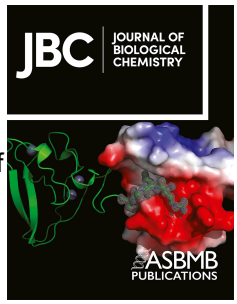

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Structural characterization and functional insights into the Type II Secretion System of the poly-extremophile Deinococcus radiodurans

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Abstract

The extremophile bacterium *D. radiodurans* boasts a distinctive cell envelope characterized by the regular arrangement of three protein complexes. Among these, the Type II Secretion System (T2SS) stands out as a pivotal structural component. We used cryo-electron microscopy to reveal unique features, such as an unconventional protein belt (DR_1364) around the main secretin (GspD), and a cap (DR_0940) found to be a separated subunit rather than integrated with GspD. Furthermore, a novel region at the N-terminus of the GspD constitutes an additional second gate, supplementing the one typically found in the outer membrane region. This T2SS was found to contribute to envelope integrity, while also playing a role in nucleic acid and nutrient trafficking. Studies on intact cell envelopes show a consistent T2SS structure repetition, highlighting its significance within the cellular framework.

Keywords

cell envelope; cryo-electron microscopy; *Deinococcus radiodurans*; lipoproteins; T2SS

Introduction

Deinococcus radiodurans is a non-motile extremophile known for its remarkable resistance to harsh conditions. Its cell envelope comprises three protein complexes (1), including the S-layer Deinoxanthin Binding Complex (SDBC), a porin that displays superoxide dismutase activity (2-9), the Radial Dimeric (RD) complex (1,10), that still remains uncharacterized, and a Type II Secretion System (T2SS). The T2SS is evenly distributed in the cell envelope of this bacterium, surrounded by

SDBC and RD copies in a structured pattern characteristic for its regularity (1,10).

T2SSs are part of a large superfamily of Type IV filament-containing systems that share homologous components, including pili and flagella (Type IV Piliation systems, T4P) (11,12). They are equally spread among Archaea and Bacteria, and a general function of substances secretion as well as DNA uptake related to competence has been assigned (13-18).

Usually, in T2SSs, several regions can be identified (13-18): I) the outer membrane region, an outer membrane-embedded core that consists of the main secretin channel (e.g., GspD or PulD, depending on the nomenclature); II) the pilotin region, a homo-oligomeric belt of an accessory pilotin (e.g., GspS, AspS, or PulS, depending on the nomenclature) localized in the periplasm and facing the Outer Membrane (OM) leaflet; III) the assembly platform, composed of several subunits (e.g., PulC, PulE, PulL, PulM, PulN), localized at the underlying Periplasmic/Peptidoglycan Region and extending into the Inner Membrane (IM); IV) the pseudopilus extrusion machinery, consisting of a helical filament that propels fully-folded substrates through the secretin channel. While various T2SS structures have been previously reported, the distinctive characteristics of the system elucidated in this study add significant value to the growing demand for comprehensive functional and structural data on secretion systems, including T2SSs.

Here, the T2SS, isolated from the wild-type strain R1 of *D. radiodurans*, is structurally described at near-atomic resolution. This case is notable due to the distinctive networking context of this machinery within its highly structured S-layer/cell envelope (1,10). Two previously uncharacterized lipoprotein subunits, DR_0940 (GspT) and DR_1364 (GspP), play significant roles. DR_0940 forms the T2SS cap, typically a domain of GspD, while DR_1364 forms a belt around the GspD β-barrel region, differing from the usual pilotin S subunits (GspS/PulS/AspS).

Additionally, a novel region named Inner Membrane-Related Region (IMRR) at the base of the complex contributes to a second gate in GspD, supplementing the typical one at the β-barrel region. The T2SS is not only involved in natural competence (19) but, as shown here, also impacts the statics of the cell envelope and its trafficking. Considering the singular networking background of this molecular machine, which is integrated into its much more structured and organized cell envelope/S-layer functional unit, these novel modular and architectural features might have implications for the cell envelope/S-layer maintenance. Subtomogram averaging and cryo-electron crystallography on intact cell envelope patches reveal a T2SS repetition with a typical p6 symmetry, consistent with that observed in the S-layer of this bacterium (20). Overall, this T2SS has distinctive features expected to be crucial in shaping the organized structure and functional properties of this cell envelope. A T2SS integrated into such an articulated supramolecular organization could be involved into specialized functions characterizing this cell envelope, most of which in this bacterium are aimed at ensuring protection at many levels (e.g., robust cell envelope structures, shielding incoming radiation,

quenching oxidative damage). During evolution, similar cases can be expected for many other S-layer-carrying bacteria including pathogens, where the presence of a highly repeated number of T2SS copies in a highly structured cell envelope can be relevant to understand pathogenic mechanisms and may turn to be useful to develop therapies against new targets. The reported findings and their associated implications contribute to enhance the overall understanding of Type II Secretion Systems, particularly in species, characterized by highly structured cell envelopes in which these complexes are integrated. This research holds significant potential for technological and biomedical applications.

