Mechanism of Uptake of α-Santonin by *Pseudomonas cichorii* Strain S

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Uptake of various solid hydrophobic substances is reported to occur by their solubilization, mediated by the cells through secretion of extracellular factors,[1-3] or via direct contact of cells with the substrate.[4,5] *Pseudomonas cichorii* strain S utilizes α-santonin, a sesquiterpene, as a sole source of carbon forming various intermediary products.[6,7] The substrate, in liquid as well as on solid media, forms conspicuous crystals, which disappear during the course of growth of the organism.[6] The present communication reports the observations on the mechanism of α-santonin uptake by *Pseudomonas cichorii* strain S.

MATERIALS AND METHODS

Organism and Growth Conditions

Pseudomonas cichorii strain S capable of utilizing α-santonin as sole source of carbon[6] was maintained on mineral medium[6,8,9] agar containing 0.4% (W/V) α-santonin.[6,8] The culture was grown in α-santonin medium incubated on a rotary shaker at 180 rpm at 28–30°C. Growth was checked at intervals turbidimetrically using Klett Summerson colorimeter and viable count.

Estimation of α-Santonin

For estimation of dissolved α-santonin, the culture broth was filtered through 0.45-μm Millipore membrane in order to remove α-santonin crystals, if present. The filtrate was extracted with chloroform (1:1). For estimation of total amount of α-santonin, consisting of crystalline and dissolved α-santonin, the aqueous medium or culture broth was extracted directly with chloroform (1:1).

The amount of α-santonin present in the chloroform extracts was estimated by the colorimetric method.[10] To the chloroform extracts, evaporated to dryness, were added 3 mL of 50% H_2SO_4 and 0.5 mL of 0.8% ferric chloride and heated for 10 min at 100°C. The reaction mixture was then cooled, diluted with 3 mL absolute alcohol, and the absorbance read at 485 nm.

Uptake of α-Santonin by Cells

Cells grown on α-santonin, glucose, benzoate, or nutrient medium were washed thrice with 0.05*M* phosphate buffer, pH 7, by centrifugation at 5000 rpm at 10°C, for 10 min, and resuspended to give an absorbance of 4.5 at 450 nm. Response of the washed cells was monitored in terms of depletion of α-santonin, using two reaction systems, involving the cells either on support or in a liquid system.

Cells on Support

Fifteen grams of silica gel of 180–200-nm mesh, washed to neutral pH with distilled water, was mixed with 1 g of α-santonin. A slurry, prepared in 50 mL mineral medium, was packed into a glass percolator column,[11] through which mineral medium was circulated with the help of an aerator. Culture of strain S grown in α-santonin medium for 24 h (2.5 mL) was pipetted on to the top of the column. Five-milliliters aliquotes were eluted at intervals for assay of α-santonin.

Liquid System

Reaction mixtures consisting of washed S cells and α-santonin, made to a volume of 3 mL, using 0.05*M* phosphate buffer, pH 7, were incubated on a reciprocating shaker at a speed of 115 strokes/min. Individual flasks were withdrawn at intervals and the contents were analysed for total and dissolved α-santonin.

RESULTS AND DISCUSSION

Growth of *Pseudomonas cichorii* S

α-Santonin Agar

Pseudomonas cichorii S grows on α-santonin agar plates and forms clear, crystal-free zones around the colonies

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(Fig. 1),[6] caused by solubilization and utilization of α-santonin. The cells when streaked on presterilized barriers such as cellophane strips (Sigma) and Millipore membrane (0.45 μm) and placed on α-santonin agar, grow within 48 h forming colonies on the barriers. The corresponding place below the barrier was marked with halo indicating dissolution of the substrate and noninvolvement of any high-molecular-weight extracellular factor in the dissolution. The batch culture supernatent placed on α-santonin agar did not show any solubilization of α-santonin crystals.

α-Santonin Liquid Medium

The growth of strain S in α-santonin medium is accompanied by visible disappearance of crystals from the medium. The quantitative analysis is depicted in Figure 2. The concentration of dissolved α-santonin was 166 μg/mL at 0 h and remained unchanged up to 12 h of incubation. The concentration of total α-santonin decreased continuously until it reached 166 μg/mL; upon further decrease, it then corresponded to that of dissolved α-santonin.

