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Marine Surfaces and the Expression of Specific Byssal Adhesive Protein Variants in *Mytilus*

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Abstract: *Mytilus* foot protein-3 (Mfp-3) is a highly polymorphic protein family located in the byssal adhesive plaques of blue mussels. Previous evidence suggested that the deposition of selected Mfp-3 variants might be influenced by the type of surface to which the mussel attaches; therefore, we undertook to rigorously investigate whether a correlation exists between surface type and Mfp-3 variants. Two hypotheses were tested in *M. galloprovincialis* and *M. edulis.* One hypothesis was that individual mussels deposit specific Mfp-3 variants on different surfaces. Laser desorption–ionization mass spectrometry was used to detect Mfp-3 variants on the underside of byssal adhesive plaques. The other hypothesis was that the transcription of specific Mfp-3s is induced by different surfaces. This was measured by using denaturing gradient gel electrophoresis to separate closely related amplified complementary DNAs among individual mussels attached to stainless steel, glass, or polyethylene surfaces. Band stabs of several Mfp-3 cDNAs were sequenced. The results clearly showed that individual mussels secreted a similar suite of Mfp-3 variants onto glass, plastic, and steel. Likewise, the expression of Mfp-3 cDNA transcripts in individual mussels revealed no clear correlation between messenger RNA expression and the type of surface. Thus, the expression and secretion of specific Mfp-3 variants do not appear to be surface-induced. These results underscore the importance of following individual mussels rather than populations in surface studies.

Key words: *Mytilus,* byssus, Mfp-3, adhesive, surface induction, transcriptional regulation.

INTRODUCTION

Sessile marine organisms rely on a secure holdfast to avoid dislodgement by the lift and drag forces of currents, waves, and tides. The holdfast in marine mussels is known as a byssus and consists of an extracorporeal bundle of threads with sticky tips or adhesive plaques. These adhere permanently and opportunistically to hard substrata (Brown, 1952; Waite, 1992; Crisp et al., 1985). Adhesive plaques in various species of *Mytilus* contain at least four protein families known as Mfps or *Mytilus* foot proteins (Papov et al., 1995; Waite, 1997). In *M. edulis* and *M. galloprovincialis,* these have the following masses: 110 kDa (Mfp-1), 46 kDa (Mfp-2), 5–7 kDa (Mfp-3), and 80 kDa (Mfp-4). All contain the posttranslationally modified amino acid, 3,4-dihydroxyphenyl-L-alanine (DOPA), as well as other unusual modifications.

Given the wide variety of surfaces that mussels can

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attach to, we are interested in exploring whether mussels can detect surface properties and, if so, whether their adhesive chemistry is specifically adapted to different surfaces. Evidence that mussels can distinguish different surfaces has already been obtained by Young (1983), who used checkerboard substrata consisting of Teflon, paraffin, polyacetal, glass, and slate to show that when given a choice, mussels definitely prefer attaching byssal threads to materials with high critical surface energies such as slate and glass. When not given a choice, mussels attach threads to any hard surface; however, plaque attachment strength (load to failure) appears to be directly correlated with critical surface energy (Crisp et al., 1985).

Does adhesion to surfaces as different as polyethylene and glass rely on the same or different bonding strategies? Answering this question depends, in part, on how accessible the interface between plaque and substratum is to chemical analysis. Matrix-assisted laser desorption/ionization–time of flight–mass spectrometry (MALDI-TOF-MS) can be used to examine the bonding face of adhesive plaques freshly secreted by a group of mussels (Ross et al., 1995; Warner and Waite, 1999). As the proteins at the exposed surface are ionized and desorbed, their mass-to-charge ratios can be determined. Preliminary MALDI-TOF-MS of adhesive plaques has shown that the predominant desorbing species belong to the Mfp-3 protein family with masses in the range of 5 to 7 kDa. Usually 3 to 5 variants of Mfp-3 were detected for any given surface, such as stainless steel, glass, and plastic.

Mfp-3 expression is localized to the distal depression of the foot near the juncture of the accessory and phenol glands (Inoue et al., 1996), and its distribution in byssus is limited to the plaques (Diamond, 1993). A high degree of Mfp-3 sequence variation is apparent in both protein and messenger RNA preparations isolated from pooled mussel feet (Papov et al., 1995; Inoue et al., 1996). A recent study reported that at least 20 different Mfp-3 mRNA transcripts occur within a single mussel (Warner and Waite, 1999). With so many potential variants in each individual and only a handful deposited onto surfaces, a strong suggestion of surface-specific variants exists.

A systematic analysis of the relation between surface type and Mfp-3 variants requires a technical approach with two capabilities: (1) detection and isolation of nanomolar amounts of material from individual mussel feet or plaques, and (2) sequence determination with comparable sensitivity. MALDI-TOF-MS can detect femtomolar levels of protein; however, because of the heterogeneity of posttranslational modifications and chain length (~50 residues), obtaining information about sequence is not feasible. Initial attempts to isolate and sequence Mfp-3 variant proteins from individual feet or plaques were not successful (Florilli, 1998).

A biased but sensitive method for obtaining variant sequence is feasible using a molecular strategy based on an amplification by reverse transcription–polymerase chain reaction of total foot RNA primed with Mfp-3-specific oligonucleotides. Because it detects the presence of mRNA and not protein, this approach is at least one step removed from the adhesion process. It does, however, address an important related question: Do surfaces have an inductive effect on transcription of Mfp-3 genes? If mussels are able to detect some characteristics of a surface, their ability to respond may rely on up-regulating transcription, translation, or secretion of a particular suite of variant proteins (Brinkmann, 1994; Sierra and Zapata, 1994; Albrecht and Tidball, 1997). The secretion and expression of Mfp-3 are compared among individual adults attached to three surfaces. If the transcription of Mfp-3 is induced by the surface type, then the expression of specific transcripts should vary between surfaces.

