MICROBIAL CELL FACTORIES

**RESEARCH**                                                                                              **Open Access**

# Double mutation of cell wall proteins CspB and PBP1a increases secretion of the antibody Fab fragment from *Corynebacterium glutamicum*

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## Abstract

**Background:** Among other advantages, recombinant antibody-binding fragments (Fabs) hold great clinical and commercial potential, owing to their efficient tissue penetration compared to that of full-length IgGs. Although production of recombinant Fab using microbial expression systems has been reported, yields of active Fab have not been satisfactory. We recently developed the *Corynebacterium glutamicum* protein expression system (CORYNEX®) and demonstrated improved yield and purity for some applications, although the system has not been applied to Fab production.

**Results:** The Fab fragment of human anti-HER2 was successfully secreted by the CORYNEX® system using the conventional *C. glutamicum* strain YDK010, but the productivity was very low. To improve the secretion efficiency, we investigated the effects of deleting cell wall-related genes. Fab secretion was increased 5.2 times by deletion of *pbp1a*, encoding one of the penicillin-binding proteins (PBP1a), mediating cell wall peptidoglycan (PG) synthesis. However, this Δpbp1a mutation did not improve Fab secretion in the wild-type ATCC13869 strain. Because YDK010 carries a mutation in the *cspB* gene encoding a surface (S)-layer protein, we evaluated the effect of ΔcspB mutation on Fab secretion from ATCC13869. The Δpbp1a mutation showed a positive effect on Fab secretion only in combination with the ΔcspB mutation. The ΔcspBΔpbp1a double mutant showed much greater sensitivity to lysozyme than either single mutant or the wild-type strain, suggesting that these mutations reduced cell wall resistance to protein secretion.

**Conclusion:** There are at least two crucial permeability barriers to Fab secretion in the cell surface structure of *C. glutamicum*, the PG layer, and the S-layer. The ΔcspBΔpbp1a double mutant allows efficient Fab production using the CORYNEX® system.

**Keywords:** CORYNEX®, *Corynebacterium glutamicum*, Protein secretion, Fab fragment, CspB, PBP1a

## Background

Recombinant antibody technologies can generate specialized whole antibodies or fragments with a myriad of potential therapeutic, diagnostic, and research applications [1]. Antibody fragments are particularly promising for clinical application because their ability to penetrate tumor cells is higher than full-length IgGs [2]. The fragment antigen-binding (Fab) molecule contains a fragmented heavy chain (HC) composed of the variable ($V_H$) and the first constant ($C_{H1}$) domains and a light chain (LC) composed of the light variable ($V_L$) and constant ($C_L$) domains. Production of recombinant Fab using microbial expression systems has been reported for several species, including *Escherichia coli* [3-8], *Pichia pastoris* [9-12], and *Saccharomyces cerevisiae* [13], but yields of active Fab have not been satisfactory.

*Corynebacterium glutamicum* is a Gram-positive, non-pathogenic soil bacterium [14,15] that has been used for industrial-scale production of amino acids such as glutamate and lysine for several decades [16,17]. *C. glutamicum*

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produces only small amounts of endogenous extracellular proteins compared with many other bacteria commonly used for protein production, a great advantage for protein purification. Thus, *C. glutamicum* is one of the most accessible and convenient bacterial species for biotechnology, but has not been used extensively for industrial production of proteins. We recently demonstrated that many heterologous proteins can be efficiently secreted in active form by the *C. glutamicum* ATCC13869 strain. Using a strong *cspB* promoter and signal peptides derived from a corynebacterial cell surface protein and the *Escherichia coli* twin-arginine translocation pathway, *C. glutamicum* ATCC13869 exhibited great potential as a host for industrial-scale production of recombinant proteins [18-24]. This protein expression system has been awarded trademark registration as CORYNEX®.

*Corynebacterium glutamicum* has a thick cell wall composed of two layers. The inner layer consists mainly of peptidoglycan (PG) and the outer layer mainly of mycolic acid. The presence of the outer layer may confer resistance against lytic enzymes, such as egg white lysozyme that catalyze hydrolysis of the β-1,4 glycosidic bond between the N-acetylglucosamine and N-acetylmuramic acid of PG [25], although this bacterium belongs to the Gram-positive category. This resistance is probably due to the function of the outer layer as a protein permeability barrier [26-29].

PG is synthesized on the outer surface of the cytoplasmic membrane by enzymes that bind to and are inhibited by β-lactam-type antibiotics such as penicillin (so that these enzymes are classified as penicillin-binding proteins, PBPs). In general, PBPs are membrane-bound proteins essential for cell wall synthesis by bacteria. They are classified into two types, high-molecular-weight PBPs (HMW-PBPs) and low-molecular-weight PBPs (LMW-PBPs). Further, HMW-PBPs are classified into class A HMW-PBPs having both a transpeptidase activity domain for crosslinking PG moieties and a transglycosylase activity domain for forming a polysaccharide chain, and class B HMW-PBPs having only a transpeptidase activity domain [30]. It is known that the class A HMW-PBPs of *C. glutamicum* are responsible for cell elongation, whereas the class B HMW-PBPs are responsible for formation of PG of septal walls at the time of cell division [30,31]. LMW-PBPs have D,D-carboxypeptidase activity and/or endopeptidase activity.

