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ORIGINAL ARTICLE

Identification of Proteins Associated with Adhesive Prints from *Holothuria dofleinii* Cuvierian Tubules

Yong Y. Peng · Veronica Glattauer · Timothy D. Skewes · Andrew McDevitt · Christopher M. Elvin · Jerome A. Werkmeister · Lloyd D. Graham · John A. M. Ramshaw

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Abstract Cuvierian tubules are expelled as a defence mechanism against predators by various species within the family Holothuridae. When the tubules are expelled, they become sticky almost immediately and ensnare the predator. The mechanism of this rapid adhesion is not clear, but proteins on the surface of the expelled tubules are widely believed to be involved. This study has examined such proteins from *Holothuria dofleinii*, sourced from adhesive prints left on glass after the removal of adhered tubules. Gel electrophoresis showed that seven strongly staining protein bands were consistently present in all samples, with molecular masses ranging from 89 to 17 kDa. N-terminal sequence data was obtained from two bands, while others seemed blocked. Tandem mass spectrometry-based sequencing of tryptic peptides derived from individual protein bands indicated that the proteins were unlikely to be homopolymers. PCR primers designed using the peptide sequences enabled us to amplify, clone and sequence cDNA segments relating to four gel bands; for each, the predicted translation product contained other peptide sequences observed for that band that had not been used in primer design. Database searches using the peptide and cDNA-encoded sequences suggest that two of the seven proteins are novel and one is a C-type lectin, while—surprisingly—at least three of the other four are closely related to enzymes associated with the pentose phosphate cycle and glycolysis. We discuss precedents in which lectins and metabolic enzymes are involved in attachment and adhesion phenomena.

Keywords *Holothuria* · Bioadhesion · Adhesive proteins · Edman degradation · De novo mass spectrometry peptide sequencing · cDNA cloning

Andrew McDevitt: Deceased, January 2011

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Y. Y. Peng · V. Glattauer · J. A. Werkmeister · J. A. M. Ramshaw (✉)
CSIRO Materials Science and Engineering, Bayview Avenue, Clayton, VIC 3169, Australia
e-mail: john.ramshaw@csiro.au

T. D. Skewes
CSIRO Marine and Atmospheric Research, Middle Street, Cleveland, QLD 4163, Australia

A. McDevitt · C. M. Elvin
CSIRO Animal, Food and Health Sciences, Carmody Road, St. Lucia, QLD 4067, Australia

L. D. Graham
CSIRO Animal, Food and Health Sciences, Julius Ave, North Ryde, NSW 2113, Australia

Introduction

A variety of applications, especially medical applications, require better adhesives for use in wet or moist environments, as most man-made adhesives bind to dry surfaces more strongly than to wet ones. Naturally inspired adhesives may provide approaches to develop new adhesives. Of particular interest have been bioadhesives that function in an aqueous environment. A range of candidates have been characterised, most notably not only the ones from a marine environment such as mussel byssus adhesive (Waite 1990; Holten-Andersen and Waite 2008) and barnacle cement (Nakano et al. 2007) but also the ones from a freshwater environment such as spiggin from sticklebacks (Jones et al. 2001). Understanding the mechanism of these natural adhesives provides opportunities for the development of novel biotechnology-based or synthetic materials of defined and uniform properties.

It has been suggested that the adhesive on the Cuvierian tubules, which provide an anti-predator defence mechanism

for certain species of holothurians (sea cucumbers) (VandenSpiegel and Jangoux 1987), could be the basis of new biotechnology-based adhesives (Flammang et al. 2005). These adhesives work in a fully submerged environment. When Cuvierian tubules are expelled, they fill with liquid, lengthen and become sticky immediately on contact with a solid surface, such as the body of a marine predator (VandenSpiegel and Jangoux 1987; Lawrence 2001). The resulting entanglement rapidly immobilises the target and—since the expelled tubules readily detach from the holothurian—affords the sea cucumber an opportunity to escape. However, the Cuvierian tubules do not stick to surfaces covered with mucins, thus avoiding the problem of self-attachment (VandenSpiegel and Jangoux 1987; Flammang et al. 2002).

Some studies have probed the mechanism of adhesion, focusing on *Holothuria forskali* and *Holothuria leucospilota* (Müller et al. 1972; Zahn et al. 1973; Flammang et al. 2002; DeMoor et al. 2003). Protease treatments and increasing concentrations of urea led to a loss of adhesion, suggesting that folded protein structures may be involved (Müller et al. 1972; Zahn et al. 1973). It has been proposed that during tubule elongation, granular cells that are internal in the pre-release tubule become located on the tubule surface and release their contents on contact with a surface (VandenSpiegel and Jangoux 1987), accounting partly or wholly for the observed adhesion. Histology has shown that these granules contain protein and lipid but lack polysaccharide (VandenSpiegel and Jangoux 1987). More recently, DeMoor et al. (2003) showed that the adhesive residue left when tubules are peeled from a surface was predominantly protein (DeMoor et al. 2003) and that different protein components were present when examined by gel electrophoresis. Studies on the present species, *Holothuria dofleinii*, have confirmed that this species also has proteins on the surface of expelled adhesive tubules (Peng et al. 2011). Studies on the adhesive properties of *H. forskali* and *H. leucospilota* (Müller et al. 1972; Flammang et al. 2002) and *H. dofleinii* (Peng et al. 2011) tubules have shown that best adhesion is found at a temperature, salinity and pH similar to that found in the organism's natural environment and that the adhesive strength is greatest with hydrophilic surfaces. Adhesive strength was decreased by lowering the pH or the NaCl concentration. Increasing concentrations of urea also led to a loss of adhesion, suggesting that native protein structures may be required (Müller et al. 1972). Some adhesion returned when the urea was removed by washing with 3.5 % NaCl (Peng et al. 2011), possibly indicating a partial refolding of adhesion-related secondary and tertiary structures.

In our previous study, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis separations revealed that the *H. dofleinii* adhesive print contained at least seven protein components (17–89 kDa) (Peng et al. 2011), all of which were unusually rich in glycine and glutamate/glutamine residues (8–17 mol% Gly, 12–16 mol% Glx) (Peng et al. 2011). The same residues also dominate the composition of tubule adhesive proteins from *H. forskali*, *H. leucospilota*, *Bohadschia subrubra* and *Pearsonothuria graeffei* (25–30 mol% Gly, 9–12 mol% Glx) (Flammang 2006). The proteins in the *H. dofleinii* adhesive print (Peng et al. 2011) are quite clearly defined. Initial studies on the print proteins from *H. forskali* (DeMoor et al. 2003) were not well-defined, but more recent studies on *H. forskali* and *B. subrubra* provide clear bands that allow comparisons between species (Flammang et al. 2009; Baranowska et al. 2011).

