Trypanosoma rangeli sialidase: cloning, expression and similarity to T. cruzi trans-sialidase

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Sialidases are hydrolytic enzymes present from virus to higher eukaryotes, catalyzing the removal of sialic acid from glycoconjugates. Some protozoa Trypanosomatidae secrete high levels of sialidase into the medium. We have now purified the secreted sialidase from Trypanosoma rangeli. Its N-terminal sequence reveals 100% identity with the corresponding region of the trans-sialidase from T. cruzi. Trans-sialidase, although homologous to viral and bacterial sialidases, displays a novel sialyltransferase activity and is involved in host cell invasion. Several homologous trans-sialidase-like genes were cloned from genomic DNA of T. rangeli, and grouped in three subfamilies. Active sialidase-encoding genes were found in one of them. The recombinant sialidase shows similar properties to those of the native enzyme, including undetectable trans-sialidase activity. Nevertheless, it has an overall identity of 68.9% with the catalytic domain of T. cruzi trans-sialidase, increasing to 86.7% admitting conservative substitutions. Only three other eukaryotic sialidases have been previously cloned, none of them showing significant homology to trans-sialidase. The isolation of a highly similar sialidase is relevant to further identify the molecular determinants allowing trans-sialidase activity. As a first approach, chimeric constructs between sialidase and trans-sialidase were generated, one of them rendering a sialidase with three times lower K_m than the natural enzyme.

Key words: eukaryotic sialidase/gene family/glycosidase/parasite/sialic acid

Introduction

Sialidases (EC 3.2.1.18) are hydrolytic enzymes that release terminal sialic acid from glycopeptides and glycolipids as an initial step in the degradation of these glycoconjugates. The viral and bacterial ones have been the most extensively studied (Roggentin et al., 1993; Vimr, 1994). Bacterial sialidases are frequently secreted into the medium, but some have an intracellular (Salmonella typhimurium, small isoform in Clostridium perfringens) or membrane-bound (Actinomyces viscosus) location (Roggentin et al., 1993; Vimr, 1994). They have been suggested to have a primary nutritional function although in some cases they might also play essential roles in pathogenesis (Corfield, 1992; Vimr, 1994). Sialidases in eukaryotic cells have been identified in the lysosomes, in the cytoplasm, and even on the surface membrane (Miyagi et al., 1993; Chou et al., 1996). However, research progress on structural and functional aspects in higher eukaryotic cells has been hampered by their instability and low activity (Ferrari et al., 1994; Sato and Miyagi, 1995).

The situation is different in a few parasitic protozoa which shed high levels of sialidase activity into the medium. Among them are the glycosylphosphatidylinositol anchored sialidase from Trichomonas foetus (Dias Filho et al., 1995) and the enzymes of African (Pontes de Carvalho et al., 1993b; Engstler et al., 1995) and American (Reuter et al., 1987; Parodi et al., 1992; Uemura et al., 1992) trypanosomes. In American trypanosomes, sialidases have been described in Trypanosoma cruzi (the agent of Chagas disease) and in T. rangeli (nonpathogenic for humans). Previous attempts to clone the sialidase gene from T. rangeli were unsuccessful (Buschiazzo et al., 1993; Smith et al., 1996). This is probably because, as we now report, the enzyme belongs to a protein family, some of whose members are enzymatically inactive. Further interest in cloning the sialidase of T. rangeli comes from its possible structural relation with the trans-sialidase of T. cruzi. This enzyme was originally described as a sialidase (Pereira et al., 1991), but later shown to be more active in reversibly transferring the α-2,3 bound sialic acid to a terminal β-galactose in glycoconjugates, than in hydrolyzing it (Parodi et al., 1992). Trans-sialidase is a virulence factor that has been involved in the mammalian-cell invasion process (Schenkman and Eichinger, 1993; Burleigh and Andrews, 1995), and it shows no significant homology to the sialyltransferases that use CMP-sialic acid as substrate. Although different from the sialidases in terms of the final acceptor of the sialic acid (Vandekerckhove et al., 1992), its sequence clearly shows that it belongs to the sialidase superfamily (Campetella et al., 1992; Roggentin et al., 1993). The structural reasons that allow trans-sialidase to preferentially transfer sialyl residues instead of just hydrolyzing them, are not known (Schenkman et al., 1994). Their identification could contribute to the engineering of glycosidases, making them more efficient in transferring monosaccharides than in the hydrolysis reaction (Ichiikawa et al., 1992). Here we report that the sialidase from T. rangeli is 68.9% identical to the trans-sialidase, increasing to 86.7% allowing conservative amino acid substitutions. Since it performs only the hydrolytic activity, (Pontes-de-Carvalho et al., 1993a, and this report) it provides a relevant tool to further localize the regions of the trans-sialidase involved in sialic acid transferase.

