



Polyamine biosynthesis in *Phytomonas*: Biochemical characterisation of a very unstable ornithine decarboxylase

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ABSTRACT

The metabolism of polyamines as well as their functions as growth regulators in plants have been extensively studied for many years. However, almost nothing is known about the biosynthesis and roles of these substances in *Phytomonas* spp., parasites of several plants. We have used HPLC and electrophoretic analyses to investigate the presence and metabolism of polyamines in *Phytomonas Jma* strain, detecting both putrescine and spermidine but not spermine. Experiments carried out by incubation of intact parasites with labelled ornithine or putrescine showed the formation of radioactive putrescine or spermidine, respectively. These results indicated that *Phytomonas Jma* can synthesise these polyamines through the action of ornithine decarboxylase (ODC) and spermidine synthase. On the other hand, we could not detect the conversion of arginine to agmatine, suggesting the absence of arginine decarboxylase (ADC) in *Phytomonas*. However, we cannot ensure the complete absence of this enzymatic activity in the parasite. *Phytomonas* ODC required pyridoxal 5'-phosphate for maximum activity and was specifically inhibited by α -difluoromethylornithine. The metabolic turnover of the enzyme was very high, with a half-life of 10–15 min, one of the shortest found among all ODC enzymes studied to date. The parasite proteasome seems to be involved in degradation of the enzyme, since *Phytomonas* ODC can be markedly stabilized by MG-132, a well known proteasome inhibitor. The addition of polyamines to *Phytomonas* cultures did not decrease ODC activity, strongly suggesting the possible absence of antizyme in this parasite.

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1. Introduction

Trypanosomatids of the genus *Phytomonas* are digenetic parasites that infect a wide variety of plants including coffee, coconut and palm oil trees. They are responsible for various diseases causing important economic losses (Dollet, 1984).

The life cycle of these pathogens involves phytophagous insects acting as intermediate hosts and plants as the main hosts (Jankevicius et al., 1989). There are three different groups of flagellated parasites included in the genus *Phytomonas* that infect different types of tissues: one group is found in the latex vessels of some laticiferous plants; the second group in fruits and seeds and a third one is found only in the phloem sap of certain Latin American plant species (Dollet, 2001). Despite the economic losses they cause, little is known about *Phytomonas* life cycle and metabolism.

The polyamines spermidine, spermine and their diamine precursor putrescine are essential constituents of both eucaryotic and procaryotic cells (Algranati et al., 1990; Igarashi and Kashiwagi,

2000). They are small aliphatic molecules, which are positively charged at physiological pH and play important roles in cell proliferation and differentiation (Heby, 1980; Heby et al., 1987). For this reason polyamine metabolism has been proposed as an important target for potential development of anti-proliferative strategies (Pegg, 1988; González et al., 1991; Müller et al., 2001; Heby et al., 2007).

Putrescine can be synthesised by two different pathways. One route involves the direct decarboxylation of ornithine through the action of ornithine decarboxylase (ODC) (EC 4.1.1.17), an enzyme specifically and irreversibly inhibited by the compound α -difluoromethylornithine (DFMO) (Metcalf et al., 1978; Hunter et al., 1991). In the alternative pathway, arginine is first converted into agmatine by arginine decarboxylase (ADC) (EC 4.1.1.19) (Cohen, 1998; Hanfrey et al., 2001). Subsequently, in bacterial systems, agmatine is transformed into putrescine and urea (Morris and Pardee, 1966; Satishchandran and Boyle, 1984), while in plants agmatine is first converted into *N*-carbamylputrescine and then into putrescine, CO₂ and NH₃ (Smith, 1975; Yanagisawa and Suzuki, 1981).

Most trypanosomatid protozoa studied to date require polyamines for their proliferation. In fact, part of the intracellular spermidine is conjugated with two molecules of glutathione, giving rise

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to bis-glutathionylspermidine (trypanothione) (Fairlamb et al., 1985; Fairlamb and Cerami, 1992). This compound, only found in the trypanosomatidae family, has an essential role in these parasites' redox equilibrium and functions as an antioxidant molecule which replaces 70% of the free intracellular glutathione (Wilkinson et al., 2000; Ariyanayagam and Fairlamb, 2001).

