Constitutive Secretion in *Tetrahymena thermophila* \(^\text{†‡} \)‡

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The growth, survival, and life cycle progression of the freshwater ciliated protozoan *Tetrahymena thermophila* are responsive to protein signals thought to be released by constitutive secretion. In addition to providing insights about ciliate communication, studies of constitutive secretion are of interest for evaluating the utility of *T. thermophila* as a platform for the expression of secreted protein therapeutics. For these reasons, we undertook an unbiased investigation of *T. thermophila* secreted proteins using wild-type and secretion mutant strains. Extensive tandem mass spectrometry analyses of secretome samples were performed. We identified a total of 207 secretome proteins, most of which were not detected in a set of abundant whole-cell protein identifications. Numerous proteases and other hydrolases were secreted from cells grown in rich medium but not cells transferred to a nutrient starvation condition. On the other hand, we detected the starvation-enhanced secretion of a small number of cytosolic proteins, suggestive of an exosome-like pathway in *T. thermophila*. Subsets of proteins from the *T. thermophila* regulated secretion pathway were detected with differential representation across strains and culture conditions. Finally, many secretome proteins had a predicted N-terminal signal sequence but no other annotated characteristic or functional classification. Our work provides the first comprehensive analysis of secreted proteins in *T. thermophila* and establishes the groundwork for future studies of constitutive protein secretion biology and biotechnology in ciliates.

Secreted proteins play fundamental cellular roles as signals for cell proliferation, survival, motility, growth arrest, or differentiation (17, 22). Secreted proteins can also function to alter the extracellular environment, for example, by acting to improve nutrient availability or hinder the growth of competing cells (2, 32). Beyond these physiological roles, the production of secreted proteins has expanding promise for contributing improvements in technology and human health (7, 23, 31). Numerous recombinant protein expression systems have been touted for high yield, efficient folding, or suitable posttranslational modification of secreted proteins, but new systems are necessary to expand the scope of protein production possibilities (14, 18).

Secreted proteins reach the extracellular environment by diverse routes of intracellular trafficking. The constitutive pathway of secretion relies on N-terminal signal peptide-mediated targeting of a nascent protein into the lumen of the endoplasmic reticulum, followed by signal peptide cleavage and a series of vesicle formation and trafficking steps that transport the secreted protein through the Golgi apparatus to the plasma membrane (13). This process is coupled with posttranslational protein modifications, including disulfide bond formation and the addition of asparagine-linked glycan chains. More recently recognized additional mechanisms of protein secretion can bypass the Golgi apparatus or even the requirement for a signal peptide (26). The existence of largely uncharted cellular pathways for protein secretion suggests that there is a much higher complexity in the content of secreted proteins, termed the secretome, than can be predicted using protein primary sequence or intracellular localization assays alone.

Ciliated protozoa are a group of single-celled organisms with aquatic habitats spanning ecological niches from saltwater ocean to the gut of domestic livestock. *Tetrahymena thermophila* is the most genetically tractable of the laboratory ciliates (9). Following their isolation from freshwater ponds of the Eastern United States, strains of *T. thermophila* have been maintained in laboratory cultures over a wide range of growth temperatures (typically 25°C to 37°C) and nutrient sources (from other organisms to standard peptone broths to chemically defined media). These and additional features, including large size, fast division time, and growth to high density, have encouraged pilot studies of *T. thermophila* as a platform for heterologous expression of secreted proteins (38).

