Involvement of the S-layer proteins Hpi and SlpA in the maintenance of cell envelope integrity in *Deinococcus radiodurans* R1

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The potential functions have been investigated of two proteins in *Deinococcus radiodurans* R1 predicted to be involved in the maintenance and integrity of the S layer: the hexagonally packed intermediate (Hpi) protein, and SlpA (DR2577), a homologue of an S-layer SlpA protein in *Thermus thermophilus*. Deletion of the *hpi* gene had little effect on the structure of the cell envelope or on shear- or solvent-induced stress responses. However, deletion of the *slpA* gene caused substantial alterations in cell envelope structure, and a significant defect in resistance to solvent and shear stresses compared to the wild-type. Ultrastructural analysis of *slpA* mutant cells indicated loss of much of the outer Hpi protein carbohydrate coat, the 'pink envelope', and the membrane-like backing layer. Together these results suggest that the SlpA protein may be involved in attachment of the Hpi surface layer to the inner cell envelope, and that SlpA may play an important role in the maintenance of cell envelope integrity in *D. radiodurans*.

INTRODUCTION

Crystalline surface layers (S layers) made of proteins or glycoproteins are a commonly observed surface structure in prokaryotes (Sleytr *et al.*, 1993). Most S-layer proteins possess a signal sequence, predominantly contain hydrophobic and acidic amino acids, and, with the exception of archaeal S layers, are low in sulfur-containing amino acids (Kuen & Lubitz, 1996; Kuen *et al.*, 1996; Sleytr *et al.*, 1993). Several strains possess more than one S-layer protein, and these may be co-expressed or selectively expressed in response to specific environmental conditions (Couture-Tosi *et al.*, 2002; Sleytr *et al.*, 1993). The S-layer protein(s) may represent up to 15 % of cell mass when present, causing a burden on the cell (Sleytr *et al.*, 1993) that may lead to S-layer loss after long-term cultivation under idealized laboratory conditions (Sleytr *et al.*, 1993; Thompson *et al.*, 1982).

A number of functions have been proposed for S layers, which in some cases have been experimentally confirmed (Beveridge *et al.*, 1997; Rachel *et al.*, 1997; Sleytr *et al.*, 1993). These include adhesion, enzyme attachment, prevention of adsorption of macromolecules, and cellular stability and rigidity (Beveridge *et al.*, 1997; Rachel *et al.*, 1997; Sleytr

et al., 1993). The surface layers of *Deinococcus radiodurans* strains SARK and R1 have been closely studied since the early 1960s, the initial interest being rooted in the extreme radiation resistance of these organisms (Thornley *et al.*, 1965). The role of S layers in this extreme resistance has not yet been demonstrated, but a role in response to radiation damage has been proposed (Gentner & Mitchel, 1975). A diagram of the cell envelope structure in *D. radiodurans* as proposed by Emde *et al.* (1980) is shown in Fig. 1. Stacked upon the inner membrane face are a peptidoglycan layer, an interstitial layer, and the 'pink envelope'. The pink envelope contains the S layer [hexagonally packed intermediate (HPI) layer and lipid-rich backing], lipids, carbohydrates, proteins, four to five carotenoids, and most likely the outer membrane (Kübler & Baumeister, 1978; Thompson *et al.*, 1982; Work & Griffiths, 1968). The outermost layer of the pink envelope is a long-chain carbohydrate coat, and in 5–10 % of the cell population it is only minimally present (Baumeister *et al.*, 1981; Emde *et al.*, 1980). Of the pink envelope components, the hexagonal network of the outer surface array has received the most attention. The S layer is predominantly made up of one protein, Hpi (Baumeister *et al.*, 1982; Peters & Baumeister, 1986). It has been proposed that hydrophobic interactions are responsible for attachment of the S layer to the outer membrane in the backing layer, as well as for the association of the S-layer units (Thompson *et al.*, 1982). Although the HPI layer itself has been characterized relatively well, the mechanism of the unusually strong attachment of the HPI layer to the rest of

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Abbreviations: HPI, hexagonally packed intermediate; S layer, surface layer; SEM, scanning electron microscopy; SLH, S-layer homology.

Fig. 1. Proposed structure of the *D. radiodurans* envelope, adapted from Emde *et al.* (1980).

the pink envelope has not yet been determined. Therefore, we performed phenotypic tests and conducted electron microscopy to determine the role of HPI and another potential S-layer protein, SlpA, in the maintenance of cell envelope integrity in *D. radiodurans*.

