



New features of the cell wall of the radio-resistant bacterium *Deinococcus radiodurans*

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ABSTRACT

We have analyzed the cell wall of the radio-resistant bacterium *Deinococcus radiodurans*. Unexpectedly, the bacterial envelope appears to be organized in different complexes of high molecular weight. Each complex is composed of several proteins, most of which are coded by genes of unknown function and the majority are constituents of the inner/outer membrane system. One of the most abundant complexes is constituted by the gene DR_0774. This protein is a type of secretin which is a known subunit of the homo-oligomeric channel that represents the main bulk of the type IV piliation family. Finally, a minor component of the pink envelope consists of several inner-membrane proteins. The implications of these findings are discussed.

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1. Introduction

Several groups of eubacteria and archaea are characterized by the presence of regular paracrystalline arrays of proteins or glycoproteins, as the main constituents of the outer envelope of their cell walls [1–3]. Such structural organization is termed, due to its location, the Surface layer, or more commonly, the S-layer [4,5]. *Deinococcus radiodurans* represents one of the most studied bacteria, not only because of its strong ability to resist high doses of ultraviolet and ionizing irradiation [6–8], but also for the intriguing features of its S-layer [9–12]. This external coat in *D. radiodurans* is considered to be the result of the assembly of a main protein named the Hexagonal Packing Intermediate (HPI), which is coded by the gene DR_2508 [13,14]. However, another protein, DR_2577, has also been reported to be involved in building the S-layer

of *D. radiodurans*. This protein is called Surface Layer Protein A (SlpA) and shares a significant homology with one of the main S-layer proteins present in *Thermus thermophilus* [15]. Electron microscopy studies have extensively described the surface views of the S-layer [11,9]. These studies have shown that the S-layer is organized in a repetition of the elementary components characterized by an internal 6-fold symmetry. These elements are organized also following a 6-fold symmetry, ultimately building the paracrystalline layer [11]. However, the S-layer of *D. radiodurans* is one part of a much more complex structure, believed to be a sequence of layers stacked upon the external surface of the outer membrane forming the so-called pink envelope [11]. Although the bacterium stains as a gram positive, genomic and bioinformatic studies combined with studies on the related *Thermus* species confirm that members of the *Deinococcus-Thermus* phylum contain two cell envelopes. These envelopes constitute an unusual cell wall which includes an outer membrane-like that represents a vestigial reminiscence of gram-negative ancestors. However, the bacterium lacks of a lipopolysaccharide layer associated with the carbohydrate coat attached to the S-layer, leading to its staining as a gram positive bacterium [16, 17]. The pink envelope is the stratification of a “backing layer”, the S-layer and a carbohydrate coat. This stratification includes the outer membrane, on which the layers sit. These layers consist of proteins, lipids, carbohydrates and carotenoids [15].

Abbreviations: HPI, Hexagonally Packed Intermediate; SlpA, Surface Layer Protein A; TGY, Tryptone/Glucose/Yeast extract broth; β -DDM, n-Dodecyl- β -D-maltoside; SDS-PAGE, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; BN, Blue native; Rf, Retardation factor; DTT, Dithiothreitol; MS, Mass Spectrometry; SLH, Surface Layer Homology; T2SS, Type II Secretion System; T3SS, Type III Secretion System; T4P, Type IV Piliation System; MW, Molecular Weight; FDR, False Discovery Rate; DPC, DNA Processing Complex

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In this work we have prepared the pink envelopes according to [12], digested them with lysozyme in order to remove the carbohydrate layer and finally solubilized the suspension by using mild detergents, thereby obtaining a solution of the outer membrane layer with the interacting S-layer elements. Samples obtained by this procedure were analyzed in terms of protein complex composition by means of native gel electrophoresis, subsequent denaturing gel electrophoresis and MS analysis.

2. Materials and methods

2.1. Bacterial strain and growth conditions

D. radiodurans strain R1 (ATCC 13939) was grown in tryptone/glucose/yeast extract broth (TGY) [18] for 24 h at 30 °C, with shaking at 250 rpm. Cells were harvested by centrifugation of 1 l cultures at 5000 ×g for 10 min at 4 °C and resuspended in 50 mM Na Phosphate pH 7.8 (Buffer A).

