Uric acid and allantoin uptake by *Bacillus fastidiosus* spores

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Uric acid enters *Bacillus fasitidiosus* spores by a constitutive carrier-mediated mechanism. The extent of uptake was proportional to the external uric acid concentration up to the limit of solubility. Most of the uric acid taken up after 2 min of incubation was not exchangeable with cold uric acid, suggesting that the uric acid was being quickly metabolized. Allantoin (an uric acid degradation product) was not incorporated by spores unles they were triggered to germinate with uric acid and the induced by allantoin. The induction of this uptake system was inhibited by chloramphenicol. The inability of spores to germinate in the sporulation medium was found to be due to the high pH of the sporulation medium after growth and sporulation.

Uric acid *Allantoin* Bacillus *Spore* *Germination* *Differentiation*

1. INTRODUCTION

Bacillus fastidiosus is a very peculiar microorganism in its nutritional requirements. It only grows in the presence of uric acid or allantoin (a uric acid degradation product) [1–3], but its germination requirements have been shown to be more stringent, since its spores only germinate in the presence of uric acid [4,5]. In addition, a heat-activation requirement for germination of manganese-supplemented spores has been reported [4]. In the course of a study on the biochemistry of germination of *B. fastidiosus* spores we decided to study the uric acid and allantoin uptake activities in dormant spores and during germination. This paper shows that these spores have a constitutive uptake system for uric acid and a germination-inducible uptake system for allantoin. In addition, the mechanism by which the spores are unable to germinate in the sporulation medium is shown.

2. MATERIALS AND METHODS

2.1. *Microorganism and culture conditions*

B. fastidiosus NCIB 10016 was obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland. Sporulation was carried out on uric acid-phosphate agar plates as described [5]. Conditions for spore obtention and germination were the same as those reported [5].

2.2. *Uric acid uptake*

For studies of uric acid uptake, spores (5 mg/ml) were incubated at 37°C with [2-^{14}C]uric acid (spec. act. 52 mCi/mmol, Amersham International). At different times of incubation, samples were centrifuged in an Eppendorf minifuge for 20 s and aliquots of the supernatants were counted for radioactivity in a scintillation fluid containing 7 g/l PPO, 0.35 g/l POPOP, 33% (v/v) Triton X-100 and 66% (v/v) sulphur-free toluene using a Beckman LS 7500 liquid scintillation spectrophotometer.

2.3. *Allantoin uptake*

Spores were germinated at 37°C in 8.9 mM uric acid and samples removed at intervals and centrifuged in an Eppendorf minifuge for 20 s. The spores were washed twice with 50 mM Tris-HCl buffer (pH 7.0) and suspended at 20 mg dry wt/ml in the same buffer containing 5 mM allantoin. The spores were then immediately incubated at 37°C and aliquots (0.5 ml) removed at intervals, cen-

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trifuged and the allantoin present in 0.1 ml of the supernatant determined as described [6].

2.4. *Dry weight determinations*

Samples were dried to a constant weight at 80°C on preweighed aluminium planchettes.

3. RESULTS AND DISCUSSION

Uric acid was taken up rapidly by spores during the first 5 min of incubation. The dependence of uric acid uptake on the external uric acid concentration is shown in fig.1. 30 s after addition of uric acid the extent of uptake was similar in both heat-activated (70°C, 30 min) and non-activated spores (fig.1B) at all concentrations of uric acid tested. After 5 min of germination the extent of uptake was clearly dependent on the external uric acid concentration (fig.1A). At low concentrations (8.9–89.2 μM) an increase in uric acid concentration did not produce a correspondingly increased uptake, but above 100 μM the uptake increased progressively in response to the external uric acid concentration. Fig.1A also shows that this increase was significantly greater for heat-activated spores and at 8.9 mM uric acid there was a 4-fold difference in the extent of accumulation by heat-activated and non-activated spores. Since the initial rates of uric acid observed for both heat-activated and non-activated spores were similar, the possibility that heat activation destroys a permeability barrier to the germinant must be discarded. In addition, the finding that heat activation produced a 4-fold increase in the extent of uric acid uptake after 5 min but did not affect initial uptake rates suggests that probably the uricase enzyme is the site for the stimulatory effect of heat activation. In a further series of experiments (not shown), the exchangeability of accumulated uric acid was measured by washing spores which were germinated in 8.9 mM uric acid twice at 37°C with either 10 mM Tris-HCl buffer (pH 7.0) or with non-radioactive 8.9 mM uric acid solution. After 2 and 5 min of germination heat-activated spores released a maximum of approx. 15% of their accumulated uric acid when washed by these procedures. The same treatment released a maximum of about 24% of the uric acid from non-activated spores at both times. These results are in marked contrast to the situation with *B. megaterium* spores, where 100% of the L-alanine germinant can be recovered by buffer washing at 2°C (M.A. Koncewicz and D.J. Ellar, personal communication). Similar experiments with *B. subtilis* showed more than 99% of the L-alanine germinant could be recovered by buffer washing [7]. The atypical nature of *B. fastidiosus* spore germination is further reinforced by the finding that uric acid uptake is temperature-dependent with no uptake occurring at 4°C (not shown). In contrast, uptake of L-alanine by *B. subtilis* spores proceeded almost as rapidly at 2°C as at 42°C [7]. Uric acid uptake by *B. fastidiosus* spores was not affected by the presence of 10-fold greater concentrations of structurally related purines (xanthine, hypoxanthine, adenine, guanine and 2,8-dihydroxypurine) or pyrimidines (uracil and thymine).

