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Glucose Influenced Degradation of α-santonin in Pseudomonas sp. Strain S (ATCC 43388)

SANGODKAR, U. M. X., MAVINKURVE, S.

Department of Microbiology, University of Goa, Goa 403005, India

Summary

Pseudomonas sp., strain S ATCC 43388 utilizes α-santonin by inducible enzyme system measurable by oxygen uptake. Cells grown on acetate or benzoate show negligible oxygen consumption with α-santonin. However, glucose grown cells show evidence of a rapid induction of santonin utilizing enzyme system indicating the implication of glucose or its metabolites in the regulation of degradation of santonin. As a consequence, growth of strain S on mixtures of glucose and α-santonin occurs at rates higher than on either of the substrates alone. Mutants with lesion in the glucose metabolism, independent of α-santonin degradation, fail to exhibit higher growth rates with the binary substrates. The results infer simultaneous metabolism of substrates.

Introduction

Alfa-santonin (santonin), a pharmaceutically important sesquiterpenoid, was until recently used as an effective anthelmintic. It has also assumed significance as a potential parent compound for antitumor drugs [1]. Microbial transformation of santonin is considered as an useful tool for obtaining newer derivatives [2—8]. The soil isolate, *Pseudomonas* sp. strain S ATCC 43388, utilizes santonin as a sole source of carbon and energy, forming a variety of transformation products during its growth [8]. Loss of santonin as the source of carbon in building the cell materials during the growth, resulted in low yields of the transformation products in the culture broth. This prompted us to search for an alternate carbon substitute to be used as supplement to the growth medium, without effecting the transformation of santonin, as an extension of our efforts of strain improvement to achieve optimal yields of transformation products. In this paper we demonstrate the inducible nature of santonin utilization. Use of glucose in the medium induced simultaneous synthesis of santonin transforming enzymes. This report has significance in view the importance of conservation of valuable amounts of parent compounds during the fermentations leading to precise microbial transformations.

* Present address: Microbial Ecology and Biotechnology, U.S.EPA, Gulf Breeze, Florida 32561, U.S.A.

Materials and Methods

Organisms and Growth Conditions: Pseudomonas sp. strain S ATCC43388 (initially identified as *P. cichorii* strain S) was isolated from soil and maintained on synthetic mineral salts medium supplemented with appropriate carbon source [9]. The medium has a composition of: K_2HPO_4, 0.63%; KH_2PO_4, 0.182%; NH_4NO_3, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.01%; $CaCl_2 \cdot 2H_2O$, 0.0075%; $FeSO_4 \cdot 7H_2O$, 0.006%; $MnSO_4$ and Na_2MoO_4, 0.6 mg of each per litre. Santonin medium was made by incorporating 0.1 to 0.5% (w/v) of santonin. The pH of the medium was adjusted to 7.0 and autoclaved at 121 °C for 10 min. The culture was routinely grown at 30 °C at high aeration rate. However for comparative growth rate studies, the culture was grown at lower aeration in the culture flasks of 250 ml capacity at 30 °C on a reciprocating shaker (120 strokes per min) and followed by using KLETT SUMMERSON photoelectric colorimeter with blue filter (No. 42). The growth rates and doubling times were calculated from the data obtained during logarithmic phase of growth. To detect the transformation products of santonin the chloroform extracts of the fermentation broth were analyzed by thin layer chromatography (TLC) [4].

Mutant Isolation: The method of mutagenesis was adapted from that used by RAHMAN and CLARKE [10] with some modifications. Culture was grown overnight in nutrient broth. Cells were harvested and resuspended in 0.1 M citrate buffer pH 6.0. N-methyl-N^1-nitro-N-nitroso guanidine (NTG, 24 μg/ml) was added to the culture and incubated at 30 °C without shaking for 30 min. The cells were harvested by centrifuging, resuspended in nutrient broth and grown overnight at 30 °C before plating on solid medium. The glucose negative mutants were selected by replica plating single colonies from nutrient agar on selective glucose agar and santonin agar plates. The presumptive mutants were further screened for reversion by plating 10^7 cells per ml of each mutant on glucose agar plates.