Trypanosomatids can obtain polyamines in different ways. *Trypanosoma brucei* does not transport polyamines; therefore putrescine synthesised by ODC is the only source of intracellular polyamines for this parasite (Li et al., 1996) and this fact probably makes *T. brucei* sensitive to DFMO treatment (Bacchi et al., 1980). *Crithidia fasciculata* and *Leishmania mexicana* transport polyamines poorly, but this mechanism is induced and becomes essential when ODC is inhibited and polyamines are depleted (González et al., 1991, 1992; González and Algranati, 1994). On the other hand, *Trypanosoma cruzi* is auxotrophic for polyamines and its survival depends exclusively on polyamine uptake from external media (Algranati et al., 1990; Carrillo et al., 1999, 2007).

In this work we have shown that promastigotes of *Phytomonas Jma* strain (which is isolated from vegetal latex) are able to synthesise putrescine only by ODC action; this enzyme has been characterised as one of the most unstable ODCs described to date and its activity is not regulated by polyamine intracellular concentrations. These facts strongly suggest the absence of antizyme in *Phytomonas Jma*. We have also demonstrated that spermidine is essential for normal proliferation of this parasite.

2. Materials and methods

2.1. Chemicals

L-Amino acids, polyamines, the ODC cofactor pyridoxal 5'-phosphate (PLP), protease inhibitors, the protein biosynthesis inhibitor cycloheximide (CH) and the spermidine synthase inhibitor cyclohexylamine (CHA) were purchased from Sigma (St. Louis, MO, USA); MG-132, a specific proteasome inhibitor, was from Calbiochem (San Diego, CA, USA); L-[1-¹⁴C] ornithine (57.1 Ci/mol), L-[U-¹⁴C] ornithine (266 Ci/mol), L-[U-¹⁴C] arginine (346 Ci/mol) and [U-¹⁴C] putrescine (110 Ci/mol) were from NEN Life Science

Products, Inc. (Boston, MA, USA). DFMO was a gift from Merrell Dow Research Institute (Cincinnati, OH, USA).

2.2. Parasite cultures

Phytomonas Jma culture, isolated from the latex of *Jatropha macrantha* (Euphorbiaceae), was originally obtained from the "Trypanosomatid Culture Collection" – University of São Paulo, Brazil (TCC-USP) (kindly provided by Dr. Camargo). Cells were grown at 28 °C in a semi-synthetic medium (SDM79) which contains only traces of polyamines (Carrillo et al., 1999), supplemented with 5% FCS. Parasite growth was followed by cell counting. All cultures were diluted to 10×10^6 cells/ml every 48–72 h, when the stationary phase was reached. Inhibitors DFMO or/and CHA were added when indicated (see Section 3.1 and Fig. 1).

2.3. Determination of polyamine intracellular concentrations by HPLC

Phytomonas promastigotes collected at the exponential or stationary phase of growth were resuspended in 0.5 ml of PBS (10^9 cells/ml); 25 μ l of 100% trichloroacetic acid were added, and after mixing and centrifugation (10 min, 12,000g) the supernatant fluid was diluted with 2 ml of M NaOH. After adding 5 μ l of benzoyl chloride and incubation for 30 min at 37 °C, the mixture was extracted twice with 1 ml of chloroform; the combined organic phase was washed with 1 ml of water and then evaporated until dry. The residue was resuspended in a mixture of H₂O–methanol (45/55 v/v). The resulting samples were injected in a Beckman ODS (C18) column for HPLC analysis, and the elution was performed with a methanol gradient (from 55% to 87%). The detection of benzoyl derivatives of polyamines was done with a UV spectrophotometer operated at 254 nm (Morgan, 1998).