In the present study, we have examined in more detail the proteins in the adhesive print from the Cuvierian tubules of *H. dofleinii*. These proteins originate from the surface of the tubule; they should include the principal proteins involved in adhesion, but other components may also be present from the disruption of the tubule structure and cell components during expansion. The protein components were once again separated by gel electrophoresis, whereupon individual protein bands were sequenced by Edman degradation of N-termini and by tandem mass spectrometry (MS/MS) of tryptic peptides. Homology searching of protein sequence databases suggested possible identities for many of the protein components. Some of the peptide sequences were then used to prepare oligonucleotide primers that allowed the amplification by PCR of segments from *H. dofleinii* tubule tissue cDNA. Cloning and sequencing of the cDNA segments afforded predicted protein sequences of substantial length, which allowed the protein bands to be identified with much greater confidence. The potential implications of some of these protein assignments are discussed.

Materials and Methods

Collection of Material

Individual *H. dofleinii* was obtained from shallow subtidal seagrass banks in Moreton Bay, Queensland, at a depth of about 1–2 m at low tide, close to the western side of Stradbroke Island (153° 26.4′ E 27° 25.13′ S to 27° 25.68′ S). Animals were held prior to use in recirculating filtered seawater tanks at 21.5–22 °C. The identification of the animals was based on morphology, spicule shape and size and 18S-RNA sequencing (Skewes and Peng, unpublished data). To collect Cuverian tubules, the *H. dofleinii* animals were held and gently stimulated underwater until tubules were expelled. Individual tubules were collected using Teflon-tipped forceps, allowed to drain briefly and transferred to a glass sheet to which they adhered. No extra pressure was applied to aid adhesion.

Isolation of Proteins and Gel Electrophoresis

Tubules on glass plates were removed by peeling, leaving the layer of adhesive and potentially other components of the

tubule wall as a print on the glass (DeMoor et al. 2003; Peng et al. 2011). This adhered material was collected by removal with a sharp razor blade and was then extracted in electrophoresis sample buffer, containing 5 % (*v*/*v*) 2-mercaptoethanol, followed by centrifugation. Protein components were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) using Invitrogen NuPAGE 4–14 % Bis–Tris gels with MES running gel buffer, at 180 V for 60 min. Molecular masses were determined by comparison to globular protein standards (BioRad) using BioRad Quantity One v.4.4.0 software. For protein identification, gels were stained by Coomassie Blue R-250.

Glycoprotein Staining from Gels

Glycoprotein staining using a periodic acid Schiff (PAS) reaction was performed after fixation of the gels in a solution of 50 % (*v*/*v*) methanol and 1 % (*v*/*v*) acetic acid for 1 h. The gels were then washed with water for 10 min. The gels were then incubated for 30 min in a solution of 1 % (*w*/*v*) periodic acid and 3 % (*v*/*v*) acetic acid, before being washed 3×5 min in water and 2×10 min in 0.1 % (*w*/*v*) sodium metabisulfite in 12 mM HCl. The gels were incubated for 30 min with Schiff reagent to stain glycoproteins in the gel a pink colour. The gels were then washed thoroughly with 0.1 % (*w*/*v*) sodium metabisulfite in 12 mM HCl.

For lectin staining, Western blotting was used to transfer the protein bands from gels to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 1 % BSA for 30 min and developed by immersion in 5 ml of 1:50 dilution of 1 mg/ml of fluorescein isothiocyanate-labelled (FITC) lectins for 60 min. After rinsing, the blot was viewed with UV illumination. The lectins used were FITC-Concanavalin A (ConA), FITC-*Dolichos biflorus* lectin (DBA) and FITC-*Datura stramonium* lectin (DSA) (Sigma, St Louis, MO).

Edman Degradation

For Edman sequencing after SDS-PAGE, Western blotting in a CAPS buffer system was used to transfer the protein bands to PVDF membranes. After staining with Coomassie Blue and de-staining, the bands of interest were excised from the blot and washed three times, alternating Milli-Q water with 50 % (*v*/*v*) methanol/water. The blot was cut into smaller pieces and loaded onto a glass cartridge for sequencing. Sequence analysis (Monash University Biomedical Proteomics Facility) used a Procise Protein Sequencer, Model 610 (Applied Biosystems) according to the manufacturer's instructions.

Peptide Sequencing by Mass Spectrometry

Individual protein bands were cut from Coomassie Blue-stained SDS-PAGE gels, destained and digested with trypsin for 16 h at 37ºC. Peptides were then extracted according to standard techniques (Casey et al. 2005). The resulting digested peptides were separated by liquid chromatography on a C18 reverse-phase column eluted with a linear gradient of acetonitrile in 0.1 % (*v*/*v*) aqueous formic acid. Eluted peptides were analysed in-line by electrospray ionisation tandem mass spectrometry in an Applied Biosystems Q-Star Pulsar instrument, and peptide fragmentation data from LC/MS/TOF was analysed to obtain de novo sequence using Analyst QS software (Applied Biosystems).

cDNA Preparation

Total RNA was extracted from tubule tissue using Qiagen RNeasy Fibrous Tissue Kit. The mRNA was converted to cDNA using Clontech Creator™ SMART™ cDNA library construction kit #K1053-1 according to the manufacturer's instructions.

Alternatively, total RNA was extracted from tubule tissue using TRIzol reagent (Invitrogen #15596-026). The mRNA was converted to cDNA using GeneRacer Kit (Invitrogen) using Superscript II Reverse Transcriptase and a GeneRacer oligo-dT primer according to the manufacturer's instructions.

