PNAS LETTER

Reply to Bharat et al.: Continuity or discontinuity, that is the question

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Bharat et al. letter (1) is an opportunity to highlight the complementarity between their findings on HPI patches (2), the outermost part of *Deinococcus radiodurans'* cell envelope (CE), and our works on intact CE patches (3, 4). CE patches have three main multi-subunit complexes (3; Fig. 1*A*), with the subunit SlpA (S-layer protein A) being the sole carrier of the S-Layer Homology domain (3–5). The SLH domain in S-layer proteins, while not always present, greatly impacts their identification and homology (6, 7).

Basis of Discussion

The work (3) focuses on two main CE complexes, Type-IV-Piliation-like-system (T4P-like) and S-layer-Deinoxanthin-Binding-Complex (SDBC), and not on HPI (3–5, 8). Our electron crystallography (cryo-EC) on intact CE patches glimpsed a surface component with p6-symmetry of which a third Radial-Dimeric (RD) complex (likely including HPI) may be part (3). We have not emphasized this aspect to avoid early speculation.

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The authors declare no competing interest.

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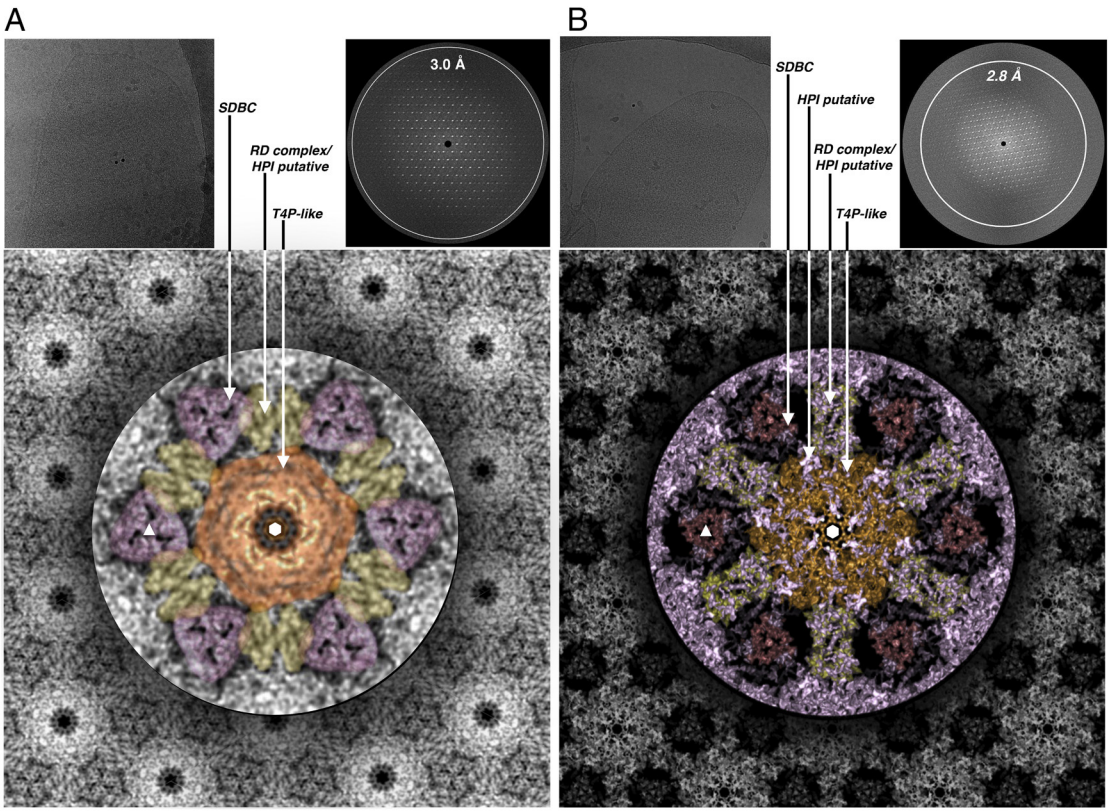