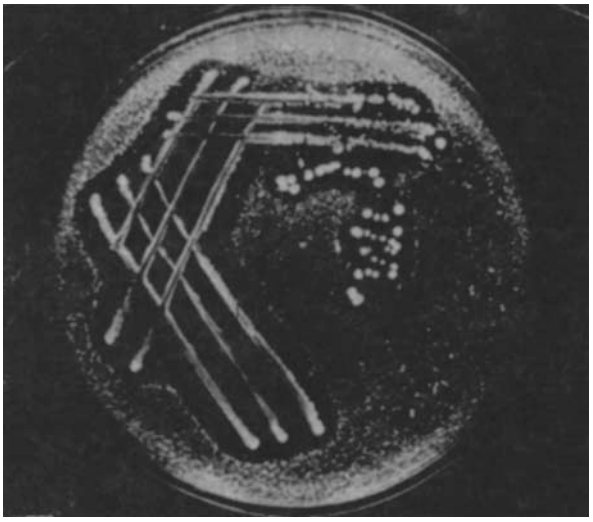

Figure 1. Halo formed around the colonies of *Pseudomonas cichorii* strain S during growth on α-santonin agar medium (ref. 6).

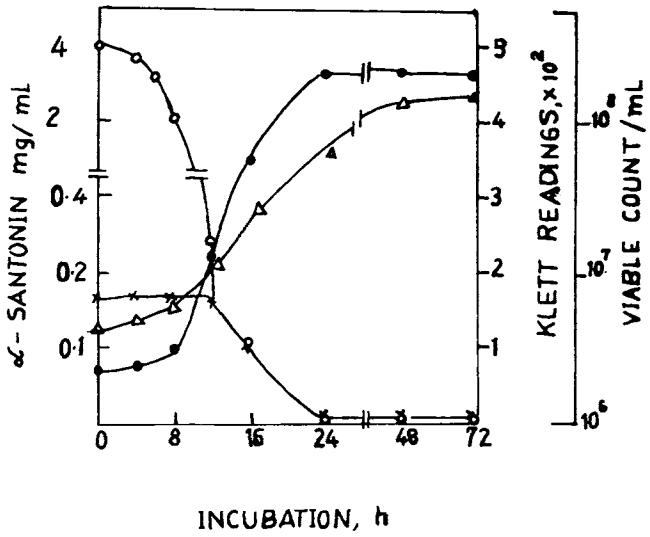

Figure 2. Depletion of α-santonin, (○) total and (X) dissolved, during growth of *Pseudomonas cichorii* strain S, monitored by (●) turbidimetry and (△) viable counts.

Depletion of α-Santonin by Resting Cells

The eluants from the column, packed with silica gel containing crystalline α-santonin, showed a constant amount of dissolved α-santonin of 166 μg/mL. When the column was inoculated with cell suspension, the eluted sample showed the initial concentration of 166 μg/mL, which was decreased to 30 μg/mL within 9 h.

When α-santonin grown cells washed in 0.05*M* phosphate buffer, pH 7, and adjusted to absorbance of 4.5 at 450 nm were incubated with saturated-α-santonin solution, an immediate depletion in concentration of α-santonin was observed (Table I). When resting cells were incubated with excess of the substrate, 1 or 2 mg/mL of the reaction mixture, the concentration of dissolved α-santonin showed a constant value of 166 μg/mL for 30 and 90 min, respectively (Table I), there being no supersaturation (more than 166 μg/mL) at any stage. These observations confirmed that *P. cichorii* S utilizes mainly the substrate made available by dissolution. Similar findings have been reported for steroids, solid paraffins, and alkanes.[13–15]

Kinetics of Uptake of α-Santonin

The above observations that cells utilized α-santonin, available in dissolved form prompted us to study the correlation between the rate of dissolution of α-santonin in the medium and its depletion by strain S. As seen in Figure 3(A), α-santonin went into the solution, in aqueous medium, at the rate of 30 μg/min/mL at ambient conditions (28–32°C), until a saturation level of 166 μg/mL was reached in 7 min.

Effect of Cell Concentration on Depletion Rate

In order to determine the rate of depletion of α-santonin from the medium, varying amounts of washed cells were

Table I. Depletion of α-santonin by resting cells of *Pseudomonas cichorii* S.

