Supporting information for

"Structural characterization and functional insights into the Type II Secretion System of the poly-extremophile Deinococcus radiodurans"

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Supporting information reported:

- Supporting text about stress assays and molecular dynamics
- Supporting text about cryo-EM methods
- Supporting figures' legends
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- **Supporting text about stress assays and molecular dynamics**

The differences assessed under stress conditions therefore suggested a loss of the cell envelope integrity in the deletion mutant likely due to the T2SS absence as a mechanical tether spanning the periplasm/peptidoglycan and behaving as a firm spacer between OM and IM. Consequently, the number of proteins released in the media after growth was also assessed and found to be increased of ~6 times in the deletion mutant, supporting the loss-integrity hypothesis (Table 1). Electromagnetic stress in the UVC was also investigated finding in both cases a higher sensitivity of the deletion mutant (Table 1).

For what concern the all-atom molecular dynamics simulations, the GspDPT/LPS system showed a stability over 300 ns with the Root-mean-square deviation (RMSD) after 100 ns reaching a plateau below 6.5 Å while the GspDPT/POPC system was stable over 60 ns (Fig. S7). Time-resolved molecular simulations on the former using the centers of mass for the different components showed stability over the simulation length. After insertion into the membrane systems, the stable complex places the GspP subunit in the periplasm, while the membrane envelops the GspT base, confirming the structural findings (Fig S2c). Conductance and ion selectivity, particularly in the presence of 1M KCl, were assessed in the GspDPT/POPC system. Under a constant electric field (1 V to -1 V), the system shows conductance exceeding 11 nS with a K^+/Cl^- ratio between ~1.3 (at V>0) and ~1 (at V<0) (Table S1), indicating slightly cation selectivity for the GspDPT/POPC system. Furthermore, simulations at 1V performed on the GspDPT/LPS system under physiological condition (0.15 M KCl) show no selectivity for cations over anions.

After stability assessment and ion transport analysis, the GspDPT/POPC system was further evaluated for the threading of ssDNA or dsDNA 24-mers (Tables 1 and S2), which were initially positioned as reported in Sup. Fig. 2c. In this system, at 1 V, ssDNA and dsDNA uptake threading took 60 and 40 ns, respectively (Movies S2 and S3). Absence of applied constraints on the DNA structure resulted in ssDNA's higher flexibility, explaining its lengthier threading time. Next, we tested the dsDNA ejection threading (Fig S2c) which, under 1V, took 20 ns (Movie S4), with the faster ejection time likely related to the forces applied. Interestingly, upon application of -1 V, the system is not ion-selective (Table S1), thus the electro-osmotic force is negligible; on the other hand, during DNA uptake the slightly cation selectivity at 1 V (K+/Cl- =1.3) produces an electro-osmotic force opposite to the electrophoretic force (29). Further simulations will be required to confirm this hypothesis. Finally, simulations showed gate stability during DNA passage, even for the dsDNA. In fact, its dimension, ~20 Å diameter, fits with the stable T2SS periplasmic gate (~26 Å) which is larger than nanopores used for ssDNA sequencing (around 10 Å), like alpha-hemolysin, alpha-aerolysin, or CsgG (30).

- **Supporting text about cryo-EM methods**

Cryo-electron microscopy data acquisition

The whole grids preparation and data acquisition was done at CEITEC (Brno, Czech Republic) according to (3). Either purified T2SS sample (5 mg/mL) or cell envelope fragments were applied to glow-discharged TEM grids (Protochips, Cu, 300 mesh, R1.2/1.3) and vitrified into liquid ethane using ThermoScientific Vitrobot Mark IV (4°C, 100% rel. humidity, 30 s waiting time, 3 s blotting time). The grids were subsequently mounted into the Autogrid cartridges and loaded to a FEI Talos Arctica (ThermoScientific) transmission electron microscope for screening prior to data acquisition. The data were collected on a FEI Titan Krios operated at 300 kV using the SerialEM software (60) and the K3 direct electron detection camera (Gatan) positioned behind the Gatan Imaging Filter. The microscope was aligned for fringe-free imaging. Single-particle data were acquired at a pixel size of 0.833 Å/px (nominal magnification of 105000×). The energy selecting slit was set to 10 eV. The data from a 5.0 s exposure were saved into 40 frames containing an overall dose of 50 $e^-/Å^2$. The dataset comprised 35000 micrographs in total. The image defocus was set to vary between −0.8 µm and −2.6 µm.

