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DIRECT OBSERVATION BY SCANNING TUNNELING MICROSCOPY OF THE TWO-DIMENSIONAL LATTICE STRUCTURE OF THE S-LAYER SHEATH OF THE ARCHAEOBACTERIUM *Methanospirillum hungatei* GP1

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Abstract

Observation of the two-dimensional (2-D) 3 nm x 3 nm lattice structure of the S-layer sheath of the archaeobacterium *Methanospirillum hungatei* is reported for the first time by scanning tunneling microscopy. The samples consisted of sheath fragments deposited on a $TaSe_2$ substrate and coated with a Pt/Ir film. In addition to confirming the 2-D structure, the images reveal some new information about the nano-scale details of the sheath structure. A lateral resolution of 1 nm was achieved, suggesting that the grain size of the Pt/Ir films was much less than for similar films deposited on a smooth metal surface.

Key Words: Scanning tunneling microscopy, two-dimensional lattice, S-layer, sheath, archaeobacterium, *Methanospirillum*.

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Introduction

The cell envelope of the archaeobacterium *Methanospirillum hungatei* (Mh) includes a cylindrical sheath, or S-layer, formed from stacked hoops, Figure 1. Diffraction studies by Stewart *et al.* (1985) suggest that the sheath consists of a paracrystalline structure with P2 symmetry and a 2.8 nm x 5.7 nm unit cell. The unit cell contains two nearly identical lobes, which means that transmission electron microscopy (TEM) and scanning tunneling microscopy (STM) images of Mh sheath are expected to show a nearly square 3 nm x 3 nm lattice of lobes. The sheath of Mh is unique among bacterial layers, due to the smallness of the lattice constant. Hexagonal (P6) symmetry, and lattice constants of 10-20 nm are more typical of bacterial S-layers and other biological membranes. Such lattices have been observed in STM studies on several different biological materials, for example: T4 polyheads by Amrein *et al.* (1989, 1991); HPI layer by Wepf *et al.* (1991); the end plugs of Mh reported by Southam *et al.* (1993), and in more detail by Xu *et al.* (1995). Resolution of the smaller two-dimensional (2-D) lattice in Mh sheath is a considerably greater challenge for STM.

Previous STM studies of Mh sheath reported by Blackford *et al.* (1989) and Beveridge *et al.* (1990) showed linear corrugations running perpendicular to the cylinder axis, corresponding to the stacked-hoop structure. The hoop widths were multiples (4-7 typically) of the expected 3 nm subunit, with deep grooves marking the boundaries between hoops (Southam *et al.*, 1993). The surface of each hoop showed a complicated structure of 3 nm repeats but the evidence for the 2-D crystal structure was not totally convincing. Some doubts remained as to the origin of the observed 3 nm repeat structure. Possible explanations were: (**1**) the 2-D crystalline structure exists only below the surface of the sheath, where it would be sampled by diffraction but not by STM, whereas the periodic pattern on the surface is obscured by an intrinsic amorphous layer, or by contamination and/or distortion effects due to air-drying of the sheath; (**2**) the observed 3 nm structure could be due to

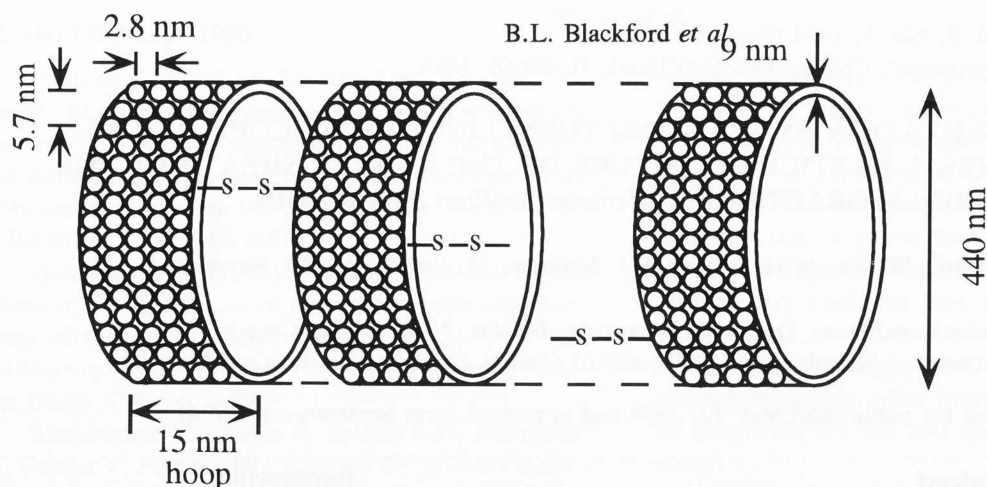


Figure 1. Drawing to show the shape and expected crystalline structure of the cylindrical sheath. The unit cell is 2.8 nm x 5.7 nm and contains two nearly identical lobes. The sheath collapses flat on the substrate for STM studies.