Results

Structure of the T2SS core

The T2SS from *D. radiodurans* R1 was isolated and resolved at ~2 Å, revealing a complex structure spanning ~173 Å in width at its widest point and ~350 Å in height, in line with the reported cell envelope thickness (1,21-23) (Figs 1, S1). The core is composed by the secretin DR_0774, a pentadecameric GspD, spanning the outer membrane and extending into the periplasm/peptidoglycan, facing the inner membrane (Figs 1, S1b, S2a). The GspD Outer Membrane Region (OMR; residues range 504-740) features a dominant 60 β-sheets β-barrel structure (~85 Å tall, ~106 Å internal diameter) (Figs 1, 2a). For each secretin monomer, two protrusions emerge: the External Upper Protrusion (EUP) in the OM (residues 703-718) and the more extensive External Bottom Protrusion (EBP) in the periplasm (residues 635-645 and 680-690) (Fig 2a). Internally, a Beta Hairpin Motif (BHM, residues 608-629) constricts the channel, creating a periplasmic gate (~26 Å diameter) (Fig 2). Below the OMR, the Periplasmic Region (PR; residues 503-156) contains the canonical N domains, N3, N2, N1, and N0 (Fig 2a). Directly beneath the periplasmic gate, a subtly discernible constriction (~67 Å diameter) related to the N3 domain is visible (Fig 2a). Another constriction at the N0 domain (~77 Å diameter) connects with the N-terminal part of GspD, the IMRR (Inner Membrane-Related Region, residues range 155-34), and identifies the N-domain-related gate (N-gate; Figs 2, S2a, S3). The GspD-IMRR is found to be peripheral to the inner membrane (~1 Å thickness by PPM3.0; Fig S2a), featuring an external diameter of ~148 Å (Fig 1) and an internal one of ~77 Å (Fig 2a). Because of the IMRR's flange shape, likely important to increase the surface of interaction with an inner membrane machinery, we named this newly identified GspD domain as Flange Domain (FD), which appears shorter but larger with respect to the N domains. The secretin DR_0774 was modelled from residue 34 (peripheral to the inner membrane) to residue 740 (outer membrane), with a 59-amino acids gap in the N3 domain and a 15-amino acids gap between the N0 and N1 domains due to a local low resolution of the map (Figs 1d, S1e). Each secretin monomer tilts ~30° along the z-axis and twists ~115° counterclockwise from the N-terminal to the C-terminal (Fig 1c). Initially classified as a PilQ, the resulting DR_0774 complex was cautiously designed as Type IV-like piliation

system (T4P-like), suggesting a potential reclassification pending further data (24). Through structural, phylogenetic, and sequence alignment analyses, this work identifies it as a T2SS, assigning DR_0774 as a GspD (Figs 3, S4). The absence of pili in *D. radiodurans* (22) further supports this conclusion.

A pentadecameric ring surrounds the base of the OMR

T2SSs typically feature a pilotin belt (PulS, GspS, or AspS) surrounding GspD. In this case, a 15-fold symmetry belt surrounds the T2SS at the OMR base (Fig 1). This arrangement relies on electrostatic interactions between belt subunits and the GspD monomers at the EBP level (Figs 2a, S5a, S5b). The belt's monomer, DR_1364, a 155-residues globular protein, has been modelled for 95 amino acids (residues 61-155; Fig S5). The DR_1364, a predicted IPT/TIG domain-containing protein (Immunoglobulin (Ig)-like fold), lacks homology and folding analogy (β-sheets vs α-helices) to known pilotins in others T2SSs (Figs S4a, S5c). This diversity stems from the GspD in this T2SS lacking the common S domain (Fig 3c) that usually interacts with the pilotin S belt (25). Instead, the new belt subunit DR_1364 is secured by the EBP. Considering the uniqueness of this subunit, we here named it GspP, adhering to the T2SS subunits nomenclature. Bioinformatic analysis confirms GspP lipoprotein features with a cleavage site at position 51-52 (c-region/site) preceded by a positively charged signal region (n-region; residues 1-37) and a hydrophobic region (h-region; residues 38-51) (Fig S5c). The h-region neighbours the cysteine in position 52 which is deputed to N-lipidation. The range of residues 52-61, while present and directed to the OM (GspP OM-link in Fig S2a), could not be modelled.

An independent subunit builds the T2SS cap

The cap domain, found on top of the OMR and only for some species, is usually made of the C-terminal region of the secretin (6) (Fig S4a). In *D. radiodurans,* the T2SS cap domain is made up of an independent subunit that robustly interacts with GspD monomers and with an extended apical part pointing upward (Figs 1, 2, S6a, S6b). Notably, a cluster of aromatic residues marks the boundary between GspD and the cap (Fig S6a). This subunit also follows a 15-fold symmetry, giving to the cap its characteristic shape (Figs 1, 2). Compared to similar structures, this cap is steeper and narrower, likely enhancing interactions with the outer membrane's structural network (Figs S2a, S4a). The cap forms an internal constriction (~80 Å diameter) at the GspD OMR level and in its uppermost part resolves into an external pore with an internal diameter of ~50 Å, finally restricting the access to the whole complex (Fig 2a). The cap subunit, identified as the DR_0940, is a 208-residues protein modelled from residue 28 to residue 199 (Figs 1d, S6). Given its uniqueness (Fig S6c), we named the DR_0940 as GspT, adhering to the T2SS subunits nomenclature. Being uncharacterised, no specific

functional properties have been assigned. GspT is predicted to be a membrane-interacting protein, likely monotopically, facing the extracellular region which in this bacterium is also of S-layer pertinence. Considering the proximity of the OM, we conducted bioinformatic analysis on the primary GspT sequence for potential lipoprotein features. Results revealed a cleavage site at position 19-20 (c-region/site) preceded by a positively charged signal region (n-region; residues 1-5) and a hydrophobic region (h-region; residues 6-19) (Fig S6c). The h-region neighbours the cysteine in position 20 which is deputed to N-lipidation (Fig S6c). Based on the above information, it is likely that the cap contributes to the stability of the S-layer assembly bridging it with the OM.