*T. thermophila* constitutive secretion has been examined primarily by using enzyme assays to detect hydrolase activities released into spent culture medium (SCM). In the only previous proteomic study of SCM, affinity purification using the protease inhibitor E-64 enriched a small family of related cysteine proteases (19). The results of functional assays suggest numerous additional activities for secreted proteins, including an autocrine growth stimulation that is essential for cell viability at low density (30). Previous biochemical studies have focused on protein secretion from cells in rich media, but in the life cycle of *T. thermophila*, nutrient starvation is a prerequisite...
for the sexual reproductive cycle of conjugation. Starved cells of any two of the seven distinct mating types communicate to initiate meiosis, gamete exchange, and the subsequent formation of a zygotic nucleus (6). Even in the absence of a complementary mating type, starved cells undergo a series of choreographed changes in cell morphology and swimming behavior (9). These cytologically visible events and many more at the biochemical level would have been selected on an evolutionary timescale to adapt the species to seasonal variation in the pond environment. Whether morphological conversions or other responses to culture conditions are accompanied by changes in constitutive secretion has not been examined.

In addition to constitutive secretion, T. thermophila has a pathway of regulated secretion that is similar in many respects to the dense core granule pathway of some vertebrate cells (16, 36). Upon stimulation, membrane-docked mucocysts discharge their contents to encapsulate the cell in a thick mucus layer thought to provide a mechanism of defense. The granule lattice (GRL) proteins give the mucocysts their crystalline appearance and compose the insoluble phase of discharged mucus, while other proteins and peptides are released as soluble factors (3, 10). The packing of the GRL proteins into mucocysts requires cleavage of a prodomain following the N-terminal signal peptide (8, 37). Strains unable to perform regulated secretion have been isolated by forward genetics following mutagenesis (27). In-depth analysis of one mutant strain, SB281, suggests that GRL protein precursors retaining the prodomain are held in an unidentified membrane compartment of growing cells that, upon starvation, is shunted to constitutive secretion (4).

Here, we investigate the T. thermophila secretome by exploiting the preliminary annotation of the ~105-Mbp macro-nuclear genome with more than 20,000 predicted genes (11, 15). We profiled the T. thermophila proteins in SCM after population growth to high density in rich medium versus an overnight interval of nutrient starvation. Two inbred wild-type strains of different mating types were compared: CU428, a stock center (http://tetrahymena.vet.cornell.edu/) and maintained at room temperature, the supernatant was collected immediately after centrifugation, leaving behind the volume near the cell pellet. The culture supernatant was then filtered (Acrodisc 0.2-μm low-protein retention filter) and concentrated 20- to 50-fold by Vivospin (10-kDa cutoff; Sartorius Stedim Biotech) centrifugation. A small fraction of the sample was examined by SDS-PAGE and staining with Coomassie G-250 (SimplyBlue SafeStain; Invitrogen), while the majority of the sample was used for proteomic analysis. For analysis of the whole-cell proteome, CU428 cells were harvested following overnight growth in Neff medium and frozen at ~8°C. The pellet was resuspended in 50 mM Tris-HCl, pH 8, with 0.1% SDS and boiled for 3 min. The sample was then brought to 0.1% trifluoroacetic acid and clarified by centrifugation. A nanoelectrospray ionization (nanoESI) interface were performed as described previously (35). Protein separation, digestion, and peptide analysis were repeated in triplicate for each sample.

Protein identification using mass spectrometry. The MS-MS data were analyzed with both the Spectrum Mill (Rev A.03.03.084 SR4; Agilent Technologies) and Mascot (version 2.2.04; Matrix Science Ltd.) search engines (28) using parameters described previously (35) with minor modifications. Data were searched against a T. thermophila database that was generated by combining the T. thermophila predicted proteome (http://www.ciliate.org/) with the Swiss-Prot database (version 51.6). In a pilot analysis using Mascot, the limited complexity of the predicted proteome of T. thermophila gave rise to a higher percentage of false positives than we have commonly observed. Discussion with Matrix Science resulted in the suggestion to concatenate the T. thermophila FASTA database with the entire Toxoplasma database of search complexity. The search criteria were set to allow two missed cleavages by a tryptic digest with no protein modifications. Validated proteins scoring 13 or better were considered valid identifications for Spectrum Mill, whereas proteins identified with a significance threshold (P value) of <0.01 were considered valid identifications for Mascot. Proteins identified from one detected peptide using a single spectrum were excluded. A complete list of peptide sequences used for protein identification is provided in Table S1 in the supplemental material. Where multiple proteins were identified from the same set of peptide data, all proteins identified are listed. An estimation of false-positive rates for both Spectrum Mill and Mascot is provided in Methods in the supplemental material.