2.4. In vivo labelling with radioactive amino acids or polyamines

Parasites at the exponential phase of growth were collected by centrifugation (10 min, 3000g), washed once with PBS and resuspended in the same buffer at a concentration of 10^9 cells/ml. L-[U-¹⁴C] ornithine, [U-¹⁴C] putrescine or L-[U-¹⁴C] arginine were

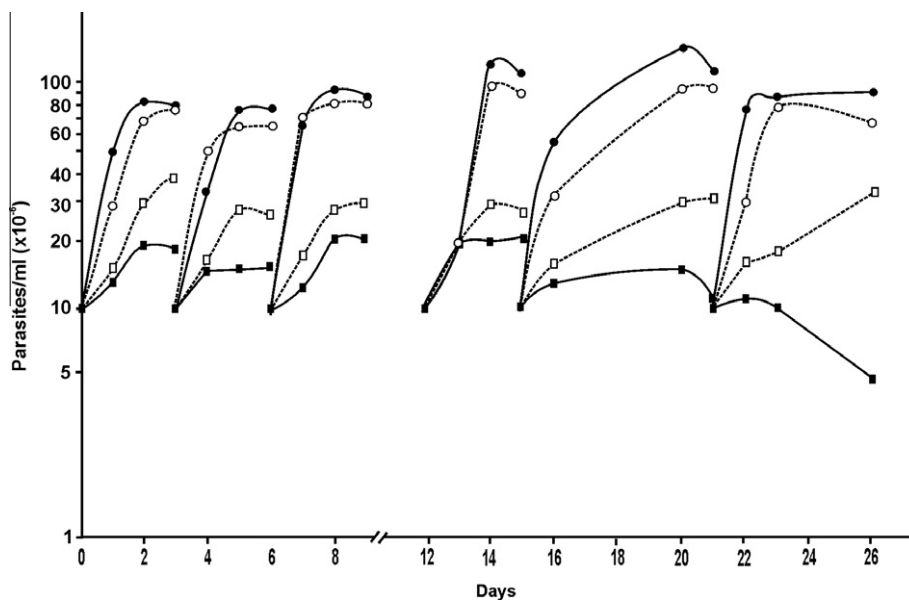


Fig. 1. Growth kinetics of *Phytomonas Jma* strain cultivated in the semi-synthetic medium SDM79. Cultures carried out in the absence or presence of polyamine biosynthesis inhibitors were diluted every 3 days to 10×10^6 cells/ml. All inhibitors were used at 5 mM concentration. Control without inhibitors (●–●); + α -difluoromethylornithine (DFMO) (○–○); + cyclohexylamine (CHA) (□–□); + DFMO + CHA (■–■).

added (5 $\mu\text{Ci/ml}$, 25 μM final concentration) and after incubation for 4 h at 28 °C, parasites were sedimented, washed with PBS and resuspended in 0.05 ml of 0.2 M perchloric acid. Cell extracts were neutralised with 2 M KOH and precipitates removed by centrifugation. The supernatant fluids were analysed by paper electrophoresis for 3 h at 600 V with pyridine acetate pH 3.5 as running buffer (Cataldi and Algranati, 1989). The radioactive compounds were detected with a radiochromatogram scanner.

2.5. Preparation of cell extracts and ODC assays

Parasites were collected at the exponential or stationary phase of growth. They were washed once with PBS and resuspended at a concentration of 2×10^9 cells/ml in buffer A (50 mM Hepes, pH 7.4, containing 1 mM DTT, 0.5 mM EDTA and 0.1 mM PLP) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM leupeptine and 0.01 mM trans-epoxy-succinyl-L-leucylamido(4-guanidino)butane (E64). Cells were lysed by addition of 0.5% Nonidet P40 at 0 °C followed by two cycles of freeze–thawing, and a subsequent sonication for two periods of 6 s each. Cell lysates were centrifuged for 10 min at 10,000g; supernatant fractions were collected and different aliquots were tested for ODC activity by incubation at 37 °C in the presence of 2 mM L-[1- ^{14}C] ornithine (5 $\mu\text{Ci/ml}$). The enzymatic reaction was performed for the indicated times and stopped by the addition of 0.5 N perchloric acid. The radioactive CO_2 released during the reaction was trapped on a piece of Whatman 3MM paper soaked with 2 M KOH and then measured in a scintillation counter.