METHODS

Bacterial strains and growth conditions. *Escherichia coli* JM 109 (Stratagene) was grown in Luria–Bertani broth or on agar plates at 37 °C in the presence of 50 µg ml⁻¹ ampicillin or kanamycin. *D. radiodurans* R1 (ATCC 13939) strains were grown in tryptone/glucose/yeast extract (TGY; Murray, 1992) at 30 °C on agar plates or in broth with shaking at 250 r.p.m. The following antibiotics were used: chloramphenicol, 3 µg ml⁻¹; kanamycin, 8 µg ml⁻¹ in agar plates and 4 µg ml⁻¹ in liquid cultures.

Chromosomal DNA preparation. A protocol for chromosomal DNA mini-preparation from *D. radiodurans* was developed, based on the larger-scale method published earlier (Udupa *et al.*, 1994), as follows. Cells were harvested from 1 ml culture in late-exponential to stationary-phase growth by centrifugation for 2 min at 16 000 g in a tabletop centrifuge. Cells were resuspended in 500 µl lysis buffer, consisting of 50 mM Tris/HCl, pH 8.0, 50 mM EDTA, 0.2 M NaCl, 2 mg lysozyme ml⁻¹, 200 µg ml⁻¹ each of proteinase K and Pronase E (Epicentre), and 0.6% SDS. Cells were incubated in this solution for 4–24 h, until lysis was apparent. Protein was removed by one or two extractions by phenol/chloroform/isoamyl alcohol (Roche), followed by one extraction by 0.5 ml chloroform. The upper layer was added to 1.2 vols 2-propanol and inverted 15 times to precipitate DNA. The DNA was collected by centrifugation or spooled onto a glass rod, washed with ice-cold 70% (v/v) ethanol, allowed to dry, and dissolved in 50–200 µl 0.1 M Tris buffer, pH 8.0.

Electroporation. A new method for generating competent cells was developed and optimized as follows. Cells were grown in 50 ml TGY to early exponential phase (OD₆₀₀=0.4–0.6), chilled on ice for 30 min, and pelleted by centrifugation at 4 °C for 8 min at 2700 g. Cells were then resuspended in 10 ml 0.1 mM HEPES buffer, pH 8.0, pelleted as above, resuspended in the same volume of buffer, pelleted again and resuspended in 5 ml of the buffer. Cells were pelleted once again and resuspended in 10 ml sterile ice-cold 10% (v/v) glycerol, pelleted again and finally resuspended in 1 ml 10% glycerol. Aliquots (100 µl) were frozen at –80 °C for up to 6 months. The highest transformation efficiencies were obtained when the cells were electroporated at 10 kV cm⁻¹ in a 0.2 cm electrode gap cuvette (data not shown). This voltage was routinely used with a capacitance of 25 µF and resistance of 200 Ω. TGY broth (1 ml) was added, following by incubation at 30 °C with shaking for 1 h (plasmids) or 12–18 h (to complete chromosomal insertion), and then plating to selective media.

Colony PCR. A sterile platinum wire was used to transfer cells from a single colony to 20 µl PCR master-mix (Invitrogen), to which 5% (v/v) DMSO was added. PCR was carried out according

to the manufacturer's instructions (Invitrogen), with an additional 5 min at 95 °C before the first cycle, and an annealing temperature of 52 °C. Primers used are listed in Table 1.

RT-PCR. The expression of DR2508 and DR2577 was confirmed by analysing RNA prepared from wild-type *D. radiodurans* R1 grown at 30 °C as previously reported (Schmid *et al.*, 2005), and carrying out PCR with the internal primers listed in Table 1. The PCR was carried out with 'Ready To Go' RT-PCR beads (Amersham) according to the manufacturer's guidelines. Product-size controls were also carried out with the chromosomal DNA template.