T2SS cell envelope distribution and localization

In *D. radiodurans,* the T2SS is integrated into a highly structured cell envelope (1,10). Indeed, in this bacterium, the regularity of the S-layer extends into the layers below and the T2SS represents a main statical component of this organization (1,10). Here, we have studied the T2SS and its architectural details in the larger structural context where it operates. Studies on intact cell envelope patches (Fig 4a; Movie S1) have been independently performed by subtomogram averaging (Fig 4b) and 2D cryo-electron crystallography (cryo-EC, Fig 4c). Results showed the regular repetition of the T2SS and its relative distribution with a p6 symmetry (Fig 4d), which, for the cryo-EC, was subsequently imposed to improve the final resolution (Fig 4c). In this bacterium, the structural unit, consisting of one T2SS, six SDBCs, and six RDs, is repeated delimiting the entire cell body (1,10). Therefore, the results here shown provide a clear overview of the astonishing complexity and regularity characterizing this cell envelope.

The T2SS contributes to cell envelope stability and trafficking

The cell envelope contributes to the trafficking and the poly-extremophile properties of *D. radiodurans* (9,10,26,27). Being highly represented in the cell envelope (1,10,24), the T2SS is expected to contribute to its stability. To investigate the functional involvement of the T2SS in the cell envelope and in the extreme resistance of this bacterium, differential effects between the *D. radiodurans* R1 wild type and its DR_0774 deletion mutant were assessed under stress conditions. Either shear stress, with frictional forces, or normal stress, with compression/expansion cycles, were tested by means of mortality post-exposure (Table 1; Fig S2b). In all cases, the deletion mutant showed to be more sensitive to mechanical stress with respect to the wild type, and with greater sensitivity to normal stress than to the shear one (Table 1; Fig S2b). In both instances, experiments demonstrate a primary cohesive role associated with the presence of the T2SS.

Considering the role of this S-layer in contributing to the UV-C resistance of *D. radiodurans* R1, we also investigated the potential involvement of the T2SS in resistance against UV-C exposure. Once

again, comparative experiments demonstrated increased sensitivity of the deletion mutant in comparison to the wild type (Table 1; Fig S2b).

Given the cohesive and secretion roles of the T2SS, we examined any disparities in terms of total protein release in the media between the wild type and the mutant. These analyses revealed a six-fold increase in the proteins released in the media for the mutant, highlighting the T2SS's involvement in S-layer stability and integrity (Table 1).

The loss of natural competence in the deletion mutant showed the pivotal role of this secretin and its T2SS in the uptake of nucleic acids (19). Here, the structural information was exploited to perform all-atom molecular dynamics simulations on the T2SS (GspDPT) inserting it in a phospholipid bilayer, either asymmetric with lipopolysaccharides (GspDPT/LPS) or symmetric with phosphatidylcholine (GspDPT/POPC), as usually done in reconstitution experiments (28). As outlined in the supplementary section, simulations showed stability of the system in both models. In particular, in the most challenging one, the GspDPT/LPS, along the 300 ns simulations in the Isothermal–isobaric ensemble (NPT), the stability of the LPS's nitrogen and phosphate atoms can be observed (Fig. S7a and b). In this model, the Root-Mean-Square-Distance from the initial structure reaches a plateau already after 100 ns, demonstrating the stability of our model (Fig. S7c). Similarly, conductance and ion selectivity, in the context of threading of ssDNA or dsDNA 24-mers, were assessed in the simpler GspDPT/POPC model system. Here, the T2SS was found to be prone to ssDNA and dsDNA uptake as well as identified as slightly cation selective (Tables 1 and S2; Fig S2c). These results are in line with other well characterized transport systems of similar size (29,30).

Discussion

Type II Secretion Systems (T2SS) are molecular assemblies that play a crucial role in cellular trafficking and cell-environment interactions. They are widely distributed in Archaea and Bacteria, including pathogenic species (11-18) and belong to a larger Type IV filament-containing superfamily, alongside pili and flagella (11,12). In *D. radiodurans*, the core secretin DR_0774 distinguishes itself as a GspD, rather than a PilQ, pointing its assembly to a T2SS more than a T4P. This observation is corroborated by the absence of pili and motility in this bacterium as well as the loss of natural competence in DR_0774 deletion mutants (19). These aspects, coupled with the involvement of two operons in constructing this machinery (Fig S4b; DR_0774 and DR_0940 are part of two different operons), underscore its active functionality within the cellular framework. These findings are further corroborated by the functional tests here reported (Table 1 and S1; Fig S2c; Movies S2, S3, S4). In *D. radiodurans,* the T2SS is highly repeated and regularly organized through the cell envelope (1,10). Subtomogram averaging and 2D cryo-EC on large cell envelope fragments further detailed this property.