the grains of the metal film used to coat the sheath for STM imaging, since similar films deposited on smooth metal substrates have grain sizes of 3 nm; (3) tip-sample convolution artifacts resulting from non-ideal tunneling tips could complicate the STM images, especially since the hoop surfaces are not atomically flat (Blackford *et al.*, 1991).

We now report STM images taken on sheath fragments which had regions where a 2-D crystalline structure of 3 nm x 3 nm was clearly revealed. In these regions the usual, less regular, surface layer of a few nm thickness was missing, perhaps due to the physical agitation of the fragmentation process. Although this supports explanation (1) given above, since improved STM tips were used, explanation (3) could also be important. A resolution of 1 nm was achieved indicating that film graininess was very small for the Pt/Ir films used, even though they were vacuum deposited at room temperature from heated tungsten filaments without any special procedures.

Experimental Procedure

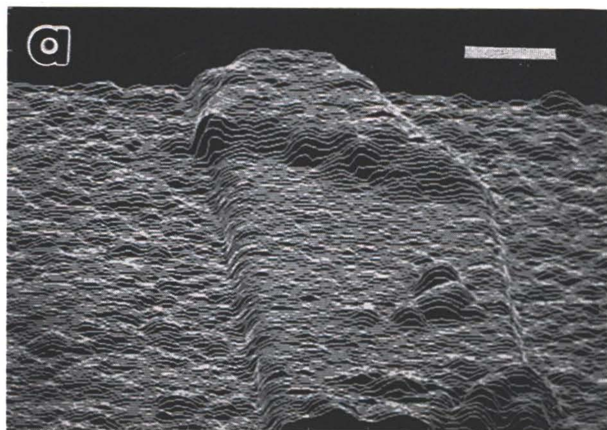
The Mh bacterium was prepared as described previously by Southam *et al.* (1993) and sheath fragments obtained by the French press method. A 2 μl drop of sheath solution in H_2O (concentration 0.1 mg/ml) was spread on a freshly cleaved $TaSe_2$ substrate and allowed to dry in air. The sample was then placed in a vacuum chamber for coating by a Pt/Ir film. The evaporation source was prepared by wrapping a 2 cm length of 0.2 mm diameter Pt/Ir (80/20) wire around a straight, triple-stranded tungsten filament made of 0.5 mm diameter wire strands. The 2 nm thick film was evaporated from the tungsten filament by Joule heating in a background pressure of 10^{-5} torr. The deposition rate was 0.1 nm/sec determined by a quartz-crystal film thickness monitor, previously calibrated by an optical interference method. The composition of the film was not determined, and could be a mixture of Pt, Ir and W in an unknown ratio.

The STM used was of the piezo-bimorph tripod design (Blackford *et al.*, 1987) housed in a dry chamber at atmospheric pressure. The tungsten tips used were etched (dc) in NaOH following the procedure described by Hacker *et al.* (1992). TEM images of these tips showed them to be sharper (radius 8 nm) than the commercially prepared tips which we used previously (Blackford *et al.*, 1991). The imaging was done in the constant current mode at a tip voltage of 0.6 volts, a tip current of 0.1 nA, and at scanning speeds of 100 to 1000 nm/sec. A typical image consisted of 128 scan lines with 256 points per line.

Results and Discussion

Figure 2a shows a low resolution, raw data image of a 300 nm wide x 600 nm long x 9 nm thick sheath fragment. The sheath is folded over on itself in the top portion of the figure. The high resolution images presented in Figures 3 to 6 below were taken on top of this fragment in the smoothest region just below the fold, corresponding to the bottom third of Figure 2b which is a magnified view of the sheath plotted by the slope shading technique. It is not known for certain whether this surface corresponds to the inner or outer surface of a complete sheath (Beveridge *et al.*, 1990), but it is thought to be an inner surface as discussed below. Note that the surface of the folded portion is considerably rougher, similar to the background roughness.

