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ORIGINAL PAPER

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Biochemical characterization of new strains of *Trypanosoma cruzi* and *T. rangeli* isolates from Peru and Mexico

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Abstract Seven trypanosome stocks isolated have been characterized by lectin agglutination, isoenzyme analysis, and the end products excreted. The stocks were isolated from different geographic areas—one from Mexico (TM5), and six from Peru, four of these isolated from different species of triatoma (TP504, TP702, TP704 and TP706), the other two isolated from the salivary glands of *Rhodnius ecuadorensis* (TRa605 and TRa606). Additionally, one strain of *Trypanosoma cruzi* isolated from a human case (strain TC-Maracay) and one strain of *T. rangeli* (TRa, Cajamarca-Peru strain), characterized and maintained in our laboratory, were used as reference strains. According to statistical study, the stocks were grouped into three clusters: (1) cluster I included the reference strain of *T. cruzi* (TC-Maracay); (2) cluster II was subdivided into two groups—subcluster IIA for the Mexican isolate (TM5) and subcluster IIB for the Peruvian ones, isolated from the salivary glands of *Rhodnius ecuadorensis* (TRa 605 and TRa 606) and the reference strain *T. rangeli* (TRa); these two new isolates were classified as *T. rangeli*; and (3) cluster III for the rest of the Peruvian isolates, which should be considered at least as a different strain from the *T. cruzi* strain Maracay. We show that the identification of *T. cruzi* and *T. rangeli* in mixed infections is readily achieved by biochemical methods. These findings identified three clusters of Mexican and Peruvian stocks that correlate with geographic origin, although assignment to a *T. cruzi* linage was not possible.

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Introduction

Chagas disease or American trypanosomiasis, caused by *Trypanosoma cruzi*, afflicts every country in South America (WHO 1983). This highly pleomorphic parasite has a complex life cycle involving a large number of triatomine insects and mammalian species. Isolation and study of *T. cruzi* populations from different origins demonstrated the presence of a large range of strains with distinct biological, biochemical and pharmacological characteristics (Rodríguez et al. 1998). This intriguing intraspecific variation has been extensively investigated by biological characterization. One widely used method is isoenzyme analysis, which has revealed different populations between the zymodeme and the transmission cycle (Miles et al. 1977, 1978; Ebert 1982; Schottelius and Muller 1984; Ebert 1985; Tibayrenc and Ayala 1988; Steindel et al. 1995). Also, lectins with different specificities for surface-membrane carbohydrate residues were used for taxonomic purposes (Miercio et al. 1980; Marinkelle et al. 1986). Recent studies of samples from various Latin American countries have shown that different epidemiological and biological characteristics are associated with the two different lineages of *T. cruzi*, that it is possible by isoenzyme typing and lectins to distinguish zymodeme I and II of *T. cruzi*, and that by lectins both inter- and intraspecific distinctions of *T. cruzi* and *T. rangeli* are given (Schottelius 1987; Fernándes et al. 1998). Thus, for a better understanding of the epidemiology and of the host–parasite relationship of *T. cruzi*, it is essential to identify strains of the parasite (Ebert 1982).

Trypanosoma rangeli, first described by Tejera (1920), is a protozoan parasite found in Central and South America, attacking a wide range of mammals (including humans), and carried by triatomine bugs. As this species

often appears together with *T. cruzi*, it is essential to characterize strains of both *T. rangeli* and *T. cruzi* for a better understanding of the interactions between the different subpopulations, this information being crucial in the Chaga disease clinic (Schottelius 1987; Steindel et al. 1991).

In a previous paper characterizing six trypanosomatids isolated from different geographical areas from South America using biochemical methods, we reported that several clusters could be distinguished; these strongly correlated with the geographical origin. Cluster I grouped isolates from Peru and the *T. cruzi* Maracay strain, while cluster II grouped the Brazilian isolates and another identified as *T. rangeli* (Cajamarca-Peru strain; Miralles et al. 2002). In the present study, we report the comparison of the results of a lectin-agglutination test, electrophoretic analysis of isoenzyme profiles and ^{1}H-NMR spectroscopy used to characterize other, new trypanosomatids isolated from different geographical areas in Mexico and Peru. Morphologically, all these have been considered members of the family Trypanosomatidae. The reference used from a human case was typified as the *T. cruzi* strain Maracay, and *T. rangeli* was characterized by Miralles et al. (2002).