Tomograms were acquired in movie mode (each tilting angle has a movie of eight frames) with a K2 direct electron detection camera (Gatan) positioned behind the Gatan Imaging Filter. CTF (Contrast Transfer Function) and ice quality were inspected in each acquired tomogram. The data set comprised 80 tomograms. Data acquisition was performed at a pixel size of 2.28 Å/px (nominal magnification of 21000x) with a dose-symmetric tilt scheme with a 3° increment (0, +3°, -3°, etc.; tilting range ± 60°), a total dose of 80 $e^-/Å^2$, and a defocus set to vary between 2 µm and 5 µm.

Single-particle data processing

The data processing for single-particle analysis was done using cryoSPARC (42) according to (3). Briefly, the movies were first corrected for the drift during data acquisition using the program MotionCor2 (61) and subsequently the CTF parameters were estimated using GCTF (62). The images with astigmatism larger than 2500 Å and estimated resolution worse than 4.0 Å, based on the CTF fit analysis, were excluded from subsequent data analysis. A set of 20 micrographs was randomly selected from the dataset for manual particle picking using e2boxer.py program (63). The manually picked particles were used to generate a starting model for automated particle picking using the program Cryolo (64). Particles were extracted from the dose-weighted micrographs using Relion 3.1 (65) and imported in cryoSPARC (42) by which, from this step forward, the data analysis has been entirely done. First, the reference-free 2D classification has been performed. Next, the damaged

particles were further removed using the *ab-initio* reconstruction task in cryoSPARC by which an *ab-initio* model obtained without symmetry imposed showed clear features of a C15 symmetry. Finally, 11000 particles were selected for the model refinement imposing the C15 symmetry which was followed by defocus refinement and another round of 3D refinement. The last step of data processing has been the local refinement of the three main regions (the cap, the beta barrel, the N domains/IMRR) that resulted in a cryo-EM map with a near-atomic resolution of ~2 Å (according to the FSC(0.143) criteria). From the obtained map, a *de novo* model was built using Coot software (43). For the three subunits, first the poly-A models were manually built, then, based also on the main subunits identified by MS (24), and finally manually assigned using Coot (43). The final model was refined with the Phenix software (44), while visualization and fittings were done using the Chimera software (45).

Cryo-electron tomography, subtomogram averaging, and cryo-electron crystallography

Cryo-electron tomography and subtomogram averaging analyses were done with the *etomo* package, *imod* and *PEET* softwares (46-48), according to (1). Briefly, tomograms were aligned using gold particles as fiducial markers and only those with minimal alignment errors were selected. For subtomogram averaging, boxes with a size of 350x350x350 voxels were selected and extracted from both in-plane and side views. Subtomograms were initially rotationally aligned, and eulerian angles were determined by systematic search over some specified range of values, with the range and the coarseness of the search being reduced in successive iterations of the search. Absolute values of cross-correlation and missing-wedge compensation were applied. No symmetrization was imposed. The Chimera software (45) was used for isosurface visualization.

The cryo-electron crystallography analyses were done with *2dx* and Focus packages (49,50) according to (1,10) with no modification.

