The Journal of Experimental Biology 205, 1807–1817 (2002)
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JEB4054E

A molecular, morphometric and mechanical comparison of the structural elements of byssus from *Mytilus edulis* and *Mytilus galloprovincialis*

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Accepted 5 April 2002

Summary

Marine mussels are renowned for their ability to produce an extra-organismic tendon-like structure that can withstand the wave forces associated with the intertidal habitat. Initial characterization of byssal properties has focused on *Mytilus edulis*, with few detailed comparisons with other mussels. *M. galloprovincialis*, a closely related species, provides an opportunity for a thorough comparison. Three full-length cDNA clones encoding the byssal collagens, precollagen D (preCol-D), preCol-NG and preCol-P, were isolated from *M. galloprovincialis*. Comparisons with *M. edulis* preCol-D, preCol-NG and preCol-P reveal a 91.3 %, 88.6 % and 90.1 % identity with the cDNA and an 89.0 %, 88.1 % and 89.0 % identity with the deduced protein sequences, respectively. Key elements are maintained between the species: in particular, modeled bends in the collagen helix due to breaks in the Gly-*X*-*Y* pattern and the location of cysteine and putative 3,4-dihydroxyphenylalanine (DOPA) residues. A potentially important difference between the two is that, in all cases, *M. galloprovincialis* byssal collagens contain additional histidine residues in their flanking domains. The significance of this may lie in the ability of *M. galloprovincialis* to utilize more metal chelate cross-links, which have been implicated in byssal thread stability.

***M. edulis* threads are typically twice the length and diameter of *M. galloprovincialis* threads and appear to contain nearly 10 % more collagen. These differences are maintained even when the different thread portions are compared. Despite differences in a number of parameters, most notably that whole *M. galloprovincialis* threads are stiffer, threads whether whole or separated into proximal and distal portions, have similar mechanical behaviors. It is apparent from this comparison that *M. galloprovincialis* and *M. edulis* are seemingly interchangeable models for byssal research.**

Key words: byssus, byssal thread, collagen, mussel, *Mytilus edulis*, *Mytilus galloprovincialis*, cDNA.

Introduction

Mytilid species inhabit near-shore oceanic environments where they are often exposed to extreme conditions including flow, dehydration and temperature. While the intertidal zone provides an abundant source of nutrients, mastery of this niche requires all organisms to develop complex adaptive strategies and structures. One way in which mussels ensure their survival is by tethering themselves to the surrounding substrata such as pilings, rocks or other shelled organisms. The nature of this attachment contrasts sharply with the rigid resistive attachment of barnacles since the mussel tether or byssus is quite flexible and allows hydrodynamic reorientation of the shell (Denny et al., 1998).

The byssus is secreted by the foot and can be morphologically separated into three distinct regions; the plaque, the thread and the stem (Bairati, 1991). The plaque, the most distal portion of the byssus, is the direct point of attachment of the mussel to its surroundings. Interesting in its own right, because of its underwater adhesive properties, the plaque's contribution to the mechanical strength of the byssus is predominantly in its bonding ability (Waite, 1983). The stem, at the most proximal end of the byssus, not only serves as an attachment point for multiple byssal threads but also mediates the direct connection between the non-living extraorganismal byssal threads and the living tissues of the mussel. Indeed, the acellular byssus originates from a fusion of retractor muscles at the base of the foot *via* the stem, in this way providing an anchor point as well as allowing for a certain degree of rotation and tension control.

The thread can be further subdivided into proximal and distal regions. The delineation of proximal and distal portions of the thread has historically relied strictly on morphological observations, with the proximal region being described as corrugated and the distal as smooth (Brown, 1952). However, such descriptive partitions may not be nearly so clear-cut. The transition zone between the two regions is almost certainly not a clean interface, but instead reflects a graded change in the

morphology and mechanical behavior of the thread. Demarcations based on the mechanical or biochemical properties of the two regions would seem to be more revealing about the actual discrimination of proximal from distal thread.

The mechanical properties of excised and separated proximal and distal thread portions indicate that the thread is a hybrid structure. The proximal portion is elastic, while the distal portion is stiff with somewhat peculiar stress-softening and self-healing properties (Vaccaro and Waite, 2001). These mechanical generalizations belie the underlying biochemistry of the disparate segments of the threads. It has been shown that the main structural components of byssal threads are a series of three collagens with block-copolymer-like domains (Coyne et al., 1997; Qin et al., 1997; Qin and Waite, 1998). Furthermore, two of these collagens are distributed in a gradient fashion along the length of the thread (Qin and Waite, 1995). Byssal precollagen P (preCol-P) is most abundant in the proximal portion of the thread with a decreasing distally directed gradient. Complementary to that is an increasing gradient of preCol-D in a proximal to distal direction. The third collagen, byssal precollagen NG (preCol-NG), is present along the entire length of the thread (Qin and Waite, 1998). Using fiber X-ray diffraction, Mercer (1952) demonstrated the existence of collagen fibers in mussel byssus. However, the fibrillar arrangement of these molecules in the thread remains unknown, leading to a number of proposed models (Qin and Waite, 1995, 1998; Vaccaro and Waite, 2001; Waite et al., 1998, 2002). In addition, it has been suggested that the fibrillogenesis of byssal collagens may involve an amino-acid-sequence-dependent self-assembly mechanism.

The initial characterization of byssal collagens from *Mytilus edulis* suggested a structure/function-type relationship, that is, the organization and mechanical properties of the underlying structural elements should reflect the properties of the byssal thread as a whole. Each of these proteins has a central collagen domain, consisting of between 437 and 521 amino acids, flanked on either side by a unique set of structural motifs (Waite et al., 1998). The N-terminal flanking domains of preCol-P consist of a histidine-rich region followed by an elastic domain, while its C-terminal side contains an acid patch, a second elastic domain and a terminal histidine-rich region. In a parallel fashion, preCol-D has both of the histidine-rich domains and the acidic cluster but the elastic domains are replaced with silk-like regions, i.e. spider ampullate silk. Four residues of 3,4-dihydroxyphenylalanine (DOPA) have been detected in the N-terminal flanking domain of preCol-D. DOPA is a prime candidate for forming cross-links in the byssal threads and is abundant in a number of other byssal-related proteins (Waite, 1999). The histidine-rich regions and the acid patch are present in preCol-NG, but the elastic domains are replaced with plant-cell-wall-like sequence motifs. A further peculiarity arises within the collagen domains of each molecule. PreCol-NG and preCol-P each exhibit single sequence breaks in the triple-helical Gly-*X*-*Y* repeat. Models indicate that these breaks cause structural bends or kinks in the collagen triple helix (Waite et al., 2002). PreCol-D has three such kinks. What role these aberrations play in the functionality of the individual molecules and the byssus as a whole remains a matter of speculation.

