantoin served equally well as substrate for growing cells, but an end product balance different from that achieved with uric acid must be expected (equations 5 and 6).

$$\text{Urate} + 1.5\ O_2 \rightarrow 5\ CO_2 + 4\ NH_3 \quad (5)$$

$$\text{Allantoin} + O_2 \rightarrow 4\ CO_2 + 4\ NH_3 \quad (6)$$

Two metabolic routes have been established in aerobic microorganisms for allantoin degradation (20–23). Both lead to release of glyoxylate, which is actually the energy source for cells growing on these nitrogenous compounds. Either 2 moles of urea or 1 mole of urea and 2 moles of ammonia are known to be formed. Urea was detected in some experiments (Table 1 and Fig. 2), but the presence of a very strong urease (Table 2) precluded determination of the molar ratio at which urea was released by whole cells. This may account for the failure of den Dooren de Jong (3) to observe urea in the original studies. The reaction represented by equation 4 would be expected in nongrowing cells if glyoxylate is degraded via pathways commonly observed in glycollate-utilizing microorganisms (9, 11). However, since ammonia is present, an oxidative assimilation of about 60% of the glyoxylate may occur as indicated by the carbon balance (Table 1). This is consistent with observations on growing cultures of other aerobic bacteria (17). Assuming a molar C/N ratio of about 4:1 in the assimilated material (14), reactions represented by equations 7 and 8 may be expected in *B. fastidiosus* for the utilization of uric acid and allantoin.

$$\text{Urate} + 0.9\ O_2 \rightarrow 3.8\ CO_2 + 3.7\ NH_3 \quad (7)$$

$$\text{Allantoin} + 0.4\ O_2 \rightarrow 2.8\ CO_2 + 3.7\ NH_3 \quad (8)$$

Equations 1 through 8 are all in accordance with the general pattern of the aerobic degradation of purines and now seem to be applicable to *B. fastidiosus* as well.

The metabolic routes by which allantoin is degraded in this organism remain to be elucidated. Glyoxylate carboligase and tartronic semialdehyde reductase, the key enzymes in the glycerate pathway, were found to be considerably lower in *B. fastidiosus* than in glycollate-grown *Pseudomonas* and *Escherichia coli* (10) and in uric acid-grown *Hydrogenomonas* (6). Alternate pathways may therefore be functioning in *B. fastidiosus*.

ACKNOWLEDGMENTS

The skillful technical assistance of Marion Andreesen is gratefully acknowledged. I thank D. Claus at the Institut für Mikrobiologie der Universität Göttingen, Germany, for strain SMG 83 and SMG 283 of *B. fastidiosus* and W. J. Payne for discussion and criticism.

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