Cloning

A panel of gene-specific degenerate primers was designed based on selected tryptic peptide sequences. Typically, peptide sequences that did not contain arginine, serine (which have high levels of code degeneracy) or Leu/Ile nor Gln/Lys (which may show mass spectrometry ambiguities) were selected. Oligonucleotides, usually 16 to 20 bases long, were synthesised commercially (Custom Oligo Synthesis, Sigma-Aldrich). For initial (touchdown) PCR amplifications, each gene-specific degenerate primer (1 μl of 100 μM) was used as the forward primer and the GeneRacer 3′-primer as reverse primer (1 μl of 10 μM). The reaction mixture also contained 5 μl of 10× reaction buffer (Invitrogen), 2 μl of 50 mM $MgSO_4$, 1 μl of 10 mM dNTPs, 2.5 U of Taq HiFi DNA Polymerase (Invitrogen) and 1 μl of first-strand cDNA produced from mRNA as noted previously, in a total volume of 50 μl. The initial PCR thermocycler programme comprised 1 cycle of initial denaturation at 94 °C for 2 min, followed by 20 cycles of {94 °C for 30 s, 68 °C for 30 s (1 °C decrease per cycle from 68 °C) and 68 °C for 3 min}, then 15 more cycles of {94 °C for 30 s, 50 °C for 30 s and 68 °C for 3 min}, and a final extension of 68 °C for 7 min. Nested PCR amplification was performed similarly but used 1 μl of the initial PCR reaction product as template. The same gene-specific degenerate primer was used, as well as other gene-specific degenerate primers, derived as above from the peptide sequences, as forward primers. The reverse nested primer was 5′ to the original GeneRacer 3′-primer. All the PCR products were

agarose gel-purified, cloned into pCR®4 TOPO Vector according to the manufacturer's instructions (Invitrogen) and then sequenced. Specific primers were designed using these new sequence data and were used with proofreading DNA polymerase (Pfu, Stratagene) to obtain a high-fidelity PCR product. In addition, a primer based on the original sequence was used for 5′-RACE to obtain additional sequence data 5′ to the original sequence using a 5′ RACE Abridged Anchor Primer (Invitrogen, #10630-010) as the 5′ sequence.

Sequencing of Cloned cDNA

For DNA sequencing, the BigDye Terminator Cycle Sequencing Ready Reaction Kit V3.1 (Applied Biosystems) was used for the sequencing reactions and products examined on an Applied Biosystems 3730 Genetic Analyser (Micromon DNA Sequencing Facility, Monash University, Clayton, AU).

Bioinformatics

Peptide sequences obtained from mass spectrometry and from cDNA clones were used for Blast-P search queries (Altschul, et al. 1997) with sequences from the National Center for Biotechnology Information/National Library of Medicine database. For short peptide queries, we focused on similarities to sequences reported for Echinodermata (Taxid: 7586), a repository that includes translations of the 23,300 genes encoded by the complete 814-megabase genome sequence data for the sea urchin, *Strongylocentrotus purpuratus* (Sodergren et al. 2006; Cameron et al. 2009). These searches automatically used the parameters recommended for short sequence queries. For cDNA-derived queries, we searched both the non-redundant protein database and then separately in Echinodermata sequences. Matches giving the most significant (i.e. lowest) 'expect value' (hereafter, *E* value) are reported; this value gives the number of matches that one might expect by chance from the database (Altschul et al. 1990). *E* values for short peptides are typically high; here, we classify as 'high confidence' any tryptic peptide match with $E<0.05$. For proposed matches, the molecular mass of the database protein is reported when the full-length sequence was available.

Results

Gel Electrophoresis

The adhesive print isolated from glass which showed a range of proteins of well-defined molecular masses were present on SDS-PAGE of reduced samples (Fig. 1). The seven strongly staining bands, H2 at 89 kDa, H3 at 70 kDa, H4 at 61 kDa, H6 at 44 kDa, H7 at 37 kDa, H8 at 26 kDa and H9 at 17 kDa, were consistently present in all tubule samples that were examined ($n>20$, derived from >10 individual animals). The collected material sometimes contained a small proportion of material that remained insoluble in the sample buffer. Examination of the adhesive prints under a microscope suggested that the samples that subsequently proved incompletely soluble in sample buffer tended to contain more fibrous material, which could derive from the collagenous wall of the tubule. In contrast, prints that contained little fibrous material typically proved fully soluble. In samples where tubule fragments are present in the adhesive print, some of the soluble protein components may well derive from the tubule wall rather than from the tubule surface, including proteins derived from rupture of cells and granules within the tubule wall. This rupture may be part of the in vivo process by which predators are ensnared by the tubules (Becker and Flammang 2010).

Band H4 has been examined as a single entity, but in some gels (Fig. 1), it appeared to comprise two components. Although each of the other bands appeared to be a single component, it is possible that two or more species of similar molecular masses were present. In many (but not all) samples, an additional band, H5, was present at 53 kDa. As it was not consistently present, it was assumed that it may not be a key component of the adhesive system and hence was not examined further.

In a few samples, an additional band (H1) was observed at 150–170 kDa. This band seemed to be more prevalent in samples where tubule fragments were present in the adhesive print and could possibly be related to the collagen that constitutes the main structural component of the tubule (Watson and Silvester 1959). In support of this hypothesis, preliminary peptide mass finger-printing of H1, followed by a broad database search not limited to echinoderm proteins, gave hits to collagen-like sequences (data not shown). Immuno-blotting using an antibody that reacts with denatured collagens from a wide range of species was also positive (data not shown). Although the apparent molecular mass of H1 is large for a monomeric collagen chain, some collagens are known to migrate anomalously in SDS-PAGE, moving more slowly than expected (Hayashi and Nagai 1980).

Previously, Flammang et al. (DeMoor et al. 2003) had shown a gel electrophoresis pattern for tubule print samples from *H. forskali*. In this case, a high background staining was present, and the bands were generally less well-defined than those in our gels of *H. dofleinii* print proteins (Fig. 1). More recently, gel electrophoresis showing better defined bands from powdered Cuvierian tubules from *H. forskali* that had been extracted by 4 M urea (Baranowska et al. 2011) and from powdered tubules from *H. forskali* and *B. subrubra* extracted in 6 M guanidine hydrochloride (Flammang et al. 2009) has been reported. The correlation between the bands observed in these studies and the present study was limited. In part, this may result from the use of different holothurian species. In

