**L-Proline is essential for the intracellular differentiation of *Trypanosoma cruzi***

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**Summary**

Using as the host cell, a proline-requiring mutant of Chinese hamster ovary cell (CHO-K1), it was possible to arrest the differentiation of amastigost forms of *Trypanosoma cruzi* at the intermediate intracellular epimastigote-like stage. Complete differentiation to the trypomastigote stage was obtained by addition of L-proline to the medium. This effect was more pronounced using the *T. cruzi* CL-14 clone that differentiates fully at 33°C (permissive temperature) and poorly at 37°C (restrictive temperature). A synchronous differentiation of *T. cruzi* inside the host-cell is then possible by temperature switching in the presence of proline. It was found that differentiation of intracellular epimastigotes and trypomastigote bursting were proline concentration dependent. The intracellular concentration of proline was measured as well as the transport capacity of proline by each stage of the parasite. Amastigotes have the highest concentration of free proline (8.09 ± 1.46 mM) when compared to trypomastigotes (3.81 ± 1.55) or intracellular epimastigote-like forms (0.45 ± 0.06 mM). In spite of having the lowest content of intracellular free proline, intracellular epimastigotes maintained the highest levels of L-proline transport compared to trypomastigotes and intracellular amastigotes, providing evidence for a high turnover for the L-proline pool in that parasite stage. This is the first report to establish a relationship between proline concentration and intracellular differentiation of *Trypanosoma cruzi* in the mammalian host.

**Introduction**

The aetiological agent of Chagas’ disease, *Trypanosoma cruzi*, is a flagellate protozoan that survives in a wide range of environmental conditions undergoing complex morphological changes throughout the life cycle in both the insect vector and the vertebrate host. Traditionally, four forms of the parasite have been described in the literature: intracellular amastigotes and bloodstream trypomastigotes in the mammalian host and epimastigotes and metacyclic trypomastigotes in the reduvid vector. In addition to these polar morphologies other intermediate forms were described in the parasite cell cycle, leading some authors to suggest a continuous transformation among stages following essentially the same path regardless if they existed in a vertebrate or in an invertebrate host (Almeida-de-Faria et al., 1999; Tyler and Engman, 2001). Particularly, an epimastigote-like form was detected in the vertebrate host and in tissue-cultured cells (Meyer and de Oliveira, 1948; Faucher et al., 1995; Almeida-de-Faria et al., 1999; Tyler and Engman, 2001). The intermediate forms appear transiently in the differentiation of amastigotes to trypomastigotes and share characteristics of both stages, although their predominant properties are those from epimastigotes, including the general morphology (Almeida-de-Faria et al., 1999).

*Trypanosoma cruzi* is capable of using carbohydrates and amino acids as carbon and energy sources. Preferentially, epimastigotes catabolize glucose and, after carbohydrate exhaustion, a shift to amino acid metabolism occurs with a large amount of ammonia excreted into the medium (Cazzulo, 1994 and references herein). Although no reserve of polysaccharides has been detected in *T. cruzi*, the parasite stores proteins in special organelles (reservosomes) that are mobilized under nutritional stress conditions (cf. Urbina, 1994). Proline is catabolized by *T. cruzi*, as well as by many other trypanosomatids, as *T. brucei* (Evans and Brown, 1972; Ter Kuile and Opperdoes, 1992), *L. donovani* (Kraussner and Flory, 1972), *H. roitmani* (Faria-e-Silva et al., 2000) and *T. congolense* (Obungu et al., 1999). Transporters for proline have been characterized from bacteria to mammalian cells, and in the particular case of *T. cruzi* two active transporters for L-proline were recently described (Silber et al., 2002). Interestingly, both transport systems are not inhibited by D-proline, an amino acid that may be present in the parasite as a
product of proline racemases, enzymes that are intracellularly located in epimastigotes and membrane-bound and shed in infective stages (Reina-San-Martin et al., 2000; Chamon et al., 2003).

L-proline or intermediates of proline metabolism support differentiation of Trypanosoma cruzi epimastigotes to metacyclic trypomastigotes (metacyclogenesis) (Homsy et al., 1989; Contreras et al., 1985) as well as the differentiation of T. congolense (Ross, 1987). Leucine and isoleucine, inhibitors of pyrroline-5-carboxylate dehydrogenase, and some di- and tricarboxylic acids as oxaloacetate, isocitrate and α-ketoglutarate inhibit proline-induced metacyclogenesis. Trypanosoma cruzi, like other organisms, is capable of catabolizing L-proline via proline oxidase to meet the necessary energy requirements (Evans and Brown, 1972; Krassner and Flory, 1972; Sylvester and Krassner, 1976).