2.2. Membrane preparation

Whole cell membrane fractions were purified at 4 °C as previously described [12] with some modifications as follows. Briefly, cells were resuspended in Buffer A, treated with DNase and disrupted using a French Pressure Cell. Unlysed cells were removed by low speed centrifugation (4 °C, 2 × 2000 ×g for 10 min). The final supernatant was centrifuged again (4 °C, 48,000 ×g for 10 min) and the pink pellet was resuspended in 10 ml of Buffer A. To remove surface polysaccharides, the membrane suspension was incubated under agitation (800 rpm) with 100 mg/ml lysozyme for 8 h at 30 °C. The membrane suspension was then washed 3 times in Buffer A by resuspension followed by centrifugation (4 °C, 48,000 ×g for 10 min).

2.3. Membrane solubilization

Before final resuspension, membranes were weighed. The mass of the membrane fraction was assumed to be the total protein mass. The measured mass was then used to calculate the detergent concentration needed for solubilization. Membranes were solubilized by agitation in 1% (w/v) n-Dodecyl-β-D-maltoside (β-DDM) at room temperature for 3–4 h with a final protein concentration of 3–5 mg/ml. The solution was then centrifuged at 48,000 ×g at 10 °C for 10 min to remove insoluble material.

2.4. Polyacrylamide gel electrophoresis

For denaturing Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), 10% (w/v) separating polyacrylamide/urea gels with 4% (w/v) stacking gels were used [19]. Samples were denatured with Rotiload (Roth) at room temperature before loading, and after the electrophoretic separation the gels were stained with Coomassie brilliant blue G250. Blue native (BN) gel electrophoresis was carried out using 3–12% (w/v) continuous gradient gels, according to [20]. The pink envelopes were mixed with 0.25 volumes of Coomassie Blue Solution (5% (v/v) Serva Blue G, 750 mM aminocaproic acid and 35% (w/v) sucrose). Electrophoresis was carried out at 205 V for 5 h at 4 °C. For 2D separation, the strips from the BN-PAGE were excised and denatured with Rotiload (Roth) at room temperature for 20 min. After denaturation the strips were placed on the top of a denaturing SDS-PAGE as described above and sealed with 0.5% (w/v) agarose in a cathode buffer. The molecular weight of the complexes isolated by the BN-PAGE was estimated by plotting the retardation factor values (Rf, length of the band migration/length of the dye front) versus the log of the molecular weight of the molecular marker (NativeMARK, Invitrogen) using a polynomial curve fit (second-order polynomial best-fit), according to

manufacturer's instructions. The obtained curve was used for calculating the apparent masses at the equivalent Rf of the BN-PAGE bands.

2.5. Electron microscopy

The pink envelopes, at a concentration of 1 µg/ml in Buffer A were negatively stained by using the droplet method with 2% uranyl acetate on glow-discharged carbon coated copper grids. Electron microscopy was performed on a CM120 electron microscope (Philips, Eindhoven, The Netherlands) operated at 120 kV. Images were recorded under low dose conditions (total dose ~ 25e-/Å²) with a 4000 SP-4K slow-scan camera (Gatan, Pleasanton, CA) at –340 nm defocus and at a magnification of 53 kx.

2.6. Mass Spectrometry

The gel bands were excised, reduced in DTT (10 mM, 56 °C, 30 min) and subsequently alkylated with iodacetamide (55 mM, 25 °C, 20 min in the dark). Following dehydration with acetonitrile, trypsin (1 ng/µl solution in 50 mM ammonium bicarbonate) was added and the gel pieces were allowed to swell on ice for 30 min. They were then digested overnight at 37 °C with shaking. After digestion, the peptide content was extracted twice with sonication (using a solution of 50:50 water: acetonitrile, 1% formic acid). The pooled extracts were placed in a clean tube and dried with a speed vacuum centrifuge. The dried pool was finally re-dissolved in 10 µl of a reconstitution buffer (96:4 water: acetonitrile, 0.1% formic acid).

