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# A quantitative proteomic analysis of the tegumental proteins from *Schistosoma mansoni* schistosomula reveals novel potential therapeutic targets

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ABSTRACT

The tegument of *Schistosoma mansoni* plays an integral role in host–parasite interactions, particularly during the transition from the free-living cercariae to the intra-mammalian schistosomula stages. This developmental period is characterised by the transition from a trilaminate surface to a heptalaminate tegument that plays key roles in immune evasion, nutrition and excretion. Proteins exposed at the surface membranes of newly transformed schistosomula are therefore thought to be prime targets for the development of new vaccines and drugs for schistosomiasis. Using a combination of tegumental labelling and high-throughput quantitative proteomics, more than 450 proteins were identified on the apical membrane of *S. mansoni* schistosomula, of which 200 had significantly regulated expression profiles at different stages of schistosomula development in vitro, including glucose transporters, sterols, heat shock proteins, antioxidant enzymes and peptidases. Current vaccine antigens were identified on the apical membrane (*Sm*-TSP-1, calpain) or sub-tegumental (*Sm*-TSP-2, *Sm*29) fractions of the schistosomula, displaying localisation patterns that, in some cases, differ from that in the adult stage fluke. This work provides the first known in-depth proteomic analysis of the surface-exposed proteins in the schistosomula tegument, and some of the proteins identified are clear targets for the generation of new vaccines and drugs against schistosomiasis.



## 1. Introduction

Schistosomiasis is one of the most important neglected tropical diseases affecting more than 200 million people worldwide (Gryseels et al., 2006; Colley et al., 2014), particularly in developing and tropical regions. Three main species of schistosomes are of major medical relevance: *Schistosoma mansoni* and *Schistosoma japonicum*, the eggs of which induce hepatosplenic inflammation and liver fibrosis when trapped in the portal system (Gryseels et al., 2006), and *Schistosoma haematobium* which is linked to bladder cancer in chronically infected people (James et al., 1974; Schwartz, 1981; Mayer and Fried, 2007). Despite the widespread use of the anthelmintic drug Praziquantel for the last 20 years in mass drug administration programs, this parasitic infection still causes a loss of 1.53 million disability-adjusted life years (DALYs) and up to 280,000 deaths annually in sub-Saharan Africa alone (van der Werf et al., 2003; King et al., 2005; Gryseels et al., 2006; Steinmann et al., 2006). Indeed, the DALYs attributed to schistosomiasis may be far greater than initially appreciated due to recent awareness of the morbidity associated with infections that were traditionally classified as "asymptomatic" (King, 2015).

*Schistosoma mansoni* presents a complex life cycle that involves a freshwater snail where the ciliated miracidium undergoes asexual replication through mother and daughter sporocyst stages, eventually shedding thousands of cercariae (Colley et al., 2014). The cercariae exit the snail into freshwater approximately 4–6 weeks after infection and quickly penetrate the skin of the human host. Once in the skin, cercariae shed their tail and transform into schistosomula, which slowly migrate through the skin before entering the blood capillaries en route to the lungs and ultimately into the portal system where they feed on blood and mature into dioecious adult worms (Miller and Wilson, 1980; Gryseels et al., 2006). Schistosome adult flukes live for 3–10 years in their definitive human hosts, mainly due to their ability to avoid immune-mediated clearance (Kusel et al., 2007). Their unique, dynamic, tegumental structure and the complex immuno-evasive

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strategies employed by these parasites help them survive in the harsh intravascular environment (Pearce and MacDonald, 2002).

The tegument of *S. mansoni* participates in different processes such as nutrition, excretion, signal transduction, osmoregulation and immune evasion and modulation, playing a key role in host–parasite interactions (Jones et al., 2004; Mulvenna et al., 2010). The great complexity of this dynamic membrane helps the parasite to transform from a free-living phase into a parasitic stage that migrates through distinct environments in the definitive host (Brink et al., 1977). By replacing its tegumental composition, in just a few hours, the parasite switches from an immune-sensitive to an immune-refractory state (Jones et al., 2004). Different studies have analysed the morphological changes that occur in the outer membrane of *S. mansoni* during development from cercariae to adult worms under different conditions. Hockley and McLaren (1971) described a trilaminate outer membrane in cercariae and a multilaminate membrane in adult worms, and then went on to implicate membrane-bound vacuoles produced by sub-tegumental cells in the formation of the heptalaminate membrane of juvenile parasites (Hockley and McLaren, 1973).

The use of in vitro transformed schistosomula was first studied by Clegg and Smithers (1972), who showed that the growth rate of schistosomula in vitro was identical to the growth rate in vivo for at least 12 days. In addition, the morphological changes that occur in the schistosomula in the first 4 days post-transformation are similar in parasites isolated from the lungs of mice and in parasites cultured in media (Samuelson et al., 1980). The similar morphological features displayed by in vitro and in vivo transformed schistosomula (Brink et al., 1977; Samuelson et al., 1980), opened the possibility of using manually transformed schistosomula for laboratory research instead of lung schistosomula which are notoriously difficult to obtain in large numbers. More recently, gene expression studies revealed the surprising similarities between mechanical- and skin-transformed schistosomula, where significant differences were detected in the expression of just 38 from ~11,000 different genes (Protasio et al., 2013). While the transcriptional changes in the first few days of schistosomula development in vitro have been characterised (Fitzpatrick et al., 2009; Gobert et al., 2010), few studies have addressed the proteome of this critical life cycle stage (De la Torre Escudero et al., 2011; Hong et al., 2011, 2013), and none have focused on the surface proteome of the two most important human schistosome species, *S. mansoni* and *S. haematobium*.