Mytilus edulis has served as the primary model organism for byssus-related studies, with a paucity of comparative studies in *M. galloprovincialis*, *M. trossulus* and *M. californianus* (Bell and Gosline, 1996). It is generally accepted that *M. edulis* is the ancestral species from which the other mytilids have evolved, with *M. galloprovincialis* and *M. trossulus* often being included in the ‘edulis complex’ (Gosling, 1992). Even though hybridization and introgression do occur amongst these groups, they are nonetheless considered to be distinct species (Beynon and Skibinski, 1996). While hybridization zones do occur, most notably, but not exclusively, in Japan (Inoue et al., 1997; Matsumasa et al., 1999), California (Rawson et al., 1999) and the British Isles (Rawson et al., 1996), each species tends to be geographically separated from the others. *M. galloprovincialis* predominantly inhabits the warmer waters of temperate latitudes, *M. edulis* is found in the colder waters of temperate latitudes and *M. trossulus* is usually found in the colder waters of the northern latitudes. Where hybridization zones do occur, *M. galloprovincialis* is frequently found in more exposed areas than *M. edulis* (Skibinski et al., 1983).

Despite genetic and physiological differences among the species (Hilbish et al., 1994), it has been suggested that the byssal threads from *M. galloprovincialis* are morphologically and mechanically similar to those of the closely related *M. edulis*. However, these studies have relied on comparisons between data from the literature that are often inconsistent (Smeathers and Vincent, 1979; Price, 1981; Bell and Gosline, 1996). No thorough comparison between the byssal threads of these two mussel species exists. Ultimately, similarities or differences in the mechanical and morphometric properties of byssal threads must be correlated with the underlying biomolecular structural components of the system, in this case the three byssal collagens.

Materials and methods

Animal collection and maintenance

Mytilus galloprovincialis (Lamarck) was collected from Goleta Pier immediately adjacent to the University of California at Santa Barbara campus. *M. edulis* L. was purchased from the Marine Biological Laboratory (Woods Hole, MA, USA). The species of individual mussels was corroborated using the polymerase chain reaction (PCR)-based method of Inoue et al. (1997). Both species were maintained in the laboratory in separate tanks with continuously circulated natural sea water. In all cases, mussels with shell lengths between 6 and 7 cm were utilized. Animals involved in thread experiments were initially stripped of old threads and then tethered onto plastic plates suspended in the seawater tanks. The production of new threads began within hours. Threads were easily removed from both the mussel and the plates with the aid of a single-edged razor blade.

RNA extraction and cDNA library construction

RNA was extracted using the Rneasy Plant Mini Kit from Qiagen (Valencia, CA, USA). In general, two freshly dissected feet from *Mytilus galloprovincialis* were used per extraction, and the manufacturer's protocols were followed after initial tissue disruption under liquid nitrogen in a mortar and pestle. Purified RNA was ultimately reverse-transcribed and packaged into a lambda ZAP Express cDNA library (Stratagene, La Jolla, CA, USA) following standard protocols. This library served as a readily available source of cDNA.

PCR and cloning of the byssal collagens

A series of PCR primers was constructed for each byssal collagen on the basis of available gene sequences from *Mytilus edulis*; the *M. galloprovincialis* cDNA library served as template in each reaction. A 3′ vector-specific primer was used to obtain the sequence of the 3′ untranslated regions.

The initial set of forward primers for preCol-D was as follows: MeDF1-ATCAACATGGTCTACAAACTC, MeDF2-CAGGACATGCCGGTAAACACGGAAC and MeDF3-GTGGTATGGGTAGACGAG. Reverse primers were designed as follows: MeDR1-GTTCCGTGTTTACCGGCATGTCCTG, MeDR2-CTCGTGGTCCTGCTGGTCCTTGT and MeDR3-AACACTTGCAGATTTTATTGATA.

Forward primers for preCol-NG were synthesized as: MeNGF1-ATGGTCCATAATTTCCTGACT, MeNGF2-TTACCAGGTGCACCCGGA and MeNGF3-AAGGAGAACTTGGACCAGTCG. The reverse primers were: MeNGR1-TCCGGGTGCACCTGGTAA, MeNGR2-CGACTGGTCCAAGTTCTCCTT and MeNGR3-AGGAACTTGCACTTTTTAT.

PreCol-P primers were as follows: MePF1-ATGGTTCGGTTTTCCCTAGC, MePF2-GAGGATTCGGTGGACCAGGTAC and MePF3-GTGGCCCAGCAGGTCCAAGA. The reverse primers were: MePR1-TTGGTCCAATTAATCCGATGA, MePR2-GAATAACACCTGGTGCTCCT and MePR3-ACGAAGACTGCAGATTTTAATA.

PCR was performed using standard conditions; buffer, dNTPs and *Taq* polymerase were from Qiagen. Reaction conditions consisted of a 30 s denaturation step at 95 °C followed by a 1 min annealing at 50 °C with a 2 min elongation at 72 °C. This cycle was repeated 35 times. PCR products were electrophoresed on 1 % agarose gels and stained with ethidium bromide. Bands of the expected size were excised and gel-eluted using the Qiagen gel purification kit and ligated into Promega's (Madison, WI, USA) pGEM-T Easy cloning vector. JM109 cells were transformed and plated, and positive clones were selected after blue/white screening. Positive clones were grown overnight in LB medium, and plasmids were purified using the plasmid purification kit from Qiagen. PreCol inserts were sequenced using vector-specific primers for M13 and SP6 primer sites at the Advanced Instrumentation center of the University of California, Santa Barbara.

RT-PCR and 5′-RACE

RNA was purified from mussel feet as previously described and used to obtain 5′ untranslated sequence information. The GeneRacer kit (Invitrogen, Carlsbad, CA, USA) was used to obtain sequence information from full-length transcripts only. In each case, previously synthesized gene-specific primers against *Mytilus edulis* sequences were sufficient for use in 5′-rapid amplification of cDNA ends (RACE) reactions when coupled with linker-specific primers. Reaction products were gel-eluted, cloned and sequenced as previously described for standard PCR reactions.

Byssal collagen sequence comparisons

Previously published *Mytilus edulis* byssal collagen sequences were obtained from the GenBank database. The following is the list of accession numbers for *M. edulis* preCol sequences; AF029249, preCol-D (Qin et al., 1997); AF043944, preCol-NG (Qin and Waite, 1998); and AF015539, preCol-P (Coyne et al., 1997). cDNA sequences were aligned and translated using Sequencher 3.0 (Gene Codes Corp., Ann Arbor, MI, USA). Comparisons between species-specific byssal collagens were performed using an online version of Clustal W from the European Bioinfomatics Institute (Thompson et al., 1994).