The presence of an intracellular epimastigote-like form of T. cruzi immediately raises the question whether this stage is an obligatory intermediate step in the differentiation of intracellular amastigotes to infective trypomastigotes. Moreover, if that is the case, there is the possibility that the differentiation process could be driven by similar mechanisms described for metacyclogenesis.

A role for L-proline in the differentiation of the intracellular epimastigote-like forms to trypomastigotes has been demonstrated herein, confirming that the intracellular differentiation of T. cruzi amastigotes to trypomastigotes occurs via the epimastigote-like form. The specific activity of the L-proline transport and the contents of free proline in the intracellular stages have been measured. As previously described for some strains (Brner et al., 1976), temperature was found to be a determining factor for the differentiation process.

**Results**

**Effect of L-proline on trypomastigote bursting**

Temperature in some strains of T. cruzi is a variable affecting vertebrate host-cell infection (Neva et al., 1961). Because in the particular case of the CL strain, optimal differentiation of amastigotes to trypomastigotes takes place in infected cells kept at 33°C instead of 37°C (Brner et al., 1976), the thermo-sensitivity of the infection system for the CL-14 clone (a clone derived from the CL strain) was evaluated. The number of extracellular motile trypomastigote forms was 7–10-fold higher when infected CHO-K1 cells were maintained at 33°C instead of 37°C (not shown). From this observation, the temperatures of 33°C and 37°C were defined as permissive and restrictive for infection respectively. Interestingly, temperature does not affect the earlier steps of the parasite intracellular cell cycle: the infection step itself and the differentiation of the trypomastigote to the amastigote form and its multiplica-

![Graph](image)

**Fig. 1.** Effect of proline and temperature on trypomastigote bursting. Infected CHO-K1 cells were maintained in DMEM-2% FCS supplemented or not with L-proline. The trypomastigotes released into the medium were counted from the fifth to tenth day post infection with Trypanosoma cruzi under the following conditions: not supplemented (∆) and supplemented with 200 μM (●) or 20 μM (●) L-proline. Inset: the total number of extracellular motile trypomastigotes collected in the medium from the fifth to the tenth day post infection was determined for cultures grown at 33°C (□) and 37°C (■) in the absence of proline or in the presence of 20 μM or 200 μM added proline. The plot represents the average number of trypomastigotes ± S.D. from three independent experiments. The data were compared for statistical significance using the Student’s t-test and P<0.05 when each condition, temperature or proline concentration, were compared to each other.

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proline was added, provided that old medium is constantly replaced by fresh medium.

Because it was observed that the temperature is a relevant variable in the differentiation of the intracellular stages of T. cruzi, the effect of L-proline at the permissive and restrictive temperature was evaluated. The CHO-K1 cells were infected at 37°C and maintained with different proline concentrations at both temperatures (33°C or 37°C). Confirming our previous results, trypomastigote bursting showed to be dependent on temperature and on L-proline concentration (Fig. 1, inset). Cultures supplemented with 200 µM L-proline released the highest number of trypomastigotes at 33°C or 37°C, but the amount of trypomastigotes obtained for each proline concentration was always much higher at 33°C than at 37°C.

Effect of L-proline on intracellular stages
In preliminary experiments, CHO-K1 cells were cultured in the absence of L-proline for 5 days previous to T. cruzi infection and the infected cells maintained in the absence or presence of 200 µM L-proline. The trypomastigote bursting was 7 × 10⁶ and 35 × 10⁶ respectively. L-proline starvation of the host cell previous to infection had no significant effect on trypomastigote differentiation. This result led us to design the following experiment in order to verify at which point of the intracellular cell cycle L-proline is essential for T. cruzi differentiation. CHO-K1 cultures were infected and maintained at 33°C in the absence of proline supplementation up to the second day post infection (see Fig. 2). At this point, when approximately 85% of the observed intracellular forms corresponded to amastigotes (Fig. 3A, a,d) culture medium supplemented with 200 µM L-proline was added to half of the culture flasks. Both cultures (supplemented or not with L-proline) were left to develop to the next well-defined intracellular epimastigote-like stage. At day 5 post infection, when 85% of the parasites were epimastigote-like forms (Fig. 3A, b,e), the medium was removed and half of the culture flasks were supplemented or not with 200 µM L-proline (Fig. 2). The number of trypomastigote released into the medium was counted in all cases (Fig. 3A, c,f). As shown in Fig. 3B, cultures that were maintained in the presence of proline in both intracellular stages (amastigote and epimastigote-like) showed the highest levels of extracellular trypomastigotes (55 × 10⁶ ml⁻¹ tryptomastigotes) when compared to cultures that received proline only at the amastigote stage (1.5 × 10⁶ ml⁻¹ parasites), similar to the cultures that were maintained in the absence of proline in all stages (1.8 × 10⁶ ml⁻¹).