Materials and Methods

Mussels used in laser desorption experiments were collected from Goleta Pier, California, CA, and identified as *Mytilus edulis galloprovincialis* using molecular markers described by Rawson et al. (1996). This is a slightly different variant from the mussels used in the molecular experiments described below. Given that it has been established that the byssal precursors for the two subspecies are nearly identical and that we are looking for mass trends in the present study, rather than correlations to sequence, the use of different subspecies should not be a problem. Getting mussels to deposit adhesive plaques exclusively onto glass or acrylic plates (plexiglas) was arranged by using specially cut plates ($10 \times 10 \times 0.3$ cm), which were tightly wrapped by 2 to 3 turns of nylon fishing line (diameter 1 mm). Mussels were tethered to these wrappings using 6-cm-long rubberbands that were loop-tied two thirds from one end. The larger loop was slip-knotted through the fishing line, while the smaller loop was dorsoventrally wrapped around the mussel. The mussel/plate combinations were then draped from a clothesline suspended across the top of the aquarium using small noncorroding spring clips. Mussels began explor-

ing the plate surface within 1 hour of immersion, and plaque deposition commenced shortly afterward. To avoid extensive protein cross-linking in the byssal plaques required the following conditions: low seawater temperatures (8°C), minimal water agitation, and plaque harvest within 24 hours of deposition. Plaques were harvested under the stereomicroscope by cutting the byssal threads a few millimeters above the plaque, then shearing the plaques from the plate surface with a new single-edge razorblade. Each collected plaque was washed with two changes of 10 ml of Q-water.

Laser Desorption Mass Spectrometry

Mass spectrometric analysis was done by matrix-assisted laser desorption ionization with time-of-flight (MALDI TOF) using a PerSeptive Voyager instrument with delayed extraction (Perkin-Elmer, Framingham, Mass.). Byssal adhesive plaques with "bottom" sides facing up were mounted onto a gold specimen plate using double-sided tape (3M), coated with matrix (1 μl × 2) and air-dried. Matrix was sinapinic acid (Sigma; 10 mg ml^{-1}) dissolved in 0.1% (vol/vol) trifluoroacetic acid in a 50/50 mixture of Q-water and high-performance liquid chromatography (HPLC)-grade acetonitrile. The samples were evacuated to at least 5×10^{-7} torr in the vacuum chamber of the spectrometer and irradiated at 337 nm with a nitrogen laser at a power setting of 2100 to 2300. Counts of molecules desorbed, ionized, and detected in the positive ion mode were averaged over 255 accumulated scans. Other conditions included an accelerating voltage of 20 kV, grid voltage at 94%, a guidewire voltage of 0.02%, and low mass gate of 800. Although MALDI-TOF has a mass accuracy that is generally better than 0.01%, some runs included a monoisotopic standard (ACTH fragment, 6964.5 $[M+H]^+$) for internal calibration.

Preparation of Adult Mussel Total RNA

Mussels (*Mytilus edulis*) were collected from submerged traps in the Roosevelt Inlet at the mouth of the Broadkill River, Delaware. Immediately after collection, individual mussels were placed in polyethylene, glass, and stainless steel containers in replicates of three. Approximately 500 ml of 1 μm-filtered, UV-sterilized seawater was added to the containers; the seawater was changed after 24 hours. After 2 days, three more individuals were collected from the docks, and the total RNA from the foot of each of the 12 mussels was extracted using a modified version of the guanidinium thiocyanate/phenol/chloroform protocol (Chomczynski and Sacchi, 1987; Coyne et al., 1997). The total RNA of the gill and mantle was also extracted from one mussel as a negative control.

Reverse Transcription

Reverse transcription (RT) was performed on 1 μg total RNA from each of the samples (Kawasaki and Wang, 1989; Sambrook et al., 1989). There were three sets of adult RNA samples, each including the 12 individuals from the four different surfaces, a no-foot control (containing gill and mantle tissue), and a no-RT control (to which no RT was added). The samples were first treated with 1 U DNase (Life Technologies) in a 10 μl 1× DNase buffer solution for 15 minutes at room temperature. The DNase was inactivated with the addition of 1 μl of 20 mM EDTA and an incubation for 10 minutes at 65°C. The samples were immediately placed on ice, and 13 μl of RT cocktail was added to a final concentration of 25 mM Tris-HCl (pH 8.4), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 0.4 mM dNTPs, and 5.4 ng/μl random hexamers. After an initial incubation at 25°C for 10 minutes, 200 U of Superscript II (Life Technologies) was added to each sample, with the exception of the no-RT control. The samples were then incubated for 30 minutes at 42°C. The reverse transcriptase was inactivated for 15 minutes at 70°C; the samples were then incubated with 1 U of RNase H for 10 minutes at 55°C. The samples were brought to a final volume of 50 μl with Q-H_2O. To ensure that the RNA was successfully reverse transcribed, the 18S ribosomal RNA was amplified using Euk A and Euk B primers (see Table 2) (Medlin et al., 1988).

Amplification of Mefp-3 Complementary DNA

Prior to denaturing gradient gel electrophoresis (DGGE), the cDNA samples were amplified in a nested PCR (Baier et al., 1993). The first round of amplification used 2 μl of the cDNA samples in a 10-μl reaction containing 1× PCR buffer, 0.8 mM dNTPs, 0.5 μM GCsigpep primer (Table 2), 0.5 μM poly(T-GA) primer (Table 2), 1.25 mM $MgCl_2$, and 0.75 U of *Taq* polymerase. The GCsigpep primer was designed based on the cDNA region coding for the signal peptides of the Mfp-3 sequences obtained from expression libraries (Floriolli, 1998); the poly(T-GA) primer was a poly(T) sequence tailed with a GA-repeat sequence. A touchdown amplification was performed under the following conditions: 1 minute denaturation at 94°C; 1 minute

annealing at 55°C, −1°C per cycle; and 1 minute extension at 72°C for 15 cycles. This was followed by an additional 20 cycles having an annealing temperature of 40°C and a final extension at 72°C for 3 minutes. The PCR products of the adult were diluted 1:100 for use in the second amplification.