While some features are in line with those found in other species, particularly the shared structural similarity from the GspD C-terminal side to the N3 domain as well as its pentadecameric assembly (Figs 3 and S4), this T2SS also presents distinctive features. Notably the typical S domain, important for the pilotin S binding and characterising the T2SS in *K. pneumoniae* and *E. coli* ETEC (S4a), here is absent (Fig 3c). Consequently, also the pilotin S is here replaced by the unrelated GspP. Interestingly, the pilotin S of these species has typically a structure built by α-helixes, while the GspP structure has a β-barrel organization. The N0 domain exhibits a distinct sequence when compared to other GspDs featuring this domain as exemplified by *K. pneumoniae* complex(Figs 3c, S4a). Additionally, the *D. radiodurans* complex displays a greater height (~350 Å of this T2SS vs ~220 Å; Fig. S4a) in comparison to known T2SS systems (12,17), including the extensively studied counterpart from *K. pneumoniae*. This aspect is likely due to the thicker cell envelope of *D. radiodurans* (1,21-23) when compared with other systems. This extension is attributed to the presence of GspT as an independent subunit building itself the cap structure, and an additional domain at the N-terminal part of GspD, the FD (Figs 2, 3a, S4a). In the cases of *E. coli ETEC* and *K. pneumoniae,* a cap formed by the C-terminal region of GspD is present, although it is different in shape and less pronounced compared to the cap build in *D. radiodurans* by the GspT, which is more extended and built by an individual subunit. Finally, the FD, a domain exclusive of *D. radiodurans'* GspD lies at the inner membrane's periphery (Fig S2a) and is likely monotopic, thereby enhancing the interaction with specific protein components in the inner membrane (24,33). The repeated T2SS structure acts as an effective spacer, stabilizing the cell envelope and maintaining cellular integrity. Resistance tests on the DR_0774 deletion mutant further emphasize its importance in withstanding mechanical stress (Table 1).

Finally, although the gate regions inside the channel were not modelled due to a local lower resolution, several details can be appreciated for both, the periplasmic and N- gates, which occur with a typical check-valve organization and are expected to have a primary role in the functionality of the complex (Figs S2a, S3). The specific function of N0 in the N-gate and its difference from the N0 of *K. pneumoniae*, the only one structurally identified to date, could result from specialization, elucidating its divergence from known T2SS systems (Figs 3c, S4a). GspT acts as an independent T2SS subunit, forming a cap toward the environment and likely serving as a bridge to the S-layer. In the previously characterized GspDs, the C-terminal part is involved in building the cap. Interestingly, this part nicely fits between GspDs including the one of *D. radiodurans* R1 (Fig 3a) even if, in the latter case, none of the domains are involved in the cap structure (Fig S4a).

The GspP, characterized by its Ig-like fold, contributes to the transversal continuity with the neighbouring complexes (1,10). The presence of such proteins in prokaryotes is associated with cell surface receptors and the inside-out evolution of cells (34,35,36).

D. radiodurans exhibits a three-dimensionally organized cell envelope with specialized multi-subunit assemblies, including the SDBC and the T2SS (1,10). This intricate supramolecular organization is a result of deep evolutionary adaptations to ensure protection against various stressors (1-10). Similar mechanisms may be widespread among bacteria, offering insights into their strategies for environmental resilience.

Experimental procedures

Cell culturing, T2SS purification and identification

Deinococcus radiodurans (strain R1; ATCC 13939) was grown in Tryptone Glucose Yeast extract (TGY) at 30°C for 24 h as described in (24). Cell envelope and T2SS isolation followed previous protocols (1,10,24). Samples' quality was assessed by Blue Native (BN) gel electrophoresis, using 3–12% (w/v) gradient gels (NativePAGE, Invitrogen) (9). Chromatography columns were regenerated and calibrated prior use by ReGenFix (https://www.regenfix.eu/) (1).

Bioinformatic analysis

SignalP-6.0 (37; https://services.healthtech.dtu.dk/services/SignalP-6.0) was used for signal peptide and cleavage site prediction. UniProt (38; https://www.uniprot.org) and DIVEIN (39; https://indra.mullins.microbiol.washington.edu/DIVEIN/index.htm) aided in multiple-sequence alignment as well as phylogenetic and divergence trees. Operons were predicted using MicrobesOnline (40; http://www.microbesonline.org/). The transmembrane regions were predicted via the PPM 3.0 web server (41; https://opm.phar.umich.edu/ppm_server).

Cryo-electron microscopy data acquisition

Grids preparation and data acquisition for Either purified T2SS sample (5 mg/mL) or cell envelope fragments were done at CEITEC (Brno, Czech Republic) according to (3). Any minor modification has been reported in Table S3. Supplemental information is provided in the SI and Fig S8.

Data processing

The data processing for single-particle analysis was done using cryoSPARC (42) according to (3), with the following differences. Any minor modification has been reported in the SI and Table S3. From the obtained and refined 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), 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).

The cryo-electron tomography and subtomogram averaging analyses were done with the *etomo* package, *imod* and *PEET* softwares (46-48), according to (1). Any minor modification and details have been reported in Table S3. The cryo-electron crystallography analyses were done with *2dx* and Focus packages (49,50) according to (1,10) with no modification.