Determination of Oxygen Uptake: The cells grown on appropriate substrates were washed thrice and suspended in 0.05 M potassium phosphate buffer pH 7.0 to a desired absorbance of 5.0 at 450 nm. The rates of oxygen consumption with the appropriate substrates were determined by conventional WARBURG manometric techniques at 30 °C [11]. Each flask contained 1.6 ml of washed cell suspension (10 mg dry weight), 0.2 ml substrate (10—50 μmoles) added initially to the side arm and 0.2 ml of 20% KOH in central well.

Results and Discussion

Very low yields of transient transformation products of santonin were obtained during the growth of *Pseudomonas* sp. strain S in batch cultures [8], because much of the carbon was converted into the cell materials. To avoid the complete loss of parent compound or the intermediates, we screened certain easily metabolizable substrates to be used as alternate source of carbon for growth so that the intermediates of santonin would be accumulated with higher yields. Strain S utilized a narrow range of organic substrates and the cells pregrown on such substrates, when transferred on santonin medium, showed a very long lag of growth. Interestingly, glucose grown culture initiated the growth on santonin medium as rapidly as santonin grown cells. This observation directed us to study the nature of induction of santonin utilization. We used oxygen uptake as the measure to determine santonin utilizing enzyme system. The resting cells of strain S grown on different substrates, were used to determine the rates of oxygen consumption. The cells grown on individual substrates showed varying respiratory responses to santonin. Fig. 1 shows the results of the cells grown on some representative substrates. Negligibly low initial manometric response to santonin by the cells grown on substrates like acetate and benzoate, followed by a long lag before resuming santonin oxidation, confirms that the santonin catabolizing enzymes are not constitutively synthesized in the cells but are induced by the substrate. Such cells pretreated with chloramphenicol (100 μg/ml) did not resume santonin oxidation. The cells grown on glucose, on the other hand, showed a rapid oxygen uptake with santonin (Fig. 1D). Similar induction of