Materials and methods

Parasite isolation and in vitro culture

The seven stocks have been recently isolated from different areas of Mexico and Peru: TM5 from *Rhodnius prolixus* in the Yucatan Peninsula of Mexico; the TP504 isolate of *Panstrongylus chinai* in the department of La Libertad in northern Peru; the TP702 isolate of *Triatoma carrioni* in the department of Amazonas in northeastern Peru; the TP704 isolate of *T. infestans* in the department of Arequipa in southern Peru; the TP706 isolate of *Triatoma herreri* from the department of Amazonas; and the TRa605 and TRa606 isolates, both from the salivary glands of two *Rhodnius ecuadoriensis* in the department of La Libertad, Peru. For comparison, we also included *T. cruzi* (TC) Maracay, originally isolated from a clinical case in the Malariological Division of Environmental Health of Maracay (Venezuela), and *T. rangeli* (TRa, Cajamarca-Peru strain), isolated from the salivary glands of *Rhodnius ecuadoriensis* (Miralles et al. 2002) and maintained in our laboratory by routine procedures (Sánchez-Moreno et al. 1995).

After isolation, the new flagellates were transferred to the biphasic culture medium Nicolle Nocloe Novi and the MacNeal medium, supplemented with minimal essential medium, and 20% inactivated fetal bovine serum. The isolates were cloned and cultured in vitro using Grace's medium (Sigma; Sánchez-Moreno et al. 1995). The parasites were inoculated at a density of 1.6×10^{6} into 5 ml of medium in 25-ml Falcon flasks, and cultured at 28°C. Aliquots were taken every 24 h for 9 days, and the number of parasites was counted in a Neubauer hemocytometer. On the last day, the cells were removed by centrifugation at 600 *g* for 10 min, and the pH of the parasite-free supernatants was measured.

Lectin-agglutination test

Lectins from *Canavalia ensiformis* (Con A; from Sigma Chemical Co. St. Louis, Mo, USA), *Vicia villosa* (VV; from Sigma Chemical Co. St. Louis, Mo, USA), *Triticum vulgaris*, a wheat-germ agglutinim (WGA; from Sigma Chemical Co. St. Louis, Mo, USA), and *Arachis hypogaea* (PNA; from Sigma Chemical Co. St. Louis, Mo, USA) were used. Parasites (in the initial growth phase) were washed three times by centrifugation (600 *g* 10 min, 4°C), and resuspended in phosphate-buffered saline, pH 7.4, containing 0.5% inactivated fetal bovine serum, at a concentration of 10^{8} parasites/ml; autoagglutinations were not observed. Tests were performed in duplicate in 96-well trays (Flow Laboratories), in which 50 µl of parasite suspension was incubated with equal volumes of lectins at different concentrations for 1 h at room temperature, for final lectin concentrations of 1, 10, 20, 50, 100, 150, 500, 750, and 1,000 µg/ml (Andrade and Saraiva 1999). Agglutination was determined by microscope observation according to Zubiaur and Alonso (1985). The agglutination tests were controlled by inhibition tests using the following sugars: *Canavalia ensiformis* plus 0.1 M α-D-glucosyl and 2% D-manose; *Vicia villosa* plus 0.1 M N-acetyl-D-galactosamine; and *Triticum vulgaris* plus 0.1 M N-acetyl-D-glucosamine. In an additional reaction, the parasites were incubated in a volume of phosphate-buffered saline, pH 7.4, equal to the volume of lectins to control for an eventual tendency of the parasites for autoagglutination (Schottelius 1982).

Isoenzyme characterization

Crude homogenates were obtained from 250 ml of culture medium containing 2×10^{7} cells/ml. Cells were harvested by centrifugation at 1,500 *g* for 10 min, washed twice in a phosphate-buffered saline (pH 7.4), and resuspended in a hypotonic enzyme-stabilizer solution containing 2 mM dithiothreitol, 2 mM E-aminocaproic acid, and 2 mM EDTA (Ben Abderrazak et al. 1993). The samples were frozen at −80°C for 15 min, and thawed at 25°C. After several freezing–thawing cycles, cell lysates were centrifuged at 8,000 *g* for 20 min at 4°C, and the supernatants stored in liquid nitrogen until used. The protein concentration was determined using the Bradford method, and storage was at a final concentration of 1 mg/ml of protein. The enzymes were separated by isoelectric focusing in a PhastSystem apparatus, using Phast-gel IEF 3-9 (Pharmacia-LKB). The following enzymes were tested: glucose phosphate isomerase (GPI; EC 5.3.1.9), isocitrate dehydrogenase (IDH; EC 1.1.1.42), malate dehydrogenase (MDH; EC 1.1.1.37),

malic enzyme (ME; EC 1.1.1.40), phosphoglucomutase (PGM; EC 2.7.5.1), and superoxide dismutase (SOD; EC 1.15.1.1). The staining procedures are described in Férnandez-Ramos et al. (1999).