Figure 3 is a high resolution line scan image viewed at 5° to the normal and slope shaded to accentuate the fine structure. It clearly shows corrugations, separated by 3 nm, running upwards to the left and having lobes spaced at 3 nm intervals along the corrugations. We interpret this as direct evidence for the 3 nm x 3 nm lobe structure expected from diffraction studies. Note that the corrugations in Figure 3 run parallel to the fold in Figure 2, which we assume to be parallel to the axis of the sheath. The corrugations are 0.1 nm in height, which may be too small due to the tip radius being larger than the 3 nm spacing, a common problem with scanning probe microscopes.



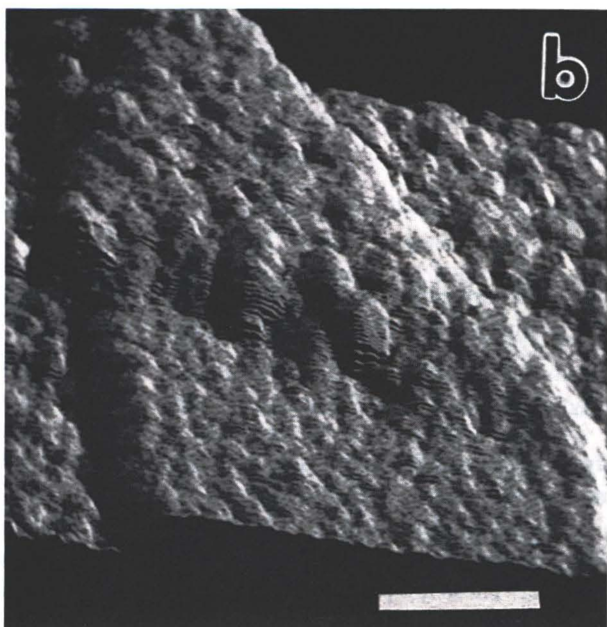
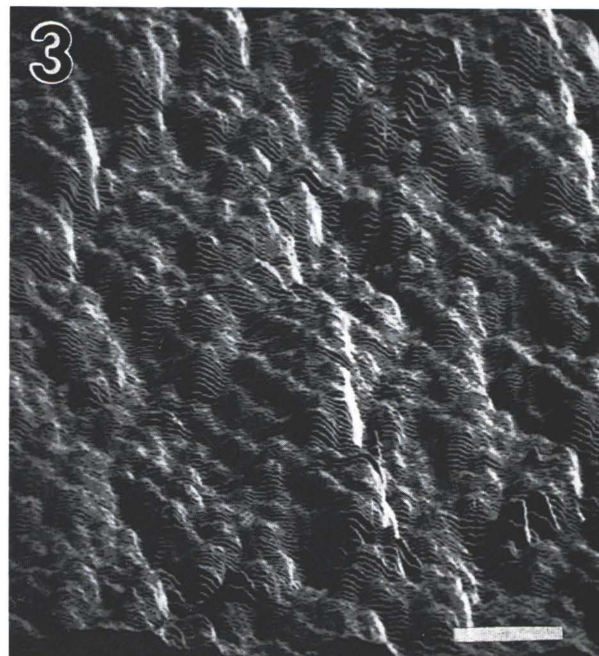


Figure 2. (a) A raw data line scan STM image of a Pt/Ir coated Mh sheath fragment. Bar: x,y: 100 nm; z: 30 nm. (b) Another scan, at higher resolution, displayed with slope shading to enhance the surface detail. Bar: x,y: 100 nm; z: 20 nm.

Figure 4 is a higher resolution image of a different area than Figure 3, which shows again the 3 nm x 3 nm lobe structure. It also shows that the corrugations are not continuous but are interrupted by a band structure running perpendicular to them, upwards to the right. We interpret these bands as due to the hoop structure of the sheath, Figure 1. The figures show that the widths of these hoops are such as to contain 3 to 5 of the 3 nm lobes, which is consistent with previous STM studies. Figure 4 also shows that the hoops are slightly tilted giving the appearance of a shingled, or fish-scale, surface