Several *C. glutamicum* strains have a surface (S)-layer outside the normal cell wall. The S-layer of many bacteria consists of a single protein assembled in two-dimensional paracrystalline arrays. The protein CspB (also called PS2) has been identified as a major secreted protein of several *C. glutamicum* strains [32,33] and forms the S-layer [34] in this species. Because of its location, the S-layer is generally involved in interaction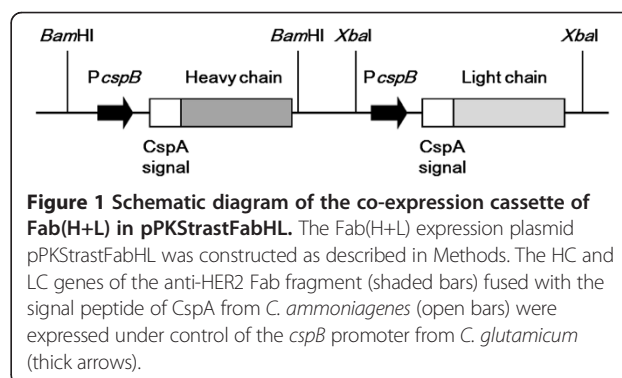s between the bacterial cell and its environment. The S-layers of several pathogenic bacteria have been reported to act as virulence factors by conferring resistance to bactericidal activity [35,36] and by adhering to the extracellular matrix proteins of the host [37]. Furthermore, the S-layer can serve as a molecular sieve and act to stabilize the bacterial cell envelope of both pathogenic and non-pathogenic bacteria [38].
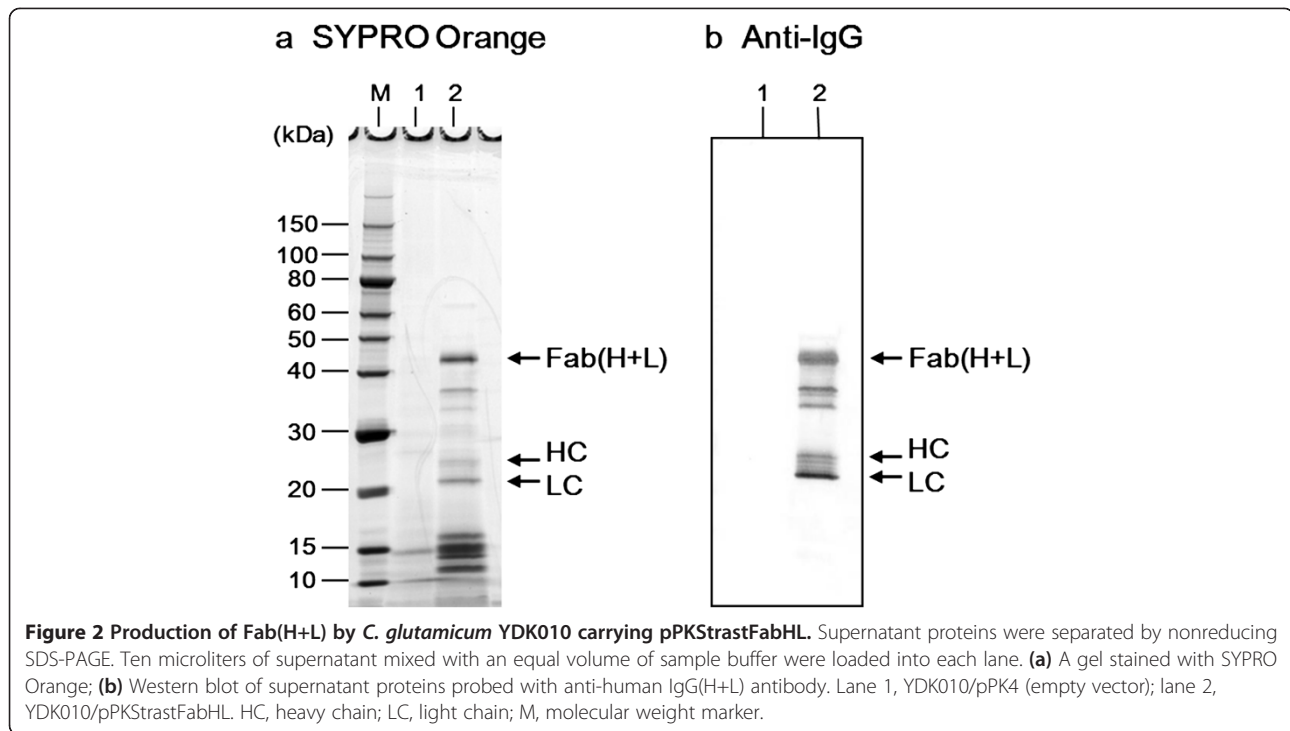
In the present study, we attempted to produce recombinant Fab using the *C. glutamicum* protein expression system CORYNEX®, but productivity was extremely low. We screened for mutations affecting the efficiency of Fab secretion and found that mutations in certain cell wall-related proteins enhanced Fab secretion, possibly by removing a physical and chemical barrier to secretion. This finding suggests that cell wall structures form a bottleneck for efficient recombinant Fab production in this expression system. The improved CORYNEX® system may enable industrial-scale Fab production.

