The first report on the sortase-mediated display of bioactive protein A from *Staphylococcus aureus* (SpA) on the surface of the vegetative form of *Bacillus subtilis*

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**Abstract**

Protein A (SpA) is one of the most important *Staphylococcus aureus* cell wall proteins. It includes five immunoglobulin (Ig)-binding domains which can bind to immune complexes through the Fc region of immunoglobulins. The binding of SpA to the polymeric supports can be used to prepare affinity chromatography resins, which are useful for immunoprecipitation (IP) of antibodies. Protein A is also used to purify many anti-cancer antibodies. In this study, SpA was displayed on the surface of *Bacillus subtilis* cells using a sortase-mediated system to display the target protein to the *B. subtilis* cell wall. A series of plasmids consisting of cassettes for cell wall-directed protein A as well as negative controls were constructed and transformed into *B. subtilis* WASD (*wprA sigD*) cells. SDS-PAGE, western blot, flow cytometry, functional IgG purification assay, and a modified ELISA assay were used to confirm the surface display of SpA and evaluate its function. Semi-quantitative ELISA results showed that the binding capacity of lyophilized Bs-SpA is 100 μg IgG from rabbit serum per 1 mg of cells under optimal experimental conditions. Low production costs, optimal performance, and the use of a harmless strain compared to a similar commercial product predict the possible use of SpA immobilization technology in the future.

**Keywords:** Surface display, *Bacillus subtilis*, Protein A

**Background**

The expression of recombinant proteins fused with signal peptides to sort and direct them to different parts of the bacterial cell surface is called surface display which has many applications such as peptide libraries screening, whole-cell bioconversion, live vaccine production, and whole-cell metal adsorbents [20].

The surface display is a suitable alternative for immobilizing recombinant proteins and enzymes on various polymeric supports and living cells because, unlike traditional physical and chemical immobilization approaches that require various steps, it is biocompatible and not time-consuming or challenging to achieve [25].

There are several ways to efficiently display heterologous polypeptides on the surface of the bacterial cells. Various surface proteins such as outer membrane lipoproteins including LamB, OmpA, and OmpF in gram-negative bacteria are used as fusion partners to express bacterial and viral antigens [3, 8, 22, 37]. Several strategies have also been used to express heterologous proteins...
on the surface of the vegetative form of gram-positive bacteria or their spores [7, 13]. Gram-positive bacteria are more rigid because they have a much thicker peptidoglycan layer than that of gram-negative bacteria. They also lack a lipid outer membrane envelope, which simplifies the extracellular secretion of heterologous proteins [32]. In addition, some species of gram-positive bacteria are safe for human health and therefore the most desirable options for use in the surface display applications as well as other biotechnology purposes [2]. Among the gram-positive bacteria, B. subtilis has more attractive properties and has many industrial and biotechnological applications [19]. Its genetics and physiology have intensively been known, and among bacteria, understanding its genetic background is second only to E. coli [25].

One of the most attractive strategies used for superficial display of homologous and heterologous proteins by a number of gram-positive bacteria is sortase-mediated immobilization.

Gram-positive bacteria express a membrane-anchored transpeptidase enzyme called sortase A that cleaves an LPXTG sorting signal in the C-terminal region of precursor proteins destined for cell wall anchoring [33]. Many of the known surface proteins in Gram-positive bacteria are covalently immobilized by sortase on the cell wall, which have cell wall anchoring properties, including the presence of an N-terminal signal peptide and a cell wall sorting signal in the C-terminal region which is highly conserved. The sorting signal consists of a conserved penta-peptide motif, LPXTG, a hydrophobic stretch of 15–22 amino acids and a short charged tail (6–7 a.a), making up a total of approximately 35 a.a. In S. aureus, the LPXTG motif serves as the recognition sequence for proteolytic cleavage between the threonine and glycine residues, followed by subsequent linkage of threonine to a branched peptide, via the penta-glycine cross-bridge amino acid group, in the peptidoglycan layer. During export via the Sec protein translocation machinery, the sorted proteins are retained within the cytoplasmic membrane due to their C-terminal hydrophobic region and the charged tail. The Sec machinery (or translocase) is a major pathway in bacteria for protein translocation from the cytosol across the cytoplasmic membrane [27].

