# Proteomic Profiling of the Planarian *Schmidtea mediterranea* and Its Mucous Reveals Similarities with Human Secretions and Those Predicted for Parasitic Flatworms*[S]

**Donald G. Bocchinfuso‡§, Paul Taylor‡, Eric Ross¶, Alex Ignatchenko‖, Vladimir Ignatchenko‖, Thomas Kislinger‖**, Bret J. Pearson§‡‡, and Michael F. Moran‡§‖§§¶¶**

**The freshwater planarian *Schmidtea mediterranea* has been used in research for over 100 years, and is an emerging stem cell model because of its capability of regenerating large portions of missing body parts. Exteriorly, planarians are covered in mucous secretions of unknown composition, implicated in locomotion, predation, innate immunity, and substrate adhesion. Although the planarian genome has been sequenced, it remains mostly unannotated, challenging both genomic and proteomic analyses. The goal of the current study was to annotate the proteome of the whole planarian and its mucous fraction. The *S. mediterranea* proteome was analyzed via mass spectrometry by using multidimensional protein identification technology with whole-worm tryptic digests. By using a proteogenomics approach, MS data were searched against an *in silico* translated planarian transcript database, and by using the Swiss-Prot BLAST algorithm to identify proteins similar to planarian queries. A total of 1604 proteins were identified. The mucous subproteome was defined through analysis of a mucous trail fraction and an extract obtained by treating whole worms with the mucolytic agent *N*-acetylcysteine. Gene Ontology analysis confirmed that the mucous fractions were enriched with secreted proteins. The *S. mediterranea* proteome is highly similar to that predicted for the trematode *Schistosoma mansoni* associated with intestinal schistosomiasis, with the mucous subproteome particularly highly conserved. Remarkably, orthologs of 119 planarian mucous proteins are present in human mucosal secretions and tear fluid. We suggest planarians have potential to be a model system for the characterization of mucous protein function and relevant to parasitic flatworm infections and diseases underlined by mucous aberrancies, such as cystic fibrosis, asthma, and other lung diseases.** ***Molecular & Cellular Proteomics 11: 10.1074/mcp.M112.019026, 681–691, 2012.***

Proteomics based approaches aimed at elucidating the proteome of cells, tissues, or organisms are becoming ever more prevalent in the postgenomic era. Many protein databases have already been compiled, with such noteworthy entries as the human, mouse, yeast, *Caenorhabditis elegans*, and *Drosophila melanogaster* proteomes (1). Although completing a protein profile of a species with a previously unannotated genome is both experimentally and bioinformatically challenging, the information can provide numerous insights into the organism's cellular processes, with disease states and models being of special interest.

Having been used in research for over 100 years (2), the freshwater planarian *Schmidtea mediterranea* (herein referred to as planarians) is an emerging stem cell model known for its remarkable ability to regenerate large portions of missing body parts (3, 4). This regenerative ability is derived from a population of pluripotent, somatic stem cells called neoblasts capable of differentiating into all cell types in planarian (5–8). Planarians are also a *bona fide* model to study cilia and cilia-driven motility, as well as developmental plasticity (2, 9). Multiple experimental methods such as RNA interference and *in situ* hybridization approaches to study gene expression, as well as cilia-specific techniques have been developed making planarians a valuable experimental model. Planarians are covered in a mucous secretion of unknown composition, which is implicated in locomotion, predator avoidance, innate immunity, and substrate adhesion (10, 11). Although the planarian genome has been recently sequenced, its lack of annotation impedes planarians' potential to serve as a model for other worm species.

From the ‡Molecular Structure and Function Program, The Hospital for Sick Children, Toronto, Canada; §Department of Molecular Genetics, University of Toronto, Toronto, Canada; ¶Stowers Institute for Medical Research, Kansas City, Missouri; ‖Ontario Cancer Institute, University Health Network **Department of Medical Biophysics, University of Toronto, Toronto, Canada; ‡‡Developmental and Stem Cell Biology Program, The Hospital for Sick Children; and §§Banting and Best Department of Medical Research, University of Toronto, MaRS Centre, 101 College Street, Toronto, ON, M5G 1L7, Canada

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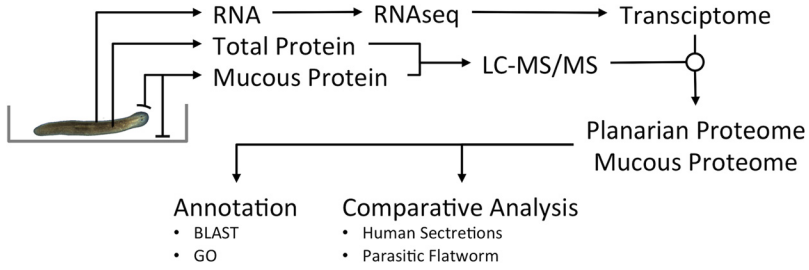


FIG. 1. **Experiment overview.** A schematic flowchart indicating the generation of a transcriptome database that was used to identify proteins following LC-MS/MS analysis of proteins isolated from whole worm or mucous preparations.

Until recently planarians had not been studied using proteomic based approaches (12, 13). In the current study, the planarian proteome and mucous fraction were characterized via high-resolution liquid chromatography-tandem MS (LC-MS/MS)[1] by using the multidimensional protein identification technology (MudPIT) method (14). A searchable protein database was constructed from sequenced planarian transcripts, and the Swiss-Prot basic local alignment search tool (BLAST), was used to annotate planarian proteins based on their similarity with known proteins in other organisms. Comparisons of planarian mucous proteins with mucosal secretions from other species including the parasitic flatworm *Schistosoma mansoni* (15) and humans revealed striking similarities (Fig. 1). These observations further establish planarians as a model organism, possibly opening new avenues for the study of parasitic infections and mucousopathies such as asthma, various lung diseases, and cystic fibrosis.

