Crystalline surface layer (S-layers) form one of the most common boundary layers of many eubacteria and archaea and are composed of self-assembling single protein or glycoprotein species. Although the ultrastructure of S-layers has been well characterised with electron and atomic force microscopy, little is known about the position of individual amino acids in the protein, and no X-ray crystal structure is available. We investigated the S-layer protein SbsB from *Geobacillus stearothermophilus* PV72/p2 to identify key residues for assembly. Twenty-three single cysteine mutants, located on the surface of the SbsB monomer, were subjected to a cross-linking screen using a sulfhydryl-reactive photoactivatable reagent. Gel electrophoretic analysis on the formation of cross-linked dimers indicated that four out of 23 mutants were located at the interface. In combination with chemical surface accessibility data, four other residues were mapped to the outer surface and three residues to the inner peptidoglycan-bound surface of the lattice. The HA epitope tag was inserted at each of the surface positions to confirm the residues position via an assembly inhibition screen as assayed by negative staining and electron microscopy. Identification of interfacial residues will enable the rational design of mutated SbsB protein for X-ray crystallization studies. Furthermore, engineered proteins carrying an insert at the outer or inner surface will provide a uniform epitope display with applications for scanning probe techniques.