- **Supporting figures' legends**

Figure S1: T2SS isolation and characterization. a) When separated by chromatography, the solubilized cell envelopes resolve into three peaks, the first of which is the T2SS (S); the T2SS purity and integrity are assessed by native electrophoresis (inset) resolving in a single band (lane S). The lane M is the molecular marker. In b), it is shown a raw micrograph with highlighted a few side/tilted (red circle) and top (red-dashed circle) views of the complex; below, representative 2D classes are shown. In c), the FSC plot of the cryo-EM single particle analysis indicates the achieved resolution (gold standard FSC 0.143) of 1.96Å. To better appreciate the variation in resolution along the structure, in d), a longitudinal cross-section of the complex has been used to show several local lower-resolution regions (2.5 Å) in the N2 and N1 domains.

Figure S2: Membranes localization and T2SS all-atom molecular dynamics. In a) a longitudinal cross-section (left) and a comprehensive cryo-EM map (center) with the fitted T2SS model are presented. The putative membrane regions are indicated with OM and IM interface. On the top-right, a detail of the OM region shows the GspP-OM link, an OM-hooking GspP N-terminal harm that remained not modelled due to lower resolution (see schematic representation at the bottom-right). In b), the effects of the stress experiments in terms of mortality post-exposure are shown for both the wild type and the DR_0774 deletion mutant under each tested condition. In c) are shown the *ab initio* configurations used for all-atoms molecular dynamics on the equilibrated GspDPT/POPC for ssDNA uptake (left), dsDNA uptake (center), and dsDNA ejection (right). The three protein subunits are indicated as orange (GspD), light green (GspP), and light blue (GspT). For the resulting movies please see Movies S2, S3, and S4.

Figure S3: The T2SS' gate systems in *D. radiodurans*. Longitudinal cross-section fitted with the T2SS model (left). The upper boxes show details of the periplasmic gate as top view (center) and side view (right). The bottom boxes show details of the N-gate as top view (center) and side view (right). The scale bar indicates 20 Å.

Figure S4: Comparison between T2SSs from different species and genes organization for the T2SS subunits in *D. radiodurans*. In a) are shown the 3D models of different T2SSs localized with respect to the OM. The main GspD subunit with related domains and accessory subunits are also shown for each T2SS. The complexes are shown in scale and from left to right are the T2SS from *D. radiodurans* (8CO1), *K. pneumoniae* (6HCG), *E. coli* ETEC (5ZDH, *Vibrio*-type), *A. hydrophila* (6I1X), and *P. aeruginosa* (5WLN). In the image are also visible the folding differences between the

GspP, characterized by only β-sheets, and the so-far-identified pilotin S in T2SSs of other species, in which only α-helices are occurring. With SL, OM, and IM are indicated the S-layer, Outer Membrane, and Inner Membrane, respectively. The scale bar indicates 100 Å. In b), the image shows the main gene localization for DR_0774 (GspD), DR_0940 (GspT), and DR_1364 (GspP), and for the first two entries also the related operons.

Figure S5: GspP structure and interaction details. In a), it is shown a detail of the extended contact surface between GspP monomers (green and red), as well as GspP (green and red) and the EBP region of GspD (yellow) monomers. The interaction between charged amino acids at the interface contributes to keeping the belt in place. In b), a detail of the side-view interaction between two GspP monomers (green and red) is also shown. The scale bars indicate 10 Å. In c), the image shows a monomer of GspP and a schematic representation of its sequence with indicated the typical regions (n, h, and c) for lipoprotein signalling and post-translational modifications. Below, it is shown a guide tree obtained by multiple-sequence alignment providing an indication of divergence between GspP and the pilotins PulS, GspS, and AspS.

Figure S6: GspT structure and interaction details. In a), it is shown a detail of the extended contact surface between GspT monomers (green and red), as well as GspT (green and red) and GspD (yellow) monomers in which a belt of aromatic residues can be glimpsed. In b), a detail of the internal surface interaction between several GspT monomers (two of which are in red and green) is also shown. The scale bars indicate 10 Å. In c), the image shows a monomer of GspT and a schematic representation of the GspT sequence with indicated the typical regions (n, h, and c) for lipoprotein signalling and post-translational modifications. Below, it is shown a guide tree obtained by multiple-sequence alignment providing an indication of divergence between GspT and the PilW, which is the Pil protein found to have the highest identity score (between 14 to 24% depending on the species).

