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Surface (S)-layer proteins of *Deinococcus radiodurans* and their utility as vehicles for surface localization of functional proteins

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ABSTRACT

The radiation resistant bacterium, *Deinococcus radiodurans* contains two major surface (S)-layer proteins, Hpi and SlpA. The Hpi protein was shown to (a) undergo specific *in vivo* cleavage, and (b) closely associate with the SlpA protein. Using a non-specific acid phosphatase from *Salmonella enterica* serovar Typhi, PhoN as a reporter, the Surface Layer Homology (SLH) domain of SlpA was shown to bind deinococcal peptidoglycan-containing cell wall sacculi. The association of SlpA with Hpi on one side and peptidoglycan on the other, localizes this protein in the 'interstitial' layer of the deinoccocal cell wall. Gene chimeras of *hpi-phoN* and *slh-phoN* were constructed to test efficacy of S-layer proteins, as vehicles for cell surface localization in *D. radiodurans*. The Hpi-PhoN protein localized exclusively in the membrane fraction, and displayed cell-based phosphatase activity *in vivo*. The SLH-PhoN, which localized to both cytosolic and membrane fractions, displayed *in vitro* activity but no cell-based *in vivo* activity. Hpi, therefore, emerged as an efficient surface localizing protein and can be exploited for suitable applications of this superbug.

1. Introduction

Surface layer (S-layer) proteins form crystalline two-dimensional arrays that cover the entire prokaryotic cell surface [1]. While they are almost ubiquitously present, there is wide diversity in their sequence and function [2–3]. When present, S-layer proteins are the most abundant proteins produced by cells, displaying oblique, square or hexagonal symmetries [1]. S-layer proteins have been reported in hundreds of bacteria with several strains possessing more than one S-layer protein [2].

The radioresistant, Gram-positive bacterium, *Deinococcus radiodurans*, is known to possess at least two S-layer proteins [4]. The organism is well known for its extreme resistance to gamma radiation, UV rays, oxidative stress and desiccation stress. Such remarkable stress tolerance has been primarily attributed to its highly efficient DNA repair system, and in more recent times to a robust protein damage protection system [5–7]. Curiosities about possible role of cell wall components of *D. radiodurans* in its radiation resistance have fuelled considerable research on the Hexagonally Packed Intermediate (Hpi) surface layer protein [8–10]. The Hpi layer is known to be the outermost proteinaceous component of the deinococcal cell wall and has been characterized to some extent [1,8,10–11]. The Hpi lattice displays hexagonal symmetry and exhibits a core concentrated around the six-fold axis [1]. The other S-layer protein of *D. radiodurans*, encoded by the open reading frame, DR_2577, is annotated as a surface-layer protein based on its homology with the S-layer protein, SlpA of the closely related, *Thermus thermophilus* [4]. The SlpA protein shows the presence of a surface layer homology motif (SLH) at the N-terminus [4]. This motif is present at the N terminus of S-layer proteins of a number of Gram positive and Gram negative organisms and binds to the peptidoglycan by recognizing secondary cell wall polymers (SCWP) [12]. Deletion of the SLH domain of *T. thermophilus* led to loss of peptidoglycan binding ability, as observed both *in vivo* and *in vitro* [13–14].

The unique architecture of S-layer protein and its location have been exploited to generate S-layer-fusion proteins for different applications in bacteria [2]. One of the key applications has been in bacterial cell surface display of relevant proteins [15–17]. Surface expression of proteins allows them better access to substrate/analyte/contaminant, enabling their exploitation for a variety of diagnostic, environmental and industrial applications, such as in whole cell biocatalysis, bioadsorption for removal of harmful chemicals and heavy metals, as biosensors, in live vaccines development, and in screening of peptide libraries *etc*. [18]. The multiple stress tolerance of *D. radiodurans* and the amenability of this organism for bioremediation technologies [19–22] make it a promising biotechnological agent for expression of desirable proteins to function under extreme environments. Therefore, development of a surface display system in this organism is highly desirable.

The cell wall of *D. radiodurans* has been under study since the 1960's [4,8,23–24]. It is now established that the complex envelope of this organism constitutes an inner membrane, the peptidoglycan, an interstitial layer which is only known to be composed of water soluble proteins, a lipid rich backing layer followed by the hexagonally packed surface layer (Hpi) and finally the outermost carbohydrate coat. The

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outermost four layers along with carotenoids form the well-known "pink" envelope of this extremophile [4]. While the exact location of the Hpi layer is known, thus far it has not been possible to affix a function to it. On the other hand, though a definitive role in maintaining deinococcal cell wall structure has been assigned to SlpA, the exact location of this protein in the complex cell envelope is rather poorly characterized. In addition, the biotechnological potential of both in surface display remains unexplored.