^{1}H-NIMR spectroscopy and metabolite identification

For the spectroscopic assessments, 5 ml of a 5-day-old culture of each isolate in Grace's medium was centrifuged at 1,500 *g* for 10 min at 4°C. The pellet was discarded, and the parasite-free supernatant was stored at −20°C until used. The ^{1}H-NMR spectra were determined by the method described in an earlier study; the chemical displacements used to identify the metabolites were consistent with those of Sánchez-Moreno et al. (1992).

Statistical study

The statistical methods were based on individual hierarchical cluster analysis, selecting the Euclidean distance to the square as the basis for measuring the associations between individuals. The Euclidean distance was calculated by the following grouping procedures: simple linkage (R_k 0.7355), average linkage among groups (R_k 0.7518), average linkage (R_k 0.7726), the centroid method (R_k 0.7586), the median method (R_k 0.7204), and the Ward method (R_k 0.7570). The cophenetic coefficient (R_k) measures the degree of distortion between relationships, means in terms of original distances between individuals, and those existing at the end of the analysis. The method with the highest cophenetic correlation was chosen as the optimal one. In the selection procedure, the average linkage between groups was considered, using the coefficient R_k of Rand, which is an index of the similarity between classifications. This analysis was made with the StatGraphics program, version 5.0.

Results

In general, all the isolates grew satisfactorily in Grace's medium supplemented with 10% IFCS (Fig. 1), reaching 7×10^6–1.8×10^7 cells/ml, depending on the isolate. In all cases, the initial concentration was 1.6×10^6 cells/ml, and growth remained exponential during the first few days of culture, becoming stationary on day 5. Figure 1 presents only the growth curves of the isolates TM5 and TP704 as well as the two reference strains, TC-Maracay and Tra. The isolates TP702 and TP706 show a growth curve similar to that of TP704, while the curve of the isolates from the salivary glands of *R. ecuadoriensis* (TRa 605 and TRa 606) resembles that of Tra, and the curve of TP504 is similar to that of TC-Maracay (data not shown).The nine isolates showed a clear agglutination

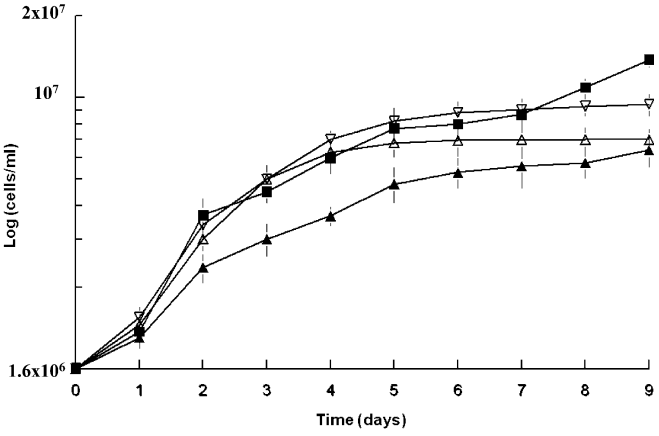

Fig. 1 Growth curves of isolates in Latin America in Grace's culture medium: *open triangles* TC, *filled triangles* TM5, *filled squares* TP704, *inverted triangles* TRa (±SD of the mean of three different experiments)

with the lectin Con A at 1 μg/ml (Table 1), although the degree of agglutination varied according to the isolate. The lectin *Vicia villosa* (VV), at all dosages assayed, agglutinated only the isolates TRa, TRa605, and TRa606 at concentrations of 20, 20, and 5 μg/ml, respectively. The wheat-germ lectin (WGA) displayed a heterogeneous agglutination pattern within the different isolates; TRa605 was agglutinated at the minimum concentration (1 μg/ml); TC, TRa, and TRa606 were agglutinated at a concentration of 5 μg/ml; and the isolates TM5, TP504, TP702, TP704, and TP706 were agglutinated at 20 μg/ml. PNA lectin agglutinated with all the isolates, except with the reference strain from Venezuela. The strong agglutination of the strain from Mexico (TM5) was noteworthy for this lectin. Cell agglutination by lectins was specific, as no spontaneous clustering of cells occurred in the absence of lectins, or in the presence of the specific saccharide inhibitors.