Generation of deletion mutants Δhpi (DR2508) and $\Delta slpA$ (DR2577). The allelic exchange vector pCM184 (Marx & Lidstrom, 2002) was modified for use in *D. radiodurans* by cutting out the tetracycline-resistance cassette and the IncP origin of transfer, and replacing them with a fragment of pI8 (Meima & Lidstrom, 2000) containing a promoter originally obtained from *D. radiodurans* SARK followed by the chloramphenicol-resistance gene, to generate pHMR173. This promoter is not found in the *D. radiodurans* R1 chromosome and was chosen to avoid undesirable recombinations within the chromosome. This vector was further modified by the addition of a minimal *groESL* promoter upstream of the kanamycin cassette but still within the *loxP* sites. The primers mini-PgroF and mini-PgroR (Table 1) were designed to amplify the 48 bp region of the *D. radiodurans* R1 *groESL* promoter containing the transcription start site and the –10 and –35 regions. Each primer was designed to contain a *Dra*III site (in bold type in Table 1) to facilitate cloning. The 72 bp PCR product was generated with the *D. radiodurans* R1 chromosomal DNA as template, cut with *Dra*III, and inserted directionally into the non-palindromic *Dra*III site upstream of the kanamycin cassette to drive kanamycin resistance in *D. radiodurans* R1. The insertion was verified by PCR. The resulting vector, pHMR186, contained multiple cloning sites on either side of the *loxP*-flanked kanamycin cassette. To generate the deletion mutants, PCR products were generated complementary to the regions upstream and downstream of *hpi* and *slpA*. Primers were specifically designed to delete the entire target genes, essentially as described by Marx & Lidstrom (2002). The PCR products were sequenced to ensure that no errors were introduced during PCR. The resulting allelic exchange vectors pHMR202 (containing *hpi*) and pHMR195 (containing *slpA*) were transformed into *D. radiodurans* R1, and colonies selected in the presence of kanamycin. To identify double-crossover recombinants, colonies were screened for chloramphenicol sensitivity on plates. Complete deletion was further confirmed by negative PCR tests with primers targeted to the 5' and 3' regions of each gene. Primer sets used in this study are listed in Table 1. We also constructed pHMR179 expressing Cre recombinase on the *D. radiodurans*–*E. coli* shuttle vector pRAD1 (Meima & Lidstrom, 2000), appropriate for generating unmarked mutants by excising the kanamycin-resistance cassette (data not shown).

Transmission electron microscopy. Cells were washed with double-distilled water and fixed in modified Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde, 8 mM CaCl₂ in 0.1 M cacodylate buffer, pH 7.4) for 2 h at 4 °C. Samples were washed in cacodylate buffer and post-fixed in 1% osmium tetroxide

Table 1. PCR primers and purposes of products

Cm, chloramphenicol; kan, kanamycin.

PCR product	Primer name	Primer sequence (5'–3')
DraIII flanked 48 bp Pgro promoter	mini-PgroF mini-PgroR	cgccacgttggtcggtcagttgacattttct gcgacacaacgtgatccgtcacggatgtagag
DR2577 flank 1, upstream of <i>slpA</i>	DR2577-1044r_ <i>SacI</i>	gacggagctcatcctgccgagcggccttac
DR2577 flank 2, downstream of <i>slpA</i>	DR2577-20r_ <i>SacII</i> DR2557 + 3543f_ <i>MunI</i>	gcagcccggtgctgcgtcgagcagcgttt aggagagatctgcttcggcgcaagtatt
DR2508 flank 1, upstream of <i>hpi</i>	DR2577 + 4553r_ <i>BglII</i> DR2508-943f_ <i>SacI</i>	gaggtcaattgttcggcggtgttttgtt cagagctcgttgccgcattgagcttggtg
DR2508 flank 2, downstream of <i>hpi</i>	DR2508-13r_ <i>SnaBI</i> DR2508 + 2846f_ <i>Asp718</i> DR2508 + 3691r_ <i>BglII</i>	cgctacgttaagcggatcggaagcagatcta cggttaccagaagtacagcaccactaaaaag cgagatctgtgaaacatcgccacgatagg
Internal PCR to verify <i>slpA</i> deletion	DR2577_278f DR2577_610r	catgaccgccgaggacatga cggcttgcaggtccaggat
Internal PCR to verify <i>hpi</i> deletion	DR2508_11f DR2508_355r	tatcgactcatggtctca gtgctaggaaccgtcgtgta
Verification of insertion by kan marker presence	kan_fwd kan_rev	aagccacgttggtctca ccgtcaagtcagcgtaat
Screen for insertion of vector DNA with Cm	cat-30f cat + 711r	ccgagcttcgacgagatt attcaggcgtagcaccag
<i>slpA</i> insertion site flank to flank end length	DR2577-1044r_ <i>SacI</i> DR2577 + 4553r_ <i>BglII</i>	gacggagctcatcctgccgagcggccttac gaggtcaattgttcggcggtgttttgtt
<i>hpi</i> insertion site flank to flank end length	DR2508-943f_ <i>SacI</i> DR2508 + 3691r_ <i>BglII</i>	cagagctcgttgccgcattgagcttggtg cgagatctgtgaaacatcgccacgatagg