The use of this type of general sortase-mediated cell wall anchoring has been the most widely used strategy for display of heterologous proteins on the surface of gram-positive bacteria [32]. However, other types of carrier proteins and various other mechanisms have also been used [1].

In B. subtilis, there are two distinct putative sortases (YhcS and YwpE) and several putative substrates, of which YhcR and YfkN have been extensively studied and first reported by Fasehee et al. [9]. YhcS is known as the major B. subtilis sortase and is responsible for covalently anchoring proteins including YhcR on the cell wall [24]. The penta-peptide recognition and cleavage motifs in the C-terminal region of the YhcR, as a natural sortase substrate, is LPDTS. In 2011, Fasehee et al. displayed the chitinase of B. pumilus on the surface of B. subtilis using protein engineering and the YhcS sortase system by fus- ing the sorting signal of the YhcR to the carboxyl-terminal region of the chitinase, thus proving the functionality of the YhcS [9].

Protein A from S. aureus (SpA) is one of the most extensively studied cell wall proteins with a molecular weight of 40–60 kDa. Its molecular weight depends on the numbers of immunoglobulin (Ig)-binding domains in the N-terminal region [14, 34]. The C-terminal part has no Ig-binding domain and contains the sorting signal to covalently binds to the peptidoglycan cross bridge [26]. This region is composed of LPXTG cleavage motif, a hydrophobic stretch of amino acids followed by a positively charged tail which are necessary for interaction and attachment of the protein to the S. aureus cell wall. The Ig-binding region includes five small three-helix-bundle domains (E–D–A–B–C) separated by conserved flexible linkers that share up to 90% amino acid sequence identity with each other [5, 36]. SpA is a valuable component used in methods such as agglutination and ELISA assay as well as western blotting in molecular biology laboratories. It can also bind to immune complexes and immunoglobulins in blood and serum, which has been widely used in biotechnology. Immobilized SpA on polymeric supports can be used as affinity chromatography resins for immunoprecipitation (IP) of antibodies from biological solutions [11, 18, 29, 30, 39].

SpA technology, especially immobilized SpA, has the advantages of interacting with the Fc region of Ig molecule without interrupting its ability to bind to antigens, leading to the development of several applications in immunological research.

The aim of this study was to introduce the structural gene of SpA along with the YhcS sortase into B. subtilis as a host for display of SpA on bacterial cell surface and the development of a biosorbent. It would provide a safer means than a similar commercial product that uses S. aureus for Ig immunoprecipitation by natural immobilization of SpA on the cell surface. In this experiment, for the first time, the native B. subtilis sortase, YhcS, as well as the sorting signal derived from its native substrate, YhcR, were used to display S. aureus protein A.

**Results**

**Expression vectors for surface display**

Different constructs including pNC1 and pNC2 were constructed as negative control plasmids and pSpA-YhcS
as a main plasmid for SpA surface display (Table 1). The plasmid constructs were first transformed in *E. coli* as a cloning host and then into *B. subtilis* as an expression host (Additional file 1: Fig. S3, S4). The constructed recombinant *B. subtilis* strains were called (Bs-NC1, Bs-NC2) as negative control strains and Bs-SpA as a main strain for SpA surface display. Bs-NC1 is a recombinant *B. subtilis* transformed with pNC1 [a recombinant pHY plasmid containing spa gene and cell wall sorting motif (CWM) but lacks the enzyme sortase]. Bs-NC2 is a recombinant *B. subtilis* transformed with pNC2 (a recombinant pHY plasmid containing spa gene but lacks the enzyme sortase and CWM). Bs-pHY is a recombinant *B. subtilis* transformed with pHY300-PLK and Bs-SpA is a recombinant *B. subtilis* transformed with pSpA-YhcS [a recombinant pHY plasmid containing spa gene, CWM and *B. subtilis* sortase (YhcS)]. More details about plasmids will be discussed in the “Methods” Section.

*B. subtilis* WASD cells were used in all experiments. WASD is a strain of *B. subtilis* that is knocked out for two surface proteases [cell wall binding protease (wprA) and sigma D (sigD)] for more efficient surface display of heterologous proteins [21].