Byssus collection

To ensure that full-length byssal threads were collected from each species, individual mussels suspended from a Plexiglas plate were killed by inserting a scalpel between the two halves of the shell and severing the adductor muscles. The stem was then removed, and single threads were isolated at their attachment point to the stem. Unless indicated otherwise, all measurements, both physical and mechanical, encompass the entire length of the thread from the stem to the point at which the thread joins the top of the plaque but did not include the plaque.

Morphometric characteristics of byssal threads

Thread dimensions were measured using a stereomicroscope (M3Z, Wild, Switzerland) equipped with a graticule. After total thread length had been determined, each thread was separated into its proximal and distal portions as determined by the point at which its corrugated appearance became smooth. The diameter of each portion was then determined at its widest point since some degree of variability in diameter is evident along the length of each segment.

Determination of collagen content and gradient in byssal threads

The percentage content of collagen in the proximal and distal portions of the byssal thread was estimated by quantifying the amount of hydroxyproline. Individual portions of either proximal or distal thread sections were placed in an ampule with 0.1 ml of 6 mol l^{-1} HCl and 0.01 ml of redistilled phenol. The threads were hydrolysed *in vacuo* for 24 h at 110 °C. Samples were then flash-evaporated at 60 °C. Amino

acids were quantitated with a Beckman System 6300 analyzer using the modified elution program described previously by Waite (1995).

Percentage collagen content was determined by assuming that all proline residues in the *Y* position of the Gly-*X*-*Y* motif are converted to hydroxyproline. Qin and Waite (1995) and Qin et al. (1997) demonstrated this tendency in both preCol-D and preCol-P from *Mytilus edulis*. From this, a sequence-determined percentage hydroxyproline value is calculated for each preCol. A mean sequence-derived hydroxyproline content was calculated for the proximal portion by averaging the hydroxyproline content of preCol-NG and preCol-P. A similar value was derived for the distal region by averaging the hydroxyproline content of preCol-NG and preCol-D. The percentage hydroxyproline content for each thread portion, determined by acid hydrolysis, was then divided by the sequence-determined mean for each thread portion. The resulting value approximates the byssal collagen content of each thread portion. This does not account for the contribution of hydroxyproline from foot protein-1 (FP-1) to acid-hydrolysed samples. The FP-1 of *M. edulis* contains 10 % hydroxyproline (Taylor et al., 1994). Given the identical consensus decapeptide repeats in FP-1 from both species (Inoue and Odo, 1994), the proportion of hydroxyproline is not likely to differ greatly in *M. galloprovincialis*.

Following the procedure established by Mascolo and Waite (1986) to determine whether a collagen gradient occurs along the length of a byssal thread based upon changes in hydroxyproline, proline and glycine content, *Mytilus galloprovincialis* threads were sequentially cut into 0.25 cm segments starting at the stem. The final segment before the plaque was often shorter than 0.25 cm. Five threads with a mean length of 2.0±0.15 cm (mean ± S.E.M.) were analyzed. Each segment was acid-hydrolysed, and the amino acids were quantified as described previously. For comparison, *M. edulis* threads were also used, but their mean length was 3.0±0.20 cm (mean ± S.E.M., N=5).

Biomechanical properties of byssal threads

Young threads, 2–3 days post-deposition, were utilized for mechanical studies. Mechanical properties were measured for whole threads and for the separated distal and proximal portions. Newly collected threads were allowed to rest in filtered sterilized sea water for up to 24 h before testing. In an effort to avoid grip slippage during extension, the ends of each thread portion were sandwiched between double-sided tape. Dehydration during testing was prevented by enclosing one end and approximately three-quarters of the thread in a polyethylene bag with filtered sterilized sea water. Both ends were then clamped into the grips of a Bionix 200 tensile tester (MTS Systems, Cary, NC, USA) equipped with a 10 N load cell. Biomechanical variables were measured using a crosshead speed of 5 mm min^{-1} and an initial gauge length of between 5 and 10 mm for distal and whole threads and between 3 and 6 mm for proximal thread portions. Threads were stretched to their breaking point. Cyclical testing of threads was also performed as described by Vaccaro and Waite (2001). Young's modulus for whole threads and the distal region was measured as the slope of the linear portion of the stress/strain curve at low strain (strain <10 %). For the proximal portion of the thread, Young's modulus was measured at the steepest portion of the stress/strain curve. Stress and strain are defined as the load per cross-sectional area (in $N\,m^{-2}$=Pa) and the change in length per initial length, respectively. No correction for the cross-sectional area was applied. The yield point offsets were measured using the 'zero-slope' method, i.e. at the first point at which the slope of the stress/strain curve is asymptotic (Ferry, 1980). The strain energy was determined by integrating the area under the curve. Statistical significance was established by single-factor analysis of variance (α=0.05).

Results

Byssal collagen sequence comparisons

cDNA sequence alignments between preCol-D, preCol-NG and preCol-P from the two species indicate an identity of 91.3 %, 88.6 % and 90.1 %, respectively. Similar identities are observed when the deduced protein sequences for preCol-D, preCol-NG and preCol-P are compared: 89.0 %, 88.1 % and 89.0 %, respectively. Figs 1–3 show the alignment of deduced protein sequences translated from the cDNA sequences of preCol-D, preCol-NG and preCol-P from *Mytilus edulis* and *M. galloprovincialis*. Sequence information is displayed to highlight the block-copolymer-like domains of each byssal collagen. Of particular note are the histidine-rich regions found in both the N- and C-terminal flanking domains of each byssal collagen.

Despite the high degree of identity for preCol-D between *Mytilus galloprovincialis* and *M. edulis*, a number of potentially important differences exist (Fig. 1). Of note is an additional histidine in the N-terminal flanking domain and two additional histidines in the C-terminal histidine-rich domain from *M. galloprovincialis*. Furthermore, the *M. galloprovincialis* sequence has fewer glycine clusters in the silk-like domains with the pattern *X*GG (where *X*=L, A, F or V), 40 *versus* 29.

Several key differences are also found in the collagen flanking domains of preCol-NG (Fig. 2). Perhaps of greatest importance is an extra poly-A run in the N-terminal plant-cell-wall-type domain of *Mytilus galloprovincialis*. It is presumed that this stretch of amino acids would serve to stiffen the molecule. Also, a single additional histidine is found in its C-terminal histidine-rich domain. Individual preCol-D and preCol-NG chains have cysteine residues whose location is maintained between the species. How these couple to form disulfide bonds is unknown, but such cross-links may prove crucial in mature byssal threads.