The nucleotide sequences reported in this article have been submitted to the GenBank with accession numbers U83180, U83181, U83182, U83183, U83240. and U83241.
Results

The N-terminal sequence of the mature sialidase from Trypanosoma rangeli is identical to the trans-sialidase from Trypanosoma cruzi.

To address the question on the structural relationship between the sialidase of Trypanosoma rangeli and the trans-sialidase of Trypanosoma cruzi, the amino terminus of the former was sequenced. The sialidase was purified from Trypanosoma rangeli culture supernatants (Table I). A high level of purification was achieved using affinity chromatography with p-phenyloxamic acid (Figure 1A) which has already been described as a potent inhibitor of this enzyme (Reuter et al., 1987). Sialidase activity was efficiently retained by the column at low ionic strength, with no detectable activity in the flow-through fraction (lane 2, Figure 1A). The enzyme eluted as a single peak with increasing ionic strength (activity peak at 0.25–0.3 M NaCl). The major protein component in this fraction migrates in SDS–PAGE according to an apparent molecular mass of 70–75 kDa (lane 3, Figure 1A). This protein was further purified by anion exchange chromatography, with an elution peak at 60 mM NaCl (data not shown), before subjecting 10–20 pmol of this highly purified sialidase to automated micro sequencing of the amino terminus. The sequence of the first 13 residues of the mature sialidase was obtained with only one uncertainty (Figure 1B), being identical to the corresponding residues in the mature trans-sialidase from Trypanosoma cruzi (Pollevick et al., 1993).

The sialidase gene of Trypanosoma rangeli is a member of a gene family. Figure 2 shows a Southern blot of restriction endonuclease digested DNA from Trypanosoma rangeli hybridized with a probe encoding the complete amino-terminal catalytic domain of the Trypanosoma cruzi trans-sialidase gene (probe TS1). A minimum of 6–10 trans-sialidase-related genes can be estimated, although an organization in tandem cannot be excluded. The digestion pattern with the enzyme Eagl resulted in a single band of 5 kb, after hybridizing with TS1 (results not shown). A genomic library was constructed using Eagl digested genomic DNA ranging from 4 to 6 kb. Eleven independent clones were isolated and named TrSA4, TrSA5, TrSA6, TrSA7, TrSA8, TrSA10, TrSA11, TrSA12, TrSA14, TrSA17, and TrSA21.

Partial sequencing of these clones was done with the antisense primer 44 (see Materials and methods) which is derived from the sequence of the trans-sialidase clone Tc-TS1N1 (Campetella et al., 1994), and primes 162 bp downstream of the codon for the first amino acid (Leu) of the mature N-terminus. These DNA sequences and the restriction patterns obtained with BssHII enzyme (not shown) allowed us to group the sialidase gene family of Trypanosoma rangeli into three subfamilies (Figure 3, Table II). Subfamily 1 includes three independent clones (TrSA5, TrSA10, and TrSA17) having more than 95% identity in DNA sequence. Subfamily 2 includes at least four independent clones (TrSA4, indistinguishable from TrSA6 and TrSA7; TrSA11; TrSA14; and TrSA21); each one can be differentiated by its restriction pattern with the enzyme BssHII in spite of having identical sequence in their 5' ends. They have 84.3% identity when compared with the predicted mature amino termini of subfamily 1 genes. The general features of a secretion signal sequence (von Heijne, 1986) can be found in the deduced N-termini from both subfamilies. The general features of a secretion signal sequence (von Heijne, 1986) can be found in the deduced N-termini from both subfamilies. Putative signal sequences were different (see Figure 3), except for the last five residues EPA(S/H)A, which could be part of the specific signal peptidase cleavage recognition site involved in maturation. Subfamily 3 includes two independent clones (TrSA8 and TrSA12) having identical sequence although different BssHII restriction patterns. They are only 42% similar to members of subfamilies 1 and 2 in the deduced N-termini but become identical to subfamily 2 members from the codon corresponding to amino acid 13. The LAP sequence, which is conserved in all the mature N-termini so far sequenced or deduced, is absent in subfamily 3 members. It should be noted that these inserts do not have the codon for a methionine upstream of the beginning of homology (see Figure 3).