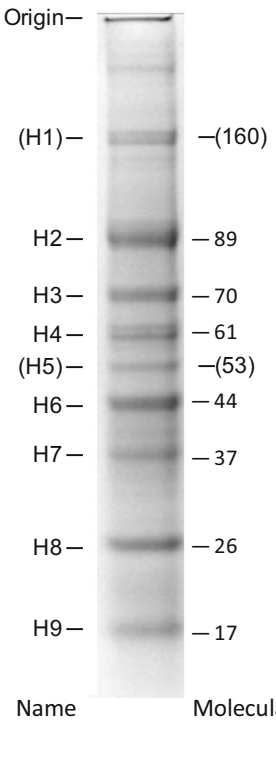

Fig. 1 Proteins from the adhesive footprint of expelled *H. dofleinii* Cuvierian tubules, separated by SDS-PAGE and stained by Coomassie Blue R-250. Molecular mass estimates are based on comparisons with globular protein standards. The selected gel shows the resolution of two bands at H4; this resolution was not always observed. It also shows strong H1 and H5 bands, which were both variable and sometimes were not readily seen

addition, different denaturant mixtures will preferentially solubilise different protein components from the adhesive prints, potentially explaining further the differences between our gel band pattern and that of other groups (DeMoor et al. 2003; Flammang et al. 2009). Nevertheless, in some cases, bands that had comparable molecular masses to those observed in *H. dofleinii* were observed in the other species, particularly for band H3 at ~70 kDa, band H6 at ~45 kDa, band H7 at ~36 kDa and band H9 at ~17 kDa.

Glycoprotein Staining of Gels

The protein bands observed during SDS-PAGE were also examined for carbohydrate content. General staining using the PAS reaction showed that only one band, H2, stained strongly for carbohydrate (Fig. 2, lane 2), while there was faint staining for the H1 band that is found in variable quantities between preparations. PVDF membranes from electroblots were also examined with FITC-labelled lectins. No reaction was observed with either FITC-DBA or FITC-DSA, which bind principally to terminal *N*-acetyl-α-D-galactosamine and oligomers of *N*-acetyl-glucosamine, respectively. On the other hand, consistent with the PAS staining, clear binding by FITC-ConA, which binds to terminal α-D-mannosyl or α-D-glucosyl residues, was observed for certain bands. Thus, FITC-ConA bound to band H2 (Fig. 2, lane 3) and also to band H1 which was present in this instance.

N-terminal Sequences

N-terminal sequences for the seven principal bands were obtained by automated Edman degradation. For bands H2, H3, H4, H6 and H8, no sequence data was obtained despite duplicate analyses. This suggests that these proteins have modified N-terminal residues, for example acetylated termini, which would block the Edman chemistry. Such a modification could be present in vivo, or it may have occurred subsequently as an artefact of isolation. However, sequences were obtained for bands H7 and H9, suggesting that modification during extraction was less likely to be the cause. The sequence observed for H7 was NH_2-Lys-Ile-Val-(Pro,Lys)-(Ile,Leu)-Leu-Thr-(Phe,Gln). This sequence did not correlate with any of the tryptic peptides identified by mass spectrometry (Table 1). The sequence observed for band H9 was NH_2-Glu-Asp-Lys-Val-Asn-Phe-Gly-Gln-(Pro,Phe)-(Lys,Asn). This sequence was also observed in one of the band H9 peptides sequenced by mass spectrometry (Table 1), indicating that the last two residues in the sequence, which were ambiguous in the Edman sequencing results, should be -Pro-Lys. Contributions from two proteins of much lower abundance were also observed during the Edman sequencing of band H9. They had sequences starting NH_2-Gly-Tyr-Glu-Thr-Phe-Val- and NH_2-Ala-Ile-Glu-Thr-Phe- before the low yields prevented identification of further residues. Neither of these minor peptides correlated with either of the other two peptides sequences found by mass spectrometry. No better-than-chance similarity (i.e. *E* value<1) to known echinoderm proteins was observed for any N-terminal sequence.

Sequences from Mass Spectrometry of Tryptic Peptides and from Translated ORFs in cDNA Clones

Each of the seven main bands was subjected to de novo sequencing of its tryptic peptides by tandem mass spectrometry in order to try to identify the proteins (Table 1). For some peptides, for example from band H2, very few potential sequence similarities to database proteins were observed, whereas for others, for example from band H3, many strong matches were observed to a single protein. In some cases, although there were possible similarities between a tryptic peptide sequence and a database sequence, the molecular mass of the parent *H. dofleinii* protein was so different to the molecular mass of the database protein that a genuine match seemed unlikely. However, it is possible that the two polypeptides are modular proteins which just have one or two domains in common; alternatively, the *H. dofleinii* band may represent a

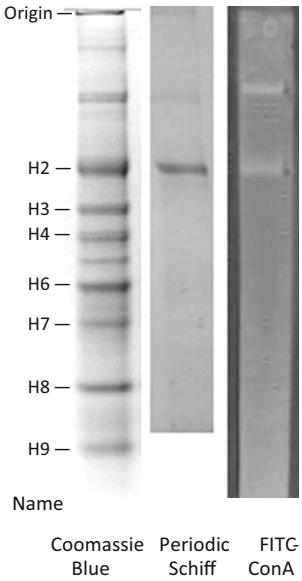

Fig. 2 Glycoproteins in the adhesive footprint of expelled *H. dofleinii* Cuvierian tubules, identified by staining of SDS-PAGE. *Lane 1*: Footprint proteins stained by Coomassie Blue (see Fig. 1). *Lane 2*: Footprint proteins stained by periodic acid/Schiff reagent, with glycoproteins staining positive (*dark bands on light background*). *Lane 3*: Footprint proteins stained with FITC-ConA, with identification of fluorescent bands (*light on dark background*) by UV illumination

proteolytic fragment of the database protein or vice versa. In almost all cases, the best matches provided by Blast-P came from translations of genes in the genome of the sea urchin, *S. purpuratus* (Sodergren et al. 2006; Cameron et al. 2009). In a few instances, other echinoderms provided the best matches, but in these cases, there were often fits for *S. purpuratus* that were nearly as good.

For band H2, most peptides did not lead to a suggested fit by Blast-P to any known echinoderm protein. Three peptides did provide potential matches, each better than expected by chance alone, but none showed a good match of molecular mass to the 89 kDa observed for band H2. However, as band H2 represented a glycosylated protein (Fig. 2), its polypeptide core could be a homolog of the best-matched protein, a predicted *S. purpuratus* peptide/nitrate transporter (77 kDa; XP_795320.2). This identification is not supported by the poor agreement between the amino acid composition of this protein and of band H2; the correlation coefficient (R=0.40, not statistically significant; Supp. Table S2) is much worse than the correlation between H2 and the average composition of the entire SwissProt database (R=0.9, P<0.0001), using SwissProt data from Tompa (2002). Overall, it seems that band H2 is probably a novel protein.