The isoenzymatic pattern of some of the isolates studied appears to reveal variations in the number of bands and their isoelectric points (Fig. 2). In general, the *T. cruzi* strain Maracay (TC) differs from the other isolates in the six systems studied (Fig. 2, line 1). The isoenzymatic pattern for the enzyme GPI (Fig. 2A) is the same as that for all the other isolates. The profile of IDH appears in Fig. 2B, reflecting in general wide variability in the number and mobility of the bands, although all the new isolates presented a band in common with the only band shown by TRa (Fig. 2B, line 9). According to the isoenzymatic profile of MDH, all the isolates shared a common band having an isoelectric point close to 9, although the isolates TP702, TP704, and TP706 (Fig. 2C, lines 4–6) presented a second band with an isoelectric point similar to the second band corresponding to TC (Fig. 2C, line 1). For the ME enzyme, an isoenzymatic profile (three bands) proved very similar in all the isolates, except for TP702 (Fig. 2D, line 4) with a single band that it shares with the rest of the isolates,

Table 1 Agglutination activities of lectins for nine *T. cruzi* isolates of different origins

Trypanosomatids isolates, origin			Minimal concentration of lectins required for agglutination[a]								Cluster
			Concavalina ensiformis ConA		*Vicia villosa* VV		*Triticum vulgaris* WGA		*Arachis hypogaea* PNA		
			(μg/ml)		(μg/ml)		(μg/ml)		(μg/ml)		
T. cruzi											
TC (reference strain)	Venezuela	Human	1	(+)		(−)	5	(+)	20	(−)	I
TM5	Yucatan, Mexico	Bug	1	(+)		(−)	20	(+)	40	(+++)	IIA
TP504	Northern Peru	Bug	1	(++)		(−)	20	(+)	40	(+)	III
TP702	Northeastern Peru	Bug	1	(++)		(−)	20	(+)	40	(+)	III
TP704	Southern Peru	Bug	1	(+)		(−)	20	(+)	40	(+)	III
TP706	Amazonas, Peru	Bug	1	(++)		(−)	20	(+)	40	(+)	III
T. rangeli											
TRa605	La Libertad, Peru	Bug	1	(+)	5	(+)	5	(+)	10	(+)	IIB
TRa606	La Libertad, Peru	Bug	1	(+)	20	(+)	1	(+)	10	(+)	IIB
TRa (reference strain)	Cajamarca, Peru	Bug	1	(+)	20	(+)	5	(+)	10	(+)	IIB

[a]Agglutination was scored from − (no agglutination at 1,000 μg/ml) to + (25%), ++ (50%), and +++ (virtually complete agglutination); average of three experiments

and TP706 with two bands (Fig. 2D, line 6). In the profile for PGM (Fig. 2E), it was found that all the isolates shared one band, although the Peruvian isolates TP504, TP702, TP704, and TP706 (Fig. 2E, lines 3–6) shared a second band with a different isoelectric point. All the isolates presented four similar bands for the