Relative quantification. A relative quantification of T. thermophila protein identifications by Spectrum Mill was performed as described previously (5) with minor modifications. Default parameters were used to generate chromatographic peak areas for each peptide precursor ion (time frame, ±15 s, and mass tolerance, ±0.001 Da). The protein relative quantification (PRQ) spectral peak intensity of all peptide precursor ions identified for each protein in each of the triplicate analyses. To normalize the data (nPTPSI), the PTPSI for each protein identified was divided by the total PTPSI for all proteins identified in the triplicate analyses of a given sample and multiplied by 100.

Annotation. The 2008 Tetrahymena Genome Database (TGD, http://www.ciliate.org) was used as the primary source of automated gene annotations, with supplemental domain and protein annotations from the TIGR T. thermophila gene annotations and GenBank. The SignalP (version 3.0) and TGD servers were used to determine the presence of a signal peptide. Proteins were assigned tentative functions using Gene Ontology (GO) project identifiers (1). The GO annotations use a controlled vocabulary, facilitating searches within TGD and across other databases.
RESULTS AND DISCUSSION

Investigation of the T. thermophila secretome. In its natural habitat, T. thermophila would experience cycles of population expansion through nutrient-rich conditions and survival through nutrient-poor conditions. To investigate whether cells respond to this type of change in part by altering the content of proteins released to the environment, we compared the SCM for cells grown in rich medium to that of cells in nutrient starvation conditions. We tested 3 strains in parallel: two distinct inbred wild-type strains (CU428 and SB210) and a strain defective in regulated secretion derived from the SB210 background (SB281). For the initial analysis, we detected SCM proteins by SDS-PAGE and staining with Coomassie G-250. Cells were pelleted gently to avoid cell lysis, and SCM was removed rapidly without recovery of the volume near the cell pellet (see Materials and Methods). Protein markers were run in lane 5, with standards at 27 and 66 kDa staining most strongly; molecular masses of standards are given at left.

Secretome validation and comparison across strains and culture conditions. Because secreted proteins constitute a minor fraction of the total cellular proteome, SCM contamination by whole-cell lysis represents a potential problem. The profiles of secretome proteins observed by SDS-PAGE were

FIG. 1. SDS-PAGE analysis of SCM from different strains (CU428, SB281, and SB210) under different culture conditions (growth to stationary phase in rich medium and starvation medium). Protein markers were run in lane 5, with standards at 27 and 66 kDa staining most strongly; molecular masses of standards are given at left.
consistent across repeat purifications and differed from the protein profiles of whole-cell samples (data not shown). To validate the secretome protein inventory defined by LC–MS–MS, we performed LC–MS–MS analysis of pelleted whole cells of the wild-type strain CU428 harvested after growth in rich medium. From this single sample, 260 distinct proteins were identified (see Table S3 in the supplemental material). This number of identifications is similar to that obtained in a methodologically similar analysis of the *T. thermophila* ciliary proteome (33). In comparing our set of 260 proteins with high representation in CU428 whole cells to the CU428 rich-medium secretome set of 156 proteins, only 15 proteins in common were identified (Fig. 2). In comparing the whole-cell set of 260 proteins to the starvation medium secretome set of 28 proteins for strain CU428, 11 proteins in common were identified (Fig. 2). When considering all 6 secretome samples combined, only 39 of 207 proteins overlapped the set of 260 abundant whole-cell proteins. More than half (58%) of the 39 proteins that comprise the overlap of the combined secretome samples and the whole-cell sample were found to possess a predicted signal sequence, suggesting that they were detected in the whole-cell sample en route to secretion. Based on these results and the additional analysis presented below, we conclude that our SCM purification, LC–MS–MS, and protein assignments yield secretome inventories specifically enriched for a proteome distinct from that of whole cells.