When comparing the byssal preCol-P protein sequences (Fig. 3), differences in histidine content are evident, with an additional three residues occurring in the N-terminal flanking regions and another three in the C-terminal flanking domains in *Mytilus galloprovincialis*. Also evident are fewer glycine

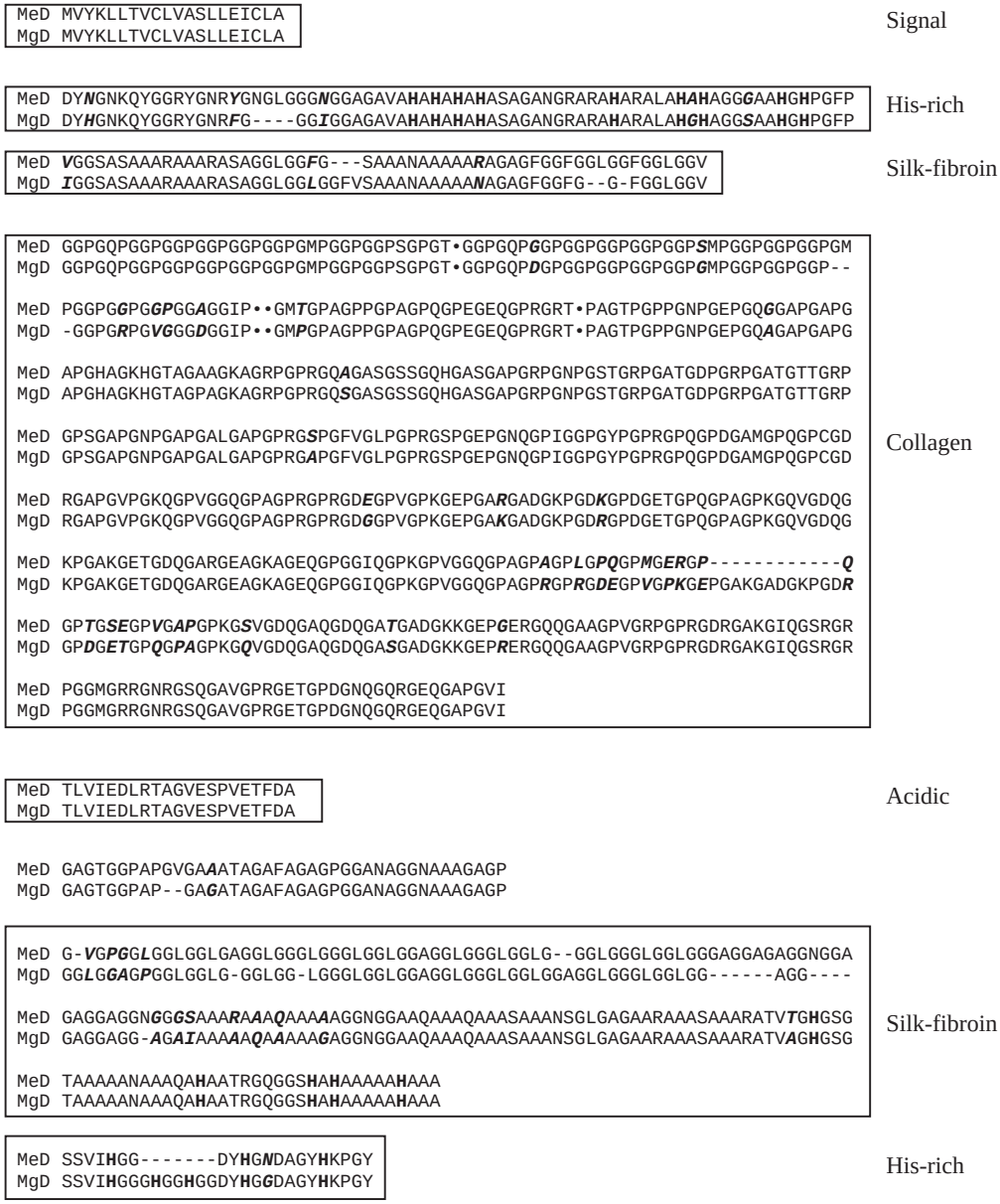

Fig. 1. Protein sequence alignment between *Mytilus edulis* (upper) and *M. galloprovincialis* (lower) precollagen D. Major domain structures are boxed and indicated. Single amino acid substitutions are indicated by bold italic type, and dashed lines indicate sequence deletions. Histidine residues that may form metal chelate cross-links are in bold type. Breaks or kinks in the collagen repeat structure are symbolized with a filled circle.

clusters with the pattern *Z*GG (where *Z*=I, F, V and A) in *M. galloprovincialis*, 31 *versus* 39 for *M. edulis*.

While these differences may prove to have functional significance, e.g. the presence of more histidine residues may give the byssal collagens from *Mytilus galloprovincialis* a greater potential to form metal-chelating cross-links, it is the similarities that may underscore the true benefit of constructing a material out of these three collagen molecules. For each molecule, the acid patch region remains unchanged except for a single isoleucine/valine difference in preCol-P. A similar absence of differences is found when comparing the collagen domains of the molecules. PreCol-NG and preCol-P have only a few substitution differences, and preCol-D from *M. galloprovincialis* has a 12-amino-acid insertion, although the integrity of the collagen sequence is maintained. Furthermore, each collagen domain has exactly the same breaks in the Gly-*X*-*Y* repeat sequence, suggesting that the molecules from both species will have similar kinked structures and properties.

Morphometric characteristics of byssal threads

Table 1 shows a comparison of the physical dimensions of byssal threads from *Mytilus edulis* and *M. galloprovincialis*. For each species, 25 individual threads were measured. Of particular note, the overall thread length in *M. edulis* is approximately double the length of *M. galloprovincialis* threads. Furthermore, the elastic proximal region makes up a higher percentage of the thread length in *M. edulis*. A similar result is seen when diameter is compared. Both the proximal and distal portions of *M. edulis* threads are approximately double the diameter of the corresponding diameters for *M. galloprovincialis* threads. Furthermore, the diameter of the