Stress assays and effects assessment

Resistance assays involved growing *D. radiodurans* R1 strains WT and ΔDR_0774 in TGY for 24 h at 25°C (OD_{650} = 1.2) (5,6). Cell cultures underwent a 10x series of 5 dilutions (from 10^{-1} to 10^{-5}) with the last 4 spotted in 3 µL volumes on 1.5% agar TGY plates. Colony Forming Units (CFU) count was performed on the 10^{-3} dilution. Shear and normal stress experiments were performed on culture suspensions by i) vortexing at 2000 rpm with a 4.5 mm orbital diameter for 30 minutes with a 1:1 (w/w) ration of 5-mm zirconium-silica beads to cultures; ii) a french pressure cell at 900 Ψ on 15 mL cultures for two cycles. Spotting was done post-exposure. Electromagnetic stress experiments were performed using a 9 W UVC lamp placed at 15 cm from the plate (irradiation rate of 2.75 J/s/m^2) for 5 minutes and then incubated for 48 h at 25°C. Spotting was done pre-exposure. Cell envelope integrity was assessed via centrifugation (5000 x *g*, 10 minutes, 4°C) and concentration/dilution in Phosphate buffer 50 mM pH 7.4, 0.05% β-DDM using a 10 kDa cutoff (Vivaspin Turbo 15, Sartorius). Proteins differences were estimated by SDS-PAGE and absorption spectroscopy at 280 nm.

Molecular Dynamics Simulations

The procedure described in (51; https://www.ks.uiuc.edu/Training/Tutorials - Membrane Protein Tutorials) for membrane-protein system preparation was followed. For both systems, GspDPT/LPS and GspDPT/POPC, the entire procedure was performed using the CHARMM-GUI server (52). The software CHARMM 36m force field for proteins (53), CHARMM 36 for lipids and DNA (54), TIP3P for water (55), and the new Multi-Site model for Ca^{2+} were used (56-59). The server Propka (60) suggested all amino acids in their standard protonation state at neutral pH. The final systems were simulated on a 20-cores CPU equipped with a single RTX-3090 GPU using Gromacs 2022.4, with a performance of 18-21 ns/day. After equilibration and 10 ns in the NPT ensemble, we started the simulations at zero voltage and thus at different voltages (Table S1). Further, we added DNA 24-mers (6A-6C-6G-6T), either ssDNA or dsDNA, placed partially in the pore above and below the filter with the orientation 3' forward (61), as reported in Fig S2c and performed DNA threading simulations.

Quantification and statistical analysis

Cell envelope and T2SS isolations for data analyses were performed on more than 40 independent preparations. All data are representative of at least three independent replicates. All attempts to

reproduce the results here presented were successful.

Supporting information

This article contains supporting information (SI). References from 62 to 67 are cited in the SI.

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Author contributions

Conceptualization, Validation, Formal analysis, Funding acquisition, Project administration, Supervision: DP, DF. Methodology: DP, DF, SM, MC. Investigation: DP, DF, SM, MC, LI, MT. Molecular dynamics simulations: SM, MC. Visualization: DP, DF, SM, MC. Writing – original draft, review, and editing: DP, DF, SM, MC LI, MT.

Competing interests

Authors declare that they have no competing interests.

Data and Code availability

Materials, data, and protocols are accessible in the manuscript and public repository Electron Microscopy Data Bank (EMDB) (EMD-16770 and PDB ID-8CO1). Data will be released upon publication. This paper does not report any original code.

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Figure legends

Figure 1: The T2SS structure of *D. radiodurans*. a) T2SS cryo-EM map showing the main external dimensions. b) T2SS model, a pentadecameric hetero-oligomer, consisting of 3 different subunits. GspD (DR_0774), in orange/yellow, forms the core divided into Outer Membrane Region (OMR), Periplasmic Region (PR), and Inner Membrane-Related Region (IMRR). GspT (DR_0940), in light blue, forms the cap region toward the S-layer. GspP (DR_1364), in light green, forms the belt at the outer membrane-periplasm interface. c) a GspD monomer (in red) tilts ~30° longitudinally and has ~ 115° transversal torsion from the top view (dashed lines). Scale bar is 50 Å.
d) a scheme of GspD (DR_0774) indicates domains, cleaved sequences, and the local low-resolution part. For GspP (DR_1364) and GspT (DR_0940), modelled regions are indicated.

Figure 2: Cross-sections and fitting of the T2SS. a) left to right: T2SS cryo-EM map cross section, model fitting, and model itself. Orange/yellow marks GspD domains: External Upper Protrusion (EUP), External Bottom Protrusions (EBP), N domains (N3 to N0), and the Flange Domain (FD). Internal pore dimensions, gates and constrictions are also indicated. b) images show top view cross-sections of the map's fitted with the model at the periplasmic (left) and the N-gates (right) heights. GspD (orange/yellow) and GspP (green) subunits at these levels are indicated. Scale bar is 50 Å.

Figure 3: GspD phylogenetic and structural comparative analyses. In a), the image presents a comparison of *D. radiodurans* GspD with others GspDs for which the atomic model is available. The analysis indicates structural similarities for the β-barrel region and the first two N domains while highlighting an increasing difference going towards the N-terminal part, which ends with the Flange Domain (FD), characteristic of the *D. radiodurans* GspD. The shown GspD are from *D. radiodurans* (8CO1), *K. pneumoniae* (6HCG), *V. cholerae* (5WQ8), *A. hydrophila* (6I1X), and *P. aeruginosa* (5WLN). The colour legend is reported in the image. In b), the image shows the phylogenetic tree of the same GspDs reported in a). In c), a multiple-sequence alignment of the same GspDs reported in a) and b) shows a good identity between the entries as well the absence of the S-domain (blue box)

in the C-terminal region of the *D. radiodurans* GspD, confirming the absence of a typical pilotin S forming the belt around the complex.