## EXPERIMENTAL PROCEDURES

*Preparation of Worm Lysates*—Lysates were generated from whole organisms of the CIW4 clonal strain of asexual *Schmidtea mediterranea*, sized matched to 2–4 mm, using a tissue homogenizer and lysis buffer containing 20 mM HEPES buffer (Cell Signaling Technology, Boston, MA) 8 M urea (EMD Chemicals, Darmstadt, Germany), 1 mM sodium orthovanadate (BioShop Canada Inc., Burlington, ON, Canada), 2.5 mM sodium pyrophosphate (Cell Signaling Technology), and 1 mM $\beta$-glycerophosphate (Cell Signaling Technology). Whole organisms were also treated with 5% NAC (Sigma-Aldrich, St. Louis, MO) in 1× PBS (BioShop) for 8 min to remove their mucous coating (16), and subsequently lysed as described above. Lysates were subsequently sonicated on continuous mode using three 30 s pulses with a sonic dismembrator (model 100; Fisher Scientific, Pittsburgh, PA). Mucosal extracts were generated by incubating whole organisms with 5% NAC in 3.3 mg/ml sodium bicarbonate (EMD, San Diego, CA) (17) as described above, and the NAC/mucous solution was extracted by pipeting. The extracted solution was concentrated using a 3 kDa molecular weight cutoff centrifugal filter (Millipore Ireland Ltd., Carrigtwohill, Ireland), and incubated with 20% acetone (Sigma-Aldrich) at −20 °C overnight to precipitate proteins. Precipitated proteins were pelleted by centrifugation at 15,000 × *g* for 10 min, and the resultant pellet was resuspended in 100 mM ammonium biocarbonate (BioShop) containing 8 M urea. Alternatively, a mucous fraction was generated by placing planarians and water (40 ml) into15 cm polystyrene Petri dishes (Sarstedt Inc., Newton, NC). Planarians were exposed to visible light for 3 h to induce motility, after which planarians were removed from the dishes. All water was collected, and the surface of each dish was washed vigorously with a solution of 8 M urea in 100 mM ammonium bicarbonate. The water and urea fractions were combined, and the resultant mixture was concentrated. All samples were reduced by using 45 mM dithiothreitol (Cell Signaling Technology) for 20 min at 60 °C and subsequently alkylated by using 100 mM iodoacetamide (BioShop) for 15 min at 23 °C in the dark. Lysates were digested overnight at 23 °C with trypsin (Thermo Scientific, Waltham, MA) in HEPES buffer containing tosyl phenylalanyl chloromethyl ketone protease inhibitor, and proteolysis was quenched with 1% trifluoroacetic acid (EMD).

*Liquid Chromatography and Mass Spectrometry Analysis*—An integrated nano-LC system (Easy-nLC; Thermo Fisher Scientific, Odense, Denmark) was used to perform a fully automated 9-cycle MudPIT analysis (14) on peptide samples from whole and NAC treated worms using inline strong cation exchange (SCX) and reversed-phase chromatography (18). Peptides from mucous extracts and the water/urea mixture were analyzed using only reversed-phase chromatography. Liquid chromatography was performed as described in the work of Taylor (18). Briefly, samples analyzed by MudPIT were loaded onto a 100 $\mu$m fused silica microcapillary column packed with 5 $\mu$m Magic C18 100 Å reversed-phase material (Michrom Bioresources Inc., Auburn, CA) and Luna 5 $\mu$m SCX 100 Å strong cation exchange resin (Phenomenex, Torrance, CA). An HPLC gradient was established consisting of 0%, 10%, 20%, 25%, 30%, 35%, 40%, 60%, and 100% ammonium acetate salt bumps, followed by a water/acetonitrile gradient. Samples analyzed using only reversed-phase chromatography were loaded onto an identical column packed only with reversed-phase resin, and subject to a water/acetonitrile gradient. Eluted peptides from both column setups were electrosprayed directly into a linear ion trap-Orbitrap Fourier transform mass spectrometer (LTQ-Orbitrap Classic; Thermo Fisher Scientific, Bremen, Germany) using a nanoelectrospray ion source (Proxeon Biosystems A/S, Odense, Denmark). MS spectra were obtained using a method that consisted of one MS full scan (400–1500 *m/z*) in the Orbitrap mass analyzer, an automatic gain control target of 500,000 with a maximum ion injection of 500 ms, one microscan, and a resolution of 60,000 (full-width half-maximum). MS/MS spectra were obtained in the linear ion trap analyzer using the six most intense ions at 35% normalized collision energy. Automatic gain control targets were 10,000 with a maximum ion injection time of 100 ms. A minimum ion intensity of 1000 was required to trigger an MS/MS spectrum. Dynamic exclusion was applied using a maximum exclusion list of 500 with one repeat count, with an exclusion duration of 40s.

*Database Creation*—Transcripts were assembled from 206 million 100 bp pairs reads from an IlluminaHiseq, 85 million 100 bp pairs of reads from an IlluminaGAxII and ~233 million 40 bp single end reads for an IlluminaGAxII. Each set of reads was assembled independently using the Trinity Assembly (19) pipeline with default parameters. The resulting transcriptome assemblies were then trimmed and assem-

[1] The abbreviations used are: LC-MS/MS, liquid chromatography-tandem MS; BLAST, basic local alignment search tool; E-value, expectation value; GO, gene ontology; MudPIT, multidimensional protein identification technology; NAC, N-acetylcysteine; SCX, strong cation exchange; SP, signal peptide.

bled together using the Velvet implementation available in Geneious (20) with default parameters. The resultant transcripts were filtered for contaminates (*i.e.* did not match genomic sequence) and transcripts which did not encode an open-reading frame of >100 amino acids (300 bp) were discarded. Finally, we compared our transcripts to *de novo* transcriptome assemblies from published data sets (13, 21, 22). Transcripts present in these assemblies which were missing from our assembly were added to our final transcriptome data set[2].