Figure S7: Stability of the model systems studied by molecular dynamics simulations. In a) and b) are shown the side and top views, respectively, of the GspDPT model embedded in LPS. In orange and blue, the visited positions respectively of phosphate and nitrogen atoms of the asymmetric bilayer from the 300 ns NPT trajectory. In c), it is shown the Root- Mean-Square deviation of the backbone atoms with respect to the initial structure, and the average oxygen-oxygen distance for opposite alanine 618 GspD residues (i-i+7 and i-i+8, i=1,15) with the error bars in grey, both during the 300 ns NPT trajectory.

Figure S8: Flow-path from data acquisition and processing to final structural results. The image

shows in a) the flow-path used for the single-particle analysis presented in this manuscript while in b) the one used for tomography acquisition followed by either cryo-electron crystallography analysis or subtomogram averaging analysis.

Table S1: Molecular dynamics simulations data. Conductance (Sigma) of the two modelled GspDPT systems calculated on independent molecular dynamics trajectories up to 60 ns. The first 20 ns were discarded from data analysis because considered as equilibration time. Probed conditions and related output for each simulation are reported. For each condition, conductance is reported as i) general, ii) specific for K^+, iii) specific for Cl^-, and iv) as ratio between the two ions (K^+/Cl^-). The block average (statistical uncertainty estimation) is indicated for each measurement.

Model System		Sigma[nS] 20-30ns	Sigma [nS] 30-40ns	Sigma [nS] 40-50ns	Sigma [nS] 50-60ns	Sigma [nS] Block average
GspDPT/POPC	**0.2 V/ KCl 1M**	11.4	11.4	12.4	11.8	11.8 ± 0.5
	K^+	5.0	6.9	6.7	7.6	6.6 ± 1.1
	Cl^-	6.4	4.5	5.7	4.2	5.2 ± 1.0
	K^+/Cl^-	0.8	1.5	1.2	1.8	1.3 ± 0.4
	0.5 V/ KCl 1M	12.7	12.4	13.1	13.1	12.8 ± 0.3
	K^+	8.1	6.4	5.6	6.7	6.7 ± 1.0
	Cl^-	-4.6	-6.0	-7.5	-6.4	6.1 ± 1.2
	K^+/Cl^-	1.8	1.1	0.7	1.0	1.2 ± 0.5
	1 V/ KCl 1M	12.2	12.5	12.5	13.0	12.6 ± 0.3
	K^+	6.6	7.0	7.2	7.0	7.0 ± 0.3
	Cl^-	-5.5	-5.5	-5.3	6.0	5.6 ± 0.3
	K^+/Cl^-	1.2	1.3	1.4	1.2	1.3 ± 0.1
	-1 V/KCl 1M	-10.8	-11.3	-11.3	-10.7	11.0 ± 0.3
	K^+	-5.6	-5.7	-5.7	-5.5	5.6 ± 0.2
	Cl^-	5.2	5.6	5.6	5.2	5.4 ± 0.2
	K^+/Cl^-	1.08	1.02	1.0	1.1	1.05 ± 0.05
Model System		**Sigma[nS] 20-30ns**	**Sigma [nS] 30-40ns**	**Sigma [nS] 40-50ns**	**Sigma [nS] 50-60ns**	**Sigma [nS] Block average**
GspDPT/LPS	**1 V/KCl 0.15M**	1.68	1.83	1.38	1.48	1.60 ± 0.2
	K^+	0.89	0.93	0.48	0.77	0.77 ± 0.2
	Cl^-	-0.68	-0.79	-0.84	-0.67	0.75 ± 0.06
	K^+/Cl^-	1.3	1.2	0.6	1.1	1.0 ± 0.3
	Ca^{2+}	0.11	0.11	0.06	0.03	0.08 ± 0.04

Table 2: DNA threading molecular dynamics simulations. The table shows the conditions assessed on the two systems by all-atom molecular dynamics simulations for DNA threading.