2.6. Stoichiometry of ODC reaction

After the ODC reaction using L-[U- ^{14}C] ornithine as substrate, the radioactive CO_2 released was measured as described above. To determine the amount of putrescine formed, an aliquot of the same reaction mixture was analysed by paper electrophoresis as previously described (Cataldi and Algranati, 1989). The radioactive compounds were identified by using pure ornithine and polyamine standards, and radioactivity measured in a scintillation counter.

2.7. Biochemical characterisation of ODC

The enzyme activity from *Phytomonas* was measured at different temperature and pH values. To test the PLP dependence of *Phytomonas* ODC, the parasite extract was prepared in buffer B (50 mM Hepes, pH 7.4, containing 1 mM DTT, 0.5 mM EDTA), similar to buffer A but without PLP, and dialysed for 3 h against the same buffer to eliminate the endogenous free PLP. The enzyme reaction was then assayed at increasing PLP concentrations. Ornithine decarboxylation was also carried out at different substrate concentrations in the absence or presence of the irreversible inhibitor DFMO. The kinetic constants V_{max} and K_m for the substrate were calculated from Lineweaver–Burk plots.

2.8. Metabolic stability of ODC and effect of MG-132

In order to study the turnover rate of ODC from *Phytomonas Jma* and the possible role of proteasome in enzyme degradation, protein synthesis of parasite promastigotes was blocked by addition of cycloheximide (CH, 50 $\mu\text{g/ml}$) to an exponentially growing culture. Another parasite culture was incubated in parallel for 16 h with 50 μM MG-132 (final concentration) before adding CH. Aliquots from both cultures were taken at different times and the residual enzyme activity was measured in all samples. Since MG-132 solutions were prepared in 0.2% DMSO, the pure solvent was added to control cultures.

3. Results and discussion

3.1. *Phytomonas* growth in SDM79 culture medium

Phytomonas Jma promastigotes grew exponentially with a doubling time of approximately 11 h in the semi-synthetic medium SDM79. After 3–4 days the culture reached the stationary phase at a cell density of $80\text{--}100 \times 10^6$ parasites/ml (Fig. 1). The kinetic characteristics of growth were similar to those previously described in Liver Infusion Tryptose (LIT) medium (Cánepa et al., 2007).

The addition of DFMO to *Phytomonas Jma* cultures did not alter the growth rate, as was previously seen in *C. fasciculata* and *T. cruzi* (Ceriani et al., 1992; Carrillo et al., 1999). However, the spermidine synthase inhibitor CHA decreased growth and the simultaneous presence of DFMO and CHA strongly affected the parasite proliferation after a few days (Fig. 1). In this case the doubling time was 23 h and the maximum cell density was 27×10^6 parasites/ml. After an extended period of incubation with both inhibitors, parasites stopped growing and then lysed. These results indicate that polyamines are synthesised by *Phytomonas Jma*, which require a critical intracellular level of spermidine for normal proliferation. The essential role of polyamines for growth was previously reported in *T. cruzi*, *Leishmania* spp. and other unrelated parasites such as *Entamoeba* spp. and *Giardia lamblia* (Gillin et al., 1984; Calvo-Mendez et al., 1993; Arteaga-Nieto et al., 1996; Carrillo et al., 2000; González et al., 2001).

3.2. Polyamine intracellular concentrations in *Phytomonas Jma*

An exponential culture of *Phytomonas* showed levels of intracellular putrescine and spermidine of 7 and 20 nmol/ 10^8 parasites, respectively. The presence of DFMO in the culture medium reduced putrescine to undetectable levels and spermidine to about 17% of control (Table 1), indicating that this residual concentration of spermidine seems to be enough to maintain the proliferation rate of these parasites even under conditions of an almost complete absence of putrescine (Fig. 1). The marked inhibition of growth observed with *Phytomonas Jma* cultivated in the presence of DFMO + CHA was concomitant with the reduction of both putrescine and spermidine to almost undetectable levels (Table 1). These results agree with the conclusion that a minimum concentration of spermidine is necessary for *Phytomonas Jma* proliferation. We could not detect spermine in *Phytomonas Jma* extracts (data not shown).