Consistent with the SDS-PAGE analysis of secretome protein contents, the maximal difference in secretome contents was observed in comparisons across culture conditions. The proteins secreted during growth in rich medium and those secreted during starvation were largely distinct, with more secretome proteins unique to the rich medium or starvation condition than were shared between them (Fig. 3). Using SB281 as an example, of the 51 rich-medium secretome proteins and 26 starvation medium secretome proteins, 12 proteins in common were identified (Fig. 3C). A greater overlap in secretome contents is evident when comparing different strains under the same culture condition (Fig. 4). Only a minority of secretome protein content is unique to any given strain in rich medium or starvation medium (the high number of protein identifications for the rich-medium secretome of CU428 makes this an exception) (Fig. 4A). Finally, we note some variance in the content of secreted proteins between the different wild-type strains of *T. thermophila*, consistent with anecdotal evidence for strain-to-strain differences in gene expression.

**Expected features of secretome proteins and protein families.** In the canonical pathway of constitutive secretion, nascent proteins are targeted for cotranslational insertion into the endoplasmic reticulum by recognition of an N-terminal signal sequence. For the identified secretome proteins, we evaluated the likelihood of an N-terminal signal sequence (see Table S2 in the supplemental material). The majority of identified secretome proteins contained a predicted signal sequence (134/207). Because this analysis relies on protein N-terminal prediction by automated gene annotation, it should be considered only a rough estimate of the actual percentage of secretome proteins targeted for secretion by the canonical pathway.

Previous analyses have reported papain family cysteine proteases in the secretome of *T. thermophila* (19, 21, 34). Indeed, regardless of strain or culture condition, papain family cysteine proteases always comprised a major fraction of the secretome proteins on a strain-by-strain basis (Fig. 5; Table S4 in the supplemental material lists these functional assignments on an
individual-protein basis). Some of the proteases are produced from top-abundance mRNAs expressed across the *T. thermophila* life cycle (25). In total, we identified 33 of the putative 114 *T. thermophila* papain family cysteine proteases (32 putative proteases and the phospholipase PLA1, which was assigned as a papain family cysteine protease by automated gene annotation; see Table S2 in the supplemental material). Although proteases as a category are well represented in the secretome under all conditions (Fig. 5), individual protease proteins have distinct specificities of secretome representation: some were detected only in rich-medium secretomes, or only in starvation medium secretomes, or under both culture conditions but only in a specific strain (see Table S2 in the supplemental material). Also, for proteases represented in most or all secretomes, variation in abundance was observed. For example, one of the cysteine proteases (TGD accession no. TThERM_881450) was detected in 5 of 6 secretomes with substantially different relative abundance: under the starvation condition, the nPTPSI for this protease in strain SB281 was 1.31, but in strains CU428 and SB210, the nPTPSI values were 8.27 and 16.6, respectively (see Table S2 in the supplemental material).

Glycosyl hydrolases are another logical class of secretome proteins. Ciliates can consume bacteria and fungi as food, which suggests that *T. thermophila* should possess a spectrum of hydrolytic enzymes that act on complex, as well as simple, sugars. We identified numerous secretome proteins annotated to have the predicted functions of glycosyl hydrolase, chitinase, or cellulase enzymes (see Table S2 in the supplemental material). These proteins were detected exclusively (by automated gene annotation) or almost exclusively (considering potential motifs in hypothetical proteins) in the secretome of cells cultured in rich medium (Fig. 5). This striking specificity suggests that *T. thermophila* alters the secretome content of carbohydrate hydrolases based on nutrient availability. In this regard, it is interesting that an abundant protein in the phagosome proteome, PHP1 (20), was detected in a subset of both the rich-medium and starvation medium secretomes (see Table S2 in the supplemental material). Phagocytosis initiates with the internalization of food particulates from the oral cavity, with...
TABLE 1. Relative quantification of secretome granule lattice protein identifications across strains and culture conditions