The present study provides insights into the relative organization of the S-layer proteins in the complex cell wall architecture of *D. radiodurans*. A non-specific acid phosphatase, PhoN from *Salmonella enterica* serovar Typhi, was employed as a reporter protein to understand interaction of the S-layer proteins with deinococcal cell wall components and to explore their utility in cell surface display of heterologous proteins in this unique organism.

2. Materials & methods

2.1. Growth media & culture conditions

D. radiodurans R1 was grown aerobically in TGY (1% BactoTryptone, 0.1% glucose, and 0.5% yeast extract) liquid medium at 32 °C under agitation (180 rpm). *Escherichia coli* cells were grown aerobically in a Luria–Bertani (LB) growth medium at 37 °C with shaking (180 rpm). The antibiotic concentration used for selection of *E. coli* JM109 transformants was 100 μg/ml of Ampicillin for recombinants carrying pRAD1 based constructs, or 50 μg/ml of Kanamycin for *E. coli* BL21 strain carrying pET29b based constructs. In case of *D. radiodurans*, recombinants were grown with 3 μg/ml chloramphenicol for pRAD1 based constructs.

2.2. Isolation of Hpi layer

The Hpi layer was isolated by extraction in 2% lithium dodecyl sulfate at 4 °C as described earlier [25]. To get a pure preparation of the Hpi layer, it was further treated with 2% sodium dodecyl sulfate at 60 °C [11] followed by washing of the Hpi layer in distilled water several times.

2.3. Identification of proteins by mass spectrometry

The proteins present in Hpi preparation were resolved by 10% SDS-PAGE and the bands of interest were manually excised from the gel. The gel plugs were subjected to destaining, reduction, alkylation, in-gel trypsin digestion and elution of oligopeptides, as described earlier [26]. The eluted peptides were subjected to mass spectrometry (UltraFlex III MALDI-TOF/TOF mass spectrometer, Bruker Daltonics, Germany). Standard ToF-MS protocol was used to acquire PMF spectra in MS mode and standard ToF-MS/MS protocol was used to acquire spectra in MS/MS mode. The Mascot searches were conducted as described earlier [27]. For MS/MS query, a peptide tolerance of 0.5 Da was used.

2.4. Construction of chimeric genes for expressing surface layer PhoN fusion proteins

In order to make the *hpi-phoN* fusion, the *hpi* ORF was PCR amplified using primers, Cd-gro and Cd-r (Table 1) as a 2.8 kb DNA fragment, digested with XbaI and NdeI and cloned upstream and in-frame with the nucleotide sequence coding for PhoN, without its signal peptide, in pRAD1. The chimeric gene was placed under control of the P_{groESL} promoter cloned in the BglII-XbaI site of pRAD1. The resultant plasmid was named pGDRF3. To construct *slh-phoN* chimera for expression in *D. radiodurans*, the sequence encoding the SlpA signal peptide along with SLH domain was PCR amplified from deinococcal chromosomal DNA, using primers SlpA-f and SLH-99r (Table 1) as a 328 bp DNA fragment. The fragment was cloned in place of the *hpi* ORF in pGDRF3 plasmid in XbaI-NdeI site, to obtain the plasmid pPSP1. The constructs were transformed into *E. coli* JM109 and *D. radiodurans* R1 cells.

Table 1
List of plasmids and primers used in the study.

Plasmids	Description of construct	Source/reference
pRAD1	*E. coli–D. radiodurans* shuttle vector, Ap^r, Cm^r, 6.28 kb	Meima R. and M. E. Lidstrom, 2000
pGRDF3	pRAD1 containing the *hpi-phoN* fusion gene with deinococcal *groESL* promoter Ap^r, Cm^r, 10.8 kb	This study
pPSP1	pRAD1 containing the *slh-phoN* fusion gene with deinococcal *groESL* promoter Ap^r, Cm^r, 8.175 kb	This study
pET16b	*E. coli* protein over-expression vector, Kan^r, 5.71 kb	Novagen
pPSP2	pET16b containing the *slh-phoN* fusion gene, Kan^r, 6.7 kb	This study
pET29b	*E. coli* protein over-expression vector, Kan^r,5.37 kb	Novagen
pPSP3	pET29b containing the *slh-phoN* fusion gene tagged to 6XHis, Kan^r, 6.4 kb	This study
pPNO1	pET29b containing *phoN* tagged to 6XHis, Kan^r 6.122 kb	Unpublished results. This lab

Primers

Name	Sequence	RE site
CD-gro	5′-CGGAGTCTAGATGAAGAAAAATATCG-3′	XbaI
CD-r	5′-ACTTCTTACATATGGACGGTTTCG-3′	NdeI
SlpA-f	5′-ATTGGGGGTTCTAGATGAAGAAAAG-3′	XbaI
SLH99-r	5′-GCGTTTTGCATATGGGTCATGTC-3′	NdeI
SLHo-f	5′-ACTCTACCATGGAGAAAAGTC-3′	NcoI
PetC-r	5′-CTTTCACCTCGAGTAATTAAG-3′	XhoI

The underlined nucleotide sequence relates to the restriction endonuclease site included in the primer.