For band H3, all but one of the peptides showed a best match to the sequence for *S. purpuratus* transketolase. Eight assignments (E value<0.25), of which five were high confidence (E value <0.037), suggested that this band is transketolase. Oligos derived from the peptide sequences allowed a segment of cDNA to be cloned and sequenced; this contained an incomplete open reading frame (ORF) encoding a 247-residue polypeptide (Fig. 3). This sequence contained 5 of the 11 peptides identified by mass spectrometry (Table 1). This protein segment has highest homology to a hypothetical transketolase protein (CAPTEDRAFT_157209; ELU00756.1) from *Capitella teleta*, a polychaete worm (77 % identity over the 247 residues of the query; E=7e-141). A comparison of the H3 sequence to the transketolase from *S. purpuratus* (NP_001229589.1; Tu et al. 2012) shows that the present fragment is located near the N-terminus of the full protein, being homologous to *S. purpuratus* residues 14 to 260 (77 % identity over the 247 residues of the query; E=5e-137). The calculated molecular mass for the *S. purpuratus* enzyme is 66 kDa, close to the 70 kDa observed for band H3. The cDNA sequence allowed clarification and/or correction of some of the peptide sequences obtained by mass spectrometry (Supp. Text A).

For band H4, few meaningful hits were found. The best of the putative assignments to a complete protein (E=0.28) fared poorly when the molecular mass of the matching protein (a protein phosphatase of 107 kDa) was compared with that observed for band H4 (61 kDa). Two peptides showed a possible match (E=1.3–1.5) to *S. purpuratus* glucose-6-phosphate isomerase (XP_001201424.2), which—at 57 kDa—has a comparable molecular mass to the 61 kDa observed for band H4. It is, therefore, possible that band H4 does contain a glucose-6-phosphate isomerase, but it is probably a mixture of this enzyme with an unidentified protein that gave rise to the non-matching peptides. The latter possibility is supported by the observation that H4 did on occasion appear to resolve into two components by SDS-PAGE (Fig. 1).

For band H6, two peptides led to proposed matches to *S. purpuratus* fructose-bisphosphate aldolase (NP_001116978.1). The longer peptide (peptide 4) gave a very high confidence match (E=8e-4) with 92 % sequence identity across the 12 residues of the query. High confidence matches were also observed for two other H6 peptides to fructose-bisphosphate aldolase from other echinoderms, *Ophiopholis* sp. KP-2011 and *Apostichopus japonicas*, as well as a low-confidence match for a third peptide to an unspecified aldolase from *Crinoidea* sp. MR-2009 (Table 1). The molecular mass for the *S. purpuratus* enzyme (39 kDa) is not far from the 44 kDa observed for band H6, and the amino acid composition of H6 is a good match for *S. purpuratus* fructose-bisphosphate aldolase (R=0.89, P<0.0001), so *H. dofleinii* fructose-bisphosphate aldolase is likely to be a major, but not necessarily the only, component of band H6.

For band H7, most peptides did not show good matches. However, two peptides had possible matches to segments of a predicted transaldolase-like enzyme from *S. purpuratus* (XP_792583.3), one with E=0.087. The molecular mass of this enzyme (36.5 kDa) is a very close match for the 37 kDa

Table 1 Blast-P analysis of mass spectrometry-derived peptide data for the seven major *H. dofleinii* Cuvierian tubule surface proteins bands separated by SDS-PAGE

Peptides	Possible protein	*E* value	Molecular mass (1)
Band H2 (89 kDa)			
1. (L/I)AQY(L/I)QK	–		
2. FGNPWEK	Glycogen phosphorylase	0.38	Fragment
3. (L/I)GQAFA(L/I)TK	–		
4. AAWDKPHGSVK	–		
5. DG(L/I)FDF(L/I)R	–		
6. DANVD(L/I)(L/I)QR	–		
7. Q(L/I)(L/I)Y(L/I)(L/I)NS(L/I)AR	Cytochrome P450	0.55	55,436
8. T(L/I)M(L/I)P(L/I)(L/I)SNSWR	**Peptide/nitrate transporter**	**0.13**	**76,657**
Band H3 (70 kDa)			
1. (L/I)SEPPNYK	**Transketolase**	**0.23**	66,028
2. EDLEGWHGK	**Transketolase**	**0.015**	**66,028**
3. AVE(L/I)AATTK	Transketolase	2.9	66,028
4. G(L/I)ED(L/I)EGWHGK	**Transketolase**	**0.018**	**66,028**
5. (L/I)GVNTDVVAVDAETK	Spectrin beta-chain like	2.8	67,622
6. Q(L/I)PNV(L/I)(L/I)DMFG(L/I)SANK	**Transketolase**	**0.010**	**66,028**
7. EGHPTPR	**Transketolase**	**0.21**	66,028
8. YLDQADYR	**Transketolase**	**0.23**	**66,028**
9. A(L/I)ED(L/I)A(L/I)FR	Transketolase	0.86	66,028
10. QA(L/I)EDVANKLR	**Transketolase**	**0.018**	**66,028**
11. VQDLVAIFDVNR	**Transketolase**	**0.037**	**66,028**
Band H4 (61 kDa)			
1. (L/I)(L/I)FN(L/I)AK			
2. FNPFDVDK	RNA-specific editase-like	1.5	76,748
3. N(L/I)(L/I)DDE(L/I)(L/I)K	ATP-binding cassette	0.48	Fragment
4. FVT(L/I)FGPSNK			
5. (L/I)(L/I)QPE(L/I)R	Ser/Thr-protein phosphatase	0.28	107,198
6. (L/I)NFTEDR	Glucose-6-phosphate isomerase	1.3	57,395
7. AV(L/I)H(L/I)A(L/I)R	Glucose-6-phosphate isomerase	1.5	57,395
8. ATATPTFAAAVFR			
9. FTT(L/I)TET(L/I)TWEER	Uncharacterized protein	0.87	55,188
Band H6 (44 kDa)			
1. SFVE(L/I)VK			
2. A(L/I)QASV(L/I)K	Fructose-bisphosphate aldolase	2.0	39,150
3. SSVPHA(L/I)TQK			
4. (L/I)AADESTGTMGK	**Fructose-bisphosphate aldolase**	**<0.001**	**39,150**
5. GVVP(L/I)QTR			
6. VTEEV(L/I)SYTYR	**Fructose-bisphosphate aldolase** (2)	**0.023**	**Fragment**
7. QPEQ(L/I)TFSYGR	Aldolase class-1 protein (3)	0.40	Fragment
8. AGYSAQDVAHATVR			
9. AVDGHTTQG(L/I)DG(L/I)AER	**Fructose-bisphosphate aldolase** (4)	**0.001**	**Fragment**
Band H7 (37 kDa)			
1. (L/I)(L/I)DQ(L/I)K			
2. (L/I)ST(L/I)DQ(L/I)K			
3. EGTE(L/I)YNYYK	Ceramide synthase 5-like	0.5	44,908
4. ISSTWEGIQAGK	**Transaldolase**	**0.12**	**36,458**
5. (L/I)DEKQFR			
6. VSTEVDAR	**Transaldolase**	**0.087**	**36,458**