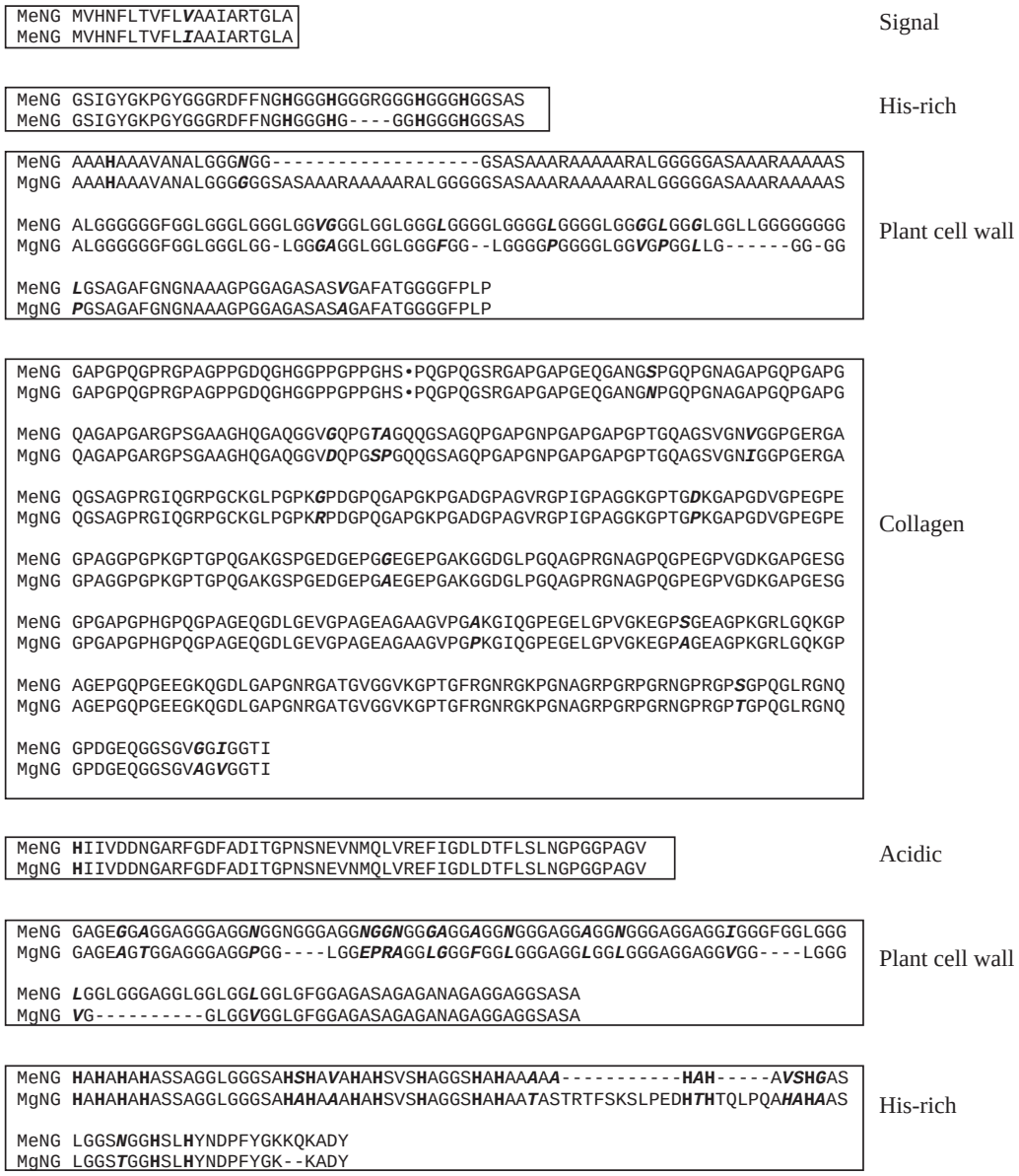

Fig. 2. Protein sequence alignment between *Mytilus edulis* (upper) and *M. galloprovincialis* (lower) precollagen NG. Major domain structures are boxed and indicated. Single amino acid substitutions are indicated by bold italic type, and dashed lines indicate sequence deletions. Histidine residues that may form metal chelate cross-links are in bold type. Breaks or kinks in the collagen repeat structure are symbolized with a filled circle.

proximal portion is 36 % and 20 % larger than that of the distal portion in *M. galloprovincialis* and *M. edulis*, respectively.

Determination of collagen content and gradient in byssal threads

The byssal collagen content of the respective proximal and distal portions of threads was calculated on the basis of the hydroxyproline content of acid-hydrolysed threads without correction for the hydroxyproline content of FP-1. Representative amino acid compositional analyses are shown in Table 2. The calculated values for byssal collagen content of the distal and proximal portions for both species are shown in Table 1. *Mytilus edulis* appears to have a larger concentration of byssal collagens in both its proximal and distal byssal thread portions compared with the same thread portions from *M. galloprovincialis*. In both cases, the distal

Table 1. *Morphometric and calculated biochemical characteristics of byssal threads from* Mytilus edulis *and* M. galloprovincialis

	M. edulis	*M. galloprovincialis*
Mean thread length (mm)	27±4.0	13±1.0
Proximal portion length (%)	34.59±4.67	21.22±2.99
Mean proximal diameter (mm)	0.16±0.02	0.08±0.009
Mean distal diameter (mm)	0.13±0.02	0.06±0.007
PreCols in proximal thread (%)	72.0±1.3	66.9±1.9
PreCols in distal thread (%)	92.5±2.1	86.9±2.4

Length and diameter measurements are means ± 1 S.D. (N=25). Precollagen (PreCol) content was calculated as described in Materials and methods; values are means ± S.D. (N=10).