*Criteria for Peptide and Protein Identification and Protein Grouping*—The complete method used for identification of peptides and proteins is described in Gortzak-Uzan *et al.* (23). Briefly MS/MS data were analyzed by using the search engine X!Tandem (CYCLONE 2010.12.01.2) (www.thegpm.org). Search analyses were performed assuming trypsin digestion allowing one missed cleavage, with a fragment ion mass tolerance error of 0.4 Da and precursor ion mass tolerance of 20 ppm. The iodoacetamide derivative of cysteine was specified as a fixed modification, whereas the oxidized form of methionine and N-terminal glutamate to pyroglutamate acid conversion were specified as variable modifications. Using a Python (version 2.6.2)-based tool a false-positive rate was calculated on the peptide level by using a scrambled version of the same database as used for initial searching. Peptides were binned into three charge states (+2, +3, +4), and X!Tandem expectation values were calculated to minimize peptides matching to decoy sequences for each charge state. The total value of reverse spectra to total forward spectra was set at 0.2%, resulting in a low number of decoy sequences in the final protein list (<0.5%). In generating a final list of proteins, only proteins identified with ≥2 unique peptides and ≥7 amino acids were accepted. Using a database grouping algorithm designed to minimize protein interference, proteins were grouped favoring parsimonious clustering (24, 25). Identified proteins were annotated with the entire UniProtKB/Swiss-Prot (519,348 entries) and UniProtKB/TrEMBL (11,636,205 entries) databases (release 2010_09), using the Swiss-Prot BLAST algorithm. BLAST homology was determined by using the best BLAST match, regardless of species, with an expectation value (E-value) inclusion threshold of 0.001. Complete information on all peptide and protein identifications, including identification probabilities and sequence coverage can be found in supplementary Table S1 and S2, respectively.

*Gene Ontology Analysis*—Gene Ontology analysis was performed with Swiss-Prot accession numbers by using the ProteinCenter software suite (version 3.8.2014, Thermo Fisher Scientific, Odense, Denmark) on March 16, 2012. Proteins were searched using the entire Ensembl human protein database as background at a false-discovery rate of 5%.

## RESULTS AND DISCUSSION

*Mass Spectrometry Analysis*—Whole planarians were homogenized, lysed, and proteins subjected to in-solution trypsin digestion as described under "Experimental Procedures". Resultant peptide solutions were analyzed by a MudPIT method combining SCX and reverse-phase LC. Eluted peptides were ionized by electrospray and injected into an LTQ-Orbitrap instrument. Identical analysis was performed on whole worms following treatment with NAC to remove the external mucous coating. The neutral pH NAC mucous extract, far less complex than the whole worm preparations, was analyzed by one-dimensional reversed-phase chromatography prior to MS/MS. Another solitary mucous fraction, the mucous trail, was produced by allowing planarians to migrate freely across the surface of a 15 cm Petri dish. Planarians were removed from the dish, and surface-adhered mucous was harvested for MS/MS analysis (see Experimental Procedures). Shown in Fig. 2 is a representative example of an MS/MS acquisition and identification for ion 817.39 (*m/z*) corresponding to the mucous peptide HGGIDLGFNMPSFGGK.

We note that a mucous extract was also produced by using an acidic NAC solution (pH 2), which is commonly used during the preparation of worms for staining and microscopic imaging. This approach was abandoned because it was suspected to result in the release of membrane and/or internal proteins as an artifact of the harsh nature of the method, which rendered worms immobile and flat as a consequence of the immediate lethality of the treatment. By contrast, the bicarbonate-buffered NAC protocol used in this study allowed for the collection of mucous while worms remained viable as evidenced by their continued motility including slow contraction of the dorsal muscles during NAC treatment, causing them to assume a bent, crescent shape.

*Annotating Identified Proteins*—A planarian transcriptome database containing greater than 25,000 planarian transcripts was used to create a protein sequence database by using an algorithm designed to translate the transcriptome into all six possible reading frames. A six-frame translation of the database was necessary because it was assembled using hundreds of millions of short sequencing reads without direction, making unknown which direction/reading frame transcripts were in. This approach is commonly used in searching mass spectrometry data with sequences in which the correct reading frame for translation is unknown (26, 27).

The efficacy of using nucleic acid sequences to search mass spectrometry data was first shown in a proof-of-principle study using known proteins (26). More recently, this aptly named "proteogenomics" method (28) has since been used with larger genomes (29–32), including full genome translations (33, 34). Integrating transcriptomic sequences with proteomic data provides an additional level of information not realized with genomic sequences in that genes can be validated on the transcriptional level, as transcriptomic sequences represent only transcribed DNA (35).

The translated database was used to analyze MS data as described under "Experimental Procedures," and the final list of protein identifications was assembled at a false-discovery rate of <0.5%. A total of 1604 planarian proteins, each identified by at least two unique peptides were identified (supplemental Tables S1 and S2). The complete list of identified proteins with annotation is available in supplemental Table S3. This data set contains the transcript accession number, the Swiss-Prot protein description, accession number, E-value, and the number of identified peptides for each protein.

An initial assessment of the mucous proteome was made by a differential analysis comparing whole worms to worms that had been treated with NAC to remove their mucous. This

[2] Manuscript in Preparation – Bret J. Pearson, 2012.