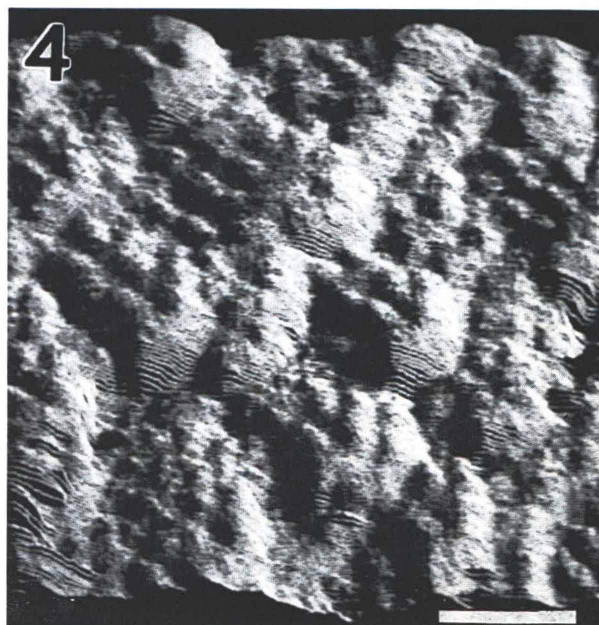


Figure 3. High resolution, slope shaded line scan image showing the 2-dimensional 3 nm x 3 nm lattice structure. Bar: x,y: 10 nm; z: 1 nm. The corrugation of the lattice structures is 0.1 nm.

Figure 4. Higher resolution image showing the 3 nm x 3 nm lattices, and also showing the hoop structure consisting of 10-15 nm wide bands running up to the right. Bar: x,y: 10 nm; z: 1 nm.

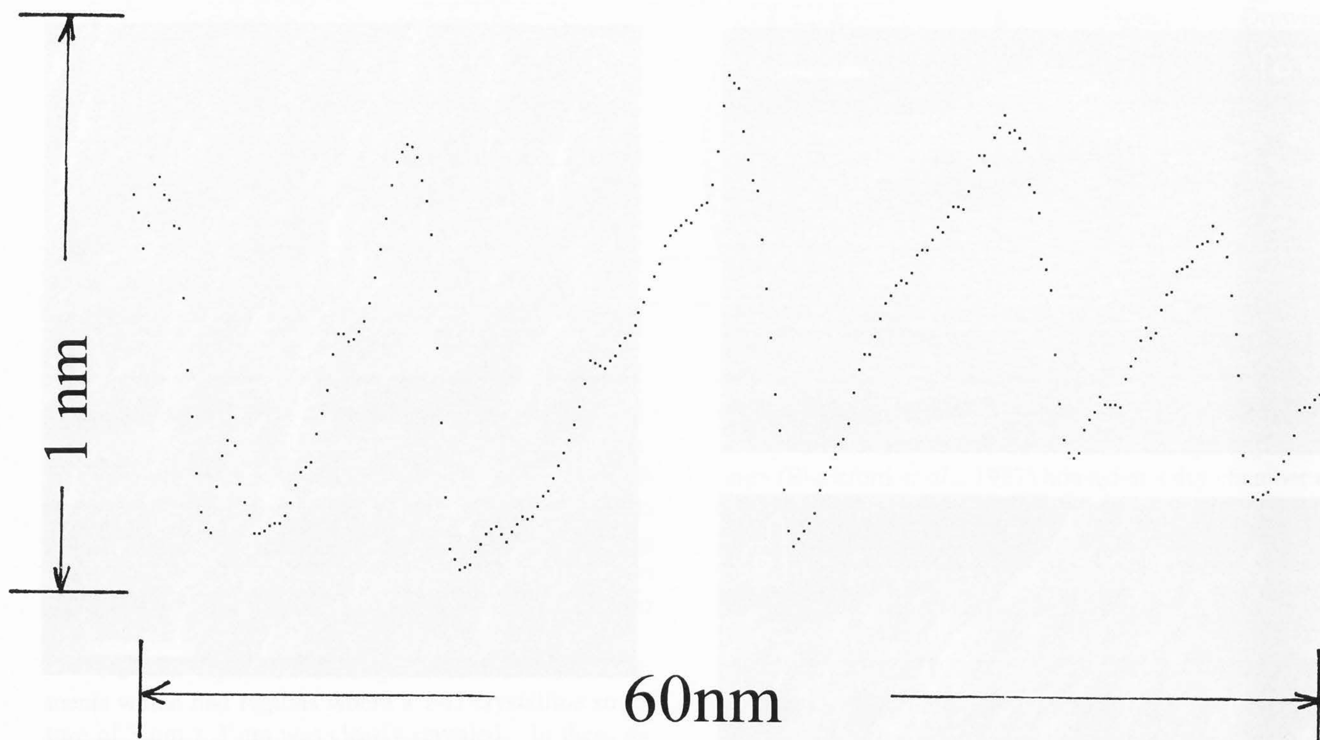


Figure 5. Cross-section running parallel to the 3 nm corrugations, and perpendicular to the hoop bands, in Figures 3 and 4. The figure shows the hoop bands to be slightly tilted giving a shingled, or fish-scale, appearance to the surface. Note the greatly exaggerated vertical scale.