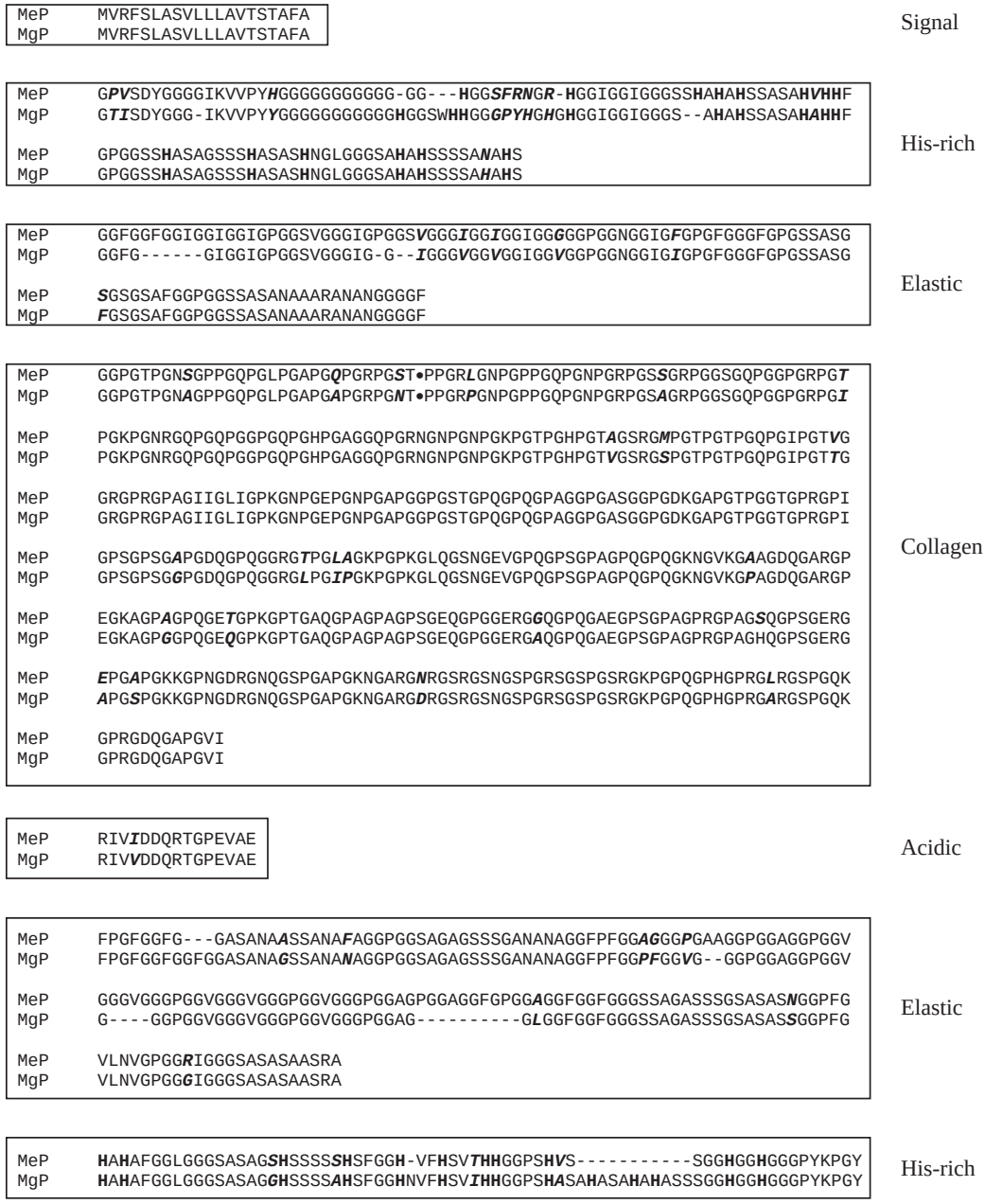

Fig. 3. Protein sequence alignment between *Mytilus edulis* (upper) and *M. galloprovincialis* (lower) precollagen P. Major domain structures are boxed and indicated. Single amino acid substitutions are indicated by bold italic type, and dashed lines indicate sequence deletions. Histidine residues that may form metal chelate cross-links are in bold type. Breaks or kinks in the collagen repeat structure are symbolized with a filled circle.

thread has approximately 20 % more collagen than the proximal region.

The manner in which this apparent difference in the proportion of collagen varies along the length of a thread was investigated by plotting the percentage composition of glycine, proline and hydroxyproline relative to location in the thread from the proximal to the distal end. Fig. 4 shows a comparison of the gradients for *Mytilus edulis* and *M. galloprovincialis* threads. As demonstrated by these plots, the collagen content of *M. edulis* threads appears to increase in a gradual fashion, whereas the gradient is much more abrupt in *M. galloprovincialis*. The inflection point in the *M. galloprovincialis* gradient suggests that the transition zone between the proximal and distal regions occurs abruptly over a length scale of a few millimeters, whereas the gradual gradient in *M. edulis* may extend over several centimeters.

Biomechanical properties of byssal threads

By testing whole threads as well as their corresponding portions under tension, several mechanical properties were measured. Representative stress/strain curves are shown in Fig. 5. Table 3 shows Young's modulus, the strain at breaking, the strength at breaking and the strain energy for proximal and distal portions from *Mytilus edulis* and *M. galloprovincialis*. For the distal region, the yield strength and strain at yield were also determined. Many of the biomechanical properties do not differ statistically between the species, as determined by single-factor analysis of variance. However, Young's modulus

Table 2. *Representative amino acid composition from hydrolysed thread portions from* Mytilus edulis *(Me) and* M. galloprovincialis *(Mg)*

	Proximal thread		Distal thread	
Amino acid	Me	Mg	Me	Mg
Hyp	3.43	3.64	5.80	4.03
As*x*	6.10	6.45	5.17	4.67
Thr	3.94	3.98	3.09	2.57
Ser	6.44	7.44	4.75	4.50
Gl*x*	5.86	6.37	5.21	5.16
Pro	9.84	9.87	7.74	6.80
Gly	28.55	25.73	34.17	34.04
Ala	9.95	9.51	16.21	19.58
Cys/2	0.58	0.52	0.13	0.09
Val	3.42	3.81	2.44	2.58
Met	0.94	0.77	0.63	0.56
Ile	2.23	2.33	1.01	1.06
Leu	3.80	3.62	2.91	2.39
DOPA	0.00	0.00	0.18	0.30
Tyr	1.69	1.85	1.50	1.37
Phe	1.97	1.75	1.09	1.09
His	3.17	3.11	2.19	2.64
Hlys	0.14	0.00	0.01	0.00
Lys	4.06	4.45	3.76	3.19
Arg	3.90	4.78	2.00	3.39

Composition is given as a percentage and is the mean of five runs with a variance of less than 10 %.

As*x* is Asp and/or Asn; Gl*x* is Glu and/or Gln. Hlys is hydroxylysine and Hyp is hydroxyproline.

DOPA, 3,4-hydroxyphenylalanine.

Table 3. *Comparison of biomechanical properties of byssal threads and thread portions from* Mytilus edulis *and* M. galloprovincialis

		M. edulis	*M. galloprovincialis*
Proximal	E_i (MPa)	**77.5±11.6**	**50.5±3.8**
	ε_b (%)	**79.0±2.2**	**108.8±3.9**
	σ_b (MPa)	46.3±11.2	35.6±1.8
	Strain energy (J cm^{-3})	18.0±5.0	20.4±1.9
Distal	E_i (MPa)	532±39	565±55
	ε_b (%)	81.4±5.5	68.1±5.5
	σ_b (MPa)	143.1±12	138.5±16
	σ_y (MPa)	50.2±4.5	47.5±3.0
	ε_y (%)	**20.4±1.6**	**13.4±1.1**
	Strain energy (J cm^{-3})	61.8±6.4	46.3±6.6
Whole	E_i (MPa)	**328.3±27**	**417.8±56**
	ε_b (%)	90.5±7.0	74.5±8.0
	σ_b (MPa)	142.8±11	123.4±14
	Strain energy (J cm^{-3})	**70.0±7.5**	**50.4±9.7**

Numbers in bold type indicate values that are significantly different from each other ($P \leqslant 0.05$).

E_i, Young's modulus; ε_b, strain at breaking; σ_b, strength at breaking; σ_y, yield strength; ε_y, strain at yield.