For over-expression of the SLH-PhoN fusion protein, the *slh-phoN* chimeric gene was PCR amplified from pPSP1 (described above) using primers SLHo-f and PetC-r (Table 1) as a 1.012 kb fragment. The PCR product was restriction digested with NcoI and XhoI and cloned into identical sites in pET16b, yielding plasmid pPSP2. In the next step, the fusion gene, along with a portion of the vector carrying the ribosome binding sequence was excised as an XbaI-XhoI product and ligated into identical sites in pET29b to obtain the plasmid, pPSP3 and transformed into *E. coli* BL21 (pLysS) cells.

2.5. In-gel zymogram analysis of acid phosphatase

Zymogram for phosphatase activity was carried out as described earlier [21]. The gel was developed using 200 μl of nitroblue tetrazolium chloride/5-bromo-4-chloro-indolyl phosphate (NBT/BCIP) (Roche Diagnostics, 18.75 mg/ml NBT and 9.4 mg/ml BCIP in 67% Dimethyl sulfoxide) mix in 20 ml of 100 mM acetate buffer pH 5.0 for 16 h. The assay was terminated by rinsing the gel in distilled water.

2.6. Western blotting and immunodetection

Proteins (50 μg) in cell-free extracts were separated by 10% SDS–PAGE, followed by electroblotting on nitrocellulose membrane. An anti-Hpi polyclonal antibody was raised in rabbit, against the Hpi polypeptides eluted from gels. The antibody was used at 1:1000 dilution for detection by a secondary antibody (anti-rabbit-IgG) conjugated to alkaline phosphatase (Sigma, St. Louis, MO).

2.7. Peptidoglycan-containing cell wall isolation from D. radiodurans

Isolation of peptidoglycan from *D. radiodurans* cells was carried out by boiling cells in sodium dodecyl sulfate which dissolves all cellular components except the peptidoglycan and any covalently linked polymers which remains insoluble and can be separated by centrifugation [14]. *D. radiodurans* cells grown to stationary phase were harvested by

centrifugation and washed in distilled water. The pellet obtained was re-suspended in 10 times the volume of 2% sodium dodecyl sulfate solution. The suspension was boiled under constant stirring conditions for 8 h continuously. This was followed by centrifugation of the suspension at 20,000 g for 40 min. The resultant pellet was re-suspended in 2% SDS and the entire process repeated two times. The final pellet obtained was washed in distilled water 6 times to remove SDS and stored at 4 °C.

Peptidoglycan concentration was routinely calculated from their diamino acid, ornithine content as described [14]. Around 10 mg dry weight of peptidoglycan was resuspended in 2 ml of concentrated HCl and transferred to a round bottom flask. The flask was placed in an oil bath at 115 °C for 6 h. The solution was vacuum dried. The residue was re-suspended in 200 μl distilled water and clarified by centrifugation at 15,000 *g* for 30 min. This solution was diluted 1:10 and used for estimation of ornithine. Ornithine estimation was carried out as described earlier [28].

2.8. Cell wall binding studies

Cell wall binding assay was carried out as described earlier [17]. Briefly, 30 μl of peptidoglycan containing cell wall (corresponding to 43 μg of ornithine equivalent) was added to 60 μl of cell extract from *E. coli* cells overexpressing SLH-PhoN or PhoN alone in 50 mM Tris–Cl, pH 8.0 and incubated at 4 °C for 2 h. This was followed by centrifugation to obtain the soluble fraction in the supernatant. The pellet was washed followed by incubation in non-reducing cracking buffer [29] at 50 °C for 15 min and again subjected to centrifugation to obtain the supernatant (insoluble fraction). The insoluble fraction, represents the protein which bound to the peptidoglycan-containing cell wall sacculi, while the soluble fraction, represents protein which did not bind to the peptidoglycan. A zymogram was carried out with the soluble, wash and the insoluble fractions.

2.9. Histochemical screening of recombinants displaying phosphatase activity

Histochemical screening of PhoN was carried out by growing cells on TGY plates containing phenolphthalein diphosphate (PDP) at 1 mg/ml and methyl green (MG) at 5 μg/ml [30]. Recombinants which display cell based phosphatase activity give rise to dark green colored colonies on PDP-MG plates, due to precipitation of methyl green resulting from the local acidification of the medium by the released phosphate.