The samples (8 µl each) were loaded on a trapping column (nanoAcquity Symmetry C₁₈, 5 µm, 180 µm × 20 mm) and subsequently separated and eluted at 0.3 µl/min with a sequential analytical column (nanoAcquity BEH C₁₈, 1.7 µm, 75 µm × 200 mm) using a nanoAcquity UPLC system (Waters). HPLC solvent A was water and solvent B was acetonitrile, both containing 0.1% formic acid. During the elution step, the percentage of solvent B increased linearly according to the following geometry: from 3% to 7% in 10 min, to 25% in 70 min and finally to 40% in 10 min. The eluted peptides were introduced directly into a mass spectrometer (Orbitrap Velos Pro, Thermo) via a Picotip emitter (New Objective) with a spray voltage of 2.2 kV and a capillary temperature of 300 °C. Full scan MS spectra were acquired in the range of 300–1700 m/z and a TOP 15 CID method was employed.

Data analysis was performed with the software MaxQuant (version 1.0.13.13) and the files obtained were used for searching in MASCOT (version 2.2.03, Matrix Science) against a species specific (*D. radiodurans*) Uniprot database with a list of common contaminants appended. The mass error tolerance for the full scan MS spectra was set at 20 ppm and for the MS/MS spectra at 0.5 Da. A maximum of 1 missed cleavage was allowed. The mascot generated .dat files were then uploaded to Scaffold for visualization of all proteins across all samples. A filter of 2 peptides per protein minimum and peptide confidence of 95% was applied. This corresponds to 2% peptide False Discovery Rate (FDR) and 0% protein FDR. The unweighted spectrum count of each protein was finally divided by its mass and the resulting index was used as an indicator of its relative contribution to each band. A cutoff for index lower than 0.25 was introduced. For each band the proteins were clustered in three groups: first group with index between 0.25 and 0.50; second group with index within 0.50 and 0.75; and third group with index higher than 0.75.

3. Results

3.1. Native gel electrophoresis (1st dimension)

The quality of the pink envelopes was previously evaluated by electron microscopy to confirm their organization in paracrystalline fragments (Fig. 1a). They were then solubilized using a mild detergent (n-Dodecyl-β-D-maltoside - β-DDM) and finally analyzed by the BN-PAGE. The constituents of the pink envelopes were resolved into

discrete bands representing several species of different molecular weights (Fig. 1b). Moreover, denaturing SDS-PAGEs were performed on the intact and solubilized pink envelopes in order to define whether some components of the sample were not solubilized after the treatment with detergent. As shown in Fig. 1c, all the components of the pink envelopes were solubilized without any preferential effect due to the detergent use.

3.2. Mass calculations

According to the migration of the molecular marker, the species separated by the BN-PAGE were found to have molecular weights spanning between 1200 kDa and 100 kDa with 10 bands identified, showing different staining intensities. In this respect, the first three bands at higher molecular weight appear to be the most representative (Fig. 1b). The apparent masses were calculated with respect to the molecular marker (Fig. 2 and inset). The first three heaviest species were found to have apparent masses of 1117 kDa, 916 kDa and 859 kDa respectively (Fig. 1b). Moreover, the remaining seven bands of lower molecular mass, range between 700 kDa and 105 kDa (Fig. 2 and inset).

3.3. Denaturing electrophoresis (2nd dimension)

In order to begin to determine the components of the complexes identified by native PAGE, second dimension denaturing PAGEs were run of the BN-PAGE lanes (Fig. 3). These experiments showed different patterns associated with each native band indicating that a correlation between the heaviest (bands I, II, III) and lightest bands was very low. Moreover, from the second dimensions it was also evident that a prominent heavy MW band was typically associated with the heaviest complexes (Fig. 3).

3.4. Mass Spectrometry analysis

In order to clarify to what extent the isolated complexes were representative of the different cell wall layers (inner membrane, outer

membrane and S-layer) and whether these complexes are associated with the S-layer rather than the underlying membrane system, we performed identification by a Mass Spectrometry (MS) of the proteins that are contained within each of the BN-PAGE bands. As shown in Table 1, each band appears to be composed of several proteins that span a broad range of molecular weights. Moreover, some of these proteins are organized into operons, whereas others are encoded by genes with unknown function (Table 1).