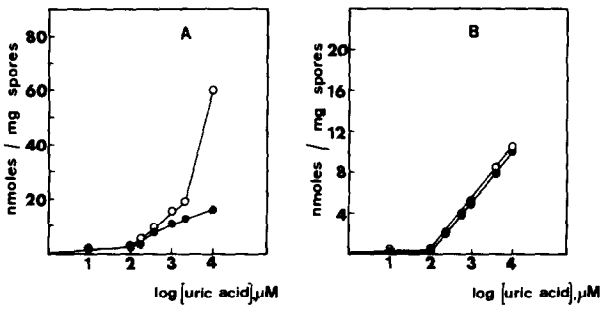

Fig.1. Influence of the external uric acid concentration on the uptake rate by spores. Heat-activated (○) and non-activated (●) spores were incubated at 37°C for 30 s (B) or 5 min (A) in the presence of different uric acid concentrations. After this period of time, the uptake rate was determined as described in section 2.

The above results strongly suggest that uric acid enters spores via some form of carrier mechanism which seems to be constitutive in the spore. This uptake system was also found when spores were formed in a sporulation medium lacking uric acid and with allantoin as the sole carbon and nitrogen source. The apparent non-exchangeable nature of the bulk of the uric acid could be the result of tight binding to sites within spores and/or to the rapid catabolism of the germinant. This latter possibility seems to be more probable in the light of a role for uricase in triggering germination of *B. fastidiosus* spores [5].

As has been mentioned, *B. fastidiosus* spores do not germinate with allantoin as germinant [4,5].

This fact cannot be explained by the absence of allantoinase in spores since this enzyme activity has been shown to be functional in spores [5]. We tested the possibility that allantoin does not permeate the spore cell wall and membrane. Dormant spores did not accumulate detectable amounts of allantoin after 3 h at 37°C. The assay method and the spore concentration employed would have detected an accumulation of 0.63 mol/mg dry wt (or 1.4×10 molecules per spore). However, when the spores were first germinated in uric acid for 30 and 60 min and then transferred to allantoin, allantoin uptake could be observed after a lag of about 45 and 30 min, respectively (fig.2). From this time, 10% of the starting allantoin concentration was taken up in 22 and 18 min, respectively. The addition of chloramphenicol (100 μg/ml) when the spores were incubated with allantoin completely inhibited the disappearance of allantoin from the medium, suggesting that de novo protein synthesis is a prerequisite for allantoin utilisation.

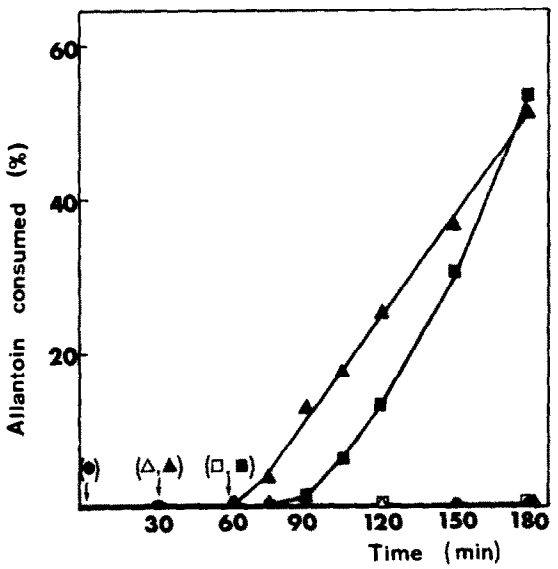

Fig.2. Allantoin uptake by spores. Heat-activated spores were germinated at 37°C in 8.9 mM uric acid. At the times indicated by the arrows, the spores were washed twice with 10 mM Tris-HCl buffer (pH 7.0), suspended in 5 mM prewarmed allantoin with (△, □) and without (▲, ■, ●) 100 μg/ml chloramphenicol, and the incubation continued. Samples were withdrawn at different time intervals, centrifuged and the allantoin present in the supernatant measured as described in section 2.

Thus the evidence favours the conclusion that during sporulation the uric acid uptake system is retained whereas the allantoin uptake system is lost. This also appears to be the case for spores produced in allantoin medium, since these spores were germinable by uric acid but not by allantoin.

B. fastidiosus spores were usually obtained from the surface of uric acid-phosphate agar plates. These plates have a whitish appearance both before and after growth and sporulation of the microorganism due to the low solubility of uric acid. Although this suggests that there may be sufficient uric acid left in the medium after growth to trigger germination, germination was not observed to occur. This cannot be explained by the absence of heat activation of these spores since, in our strain, the requirement for heat activation of spores formed in the presence of manganese was not absolute and spores germinate without this treatment although with a lower germination rate. The mechanism by which germination of the newly formed spores was prevented was investigated as follows: the microorganism was grown on cellophane films placed on the surface of the agar in the plates. When sporulation was complete the spores were harvested from the cellophane by washing it with deionized water. After melting the underlying agar in a boiling water bath, the pH was measured and found to be between 9 and 10. At this alkaline pH germination of *B. fastidiosus* spores does not take place [5]. However, when this medium was neutralized using KH_2PO_4 and allowed to solidify, spores were able to germinate normally. To take into account the possibility that this experiment had destroyed a heat-labile germination inhibitor, the underlying agar from a second set of plates upon which sporulation had been completed was extracted with 50 mM Tris-HCl buffer (pH 7.0) and the extract tested for its effect on germination. No inhibitory effect on germination was observed. Thus these results proved that the inability of spores to germinate in the sporulation medium is due to an alkalinization of the sporulation medium after growth and sporulation. An alternative mechanism to inhibit germination of newly formed spores could be the formation of oxonate as a consequence of the oxidation of uric acid in alkaline solution. Oxonate is a po-

tent uricase inhibitor [6]. However, such a mechanism is ruled out by our observation that germination of *B. fastidiosus* spores in 3 mM uric acid was not inhibited by oxonate concentrations as high as 60 mM.

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