Transport and content of proline in vertebrate host stages
The capacity of the intracellular stages to consume L-proline to support growth and differentiation must be reflected in the ability to transport the amino acid from the extracellular medium to the cytoplasm. In this context, the L-proline transport activity was evaluated in the three developmental stages found in culture cells infected by T. cruzi: intracellular amastigotes, intracellular epimastigote-

Fig 2. Experimental design to evaluate the effect of L-proline on different intracellular stages of T. cruzi. Symbols to the right of the figure correspond to symbols in Fig. 3B.

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The role of proline on the intracellular cycle of *Trypanosoma cruzi*. The intracellular amastigote forms corresponding to the second day post infection (a, e) showed higher capacity to transport L-proline when compared to amastigotes or trypomastigotes, being 30-fold higher compared to trypomastigotes (Fig. 4). The intracellular concentration of free proline was determined in three independent experiments by the ninhydrin method gave similar results. Intracellular epimastigote-like forms have the lowest amount of intracellular free proline (0.45 ± 0.06 mM) compared to trypomastigotes (3.81 ± 1.55 mM) or amastigotes (8.09 ± 1.46 mM) (Table 1).

**Discussion**

The role of proline on the *T. cruzi* intracellular cycle was studied using the proline auxotrophic CHO-K1 cell line as the host cell for the infection system. This cell is defective in the conversion of glutamic acid to glutamic semialdehyde, an early step in the proline synthesis pathway (Kao and Puck, 1967). Thus, the effect of L-proline on the intracellular cycle of the parasite is significant, with a specific accumulation of the epimastigote-like forms. It was found, however, that complete differentiation of these forms to trypomastigotes needed L-proline, in addition to the temperature switching. This effect was confirmed in kinetic experiments where L-proline was added along the parasite intracellular cycle with host cells starved for L-proline previously to the infection step, to minimize the intracellular pool of proline and related intermediate compounds. The effect is specific for L-proline because: (i) glucose, an ubiquitous source of carbon and energy, is present throughout the experiment, and (ii) L-arginine and other amino acids as glutamine, members of the 'glutamate family', are abundant in the culture medium.

This effect on the differentiation of intracellular epimastigote-like forms to trypomastigotes concurs with the observation that L-proline is essential for the *in vitro* differentiation of these stages.

**Table 1.** Proline concentration in different stages of *Trypanosoma cruzi* intracellular differentiation. Parasites were grown in CHO-K1 cells in a 200 μM proline-containing DME medium plus 10% FCS. Results are the average of three independent experiments ± S.D.

<table>
<thead>
<tr>
<th>Stage</th>
<th>[Proline] (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular amastigote</td>
<td>8.09 ± 1.46</td>
</tr>
<tr>
<td>Intracellular epimastigote-like</td>
<td>0.45 ± 0.06</td>
</tr>
<tr>
<td>Trypomastigote</td>
<td>3.81 ± 1.55</td>
</tr>
<tr>
<td>CHO-K1 cells</td>
<td>0.69 ± 0.06</td>
</tr>
</tbody>
</table>

a. Student’s t-test: *P* < 0.005 when amastigotes are compared to epimastigotes and host-cells; *P* < 0.05 when all data are compared to each other.

Fig. 3. A. Time-course of intracellular cycle of *Trypanosoma cruzi*. (a, d) Intracellular amastigote forms corresponding to the fifth day post infection (c, f) intracellular trypomastigote forms on the seventh day post infection. (a, b, c) Phase-contrast microscopy; (d, e, f) Fluorescence microscopy. For fluorescence microscopy parasites were incubated with a polyclonal antibody against *Trypanosoma cruzi* followed by FITC-conjugated secondary antibody and nuclei were stained with rhodamine (Molecular Probes) and can be seen in red.