3.3. Polyamine biosynthesis in *Phytomonas Jma*

When growing cultures of *Phytomonas Jma* were incubated with L-[U- ^{14}C] ornithine, radioactive putrescine was formed (Fig. 2A); pre-incubation with DFMO for 2 days greatly decreased the labelled putrescine as shown in Fig. 2B. Although the presence of spermidine was not detectable in this experiment, the synthesis of this polyamine was observed when parasite cultures were supplemented with radioactive putrescine (Fig. 2C). Our results

Table 1
Polyamine intracellular concentrations in *Phytomonas Jma* strain.

Culture conditions	Putrescine (nmoles/ 10^8 cells)	Spermidine (nmoles/ 10^8 cells)
Control culture ^a	7 \pm 2	20 \pm 3
+DFMO ^b	<0.08	3.4 \pm 0.5
+DFMO + CHA ^c	<0.3	<0.1

Phytomonas were cultivated in SDM79 medium without (control)^a or with 5 mM α -difluoromethylornithine (DFMO)^b or DFMO + cyclohexylamine (CHA) (5 mM each)^c.

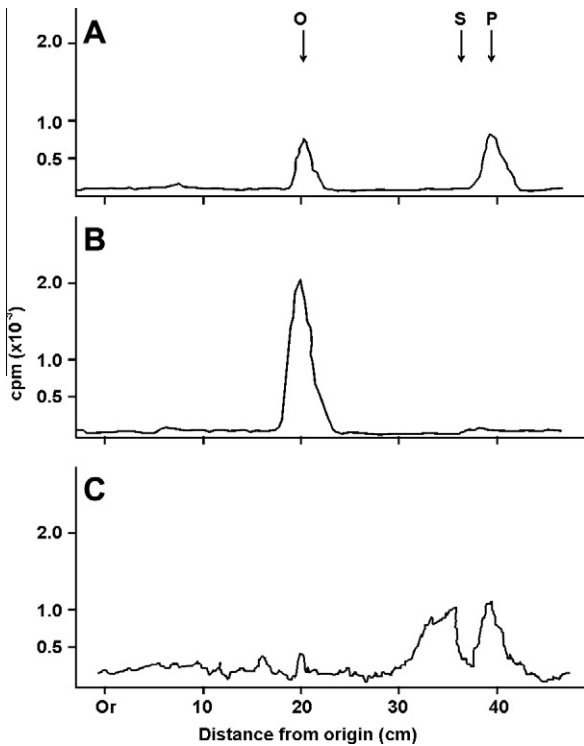


Fig. 2. Polyamine biosynthesis in *Phytomonas Jma* strain. Exponentially growing parasites were collected and incubated with L -[U - ^{14}C] ornithine or [U - ^{14}C] putrescine as indicated in Section 2. Cell extracts were then analysed by paper electrophoresis. Radioactive products were detected with a radiochromatogram scanner. (A) Control culture of *Phytomonas*. (B) Parasite culture was incubated with 5 mM α -difluoromethylornithine (DFMO) for 48 h before the addition of the radioactive amino acid. (C) The parasite culture was incubated with radioactive putrescine (for 8 h). O, S and P indicate the position of standard ornithine, spermidine and putrescine, respectively. cpm, counts per minute.

demonstrate the presence of ODC and spermidine synthase enzymatic activities in *Phytomonas Jma* promastigotes. Radioactive agmatine did not appear after addition of L -[U - ^{14}C] arginine to *Phytomonas* cultures (data not shown), indicating very low levels or the absence of ADC activity in these parasites.

3.4. Biochemical characterisation of *Phytomonas Jma* ODC

Ornithine decarboxylation was detected in *Phytomonas* cell extracts. This ODC activity showed the expected 1:1 stoichiometric

relationship between putrescine synthesised and CO_2 released during the reaction. The same parasite extracts were also able to decarboxylate lysine with a rate 100-fold less than the ornithine decarboxylation, as was previously described for ODC from other organisms (Eichler, 1989).

The specific activity of the enzyme was maximal (7.5 nmol/h/mg protein) at the early log phase of growth, concomitant with the maximum proliferative capacity of the culture, and decreased markedly at the stationary phase (Fig. 3A). Based on these findings, subsequent studies on ODC were carried out in extracts from early log phase cells.