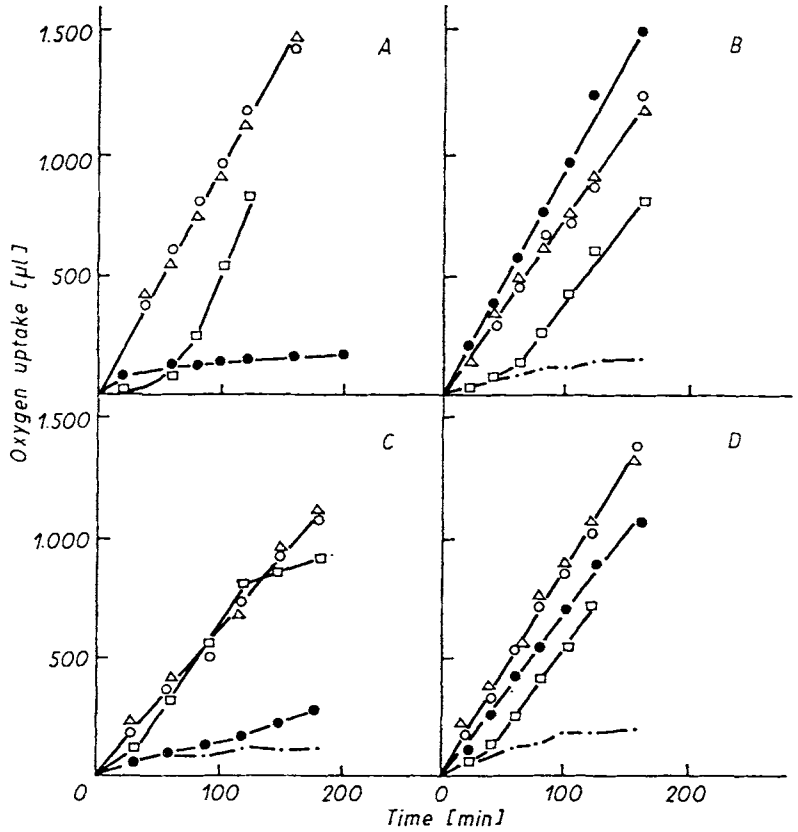

Fig. 1. Oxygen uptake by *Pseudomonas* sp. strain S grown on (A) acetate, (B) santonin, (C) benzoate and (D) glucose. Substrates: ·, endogenous; ○, acetate; ●, santonin; □, benzoate and △, glucose

dissimilatory enzymes for aromatic compounds in the strains of *P. putida* grown in the binary mixtures, containing glucose and the substrate, was reported in the past [12]. Nevertheless, the synthesis of the dissimilatory enzymes for santonin in the cells grown on glucose alone appears to be an intriguing phenomenon. It is likely that the gratuitous synthesis of the santonin degrading enzymes lays an additional biosynthetic burden, which the cells can afford with the substrates yielding high energy, such as glucose, but not with simpler substrate like acetate. The benzoate grown cells did not induce santonin oxidation presumably because the whole set of pathway for degrading aromatic compounds appears to be independent from santonin degradation. There is also no evidence of any aromatic metabolite formed from santonin [8].

To detect whether synthesis of the dissimilatory enzymes takes place simultaneously for glucose as well as santonin, we studied the growth rates of strain S on medium containing binary mixtures of glucose and santonin. The doubling time of strain S in santonin medium reduced considerably from 347 min to 159 min on incorporation of glucose in increasing concentrations (Tab. 1). Also, during the growth on binary mixtures diauxic growth was never observed. During the course of chemical mutagenesis of strain S with NTG, we could isolate glucose negative mutants, at a very high frequency, that were independent of mutations in santonin degradation. We used one such mutant, strain

Tab. 1. Growth rates of *Pseudomonas* sp. strain S and its mutant strain B110 on binary mixtures of santonin (10 mM) and glucose in mineral medium at 30°C

Glucose (mM)	Strain S[a]	Strain B110
	Doubling time [min]	
0	347	348
2	315	388
4	213	351
6	201	378
8	181	351
10	159	387
16	166	370

[a] Doubling time of strain S on glucose (10 mM) alone was 347 min

B110, which does not grow on glucose but grows on santonin at the same rate as the parent strain S. The TLC analysis of the culture broth of strain B110 also showed identical transformation products of santonin as those formed by strain S (results not shown). However, unlike wild type strain S, it did not show increase in growth rate when inoculated in the medium containing the mixture of glucose and santonin (Tab. 1).

At present we do not know whether glucose itself, or any of its metabolites, acts as an inducer of santonin dissimilation. Ordinarily in presence of variety of substrates diauxic growth occurs because the cells dissimilate one of the nutrients and suppress the synthesis of the enzymes for the metabolism of others [13–15]. *P.* sp. strain S appears to metabolize both the substrates simultaneously, corroborating the findings of REBER and KAISER [12] on simultaneous metabolism of the components in a binary mixture by *P. putida*, and thus contributing to the apparent increase in the growth rate to values higher than on individual substrates. The possibility of the presence of two diverse sets of genes, those of glucose on chromosome and those of santonin metabolism on plasmid, cannot be ruled out at this stage. We have recently detected a high molecular non-transmissible plasmid in strain S for which the function is not yet assigned due to the difficulties in getting cured strains (unpublished). Such distribution of genes, if present, would make the regulatory mechanisms of the two dissimilatory pathways function independently, thereby increasing the growth rate. Identification and characterization of the mutation in glucose utilization in strain B110 should help in understanding the precise role of glucose in induction of santonin utilizing enzymes. The use of glucose as co-substrate and glucose grown cells have been convenient in getting higher yields of transformation products of santonin in our batch fermenters by diverting the loss of parent substrate and the intermediates from building the cell mass [8].

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