Model System	Atoms n.	Equilibration time	Isothermal-isobaric (NPT) ensemble	Electric field	1M DNA at +1V	1M DNA at -1V
GspDPT/LPS 0.15M KCl Ca^{2+}	~ 820000	3+5 ns	300 ns	1V 60 ns	NO	NO
GspDPT/POPC1 M KCl	~ 945000	3+10 ns	60 ns	1.0V 60 ns 0.5V 60 ns 0.2V 60 ns -1V 60 ns/	ss/dsDNA uptake	dsDNA ejection

Table S3: cryo-EM data collection and processing. The table reports the information about data collection (single-particle and tomography) and data processing (single-particle, subtomogram averaging, and cryo-electron crystallography).

Single-particle data collection and processing	
Microscope	FEI Titan Krios
Detector	Gatan K3 (post-GIF 20 eV)
Voltage (keV)	300
Spherical aberration	2.7
Magnification	105 000 x
Electron exposure ($e^-/Å^2$)	55.0
Defocus range (μm)	-0.8 to -1.6
Pixel size (Å)	0.83
Micrographs (total no.)	35 000
Particles used for reconstruction (no.)	11 000
Symmetry imposed	C15
Gold standard resolution (Å)	1.9
FSC threshold	0.143
Software used for data processing and local refinement	CryoSparc
Number of atoms/residues/chains	
GspD (DR_0774)	4 589/609/15
GspP (DR_1364)	683/95/15
GspT (DR_0940)	1 040/142/15
Global validation metrics	
Clashscore	7
Ramanchandran outliers	0.1%
Sidechain outliers	0.3%
Software used for real space refinement	Phenix
Model-to-map fit CC_mask	0.783
Geometry restrains deviations	
Bond	0.006
Angle	0.749
Molprobity Statistics Ramanchandran angles (%)	
Outliers	0
Allowed	8
Favored	92

Tomography data collection and processing (tomo & subtomo)	
Microscope	FEI Titan Krios
Detector	Gatan K2 BioQuantum (post-GIF 20 eV)
Voltage (keV)	300
Spherical aberration	2.7
Magnification	21 000 x
Frame and total dose ($e^-/Å^2$)	0.3 and 96
Defocus range (μm)	-2 to -5
Pixel size (Å)	2.28
Tomograms (no.)	80
Tilting range	± 60°
Tilt scheme	dose-symmetric (0,+3°,-3°,...)
Symmetry imposed	none
Resolution (Å)	8
Software used for data processing	eTomo (imod and PEET)
Subtomogram box volume size	350x350x350 voxels
Averaged particles (no.)	68 000

cryo-electron crystallography data processing	
Plane group symmetry	p6
Unit cell dimensions	a (Å) = 190; b (Å) = 190; c (Å) = 200 γ (°) = 120
Micrographs (no.)	73
Tilt angle distribution	± 45°(0,+3°,-3°,...)
Defocus range (Å)	39 500 to 123 738
Magnification	21 930x
Index quality range (IQ)	1-6
Resolution cutoff in-plane (Å)	4
Resolution cutoff in-z (Å)	10
Software used for data processing	2dx package

Movie S1: Representative reconstructed tomogram of a typical cell envelope patch from *D. radiodurans* R1.

Movie S2: Molecular dynamics simulation of the ssDNA uptake threading for the GspDPT/POPC system.

Movie S3: Molecular dynamics simulation of the dsDNA uptake threading for the GspDPT/POPC system.

Movie S4: Molecular dynamics simulation of the dsDNA ejection threading for the GspDPT/POPC system.