**Table 1** Plasmids and strains

| Plasmids and strains | Relevant properties | References |
|----------------------|---------------------|------------|
| Plasmids             |                     |            |
| pHY300-PLK           | *B. subtilis* protein expression vector; Ap', TC' | NIGEB collection |
| pChiM                | Recombinant pHY plasmid containing Chitinase (ChiS), cell wall sorting motif and sortase (YhcS) | Previous study |
| pSpA-YhcS            | Recombinant pHY plasmid containing spa gene, cell wall sorting motif (CWM) and *B. subtilis* sortase (YhcS) | This study |
| pNC1                 | Recombinant pHY plasmid containing spa gene and cell wall sorting motif but lacks the enzyme sortase | This study |
| pNC2                 | Recombinant pHY plasmid containing spa gene but lacks the enzyme sortase and CWM | This study |
| Strains              |                     |            |
| *E. coli* (DH5α)     | F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG φ80lacZΔM15 Δ(lacZYA-argF)U169, hsdR17(rK− mK+)λ− | Invitrogen |
| *B. subtilis* (WASD) | trpC wprA::kan sigD::cat | NIGEB collection |
| Bs-pHY               | *B. subtilis* (WASD) transformed with pHY300-PLK | This study |
| Bs-SpA               | *B. subtilis* (WASD) transformed with pSpA-YhcS | This study |
| Bs-NC1               | *B. subtilis* (WASD) transformed with pNC1 | This study |
| Bs-NC2               | *B. subtilis* (WASD) transformed with pNC2 | This study |

**Fig. 1** Confirmation of cell wall separation from protoplast using gram staining. **A** *B. subtilis* cells before lysozyme treatment and **B** *B. subtilis* cells after lysozyme treatment and cell wall separation
lose their rod shape and become spherical after separat-
ing the cell walls, because the bacterial peptidoglycan
preserves the specific shape of each bacterial species.
This indicates that the cell wall layer that maintained the
bacterial rod shapes has been removed. In addition, prior
to treatment with lysosome, the cells have a thick layer of
peptidoglycan in the cell wall that retains the dye, crystal
violet. After treatment with lysosome and removal of the
peptidoglycan, the crystal violet will be washed out on
addition of alcohol, and the cells turn red after addition
of safranin.

Protein fractions from the cell wall and protoplast of
the recombinant B. subtilis WASD including negative
controls were evaluated by SDS-PAGE and western blot
analysis to determine their Protein A content. In order
for cell wall proteins not to be contaminated with the
cytoplasmic and protoplasmic proteins and to be able to
prove that the protein of interest (SpA) is immobilized
on the cell wall (compared to controls), we have to treat
the cells with lysozyme to gently shave and separate the
cell wall-anchored fraction from protoplast proteins. As
proteins are first translated in the cytoplasm and then
are transported out of the cell through the Sec secretory
pathway, a small percentage of these proteins are also
found inside the cytoplasm. As shown in Fig. 2, Protein
A of about 50 kDa was observed in the cell wall samples
isolated from surface-engineered Bs-SpA and also in a
very small amounts on the surface of Bs-NC1 cells. Pro-
tein A was found mostly in the protoplasts of the negative
control bacteria.

Negative control plasmid construct pNC1 lacks the
native B. subtilis sortase, YhcS, but has the LPDTS sort-
ing motif that have been shown in our previous publi-
cation [9] that is identified by YhcS. Although Bs-NC1
strain has been transformed with the plasmid that lacks
the overexpressed YhcS, the native endogenous sortase is
able to identify this motif and immobilize a small amount
of protein A onto the cell surface. However, the amount
of surface-stabilized protein A by this control strain is
lower than that of the Bs-SpA strain in which YhcS is
overexpressed.

Further confirmation of this result, seen in the lane
3 and 4 for the Bs-NC2 strain and the Bs-pHY strain,
respectively. These strains lack the sortase cleavage and
recognition motif and therefore the protein A is not
immobilized on their surface.

On the other hand, western blot analysis was also per-
formed on proteins extracted from protoplast of different
bacterial strains, the results of which are shown in Fig. 3.
Lane 1 in Fig. 3, corresponding to proteins extracted
from protoplasts of the Bs-SpA, shows a weak band
which indicates a small amount of protein A in its proto-
plast fraction.