2.10. Bioinformatic analysis

The sequence alignment of proteins was carried out using the web-based ClustalW software (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

3. Results

3.1. The Hpi and SlpA proteins of D. radiodurans are closely associated in the cell envelope

A translucent pink membranous pellet obtained in the Hpi preparation consisted of four major bands (123 kDa, 104 kDa, 91 kDa and 58 kDa) and several minor bands (84 kDa to 14 kDa) as shown in Fig. 1a.

The Hpi protein is expected to be a 99 kDa protein but the preparation also had one band of higher mass of 123 kDa. Further, on treating the isolated Hpi fraction with 2% SDS at 60 °C, the 123 kDa band disappeared completely, as shown in Fig. 1a.

Results from peptide mass fingerprinting of the various polypeptides excised from the gel, revealed that the band which always co-elutes with the Hpi layer was in fact SlpA (Fig. 1b). Other lower molecular mass bands, from 104 kDa up to 58 kDa, were all identified as Hpi proteins (Fig. 1b) and appear to be products of specific cleavage of the Hpi protein.

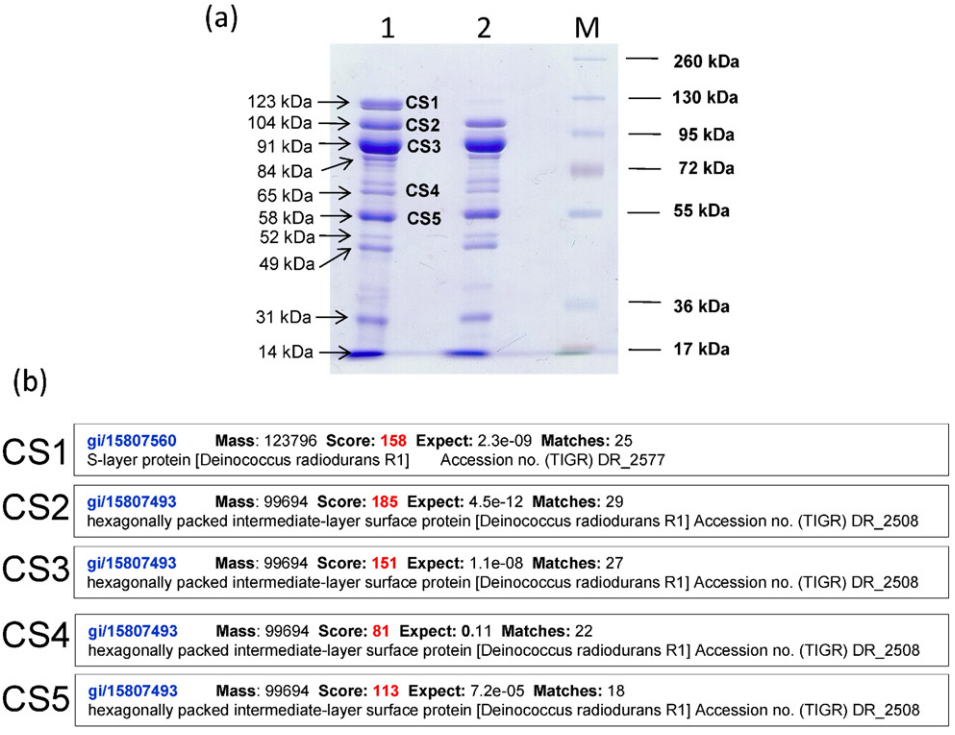

Fig. 1. Isolation of the Hpi layer and identification of polypeptides obtained by peptide mass fingerprinting. (a) Hpi layer isolated from *D. radiodurans* was treated with SDS at 60 °C for 15 min. Proteins (30 μg), isolated before (lane 1) or after (lane 2) the SDS treatment were resolved by 10% SDS-PAGE and stained with CBB. Lane M depicts molecular mass standards co-electrophoresed on the same gel. (b) Five of the polypeptides (marked CS 1 to CS 5) from Hpi preparation were processed for identification by MALDI-TOF-MS. The corresponding MASCOT search results for each polypeptide are shown.