The 25-μl nested PCR reaction contained 1 μl of the diluted PCR products, 1× buffer, 0.5 μM GCsigpep primer, 0.5 μM Mefp-3.3UCA primer (Table 3), 1.25 mM $MgCl_2$, and 0.625 U of *Taq* polymerase. The Mefp-3.3UCA primer was designed based on the 3′-untranslated region of the known Mfp-3 sequences. Using primers specific for the cDNA of the signal peptide and 3′-untranslated regions ensured that the entire cDNA sequence of the functional protein would be amplified. The reaction was initially heated at 94°C for 1 minute, at which time the Mfp-3.3UCA primer was added, followed by a 1-minute denaturation at 94°C, a 1-minute annealing at 52°C, and a 30-second extension at 72°C for 30 cycles.

Denaturing Gradient Gel Electrophoresis

DGGE analysis was used to determine the variation of expression among the mussels attached to the different surfaces (Myers et al., 1985, 1989). The gels were composed of 6% polyacrylamide/bisacrylamide (37.5:1), 0.8% (vol/vol) TEMED, and 0.8% (vol/vol) ammonium persulfate; the denaturing gradient was 25% to 45% (vol/vol) of both 7 M urea and formamide. To avoid overloading the gel, the amount of sample loaded varied from 2.5 to 7.5 μl depending on the intensity of the amplified products. The samples electrophoresed at a current of 130 V in 1× TAE (0.04 Tris-acetate; 1 mM EDTA, pH 8.0) at 60°C for 3.5 to 4 hours.

The bandstab protocol of Wilton et al. (1997) was used to determine the sequences of the Mfp-3 amplified products from the first set of adult mussels. A pipet tip was used to stab individual bands of the DGGE gels; after immersing the pipet tip in the previously described second-round PCR mixture, the sequences were reamplified under the same nested conditions for 25 cycles. DGGE analysis of the PCR products was performed to ensure that a single sequence was successfully amplified. When single bands were not obtained, the bandstab, amplification, and electrophoresis were repeated a second time. The successfully amplified products were then cleaned using the QIAquick PCR Purification kit (Qiagen). Cycle sequencing was performed on 1 to 2 μl of the purified PCR products using the ABI PrismTM Dye Terminator Cycle Sequencing Kit with AmpliTaq DNA Polymerase, FS (Perkin-Elmer) (Sanger et al., 1977; Prober et al., 1987). Forward sequences were obtained using the GCsigpep primer and, if clarification was necessary, the Mfp-3.5 primer. The reverse sequences were obtained using the Mfp-3.3UCA primer. The sequences were then determined by the ABI Prism 310 capillary sequencer as per the manufacturer's instructions. AutoAssembler (Perkin-Elmer) and Sequence Navigator (Perkin-Elmer) were used to analyze and align the sequences.

Results

MALDI-TOF of Adhesive Plaques

The results of tracking the presence of proteins at or near the interface of adhesive plaques taken from nine mussels on three different surfaces are shown in Figure 1 and Table 1. Several general features are noteworthy. First, three to five Mfp-3 variants are readily detectable on each plaque. In fact, Mfp-3 is the only protein consistently detected on the plaque surface. Second, each Mfp-3 variant is heterogeneous with regard to how much additional oxygen (atomic mass, 15.9994 Da) it contains. This oxygen is known to be invested in the posttranslational hydroxylation of Tyr and Arg to DOPA and 4-hydroxyarginine in Mfp-3 (Papov et al., 1995), respectively. Third, the level of hydroxylation in the most abundant molecular species in each variant varies in individual mussels.

Figure 1 illustrates the complexity of Mfp-3 in adhesive plaques of three individuals (mussels 2, 4, and 7). The variants are labeled A to E, and each consists of increasing oxygenation levels (left to right) with the prevailing species indicated by a mass number in Table 1. Figure 1 shows that despite their complexity, Mfp-3 patterns pertaining to a particular mussel persist in any plaque made by that mussel for attachment to any surface. For example, for mussel 2, variant D dominates all surface types although there are slight shifts in the mass of the prevailing species: e.g., steel (6154), plastic (6187), and glass (6199). The latter differences are minor and may be attributed to deprotonation (−1 Da/res DOPA), dehydrogenation of DOPA to *o*-quinone (−2 Da/res DOPA), or hydroxylation (+16 Da/res DOPA or Arg), all of which are very likely. Variant A is consistently the second most abundant on the surfaces;