Those clones encoding sequences identical to that of the mature amino terminus of the native sialidase (Figure 1) were expressed in the plasmid pTrcHisA in Escherichia coli, to identify the gene/s encoding active sialidase. Three clones were identified (pTrcTrSA4, pTrcTrSA14, and pTrcTrSA21), all of them expressing enzymatically active recombinant proteins. They were all members of the same subfamily (subfamily 2; Figure 3 and Table II). Clone pTrcTrSA4 was further

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### Table I. Purification of secreted sialidase from Trypanosoma rangeli

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Culture supernatant</td>
<td>65</td>
<td>18.4</td>
<td>0.28</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. Oxamic acid-agarose</td>
<td>1.6</td>
<td>16.8</td>
<td>10.5</td>
<td>91.3</td>
<td>37.5</td>
</tr>
<tr>
<td>3. MonoQ</td>
<td>0.69</td>
<td>15.9</td>
<td>23.04</td>
<td>86.4%</td>
<td>63.78</td>
</tr>
</tbody>
</table>

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![Figure 1](https://example.com/image1.png)

**Fig. 1.** SDS–PAGE of oxamic acid–agarose purification (A) and N-terminal sequence (B) of the sialidase from *Trypanosoma rangeli*. (A) Crude culture supernatant of *Trypanosoma rangeli* (lane 1), p-phenyloxamic acid-agarose column flow-through fraction (lane 2) and eluate fraction with sialidase activity from the same column (lane 3) were electrophoresed on a 10% SDS–PAGE under reducing conditions. The gel was stained with Coomasie brilliant blue R-250. (B) N-Terminal amino acid sequence obtained from purified sialidase from *Trypanosoma rangeli* (Trypanosoma rangeli SA) is compared with the N-terminus of the trans-sialidase from *Trypanosoma cruzi* (Trypanosoma cruzi TS, as reported in Pollevick et al., 1993).
used as the source of recombinant sialidase. The protein product was partially purified by affinity chromatography with p-phenyloexamic acid coupled to agarose, showing identical elution pattern and molecular size as the native enzyme. Kinetic parameters show a hyperbolic Michaelis behavior, with similar $K_m$ values obtained when the recombinant and native enzymes were assayed using MUNANA as substrate (Table III). $V_{max}$ was found to be five times higher in the recombinant version, possibly due to the presence of a mixture of enzymatically active and inactive molecules in the preparation obtained from parasite supernatants. No trans-sialidase activity could be detected when incubating the recombinant sialidase with [p glucose $^{1-14}C$] lactose and NeuAcα2→3 lactose, even raising the molar ratio of sialic acid donor:acceptor to 100:1. To compare the specific activity of the $T.rangeli$ enzyme with that of the trans-sialidase from $T.cruzi$ assayed as a sialidase, equivalent amounts of both recombinant enzymes were incubated with different concentrations of MUNANA as substrate. The sialidase and trans-sialidase had a $V_{max}$ of 105 and 8 μmol-min$^{-1}$-mg$^{-1}$, respectively.

The structure of the sialidase from $T.rangeli$

Clone TrSA4 was completely sequenced. The deduced protein sequence is shown in Figure 4. The translation of both strands of the DNA sequence of clone TrSA4 in all six reading frames was used to search homologous sequences in a nonredundant protein database (GenBank CDS translations + PDB + SwissProt + SPupdate + PIR; Total: 224,269 sequences), using the BLAST algorithm. As expected, high scoring segment pairs correspond to homologous sequences from $T.rangeli$ (U46073, U46072, L14943; Buschiazzo et al., 1993; Smith et al., 1996), members of the trans-sialidase family of $T.cruzi$ (Pollevick et al., 1991; Uemura et al., 1992; Cremona et al., 1995), and bacterial sialidases (Roggentin et al., 1993).

The first ATG codon present in the ORF is embedded in a favorable context for translation initiation (Kozak, 1989). The presence of a putative signal peptide, absent in the purified protein, is consistent with the fact that this enzyme is secreted by the parasite. The following codons perfectly match with the 13 amino acids sequenced from the mature N-terminus. The predicted molecular mass of the protein is 71.9kDa, as expected from the purified native sialidase.