Time (min)	Cells incubated with α-santonin		
	166 μg/mL	1000 μg/mL	2000 μg/mL
	Amount of dissolved α-santonin (μg/mL)		
0	166	166	166
5	67	166	166
25	65	166	166
30	<6	166	166
60	<6	45	166
90	<6	25	166
110	<6	—	65
120	<6	10	45

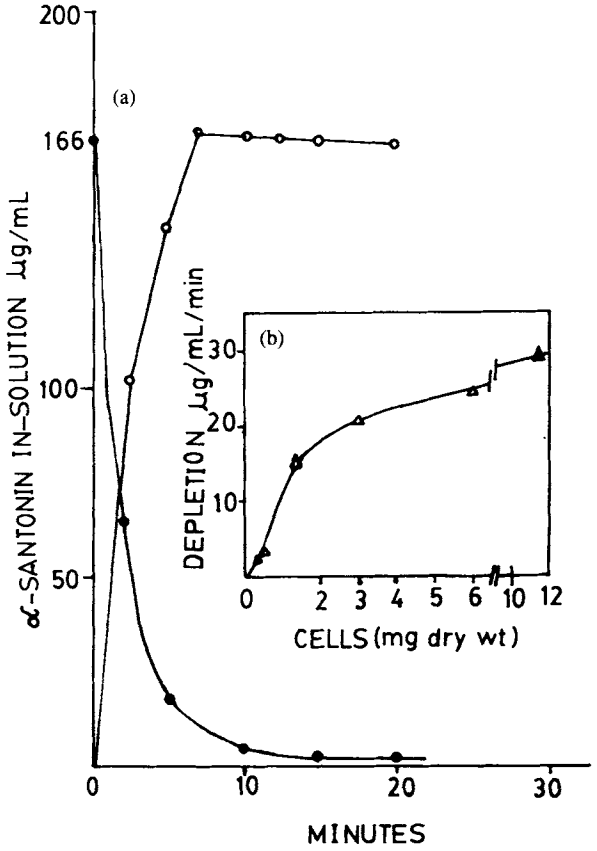

Figure 3. (A) Dissolution of α-santonin in mineral medium, α-santonin (5 mg) in 10 mL water, incubated at 115 strokes/min at room temperature. Aliquotes were filtered (0.45 μm Millipore filter) and (○) estimated for dissolved α-santonin. The depletion of dissolved α-santonin (●) by *Pseudomonas cichorii* S, is also shown. (B) Rate of depletion of α-santonin by (△) varying amounts of cells is also shown.

incubated with the saturated solution of α-santonin. Figure 3(B) shows that the rate of depletion increases initially with the increasing amount of cells and levels off at a maximum rate of 29.93 μg of α-santonin min/mL with 11.62 mg dry wt cells. The profile shown in Figure 3(A) illustrates that the rate of depletion of α-santonin by cells corresponding to 11.62 mg dry wt from the medium is nearly equal to the rate of dissolution of crystalline α-santonin in the medium. It should be noted here that the quantity of cells (11.62 mg dry wt) is five times more than the number of cells at stationary phase in batch culture,[8] therefore the rate of dissolution of α-santonin is not a limiting factor in a growing culture at ambient conditions, wherein the substrate is replenished by a physical phenomenon of dissolution of the crystals as has been reported in the utilization of *n*-docosane and *n*-tetracosane.[10] Since the substrate–cell interaction in *P. cichorii* takes place only with dissolved α-santonin, the excessively high concentration of the substrate in crystalline form would not exhibit any toxic effect on cells. Thus, α-santonin at concentration as high as 5% is reported to be tolerated by the cells.[6,8]

Mechanism of Interaction of *P. cichorii* S with Santonin

Sodium azide ($10^{-3}M$) and α-α' dipyridyl ($10^{-3}M$) did not affect the viability of the cells, but inhibited the cell–santonin interaction (Fig. 4), implying the involvement of oxidative metabolism in the process. The inhibitory effect of EDTA (Fig. 4), attributed to the chelation of divalent metal ions,[16] corroborated the oxygen uptake data, wherein the cells were treated with 1 mM EDTA in 0.1mM Tris HCl buffer for 20 min prior to manometric studies (Fig. 5).