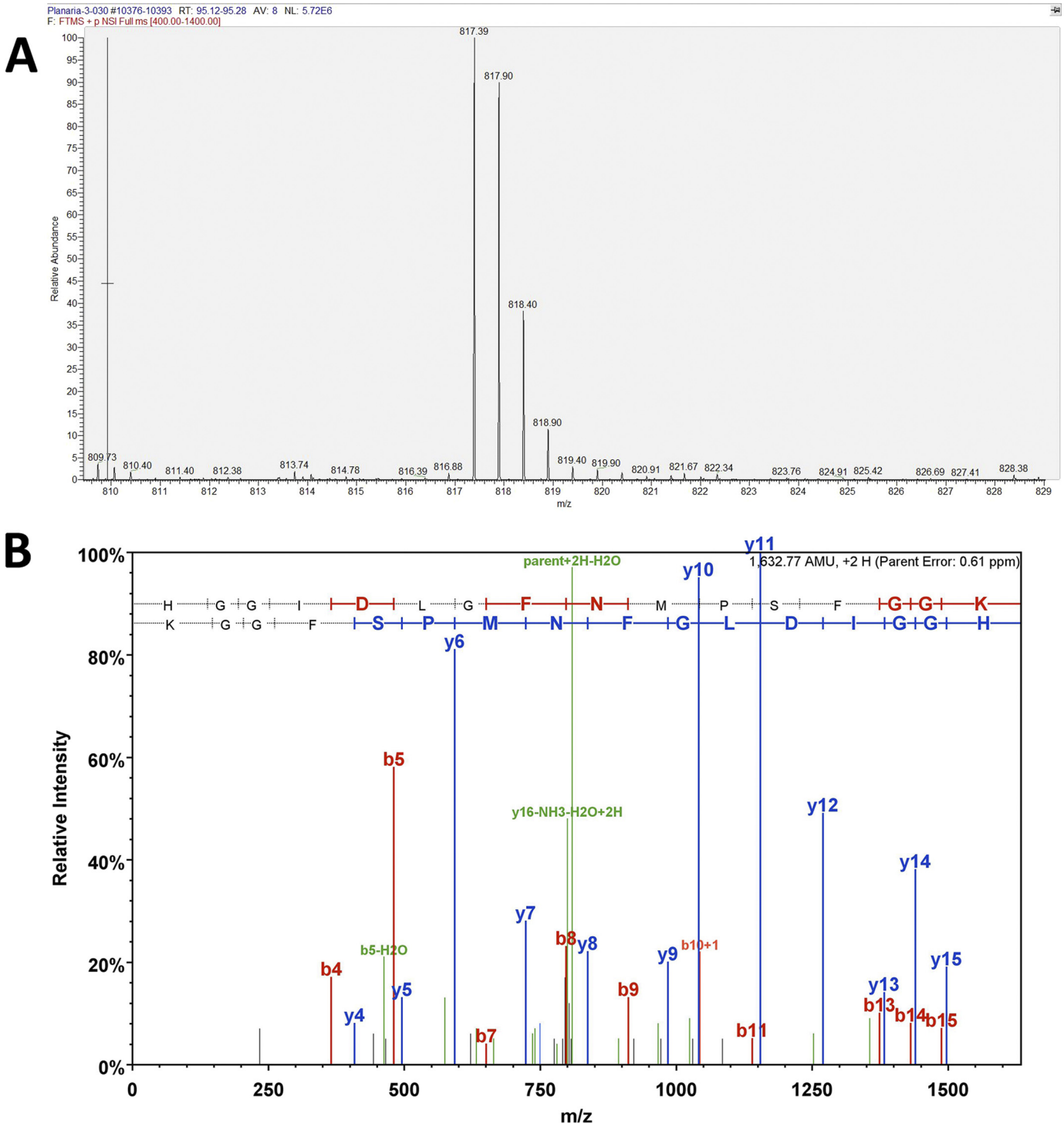


FIG. 2. **Orbitrap-FT data for the peptide HGGIDLGFNMPSFGGK at *m/z* 817.** 39. *A*, The MS spectrum for the doubly charged peptide at 817.39. *B*, The fragmentation spectrum (MS/MS) with the fragment ions annotated.

indicated 236 NAC-sensitive proteins that were unique to whole, untreated planarians, and seven unique to the NAC-treated worms (Fig. 3*A*, supplemental Table S3). These numbers suggest that NAC-treated worms had fewer proteins because of the effective removal of their mucous fraction, but also suggests that, because of the sampling nature of the MS/MS protocol, the recorded protein lists have not fully accounted for the entire worm proteome. MS analysis of the buffered NAC extract revealed 249 proteins (supplemental Table S3). The majority of NAC extract proteins (247) were

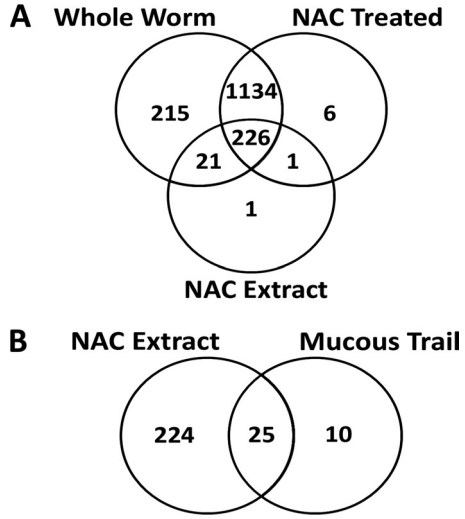


FIG. 3. **Venn diagrams depicting overlap in nonredundant peptides among analyzed fractions.** *A*, Overlap between whole worm, NAC-treated worms, and NAC extract samples. *B*, Overlap between NAC extract and mucous trail samples.

detected in whole worms, and a fewer number (227) were found in the NAC-treated worms (Fig. 3*A*). This suggests the NAC treatment was enriched for mucous proteins, but that many mucous proteins may not reside exclusively in the mucous compartment.