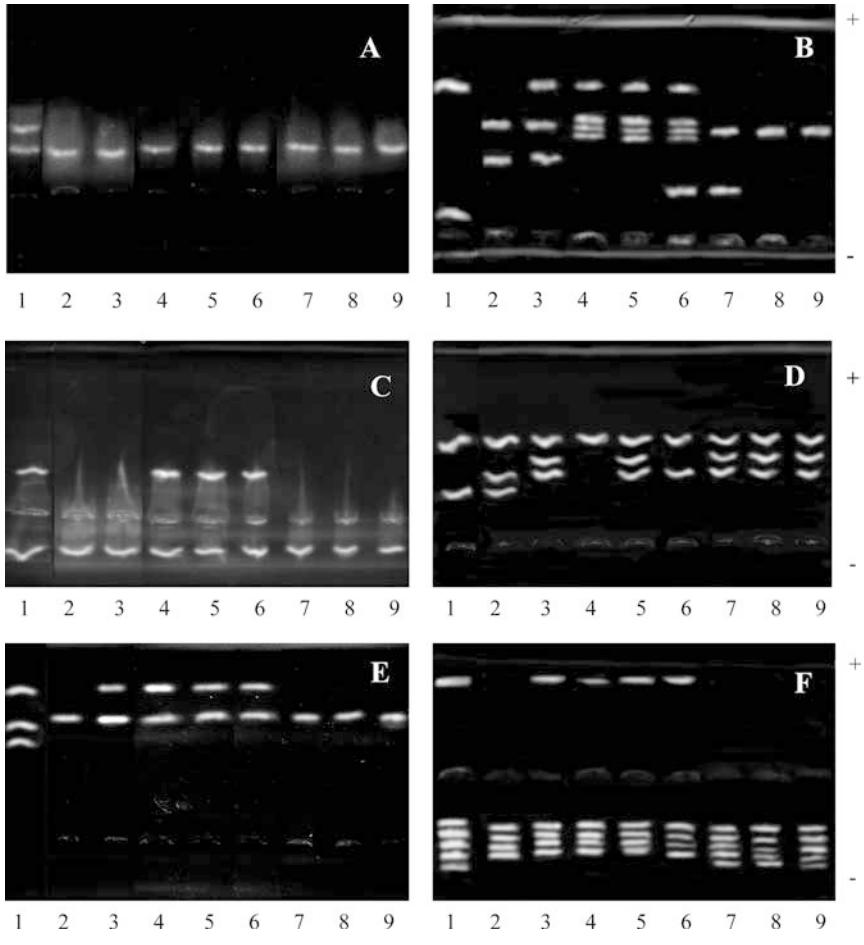

Fig. 2A–F Isoenzyme profiles of *1 T. cruzi*, Maracay strain, *2* isolate TM5, *3* isolate TP504, *4* isolate TP702, *5* isolate TP704, *6* isolate TP706, *7* isolate TRa606, *8* isolate TRa605, and *9* isolate TRa. Enzymes are **A** glucose phosphate isomerase (GPI), **B** isocitrate dehydrogenase (IDH), **C** malate dehydrogenase (MDH), **D** malic enzyme (ME), **E** phosphoglucomutase (PGM), and **F** superoxide dismutase (SOD)