However, in protein samples extracted from the proto-
plast of Bs-NC1 and Bs-NC2 strains, higher amounts of
protein A are present in protoplast samples, indicating
that proteins synthesized within the cytoplasm cannot be
transported to the cell surface and are accumulated in the
cytoplasm. These results demonstrate the efficiency of
the developed surface display system in this study and the
efficiency of sortase YhcS used in recombinant Bs-SpA.
The Bs-pHY control strain, as expected, does not express
any protein A in its cytoplasm.

**Flow cytometry analysis of SpA display on the surface of B. subtilis strains**
Quantification of the SpA expression on the surface of
the recombinant B. subtilis cells was evaluated by flow
cytometry. The results showed that the percentage of
fluorescent Bs-NC1 strains, which was used as a nega-
tive control, and Bs-SpA strains, which showed in above-
mentioned experiments to expressed the highest amount

![Fig. 2 Confirmation of the presence of Protein A on the surface of the B. subtilis cells using western blot analysis of protein extracted from the cell wall fractions. Lane 1: cell walls fraction from Bs-SpA; Lane 2: cell wall fraction from Bs-NC1; Lane 3: cell wall fraction from Bs-NC2; Lane 4: cell wall fraction from Bs-pHY. Lane 5: protein marker](image)

![Fig. 3 Western blot analysis using protoplast samples isolated from various B. subtilis strains developed in this study. Lane 1: protoplasts fraction from Bs-SpA; Lane 2: protoplasts fraction from Bs-NC1; Lane 3: protoplast fraction from Bs-NC2; Lane 4: protoplast fraction from Bs-pHY](image)
of the protein A on the cell surface, were about 8% and 28%, respectively. Significant increase in fluorescence in the Bs-SpA strains compared to the negative control strain is another confirmation of the efficiency of this surface display system and the expression of protein A on the surface of the developed strain (Fig. 4).

Whole cell SpA assay
The functional analysis of protein A on the surface of different B. subtilis strains were evaluated by their ability to bind IgG and immunoprecipitate them from rabbit serum. The result showed that the surface-modified bacteria displaying protein A are able to purify the IgG from other serum proteins just like commercial protein A-agarose beads (Fig. 5).

The Experiments were carried out with 5 and 10 mg of the lyophilized recombinant bacterial strains and according to test results and considering the ease of use and cost reduction, 5 mg of freeze-dried strains was chosen. ELISA results showed that the binding capacity of lyophilized Bs-SpA is 100 μg rabbit IgG per 1 mg of cells under the optimal experimental conditions.

The amount of IgG purified by the recombinant strain was approximately 55% of the IgG amount purified by the commercial protein A-agarose in the functional assay using equal amount of both strain and agarose beads in terms of weight.

Preparation of protein A-agarose as a commercial product includes the preparation of beads, activation of its surface functional groups, and on the other hand, expression and purification of the recombinant protein A, and then the final step of protein A immobilization on the surface of agarose beads in a covalent form. Considering the various steps and problems of preparing protein A-agarose, as well as the lower cost of using the recombinant strain and the simplicity of its preparation for antibody purification, and cost-effectiveness, justifies its lower efficiency in antibody purification efficiency compared to protein A-agarose.

Fig. 4 Flow cytometry analysis, in the FACS format, of surface modified and negative control recombinant B. subtilis cells. a Bs-NC1 strain as a negative control, b Bs-SpA strain. Vertical line denotes the number of cells and horizontal line is the strength of fluorescence. M1 shows a gate to detect only cells that fall in a positive area. All the cells outside this gate are ignored as a background. The cells that are located in M1 region represent the cell that display the SpA protein on their surface.

Fig. 5 Purification of rabbit serum IgG by recombinant B. subtilis strains. IgG heavy chain is about 50 KDa and IgG light chain is about 23 KDa. Lane 1. Protein marker; Lane 2. Supernatant from serum incubation with the commercial protein A-agarose as a positive control; Lane 3. Supernatant from incubation of serum with the recombinant Bs-SpA strain; Lane 4. Supernatant from serum incubation with the recombinant Bs-pHY strain as a negative control.
Reusability of Bs-SpA

Matrix recycling and its reusability for many times is one of the most important advantages of protein surface display in industry. Therefore, the expenses regarding protein expression, purification, and immobilization can be decreased. After elution of the bound IgG from the Bs-SpA, they were re-used to bind the IgG in the rabbit serum several times. The amount of antibody bound to the Bs-SpA was evaluated for each round of IgG binding/elution, and the activity was compared with the initial activity, which was assumed as 100%. The residual activity of the immobilized protein A was found to decrease after multiple uses. As shown in the Fig. 6, after 6 uses, about 50% of the IgG-binding activity is still preserved.