| Protein | TTHERM | SB281 Rich medium | SB210 Starvation medium | CU428 Starvation medium | SB281 Starvation medium | SB210 Starvation medium | CU428 Starvation medium |
|---------|--------|-------------------|-------------------------|------------------------|-------------------------|-------------------------|------------------------|
| GRL1    | 527180 | 0.22              | 0.01                    | 6.98                   |                        |                         |                        |
| GRL2    | 473020 | M                 |                         |                        |                        |                         |                        |
| GRL3    | 624730 | 0.26              | 0.03                    | 3.25                   |                        |                         |                        |
| GRL4    | 624720 | 0.93              |                         |                        | 11.56                   | 1.37                    |                        |
| GRL5    | 378800 | 0.4               |                         | 9.02                   | 1.43                    | 0.62                    |                        |
| GRL7    | 522600 | 38.98             | 0.03                    | 9.88                   |                        |                         |                        |
| GRL8    | 1055600| 0.11              |                         | 3.89                   | 1.03                    |                        |                        |
| GRL9    | 1018540| 0.4               |                         | 9.02                   | 1.43                    | 0.62                    |                        |

*Numbers in these columns are nPTPSI values as determined by Spectrum Mill. Bold values indicate proteins that were detected by both Spectrum Mill and Mascot. Proteins identified only by Spectrum Mill are not bold. Proteins identified only by Mascot are indicated by an M.*

subsequent vesicle trafficking to release waste at the opposite pole of the cell. The detection of PHP1 in 2 out of 3 starvation medium secretomes (SB210 and SB281) suggests that the release of processed phagosome contents continues after cells are transferred to a culture condition lacking food particulates. Alternately, PHP1 could have additional localizations and/or are transferred to a culture condition lacking food particulates.

The detection of PHP1 in 2 out of 3 starvation secretomes (SB210 and SB281) suggests that the release of processed phagosome contents continues after cells are transferred to a culture condition lacking food particulates. Alternately, PHP1 could have additional localizations and/or are transferred to a culture condition lacking food particulates.

**Cross-traffic of constitutive and regulated secretion.** Induced secretion of *T. thermophila* mucocyst contents results in the production of a rigid matrix surrounding the cell, creating a physical barrier between the cell and its environment (16, 36). Our cell culture and harvesting conditions avoided the known inducers of regulated secretion, and any released matrix of mucus should have pelleted with cells rather than being recovered with soluble components of SCM. Nonetheless, the combined secretome identifications detected 8 of the 10 GRL proteins that form the polygonal lattice of discharged mucus (10). Each of the 6 GRL proteins characterized as biochemically most abundant was detected in more than one secretome sample (GRL1, -3, -4, -5, -7, and -8), as was one of the two relatively uncharacterized GRL protein family members thought to be at low abundance (GRL9; GRL10 was not detected). In the quantitative analysis of constitutive secretome contents, GRL9 was not in low abundance relative to the levels of the other GRL proteins (Table 1). One of the two characterized GRL proteins thought to be minor contributors to mucocyst content was also identified (GRL2; GRL6 was not detected), but only in the rich-medium secretome of strain SB281.