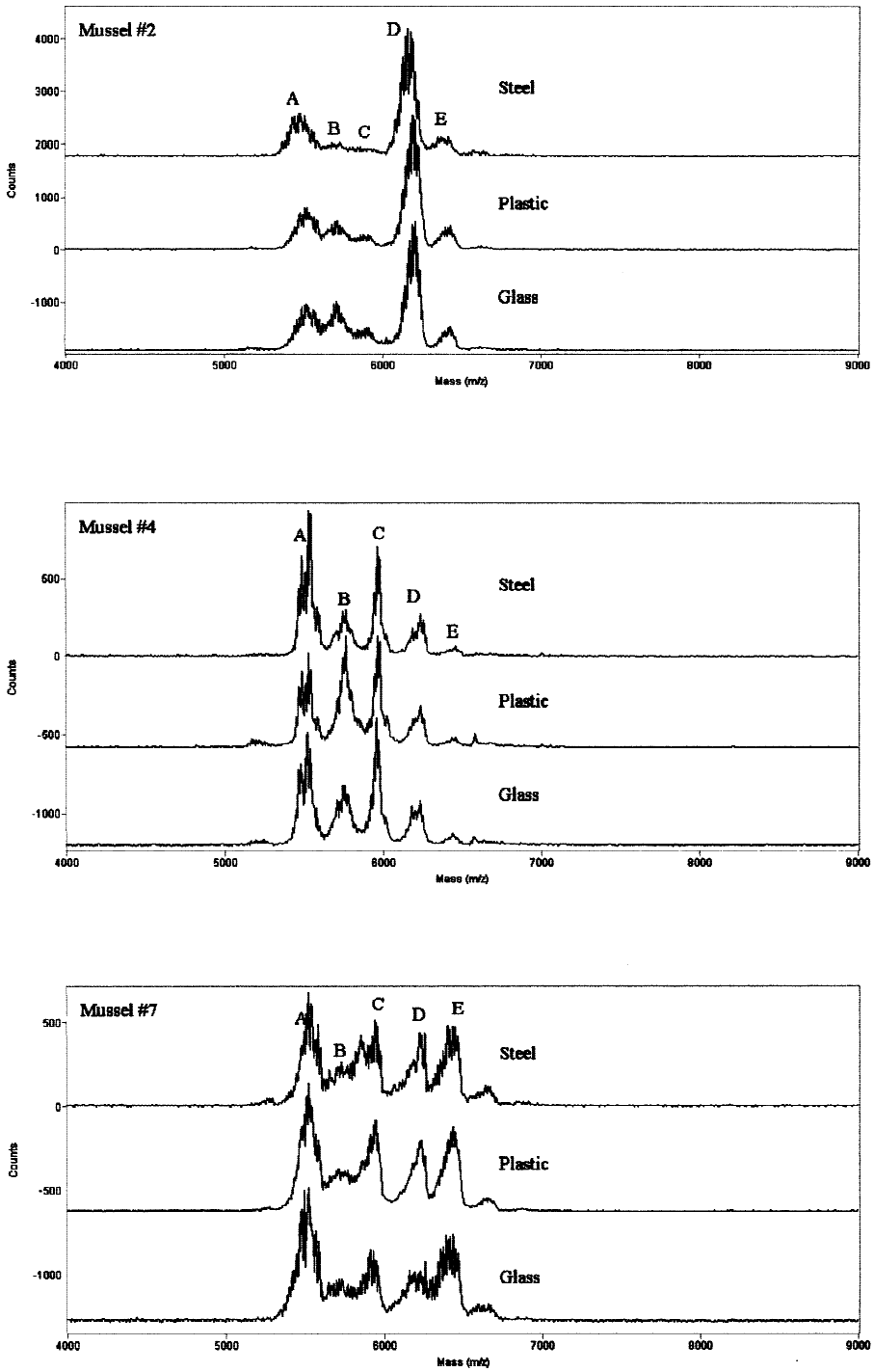

Figure 1. Matrix-assisted laser desorption ionization–mass spectrometry with time of flight of the bonding faced of byssal adhesive plaques from *M. galloprovincialis.* Plaques were prepared for laser irradiation as described in the "Materials and Methods" section using sinapinic acid as matrix. All plaques are from individual mussels (2, 4, and 7) exposed to three different surfaces: steel, plexiglas plastic, and glass. Letters A to E denote variants. The *m/z* for the highest peak in each cluster is identified in Table 1.

variants B, C, and E are in third place. Variant A is dominant on all surface types in mussels 4 (Figure 1), 5, 6, 7, and 9 (Table 1). Variant D prevails in mussels 2 and 8, while E prevails in mussel 3. The data for all nine mussels are summarized in Table 1. The trends are self-evident and not improved by statistical analysis.

RT-PCR of Mfp-3

Three sets of individual mussels (*Mytilus edulis*) were used to examine the effect of surface on the expression of Mefp-3. Each set included 12 individuals; 3 individuals each were placed on stainless steel (S), glass (G), and polyethylene (P),

Table 1. Fp-3 Variants (A to F) Deposited by Nine Mussels onto Steel, Plastic, and Glass Surfaces.*

	Surface		
Mussel	Steel	Plastic	Glass
1	**A-5505**	**A-5525**	**A-5515**
	B-5757	B-5761	B-5754
	C-5926	*C-5942*	*C-5937*
	D-6159	D-6203	D-6197
	—	E-6435	E-6451
2	*A-5475*	*A-5520*	A-5521
	—	B-5707	*B-5703*
	—	—	C-5897
	D-6154	**D-6187**	**D-6199**
	E-6409	E-6426	E-6422
3	*A-5487*	*A-5491*	*A-5491*
	—	—	—
	—	—	—
	D-6230	D-6236	D-6236
	E-6463	**E-6468**	**E-6468**
	F-6676	F-6680	F-6674
4	**A-5522**	**A-5523**	**A-5517**
	B-5760	*B-5758*	*B-5748*
	C-5957	**C-5959**	**C-5953**
	D-6234	D-6230	D-6226
	—	—	—
5	**A-5442**	**A-5442**	**A-5440**
	—	—	—
	C-5858	C-5909	C-5891
	D-6180	*D-6170*	*D-6158*
	—	—	—
6	**A-5461**	**A-5462**	**A-5468**
	B-5688	B-5675	B-5690
	C-5929	C-5931	*C-5938*
	D-6219	D-6229	**D-6234**
	E-6425	D-6444	D-6448
7	**A-5519**	**A-5521**	**A-5517**
	—	—	—
	C-5939	C-5934	C-5907
	D-6228	D-6224	D-6254
	E-6401	*E-6434*	*E-6432*
8	*A-5521*	*A-5522*	*A-5521*
	B-5753	B-5745	B-5743
	C-5957	C-5957	C-5955
	D-6252	**D-6254**	**D-6252**
	E-6459	E-6461	E-6476
9	**A-5442**	**A-5444**	**A-5437**
	B-5617	—	B-5651
	C-5875	**C-5906**	*C-5906*
	D-6155	*D-6168*	*D-6187*
	E-6318	E-6350	E-6346

*Dominant variant (boldface), strong second (italics), and less abundant (normal). Numbers represent maximal *m/z* peak for each variant cluster.

and another 3 were collected from the docks (W for wild type). Mussels are identified according to the surface to which they were attached, and each of the three individuals from a particular surface was also labeled 1–3, 4–6, or 7–9 for the first, second, and third sets, respectively. After performing the RT reaction for each set, the 18S ribosomal cDNA was amplified to ensure that the Mfp-3 cDNA was amplifiable. Only G2 and P8 failed this test (data not shown); therefore, these individuals were not included in the Mefp-3 amplification.