The presence of proteins carrying characteristic domains helps to define the location of specific complexes. For example, the S-layer proteins or outer membrane proteins can be tentatively identified by the presence of specific domains such as the Surface Layer Homology (SLH) domain (Table 1) [19,21]. Accordingly, 7 of the identified proteins were found to be either associated with the outer membrane/S-layer region or contain typical domains associated with this region of the cell wall (Table 1). These proteins appear to be mostly related to the heaviest complexes (Fig. 1b, bands I, II and III; Table 1). In addition, MS analysis has also shown that, of the total proteins identified, 6 are associated with the inner membrane. In particular, the V-type ATPase complex subunits and a sugar ABC transporter were found to be the most representative components of this compartment, suggesting that the pink envelopes could also contain the inner membrane. Furthermore, these 6 proteins were also found to be almost exclusively associated with the lightest complexes isolated by the BN-PAGE (Fig. 1b and Table 1). However, of the total proteins identified, only 1 could be assigned to be extracellular and 3 to the periplasm, while for the remaining 20 proteins it was not possible to determine their localization and function.

Finally, it should be noted that the protein DR_0774 was found to be present in these data. According to the BN-PAGE (band I) and MS analysis DR_0774 appears to be characteristic and exclusive to band I (Fig. 1b, Fig. 3 and Table 1). The protein DR_0774 is homologous to a type of secretin which is known to be organized into dodecameric complexes constituting the main bulk of the Type II Secretion System (T2SS), Type III Secretion System (T3SS) and Type IV Pili System (T4P) [22,23]. This protein is annotated in Uniprot (www.uniprot.org) as the general (type II) secretion pathway protein D (GspD), a secretin

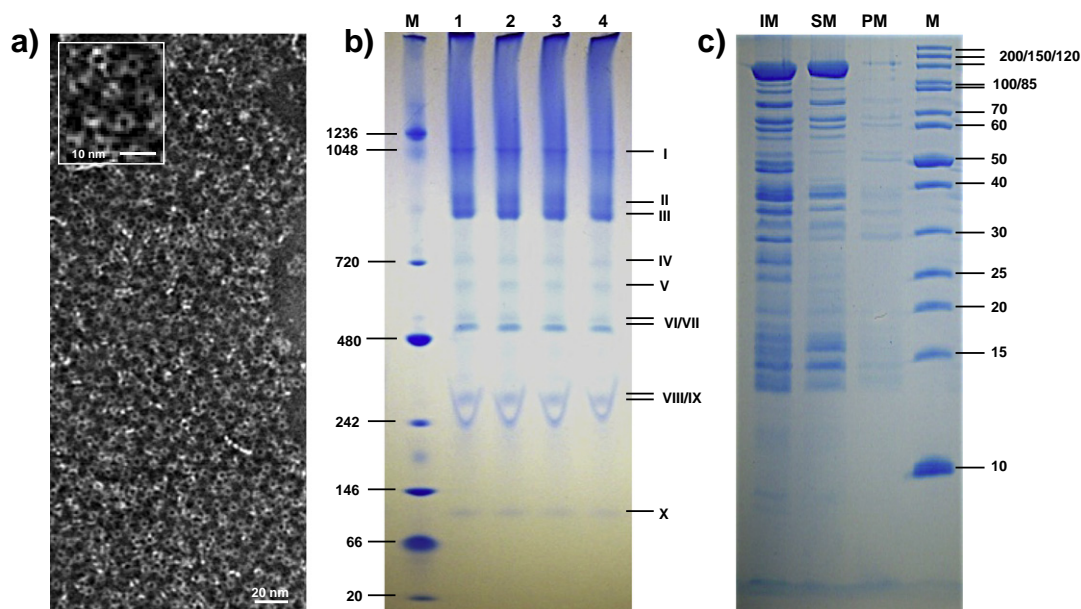


Fig. 1. a: Control electron micrograph representing a negatively stained fragment of the pink envelope. Inset representative detail of an elementary unit with typical 6-fold symmetry. b: BN-PAGE of the pink envelopes solubilized using n-Dodecyl- β -D-maltoside. The lane M indicates the standard and the equivalent molecular weights; the lanes from 1 to 4 represent the pattern of separation on solubilized pink envelopes. The numbers from I to X indicate the resolved complexes. The lanes from 1, 2, 3 and 4 were loaded with 1, 1.5, 2, and 2.5 μ l of sample respectively. c: SDS-PAGE of the pink envelopes in several stages of preparation. IM indicates the integral membranes (not solubilized), SM is the solubilized membrane after centrifugation and PM is the fraction not solubilized, equivalent to the pellet resuspended after solubilization and centrifugation. Lane M indicates the standard with the corresponding molecular weights.