Mucous-producing species are known to display bilateral secretion, secreting biochemically different mucosal fractions (36, 37). These fractions function independently of one another, consisting of a "trail," left behind during locomotion, and a portion more closely associated to the animal's exterior, used in times of inactivity. In gastropods, this "inactive" portion contains up to 2.7 times more protein by mass than the trail portion (36). Consistent with this, the mucous fraction recovered from Petri dishes previously inhabited by planarians was found to contain only 35 proteins, with 25 overlapping with the NAC extract (Fig. 3*B*, supplemental Table S3), and may represent a distinct "trail" subset of the planarian mucous proteome. Collectively, 452 nonredundant proteins were implicated as mucous proteins by their presence in the NAC extract, mucous trail fraction, or NAC-sensitive worm association.

Proteins identified by MS were annotated by comparing identified protein sequences to characterized proteins from all available species using the Swiss-Prot BLAST algorithm (supplemental Table S3). The top BLAST entry corresponding to each identified protein was used to annotate protein hits, with an E-value inclusion threshold of 0.001. In total, 1252 identified proteins were matched to a BLAST entry, whereas 352 had no BLAST match. Of the 249 proteins identified in the buffered NAC extract, 189 had a corresponding BLAST match, as did 22 of the 35 mucous trail fraction proteins. Twenty-two mucous trail proteins were also found in the buffered NAC extract, whereas 34 mucous trail proteins were also identified in untreated planarians. In total, out of all 452 candidate mucous proteins, a number that includes the 236 NAC-sensitive proteins from whole worms, 299 had a corresponding BLAST match.

*Comparing Mucous Proteins to Published Secretomes*—Identified planarian mucous proteins were compared with member proteins of published secretomes, from human mucous (38–40), and human tear fluid (41). Strikingly, 119 planarian mucous proteins, which group into 70 related protein families, appeared to be orthologs or very similar to proteins identified in these characterized secretomes. Table I shows the overlap between identified NAC extract, mucous trail, and NAC-sensitive proteins with proteins from published secretomes described above (see supplemental Table S4 for Swiss-Prot identifiers). This provided validation to our annotation of planarian mucous proteins, and to the best of our knowledge for the first time revealed mucous proteins conserved across diverse species. Comparatively, nasal mucous shared eight proteins with the NAC extract, two "NAC-sensitive" proteins, and two proteins with mucous trail. Olfactory cleft mucous shared seven proteins with mucous trail, 31 proteins with the NAC extract, and seven similar to NAC-sensitive proteins. Cervical mucous shared two proteins with mucous trail, 35 proteins with the NAC extract, and 13 "NAC-sensitive" proteins. Tear fluid shared 8 proteins with mucous trail, 77 proteins with the NAC extract, and had 34 "NAC-sensitive" proteins. Collectively, this represents a 7%, 40%, 47%, and 20% overlap for nasal mucous, olfactory cleft mucous, cervical mucous, and tear fluid respectively.

Some overlapping proteins most likely play specific roles within the mucous environment. For example, collagen, which is present in different isoforms in both planaria mucous and tear fluid, is hygroscopic in nature, consequently serving as an external emollient (42). Peroxiredoxins, which serve as antioxidants in mucous (43), are both found in planaria mucous and other secretomes (Table I). In other worm species, such as the annelid *Laeonereisacuta*, antioxidant proteins play a substantial role in protecting the worm against environmental reactive oxygen species (44). Specifically, *L. acuta* secrete large amounts of mucous that contains the antioxidant species catalase, superoxide dismutase, and glutathione peroxidise. These enzymes intercept or degrade environmental peroxyl and hydroxyl radicals originating from organic matter in their aqueous environments (45). Likewise, the antioxidant activity of the mucosa covering respiratory tract epithelial cells in humans has been shown to be crucial for protecting against radical damage from environmental pollutants and bodily microorganisms (46).

*Gene Ontology Analysis*—GO classification remains an effective method to analyze previously unexamined proteomes (41), as is the case with planarian. In order to perform GO analyses (47), identified planarian proteins were annotated with homologous human matches, using the Swiss-Prot BLAST algorithm. GO analysis was performed

TABLE I

*Overlap between identified NAC extract, mucous trail, and NAC-sensitive proteins with proteins from published secretomes*