Discussion

Protein A has five Ig binding domains through which can bind to the Fc region of antibodies. This property is used in the pharmaceutical and biotechnology industries for the purification of antibodies with therapeutic applications as well as the purification of antigen–antibody complexes by immunoprecipitation and then proteomic analysis. This property of SpA has been used as immobilized on the polymeric surfaces as well as on various surfaces of living cells by surface display.

The first use of S. aureus cells carrying protein A as an IgG-binding agent was demonstrated by Jonsson et al. [15] which provided a process for quantifying Alpha-fetoprotein (AFP) tumor marker in human serum. Kesler then published two papers on the benefits of protein A-displaying staphylococci as a bioadsorbent for IgG in the immunoprecipitation process [16, 17].

In 1994, Djojonegoro et al. developed a filamentous bacteriophage M13-based display system for a single Ig-binding domain of the S. aureus Protein A. By expressing the B domain of protein A on the bacteriophage surface, the phage was able to interact with the immobilized IgG molecules that could be used to purify phage [6].

In the present study, unlike our previous study, which was based on B. subtilis spores surface display, the vegetative form of the B. subtilis were used as a safe vehicle to display the S. aureus protein A [10]. Spore surface display system has some drawbacks, including the fact that the process of separating spores is a complex, time-consuming, and relatively expensive process, and it is also relatively difficult to obtain large amounts of pure spores.

On the other hand, the surface display system developed based on the vegetative form of the B. subtilis has not been studied as much as the spore display system [19] and few studies have been reported in which the system based on the sortase and sortase substrates to display heterologous proteins [24]. In particular, this vegetative surface display has been reported based on sortase of other gram-positive bacteria such as S. aureus and L. monocytogenes [28], but the vegetative surface display system based on B. subtilis sortase and its native substrate has not been reported, except for a report by Fasehée et al. [9].

Herein we described the display of protein A on the surface of B. subtilis cells using Yhcs sortase and its native substrate, YhR. The recombinant strain of B. subtilis developed in this study (Bs-SpA) is used as a formalin-fixed and heat-killed.

The strain developed as a bioadsorbent in this study, compared to the similar commercial products developed by different companies like Merck, that uses S. aureus naturally displaying protein A, is a safe and probiotic strain and has many advantages over those commercial products.

The cost reduction and optimal performance of Bs-SpA predict the potential application of this immobilization technology in the relevant immunological and biotechnological research. This product is routinely used for IgG purification and immunoprecipitation assays.

In this study, we did not use the S. aureus sortase because it is not functional in the B. subtilis host as the cross-bridge of lipid II precursors differs in structure between bacterial species. In B. subtilis and L. monocytogenes, the cross-bridge amino group is derived from the side chain of m-diaminopimelic acid (m-Dpm). In contrast, the S. aureus cross-bridge contains five glycine residues that are tethered to the L-amino group of lysine within lipid II [C55-(PO3)2-MurNac(L-Ala-D-iGln-Lys(NH2-Gly5)-D-Ala-D-Ala)-GlcNAc]. Sortase A enzymes of different bacterial species have evolved to recognize cross-bridge structures of that species [33].

Fig. 6 The reusability of Bs-SpA. After 6 uses, the SpA activity was reduced to about a half and after 10 times uses, it was reduced to a quarter.
Conclusions
Display of proteins and peptides on microorganisms (bacteria, yeast, and phages) is becoming a leading technology due to low cost and its various applications. The non-pathogenicity of *B. subtilis* as a safe surface make this technology applicable to food and biological industries. Also, the advantages of easy purification and recycling of immobilized enzyme can greatly reduce the cost of industrialization. The surface-engineered, formalin-fixed and heat-killed strain of *Bs*-SpA prepared in this study, expresses protein A on the surface of its vegetative form and is able to bind and purify immunoglobulins and can be a safe alternative to the biological products on the market offered by companies. In this research, Yhcs sortase system was used to display protein A on the surface of *B. subtilis* cells. The genetic method used in this study has other advantages, including the fact that this method eliminates the need for the complex and time-consuming steps, including the preparation and purification of resins and polymer surfaces, surface activation, protein purification and immobilization. It is also very cost-effective, especially for laboratories that perform large number of immunoprecipitation tests.