in buffer for 2 h at room temperature. Following three 5 min wash steps, cells were embedded in 1.5 % Noble agar and dehydrated in a graded series of ethanol (35, 50, 70, 80, 90, 95, 100 %). Blocks were then infiltrated at three concentrations of Spurr's reagent using propylene oxide as the transition solvent, and ending in 100 % Spurr's resin. Thin sections of samples were stained with 7 % uranyl acetate and Reynolds lead citrate for 20 and 10 min, respectively. Samples were viewed using a JEOL 1200 Ex II transmission electron microscope operated at 80 kV.

Scanning electron microscopy (SEM). Cells were fixed as described above, washed three times for 5 min with double-distilled water, spotted onto plastic cover slips coated with 1 % poly-L-lysine, dehydrated in a graded series of ethanol to 100 %, and critical point dried. Samples were sputter coated with gold/palladium, and viewed with a JEOL JSM 6300F scanning electron microscope at 15 kV.

Protein analysis. Overnight cultures of *D. radiodurans* R1 and HMR195 were left without shaking for 12–16 h to allow natural settling of cells. After settling, the supernatant of the *D. radiodurans* HMR195 mutant was cloudy and contained visible flocs, whereas that of the wild-type strain was clear. Proteins were extracted from 3 ml of the supernatants using chloroform:methanol (1:2). The *D. radiodurans* R1 wild-type pellet was resuspended in 25 µl SDS-PAGE loading buffer (Sambrook *et al.*, 1989), and the HMR195 pellet was resuspended in 50 µl loading buffer, and both were boiled for 5 min at 95 °C, after which equal volumes were loaded onto an 8 % polyacrylamide gel along with molecular mass markers (Fermentas). Three of the most prominent bands from the *D. radiodurans* HMR195 supernatant were cut out of the gel.

Gel slice digestion and protein identification were performed by the Proteomics and Spectroscopy Laboratory at the Fred Hutchinson Cancer Research Center, Seattle, WA, by the following method.

Proteolytic digestion of Coomassie-stained gel slices was carried out as described by Shevchenko *et al.* (1996). Following digestion, samples were desalted using a microC18 ZipTip (Millipore) and dried. Samples were then resuspended in 7 µl 0.1 % trifluoroacetic acid (TFA) and analysed by liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI MS/MS) with an LCQ DECA XP mass spectrometer (ThermoElectron), using an instrument configuration described by Gatlin *et al.* (1998). Data were collected in a data-dependent mode in which a MS scan was followed by MS/MS scans of the three most abundant ions from the preceding MS scan. MS data were searched against the *D. radiodurans* protein database (White *et al.*, 1999), using the software search algorithm COMET (Institute for Systems Biology). Protein identifications were considered valid if at least two peptides were matched to a protein and if the peptide matches had raw scores greater than 200 for +1 ions, 300 for +2 ions, and 300 for +3 ions, Z scores greater than 4, and percentage ions of greater than 15 %.

Shear stress survival assay. Overnight cultures of *D. radiodurans* R1 and the deletion mutants in *hpi* (HMR202) and *slpA* (HMR195) were diluted to OD₆₀₀ 0.5 in 1 ml 1 mM HEPES buffer with 500 µl of 0.1 mm zirconium–silica beads added. Cell suspensions were exposed to shear stress by vortexing for varying lengths of time (0, 30, 120 or 240 s). Survival was assessed by serial dilutions, spotting 5 µl aliquots of each dilution in triplicate onto TGY plates. c.f.u. were calculated from the spot of each dilution series with countable colonies. Because of the aggregation of *slpA* mutant cells, the survival rate was underrepresented at 0 min, but after samples were vortexed for 30 s, the colony count increased. To adjust for this factor, all cultures were normalized to the c.f.u. counts after 30 s of vortexing.