Typical sialidase motifs are conserved in the $T.rangeli$ enzyme: (1) the FRIP region, as identified by Roggentin et al., 1993, close to the N-termini of all sialidases; (2) the presence and overall spacing of four Asp boxes (SXDQGXTW) (Roggentin et al., 1989); and (3) all the amino acids shown to be involved in the catalytic site of the sialidase from $S. typhimurium$ (Crennell et al., 1993). This last feature implies a greater relatedness of the sialidase from $T.rangeli$ with the sialidase from $S. typhimurium$, than with the viral enzymes (see Crennell et al., 1993, for a comparative analysis with the catalytic site of influenza sialidase). As has been postulated, this high similarity in the structure of the catalytic sites probably implies similar catalytic mechanisms (Crennell et al., 1993). Together with these conserved elements of the sialidase superfamily (Roggentin et al., 1993), the $T.rangeli$ enzyme also contains a region near the C-terminus that encodes a sequence found in type III module of fibronectin (Fn3) (Pereira et al., 1992; Cremona et al., 1991; Uemura et al., 1992; Pollevick et al., 1993, for a comparative analysis with the catalytic site).
or trans-sialidase activities chimeric exchange constructs were

geli, As a first approach in identifying regions that define sialidase enzymes.

gave a negative result. Therefore, in spite of the highly con-

fully inhibit trans-sialidase activity (Leguizamon et al., 1994), this domain in both recombinant proteins was exchanged by using a BsiWI site conserved in both genes. The trans-sialidase with the Fn3 domain of sialidase completely lost both trans-sialidase and sialidase activities (results not shown). On the other hand, and although recombinant chimeric version of the sialidase with the Fn3 of the trans-sialidase does not acquire trans-sialidase activity, it does remain fully active as a sialidase. In fact, the chimeric version was reproducibly more active than intact recombinant sialidase with an apparent $K_m$ value three times lower and approximately three times higher $V_{max}$ (Table III).

**Discussion**

We have demonstrated that the sialidase of *T.rangeli* is homologous to the trans-sialidase of *T.cruzi*. The N-terminal sequence of the native enzyme purified from *T.rangeli* culture supernatants revealed 100% identity with the corresponding region of the mature trans-sialidase (Figure 1). This amino-terminal sequence is different from any other known sialidase, including the other three eukaryotic sialidases so far cloned: the cytolic forms of rat (Miyagi et al., 1993) and Chinese hamster (Ferrari et al., 1994), and the sialidase L of the leech *Macrobdella decorra* (Chou et al., 1996). Thereafter, we were able to isolate three different genomic clones from *T.rangeli*, homologous to the trans-sialidase gene, that express fully active sialidase in *E.coli*. The recombinant enzyme was analyzed, showing similar properties when compared with the native enzyme (Table III). The complete DNA sequence shows the features expected from the native secreted enzyme: calculated molecular mass, deduced mature N-terminal sequence, and the presence of a putative secretion signal.

These sialidase genes are members of a sialidase gene family since six other homologous genes that express undetectable sialidase activity were identified. What is the reason for having several genes encoding active sialidases and inactive sialidase-like proteins, is not known. But, this organization closely resembles what happens in *T.cruzi*, where the trans-sialidase-related proteins are also grouped in families containing enzymatically active and inactive members (Campetella et al., 1992; Uemura et al., 1992; Schenkman et al., 1994; Cremona et al., 1995). The *T.rangeli* sialidase family can be divided in three subfamilies, according to multiple alignment of DNA sequences. Only genes included in subfamily 2 were found to express active sialidase. Genes of sialidase subfamilies 1 and 2 encode putative signal peptides, consistent with the fact that sialidase is actively secreted, and suggesting that inactive sialidase-related molecules could follow the same pathway. Genes included in the third subfamily (3) have no sequences coding for a starting Met, although 100% identity was shown.

1991). Included in this Fn3 region is the last, partially conserved, Asp box and a conserved motif characteristic of the trans-sialidase family: VTVXNVXLYNR (Cross and Tackle, 1993).

Figure 4 also shows an amino acid alignment of the deduced protein sequences of the sialidase from *T.rangeli* with the catalytic domain of the trans-sialidase from *T.cruzi* (TcTS1N1; Campetella et al., 1994). The overall identity with trans-sialidase is of 68.9%, increasing to 86.7% allowing conservation of amino acid substitutions. At variance with trans-sialidase, the *T.rangeli* enzyme lacks any repetitive region on the C-terminus. Differences between sialidase and trans-sialidase are evenly dispersed along the proteins, with no particular region showing grouped deletions, insertions or substitutions, differentiating both molecules.