Table 1 (continued)

Peptides	Possible protein	*E* value	Molecular mass (1)
Band H8 (26 kDa)			
1. YFAESLKPLMEDEDGV	**Uncharacterized protein**	**0.13**	**196,679**
2. NDELDAQEFNR	LRR and PYD domain protein	0.74	Fragment
Band H9 (17 kDa)			
1. EDKVNFGQPK	Uncharacterized protein	1.7	Fragment
2. EGDFVWSDGK	**C-type lectin A chain** (5)	**0.038**	**16,032**
3. LLYISSIEEFK	ADP-dependent glucokinase	0.40	52,854

Peptide column: Leucine (L) may equally well be isoleucine (I) as these have the same mass. Both residues are indicated, except where cDNA sequences retrospectively allowed a specific assignment. Other ambiguities are indicated in Supp. Text B. *Molecular mass column*: (1) When only a fragment is available in the database, no molecular mass is given. *Possible Protein column:* Best matches are all from *S. purpuratus*, except where noted: (2) *Ophiopholis* sp. KP-2011, (3) *Apostichopus japonicus*, (4) *Crinoidea* sp. MR-2009, (5) *Cucumaria echinata*. *Expect value (E value) column:* The lower the *E* value, the greater the significance of the reported match. Best matches that had an *E* value of >4 are not shown. Bold type in this and in other columns indicates matches where an *E* value of <0.25 was indicated, compared to a value of 1 (where one match of this kind is expected simply by chance)

observed for band H7. The possible identity of band H7 as a *H. dofleinii* transaldolase (or transaldolase-like protein) is supported by the very good agreement of the amino acid composition of this band with that of the transaldolase-like enzyme from *S. purpuratus*; the correlation coefficient (R=0.94, P<0.0001) is the highest of the 12 coefficients that we calculated (Supp. Table S2).

For band H8, sequences for only two peptides were obtained. Both peptides gave low-confidence matches to database proteins (Table 1); for the better fit (peptide 1), the molecular mass match for the parent proteins was very poor. Oligos derived from the peptide sequences allowed a segment of cDNA to be cloned and sequenced. The nucleotide sequence (Fig. 4) contained the C-terminal segment of an ORF, corresponding to a 72-residue polypeptide, followed by a 3′ untranslated region (UTR) and polyA tail. The amino acid sequence contained both of the H8 peptides sequenced by mass spectrometry and allowed clarification and/or correction of some of those sequences (Supp. Text A). The modest correlation between its amino acid composition and that of band H8 probably reflects the fact that the cDNA-derived sequence (R=0.62, P=0.011) encompasses only ~20 % of the total polypeptide (Supp. Table S1), the lowest coverage of the three clones. Database searches with the cDNA-derived polypeptide segment did not identify any likely matches, the best (E=2.0) being to a predicted histidine kinase of 79 kDa from *Kurthia* sp. JC30 (WP_010288503), as compared to 28 kDa for H8. The best match to a credible echinoderm protein (E=0.84) was to a predicted exportin-6-like *S. purpuratus* protein of 41.7 kDa (XP_001190226.2). However, this identification is not supported by the poor agreement between the amino acid composition of this protein and of band H7; the correlation coefficient (R=0.45, not statistically significant; Supp. Table S2) is much worse than the correlation between H7 and the average composition of the entire SwissProt database (R=0.88, P<0.0001). Overall, these data suggest that band H8 probably represents a novel protein.

For band H9, three peptide sequences were obtained by mass spectrometry. One of these peptides (peptide 1) corresponds to the N-terminal sequence identified by Edman degradation analysis, but it did not show similarity to any known echinoderm protein. One of the other two tryptic peptide sequences showed a high confidence match to a chain of the *Crinoidia* sp. MR-2009 C-type lectin CEL-1 (Table 1), a Ca^{2+}-dependent carbohydrate-binding protein specific for GalNAc residues (Hatakeyama et al. 2002). Oligos derived from the peptide sequences allowed a segment of cDNA to be cloned and sequenced; this contained a 5′-UTR, a complete ORF, a 3′-UTR (579 nucleotides) and a polyA tail sequence (Fig. 5). The predicted translation product of the

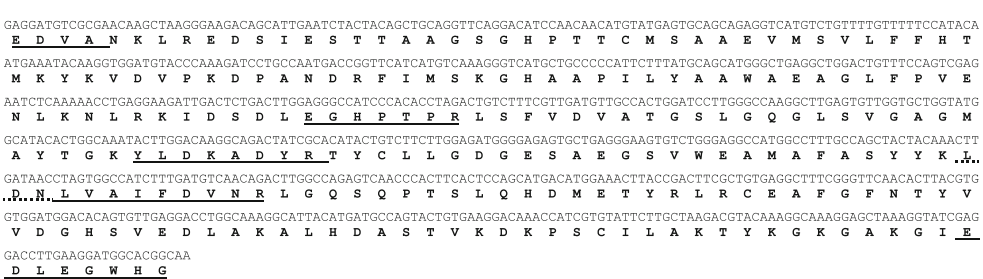

Fig. 3 Partial cDNA sequence and predicted amino acid sequence for a segment of band H3. Peptide equivalents in Table 1 are *underlined*

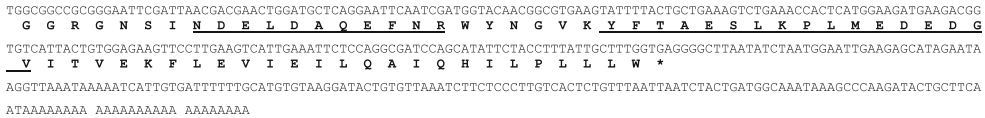

Fig. 4 Partial cDNA sequence and predicted amino acid sequence for a segment of Band H8. Peptide equivalents in Table 1 are *underlined*

ORF (165 residues) contained all the three of the observed tryptic peptides and included an additional 20-residue segment prior to the N-terminus determined by Edman degradation. In agreement with this, the algorithm SignalP v4.1 strongly predicted residues 1–20 of the full-length protein to be a secretory signal peptide (Supp. Fig. S1). The cDNA-derived protein sequence allowed clarification and/or correction of some of the peptide sequences obtained by mass spectrometry (Supp. Text A).