Growth in the presence of toluene. Cells were streaked from frozen stocks onto TGY plates containing antibiotics, as appropriate. A single colony was grown overnight at 30 °C with shaking in 2 ml

TGY broth with antibiotics if needed, and 0.25–1 ml was transferred to 25 ml TGY without antibiotics in a 250 ml flask. After growth overnight, the OD was measured using a Klett colorimeter (Klett–Summerson) that had been calibrated to a spectrophotometer at 600 nm. Cultures were diluted in Klett flasks to the OD corresponding to early exponential phase (a Klett value of 20–25, OD₆₀₀ of about 0.16–0.2) in 12.5 ml TGY broth, and were capped with rubber stoppers. The initial OD was recorded and deviations in readings due to the vials were subtracted from this and subsequent readings. The cultures were grown with shaking at 30 °C and checked hourly until reaching a density of 40–45 Klett units. At this point, the vials were briefly removed from the shaker to add the appropriate amount of toluene, restoppered tightly, and the stoppers were sealed with Parafilm to prevent loosening. The stoppers were not removed after this time, and measurements were taken using the Klett colorimeter until readings stabilized in stationary phase.

RESULTS

Identification of putative S-layer genes

The *hpi* gene (*DR2508*) has been annotated in the genome of *D. radiodurans* R1 (White *et al.*, 1999) by its homology with the *hpi* gene from the *D. radiodurans* SARK strain (Peters & Baumeister, 1986). To identify additional candidates for S-layer proteins, we searched the *D. radiodurans* R1 genomic sequence for genes containing the S-layer homology (SLH) domain (White *et al.*, 1999). Among several other putative S-layer-related protein genes, *DR2577*, *DR1124* and *DR0115* were identified as containing domains resembling the annotated SLH domain. The corresponding gene products are predicted to have molecular masses of 124, 43 and 31 kDa, respectively. *DR2577* revealed homology to an S-layer protein (SlpA) from a related organism, *Thermus thermophilus* HB8 (25 % identity and 37 % similarity at the amino acid level), suggesting that *DR2577* was a candidate for a secondary S-layer protein in *D. radiodurans* R1. *DR2577* was therefore chosen for further experimentation. For the purpose of this study, we henceforth refer to *DR2577* as *slpA*.

Deletion of the *hpi* and *slpA* genes

We first tested the expression of *hpi* and *slpA* in *D. radiodurans* R1 via RT-PCR, and obtained positive results (data not shown), indicating that *hpi* and *slpA* are actively expressed in *D. radiodurans* wild-type. In order to determine the possible roles of Hpi and SlpA in cell growth, morphology, and resistance to shear and solvent stresses, deletion mutants in both genes were generated, resulting in the mutants HMR202 (defective in *hpi*) and HMR195 (defective in *slpA*). We further attempted to generate a double *hpi/slpa* mutant, but these efforts were not successful, possibly due to alterations in the cell envelope that might have either interfered with cell competency or reduced viability during electroporation and CaCl₂ treatment (data not shown).

Growth phenotypes of the HMR202 (*hpi*) and HMR195 (*slpA*) mutants

When grown in TGY broth at 30 °C, the HMR202 (*hpi*) mutant and wild-type demonstrated equivalent doubling times, whereas that of the HMR195 mutant was about twice the doubling time of the wild-type (Fig. 2), although a similar OD was eventually reached after several days of growth. Mutant HMR195 (*slpA*) revealed a tendency toward clumping, with the clumps resembling highly disordered masses of cells under phase-contrast light microscopy (data not shown). Furthermore, we observed that one of the pair of dividing cells in *D. radiodurans* tetrads would often be much smaller than the other. This suggested that normal cellular division was impaired in the *slpA* mutant strain (data not shown). The colony morphology of mutant HMR195 (*slpA*) also differed from that of the wild-type: the normally smooth and shiny colonies instead appeared rugose and powdery, and were easily broken up with an inoculating loop.

Electron microscopy of *D. radiodurans* R1 and the HMR202 (*hpi*) and HMR195 (*slpA*) mutants

Thin section microscopy (Figs 3, 4, 5 and 6) and SEM (Fig. 7) were used to visualize the effects of the mutations on *D. radiodurans* whole-cell and envelope morphology. The HMR202 (*hpi*) mutant did not appear significantly altered in thin section micrographs. The most obvious change was that the peptidoglycan layer seemed to stain more completely and appeared more dense, while the areas surrounding the peptidoglycan layer appeared less dense than in the wild-type cells. It also appeared, in general, that the outer layer containing the backing layer, the HPI layer and the carbohydrate coat was not as thick in this mutant as in the wild-type cells. SEM also revealed few differences

Fig. 2. Growth of *D. radiodurans* R1 (○), and the *hpi* (□) and *slpA* (△) mutants in TGY broth.