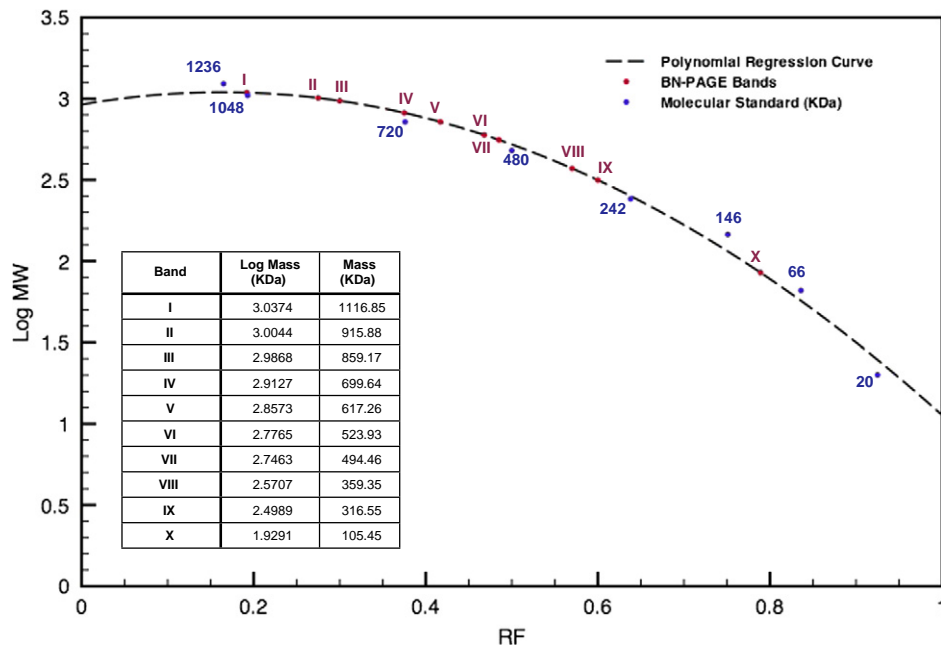


Fig. 2. The plotted retardation factors (RF) versus the Log of the molecular weight using a polynomial curve fit (second-order polynomial). The numbers in blue represent the mass (kDa) of the molecular marker components, the numbers in red indicate the complexes resolved by the BN-PAGE. The inset below on the left indicates the calculated Log MW and the equivalent masses for each of the complexes.

typically involved in the T2SS and T3SS. In the Prokaryotic Operon Database (ProOpDB - <http://operons.ibt.unam.mx/OperonPredictor/>) the locus of DR_0774 is clustered with the genes DR_0770, DR_0771, DR_0772 and DR_0773 (Supl. Fig. 1a). Each of these genes is putatively assigned as, or has high homology with, the *Deinococcus gobiensis* and *Deinococcus geothermalis* genes that code for the pilins PilQ, PilM, PilN, PilO and PilW respectively and that, as for *D. radiodurans*, appear to be clustered into the same operon (Supl. Fig. 1a). Further analysis on DR_0774 indicates higher homology with the PilQ of *D. gobiensis* and *D. geothermalis* rather than with the GspD of the same species (Supl. Fig. 1b, c). These data could suggest that DR_0774 may be a pilin, and due to the operonic background it could be involved with the T4P system rather than the T2SS or T3SS. The presence of this complex in the cell wall and its opening to the extracellular compartment would imply a passage through the S-layer with which it may interact. Additionally, MS analysis performed on the prominent heaviest band of the second dimension denaturing PAGEs (Fig. 3), has shown that this protein is DR_2577, a putative S-layer protein which is present in

the S-layer at high levels and that may represent a primary, if not the main, component of the pink envelopes. Consistently, the presence of the protein DR_2577 was also confirmed by MS in the three heaviest complexes separated by the BN-PAGE (Table 1).