The schistosomula stage is critical for sexual maturation and parasite establishment, and its surface plays an important role in host–parasite interactions, being the most susceptible target for vaccines and drugs against *Schistosoma* spp. (Jones et al., 2004; Loukas et al., 2007). Using a combination of tegumental labelling and high-throughput quantitative proteomics techniques we have identified a number of proteins that are highly expressed on the tegument of *S. mansoni* schistosomula at different stages of development, and highlight the utility of some of these proteins for the design of novel therapeutics against this important neglected tropical disease.

## 2. Materials and methods

### 2.1. Parasite material

*Schistosoma mansoni* (Puerto Rican strain) -infected *Biomphalaria glabrata* snails were provided by the National Institute of Allergy and Infectious Diseases (NIAID) Schistosomiasis Resource Center for distribution through BEI Resources, NIAID, National Institutes of Health (NIH), USA: *S. mansoni*, Strain NMRI Exposed *B. glabrata*, NR-21962. Cercariae were collected from the snails as described previously (Tucker et al., 2013), and immediately transformed into schistosomula by vortexing followed by a Percoll gradient to separate tails from bodies (Colley and Wikel, 1974; Tucker et al., 2013). Transformed schistosomula were incubated in Basch medium at 37 °C, 5% $CO_2$ (Basch, 1981) at a density of ~30,000 schistosomula in 4 mL of medium for 3 h, 2 days and 5 days. As a control, non-cultured schistosomula were processed immediately after transformation from cercariae.

### 2.2. Biotinylation of schistosomula tegument

The method used for tegumental biotinylation is a modified version of the method followed by Mulvenna et al. (2010). Approximately 60,000 schistosomula were used at each time point studied. The parasites were cultured for defined periods as described in Section 2.1, washed in Hank's Buffered Salt Solution (HBSS; Invitrogen, USA) twice at room temperature (RT) and incubated with 1 mM EZ-Link Sulfo-NHS-SS-Biotin (Thermo Fischer Scientific, USA) for 30 min at 4 °C. Biotin was then removed and parasites washed three times at RT in RPMI 1640 with free amino acids (Invitrogen) to quench any remaining biotin. The parasites were then snap-frozen in liquid nitrogen and kept at −80 °C until further use. Teguments were removed using the freeze/thaw/vortex technique (Roberts et al., 1983), where schistosomula were slowly thawed on ice, washed in TBS (10 mM Tris/HCl, 0.84% NaCl, pH 7.4) and incubated for 5 min on ice in 10 mM Tris/HCl, pH 7.4, before vortexing for five 1 s bursts. The tegumental extract was pelleted at 1000*g* for 30 min and solubilised three times in 200 µl of solubilising solution containing 0.1% (w/v) SDS, 1.0% (v/v) Triton X-100 in 40 mM Tris, pH 7.4 with pelleting at 15,000*g* between each wash. The washes were combined and incubated with 240 µl of streptavidin-agarose beads (GE Healthcare, UK) for 2 h at RT with gentle head-over-head mixing. After pelleting the beads, the supernatant was collected and proteins that were not bound to streptavidin beads were retained as the "unbound" fraction. Proteins bound to streptavidin were eluted by incubating the beads three times with 300 µl of 2% SDS for 10 min at 95 °C followed by vortexing for 2 min. Proteins eluted from streptavidin in SDS were retained as "biotinylated" proteins.

### 2.3. Immunofluorescence and microscopy analysis

For microscopy purposes, parasites were biotinylated with 1 mM EZ-Link Sulfo-NHS-SS-Biotin as described in Section 2.2. Parasites were then washed three times in HBSS, incubated with streptavidin-FITC for 30 min at RT and washed twice more with HBSS. Negative control parasites were not labelled with biotin and were instead incubated with just streptavidin-FITC. Biotinylated parasites that were not subjected to streptavidin-FITC were also analysed to detect autofluorescence from the biotin label. Samples were visualised using a Zeiss AxioImager M2 ApoTome fluorescence microscope (Zeiss, Germany) equipped with an AxioCam MRn at 60× magnification.

To visualise the extent of biotin internalisation in schistosomula, biotinylated and unbound (control) parasites were fixed in 4% paraformaldehyde in 0.1 phosphate buffer, pH 7.4, for 30 min at RT followed by thorough washing in PBS. Samples were embedded in paraffin, cut and stained with streptavidin-Alexa-555 and counterstained with DAPI before visualisation with a LSM 780 confocal microscope (Zeiss) at 100× magnification.

### 2.4. Protein digestion and iTRAQ labelling

Two biological samples for each time point were processed and analysed as follows: iTRAQ labelling, reduction, alkylation and digestion was performed on biotinylated and unbound samples



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