Values are means ± S.D. (N=10).

is higher for the proximal portion of *M. edulis* compared with *M. galloprovincialis*, while the strain at breaking is lower. This is indicative of a stronger, stiffer proximal region in *M. edulis*. For the distal region, only the yield strain is statistically different, with *M. edulis* having a higher strain, which is also indicative of its increased toughness. Cyclic testing of thread portions was performed as in Vaccaro and Waite (2001) with threads repeatedly loaded to 50 % of their initial length to determine whether there was a difference in the hysteresis or recovery of initial modulus in threads from the two species. No significant differences were observed (data not shown). The mechanical properties of whole byssal threads from each species are very similar, with *M. galloprovincialis* having slightly, but significantly, lower strain energy.

Discussion

The importance of a well-designed byssus is paramount to the survival of mytilids in the high-energy intertidal zone. Structural fidelity has been achieved in mytilids by constructing a thread with a collagenous core and a protective cuticle. The protective coating, the DOPA-rich protein FP-1, together with the adhesive plaque proteins are interesting in their own right, but their contribution to the mechanical stability of the thread is unknown. The main structural determinants of the byssus are the collagen-like molecules known as preCol-D, preCol-NG and preCol-P. These molecules have graded distributions along the length of the thread, with preCol-P predominating in the proximal portion and preCol-D predominating in the distal portion. Furthermore, gradients of individual preCol variants may also exist within the thread, as has been shown for preCol-P by Coyne and Waite (2000). It is presumably the nature of this gradient and the interactions these molecules have with one another, with the non-gradient collagen preCol-NG and with other matrix proteins such as PTMP1 (C. Sun, J. M. Lucas and J. H. Waite, in preparation) that account for the characteristic mechanical properties of the whole threads and of the individual thread portions.

It has been proposed that metal ions, possibly Zn^{2+}, Cu^{2+} and Fe^{2+}, play a significant role in the structural integrity of the thread. An exact comparison between the metal composition of byssal threads from *M. edulis* and *M galloprovincialis* has not been made. However, Vaccaro and Waite (2001) demonstrated using metal chelators, such as EDTA, that such ions are essential for the normal recovery of threads under cyclic tension. No cross-links have been isolated from byssal threads; however, McDowell et al. (1999) have detected 5,5′-di-DOPA in the byssal plaques of *Mytilus edulis* using rotational echo double-resonance nuclear magnetic resonance. Waite (1990) has suggested that similar di-DOPA- or other quinone-based cross-links might also be present in byssal threads. Such cross-links may exist between byssal

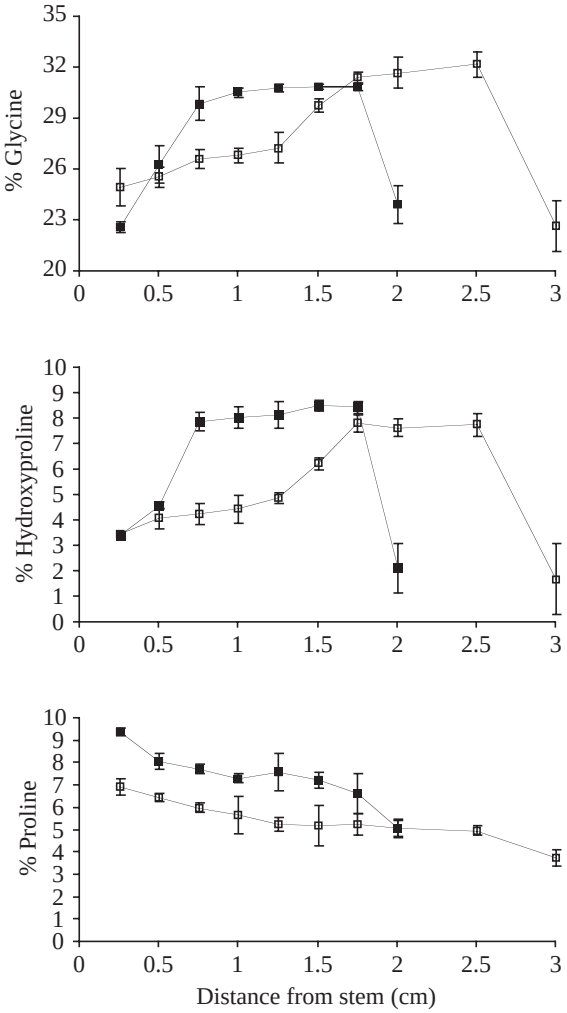

Fig. 4. Distribution of the amino acids glycine, hydroxyproline and proline in hydrolysed byssal threads from *Mytilus edulis* (□) and *M. galloprovincialis* (■). Each point represents the mean of five hydrolysed samples taken at regular distances from the thread's attachment point to the stem. Values are means ±1 S.D. The 2 cm and 3 cm points represent the plaques from *M. galloprovincialis* and *M. edulis*, respectively.

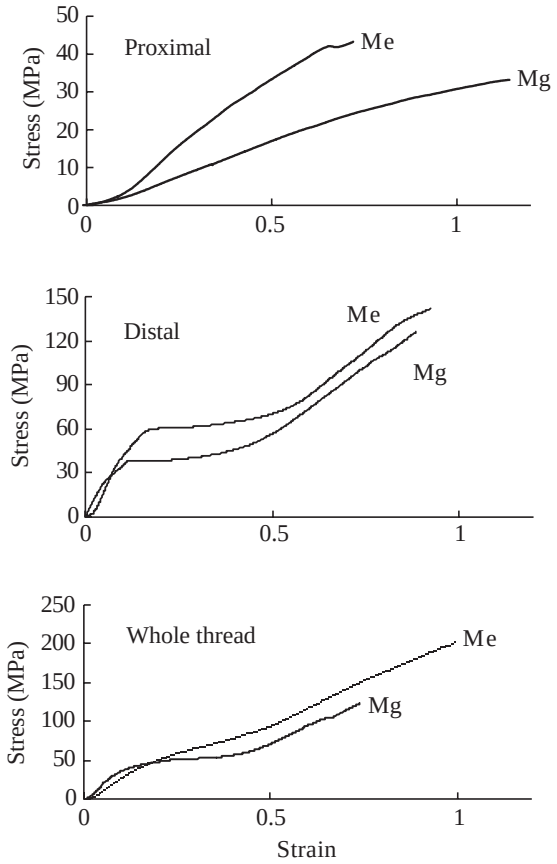

Fig. 5. Representative stress/strain curves for proximal, distal and whole threads of *Mytilus edulis* (Me) and *M. galloprovincialis* (Mg). Threads were pulled to breaking.

collagen molecules because DOPA has been detected in preCol-D and is possibly a component of the other preCol variants. It has been surmised that, at least in the case of the byssal collagens, metal chelate complexes represent a significant cross-linking alternative. Such complexes involving histidine, DOPA or even cysteine residues would differ from di-DOPA in their reversible, sacrificial breakage under tension (Thompson et al., 2002).