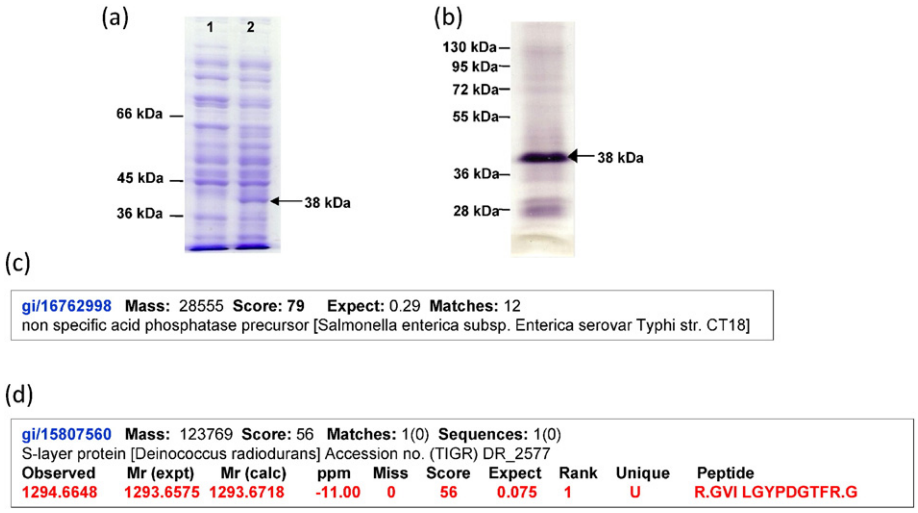

Fig. 2. Overexpression and activity of the SLH-PhoN in recombinant *E. coli* cells. (a) Proteins (30 μg) extracted from SLH-PhoN expressing *E. coli* cultures, incubated without (Lane 1) or with (Lane 2) IPTG, were resolved by 10% denaturing PAGE and stained with CBB. (b) Proteins (30 μg) extracted from SLH-PhoN expressing cultures were resolved on 10% denaturing gel. The gel was renatured in 1% Triton X-100 in 100 mM acetate buffer, pH 5.0 and developed in NBT–BCIP solution. Protein identified for the 38 kDa band (c) shown in Fig. 2(a) and the 1294 Da peptide released upon its trypsinization (d).

3.2. The SLH domain of SlpA protein binds the deinococcal peptidoglycan-containing cell wall sacculi

The nucleotide sequence coding for the N-terminal SLH domain was fused to the *phoN* gene, and over-expressed. The region of the DR_2577 ORF of *D. radiodurans* constituting the SLH domain was determined (Fig. S1) by the conserved sequence generated from literature [31] and by determining the homology between SlpA protein of *T. thermophilus* and DR_2577 using the Clustal W web-based software. The alignment of the sequences showed good homology from 35th amino acid of the deinococcal SlpA protein till 150 amino acids from the N terminal region. In this region, however, the signature homology sequence extended between 36 and 99 amino acids of the SlpA protein and is highlighted in gray (Fig. S1). Therefore, the nucleotide sequence encoding the first 99 amino acids of the SlpA protein, inclusive of its signal peptide, was combined with *phoN* ORF and used to over-express the fusion protein in *E. coli.*

PhoN without its signal peptide, a 26 kDa protein, was expressed as a 38 kDa protein, when fused to the sequence coding for the SLH domain along with its C-terminal His-tag (Fig. 2a). The fusion protein showed phosphatase activity on a zymogram (Fig. 2b). The identity of the 38 kDa protein (Fig. 2a) was further confirmed by peptide mass fingerprinting using mass spectrometry (Fig. 2c and d). Since the phosphatase enzyme constituted a larger portion of the fusion protein, the first hit generated on the MASCOT software was the non-specific acid phosphatase from *S. enterica* serovar Typhi (Fig. 2c). In order to confirm presence of the small SLH domain, a 1294 Da peptide was chosen for MS/MS analysis because it matched theoretical trypsin digest of SLH domain, but did not match the acid phosphatase from *S. enterica* (data not shown). The MS-MS spectrum of 1294 Da peptide matched with the SLH domain of SlpA protein (Fig. 2d), thereby confirming the expression of desired SLH-PhoN fusion protein.

Peptidoglycan along with covalently linked polymers including SCWP was isolated from *D. radiodurans*, as described in the Material and methods section, wherein this cell wall fraction is reported to remain insoluble while the remaining cell components are solubilized and removed through centrifugation. Ornithine, an amino acid known to be present in the deinococcal cell wall, was estimated to be present at around 61 μg ornithine/mg of peptidoglycan (6.1% of the deinococcal peptidoglycan dry weight), which is in good agreement with the values reported in literature for peptidoglycan from *T. thermophilus* (6.7%) [14].