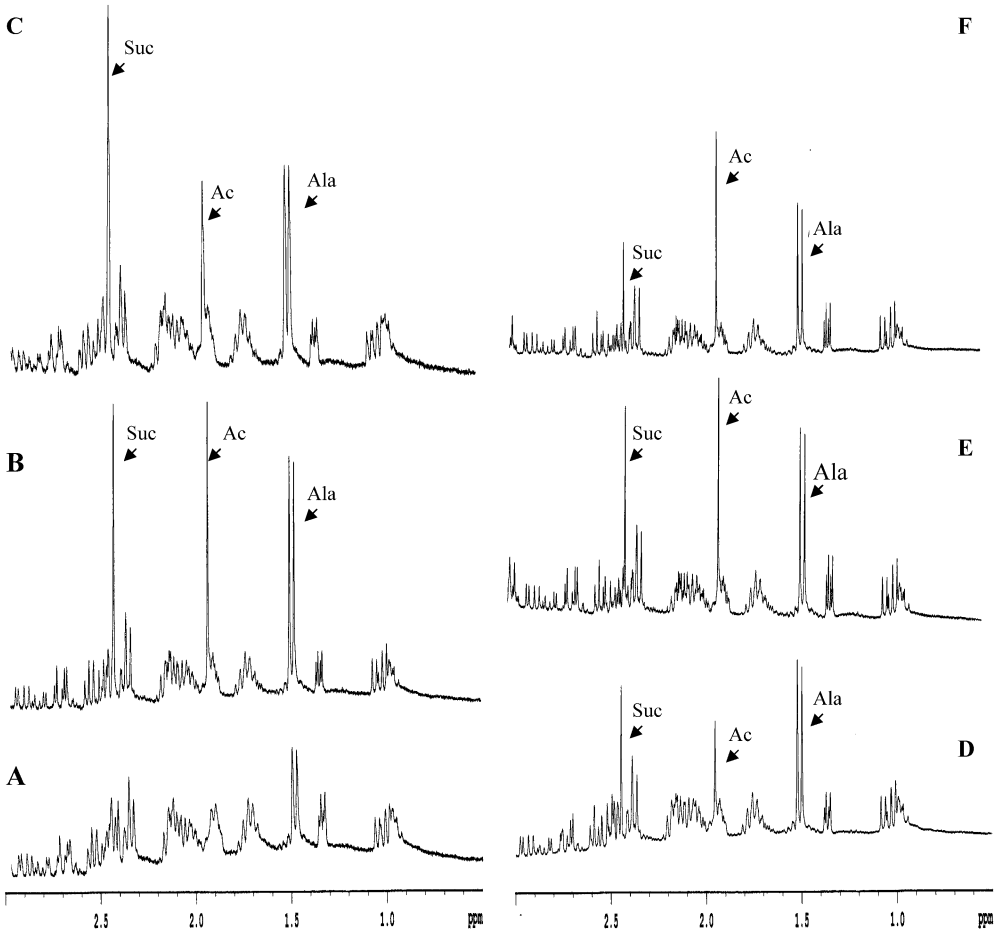

Fig. 3A–F ^{1}H-NMR spectra of the isolates culture medium. **A** Fresh culture medium before inoculation of cells; **B** spectra obtained for the cell-free culture media of the *T. cruzi* strain Maracay; **C** isolate TP704; **D** isolate TRa; **E** isolate Tra606; **F** isolate TM5. *Suc* Succinate, *Ac* acetate, *Ala* alanine

enzyme SOD (Fig. 2F); the isolates TRa606, TRa605, and the reference strain TRa also presented a fifth band, with an isoelectric point of close to 9, which they shared with the reference strain TC (Fig. 2F, lines 7–9, and line 1). In addition, this reference strain shared a band having an isoelectric point of 3.6 with the isolates TP504, TP702, TP704, and TP706 (Fig. 2F, lines 3–6, and line 1).

To identify the main metabolites excreted into the medium, we used ^{1}H-MNR spectroscopy to analyze the composition of the medium in which the different isolates had been grown for 5 days, as well as the cell-free medium used as control (Fig. 3). The additional peaks visible in the spectra corresponding to the media used to grow flagellates, in comparison with the control spectrum (Fig. 3A), indicated excreted metabolites. The ^{1}H MNR study indicated that for all the isolates the main metabolites excreted were succinate (Suc, 2.3 ppm), acetate (Ac, 1.8 ppm), and alanine (Ala, 1.4 ppm). Quantitative differences appeared between isolates; for example, for TP704, the main metabolite was succinate (Fig. 3C); acetate proved to be the main metabolite for TC, TRa 606, and TM5 (Fig. 3B, E and F, respectively); and alanine was the main metabolite for the isolates TRa (Fig. 3D) as well as TRa605, TP504, TP702, and TP706 (data not shown).

Discussion

The results of the lectin-agglutination test showed that the composition of carbohydrates of the membranes of the trypanosomes varied from one isolate to another. The nine isolates agglutinated with the lectin Con A, indicating that all had molecules of α-D-glucose and α-D-mannose in the composition of their membranes, although in the isolates TP504, TP702, and TP706 these molecules were more numerous than in the others, or presented a different arrangement that permitted agglutination. The lectin from *Vicia villosa* agglutinated only with trypanosomes isolated from the salivary glands of *R. ecuadoriensis* (TRa605 and TRa606) and the reference strain TRa, indicating that *N*-acetyl-galactosamine was among the sugars of its membrane, in addition to presenting a strongly marked agglutination at a very low concentration, this being evidence of an abundance of this sugar. These data agree with those of other authors (Acosta et al. 1991), who determined that VV lectin only selectively reacts with *T. rangeli* strains. The wheat-germ lectin (WGA) displayed a heterogeneous agglutination pattern within the different isolates, and thus the presence of *N*-acetyl-D-glucosamine residues among the surface sugars was highly variable.

PNA lectin clearly separates the TC reference with regard to the rest of the isolates from Peru and Mexico.

Different authors have found that strains from Venezuela and Brazil, including type 2, were PNA types, as they agglutinated with *Arachis hypogaea* lectin but not with WGA lectin (Vivas et al. 1979, Schottelius 1982), as occurred with our reference strain TC. The Mexican isolate (TM5) presented a strong agglutination with PNA lectin, which distinguishes the Peruvian isolates, demonstrating that neuraminic acids exist in abundance on the surface of the epimastigote from these isolates. According to statistical analysis by the group average method, the Euclidean of the lectin-agglutination test grouped the different isolates into three clusters: the first cluster was formed by the reference TC strain; the second by TM5, TP504, TP702, TP704, and TP706; and the third by TRa, TRa605, and TRa606 (data not shown).