The predicted 145-residue sequence for the mature H9 protein has a calculated molecular mass of 16.3 kDa, in good agreement with the 17 kDa estimated from the SDS-PAGE. Database searches with the full-length cDNA-derived protein sequence gave as the top-ranked hit a match to the C-type lectin (AFW17073.1; 19 kDa) of *Trachidermus fasciatus*, an estuarine fish native to Asia (32 % identity over 139 residues of the query; E=2e-13) (Supp. Fig. S2). Other very strong matches were to the C-type lectin domain of mouse CD209 antigen-like protein E and to the C-type lectin domain of the aggrecan core protein-like proteins from cichlid fish (E=2e-12 to 5e-13). The closest echinoderm match (E=5e-14) is to a predicted C-type lectin from *S. purpuratus* named echinoidin-like isoform 2 (XP_003726044.1; 18-kDa mature protein, after removal of 26-residue signal peptide predicted by SignalP v4.1) (Supp. Fig. S2). The surprisingly poor agreement between the amino acid composition of band H9 and the mature polypeptide encoded by the cDNA ORF (R=0.65, P=0.006; Supp. Table S1) as well as between the composition of band H9 and the best-matching *S. purpuratus* protein (R=0.50, P=0.047; Supp. Table S2) suggests that the amino acid analysis of this band includes several proteins (Peng et al. 2011). Overall, the good match of predicted to observed molecular masses, the presence of all tryptic and N-terminal peptide sequences in the ORF and the high homology of the cDNA-predicted mature protein to C-type lectins leave little doubt that this protein is indeed a protein of this type.

Discussion

In the present study, we have extended the information on Cuvierian tubule composition through examination of the proteins deposited onto an artificial substratum from the surface of the sticky, extended tubules. Gel electrophoresis showed that there were seven significant, strongly staining protein bands consistently associated with the adhesive print left by tubules extended from *H. dofleinii*.

For two of the seven proteins, H2 and H8, Blast-P searches found no significant similarities with any characterised proteins, suggesting that these probably represent hitherto unidentified echinoderm proteins. As such, they may be candidates for further investigation as potentially adhesive proteins. One of the other proteins, H9, yielded tryptic peptides whose sequences appeared to have homology to a known protein, lectin CEL-1, encoded in the genome of the closely related organism, *S. purpuratus* (Sodergren et al. 2006; Cameron et al. 2009). When cDNA segments were PCR-cloned from *H. dofleinii* using degenerate primers designed from H9 peptide sequences, the predicted polypeptide sequence confirmed that this tubule print protein was a Ca^{2+}-dependent C-type lectin. All of the invariant cysteines (which are required to form two disulfide bonds) are present, and some of its predicted ligand-contacting residues are shared with its closest database matches (Supp. Fig. S2). There can be little doubt that this protein is a functional lectin.

The identification of H9 as a C-type lectin suggests that carbohydrate recognition and sugar binding are possible contributors to tubule adhesion. Some adhesive-secreting fungi entrap nematodes using lectins (Rosenzweig et al. 1985). We also note that mRNAs encoding lectins were abundant in the adhesive slime gland of the onychophoran *Euperipatoides rowelli* (Haritos et al. 2010), a terrestrial velvet worm that uses a jet of sticky quick-setting gel to immobilise its prey. In addition, galectin-like proteins were identified in the sticky

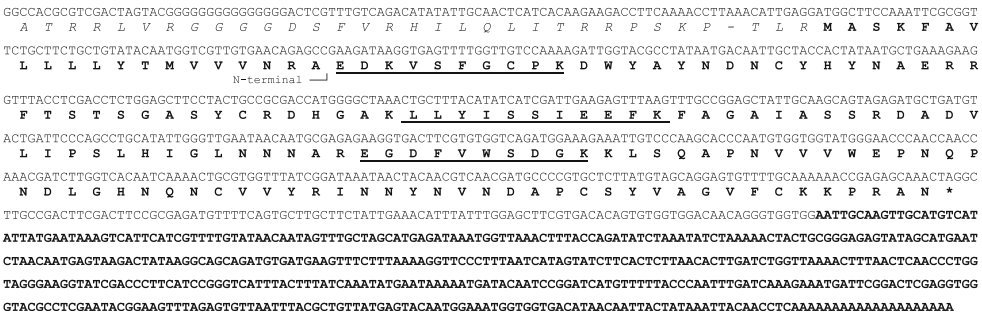

Fig. 5 cDNA sequence and predicted amino acid sequence for a segment of band H9. Peptide equivalents in Table 1 are *underlined*

dermal secretion of the frog *Notaden bennettii* (Vaughan and Peng, unpublished data), an elastomeric adhesive whose ability to bond in wet environments has raised the hope of a new type of surgical adhesive (Graham et al. 2005, 2010).

Four of the remaining proteins, H3, H4, H6 and H7, yielded tryptic peptides whose sequences also appeared to have homology to well-known proteins in *S. purpuratus* (Sodergren et al. 2006; Cameron et al. 2009) or other echinoderms, these being transketolase, glucose-6-phosphate isomerase, fructose-bisphosphate aldolase and a transaldolase-like protein, respectively. For one of these, H3, a cDNA segment was PCR-cloned from *H. dofleinii* using degenerate primers designed from its peptide sequences. The predicted polypeptide sequence of the cloned ORF was indeed that of a transketolase.

Interestingly, all four protein identifications involve enzymes present in the pentose phosphate cycle or glycolysis, a grouping that strengthens the only assignment not supported by a better-than-chance *E* value (namely, H4 as glucose-6-phosphate isomerase). Our overall assessment of *H. dofleinii* band identities, and the likely extent of coverage represented by the corresponding cDNA clones, is summarised in Table 2.