In cell wall binding assays (Fig. 3), the insoluble fraction (I) represented the protein bound to the peptidoglycan-containing cell wall sacculi. A zymogram was carried out with the soluble, wash

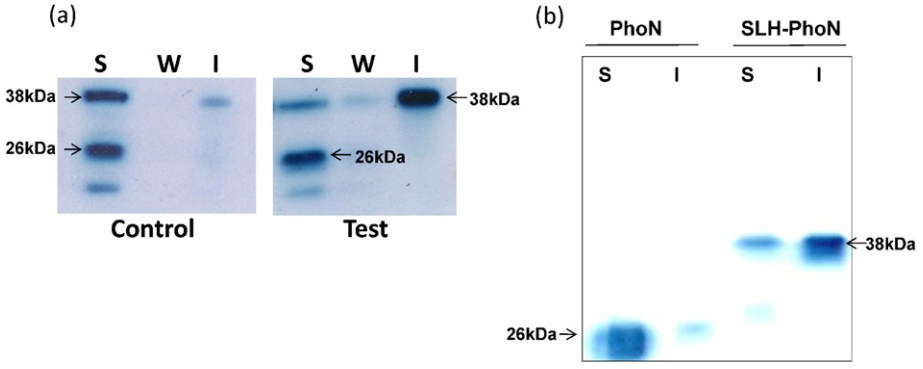

Fig. 3. Binding of SLH-PhoN fusion protein to peptidoglycan-containing cell wall. (a) Cell-free extracts from recombinant *E. coli* cells expressing SLH-PhoN fusion protein were incubated in the absence (Control) or presence (Test) of deinococcal cell wall at 4 °C, following which soluble fraction (S) was separated by centrifugation. A wash (W) was given and the insoluble fraction (I) obtained was incubated at 50 °C for 15 min in non-reducing Laemmli's buffer. All the fractions were resolved by 10% non-reducing SDS-PAGE and developed for phosphatase activity. (b) Peptidoglycan containing deinococcal cell wall was incubated with cell-free extracts of *E. coli* expressing either PhoN or SLH-PhoN fusion protein and processed as described in (a) above.

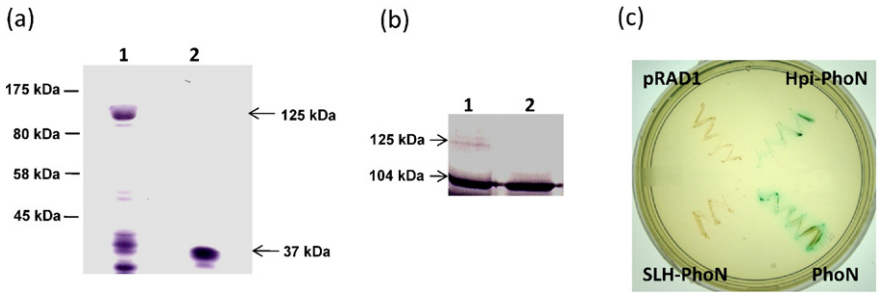

Fig. 4. Phosphatase activity of fusion protein in recombinant *D. radiodurans* cells. (a) Zymogram showing *in-gel* phosphatase activity of proteins (20 μg) extracted from *D. radiodurans* cells expressing Hpi-PhoN (Lane 1) or SLH-PhoN (Lane 2). (b) Proteins (50 μg) were separated by 10% SDS-PAGE, electroblotted and immunodetected using an anti-Hpi antibody. (c) Recombinant *D. radiodurans* cells were patched on TGY with chloramphenicol (3 μg/ml), phenolphthalein diphosphate (PDP) (1 mg/ml) and methyl green (MG) (5 μg/ml). The recombinants positive for whole cell phosphatase activity appeared as green colored streaks. *D. radiodurans* cells carrying the empty vector pRAD1 served as negative control.

and the insoluble fractions of the cell wall binding assay. Most of the activity for SLH-PhoN fusion protein was found in the insoluble fraction while in control, where no peptidoglycan was added, almost all the phosphatase activity was present in the soluble fraction (Fig. 3a). A smaller degradation product of the SLH-PhoN protein, which appeared in zymograms, did not bind the peptidoglycan-containing cell wall sacculi. A similar binding assay carried out with cell-free extracts containing PhoN (without SLH domain), as a negative control, also did not show binding (Fig. 3b).

3.3. The Hpi-PhoN fusion protein localizes exclusively to membrane fraction while SLH-PhoN protein is present both in the cytoplasm and membrane bound fraction

A zymogram using protein extracts from recombinants carrying S-layer-PhoN chimeras, revealed an activity band of the expected size of 125 kDa for the Hpi-PhoN protein as compared to 37 kDa for SLH-PhoN protein in recombinant *D. radiodurans* cells (Fig. 4a). The Hpi-PhoN fusion protein was further confirmed by immunodetection of a 125 kDa band, in addition to the 104 kDa band for Hpi protein, using an anti-Hpi antibody (Fig. 4b). *D. radiodurans* cells carrying the *hpi-phoN* chimeric gene displayed green colored colonies on histochemical plates, while those bearing *SLH-phoN* did not (Fig. 4c).

Fractionation studies showed that phosphatase activity could be detected only in the membrane bound fraction of recombinant *D. radiodurans* Hpi-PhoN cells, predominantly as an expected 125 kDa band (Fig. 5a). No phosphatase activity of Hpi-PhoN was detected in the cytosolic fraction. In the case of SLH-PhoN expressing cells, around 65% of the phosphatase activity was associated with the cell envelope and rest in the cytosol (Fig. 5b).