We expected to detect GRL proteins in the starvation medium secretome of strain SB281, based on previous characterization of the mutagenesis-induced defect in regulated secretion in this strain (4, 27). Indeed, GRL proteins were best represented and highest in relative abundance in the SB281 secretomes. The SB281 rich-medium secretome included all 8 of the GRL proteins identified in the combined secretomes (8 of 51 total identifications), and the SB281 starvation medium secretome included 7 of these GRL proteins (7 of 26 total identifications). The SB210 secretomes included only a single GRL protein in rich medium (1 of 54 total) or 4 in the starvation condition (4 of 29 total), and the CU428 secretomes included only 2 GRL proteins in rich medium (2 of 156 total) or 2 distinct GRL proteins in the starvation condition (2 of 28 total). By quantitative analysis as well, the GRL proteins were dramatically more abundant in the secretomes of SB281 than in the secretomes of the wild-type strains (Table 1). Because strain SB281 does not produce mucocysts, GRL protein secretion cannot result from a spontaneous background of mucocyst discharge. Recently, an unanticipated complexity of mucocyst cargo trafficking steps has been recognized that may provide the cellular pathway(s) for shunting of mucocyst cargo to constitutive secretion (29). We suggest that, in all of the strains, GRL proteins precluded from mucocyst condensation may be constitutively secreted in soluble form. However, the GRL proprotein regions removed from the mature polypeptides by processing were detected only in the SB281 secretomes (in the starvation condition for GRL1 and in both the starvation condition and rich medium for GRL3), where the overall detection of GRL proteins was maximal. Due to the limited peptide sequence coverage of GRL proteins in the secretomes of the wild-type strains, the protein processing state and the secretion mechanism both remain uncertain.

In addition to the GRL family of structural lattice subunits, mucocysts harbor soluble cargo proteins, defined by their ability to be released from the insoluble GRL protein lattice by diffusion. The soluble cargo proteins share a C-terminal crystalline fold (CCF) that is common to a *T. thermophila* family of 10 annotated CCF proteins, Igr1, and the granule tip (GRT) proteins Grt1 and Grt2 (3). The combined secretomes include 5 of the 10 annotated CCF proteins and both GRT proteins but not Igr1. All 5 of the CCF proteins identified in any secretome were detected in the secretomes of SB281; 4 CCF proteins were detected in the rich medium secretome and 1 distinct CCF protein was detected in the starvation medium secretome. The CU428 rich-medium secretome contained 2 CCF proteins, and the starvation medium secretome contained none, while the rich-medium and starvation medium secretomes of SB210 each contained 1 distinct CCF protein. Curiously, unlike the GRL and CCF proteins, GRT proteins were detected only in the starvation medium secretomes of SB210 and SB281. We conclude that although several protein families share the biological destiny of becoming mucocyst cargo, their detection as constitutively secreted proteins shows a differential dependence on strain background and culture conditions.

**Novel secretome contents.** The largest number of proteins in the unique-protein inventory of the combined secretomes, constituting 72 of 207 total identifications, had an automated an-
notation of hypothetical or conserved hypothetical protein. Most of these hypothetical proteins were identified in a single secretome (see Table S2 in the supplemental material). Considering each of the 6 secretome samples individually, hypothetical proteins with unknown function were the dominant category only in the SB210 rich-medium secretome (Fig. 5). The biology of T. thermophila suggests that the secretome should contain factors with autocrine growth-promoting activity (30). Curiously, several hypothetical proteins share an automated annotation of “growth factor, receptor” domain (see Table S2 in the supplemental material). Also, a number of hypothetical proteins are peptide sized, particularly after removal of the N-terminal signal peptide. These gene products are excellent candidates for mediating cell-to-cell signaling. The large number of secretome protein identifications made in this study precludes an in-depth study of their functions within the scope of this work.

Beyond the majority of secretome proteins with an N-terminal signal sequence for secretion and the many uncharacterized proteins that may have this signal but appear to lack it due to automated-gene-finder inaccuracy, the T. thermophila secretome includes a few identifications of proteins with known function and/or localization in the cytosol. For example, the heat shock family protein folding chaperone Hsp82 was detected in the starvation medium secretome of strain CU428. The prediction of a signal peptide in T. thermophila Hsp82 is weak and not consistently positive across all signal peptide prediction algorithms. Provocatively, Hsp90 family proteins can be secreted from mammalian cells in a signal sequence-independent manner (24, 39). In addition to Hsp82, starved cells also secreted 14-3-3 signaling proteins not detected in the secretome of any strain in rich medium (see Table S2 in the supplemental material). The previously characterized 14-3-3 protein FTT18 was detected in the starvation medium secretome of all 3 strains. Automated annotation assigned this protein a signal sequence at predicted amino acid 173, but the GenBank record of a cloned mRNA and supporting expressed sequence tag (EST) data establish an alternate protein model lacking a signal peptide (40). Of the 3 FTT18 peptides identified in this study, one is within both gene models, one is only in the GenBank model, and the third spans the alternative splice site distinguishing the two models, directly confirming the GenBank model.