**Serological reactivities**

Due to their high homology, it was of interest to determine if sera from *T.cruzi*-infected rabbits or from mice immunized with the catalytic domain of the trans-sialidase, that are able to fully inhibit trans-sialidase activity (Leguizamon et al., 1994), may also inhibit the sialidase from *T.rangeli*. Trans-sialidase–neutralizing antibodies failed to inhibit the *T.rangeli* enzyme since with as little as 1 pg of recombinant sialidase, there was no detectable inhibition when using 5 μl of pure antiserum. Conversely, 5 ng of trans-sialidase were completely inhibited with 5 μl of a 1:100 dilution of each antiserum. Also, a rabbit antiserum raised against the purified native sialidase of *T.rangeli*, inhibited the enzyme while incubation with trans-sialidase gave a negative result. Therefore, in spite of the highly conserved sequence, antibodies were able to discriminate both enzymes.

**Chimeric proteins**

As a first approach in identifying regions that define sialidase or trans-sialidase activities chimeric exchange constructs were

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Cloned membersa</th>
<th>DNA similarity within subfamiliesb (%)</th>
<th>DNA similarity among subfamilies (%)</th>
<th>Recombinant sialidase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TrSA5, TrSA10, TrSA17, TrSA4</td>
<td>&gt;95</td>
<td>69.6</td>
<td>57.8</td>
</tr>
<tr>
<td>2</td>
<td>TrSA11, TrSA14, TrSA21</td>
<td>100c</td>
<td>69.6</td>
<td>65.7</td>
</tr>
<tr>
<td>3</td>
<td>TrSA8, TrSA12</td>
<td>100c</td>
<td>57.8</td>
<td>65.7</td>
</tr>
</tbody>
</table>

aGenBank accession numbers U83180, U83181, U83182, U83183, U83240, and U83241.
bCalculated among the members of each subfamily comparing only the 5' region of the ORF's (see Materials and methods).
cThese clones are different according to BssHII restriction patterns.
dAsp box and a conserved motif characteristic of the trans-sialidase family: VTVXNVXLYNR (Cross and Tackle, 1993).

<table>
<thead>
<tr>
<th>Protein</th>
<th>App. $K_m$ (μM)</th>
<th>$V_{max}$ (μmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>220</td>
<td>23.4</td>
</tr>
<tr>
<td>Recombinant</td>
<td>180</td>
<td>105</td>
</tr>
<tr>
<td>Recombinant chimeric*</td>
<td>60</td>
<td>320</td>
</tr>
</tbody>
</table>

*Chimeric construct containing the C-terminal Fn3 domain of the trans-sialidase from *T.cruzi*. **Table III. Kinetic parameters of native and recombinant sialidases from *T.rangeli* using MU-NANA as substrate**
Fig. 4. Deduced amino acid sequence of the sialidase from *Trangeli* and alignment with the trans-sialidase from *T. cruzi*. The deduced amino acid sequence of the sialidase from *Trangeli* (genomic clone TrSA4) is aligned with the catalytic domain of the trans-sialidase from *T. cruzi* (TcTS; Castello et al., 1994), using the Clustal method with PAM250 residue weight table (Higgins et al., 1996). Residues that differ from *T. rangeli* sialidase are boxed. Numbering of amino acids starts from the mature amino termini as determined by protein sequencing. The conserved FRIP sequence of the sialidase from *E. coli* and *T. cruzi* lacks trans-sialidase activity) with the trans-sialidase from *T. cruzi* provides us with a relevant tool for the future understanding of how sialic acid is transferred to a molecule other than water.

Materials and methods

Sialidase activity was assayed in 50 μl of 50 mM 2-(N-morpholino) ethane-sulfonic acid buffer (MES; Sigma) pH 6 in the presence of 1 mM 4-methylumbelliferyl-N-acetyl neuraminic acid (MUNANA; Sigma). The quantity of enzyme used was always adjusted to measure initial velocity in 15 min at 25°C, except when screening for genomic clones expressing a recombinant sialidase, where reactions were extended for 2 h at 37°C. Reactions were stopped with 3 ml 0.1 M Tris-HCl buffer pH 10 and the amount of 4-methylumbelliferylboron released was measured with a Jasco spectrophotometer (model FP-770, Japan Spectroscopic Co., Ltd., Tokyo, Japan). Equivalence to moles of sialic acid was done using 4-methylumbelliferyl (MUCAN; Sigma) as standard. One unit is defined as the amount of sialidase which liberates 1 μmol of MU per min at 25°C. For determination of kinetic parameters, sialidase activity was continuously monitored in the spectrophotometer (Ex. 366 nm; Em. 448 nm), setting the reactions with different concentrations of MUNANA (1 μM to 10 mM) in a final volume of 0.15 ml 50 mM MES buffer pH 6. Mean values were obtained from four independent experiments performing reactions at 25°C. V_max values are expressed as μmol MU/min−1·mg protein−1.