B. Trypomastigote bursting from the fourth to the tenth day postinfection in 5 ml cultured medium that were maintained in presence of L-proline (●), cultures that were L-proline supplemented in the intracellular epimastigote-like stage (■), cultures that were L-proline supplemented in the intracellular amastigote stage but were free from L-proline on the intracellular epimastigote-like stage (▲) and cultures that were not L-proline supplemented (▲). Each experimental point is the average number of trypomastigotes ± S.D from three independent experiments. Data from sixth to eighth day period were pooled for each curve and compared to each other using the Student’s t-test (*P* < 0.01 or lower).

Fig. 4. L-Proline transport activity in the three developmental stages found in cultured cells infected by *Trypanosoma cruzi* intracellular amastigotes (□), intracellular epimastigote-like forms (■) and trypomastigotes (●).

ferentiation of epimastigotes to trypomastigotes (metacyclogenesis) occurring in an axenic defined medium with a composition similar to the insect vector urine (Contreras et al., 1985). L-proline has been proposed as the source of fuel for parasite cell remodeling (Homsy et al., 1989).

Because proline homeostasis is a function of proline supply, transport across the membrane and metabolism, the contribution of L-proline transport into the parasite cytoplasm, conceivably the earliest step to make it available for consumption, was measured. It was found that intracellular epimastigote-like forms, requiring L-proline to accomplish differentiation to trypomastigotes, have the highest transport activity among the vertebrate parasite stages. Biphasic curves have also been found for intracellular epimastigote-like forms and trypomastigotes when activity was measured against growing L-proline concentrations, similar to the high affinity (Km = 0.35 mM) and low affinity (Km = 1.36 mM) L-proline transport systems described by our group in epimastigotes grown in liquid axenic medium (Silber et al., 2002). Measurement of the intracellular concentration of free proline among the T. cruzi stages belonging to the vertebrate phase of the cycle showed that amastigotes have the highest intracellular concentration (8.09 mM), followed by trypomastigotes (3.8 mM), both higher than that found in non-infected, host cells (0.69 mM). In the particular case of the intracellular epimastigote-like forms, the intracellular proline concentration of 0.45 mM was approximately 20-fold lower than that in amastigotes and 1.5-fold lower than the concentration of the amino acid in the host cells. Conceivably, the large proline pool accumulated in amastigotes is used for the beginning of their differentiation to epimastigote-like forms. The latter, in turn, need to somehow activate their transporters in order to maintain a steady supply of proline for completion of the differentiation process to trypomastigotes. In fact, the data suggest that the high affinity L-proline transport is fully active in intracellular epimastigote-like and trypomastigote forms. In particular, the epimastigote-like forms have the highest L-proline transport activity.

Taking into account the relatively lower amount of proline in the urine of Rhodnius (0.087 mM) (Maddrell and Gardiner, 1980) and in CHO-K1 cells (0.69 mM), the importance of, at least, the high affinity proline T. cruzi transporter becomes evident when one considers the differentiation of axenically grown epimastigotes to metacyclic trypomastigotes (Contreras et al., 1985) and intracellular epimastigote-like forms, herein described, to trypomastigotes. Altogether, the present evidence points to proline as a key metabolite for parasite remodeling to the trypomastigote stage, thus completing the parasite cycle in the invertebrate as well as the vertebrate host cell.

It is possible that the low proline transport activity found in amastigotes could account for the accumulation of a large free proline pool, provided that at this stage proline is not used for other purposes. However, one can hypothesize that the high free proline concentration found in amastigotes could be a consequence of a high rate of endogenous protein breakdown. The source of this putative hydrolyzable protein remains to be determined since in spite of the fact that T. cruzi is able to stock proteins in organelles called reservosomes they only exist abundantly in axenically grown epimastigotes. In fact, reservosomes are drastically reduced in size during in vitro differentiation of epimastigotes to metacyclic trypomastigotes and are absent in trypomastigotes (Soares et al., 1989) and intracellular epimastigotes (Almeida-de-Faria et al., 1999). Another possibility for the amastigotes to obtain a supply of proline would be to export proteases to the host-cell cytoplasm and import proline-containing hydrolysed peptides through specific peptide transporters as it occurs in Lactococcus lactis (Smid and Konings, 1990; Lanfermeijer et al., 2000). That possibility should be explored as T. cruzi contains a cisteryl protease (Monteiro et al., 2001; Cazzulo, 2002) and a specific prolyl oligopeptidase (Santana et al., 1997; Grellier et al., 2001) that cleaves proteins at the carboxyl side of proline residues. Trypanosoma cruzi trypomastigotes, which remain mostly in blood, may catabolize glucose considering its abundance in the extracellular medium. In that situation, proline consumption may be facultative as it occurs with axenically grown epimastigotes in the presence of added glucose.