The nested PCR for the amplification of Mfp-3 cDNA resulted in products ranging in size from 290 to 310 bp. The PCR products from the first set were approximately 310 bp in length when electrophoresed in the 1.2% agarose gel regardless of the surface to which they were attached; this is the same size as the PCR product from the positive control pA100, a plasmid containing a cDNA insert of Mfp-3 (Figure 2A) (Floriolli, 1998). Similarly sized bands were also present in the second and third sets; however, another band of approximately 290 bp was present in individuals S5, S6, G4, P5, P6, W5, and W6; P7 also had another band, this one at approximately 330 bp (Figures 3A and 4A).

DGGE Analysis

Given the uninformative results of the conventional electrophoretic analysis in agarose, all samples were subjected to DGGE in order to determine if sequence differences existed among the amplified Mefp-3 cDNA of individuals on specific surfaces (Myers et al., 1985, 1989). The results, while vastly improving the resolution of electrophoretic species, may be complicated by the presence of heteroduplex bands, which must ultimately be identified by sequencing. Each set of individuals was analyzed separately to avoid any uncontrolled variables that may have occurred in a comparison between gels.

The amplification products of the individuals from the first set of mussels resulted in a total of 14 bands with unique mobilities in the DGGE polyacrylamide gel (Figure 2B). The number of bands among the first set of individuals varied from three to six per individual. For instance, the S1 sample has three bands, whereas, the S3 sample has six. The +6, −4, and −5 bands of S3 are extremely faint but are present in the gel. Of the 14 different bands, there are 6 that are unique to certain individuals: S3 has three unique bands, the +2, −4, and −5 bands; the remaining unique bands, +7, −1, and −6, are present in W2, W1, and G3, respectively. Three bands are found in the products of individuals from a single surface as well as in samples of individuals that were collected from the docks. For instance, the +6 band is present in W1, W3, and S3, and the +5 band is an amplified product identified in individuals G3 and

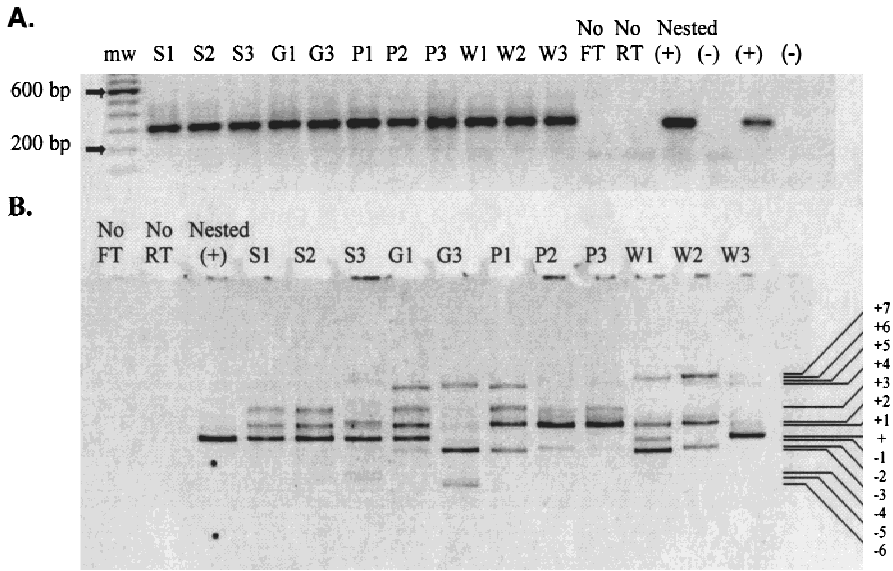

Figure 2. Migration of nested PCR products from the first set of adult mussels (*M. edulis*) attached to stainless steel (S), glass (G), polyethylene (P), and the docks (W). The individual mussels for each surface are labeled 1, 2, and 3. The NoFT (no foot), NoRT (no reverse transcriptase), nested(−) (nested, no template), and (−) (no template) samples served as negative controls; the nested(+) and (+) were samples of amplified pA100. The (+) and (−) were run through the second round of amplification only. **A:** PCR products electrophoresed through a 1.2% agarose gel; (mw) indicates the 100-bp molecular weight marker. **B:** PCR products electrophoresed through a 6% polyacrylamide/bisacrylamide (37.5:1), 25%–45% denaturing gradient gel. The band numbers on the right represent a summary of the different bands present in this gel. The + band is the PCR product of pA100. Bands with slower migrations are positive; those with faster migrations are negative. The increasing absolute values indicate an increased distance from the + band.

W2. The −2 band is present in the samples from W2, P1, and P2, but not in P3. A few bands are present in every individual of a given surface; however, these bands are also found in the samples from individuals of other surfaces. Every individual placed on steel has the + and +1 bands; the +1 band is also present in the polyethylene and dock samples as well as in G1, and the + band is found in G1 and W3. The polyethylene individuals each have the +3 band, but this band is not unique to the individuals on this surface; the +3 band is also present in the S1, S2, and G1 samples. The individuals placed on glass also have an amplified product common among them; however, the −3 band is also found in P1 and W1. No bands are unique to every individual of a specific surface.

In the second set of individuals, labeled 4–6, the number of bands per individual varies from 3 to 6 (Figure 3B). There are three unique bands: one, the +5 band, exists in the W4 sample; and the −2 and −5 bands are present in the S5 sample. The +6 band is found in the S6 and W4 samples; the −1 band is also present in S6 as well as in each sample collected from the docks. Bands +2, +3, and +4 are only present in individuals G5 and P4. These samples are nearly identical except for the −3 band, which is present in P4. This band is also found in G6 and W6. The remaining bands are present in samples from each surface; however, they are not necessarily present in each individual. For instance, the −4 band occurs in S4, S6, G4, P5, P6, and W5; the + band is present in S4, S6, G4, P4, and W4; and the +1 band, which is the most common for this set of individuals, is present in all samples except S5, S6, and W4. As with the first set of mussels, no bands are unique to each individual on a specific type of surface.