It is believed that with further research on *B. subtilis* surface display, this technology will play an important role in more fields in the future.

Methods
Plasmids, bacterial strains and growth conditions
The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strain DH5α and *B. subtilis* strain WASD were used as the cloning and expression host for protein A surface display, respectively. *E. coli* cells were propagated at 37 °C on LB agar plates. For protein production, *B. subtilis* cells were cultured in superrich medium (SRM) (Bacto tryptose, 25 g/liter; yeast extract, 20 g/liter; dipotassium phosphate, 3 g/liter; glucose, 4.5 g/liter; pH 7.5) at 30 °C for 14 h and harvested for analysis.

DNA techniques
The sortase-mediated system was used for displaying protein A on the bacterial cell wall. To achieve this goal, the following constructs, including various controls, were designed in such a way that the cleavage motif and sortase were located adjacent to each other in a cassette (Fig. 7). Meanwhile, the start codon of the sortase and the stop codon of the modified substrate overlapped. It should be noted that a native signal peptide of protein A is used in these constructs, since an N-terminal signal peptide is necessary for the Sec protein translocation machinery as described in the introduction.

The negative control constructs (pNC1 and pNC2 related to Table 1) were designed using restriction enzyme digestion by enzymes cleavage sites embedded in the main construct or by designing primers and amplification by PCR (Additional file 1: Table S1 for oligonucleotide sequences).

The plasmid PHY300 was used as a shuttle vector for cloning the constructs in the *E. coli* (DH5α) and then for expression in the main host, *B. subtilis* (WASD). In this plasmid ampicillin and tetracycline are selection markers recognized by *E. coli* and *B. subtilis*, respectively. The tetracycline is also used as an expression inducer.

After ligation, the constructs were transformed into the competent *E. coli* (DH5α) cells by the thermal shock method [31]. The standard method of calcium chloride was used for this purpose. Several different methods such as plasmid extraction and enzymatic digestion, were then used to verify the recombinant clones. After confirmation of cloning, the recombinant plasmids were extracted from the primary host. *B. subtilis* transformation was performed by the Spizizen competent cells [35] and the natural transformation method. In this method, the bacteria’s culture becomes more deficient in different stages, so that *B. subtilis* forced to absorb DNA from the environment [23]. In addition to these constructs, plasmid PHY300 with no insert was transformed into the bacteria and used as another negative control (refer to the Additional file 1 for more detail about plasmids construction and strain development).

Protein A expression on the cell wall of the recombinant *B. subtilis*
For this purpose, *Bs*-pHY, *Bs*-SpA, *Bs*-NC1, and *Bs*-NC2 were grown in the SRM culture containing tetracycline (final concentration of 25 μg/ml) at 30 °C for 14 h and 180 rpm. Since the target gene is cloned downstream of the tetracycline resistance gene in the plasmid PHY300, it is under the control of its promoter and is constitutively expressed.

Extraction of cell wall-bound proteins using lysozyme
To confirm that the protein A is immobilized on the cell surface of *B. subtilis* and covalently linked to its cell wall, the bacterial cell wall was isolated from the protoplast as a first step according to Welbull’s article with some modification as follows and the presence of protein A was investigated in this fraction [38]. The cells from a late log-phase *B. subtilis* culture were collected by centrifugation, washed, pelleted, and finally resuspended in 150 μl protoplast buffer (20% sucrose, 50 mM Tris pH 7.5, 15 mM MgCl₂ [pH 7.6], 0.2 mg/ml Lysozyme). Samples were incubated at 37 °C for 30 min. Protoplasts were separated from the extracted cell wall fraction by centrifugation.
at 5400 × g for 7 min. The resulting protoplast pellet was resuspended in 150 µl of protoplast buffer. For checking the accuracy of the method, bacterial cells were stained using Gram’s method and were observed under the light microscope [4]. The cell wall and protoplast fractions were used for further analysis, such as ELISA and western Blotting.