with an amorphous material (Southam *et al.*, 1993) at the boundary between hoops. This is shown more clearly in the line scan of Figure 5, running parallel to the 3 nm corrugations. This is a newly discovered feature of the sheath, which could have implications for the bonding between hoops. Or, it may simply be an artifact of the drying process; further investigation is needed.

Figure 6 is a top view of yet another area, showing more clearly that the 3 nm corrugations running up to the left are continuous only across the width of a hoop, in most cases. This observation is consistent with, and gives some support to, the hoop model proposed by Stewart *et al.* (1985).

The absence of deep grooves between the hoops in Figures 2 to 6 suggests that it is the inner sheath surface which is being imaged, since deeper grooves are often seen in images of the outer sheath surface, as shown by Southam *et al.* (1993).

Figure 7 gives further evidence for the existence of the 2-D lattice structure. It is a high resolution image taken on a different sheath fragment from that in Figure 2, and is probably an outer sheath surface because of the relatively deep grooves between the hoops. In some areas, indicated by the white arrowheads, a 3 nm x 3 nm pattern is clearly visible. The usual amorphous layer, such as in the lower right of Figure 7, is missing in the areas where the 2-D pattern is visible.

Our claimed resolution of 1 nm is based on the appearance in the images of numerous structures of about this size. These are particularly noticeable in the bottom left of Figure 4, and can also be seen in Figure 3.

Thus, the STM has yielded the following possible new insights about the nano-scale details of Mh sheath which could not be obtained from TEM or diffraction: (1) the existence of an amorphous layer on the sheath surface which often obscures the 2-D lattice to STM; (2) the fish-scale-like arrangement of the hoops; and (3) the lack of continuity of the 3 nm corrugations from one hoop to the next, in many instances.

Summary and Conclusions

The two-dimensional 3 nm x 3 nm crystal structure of the sheath of *Methanospirillum hungatei* has been directly observed by STM for the first time. A resolution of 1 nm was achieved, indicating that the grains of the Pt/Ir coating film were much less than the 3 nm expected for similar films deposited on a smooth, hard surface. This suggests that the metal atoms are less mobile on the soft biological structures, thereby inhibiting grain growth. Since the films were deposited at room temper-

ature without any special procedures we conclude that the elaborate film procedures reported in the literature, (Amrein *et al.*, 1989, 1991; Wepf *et al.*, 1991), are not always necessary to achieve 1 nm resolution on biological materials with STM. Also, we are now more confident that the 3 nm substructures seen in previous STM studies on Mh sheath, and end plugs, are probably real and not an artifact of the coating film.

We conclude that STM imaging of coated biological membranes at the 1 nm resolution obtained in this study can give useful 2-D and 3-D structural information which is difficult to obtain otherwise.

Acknowledgements

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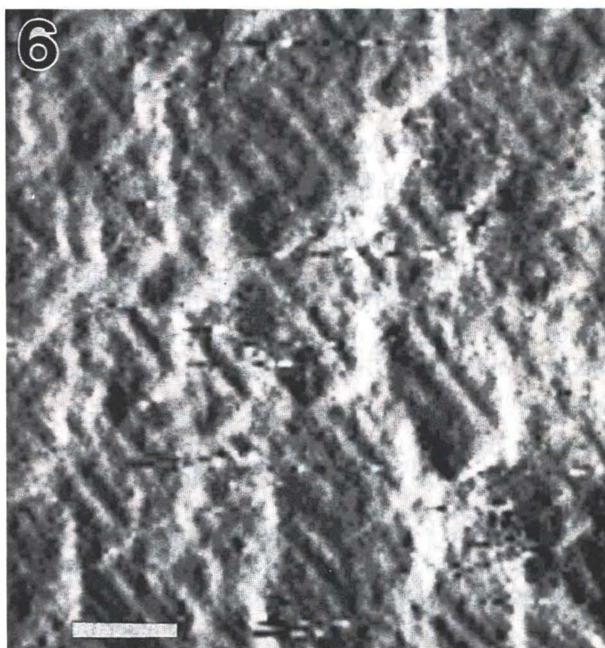