The kinetic parameters of ODC calculated from a Lineweaver-Burk plot indicated a V_{max} value of 8 ± 2 nmol/h/mg of protein and an apparent K_m for ornithine of 0.4 ± 0.1 mM. These values were of the same order as those obtained for ODC from the related parasite *C. fasciculata* (Ceriani et al., 1992).

DFMO inhibited the ODC reaction in a dose-dependent way, with a half maximal inhibitory concentration (IC_{50}) of 0.2 ± 0.05 mM (Fig. 3B). The inhibitory effect of DFMO on ODC activity could explain the reduction of endogenous polyamine levels previously observed (Table 1 and Fig. 2).

The ODC reaction performed *in vitro* under different conditions indicated that optimal temperature and pH were 45 °C and 6.4, respectively. ODC activity from *Phytomonas Jma* was partially dependent on PLP concentration, a cofactor for most of cellular decarboxylases (Poulin et al., 1992). PLP increased the catalytic activity approximately fivefold, with an $S_{0.5}$ value of 10–12 μ M (Fig. 3C).

3.5. Metabolic stability of ODC and effect of MG-132

Experiments on enzymatic stability of *Phytomonas Jma* ODC showed an extremely short half-life of 12 ± 2.5 min (Fig. 4A). This enzyme seems to be one of the most unstable ODCs described in kinetoplastids and other organisms (Hayashi and Murakami, 1995). A high turnover has been proposed as essential for rapid and dramatic changes in the enzyme and product levels (Berlin and Schimke, 1965). The *Phytomonas* ODC short half-life can explain why, despite the enzyme sensitivity to DFMO, cell proliferation is not affected by this inhibitor. The parasite might continuously produce new molecules of the enzyme which, before being inactivated, might synthesise minimal putrescine levels able to maintain the critical spermidine concentrations needed for normal proliferation, as we have proposed for other trypanosomatids (Carrillo et al., 2000). On the other hand, it is known that ODC of

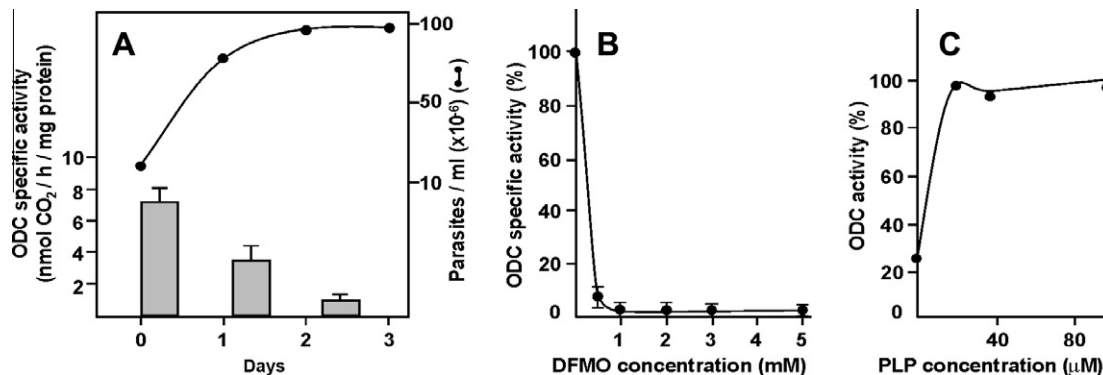


Fig. 3. Ornithine decarboxylase (ODC) activity in cell extracts of *Phytomonas Jma* strain. (A) ODC-specific activity measured in extracts obtained at different stages of parasite growth. (B) ODC activity in extracts of exponentially growing *Phytomonas*. Assays were carried out in the absence or presence of α -difluoromethylornithine (DFMO) at different concentrations. (C) ODC activity assayed *in vitro* at different concentrations of pyridoxal 5'-phosphate (PLP). Values in B and C are given as percentages of maximum activity. All other details are as indicated in Section 2.