Most of the assignments in Table 2 come as a surprise; we had expected that any *H. dofleinii* print proteins would have similarities to secreted structural/fibrous proteins or to proteins found in bioadhesives from other marine organisms but had not anticipated that they might be globular proteins identical or closely related to intracellular enzymes responsible for central metabolism and energy generation. We cannot exclude the possibility that these enzyme-like proteins are cytoplasmic contaminants arising from cell rupture and that they do not actually participate in tubule adhesion. However, it is difficult to understand why so many contaminants in an extracellular print should be intracellular enzymes involved in sugar metabolism. We note that the recruitment of metabolic enzymes for structural purposes is not without precedent. For example, many vertebrate eye lens crystallins are closely related or identical to metabolic enzymes such as lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and α-enolase (Piatigorsky 2007), while transketolase (the identity assigned with confidence to *H. dofleinii* band H3) is in mammals considered to be a corneal crystallin (Piatigorsky 2007). A further possibility, explored in the next paragraph, is that the enzyme-like *H. dofleinii* proteins are not just structural but functional components of the tubule print.

In bacteria, some metabolic enzymes are displayed on the outer cell surface, where they are thought to be involved in cell attachment rather than catalysis. For example, glucose-6-phosphate isomerase (the enzyme to which *H. dofleinii* band H4 may be related) features as a surface antigen in *Streptococcus agalactiae* (Hughes et al. 2002), and fructose-bisphosphate aldolase (the putative identity of *H. dofleinii* H6) is surface-localised in *Neisseria meningitidis*, where it mediates the adhesion of this pathogen to the cells of its human host (Tunio et al. 2010). In addition, a transaldolase (the enzyme to which *H. dofleinii* band H7 shows similarity) is exposed on the cell surface of *Bifidobacterium bifidum* and serves to adhere this bacterium to the human gut lining (González-Rodríguez et al. 2012). None of these bacterial proteins possess identifiable secretory peptides (Tunio et al. 2010; González-Rodríguez et al. 2012). González-Rodríguez et al. (2012) observe that many enzymes of carbon catabolism, either from prokaryotic or eukaryotic cells, can be displayed on the cell surface (presumably via nonclassical secretion or translocation mechanisms) where they perform "moonlighting" functions in adhesion and attachment. Convergent evolution can lead to evolutionarily distant organisms adopting similar molecular strategies for adhesion (Graham et al. 2013). Accordingly, the possibility that some holothurians may exploit an adhesive process similar to the bacterial paradigm deserves serious consideration.

Table 2 Summary of most likely band identities and probable coverage of associated cDNA clone (if any)

Band	Assignment	Likely extent of cDNA clone (%)[a]
H2	Novel	–
H3	Transketolase	40
H4	(Glucose-6-phosphatase?)	–
H6	Fructose bis-phosphate aldolase	–
H7	Transaldolase-like	–
H8	Novel	20
H9	C-type lectin	100

[a] From Supp. Table S1

In all cases where a potential or definite identification of the protein was made, we should bear in mind that additional peptides with unrecognised sequences were also present in the tryptic digest. For band H4, electrophoresis had indicated that two equally abundant bands were probably present; for band H9, the amino acid composition of the band matched that of the cDNA-predicted mature protein less well than expected. For the others, while the band appeared homogeneous in one-dimensional SDS-PAGE, other proteins appear to have been present at lower levels. A single preliminary 2D-PAGE separation of the tubule proteins (not shown) was complex and did not help to clarify the situation; most of the major proteins resolved into horizontal chains of four to six spots, presumably charge isoforms arising from post-translational modifications.

The efficacy of the holothurian tubule adhesive system, including its rapid action underwater, makes an understanding of its mechanism highly desirable; biomimicry, either through biotechnology and/or synthetic chemistry (Flammang and Jangoux 2004), could then lead to the development of new

high-performance medical adhesives. Some workers have proposed that the granules that rupture to release proteins and lipids during the expulsion of the tubules (VandenSpiegel and Jangoux 1987) contain a ~10-kDa protein monomer which polymerises to form the proteins with higher molecular masses (Flammang and Jangoux 2004; Flammang 2006) that are observed in tubule adhesive prints (DeMoor et al. 2003). We previously observed that the molecular mass intervals of the *H. dofleinii* proteins are not particularly suggestive of homopolymerisation of a small protein monomer (Peng et al. 2011), and our present identification of different peptides from different protein bands—with no tryptic peptide shared by two or more bands—argues strongly against them being homopolymers of different length.

The identification of a C-type lectin in the *H. dofleinii* tubule print raises the possible involvement of carbohydrate recognition in tubule adhesion, and this theme may be extended by the enzyme-like proteins that we found to be associated with the tubule print, whose active sites are also designed to bind sugars. Future work should address the question of whether or not the enzyme-like proteins associated with the *H. dofleinii* adhesive print are catalytically competent (which would suggest that they are either cytoplasmic contaminants or the result of gene sharing) or whether they have lost their metabolic function in favour of a structural and/or ligand-binding role (i.e., paralogs resulting from gene duplication). An alignment of the polypeptide from the band H3 cDNA clone with the corresponding segment of yeast transketolase (Supp. Fig. S3) shows that invariant residues essential for catalysis are present in the *H. dofleinii* protein; the residues that contribute to cofactor and metal binding are also identical or well conserved, so this protein may well be enzymatically competent.

While a number of glycolytic enzymes are known to bind to cytoskeletal filaments and membranes (Piatigorsky 2007), and while we have cited instances where metabolic enzymes feature in attachment and adhesion phenomena, we recognise that the mammalian counterparts of the enzymes implicated in the *H. dofleinii* adhesive print are not regarded as promiscuously 'sticky'. However, one should remember that these proteins are normally studied under mammalian physiological conditions (0.9 % NaCl), an environment in which the *H. dofleinii* tubules are no longer adhesive (Peng et al. 2011). Future work could also address the question of whether, at the salt concentration of seawater (3.5 % NaCl), some or all of these mammalian metabolic enzymes exhibit stickiness or develop other non-specific adhesive properties.

In closing, it is worth remembering that Flammang and Jangoux (2004) noted differences in the sizes and compositions of surface (adhesive) proteins in *H. forskali* and *H. maculosa*, which led them to suggest that the adhesive mechanism may differ between species. We should therefore exercise caution when generalising from studies conducted on a single species of holothurian.

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