Figure 4: T2SS organization in the cell envelope. a) image shows a slice of a tomogram with typical T2SS repetition; zoom-in insets highlight details in top and side views. Scale bar indicates 500 Å. b) typical subtomogram averaging: top view (upper-left corner) and side views from different angles (bottom-left corner); red- and blue-dashed lines in top view indicates side-view orientations. FT analysis of tomogram (upper-right corner) and the top view of 3D subtomogram averaging volume (bottom-right corner) are also shown. c) cryo-electron crystallography results: left- Fourier coefficients plot from FT analysis of cell envelope patches; right- top view of the resulting 3D cryo-EC map with the central T2SSin orange. d) top (left) and side (right) views scheme illustrating the T2SSs organization in *D. radiodurans* cell envelope. The central T2SS is a map with the model fitted. Scale bars in b), c), and d) indicate 200 Å.

Table 1. Summary of the functional assays: Comparative analysis of wild type vs. DR_0774 deletion mutant to assess DR_0774 and its T2SS functionality.

	Assay/Prediction		Method	Functional properties	ΔpilQ vs WT		Reference
					qualitative (specific property)	quantitative	
Electromagnetic stress	Spotting pre-exposure	UV-C exposure	Hg lamp (9 W)	UV-C resistance	decreased	+30.36% (mortality)	this work
Mechanical stress	Spotting post-exposure	Shear exposure	Zirconium-silica beads/Vortexing	Mechanical tethering (spacer)*	decreased	+15.25% (mortality)	this work
	Spotting post-exposure	Pressure exposure	French pressure cell/Compression-expansion	Mechanical tethering (spacer)*	decreased	+85.40% (mortality)	this work
Cell properties	Electrophoresis	Protein secretion/ Cell envelope integrity	Cell growth/ Supernatant analysis	Protein release in the media	increased	~6x	this work
Trafficking	Spotting post-exposure	DNA uptake	pRAD1 plasmid/Transformation	Natural competence	precluded	0	Ref. 19
	Molecular dynamics	ssDNA trafficking	Molecular dynamics	Natural competence	n.a.	n.a.	this work
	Molecular dynamics	dsDNA trafficking	Molecular dynamics	Natural competence	n.a.	n.a.	this work
	Molecular dynamics	Ion transport	Molecular dynamics	Nutrient uptakes	n.a.	n.a.	this work

* This function is specified to distinguish from the one of mechanical tethering as a turnbuckle for the SDBC[3].