The results clearly revealed a more exterior localization of Hpi-PhoN, compared to SLH-PhoN, in *Deinococcus* cell envelope.

4. Discussion

The Hpi layer is the outermost proteinaceous layer in *D. radiodurans* [4–5], which interacts with the lipid rich backing layer [1] and yields multiple bands on denaturing polyacrylamide gels, even when protease inhibitors are used [11]. The Hpi protein is a 99 kDa protein, but has always been obtained as 104 kDa band [10–11] possibly due to glycosylation of the protein [32]. The multiple bands have been speculated to be a result of *in vivo* degradation of Hpi [11]. The results presented in Fig. 1 demonstrate that the various peptide bands obtained on resolving the Hpi layer by SDS-PAGE are discreet *in vivo* cleavage products of the Hpi protein. The observation that a ~123 kDa protein co-purified with the Hpi layer, but disappeared on treatment of the Hpi with SDS at 60 °C has been reported before [10] and has been established as the SlpA protein of *Deinococcus*, in this study (Fig. 1). The co-elution of SlpA with Hpi protein confirmed that these proteins are tightly associated in the cell envelope of *D. radiodurans*. It shows either a direct interaction of SlpA with the Hpi protein or an interaction with the lipid rich backing layer suggested earlier [5], remnants of which may co-isolate with Hpi layer. A recent study has also indicated a structural association between SlpA and Hpi in *Deinococcus* [33].

Earlier studies in *T. thermophilus* showed that a single SLH domain found at the N terminus of the SlpA protein bound a cell wall polymer covalently attached to the peptidoglycan [13–14]. This polymer was speculated to be an envelope of thick layer of unstructured material between the peptidoglycan and the S-layer — outer membrane complex. Similar uncharacterized layer was visualized in *D. radiodurans* cells by TEM and an antibody raised against S-layer-attached cell wall fragments of *T. thermophilus*, αSAC, showed cross reactivity with cell walls of *D. radiodurans* [14]. SlpA mutants of *T. thermophilus* resulted in peeling off of the outer membrane from the peptidoglycan layer, suggesting that the SLH domain anchors the outer membrane to the bacterial cell

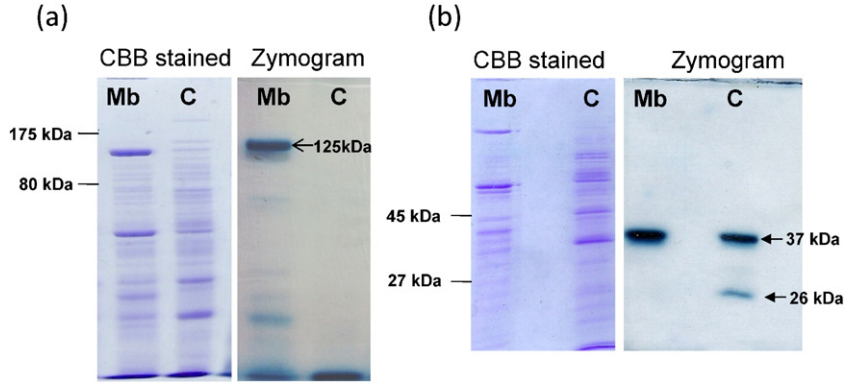

Fig. 5. Localization and cell-based activity of the fusion proteins in recombinant *D. radiodurans* cells. Recombinant cells expressing (a) Hpi-PhoN or (b) SLH-PhoN fusion proteins were sonicated and cytosolic (C) and membrane bound (Mb) fractions were separated by centrifugation. Proteins (20 μg) were resolved by 10% non-reducing SDS-PAGE. Gels stained with CBB or processed for zymogram are shown.

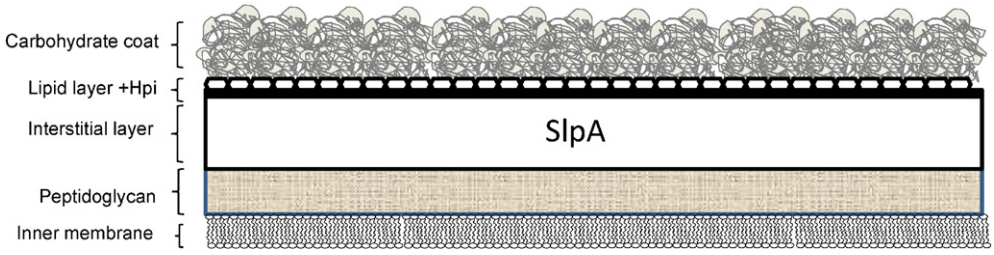

Fig. 6. The cell wall structure of *D. radiodurans*, with the likely location of SlpA protein marked.

wall [14]. Similar results have been reported in the SlpA mutant in *D. radiodurans* [4].