It should be interesting to analyse the free amino acid pool and the transport activity in the host cell during the infection cycle in order to get a clearer picture of the carbon source available for each intracellular stage of the parasite and the interconnection between the parasite and the host metabolisms. In this context, it is important to consider that an upregulation of the transcripts for the high affinity glutamate transporter in the host cell after 24 h post infection by T. cruzi was detected (Avalos et al., 2002). Other carbon sources, mainly glucose, have to be considered to understand when the parasite switches between glucose or proline for better adaptation to different host environments.

Although the ability to metabolize glucose and amino acids, mainly proline, as the primary carbon source is well documented in trypanosomatids, as Leishmania and Trypanosomes (Urbina, 1994), the mechanism triggering either one or the other metabolic pathway is unknown. Glucose sensors that generate a signal for induction of genes encoding hexose transporters were described in Saccharomyces cerevisiae (Ozcan et al., 1998). Proline induction of its transporter has also been described in Aspergillus nidulans (Tazebay et al., 1997) as well as circadian regulation of mRNA level of enzymes related to the metabolism of proline in Arabidopsis thaliana (Hayashi
et al., 2000). Conformational changes of the transcriptional regulator of gene expression are the key events in proline utilization by *Saccharomyces cerevisiae* (Etages et al., 2001).

Although the main role of proline in *T. cruzi* is considered to be a source of energy and carbon skeletons, other possibilities cannot be ruled out. The recent description of proline racemase isofoms (Reina-San-Martin et al., 2000; Chamond et al., 2003) points out to the importance of proline in other metabolic routes. Whereas proline racemase is intracellular in epimastigotes, the enzyme is membrane-bound and secreted into the medium in infective forms (Chamond et al., 2003). At first sight, proline racemase should not be involved in the differentiation mechanism since the concentrations of proline in the host-cell and in the different trypanosome stages are much lower than the reported values for the Km of proline racemase (29–75 mM) measured in epimastigotes. However, one can not rule out the possibility that L- and D-proline enantiomers may exist in equilibrium mainly in the 8 mM free proline pool found in amastigotes. More experimental work is needed to establish a role for proline racemase in *T. cruzi* differentiation.

In short, one may conclude that: (i) the intracellular epimastigote-like forms are an obligatory intermediate step in the differentiation of amastigotes to trypomastigotes, confirming previous claims (Almeida-de-Faria et al., 1999); (ii) L-proline is essential for the differentiation of the intracellular epimastigote-like forms to trypomastigotes; and (iii) the differentiation of the intracellular epimastigote-like forms to trypomastigotes in the CL strain of *T. cruzi* is modulated by temperature, the permissive one being 33°C. The temperature dependence of the CL14 clone herein observed as described for the CL strain (Brener et al., 1976) is the most likely explanation for the low parasitemia observed when experimental animals are infected with the CL-strain or CL-strain derived clones of *T. cruzi*. That observation should be taken into account mainly by the laboratories studying functional genomics in *T. cruzi* as sequencing of the parasite genome is being asayed (Nycomed Pharma AS) for 10 min at 4300 rpm.

Employing temperature sensitive *T. cruzi* clones and host-cells auxotrophic for proline as described herein provide the investigator, for the first time, with a synchronous system to examine in more detail the intracellular differentiation of *T. cruzi*.

**Experimental procedures**

**Cell culture**

The Chinese Hamster Ovary cell line CHO-K1 was purchased from the ATCC (CCL 6.1) and routinely cultivated in RPMI medium (Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS), 0.15% (w/v) NaHCO3, 100 units ml−1 penicillin and 100 μg ml−1 streptomycin at 37°C in a humid atmosphere containing 5% CO2. Whenever a proline free medium was necessary, DME was employed (supplemented with 2% FCS).

**Cell viability**

CHO-K1 was grown on coverslips under the conditions indicated in each experiment. The viability of cells infected or not with *T. cruzi* was evaluated every day by incubation at 37°C for 30 min with ethidium homodimer or calcein AM (Molecular Probes), respectively, followed by fluorescence analysis.