The sequence of the preCols makes them strong prospects for polyvalent metal interactions given the histidine-rich regions found in both the N and C termini of all three preCols, the DOPA residues in preCol-D and the cysteine residues in preCol-D and preCol-NG. If the sheer number of histidines is any indication of cross-linking potential, then *Mytilus galloprovincialis* would be expected to produce stronger, stiffer threads by virtue of having more histidines in the flanking domains of all its preCols. Comparisons of the mechanical properties of threads from *M. edulis* and *M. galloprovincialis* partially support this conclusion. When the performance of intact threads is considered, *M. galloprovincialis* threads have a higher Young's modulus that is indicative of a more cross-linked or crystalline structure. This value is somewhat offset by the higher strain energy of *M. edulis* threads; however, because of variance in the values, the difference in strain energy is not as significant. Overall, mechanically, the threads appear to be fairly similar with only slight differences between the two species. Something more must be going on.

Mytilus galloprovincialis threads are shorter and slimmer than those of *M. edulis* which, in conjunction with having a lower concentration of collagen, may mitigate any increased cross-linking advantage. Perhaps what is achieved in both cases is a set of mechanical properties influenced by evolutionary pressures whereby *M. galloprovincialis* has found the need to construct a thread with a lower potential for extension. Recent mathematical modeling of marine organisms (such as mussels) with flexible attachments suggests that 'going with the flow' does not necessarily reduce dislodgment forces under all conditions (Denny et al., 1998). In particular, the strategy of making extensible byssal threads under high-

flow regimes to reduce forces due to drag and lift could, in some instances, increase inertial forces on the mussel because of momentum. As the degree of byssal extension will have a direct bearing on the consequent momentum and inertial energy of the systems, thread length represents perhaps the simplest way for species to adapt their holdfast to flow.

Although sequence comparisons tend myopically to focus on differences, it may be the similarities in this case that prove to be most significant. The flaws in the collagen triple helix are maintained between the species. Modeling of the collagen domains has revealed that these flaws correspond to kinks in the collagen structure, thus requiring precise lateral packing for fibers to form or allowing space for requisite matrix proteins (Waite et al., 2002). Imperfections in invertebrate collagens are not uncommon. Flaws in the triple helix of collagens have been found in the tubeworm *Riftia pachyptila* (Mann et al., 1992), the cnidarian *Hydra vulgaris* (Fowler et al., 2000) and the marine worm *Arenicola marina* (Sicot et al., 1997). In each case, the imperfections have been either modeled or clearly demonstrated by rotary shadowing electron microscopy as kinks or bends in the triple helix. The function of such bends remains unknown, but clearly they are well-tolerated and presumably designed to achieve some sort of structural integrity. The exact nature of the structural packing of byssal collagens remains elusive, but it is almost certainly key to understanding the mechanical properties displayed by byssal threads.

In all three sets of byssal collagens, the acidic domains remain virtually unchanged between species, except for a single isoleucine/valine difference in preCol-P. This lack of variability indicates a high degree of evolutionary pressure to maintain this sequence among species. It has been suggested that the acid patch in spider silks may coordinate the self-assembly of these fibers (Hayashi et al., 1999; Xu and Lewis, 1990). While only conjecture, a similar role is conceivable in mussel byssus. Furthermore, the *X*GG (where *X*=L, A, F or V) repeat pattern found in the silk-fibroin domains of preCol-D has been implicated in forming the extended flexible secondary structure of flagelliform silks and may provide both silk and byssus with their abilities to stretch and recoil (Hayashi and Lewis, 2001).

Suresh (2001) reviews the benefits of constructing graded materials and discusses how many biomaterials may be capable of serving as models for the construction of synthetic materials. Byssal threads clearly fall into the category of graded materials. Through the manipulation of gradients at the macro, micro and molecular levels, mytilids have constructed a material, the byssus, that adheres under water, can resist periodic exposure to the sun and air, yet is able to maintain its ability to stretch and 'self-heal' in the face of repeated deformation by waves (Waite et al., 2002). At the macroscopic level, the plaque, the distal and proximal thread, the stem and the retractor muscle form a graded series of differing materials that are carefully transitioned to produce a functional thread. At the microscopic level, collagen fibers are aligned and interspersed with matrix proteins in such a way that their interactions mediate stresses along the thread and allow for some degree of reorganization or 'self-healing'. Finally, at the molecular level, individual preCol molecules of at least three types, each with possible multiple variants, are carefully titrated along the length of the thread to achieve the desired degree of inter- and intramolecular cross-linking. A comprehensive understanding of the structure may prove to be an ideal model for the synthesis of materials with similar 'self-healing' properties.

Mytilus edulis and *M. galloprovincialis* represent only two of the many mytilid species. It is clear from this comparison that the species are largely interchangeable as a model for byssal studies, especially with regard to conventional biomechanical variables. Comparison of these mechanical properties with those of the byssus of other mytilids is intriguing but must be undertaken with some caution. Values in the literature for *M. californianus*, *M. trossulus*, *M. edulis* and *M. galloprovincialis* are often inconsistent (Bell and Gosline, 1996; Price, 1981; Smeathers and Vincent, 1975). Similarly, comparisons with the values reported here do not correspond precisely with those reported in the literature. While the basis for these inconsistencies is speculative, sample size, mussel health, reproductive stage, thread age, equipment limitations and the precise separation of threads into distal and proximal portions are all possible factors contributing to disparities. Even with that caveat, *M. californianus* threads (Young's modulus 868±181.2 MPa) are 2–3 times stiffer than those of *M. edulis* and *M. galloprovincialis* irrespective of the values with which they are compared (Bell and Gosline, 1996). Furthermore, mechanical values for mussels in the 'edulis complex' are much more similar to each other than they are to more distantly related mytilids.

With these mechanical considerations, it would be both telling and interesting to know how the byssal collagens of other mytilids compare with those of these two species. *Mytilus californianus*, in particular, is notorious for producing very long, thick, tough threads (Bell and Gosline, 1996). Information about whether such remarkable threads are built upon similar underlying structural components with similar distributions and assemblages would undoubtedly lead to further refinements to the functional and structural models of these unique extraorganismic fibers.

The sequence data reported here have been submitted to GenBank under accession numbers AF448526 (MgpreCol-D), AF448524 (MgpreCol-NG) and AF448525 (MgpreCol-P).

The authors thank the Biomaterials Program at NICFDR for financial support (DE 10042).

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