4. Discussion

4.1. Emerging details of the composition of the pink envelope

The results shown here indicate that complexes ascribed to bands I, II and III appear to be a relevant component of the pink envelope. These complexes are not homo-oligomeric as one might expect when considering the S-layer components of the pink envelope and the dominance of the single protein HPI (DR_2508) [13,14]. Instead, large hetero-oligomeric complexes are observed that are most likely juxtaposed contributing in the stability of the cell wall and its components. The isolation of complexes in which the inner membrane proteins, the outer membrane proteins and the S-layer proteins can be

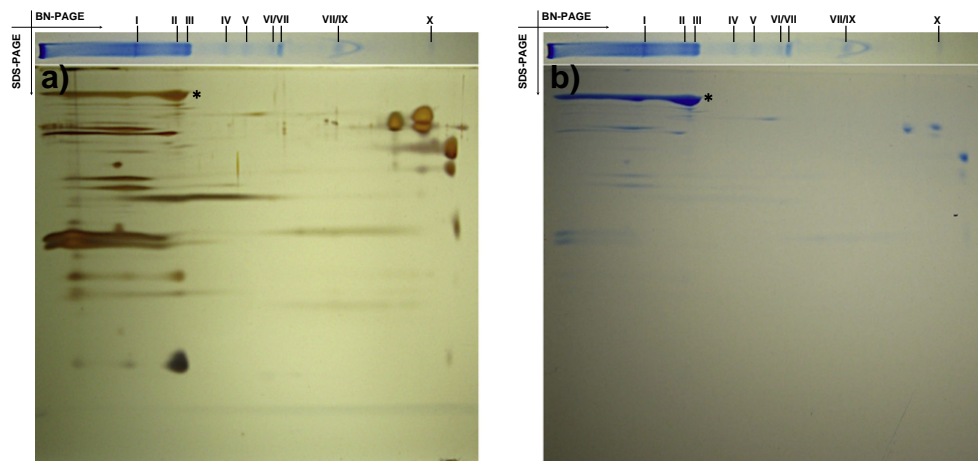


Fig. 3. Second dimension SDS-PAGE starting from the BN-PAGE lanes. Two gels were run under equivalent denaturing conditions but were stained in silver (left) and in Coomassie (right). The asterisks indicate the band that was analyzed by MS and was identified as DR_2577.

Table 1
Proteins identified by MS and their relative genes. The complexes VIII and IX were analyzed together due to the closeness in the BN-PAGE bands. Using the gene ontology each protein is classified according to its cellular compartment. The name and the possible presence of conserved domains are shown. The dotted boxes indicate genes organized into operons. Source: UniProt (<http://www.uniprot.org/>). The bold + indicates the complex in which a specific protein has the highest index (I).

	COMPLEX *											
	I	II	III	IV	V	VI	VII	VIII/IX	X			
GENE											Protein function	Protein localization
DR_0116	+	+	++	++							Uncharacterized protein (conserved site: Outer membrane insertion C-terminal signal, omp85 target)	Outer membrane
DR_0285						+	+				Uncharacterized protein	Unknown
DR_0379		+	+								Outer membrane protein	Outer membrane
DR_0456								+			Biopolymer transport protein (domain: MotA/TOLQ/ExbB proton channel family)	Membrane (outer or inner?)
DR_0486		++	+++	+	+	+	+++	+			Uncharacterized protein	Unknown
DR_0505	+	++	+								5'-nucleotidase family (domain: 5'-nucleotidase C-terminal, http://www.ebi.ac.uk/interpro/entry/IPR008334)	Unknown
DR_0564				+	+	+	+				Amino acid ABC transporter (domain: extracellular solute binding protein, http://www.ebi.ac.uk/interpro/entry/IPR001638)	Outer membrane (periplasmic side)
DR_0644		+	+++								Uncharacterized protein	Unknown
DR_0691	+	+									Uncharacterized protein	Unknown
DR_0695	-	-	-	-	-	-	+	-	-	-	V-type ATP synthase (I subunit)	Inner membrane
DR_0698	-	-	-	-	-	-	+	-	-	-	V-type ATP synthase (C subunit)	Inner membrane
DR_0753								++			Uncharacterized protein	Unknown
DR_0774	+										General secretion pathway (D protein)	Inner membrane/Outer membrane
DR_0940	++							+			Uncharacterized protein	Unknown
DR_0951			+		+			+			Succinate dehydrogenase (iron-sulfur subunit)	Periplasm
DR_0972								+	+		Uncharacterized protein	Unknown
DR_0986								+	+		Extracellular solute binding protein	Inner membrane/Periplasm
DR_1124	+	+	+	+		+					SLH family protein	S-layer/Outer membrane
DR_1180			+								Uncharacterized protein	Unknown
DR_1306								+			Uncharacterized protein	Unknown
DR_1483	+	++	++	+++	+	+	+++				Uncharacterized protein	Unknown
DR_1490	+	+	+								Uncharacterized protein	Unknown
DR_1546						+		+			Superoxide dismutase	Inner membrane/Periplasm
DR_1768	+++	+++	+++	+	+	+	++	+			Uncharacterized protein	Unknown
DR_1931			+								Uncharacterized protein	Unknown
DR_2153							+				Sugar ABC transporter (ATP binding protein)	Inner membrane/Periplasm
DR_2310				++		++	+				Uncharacterized protein (domain: metallo peptidase catalytic domain; http://www.ebi.ac.uk/interpro/entry/IPR024079)	Unknown
DR_2508				+							Hexagonally Packed Intermediate (HPI)	S-layer
DR_2542								+	+		Peptidyl-prolyl cis-trans isomerase	Unknown
DR_2577	+++	+++	+++								S-layer protein putative	S-layer
DR_A0202						+		+			Superoxide dismutase	Periplasm
DR_A0207				+							Uncharacterized protein (domain: glycoside hydrolase superfamily; http://www.ebi.ac.uk/interpro/entry/IPR017853)	Unknown
DR_A0281	++	+	+	+							Uncharacterized protein	Unknown
DR_A0282	++	+	+	+							Uncharacterized protein	Unknown
DR_A0283	+										Probable subtilase-type serine protease (domain: peptidase, C-terminal; http://www.ebi.ac.uk/interpro/entry/IPR007280)	extracellular compartment (putative)
DR_B0007		+									Metal binding protein (ABC transporter periplasmic binding domain; http://www.ebi.ac.uk/interpro/entry/IPR002491)	Periplasm (putative)

