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Detoxification of dihydroxypyridine by the rumen bacterium *Synergistes jonesii*

Degradación de dihidroxipiridina por la bacteria ruminal *Synergiste jonesii*

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Abstract

There are rumen bacteria capable of degrading toxic dihydroxypyridine compounds derived from mimosine, a non-protein amino acid found in *Leucaena leucocephala*. The degrading activity can be lost reversibly or irreversibly. In our study we determined the extension of bacterial growth and the degradation of 2,3 DPH under different conditions. The degradation of 2,3 DPH began once the late exponential phase of cultivation has been reached, and ended in 3 days. If additional 2,3 DHP is added to the degraded solution (once the original 2,3 DHP had been degraded), the total degradation occured in 12 h. This activity was observed in saline solutions with pyruvate, in rumen fluids and argenine, but not in solutions supplemented with AGV or vitamins. Under strict anaerobic conditions growth and degradation were optimum. Airation of the samples diminished both growth and specific degradation activity. The maintenance of cultures without 2,3 DHP brought about loss of the degradation activity, which was recovered in some cases in re-cultivation in mediums with 2,3 DHP content, and a long lag phase (4 weeks) in the degradation activity (even when growth was normal). The presence of plasmides could not be confirmed. These results are relevant when manipulating laboratory degradation cultures. The degradation activity is present in extracts free of cells in two systems: An atmosphere of N2 and pyruvate (10mM) or an atmosphere of H2 and methyl viologen (0,5 mM). The products of the degradation process with 2,3 DHP are still unknown.

Key words: Rumen bacteria, *Synergistes jonestii*, dihydroxypyridine degradation, *Leucaena*.

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Resumen

Existen bacterias del rumen capaces de degradar piridinedioles toxicos de la mimosina, un aminoacido no proteico presente en *Leucaena leucocephala*. La actividad degradadora puede ser perdida temporal o permanentemente. En nuestro estudio, hemos determinado la extensión del crecimiento bacteriano y de la degradación del 2,3DHP bajo diferentes condiciones. La degradacion del 2,3 DHP comenzó una vez que se alcanzó la fase exponencial tardía del cultivo, y culminó en 3 días. Si se añadía al cultivo degradador 2,3 DHP adicional (una vez que el 2,3DHP original había sido degradado), la degradación total ocurría en 12 h. Esta actividad fue observada en medio de solución salina suplementada con piruvato, licor ruminal y arginina, pero no en la suplementada con AGV o vitaminas. Bajo condiciones de anaerobiosis estricta se observaron crecimiento y degradación óptimos. Aireación de las muestras disminuyó tanto el crecimiento como la actividad degradadora específica. El mantenimiento de cultivos en ausencia del 2,3 DHP provocó una pérdida de actividad degradadora, recuperada en algunos casos luego del recultivo en medio contentivo de 2,3DHP, y una larga fase lag (4 semanas) en la actividad degradadora (aunque el crecimiento fue normal). La presencia de plásmidos no pudo ser comprobada. Estos resultados son relevantes a la hora de manipular cultivos degradadores en el laboratorio. La actividad degradadora está presente en extractos libres de células bajo dos sistemas: atmósfera de N_2 y piruvato, (10mM) o atmósfera de H_2 y methyl viologen (0,5mM). Los productos de la degradación del 2,3 DHP aún se desconocen.

Palabras claves: Bacterias ruminales, *Synergistes jonesii*, degradación de dihidroxipiridina, *Leucaena*.

Introduction

There are rumen bacteria capable of degrading toxic dihydroxypyridine compounds derived from mimosine, a non-protein amino acid found in the toxic legume *Leucaena leucocephala*. If these bacteria are absent in the rumen, intoxication of the ruminant follows the consumption of the plant (7).This occurs in ruminants from Australia and India. Ruminal bacteria capable of DHP degradation have been isolated from animals in Venezuela (4, 5) and Hawaii (1) places where animal consumption of the plant does not lead to intoxication.

Activity of degradation of the 2,3 dihydroxypyridine (2,3 DHP) varies according to growth conditions but sometimes, activity can be reversibly or irreversibly lost. Several isolates have been found to have lost DHP-degrading activity in our laboratory. Our *Clostridium* degrader as well as other unidentified 3,4DHP-degrading strains were found to have lost activity after two years storage at -70ºC. Similarly, active mixed cultures from the rumen of sheep fed *Leucaena* were found to have lost the degrading capacity after having been kept in the refrigerator (4ºC) for several weeks. The objective of this work was to de-

termine the *in vitro* conditions for bacterial growth and degradation of 2,3 DHP by the rumen degrader *Synergistes jonesii.*

Materials and methods

An active degrader strain of *Synergistes jonesii* (strain 78-1, kindly donated by M. Allison) was used for the experiments. Anaerobic cultures in Hungate tubes (Bellco Glass, Inc.) under CO_2)were grown on medium 98-5 without carbohydrates and containing phytone (3% w/v) and filter-sterilised 2,3 dihydroxypyridine (final concentration 2mM or 4,5mM). Clarified rumen fluid from cows consuming straw (*Pannicum maximum*) and molasses, was prepared by filtration of the liquor in double gauze, autoclaving and centrifuging at 10,000g for 20 min. Saline medium contained mineral solution I and II (15% v/v), Na_2CO_3 (0,4% w/v), cysteine HCl (0.05% w/v), and was supplemented with either phytone (3% w/v), pyruvate (10mM), clarified rumen fluid (30% v/v) or arginine (10mM). Media were dispensed (10 ml) in Hungate tubes plugged with a rubber vinyl stopper and gasified with O_2 -free CO_2 and autoclaved (115ºC for 15 min). For all the experiments, a culture in early stationary phase grown on modified media 98-5 containing 4,5mM 2,3 DHP that had started degradation of the DHP, was used as inoculum (0,2mL) in 10mL medium.