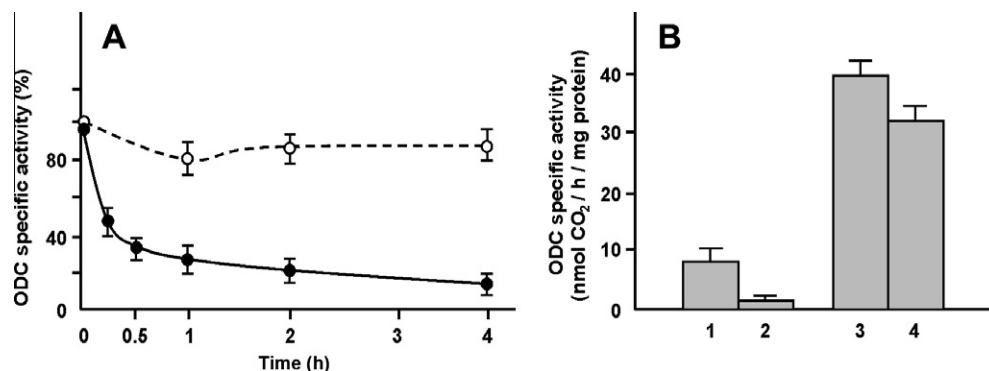


Fig. 4. Stability of *Phytomonas* ornithine decarboxylase (ODC) and effect of the proteasome inhibitor MG-132 on the metabolic turnover. (A) Cultures of *Phytomonas Jma* strain were incubated for 16 h in the presence of DMSO (●-●, control) or 50 μ M MG-132 (○-○). Cycloheximide (CH) (50 μ g/ml) was then added to both cultures and aliquots were taken thereafter to measure the remaining enzyme-specific activity. (B) Parasite cultures were treated as in (A) and ODC-specific activity was measured at the time of CH addition (bars 1 and 3) and 3 h later (bars 2 and 4). ODC activity in the absence (bars 1 and 2) or presence (bars 3 and 4) of MG-132. All values are the average \pm SD of three experiments.

T. brucei is stable and this parasite growth is permanently blocked by DFMO. For this reason, this drug has been used as a cure for acute infections of mice and human sleeping sickness (Bacchi et al., 1980; Van Nieuwenhove et al., 1985).

Some inhibitors of proteases such as E64 and leupeptine did not affect the stability of ODC (data not shown). In contrast, the proteasome inhibitor MG-132 (Lee and Goldberg, 1996, 1998) stabilized the enzyme indicating that the proteasome is involved in *Phytomonas Jma* ODC degradation (Fig. 4A and B) as was previously found for mammalian cells (Murakami et al., 1992).

A pre-incubation of *Phytomonas Jma* cultures with exogenous 0.1 mM putrescine or spermidine for different times did not show any effect on the ODC activity. This result rules out the possibility that polyamines might regulate the ODC turnover through any of the alternative feedback mechanisms observed in other organisms, such as the antizyme regulation of mammalian ODC (Canellakis et al., 1985; Matsufuji et al., 1995).

We have shown that exponentially growing *Phytomonas Jma* contained putrescine and spermidine intracellular levels at a ratio of approximately 1:3 (7 and 20 nmoles/ 10^8 cells, respectively). These polyamines were synthesised from ornithine through the action of *Phytomonas* ODC and spermidine synthase. Our results also indicated very low levels or absence of ADC enzymatic activity in this parasite.

Phytomonas ODC was characterised by the stoichiometry of the catalysed reaction, the effects of specific inhibitors and the activation induced by PLP as a cofactor.

Measurements of the enzyme half-life have indicated that this is one of the most unstable ODCs described to date. It is interesting that, to our knowledge, this is the first report of a digenetic parasite containing an unstable ODC. The stabilization of the enzyme observed in the presence of MG-132 (a proteasome inhibitor), evoked a marked increase in total activity. This result supports the involvement of proteasome in *Phytomonas* ODC degradation. The addition of putrescine and/or spermidine to parasite cultures for 48 h did not affect ODC activity, strongly suggesting the absence of antizyme-regulated mechanisms in *Phytomonas Jma*.

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