*Unweighted spectrum count / MASS (kDa) = i; + = 0.25<i <0.50; ++ = 0.50<i <0.75; +++ = i>0.75 (for details see section 2.6.)

observed suggests that the S-layer and the underlying membrane system may interact with each other, forming complexes that are shared between the layers of the cell wall. The presence of DR_0774 and other proteins hints at a complex that could span both the inner and the outer membranes. According to this suggestion, there is some indication that may prove the extension of this interaction to the inner membrane layer. From the MS analysis of the Band I (BN-PAGE) the presence of 6 proteins which are also characteristic components of a previously isolated DNA Processing Complex (DPC) from the *D. radiodurans* [24] can be observed. Moreover another 6 proteins typical of the DPC are also found in this analysis but are not reported in our list due to their very low mass indices (see Section 2.6 and Supl. Table 1). Performing an analysis on the possible location of these proteins, it was found that the DPC is composed of both globular and transmembrane proteins some of which contain a signal peptide (Supl. Table 1). Between them several are candidate to be cytosolic (e.g. DR_1768) and one to be localized in the inner membrane (DR_1483) (Supl. Table 1). The extension of this complex could involve the S-layer considering the presence of the protein DR_1124 that contains a SLH domain. The DPC is composed of 24 proteins; the 12 missing proteins in our analysis are mainly globular and could be lost during the preparation that is typically developed for membrane protein isolation (Supl. Table 1).

The expected mass of the PilQ dodecamer is 940 kDa; therefore, as confirmed by MS analysis, other proteins must contribute to the complex of band 1 (1120 kDa). Taking this into account the residual mass associated with the complex in band 1 fits with a contribution of the 6 DPC subunits (DR_0116, DR_0505, DR_0691, DR_1124, DR_1483, DR_1768) equivalent to ~190 kDa and the remaining proteins (DR_0940, DR_0281, DR_A0282 and DR_A0283) equivalent to ~150 kDa. The protein DR_2577 is excluded as it is most likely not part of the complex but is present in the band as a result of its smearing along with the lane of the BN-PAGE. This would bring a calculated mass of 1280 kDa that is heavier with respect to the apparent mass of 1120 kDa. However, the error during the size mass estimation of complexes resolved by native PAGE can be significant (up to $\pm 15\%$), since the complex during migration maintains its native conformation [20]. This implies that the residual components, even together (340 kDa), are unlikely to constitute a separate complex with a mass equivalent to the PilQ dodecamer that could co-migrate in the same BN-PAGE band.