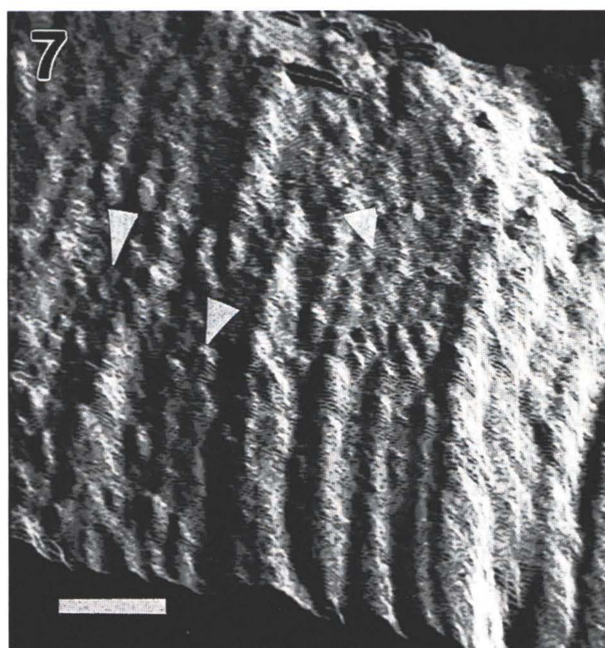

Figure 6. Top view showing that the 3 nm corrugations are continuous across a hoop, but are generally not continuous over long distances. Slope shaded. Bar: x,y: 10 nm.

Figure 7. STM image from another sheath fragment showing further evidence for the 3 nm x 3 nm lattice structure, which is exposed in several locations shown by the arrows. Probably the *outer* surface of sheath. Bar: x,y: 10 nm; z: 7 nm.

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Discussion with Reviewers

E. Weilandt: I am still wondering about the background roughness of the substrate as visible in Figure 2. Normally one chooses very smooth substrates to avoid artifacts. As you say in the text, it is rougher than the smooth regions on the sheath. What do you think is causing this large corrugation? In your opinion, what is the reason you get a much better resolution on the sheath than on the substrate?

Authors: The $TaSe_2$ substrates used are atomically flat when imaged bare. We think that the background roughness comes from tiny fragments of the bacterium which are always present in the solutions. For some reason, which we do not understand, these tend to attach preferentially to the substrate and not to the sheath. We have always found this in our STM studies on this bacterium and have mentioned it in previous publications (for example, Blackford *et al.* 1989, 1991).

E. Weilandt. In my opinion, the small corrugation of the lobes could also be due to the metal coating of the sample. It is difficult to understand how you could resolve a 3 nm structure when there is a 2 nm thick coating on the sample. How can you be sure that you are not mainly observing the grain structure of the Pt/Ir film?

Authors: We do not see why a 2 nm thick coating should necessarily obscure a 3 nm (2-D) periodicity of the underlying surface. It is even possible that decoration effects occur with the grains growing at preferred sites, thus emphasizing the 2-D structure. However, this does not mean that the 2-D structure is due to the coating, but is perhaps amplified by it. Also the 2-D structure is not seen in areas where the amorphous layer exists, as shown in Figure 7. If it was mainly due to the coating, it should be seen everywhere.

R. Guckenberger. What is the reasoning behind the value of 1 nm quoted for the achieved resolution? You have seen a periodicity of 3 nm, thus you can quote a resolution of 3 nm.

Authors: As stated in the text, the reason for the quoted 1 nm resolution is the appearance in the images of numerous structures of about this size.

M. Amrein: It is widely believed (and proven in many cases) that air-drying a protein sample causes strong structural alterations?

Authors: We agree that this could be a factor, as mentioned in the text. However, the protein sheath we studied is particularly tough, as found by previous scanning electron microscopy (SEM) studies. Also the recent scanning force microscopy (SFM) studies of Xu *et al.* (this issue) measured the Young's modulus of the sheath to be about 20% that of steel. We believe therefore that such structural alterations should be minimal in this case.

M. Amrein: From evaporation sources composed of 80% Pt and 20% Ir, almost solely Pt evaporates until the ratio has become reversed. From our experience, the resulting films behave just like pure Pt films (and are pure Pt films). Do the authors have results that prove otherwise?

Authors: We did not measure the composition of our films. We agree that they could be mostly Pt, or a mixture of Pt and Ir, and possibly W from the filament. A knowledge of the film composition would be nice to have, but is not necessary.