Fig. 3. Thin section electron micrograph of (a) *D. radiodurans* R1 tetrad and (b) magnification of the membrane. Layers corresponding to those in Fig. 1 are indicated.

between the HMR202 mutant and the wild-type strain. The shape of the cells did not seem affected, and the overall tetrad shape appeared similar. However, the surfaces of the HMR202 mutant cells appeared smoother than in the wild-type. In particular, they lacked the ‘mushroom shapes’ or ‘beads’ (Baumeister & Kübler, 1978; Thornley *et al.*, 1965), which can clearly be seen in the SEM image of wild-type cells (Fig. 7a).

In contrast, the envelope of the HMR195 (*slpA*) mutant was clearly compromised. As seen from thin section micrographs (Fig. 5), the outer layers appeared to be detached from the cell and peeling in places. However, we observed that the peptidoglycan layer and perhaps some of the interstitial layer remained attached (Fig. 6b). SEM also

showed dramatic differences between the HMR195 mutant and wild-type cells (Fig. 7c). In summary, the electron microscopy images suggested that the mutant cells were shedding exterior layers of the cellular envelope.

Analysis of supernatant proteins in the HMR195 mutant

If left without shaking overnight, *D. radiodurans* R1 wild-type cells settle at the bottom of the flask, leaving a visually clear supernatant. This is also true of *D. radiodurans* HMR202 (*hpi* mutant). However, in *D. radiodurans* HMR195 (*slpA* mutant) culture, flocs of material were visible, even after several days without shaking. To examine this material, proteins were extracted from the supernatant,

Fig. 4. Thin section electron micrograph of (a) *D. radiodurans* *hpi* mutant tetrad and (b) magnification of the membrane.

Fig. 5. Thin section electron micrograph of (a) *D. radiodurans* *slpA* mutant tetrad and (b) magnification of the membrane.

concentrated, and analysed by SDS-PAGE along with the $2 \times$ concentrated supernatant from the *D. radiodurans* R1 culture. Much more protein was extracted from the HMR195 mutant supernatant compared to the wild-type supernatant, as seen on the gels (Fig. 8). In particular, two bands of around 100 kDa, and one band of around 35 kDa, seemed to be significantly and specifically enriched in the mutant supernatant. The two 100 kDa bands (labelled 1 and 2 in Fig. 8) and the 35 kDa band (labelled 3) were removed from the gel and analysed by MS (Table 2). Bands 1 and 2 were both identified as the Hpi (DR2508) protein. The 35 kDa band was very clearly dominated by the DR1185 protein, annotated as an S-layer-like, array-related protein in the genomic database (White *et al.*, 1999). Together with the electron microscopy results, this suggests that the flocculent material in the *slpA* mutant supernatants consists of shed membrane components.

Stress resistance of the HMR202 and HMR195 mutants

To understand the involvement of Hpi and SlpA proteins in maintenance of cellular integrity, the resistance of mutant cells to solvents and the ability to survive shear stress were studied. Fig. 9 shows the survival curves at 30 s to 4 min of continuous vortexing. Because disassociation of cellular clumps in the HMR195 mutant culture occurred during the first 30 s of vortexing, survival was normalized to total c.f.u. after 30 s. *D. radiodurans* R1 showed no appreciable loss of survival over this first 30 s time period (data not shown). The survival of the HMR202 (*hpi*) mutant was not significantly lower than that of the wild-type strain after 4 min of vortexing, whereas in contrast, the survival of the HMR195 (*slpA*) mutant dropped one and a half orders of magnitude, suggesting that SlpA contributes to shear stress resistance.

Fig. 6. Magnified thin section micrographs comparing *D. radiodurans* *hpi* and *slpA* mutant envelopes.

Fig. 7. Scanning electron micrographs of the surfaces of (a) *D. radiodurans* R1, (b) the *hpi* mutant and (c) the *slpA* mutant. The images have been colourized to display surface features. Bars: (a) 100 nm; (b) 1 µm; (c) 100 nm.

When grown with 0.5 % toluene, the HMR202 (*hpi*) mutant showed a slower exponential-phase growth rate and a lower cell density in the stationary phase compared to wild-type (Fig. 10). In contrast, the HMR195 (*slpA*) mutant was again dramatically affected, showing no significant growth over 60 h after the addition of toluene. Taken together, these results suggest that SlpA may play a significant role in

Fig. 8. SDS-PAGE of the supernatants of the *D. radiodurans* *slpA* mutant culture and the R1 wild-type culture.

maintenance of cell integrity, whereas Hpi appears to play a minor role.