**SDS-PAGE and western blot analysis**

Cell wall and protoplast fractions from a specific number of obtained bacterial cells were analyzed by SDS-PAGE to determine their protein A content. Due to the limited surface loading capacity of each cell and to further confirm the surface expression of protein A, its expression was also determined by western blot analysis using a specific antibody. For western blot analysis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes and treated with a rabbit anti-goat IgG antibody bound to horseradish peroxidase (HRP) in a 1:500 dilution. The blot was developed with hydrogen peroxide and 4-chloronaphthol as a substrate.

**Evaluation of cell surface binding of SpA by flow cytometry**

Quantitative determination of SpA expression on the surface of *B. subtilis* cells was examined by flow cytometry. For this purpose, the cells were washed three times with phosphate-buffered saline (PBS), resuspended in 1 mL PBS solution containing rabbit antibody conjugated to FITC (1:1000) and incubated on ice for 1 h. Cells were washed again three times with PBS, and resuspended in 500 µL of PBS. Fluorescent signals created on the surface of the control and SpA-conjugated cells were analyzed using a fluorescence-based flow cytometer (FACSCalibur, BD, USA). The Cell Quest ver. 1.0 software was used for data analysis.

**Bacterial fixation with formaldehyde**

Bacterial formaldehyde fixation was performed in such a way that cell surface proteins are preserved. Briefly, after growing the bacteria, they were centrifuged, the supernatant was discarded, and the bacterial pellet was dissolved in 100 ml PBS + 0.02 sodium azide. The pellets were then washed twice and weighed. Bacteria were dissolved...
in PBS 10% wt/vol and formaldehyde was slowly added to the final concentration of 1.5%. The bacterial suspensions were placed on a stirrer for 90 min at room temperature. After centrifugation, the bacterial pellets were dissolved in 10% w/w PBS and incubated at 80 °C for 5 min with gentle rotation. The bacterial suspensions were then placed in cold water and after several washing steps as before, they were kept at 4 °C until testing.

**IgG binding assay using surface-engineered bacteria**

The ability of surface-modified *B. subtilis* to bind and purify IgG was analyzed by the binding assay using rabbit serum. 30 ml of the surface-modified bacteria or 10 mg of their dried powder were resuspended and washed with 1 ml of starting buffer [100 mM Tris–HCl (pH 8)]. The pH of rabbit serum was adjusted to 7.5–8 with 1 M Tris and added as standards. After blocking with 1% (w/v) Bovine Serum Albumin (BSA) and a final washing step, an HRP-conjugated anti-rabbit antibody at 1:2000 dilution was added to each well, and the development of color reaction was carried out by adding ABTS [2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid)-di ammonium salt] substrate. The absorbance was determined by the ELISA reader at 405 nm. The standard curve was plotted and the concentration of purified antibodies was calculated.

**Reusability of Bs-SpA**

*B. subtilis* cells can be easily purified and isolated from the culture by centrifugation. IgG purification from rabbit serum and its quantification was used for reusability analysis of the Bs-SpA. At the end of each cycle of reusing, the Bs-SpA was removed from the reaction and washed three times with 10 mM Tris buffer (pH 8), after which a sample of new serum was added to the Bs-SpA cells to start a new cycle. The relative activity was calculated by defining the activity of the first reaction as 100%.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s12934-021-01701-4.

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**Figure S1.** pChiM is a plasmid containing cell wall sorting motifs of *B. subtilis* (CWM), sortase cleavage motif (LPDTS) and the enzyme sortase (YhcS). This plasmid was used as a template for making other constructs prepared in this study. **Figure S2.** Schematic representation of SpA display constructs. Plasmids were constructed to express the SpA with or without the C-terminal cell wall motif (CWM) of YhcR, and with or without the sortase YhcS. **Figure S3.** Diagrammatic representation of the constructs used in this study as control plasmids (pNC1 and pNC2) and main construct (pSpA-YhcS). **Figure S4.** The process of making constructs and their transformation. Table S1. Oligonucleotide primers sequences used in this study.

**Acknowledgements**

This research was supported by the National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.

**Authors’ contributions**

GA designed the work, interpreted the data and revised the manuscript; SG has done the practical part and prepared the primary draft of the manuscript. Both the authors read and approved the final manuscript.

**Funding**

No funding.

**Availability of data and materials**

Not applicable.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.
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