Figure 1

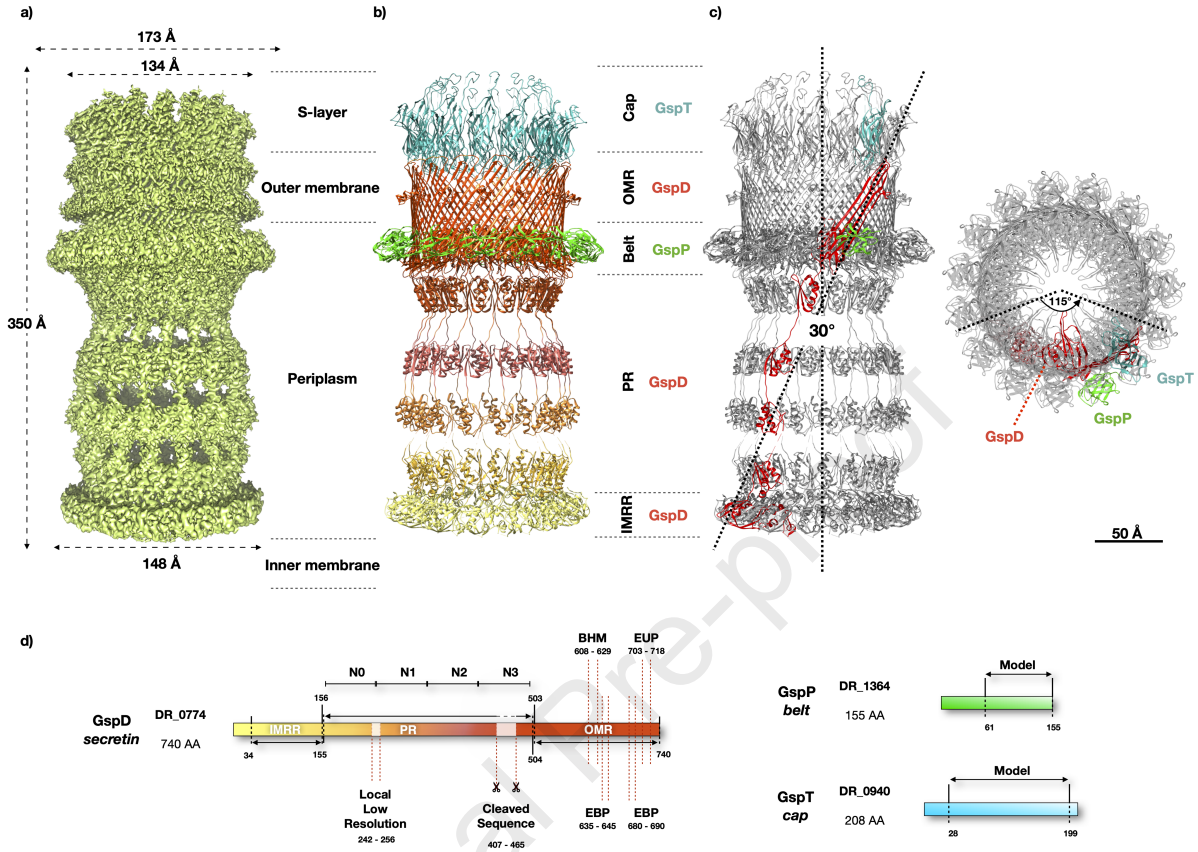


Figure 2

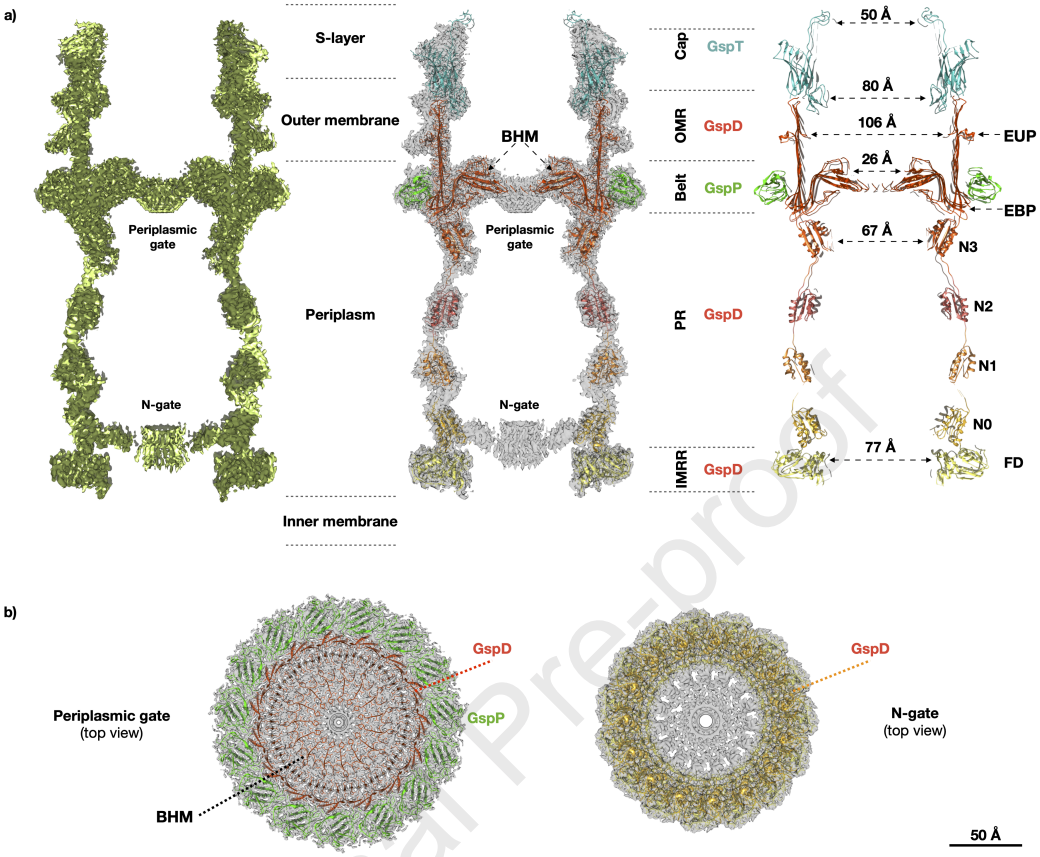


Figure 3

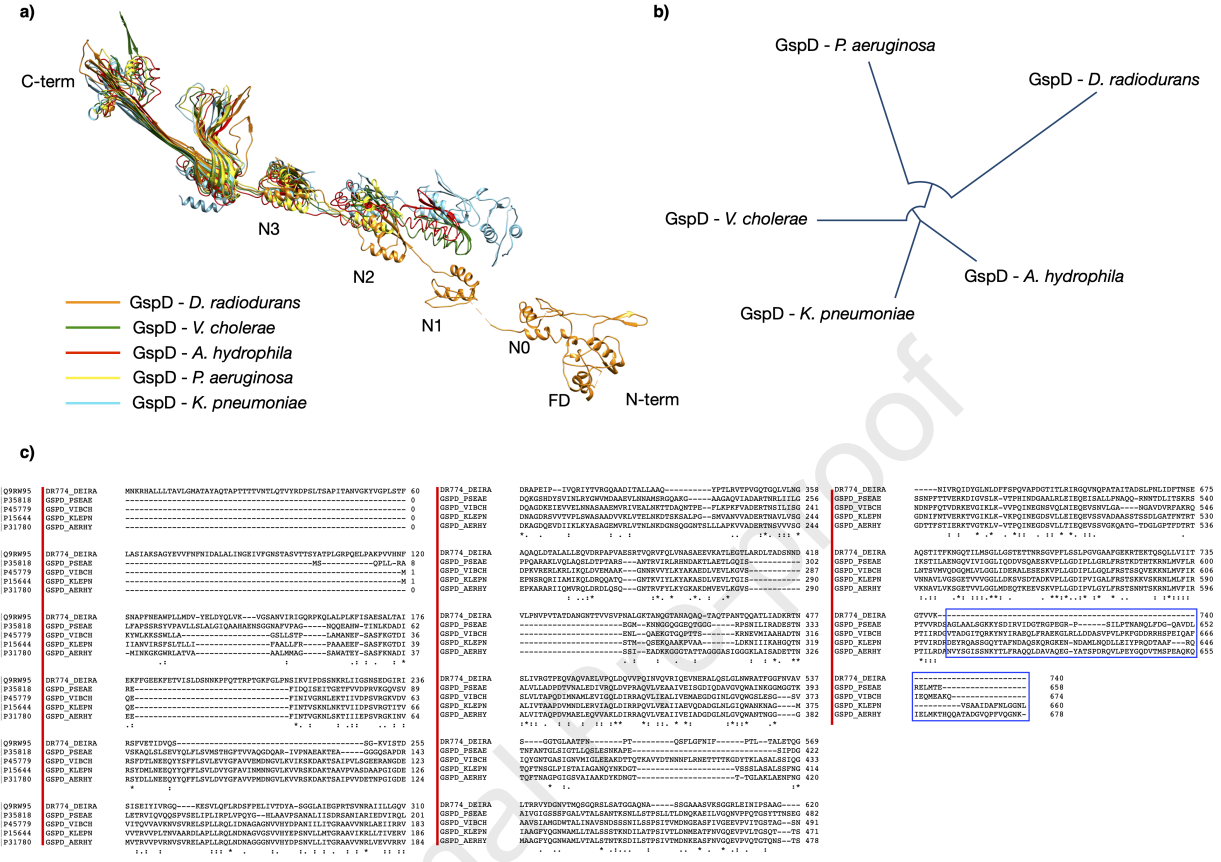
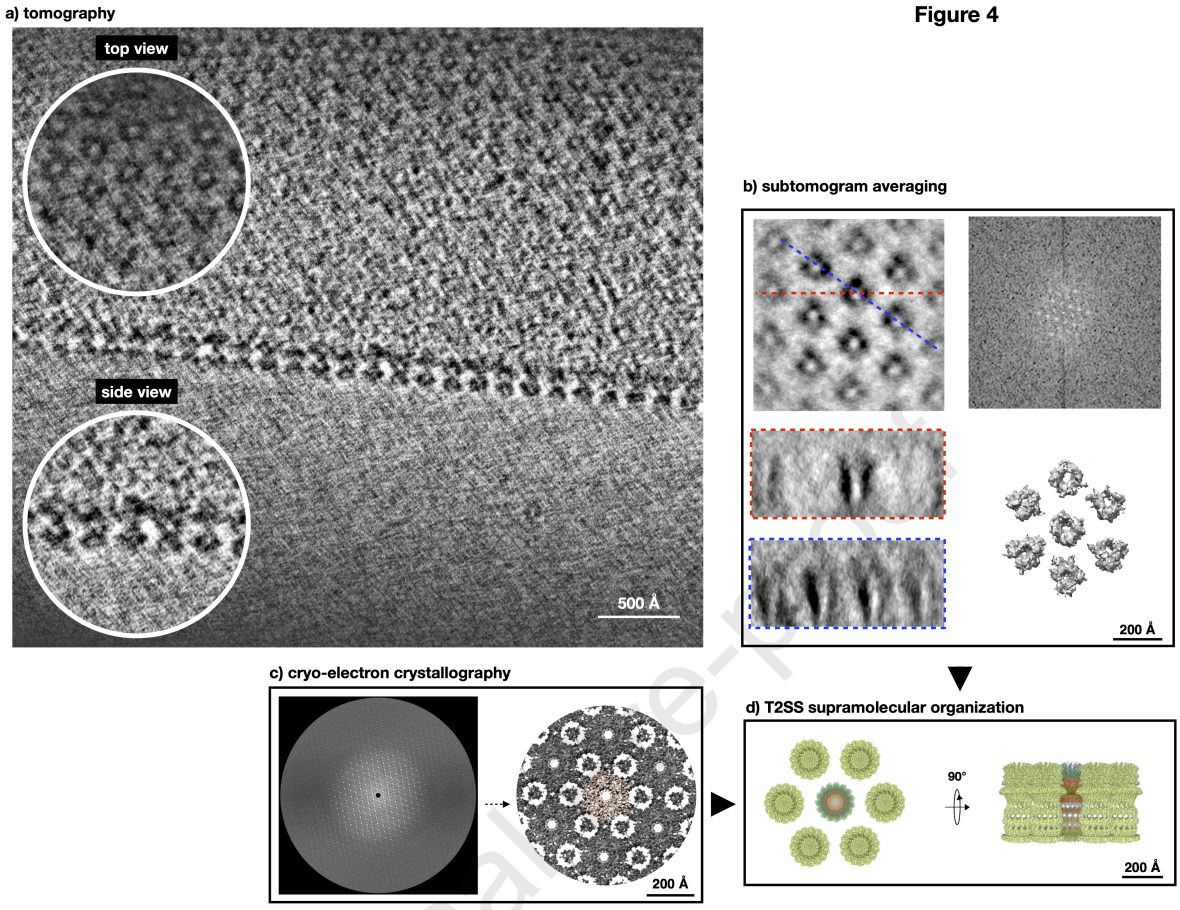


Figure 4



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Author contributions

Conceptualization, Validation, Formal analysis, Funding acquisition, Project administration, Supervision: DP, DF. Methodology: DP, DF, SM, MC. Investigation: DP, DF, SM, MC, LI, MT. Molecular dynamics simulations: SM, MC. Visualization: DP, DF, SM, MC. Writing – original draft, review, and editing: DP, DF, SM, MC LI, MT.