Fig. 1. cryo-electron crystallography of intact cell envelope patches and related maps. In (*A*), the image shows a cryo-EM micrograph of an intact cell envelope patch with the typical patterned regularity (*Top-Left*). On the *Top-Right*, the Fourier transform (FT) analysis shows Fourier coefficients beyond 3.0 Å resolution confirming the regular repetition of the cell envelope complexes. On the *Bottom*, the resulting 2D-projection map shows an overview (grey scale) and a magnified view (colored) of the proteinaceous functional/structural unit: a T4P-like (orange) is surrounded by 6 SDBC (pink) and 6 RDs (yellow). In (*B*), the image shows a micrograph (*Top-Left*) of an intact cell envelope patch (implemented with respect to *A*) showing Fourier coefficients beyond 2.8 Å resolution (*Top-Right*). On the *Bottom*, a top view of the resulting 3D map shows an overview (grey scale) and a magnified view (colored) of the different complexes: a T4P-like (orange) is surrounded by 6 SDBC (dark pink) and 6 RDs (yellow). The upper proteinaceous net (light pink) is also shown and its interaction with the T4P-like's top and the RD is glimpsed. This net as well as the RD are hypothesized to be HPI (3), in agreement with ref. 2. See refs. 3 and 8 for more details on the analyses here reported. The hexagon and triangle indicate the local p6- and p3-symmetry axes, respectively.

The milestone studies cited by ref. 1 focused on the S-layer while ours on intact CE patches (3; Fig. 2). Those authors solubilize proteins with 2% SDS, sometimes even at high temperatures, both standards for protein denaturation rather than close-to-native isolation. None of those works report methods to exclude denaturation/refolding/reassembling which, for S-layer proteins, can lead to artifacts (9, 10). Importantly, none of those works, including ref. 2, present a biochemical characterization or use an inclusive identification method (mass spectrometry). Not least, some of those works, including ref. 2, don't use the *D. radiodurans* R1 strain, which makes a difference for S-layer studies (e.g., ref. 11). All our works are performed on the R1 strain.

Is HPI the Sole Component of this S-Layer?

As in ref. 3, we would like to emphasize that S-layer boundaries are defined by continuity between different complexes/layers, rather than discontinuity. SDBC (3, 5, 8) and T4P-like (3, 8) provide continuity in the HPI p3- and p6-symmetry axes, respectively (Fig. 1). This continuous regularity pervades the CE layers for 35 nm, as indicated by cryo-EC FT-analyses and 3D map (3, 8; Fig. 1*B*). Our interpretation aligns with the extensive characterization of these samples (3–5, 8; Fig. 2) and also fits with ref. (2) suggesting that under the HPI layer, there are other complexes aligned at the main centers of symmetry (Fig. 1).

SlpA Stoichiometry, Function, and Deletion

The much higher expression of SlpA, the main SDBC component, compared to HPI has been extensively investigated (3–5, 7, 8; Fig. 2). In contrast, refs. 1 and 2 offer limited evidence on the S-layer extension to the underlying CE layer(s) and insufficiently justify the exclusion of SlpA, typically linked to the S-layer.

Biochemical data and cryo-EC maps strongly support our findings (3–5, 7, 8). Previous works on the SlpA-deletion mutant showed the compromised CE/S-layer integrity (7; Fig. 2*D*), providing evidence for a tethering function. This phenomenon is not observed in ref. 2, which fails to provide an explanation. For isotropic reasons, a model with a regularly repeated SDBC (3) is more plausible for tethering.

In summary, these results mutually reinforce each other. Upon clarifying strain comparability and some minor issues, the presence of HPI on the cell envelope's surface, forming a seamless paracrystalline structure extending into the underlying layers, aligns perfectly with our observations.