The isoenzyme patterns of the three isolates from the salivary glands of *R. ecuadoriencis* are more homogeneous for the six enzymatic systems tested. The TC strain from Maracay showed differences in the loci of the six enzymatic systems with respect to the rest of the isolates from Peru and Mexico. The Peruvian isolates (TP702, TP704, and TP706) showed great similarity in the enzymatic loci GPI, MDH, ME, PGM, and SOD. Comparing our results with those of Ebert (1982), we find a strong coincidence in the profiles of some of the enzymes assayed (PGM, MDH, and ME), implying that these Peruvian isolates may be correlated to the *T. cruzi* isolates included within the zimodene Z1 by this author.

The statistical study of these results grouped the nine isolates into three clusters: the first includes the reference TC strain; the second includes the isolate from Mexico (TM5), the Peruvian isolates (TP504, TRa605, and TRa606), together with the strain isolated from *R. ecuadoriensis* of Peru and used as the reference strain of *T. rangeli* (TRa); and the third includes the rest of the Peruvian isolates (TP702, TP704, and TP706).

The members of the family Trypanosomatidae are incapable of completely degrading carbohydrates, even in the presence of oxygen, producing CO_2 and dicarboxylic acids (Sánchez-Moreno et al. 1995). In addition, the relative proportion of these end products varies among subspecies of *T. cruzi* (Urbina et al. 1993).

By ^{1}H-MNR spectroscopy, we identified the main metabolites excreted into the culture medium by the isolates. The analysis indicated that for all the isolates, the main metabolites excreted were succinate, acetate, and alanine, the proportion varying according to the isolate. Several authors have identified these same metabolites in other *T. cruzi* strains, such as the strain Tulahuen (Cazzulo 1994), strain Y (Rogerson and Gutteridge 1980), and the Bolivia strain of our group (Penin et al. 1998). Furthermore, other *T. cruzi* isolates from Brazil, characterized in a previous study, excreted other metabolites (Miralles et al. 2002). The differences found when the seven isolates were compared with the reference strains indicated changes in the metabolic strategies of the trypanosomes in degrading energy substrates, and showed that certain energy pathways are more developed in some strains than in others (Miralles et al. 2002).

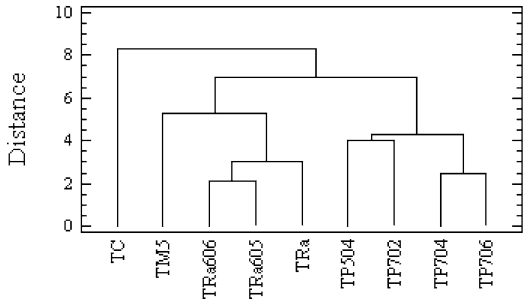

Fig. 4 Dendrogram based on individual hierarchical cluster analysis (program Stat-Graphics version 5.0)

The statistical analysis of all the results together (Fig. 4) provides a more real phylogenetic approach, and in this way the isolates were grouped into three clusters. Cluster I is the Maracay *T. cruzi* strain, which clearly differentiates the rest of the isolates. Cluster II can be divided into subcluster IIA, including the strain isolated from *R. prolixus* in the Yucatan Peninsula in Mexico, and subcluster IIB, including the isolates from the salivary glands of *R. ecuadoriensis* from the department of Libertad in Peru, together with the reference strain *T. rangeli*. This indicates that these isolates (TRa605 and TRa606) should be considered as belonging to the species *T. rangeli*. In support of this hypothesis, *T. cruzi* and *T. rangeli* differ in the isolation site of the flagellate, which in this case is *T. rangeli*, almost invariably in salivary glands (Grisard et al. 1999).

The third cluster includes the other four isolates from Peru (TP504, TP702, TP704, and TP706). Being phylogenetically distant from the Maracay strain of *T. cruzi*, these should be considered as belonging to another strain, although phylogenetically they have been recently separated from the TRa strains. The biochemical differences found suggest that the *T. cruzi* isolates are markedly heterogeneous, even when they come from the same host species and the same geographical region (Moreno et al. 2002). According to Tibayrenc et al. (1986), groups of clones that are biochemically similar in terms of their biological properties can be found. On the other hand, different clones with the same origin adapted to jungle, peridomestic, and domestic cycles would have conserved biochemical/enzymatic properties but would show different biological behavior.

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