DISCUSSION

The structure of the *D. radiodurans* cell envelope has been under study for almost 40 years. The order of the layers of the envelope and the nature of some of these layers have been determined (Baumeister *et al.*, 1986; Emde *et al.*, 1980; Peters *et al.*, 1987), but several questions remain regarding the structure, and little is known about the potential functions of the S layer. In this study, we mutated genes encoding two of the S-layer proteins, *hpi* and *slpA*, and studied phenotypes of the resultant mutants. Although spontaneous mutants lacking an HPI layer have been described in *D. radiodurans* SARK (Thompson *et al.*, 1982), this is the first study conducted with a genetically defined *hpi* mutant. The phenotype of the HMR202 (*hpi*) mutant indicates that the HPI layer is required for the formation of external features of *D. radiodurans* R1. The rippled 'grape-skin' appearance of *D. radiodurans* was first described in 1965 by Thornley and co-workers (Thornley *et al.*, 1965). This topography has also been described as 'irregular granules' or 'small bead-like structures' on the surface of the cells (Baumeister & Kübler, 1978). The *hpi* mutant generated in this study appeared to lack both of these structures, while they appeared to be present in the *slpA* mutant. Whether the bead structures fulfil some function or are simply remnants of S-layer irregularities at the points of division remains unclear. However, the HPI layer does seem to be involved in both the outer cell smoothness and the formation of small bead-like structures on the outer surfaces of *D. radiodurans*. Likewise, the functions tested in this mutant, resistance to vortexing (as a measure of shear stress) and resistance to toluene, were only minimally affected, suggesting that Hpi does not play a major role in these outer-layer-related functions.

Table 2. Identification of proteins enriched in the supernatant of the *slpA* mutant

Band numbers correspond to those shown in Fig. 8.

Band	Protein	Molecular weight (kDa)	Amino acid coverage (%)	Description
1	DR2508	99.4	44.3	Hpi
2	DR2508	99.4	43.4	Hpi
3	DR1185	36.7	79.8	S-layer-like array-related protein

The structural phenotype of the HMR195 (*slpA*) mutant was much more dramatic. From the microscopy studies, it appeared that layers were peeling from the surface of the mutant cells. Tests for supernatant proteins in the HMR195 mutant revealed that these layers must have contained Hpi and DR1185, a protein annotated as an S-layer-like, array-related protein (White *et al.*, 1999). This phenotype is similar to that of the SlpA mutant of *T. thermophilus* HB8 (Olabarria *et al.*, 1996). In *T. thermophilus* HB8, SlpA is an S-layer protein possessing the SLH domain near its N terminus, which is implicated in having a role in attaching to the peptidoglycan layer (Olabarria *et al.*, 1996). The removal of this SLH domain has been shown to result in shedding S layers (Olabarria *et al.*, 1996). In *D. radiodurans*, the S-layer protein Hpi does not contain an SLH domain. However, our results suggest that *D. radiodurans* SlpA may provide this anchoring function. As it is not possible to identify the HPI layer in thin sections (Thornley *et al.*, 1965), and due to difficulties in determining the structure of such an extremely perturbed cell envelope, we were not able to directly discern which layers were peeling off. However, it is clear that the layers peeling away from the cell surface do not have the original curvature of the cell wall. It has been previously determined that the backing layer of the pink

Fig. 9. Survival of *D. radiodurans* R1 and the S-layer mutants after vortexing. ○, R1; △, Δ *slpA*; □, Δ *hpi*.

Fig. 10. Growth of *D. radiodurans* R1 and the S-layer mutants after 0.5% toluene shock. ○, R1; △, Δ *slpA*; □, Δ *hpi*. Arrow, addition of toluene.

envelope, rather than the HPI layer, provides the rigidity and the curvature of the cell envelope (Baumeister *et al.*, 1981), suggesting that the SlpA protein interacts with the backing layer. The *slpA* mutant of *D. radiodurans* was also strikingly more sensitive than the wild-type and *hpi* mutant to both shear and toluene stress, resistance to which is known to be imparted by the cell envelope, thus underscoring the importance of SlpA in outer layer integrity.

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