Among the third set of individuals, there are six different migratory bands varying from 1 to 3 per individual (Figure 4B). The +1 band is common among the surfaces; however, this band is not found in the G9 and W9 samples. The +band is found in individuals from every surface except polyethylene (which only includes two individuals), but not every individual placed on a given surface has this particular cDNA; S9, G8, and W8 do not have this band. The +3 and

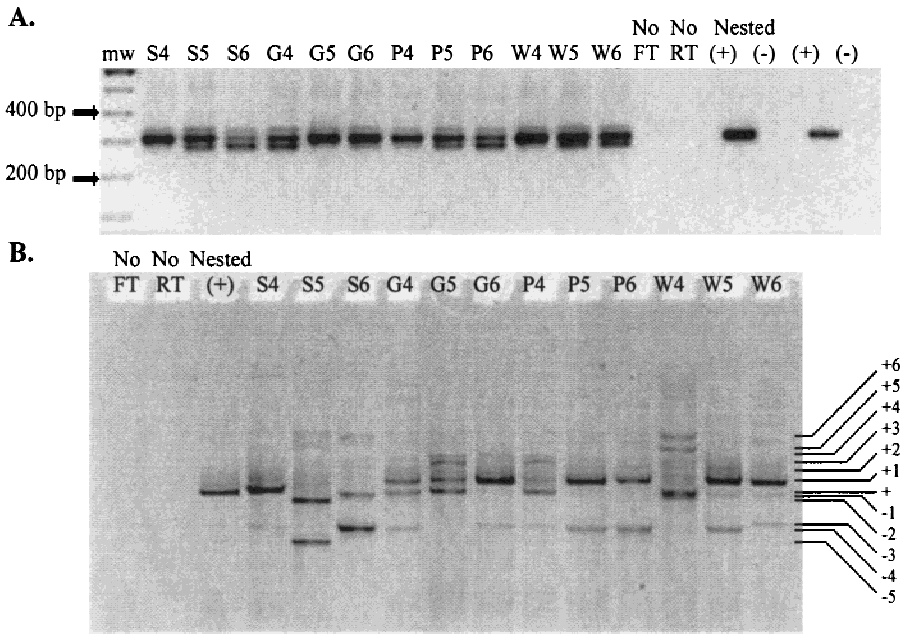

Figure 3. Migration of nested PCR products from the second set of adults. The individual mussels for each surface are labeled 4, 5, and 6. **A:** See Figure 1A. **B:** The band notation, indicated on the right, represents the migrations of the bands within this gel. See Figure 1B for further explanations.

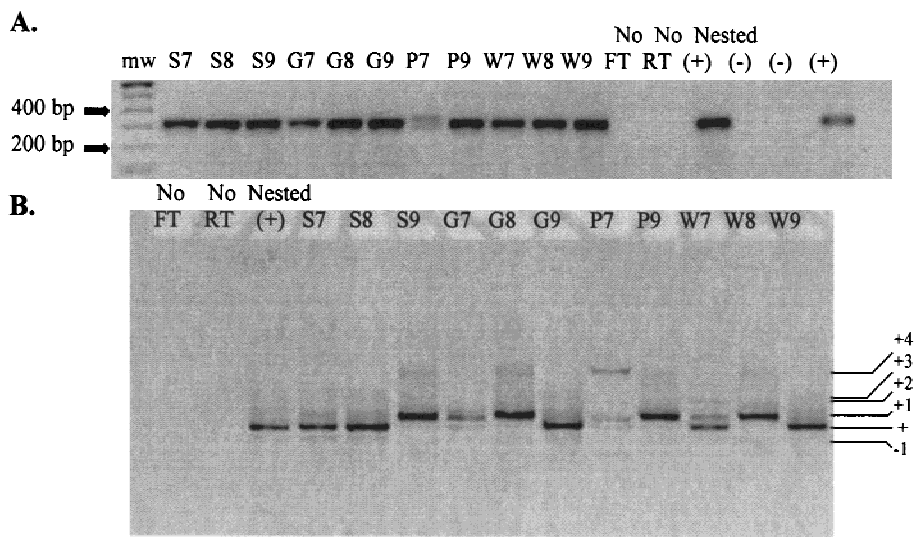

Figure 4. Migration of nested PCR products from the third set of adults. The individual mussels for each surface are labeled 7, 8, and 9. **A:** See Figure 1A. **B:** The band notation, indicated on the right, represents the migrations of the bands within this gel. See Figure 1B for further explanations.

+4 bands are present in S9 and G8; +4 is also present in P7. Two unique bands, +2 and −1, are found in the W7 and S8 samples, respectively. Again, no band is both unique to a surface and present in each individual attached to that surface.

The greatest amount of variation in each set seems to exist among the individuals. Within each surface, the bands very in number from as few as 1 to as many as 6. The + and +1 bands are the most common in each gel. Although each gel has sequences with faster and slower migrations than these bands, there is little correlation between the relative mobility of the bands and a specific surface. For instance, no band migrating slower than the +1 band was detected in G4 and G6, yet one such was found in G5. Also, no band that migrates faster than the + band was detected in G5, but one was present in G4 and G6. Other individuals seem to have a mixture; any pattern among the individuals is difficult to determine.