Trans-sialidase activity was assayed as described elsewhere (Buschiazzo et al., 1996). Briefly, the enzyme was incubated in 20 mM Tris-HCl buffer pH 8, for 15 min at 25°C, in the presence of 0.4 nmol [D glucose l-14C]lactose (54 mCi/mmol; Amersham, Little Chalfont, Buckinghamshire, UK) as acceptor and 30 nmol NeuAcα2-3 lactose (Sigma) as donor of sialic acid in a final
volume of 30–50 μL. The reaction was stopped and radioactivity bound to QAE Sephadex (Pharmacia Biotech., Uppsala, Sweden) was measured in a β-
spectillation counter.

Comparison of the sialidase activity in sialidase and chimeric sialidase/
trans-sialidase recombinant molecules, were done with equal mass of oxamic
acid-purified recombinant enzymes, after quantification of bands in Coo-
massie-stained SDS-PAGE and Western blots (not shown).

Sialidase purification and protein sequencing

T. rangeli parasites were grown at a concentration of 5 x 10^3 to 10^4 parasites/ml. The parasites were collected, and the supernatant dialyzed extensively against Tris-HCl pH 8, using as template a PCR fragment containing the complete amino terminal
catalytic domain of a trans-sialidase gene (clone IN 1, Campetella
et al., 1989). The ligation product was used to transform XLI-Blue mrf
1989) encoding the catalytic
amino terminal domain of the trans-sialidase of T. cruzi, was subcloned in E.coli-digested pTrcHisA plasmid (Invitrogen), resulting in plasmid pTrcTS611/2. Exchange of the carboxyl termini of the trans-sialidase of
T. cruzi and the sialidase of Trangeli, was accomplished performing the corre-
sponding DNA exchange between recombinant plasmids pTrcTS611/2 and
pTrcTS4 by restriction with enzymes BsiWI and Seal (New England Bio-
labs).

DNA isolation

Total DNA from culture forms of T. cruzi and T. rangeli parasites was isolated using conventional proteinase K, phenol/chloroform methods as described pre-
viously (Sambrook et al., 1989).

Southern blots

Total DNA of parasites was digested with the indicated restriction enzymes and 5 μg of digest was electrophoresed in a 0.7% agarose gel, alkali-denatured, transferred by capillarity to a Hybond-N nylon membrane (Amersham) and UV cross-linked (Stratagene, La Jolla, CA). Radioactive probe TSI was gen-
erated by random priming with Klenow DNA Pol (Sambrook et al., 1989) using as template a PCR fragment containing the complete amino terminal
catalytic domain of a trans-sialidase gene (clone IN1, Campbell et al., 1994). Filters were hybridized at 65°C with the [32P]-labeled probe TSI, using a hybridization solution containing 0.5% SDS, 5x Denhardt’s solution, 100 μg/ml denatured sonicated salmon sperm DNA and 3x SSC (1x SSC: 150 mM NaCl in 30ml) in the same buffer.

Library construction and screening

Total DNA of T. rangeli was digested with restriction enzyme EagI (New England Biolabs Inc., Beverly, MA). Digestion product was separated by agarose gel electrophoresis, and fragments corresponding to 4–5 kbp were ligated into EagI-digested, dephosphorylated pBluescriptKSII+ plasmid (Stratagene). The ligation product was used to transform XLI-Blue mrf E.coli
cells (Stratagene) by electroporation using a GenePulser apparatus (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer’s instructions. This library was screened by colony hybridization using the [32P]-labeled probe TSI.

DNA sequencing and analysis

Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp., Cleve-
land, OH) was employed with double-stranded templates, according to the manufacturer’s instructions. Sequence from the 5’ region of the ORF of several members of the sialidase gene family was obtained from genomic clones using oligonucleotide 44 (5’ TTTCGTAGCGAGCGTCC 3’) as the primer.
References


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Cloning and expression of sialidase genes from *T.rangeli*