Conditions of incubation were 38ºC, statically. Growth of cultures was monitored as absorbance at 600nm in a spectrophotometer (Spectronic 20D, Bausch & Lomb). Methods for detection of plasmid DNA in *S. jonesii* were those of Anderson and McKay (2) and Azad *et al.*, (3). Degradation of 2,3 DHP was assessed by a colorimetric method Matsumoto and Sherman (8) which detects the presence of a chromogenic complex between the 2,3 DHP and Fe^{3+}.

To asses the effect of aeration, growth of *S. jonesii* and degradation of 2,3 DHP was monitored (3 replicates) in anaerobic or partially aerated tubes. Aerated tubes were those inoculated by opening the cap to air, and then closed, or anaerobically inoculated to which 4 ml of gas phase were replaced by filtered air (0,22µm millipore).

To determine whether cultures in stationary phase (resting cells) were able to further degrade additional 2,3 DHP, cultures where grown on the modified 98-5 medium containing 4,5mM 2,3DHP, for 5 days. Once all 2,3 DHP was degraded (after 5 days of incubation), 1ml of a solution 20mM 2,3 DHP in saline medium was added to the cultures (final concentration 2mM; n= 6) and 2,3DHP degradation was monitored..

To determine the effect of different media on the degradation of 2,3 DHP by resting cells, pellets of 5 days cultures (after centrifugation at 1340 g for 10 min), ussualy reaching an ODmax of 0,35, were resuspended to their original cell density, in resuspension media containing 2mM 2,3 DHP. These media were: modified 98-5; saline medium supplemented with

either clarified rumen fluid (30% v/v), phytone (Bacto Dickinson, 3% w/v), pyruvate (50mM), arginine (10mM), VFA, essential amino acid mixture (5% v/v, Microbiological Asoc., Maryland, USA), or vitamin solution (2% v/v).

Cell free extracts were obtained from 12 L cultures, after passing the cell pellets through a french press and ultracentrifugin the supernatants. Hydrogenase activity in the extracts was determined by reduction of methyl viologen.

Results and discussion

S. jonesii grew on media modified 98-5 and saline-phytone, while saline media containing pyruvate, rumen fluid or arginine, did not support growth. Degradation of 2,3 DHP in modified 98-5 typically occurred once an optical density of the cultures of about 0,2 was reached, and complete degradation was achieved between days 2 and 5 of incubation. Specific activity, as defined as mM 2,3 DHP degraded OD^{-1} min^{-1} was 3.29. If additional 2,3 DHP was added to the culture at day 5 (after no 2,3 DHP was left in the original culture), then degradation occurred within the first 12 hours and specific acticity increased to 547,75, over 150 fold, with no further growth.

Resting cell pellets were able to degrade 2,3 DHP with a similar specific activity to that of stationary cultures when resuspended in either modified 98-5, saline-phytone, saline-pyruvate, rumen fluid or arginine, with no further growth observed. Resuspension of pellets in saline-vitamin solution, saline-VFA, or saline-amino acids did not allow degradation of 2,3 DHP (2 mM).

Aerobic conditions did not allow grow of *S. jonesii,* and partial aeration decreased growth, and degradation activity. Specific acitivity decreased from 3,49 in anaerobic cultures, to 2,04 with 4 mL air, and 1,33 in cultures inoculated in open air .

We have found lost of 2,3DHP degradation activity in cultures maintained in the incubator for two months in medium lacking 2,3 DHP. Refrigerated cultures also lost activity in variable time periods. Inactive cultures were capable of growing in medium containing 2,3 DHP. Contamination of these cultures as possible cause of loss of acticity was discarded. One of the cultures inactivated after two months in the incubator recovered the activity after 34 days of incubation in medium containing 2,3 DHP (the complete degradation of the medium 2,3DHP occured within the last week week of incubation).Cultures stored in the refrigerator in the absence of 2,3 DHP for more than 4 months, irreversibly lost the capability to degrade 2,3 DHP.

The induction of temporary or permanent loss of activity by the absence of the toxin in the culture medium could not be explained by the presence of plasmids, which were not found in *S. jonesii.* Catabolite represion remains to be tested to explain the dissapearance of activity.This phenom-

enon is highly relevant when performing screenings of 2,3 DHP-degrading activity in rumen contents and in the *in vitro* manipulation of active cultures. In *in vivo* conditions, the relatively common observation of a progressive dietary change when introducing a toxic plant in the diet to avoid intoxication, would support the hipothesis of increase of 2,3DHP-degrading activity induced by the presence of 2,3 DHP. However, evidence of persistence of 2,3 DHP-degrading bacteria has been reported in cattle after six months fed a diet lacking *Leucaena* (6).

The 2,3 DHP is degraded in cell free systems, under both N_2 or H_2 atmosphere, given the system contains pyruvate or methyl viologen, respectively. Adition of NADH or cytochrom C did not alter degradation activity in any of the systems. The results suggest that neither NADH is a reducing intermediate, nor cytochrom C is an electron transporter.

From the results reported in this work, we conclude that degradation of the 2,3 DHP occurs after late exponential growth, being highest in stationary cultures. The lack of 2,3DHP in culture medium causes loss of degrading activity, which can be in some cases reverted, by exposure to 2,3DHP. This phenomenon is relevant to the conditions of *in vitro* manipulation of active degrader cultures but its relevance in *in vivo* conditions is not clear.

Acknowledgements

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