**Parasites**

Epimastigote forms of *Trypanosoma cruzi*, CL-14, a clone derived from the CL strain (Brener and Chiari, 1963) were cultured in liver infusion-tryptose medium (LIT) supplemented with 10% FCS at 28°C (Camargo, 1964). The parasites were kept in exponential growth by subculturing every other day. Intracellular forms and trypomastigotes were obtained by infection of CHO-K1 cells with trypomastigotes, as described (Almeida-de-Faria et al., 1999). Amastigotes and intracellular epimastigotes were purified from CHO-K1 on day 2 and around day 4–5 post infection respectively. Trypomastigotes are collected in the extracellular medium from seventh day on. When necessary, the intracellular forms were purified after disrupting the infected cells with a rubber policeman. After centrifugation, the pellet was suspended in RPMI medium and purified by centrifugation on 5 ml lymphoprep (Nycoderm Pharma AS) for 10 min at 4300 rpm.

**Effect of L-proline on trypomastigotes bursting**

CHO-K1 cells were infected with trypomastigotes in RPMI medium supplemented with 2% FCS. After 3 h at 37°C, free trypomastigotes in the medium were removed by washing with phosphate-buffered saline (PBS), and incubated with 5 ml of DME medium (Sigma), supplemented or not with different concentrations of L-proline (20 or 200 μM) at 33°C or 37°C. Depending on the experiment, intracellular forms or motile trypomastigotes recovered every day from the supernatant were counted in a haemocytometer.

**Transport assays**

Transport assays were performed as previously described (Silber et al., 2002). Different stages of *T. cruzi* were washed three times by centrifugation and resuspended in phosphate-buffered saline (PBS) pH 7.4 (intracellular amastigotes and epimastigotes forms) or RPMI buffer solution (trypomastigotes) and distributed in aliquots of 100 μl (2 x 107 parasites). Transport assays were initiated by the addition to the experimental tube of 100 μl of the desired dilution of L-proline in PBS in the presence of 0.5 μCi of

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L-[2,3H]-proline. V0 was measured at 28°C for 30 s by incorporation of radiolabelled L-proline in two proline concentrations: 0.75 mM or 3.0 mM. In all cases proline transport was stopped by addition of 800 μl of cold 50 mM L-proline in PBS followed by rapid washing by centrifugation at 10 000 g for 60 s.

Intracellular proline quantification

Two methods have been used to quantify free intracellular proline. Parasites (1 × 10^6) were washed in cold PBS, suspended in 1 ml of PBS and disrupted by sonication. The lysate was centrifuged (100 000 g, 30 min), and the supernatant was freed of proteins by TCA precipitation followed by centrifugation at 20 000 g for 30 min. Measurement of intracellular proline was made in the remaining supernatant solution, as described (Bates, 1973). Briefly, 200 μl of the supernatant were incubated with 200 μl of acid-ninhydrin (0.25 g ninhydrin dissolved in 6 ml glacial acetic acid and 4 ml 6 M phosphoric acid) and 200 μl of glacial acetic acid for 1 h at 100°C. The tubes were then transferred to an ice bath to stop the reaction and the mixtures extracted with 400 μl of toluene. The toluene phase was separated and the absorbance was read at 520 nm. The results obtained with the ninhydrin method were of the same order of magnitude as those obtained by the amino acid analyzer as follows: the amino acid contents analyzed were performed in a Shimadzu HPLC-LC10A/C-R7A system (Shimadzu Corporation, Tokyo, Japan). The pH of the sample solution was adjusted to about 2.20 by adding 6 N hydrochloric acid and dried in a Speed Vac Plus systems. The sample was dissolved in 0.2 N sodium citrate, pH 2.2 and the amino acid contents analysed. The values were means of three determinations and were expressed in nanomoles of proline present.

Determination of water content

Water content was measured by a gravimetric method. Cells (1 × 10^6) were washed in cold PBS and centrifuged (10 000 g, 15 min). The pellets were weighed before and after an overnight lyophilization and the difference was assumed to correspond to the loss of water. Because the weight loss was determined at room temperature, the equivalence 1 g = 1 ml was utilized as an approximation. The measured water volume was 8.06 μl per 10^6 cells. This value was found to be consistently higher (2.5-fold) than that reported by Rohloff et al. (2003) who employed a more precise method for water determination (Y strain). Thus, all proline concentrations herein described were corrected using that factor.

Protein determination

The amount of protein was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

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References