The presence of specific cytoplasmic proteins in the starvation medium secretomes and the lack of extensive overlap between CU428 whole-cell and secretome proteins detected by LC–MS-MS suggest that T. thermophila exploits a noncanonical pathway of protein secretion, with starvation potentially upregulating the formation of a noncanonical secretory compartment or its fusion with the plasma membrane. Conditional secretion of cytoplasmic proteins has precedent in recent studies of a stress- and signal-induced exosome secretion best characterized in mammalian cells (12, 26). In addition to Hsp82 and 14-3-3 proteins, other secretome proteins lacking an N-terminal signal peptide that are obvious candidates for exosome cargo are calmodulin, 2 EF-hand proteins, and 2 translation elongation factors (see Table S2 in the supplemental material). Notably, all of these were detected specifically in starvation medium secretomes, despite the identification of more proteins overall in the rich-medium secretomes (Fig. 3). Our secretome inventories suggest the hypothesis of an exosomelike pathway of protein secretion in T. thermophila, but additional cellular and genetic studies will be necessary to validate this hypothesis and to address the physiological significance of noncanonical protein secretion.

Overall perspectives. Proteases, glycosyl hydrolases, chitinases, and lipases were highly represented in number and in proportion of the secretome for cells grown in rich medium (Fig. 5; see Table S2 in the supplemental material). It is important to note that the conditions used to optimize protein yield in the rich-medium secretomes exploited cultures of T. thermophila grown to stationary phase, a maximal density that depends on the degree of aeration. Cells are likely to send and receive signals that dictate the stationary-phase cessation of cell division. Coordinate with these events, cells may also increase the secretion of enzymes that would otherwise be internally compartmentalized. At high cell density, this speculative strategy could maximize the ingestion of nutrients by the community as a whole. Independent of the biological motivation, the identification of a wide array of secreted hydrolases has implications for the use of T. thermophila as a heterologous protein expression system. It will be important to optimize culture conditions and strain backgrounds to improve T. thermophila as an expression platform for recombinant protein secretion.

The results of unbiased analysis of secretome contents by LC–MS-MS suggest that GRL and CCF proteins are secreted constitutively. It seems logical that subunits produced in excess of mucocyst capacity would be aborted to constitutive secretion. Nonetheless, it remains curious that different classes of mucocyst proteins showed differential representation in the constitutive secretome. Most striking is the observation that GRT proteins with polarized mucocyst localization were detected only in the secretomes of starved cells. It remains to be determined whether this specificity reflects a simple difference in protein expression level or a more complex biological regulation, for example, a cortical reorganization necessary to accomplish the starvation-induced change in cell morphology. The physical and functional implications of this protein class-dependent and condition-dependent cross talk between T. thermophila constitutive and regulated secretion pathways will be fascinating to explore in additional detail as a paradigm for specificity in protein sorting.

Our proteomic surveys uncovered a wealth of novel secretome content. We identified numerous hypothetical proteins with a predicted N-terminal signal peptide, some with specificity for a particular strain background and/or culture conditions. To our surprise, in the starvation condition secretomes, we also identified known cytoplasmic proteins lacking a signal peptide. These evolutionarily conserved multifunctional proteins are likely to be secreted by a noncanonical exosome-like pathway that would also contribute lipids and other molecules to the extracellular environment (12, 26). The diverse properties of secretome proteins, along with the large number of proteins identified in the inventory overall, suggest that T. thermophila has evolved extensive interactions with its community and culture environment. These interactions would be consistent with ciliate adaptation to diverse aquatic niches as part of an overall strategy for evolutionary prosperity.
CONSTITUTIVE SECRETION IN TETRAHYMENA THERMOPHILA

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