4.2. DR_2577 is a dominant component of the envelope

From the MS analysis performed on the first dimension (Fig. 1b) and the second dimension (Fig. 3) PAGEs the protein DR_2577 is identified as one of the more abundant in the sample. DR_2577 is a surface layer protein that shares homology with SlpA (Surface Layer Protein A) from the *T. thermophilus* S-layer, that in this organism represents one of the main S-layer proteins [15,25,26]. Moreover, the relevance of SlpA for the stability of the *D. radiodurans* S-layer has been already demonstrated [15], suggesting an important role for this protein. Although also present in the complexes of band I, this protein characterizes the complex of bands II and III (Fig. 1b, Fig. 3, Table 1) and is the protein mainly responsible for the observed smearing in the first third of the BN-PAGE lanes. Such behavior may be due to a tendency to aggregate, or to auto-assemble which is typical for the S-layer proteins [27].

4.3. The presence of the DR_0774: possible interaction with the S-layer and implications for symmetry

Trafficking across the cell wall must occur through the bacterial S-layer. This implies that, owing to the porosity imposed by S-layer periodicity, the exchange between cell and environment through the pores of the inner and outer membranes must be integral with the S-layer regularity. This observation appears to be consistent with a situation where the three-dimensional extension of symmetry into the

pink envelope or at least into its outer membrane may be needed in order to satisfy the physiological requirements of cell trafficking [28]. This conclusion fits well for the presence of the protein DR_0774, which in vivo is found to form dodecamers able to span the outer membrane [22,23]. Moreover, the homology of DR_0774 with the pilin PilQ and its association with an operon of pilins make this protein a likely candidate to be part of a T4P system [29,30]. In agreement with this, DR_0774 is contained not only within the heaviest complex of the BN page but also is a major component of this band, suggesting that it could take part in the architecture of the pink envelopes (Fig. 1b). However, it must be stressed that only one protein of the operon, the DR_0774, is found in the MS analysis. Therefore, it cannot be stated whether the absence of the other expected proteins is due to the experimental procedure or if there is a physiological implication. In every case DR_0774 could bring a strong continuity of the two-dimensional S-layer stability on the plane of the outer membrane, behaving as a pillar. Moreover, it would also be the main channel through which trafficking is managed [22]. In this architectural organization, this complex could span the outer membrane, connecting itself into the S-layer pore.

4.4. The role of the inner membrane into the three-dimensional network of the cell wall

The pink envelope appears to include a broad group of inner membrane proteins (Fig. 1b and Table 1 — complex I and complexes from IV to X). This finding would suggest that the inner membrane compartment is a relevant fraction of the pink envelope and that the pink envelope probably represent parts of the cell wall fragmented transversally.

5. Conclusions

After detergent treatment, the pink envelopes of *D. radiodurans* can be separated into several complexes by the BN-PAGE and further characterized by MS. The components of the separated complexes originate either from the outer-inner membrane system and/or from the S-layer. By the estimation of the amount and the presence of the different constituents, the pink envelope seems to retain the S-layer in its almost intact organization and the two membranes with a variable degree of integrity. The relevant amount of the protein DR_0774 may contribute at the cohesion between the S-layer and the outer membrane by forming homo-oligomers in agreement with the presence of the complex I identified by the BN-PAGEs. Homology studies and the organization of DR_0774 into an operon of pilins suggest that this protein may be identified as a PilQ rather than the alternative GspD. In light of these facts, DR_0774 and the rest of the genes in its operon (PilM, N, O, W) may constitute the bulk of a T4P system. Moreover, another important feature that emerged from the analysis of the pink envelopes is that the S-layer protein DR_2577 (SlpA) was found to be one of the most representative proteins of the cell wall and characteristic of the complexes II and III identified by the BN-PAGEs. Some of the proteins constituting the complex in band I represent a sub-complex of the DPC that is associated with the T4P. This sub-complex is composed of several sub-units and seems to be extended through the membranes to the cytosolic side. Similarly to the remaining complexes associated with bands IV to X the DPC sub-complex would involve also an inner membrane component suggesting that the pink envelopes could also retain parts of the inner membrane representing in its integral parts the entire cell wall fragmented transversally. Finally, the possible presence in vivo of the DPC-T4P supercomplex would be consistent with the known role of the T4P as a DNA translocator [31] through which the bacteria are believed to exchange DNA. In this context the DPC would be extremely important for DNA processing during trafficking.

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