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References	Characterization	Gene	Complex	Name
3,4,5,8	1-10	DR_2577	SDBC	SlpA
3,4,8	1-3,5,6,8	DR_2508	RD	HPI
3,4,8	1-8	DR_0774	T4P-like	PilQ

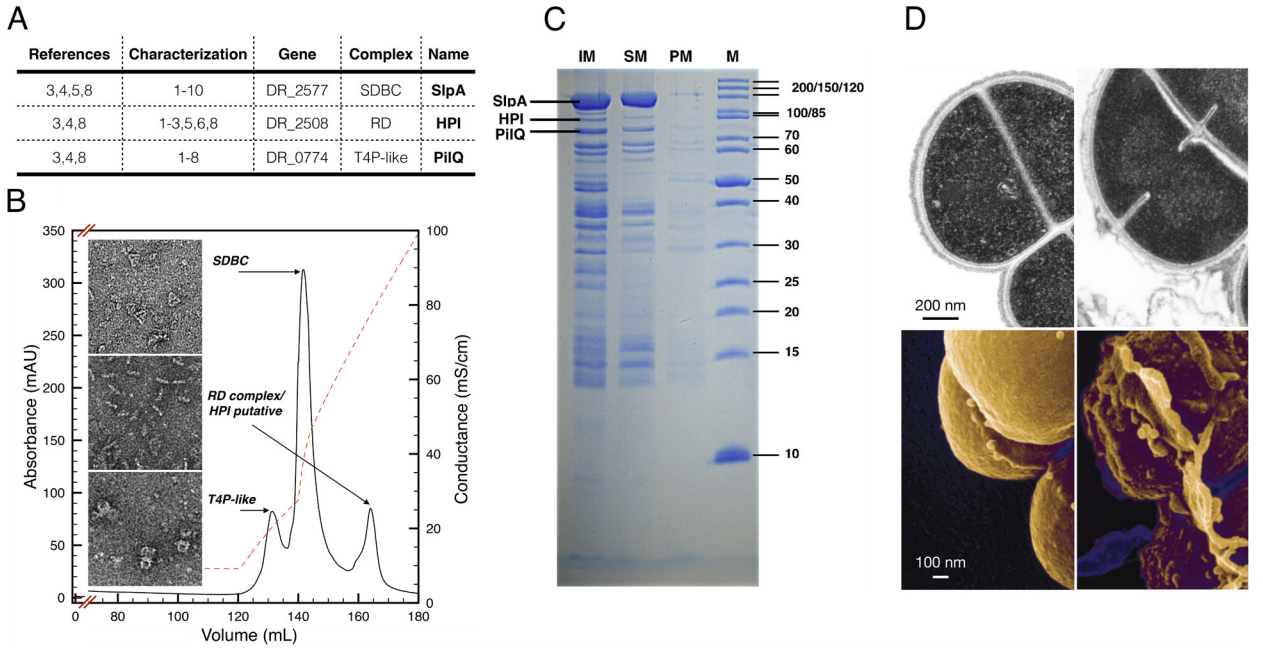

Fig. 2. Characterization of the cell envelope complexes. In (*A*), the table summarizes information and characterization of the main subunits for each of the three main cell envelope complexes: SlpA for the SDBC, PilQ for the T4P-like, and the HPI for RD. The characterization of the three complexes isolated from intact cell envelope patches includes: 1) denaturing electrophoresis; 2) native electrophoresis; 3) ion-exchange chromatography; 4) size exclusion chromatography; 5) mass spectrometry (MS) on native gels; 6) MS on denaturing gels; 7) MS on liquid samples obtained by chromatography; 8) electron microscopy (micrographs); 9) electron microscopy single-particle analysis (cryo-EM); and 10) functional assays. For each complex, the main references are cited. In (*B*), the image shows a chromatogram (anionic exchange) where the solubilized cell envelope patches have been resolved into three main peaks, each one corresponds to the three-cell envelope complexes as shown by the micrographs in the insets. The red dashed line is the conductance, the black-continuous line is the absorbance at 280 nm. In (*C*), the image shows denaturing electrophoresis of the intact (lane IM), solubilized (lane SM), and not-solubilized fraction after mild detergent treatment (lane PM) of the isolated cell envelope patches. The lane M is the molecular marker. The indicated bands of SlpA, HPI, and PilQ have been identified by MS. In (*D*), the image shows thin sections micrographs (*Top*) and scanning electron micrographs (*Bottom*) of *D. radiodurans* R1 wild-type (*Left*) and the SlpA-deletion mutant (*Right*) as presented in ref. 7. For more details on the analyses here reported, please see refs. 3–5, 7, and 8.

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