| | Putative human orthologs identified in *S. meditteranae Mucous* | Mucous fraction | | | Related proteins identified in human secretomes | | | |
|---|---|---|---|---|---|---|---|---|
| | | NAC-Sensitive | NAC extract | Trail | Tear fluid (de Souza et al. 2006, Ref. 41) | Olfactory cleft mucous (Débat et al. 2007, Ref. 39) | Cervical mucous (Panicker et al. 2010, Ref. 40) | Nasal mucous (Casado et al. 2005, Ref. 38) |
| 1 | 14-3-3 protein epsilon, zeta | | ● | | 14-3-3 epsilon, zeta/delta, beta/alpha | | 14-3-3 epsilon, sigma, zeta/delta | |
| 2 | 40S ribosomal protein: S8, S9, S11, S12, S14, S15, S21, S23, S27, 60S: P1, L6, L7a, L8, L17, L27, L32, L34, L35a | ● | ● | | 60S acidic RP P0, 40S RP S3, S27a, Similar to 40S RP SA | | | |
| 3 | Actin, Actin-2 | | ● | ● | Actin-like protein 2, Actin, cytoplasmic 2, Actin-like protein 3 | Actin 1, 2 | Beta-actin | Actin, alpha-2 |
| 4 | Actophorin | | ● | | Cofilin | | | |
| 5 | Adenosylhomocysteinase | | ● | | Adenosylhomocysteinase | | | |
| 6 | Adenylyl cyclase-associated protein 1 | | ● | | Adenylyl cyclase-associated protein 1 | | | |
| 7 | Alpha-1, 2 macroglobulin | | ● | | Alpha-2-macroglobulin precursor | | | $\alpha$2, $\beta$2-macroglobulin |
| 8 | Annexin A7 | | ● | | Annexin A1, A2, A3, A4, V, Splice Isoform 1 of Annexin A7 | Annexin A1, A2, A3, A7 | Annexin A1, A2, A3, A5 | Annexin A2 |
| 9 | Basement membrane proteoglycan | | ● | | BM heparan sulfate proteoglycan precursor | | | |
| 10 | Calcium-binding protein 16 kDa, 20 kDa | | ● | | Calcium-binding protein A4, 45 kDa precursor | | | |
| 11 | Calreticulin | | ● | | Calreticulin precursor | Calreticulin (precursor) | | |
| 12 | Calumenin | ● | | | Splice Isoform 1 of Calumenin precursor | | | |
| 13 | Catalase | | ● | | Catalase | | Catalase | |
| 14 | Chitinase 4 | | ● | | Chitinase 3-like protein 2 precursor | | | |
| 15 | Collagen alpha-1I, 1II, 1V, −2 I, V | ● | ● | | Collagen alpha 1(VI) chain precursor | | | |
| 16 | Coronin-1C | | ● | | Coronin-1A | | | |
| 17 | Cystatin-A | | ● | | Cystatin C precursor | Cystatin SN | Cystatin A, B | |
| 18 | Dihydropyrimidine dehydrogenase | ● | | | Dihydropyrimidine dehydrogenase precursor | | | |
| 19 | Dipeptidyl peptidase 1, 3 | | ● | | Dipeptidyl peptidase 4 | | | |
| 20 | DJ-1 | | ● | | DJ-1 | | | |
| 21 | Dynein light chain 1, 8 | ● | ● | | Dynein heavy chain | | | |
| 22 | Elongation factor 1$\alpha$, 2 | | ● | | Elongation factor 1-alpha, delta, gamma, 2 | Elongation factor Tu (precursor) | Elongation factor 1-alpha 1 | |
| 23 | Enolase | | ● | | Alpha-enolase | $\alpha$-Enolase | | |
| 24 | Eukaryotic translation initiation factor 3D | ● | | | EIF 3, 4A-I | | | |
| 25 | Fatty-acid binding protein | | ● | | | | Fatty-acid binding protein | |
| 26 | Filamin-1 | ● | | | Filamin A, SI 1of Filamin B | | | |
| 27 | Fructose-1,6-bisphosphatase 1 | | ● | | Fructose-1,6-bisphosphatase | | | |
| 28 | Fructose-bisphosphate aldolase | | ● | | Fructose-bisphosphate aldolase A | | | |
| 29 | Gelsolin-like protein 1, 2 | | ● | | Gelsolin precursor | | | |
| 30 | Glucose-6-phosphate isomerase | | ● | | Glucose-6-phosphate isomerase | | | |
| 31 | 78 kDa glucose-regulated protein | | ● | | Glucose-regulated protein precursor | Glucose-regulated protein 78 kDa | | |
| 32 | Glutathione S-transferase, mu 1, mu 28 | | ● | | Glutathione S-transferase | Glutathione S-transferase A1, P | Glutathione-S-transferase | |
| 33 | Glyceraldehyde-3-phosphate dehydrogenase | | ● | | Glyceraldehyde 3-phosphate dehydrogenase | Glyceraldehyde-3-phosphate dehydrogenase | | |
| 34 | Glyoxylate/hydroxypyruvate reductase | | ● | | Glyoxylate/hydroxypyruvate reductase | | | |
| 35 | Golgi apparatus protein 1 | ● | | | Golgi apparatus protein 1 | | | |
| 36 | Guanine nucleotide-binding protein beta-1 | | ● | | Guanine nucleotide-binding protein beta-2 | | | |