There are individuals, however, that have common migration patterns. In the first set, the S1 and S2 individuals

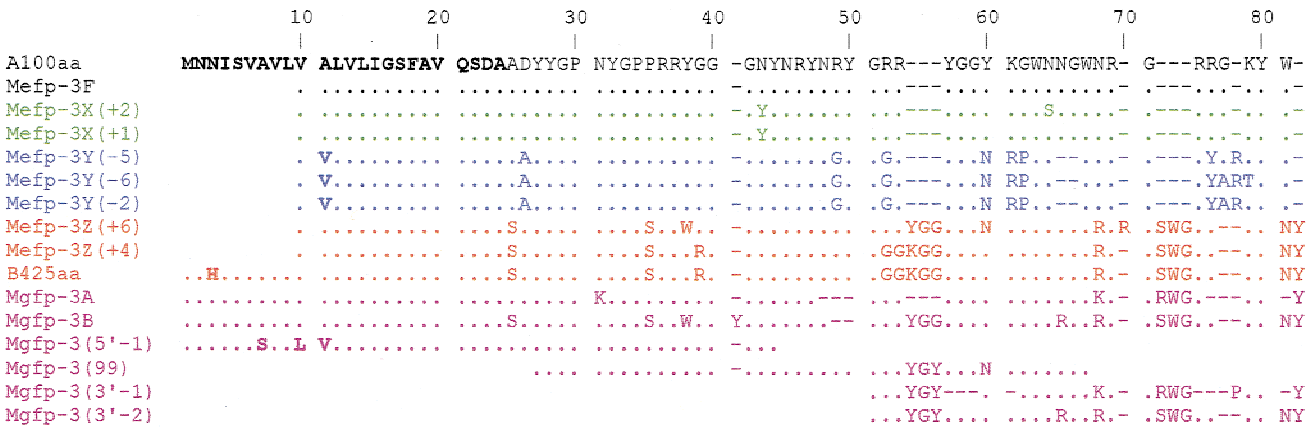

Figure 5. Protein variants translated from the cDNA sequences of the bandstabs. A100aa (from pA100), B245aa, and the Mgfp-3 sequences are included for comparison (Inoue et al., 1996; Floriolli, 1998). The boldface indicates amino acids of the signal peptide region; a dot (.) indicates an amino acid common to the A100aa sequence; a dash (-) indicates a gap introduced to preserve alignment. The colors represent a specific protein group: black, Mefp-3F; green, Mefp-3X; blue, Mefp-3Y; red, Mefp-3Z; purple, Mgfp-3.

have identical patterns, as do P2 and P3 (Figure 2B). In the second set of individuals, P5 has a pattern identical to P6; S4 and G4 also have the same pattern (Figure 3B). In the third set of individuals, there is less variation in the migration patterns than there is in the first two sets (Figure 4B). The migration patterns of S7 and G7 are identical, as are those for S9 and G8. G9 and W9 each had a single band, the + band; P9 and W8 also had a single band, the +1 band. The remaining individuals have unique patterns; however, some band patterns are often found in those of other individuals. For instance, were it not for the presence of the + band in G1, it would have a pattern identical to P1. The pattern found in G6 is present in P4 and W6; that of P5 (which is identical to P6) is found in S4, G4, and W5. G5 and P4 also have nearly identical patterns; the −3 band is present in P4 but not in G5. P7 has two of the three bands present in S9 and G8. There is no clear pattern of expression, and even though some individuals from a given surface share an identical pattern, the pattern is not consistent throughout all the individuals of the surface within each gel.

Mfp-3 Sequences from DGGE Bands

Bands of different mobility on DGGE gels suggest that cDNA sequence differences may exist among the individual mussels. In order to determine the actual differences in the protein sequences, 24 bands from the first set of individual mussels were stabbed, reamplified, and then electrophoresed on a DGGE gel to ensure the presence of a single band that could then be sequenced (Wilton et al., 1997).

Figure 4 summarizes the results of the DGGE bandstabs. After the reamplification of the 24 bands, 10 of the PCR products resulted in a single band and, thus, could be sequenced. Five of the PCR products were sequenced after a second round of bandstabbing and reamplification. One bandstab did not amplify; two of the PCR products were not pure and did not result in clean sequences. The remaining reamplified cDNA samples, when electrophoresed, resulted in two bands of faster mobility, suggesting that the original band was a heteroduplex. These heteroduplex bands included S1(+3), G1(+3), P1(+3), P2(+3), P3(+3), and W1(+1). The W1(+1) band was the only stabbed +1 band that did not result in a single sequence. A total of 15 cDNAs were obtained, and the expected protein sequences were determined (Figure 5).

The sequences can be divided into four groups, Mefp-3F, Mefp-3X, Mefp-3Y, and Mefp-3Z (Figure 5). The Mefp-3F group is so named because the deduced mature proteins of the cDNA bandstabs were identical to the original Mefp-3F protein variant (Papov et al., 1995). The Mefp-3F sequences include S1(+), S3(+), G1(+), G3(−3), and W1(−3) (Figure 4). The cDNA sequences of the positive control, pA100, and the (+) bands are identical (data not shown); this supports the suggestion of their identical migration pattern in the DGGE gel (Figures 2B, 3B, and 4B). The (−3) bands have migration patterns that differ from the (+)

bands owing to a single base change in each sequence at different positions within the 3′-untranslated region (data not shown).

The Mefp-3X group differs from the Mefp-3F by only 1 to 2 amino acid changes, and includes the S3(+2), G1(+1), P1(+1), P3(+1), and W2(+1) cDNA sequences. Only the S3(+2) sequence has a DGGE migration pattern that differs from the others of this group owing to the variations in its cDNA sequence. The group has a common amino acid variation in comparison with Mefp-3F; i.e., a tyrosine replaces an asparagine at position 43. Mefp-3X(+2) has an additional serine substitution at position 64.

The third group of sequences, Mefp-3Y, subsumes the cDNA sequences of S3(−5), G3(−6), and P2(−2). These differ from Mefp-3F by 8 to 9 amino acid substitutions. These include an alanine at amino acid position 77 in Mefp-3Y(−6) and Mefp-3Y(−2), and threonine in Mfp-3Y(−6) at amino acid position 79. All variants of Mefp-3Y have in common a proline at position 62; an alanine at position 26 is also unique to this group. The remaining substitutions and insertions are of residues commonly found among Mefp-3 variants.