The SLH domains occur in variable numbers in many types of proteins of phylogenetically unrelated bacteria [1]. S-layer proteins from Gram-positive bacteria typically possess three SLH motifs with 10–15 conserved amino acids [12]. The SLH-PhoN fusion protein provided an easy tool to study cell wall binding by SLH domain in *D. radiodurans*.

In *D. radiodurans*, the SLH-PhoN fusion protein bound peptidoglycan containing cell-wall sacculi while PhoN did not, proving that cell wall binding resulted exclusively from SLH domain present in a single copy at the N terminus of SlpA (Fig. 3). The non-covalent association of the SlpA protein with the Hpi layer at one end and peptidoglycan at the other, forms a continuum in the interaction between different layers of the deinococcal cell envelop unraveling its location and orientation. It suggests that the SlpA protein must span the space extending between the peptidoglycan and the backing layer of the deinococcal cell envelope, *i.e.* the interstitial layer. Considering that SlpA is one of the most abundant proteins produced in *D. radiodurans* cells, it should occupy a significantly large volume in the cell envelope. It is, therefore, tempting to speculate that the interstitial layer may actually be composed of the SlpA layer and possibly the secondary cell wall polymers attached to the peptidoglycan (Fig. 6). The association of SlpA and Hpi further substantiates the popular notion that the lattice structure of the Hpi layer may continue down to the rest of the outer membrane to facilitate cell trafficking [33–34].

To date, surface display in *D. radiodurans* has not been attempted. For this purpose, the Hpi protein seems to be an automatic choice due to its location as a penultimate layer in the multi layered cell wall [5]. The entire protein was used for fusion with PhoN. Further, since the C terminal of the Hpi protein is known to be exposed to the outer surface [35], PhoN was fused to C terminus of the Hpi protein in the present work. The other S-layer protein, SlpA is a rather large protein (123 kDa) which is poorly characterized. Therefore, the utility of the small SLH domain of SlpA protein in membrane anchoring was explored as has been done in some Gram positive organisms, mentioned earlier.

The Hpi-PhoN fusion protein was exclusively present in the membrane bound fraction of recombinant *D. radiodurans* carrying the chimeric gene and displayed phosphatase activity both *in vivo* and *in vitro*. The result demonstrates that Hpi-PhoN accurately localizes as a functionally active fusion protein in deinococcal cell envelope. Thus, Hpi emerged as an efficient membrane targeting vehicle for surface display in *D. radiodurans* in this study. Considering that the Hpi layer is a covalently cross-linked layer whose structure is preserved in the presence of sodium dodecyl sulfate and urea up to a temperature nearing 100 °C, this protein serves as an excellent stable carrier for surface display [10]. The fact that Hpi-PhoN constituted a rather small fraction of the total Hpi protein (Fig. 4b), can be improved upon in future, by expressing this construct in a Δ*hpi* background to avoid competition with resident Hpi in surface localizaion.

Multiple copies of the SLH domain have served as excellent surface display vehicles in Gram positive organisms such as *Bacillus*, where cell wall architecture is less complicated and constitutes only the peptidoglycan layer immediately followed by S-layer as the outermost component [17,36]. *D. radiodurans* recombinant cells bearing *slh-phoN* fusion construct, failed to show cell based PhoN activity on histochemical plates or in pNPP assay in solution. But cell-free extracts showed activity in zymograms. Lack of cell-based *in vivo* PhoN activity in recombinant *D. radiodurans* may be due to the close interaction of the SLH domain with the SCWP of peptidoglycan resulting in the protein being buried deep inside the peptidoglycan and possibly interfering with proper folding. In addition, the SLH-PhoN fusion protein was probably capable of anchoring only partially to the deinococcal cell wall.

D. radiodurans has found applications in bioremediation and vaccine development [7,19,21–22,37–39]. Both these fields would benefit from development of surface display systems described in this study. Making surface layers composed exclusively of the fusion protein is an important future direction. The recombinant fusion layer so formed would offer the advantage of high density of relevant protein in precise orientation which in turn could find several applications especially in diagnostics.

5. Conclusion

The association of SlpA with Hpi on one side and peptidoglycan on the other, localizes this protein in the 'interstitial' layer of the deinococcal cell wall. The work provides insights into the organization of two surface layer proteins (Hpi and SlpA) in the cell wall of *D. radiodurans* and demonstrates utility of Hpi as an efficient surface localizing protein that can be exploited for suitable applications of this organism.

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Transparency document

The Transparency document associated with this article can be found, in the online version.

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