TABLE I—*continued*

| | Putative human orthologs identified in *S. meditteranae Mucous* | Mucous fraction | | | Related proteins identified in human secretomes | | | |
|---|---|---|---|---|---|---|---|---|
| | | NAC-Sensitive | NAC extract | Trail | Tear fluid (de Souza et al. 2006, Ref. 41) | Olfactory cleft mucous (Débat et al. 2007, Ref. 39) | Cervical mucous (Panicker et al. 2010, Ref. 40) | Nasal mucous (Casado et al. 2005, Ref. 38) |
| 37 | Heat shock protein 10 kDa, 60 kDa, 40-3, cognate 70, −3, 71 | ● | ● | | HSP $\beta$, $\beta$170 kDa 1B, 4, 90 $\alpha$2, HS cognate 71 kDa | HSP 27, 60, 70, HSC 70 | HSP 70 1, 1L, 5, 6, 8, HSP beta-1 HSP 90-alpha, beta | |
| 38 | Heterogenous ribonucleoprotein K, U1 small nuclear ribonucleoprotein A | ● | | | SI RNP D0, RNP F | Hetero nuclear RNP K | | |
| 39 | Histone H1-gamma, H2B, H3 | ● | ● | | Histone H2A.e | | Histone H4 | Histone H2B, H4 |
| 40 | Inorganic pyrophosphatase | | ● | | Inorganic pyrophosphatase | | | |
| 41 | Isocitrate Dehydrogenase $\alpha$ (probable) | ● | | | Isocitrate dehydrogenase | Isocitrate dehydrogenase | Isocitrate dehydrogenase $\alpha$ | |
| 42 | Major Vault Protein | | ● | | Major vault protein | | | |
| 43 | Malate dehydrogenase | | ● | | Malate dehydrogenase | | | |
| 44 | $\alpha$-Mannosidase | ● | | | $\alpha$-Mannosidase II | | | |
| 45 | Matrix metalloproteinase-19 | | ● | | Matrix metalloproteinase-9 precursor | | | |
| 46 | Myosin heavy chain, light chain 2 | | ● | | Myosin heavy chain | | | |
| 47 | Peptidase inhibitor 16, Kunitz-type serine protease inhibitor 6 | | ● | ● | Protease C1 inhibitor precursor | | | |
| 48 | Peptidyl-prolyl cis-trans isomerase B, FKBP2 | ● | ● | | Peptidyl-prolyl cis-trans isomerase A, C | | Peptidyl-prolyl cis–trans isomerase A | |
| 49 | Peroxiredoxin-6 | | ● | | Peroxiredoxin 1, 4, 5, 6 | Peroxiredoxin 1, 2, 5, 6 | Peroxiredoxin-1, 5 | Thiol-specific antioxidant protein |
| 50 | Phosphoglycerate kinase | | ● | | Phosphoglycerate kinase 1 | | Phosphoglycerate kinase 1 | |
| 51 | Plastin-1 | | ● | | Plastin 3 variant, L-plastin | | Plastin-1d, 2e | |
| 52 | Profilin-4 | ● | | | Profilin-1 | | | |
| 53 | Prominin-1 | ● | | | Prominin-1 precursor | | | |
| 54 | Protein disulfide-isomerase 2, A3, A4 | ● | ● | | Protein dissulfide-isomerase A6 | Protein disulfide-isomerase A3 | Protein disulfide isomerase precursor | |
| 55 | Puromycin-sensitive aminopeptidase | | ● | | Puromycin-sensitive aminopeptidase | | | |
| 56 | Rap-2A | ● | | | Rab-1A | | | |
| 57 | Rab GDP dissociation inhibitor beta | | ● | | Rab GDP dissociation inhibitor beta | | | |
| 58 | Rho GTPase activating protein 1 | ● | | | Rho-GTPase-activating protein 1 | | | |
| 59 | Septin 4, 7 | ● | | | Septin 2, 7 | | | |
| 60 | Serine/threonine-protein phosphatase PGAM5 | ● | | | Serine-threonine phosphatase 2A, PP-1 | | | |
| 61 | Spectrin $\alpha$, $\beta$ chain | ● | ● | | Splice Isoform 1 of Spectrin $\alpha$ chain | | | |
| 62 | Stress-induced-phosphoprotein 1 | | ● | | | Stress-induced-phosphoprotein 1 | | |
| 63 | Superoxide Dismutase [Cu-Zn] | | ● | ● | | Superoxide dismutase [Cu-Zn] | Superoxide dismutase [Mn] | |
| 64 | Syntenin-1 | | | ● | Syntenin-1 | | | |
| 65 | Thioredoxin | | ● | | Thioredoxin | | Thioredoxin | |
| 66 | Thymidine phosphorylase | | ● | | Thymidine phosphorylase precursor | | | |
| 67 | Triosephosphate isomerase | | ● | | Triosephosphate isomerase 1 variant | Triosephosphate isomerase | Triosephosphate isomerase | |
| 68 | Tropomyosin | | ● | | | Tropomyosin $\alpha$3 | Tropomyosin-1$\alpha$, $\beta$ | |
| 69 | Tubulin alpha-1B, 2/4, beta-2, −2C | | ● | ● | Tubulin alpha-1, alpha-3, beta-2 | Tubulin alpha-1, 6, 8, beta-2 | | |
| 70 | Ubiquitin-1 | ● | ● | | | | Ubiquitin | |

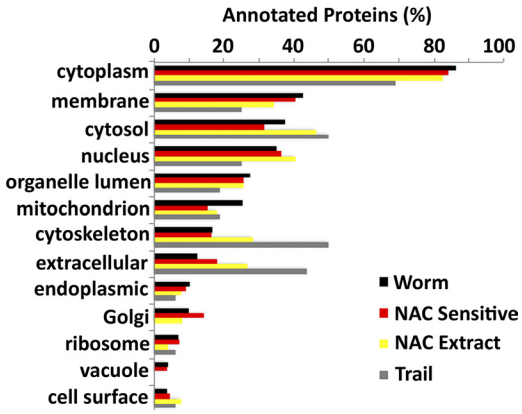


FIG. 4. **GO analysis examining cellular compartmentalization of annotated proteins identified in whole worms, the NAC-sensitive fraction, the NAC extract, and the mucous trail fraction.**

on all identified planarian proteins with a human BLAST match, including individual analyses for whole planarian, the NAC extract, and the mucous trail fractions. Analyses were performed by using the ProteinCenter software suite (Thermo Fisher Scientific), with statistical analyses done on the GO classifications of molecular function, cellular compartmentalization, and biological process. Results for cellular compartmentalization are shown in Fig. 4, whereas molecular function and biological process results are shown in supplemental Fig. S1.

Enrichment for extracellular proteins in the NAC extract is evident upon examination of the compartmentalization classification in comparison to the entire planarian proteome. Although 12% of proteins in the entire planarian proteome were classified as having an extracellular localization, 18% (43/236) of the NAC-sensitive set of proteins, more than a quarter (27%; 67/249) of the NAC extract proteins, and greater than 40% (15/35) of the mucous trail proteins were annotated as extracellular, with an overrepresentation of extracellular proteins over the background Ensembl human protein database. This demonstrates a clear enrichment for extracellular proteins in planarian mucous, as is expected for an extracellular fraction. Not all identified planarian mucous proteins hold GO annotations for the extracellular region, which is true for other published secretomes. This raises questions about the mechanism by which these proteins enter the extracellular compartment, but may also be an indication, at least for the present study, that the fractionation methodology was not perfected.