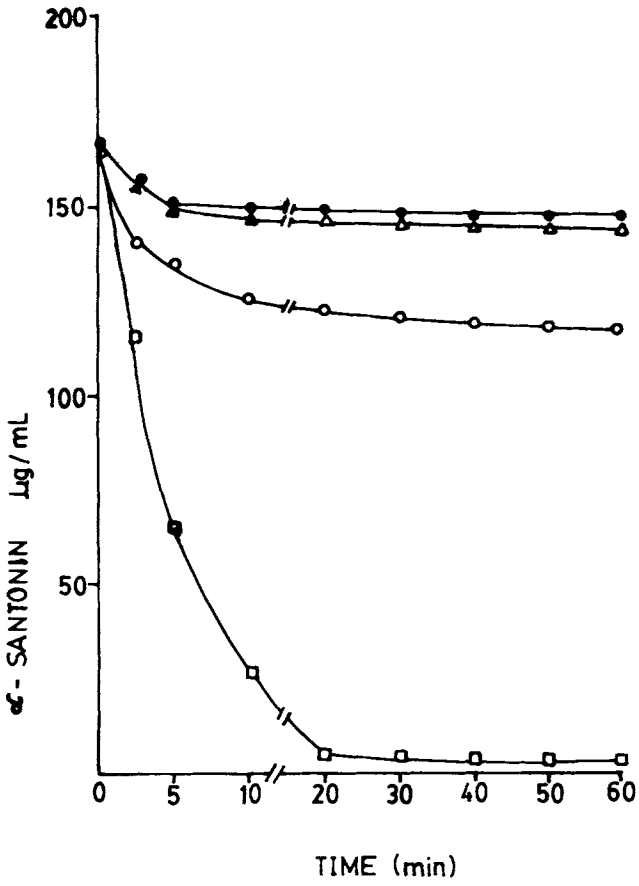

Figure 4. Effect of inhibitors on uptake of α-santonin by *Pseudomonas cichorii* S. Santonin-grown resting cells, suspended in buffer, were incubated with α-santonin and inhibitor: (□) Control, (○) EDTA of $10^{-3}M$, (△) α-α' dipyridyl of $10^{-3}M$, and (●) sodium azide of $10^{-3}M$.

Gram negative cells often possess specific factors for substrate transport in their envelopes. The presence of such factors is inferred from loss of activity on osmotic shocking[17] or cation and pH manipulation[18] and its simultaneous appearance in supernatant fluid. Resting cells of strain S, treated with 0.2mM $MgCl_2$[18] for 20 min gave an oxygen uptake similar to that given by untreated cells (Fig. 5). The shock fluids incubated with α-santonin crystals also failed to show dissolution. Thus, the factors interacting with dis-

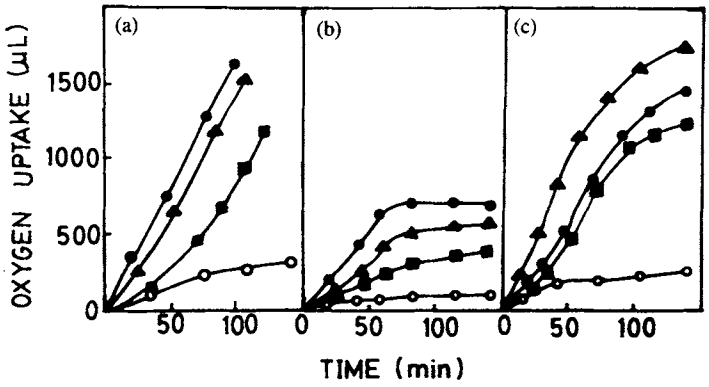

Figure 5. Effect of pretreatment of *Pseudomonas cichorii* S on oxygen uptake: (A) untreated control, α-santonin grown cells, suspended in 0.05*M* phosphate buffer, pH 7.0, treated with (B) 1m*M* EDTA (C) 0.2m*M* Mg^{2+}, for 30 min washed in phosphate buffer and used for oxygen uptake with (●) α-santonin, (▲) acetate, (■) benzoate, and (○) endogenous.

solved α-santonin are probably, bound firmly to the cell envelope.

CONCLUSIONS

Foregoing observations indicate that *Pseudomonas cichorii* S cells utilize α-santonin, available in solution, dissolved at a rate of 30 μg/mL/min at ambient temperature (28–32°C). The rate of uptake of α-santonin by the cells is influenced by concentration of cells and the substrate. Uptake of α-santonin appears to involve oxidative metabolic process, requiring divalent metal ions.

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