Mefp-3Z differs from Mefp-3F by 12 to 15 amino acid substitutions. The group most resembles B425, a previously sequenced cDNA (Floriolli, 1998) as well as the cDNAs for Mgfp-3 reported by Inoue et al. (1996). G1(+4) and B425 are identical except for a single base deletion in the 3′-untranslated region (data not shown). The second sequence of this group, 3S(+6), translates into three unique amino acid substitutions found at positions 38, 54, and 70. This protein sequence also differs from Mefp-3Z(+4) at amino acids 39, 52, 53, and 60. Of these four differences, three are identical to the Mefp-3F sequence, and the fourth, at position 60, is common to the Mefp-3Y group.

The deduced Mfp-3 protein sequences show no clear correlation with the type of surface (Table 2; Figure 4). Four of the eight protein sequences were found in one individual mussel, S3; three sequences were present in G1. Each group of sequences was expressed in individuals from at least two surfaces; Mfp-3X and Mfp-3Y were expressed in individuals placed on each of the three test surfaces. Because the (−3) cDNA bands each translated into Mefp-3F, it is likely that the (−3) bands present in P1 and W1 also translate into Mfp-3F (Figure 2B; Table 2). The Mfp-3Z(+4) sequence may be expressed in P1 because this individual had a cDNA band with the same migration. Mfp-3Z(+6) and Mfp-3Y(−2) may also be expressed by individuals collected from the docks. Although the W1(+1) had a migration identical to that of the cDNA of Mfp-3Y(+1), W1(+1) was a heteroduplex; only sequencing can actually distinguish such ambiguities. The three sequences of Mfp-3Y were from bands unique to a single individual from different test surfaces.

Table 2. Primers Used in the RT-PCR and Sequencing Reactions

Primer	Sequence
Poly (T-GA)	GAGAGAGAGAGACTCGAG(T_{18})
GCsigpep	CGCCCGCCGCGCCCCTATCAGTGTCGCAGTGTTG
Mefp-3.3 UCA	CCACCACACATTAAACTTTATTATATATC
Euk A	AACCTGGTTGATCCTGCCAGT
Euk B	GATCC(A/T)TCTGCAGGTTCACCTAC

Discussion

Mfp-3 Variants and Surface Types

This study supports the conclusion that Mfp-3 variants are consistently detected by MALDI-TOF-MS at or near the plaque-substratum interface, and that Mfp-3 variants do not appear to be correlated with surface type. This point is particularly important since previous results based on limited data and random sampling (Ross et al., 1995; Warner and Waite, 1999) suggested that mussels might tailor their adhesion to different surfaces by selecting a different suite of Mfp-3 variants for each distinct surface. By tracking the attachment of individual mussels on steel, glass, and plastic substrates, we found that each mussel appeared to have a unique Mfp-3 "footprint" that persists regardless of the substratum. We remain puzzled by why the variants in these outprints are a small subset of the more than 20 possible for an individual mussel (Warner and Waite, 1999). Perhaps this is a problem of resolution or relative detection limits.

Transcription of Mfp-3 is not likely to be surface-induced either. Analysis of Mfp-3 cDNAs among each set of surface-adapted mussels by DGGE shows no clear correlation between surface type and transcription. There were samples such as S-1 and S-2 with cDNA sequences of common mobility; however, these similarities were not consistent for all individuals placed on identical surfaces. Moreover, cDNAs with identical mobilities did not always have the same sequence. It could be argued that because marine

surfaces are likely to be patchy rather than clean (Dobretsov and Railkin, 1996; Wimpenny, 1996), some variation in transcription patterns might be expected. However, variation in Mfp-3 deposition onto several surfaces was not evident by MALDI-TOF within the same individual. The nature of surface patchiness and the extent to which it can be removed by mussels remain controversial (Waite, 1992). Although unlikely to put the matter of surface-induced adhesion to rest, the present results suggest that individual mussels have only one formula for sticking, and this is generally applied to all surfaces. Differences between individuals most likely reflect extent of posttranslational hydroxylation and, perhaps, multiple alleles.

Mfp-3 Sequence Variation

Despite the lack of correlation between Mfp-3 expression and surface type, we sequenced some of the putative Mefp-3 variants from bandstabs of DGGE gels. Eight Mefp-3 sequences, including Mefp-3F, were identified by cDNA analysis. This brings to 16 the number of distinct mature protein Mefp-3 variants detected in *M. edulis.* The number is certainly larger if variations in the signal sequence are included. The deduced variants have several characteristics in common. Each is rich in glycine (24–28 mole %), tyrosine (18–23 mole %), arginine (17–20 mole %), and asparagine (10–15 mole %). Tryptophan and proline (3–4 and 2–4 residues per sequence, respectively) are also consistently present in each Mefp-3 variant.

The amino-termini of the Mefp-3 family are the most conserved. Amino acid sequences that do not contain substitutions include YYGPNYGP, RR, GG, YNRYN, and YG (Figure 4). Not surprisingly, these regions are also highly conserved within the Mgfp-3 sequences (Inoue et al., 1996). The carboxyl-termini have a higher degree of variability. Most of the amino acid substitutions and insertions are of residues common to the family, such as tyrosine, glycine, asparagine, and arginine. One exception is the occurrence of threonine in Mefp-3Y(−6). The fact that threonine was detected in a single HPLC fraction of purified Mefp-3 suggests that this residue is rarely present in the protein family (Papov et al., 1995). Threonine replaces the single lysine in the sequence of Mefp-3Y(−6). This is intriguing because lysine is rarely if ever substituted in other Mfps (Laursen, 1992; Inoue et al., 1995).

Because of the high glycine content, all Mfp-3s are likely to be flexible proteins with open conformations (Chou and Fasman, 1978). These structures would enable DOPA residues, in particular, to come into contact with other proteins and surfaces. A recent study has reported that the adhesive strength of biomimetic adhesive polypeptides in vitro is directly related to DOPA content (Yu et al., 1999).

The molecular mechanism for generating varied Mfp-3 sequences is unclear at this time. Given that nothing is known about the genomic sequence of Mfp-3, the field of possibilities is simply too broad to entertain reasonable speculation at present.

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