Analysis of signal peptide (SP) sequences reinforced the conclusion that the mucous fractions were enriched for secreted/extracellular proteins. Although the whole planarian proteome is comprised of 13% SP-containing proteins, the NAC extract had 18% SP proteins, and 20% of the set of NAC-sensitive proteins contained an SP sequence. The mucous trail fraction showed the greatest enrichment for SP-containing proteins at 30%. As SPs target proteins through the secretory pathway (48), enrichment for SP-containing proteins again serves to validate the identified proteins as bona fide mucous proteome constituents. Although not all the identified mucous proteins are predicted to contain an SP, this alone does not affect their legitimacy as extracellular constituents, as not all extracellular proteins contain this feature (49, 50).

GO analysis of the NAC extract revealed an underrepresentation of membrane-associated proteins, as referenced against the Ensembl human protein database. The low level of membrane- localized proteins supports the conclusion that the NAC treatment did not significantly disrupt membrane-associated proteins, causing them to partition into the NAC fraction as artifacts.

*Clinical Implications for the Planarian Mucous Model*—Given its significant overlap with proteins found in human secretions, the planarian mucous proteome may prove to be a useful model in human disease studies. Planarians have already proven to be an effective model in the study of growth and development, and have found acclaim as a stem cell model in studying regeneration.

Diseases associated with mucous pathology are prevalent in humans and other animals (51) and may benefit from research based on mucous models, especially in the development and testing of therapeutic agents. In the treatment of mucous hypersecretion, a condition correlated with asthma and poor prognosis in lung disease (52), practitioners sometimes rely on the use of unproven products that would benefit from testing in a model system (53). The use of mucous models is also important in the development of drugs that pass through mucosal layers, but do not necessarily target the mucous itself. Many drugs bind to and interact with mucous, affecting drug uptake, release, and overall efficacy (54). This is perhaps especially important in cystic fibrosis, a disease hallmarked by thick, dense mucous, which impedes drug delivery and diffusion (55).

Mucous models hold therapeutic importance in oral health care, where the protective and emollient properties of mucous are of particular interest. This is primarily evident in the treatment of mouth dryness, a common condition for which contemporary therapies do not sufficiently emulate natural saliva (56). The biochemical properties of mucous are also of commercial interest, as mucosal substances are used in coating biomaterials for low friction coefficient implants (57). Such commercial applications may not only benefit from planarian as a mucous model, but from planarian mucous or synthetic derivatives.

The planarian mucous proteome shares many proteins with tear fluid, making planarian a pertinent model for studying tear fluid in addition to mucous. Tear models hold practical for studying both the physical and chemical properties of tears, something which has shown to be important in the research and development of many commercial applications (58). Disease studies can also benefit from a proteomics-defined tear fluid model, as many ocular diseases result from irregularities

in the tear fluid proteome. Specifically, conditions such as diabetic dry eye disease have been linked to decreased reactive oxygen species protection (59), resulting from changes to protective proteins such as peroxiredoxins, found in both human tear fluid (60) and planarian mucous.

The nonparasitic flatworm *S. mediterranea* may serve as a model for parasitic flatworms, or flukes, such as *Schistosoma mansoni*, which is a significant parasite of humans, associated with intestinal schistosomiasis. Therefore, the proteins identified in our study were systematically compared with an *S. mansoni* gene database, annotated with proteins similar to *S. mansoni* queries using the Swiss-Prot BLAST algorithm (15). Of the 1604 *S. mediterranea,* proteins identified herein 1369 were also found in the *S. mansoni* proteome, representing an overlap of 85%. Interestingly, the proteins we identified in mucous fractions (NAC extract; trail mucous; NAC-sensitive) were also similar to proteins in the *S. mansoni* parasite, with overlap exceeding 75% (82%, 77%, 78%, respectively) (supplemental Table S5). The high overlap between the *S. mediterranea* and *S. mansoni* proteomes further establishes planarians as a model to study *S. mansoni* and other parasitic flatworms such as *Schistosoma japonicum*, which themselves present numerous experimental challenges (61). The extensive overlap between *S. mansoni* gene products and planarian mucous proteins is also noteworthy given that the pathogenicity of some parasitic worm species is driven by secretory products released into the host environment (62). Some of these proteins, such as serine and metallo proteases, and nucleoside diphosphate kinase were also found in planarian mucous. We suggest the potential to use planarians to identify and validate conserved flatworm mucous proteins as targets against which new drugs or therapeutic modalities may be developed.

## CONCLUSIONS

This study has provided annotation for the planarian proteome and mucous subproteome, broadening the potential of an already established model system. Annotation of the mucous proteome creates abundant possibilities for examining both the physiological and biochemical functions of mucosal proteins within the context of the mucous environment. Given the wide range of functions of planarian mucous, from locomotion and substrate adhesion, to predation and innate immunity, it is quite possible that mucosal proteins carry out these responsibilities as a function of previously undocumented mechanisms and properties. Lastly, many identified planarian proteins in this report share no significant BLAST match establishing the need for further genome and proteome annotation and functional characterization. These proteins may prove especially interesting if they are associated with tissue regeneration or other biological properties that distinguish planarians as a model system.

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S This article contains supplemental Fig. S1 and Tables S1–S5.

¶¶ To whom correspondence should be addressed: The Hospital For Sick Children, MaRS Centre, East Tower, Room 9-804, 101 College Street, Toronto, ON, M5G 1L7, Canada. Tel.: 001 647 235-6435; E-mail: m.moran@utoronto.ca.

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