## Results

### Secretion of antibody Fab fragments by *C. glutamicum* YDK010

Secretion of the Fab(H+L) fragment of the anti-HER2 antibody "trastuzumab", used for targeted therapy of HER2$^+$ breast cancer, was first assessed in the YDK010 strain. Bacteria were transformed with the pPKStrast-FabHL plasmid containing HC and LC genes of the Fab region fused with the signal peptide derived from the *cspA* gene of *C. ammoniagenes* under the control of the *cspB* promoter of *C. glutamicum* (Figure 1 and Additional file 1: Figure S1). The transformant was then cultured in 4 ml of MMTG medium at 30°C for 96 h and the culture supernatant was analyzed by non-reducing SDS-PAGE and Western blotting with anti-human IgG(H+L) antibody. A secreted protein of about 45 kDa, corresponding to the molecular weight of Fab(H+L), was detected in the culture supernatant (Figure 2). This band was subjected to N-terminal amino acid sequencing, and, as expected, the N-terminal amino acid sequences of both HC (EVQLV) and LC (DIQMT) of the Fab(H+L) were detected. This result indicated that the signal peptide of CspA fused to



**Figure 1 Schematic diagram of the co-expression cassette of Fab(H+L) in pPKStrastFabHL.** The Fab(H+L) expression plasmid pPKStrastFabHL was constructed as described in Methods. The HC and LC genes of the anti-HER2 Fab fragment (shaded bars) fused with the signal peptide of CspA from *C. ammoniagenes* (open bars) were expressed under control of the *cspB* promoter from *C. glutamicum* (thick arrows).

**Figure 2 Production of Fab(H+L) by *C. glutamicum* YDK010 carrying pPKStrastFabHL.** Supernatant proteins were separated by nonreducing SDS-PAGE. Ten microliters of supernatant mixed with an equal volume of sample buffer were loaded into each lane. **(a)** A gel stained with SYPRO Orange; **(b)** Western blot of supernatant proteins probed with anti-human IgG(H+L) antibody. Lane 1, YDK010/pPK4 (empty vector); lane 2, YDK010/pPKStrastFabHL. HC, heavy chain; LC, light chain; M, molecular weight marker.

both HC and LCs had been correctly processed for secretion and that HCs and LCs formed a heterodimer in the culture supernatant. However, accumulation of Fab (H+L) in the culture supernatant was barely detectable by Coomassie brilliant blue (CBB) staining. The secreted Fab yield was estimated to be approximately 11 mg/l. Protein bands of about 24 and 21 kDa, corresponding to the monomeric HCs and LCs, respectively, were also detected. Indeed, these bands had the expected N-terminal amino acid sequences of HC and LC. Other minor protein bands at 34–37 kDa were detected and may represent degradation products of Fab(H+L).

### Enhanced Fab secretion in the *pbp1a* deletion mutant *C. glutamicum* YDK010

To improve the productivity of Fab(H+L) by the *C. glutamicum* YDK010 strain, we first investigated the effects of PBP gene deletion on the Fab secretion, as the PG layer synthesized by PBPs could function as a barrier to Fab secretion. Genome sequence data suggest that *C. glutamicum* has at least nine PBPs, of which the class A HMW-PBPs PBP1a and PBP1b and the class B HMW-PBPs PBP2a, PBP2b, and FtsI (PBP3) are major PG synthases [30]. Of these, only FtsI is essential for *C. glutamicum* growth [39], whereas the other four HMW-PBPs are dispensable [30]. The LMW-PBPs PBP4, PBP4b, PBP5, and PBP6 were characterized as carboxypeptidases and lactamases on the basis of sequence similarity analysis [30]. In this study, we investigated the effects of deleting each of these nonessential HMW-PBPs on Fab(H+L) secretion

(Figure 3). Deletion of *pbp1a*, encoding the class A PBP1a, resulted in approximately 5.2 times higher secretion of Fab(H+L) than in the parent strain. In contrast, deletion of *pbp1b* had no effect on Fab(H+L) secretion. Similarly, deletion of *pbp2a* and *pbp2b* individually did not increase Fab(H+L) secretion (data not shown).

### Effect of *cspB* mutation on secretion of Fab(H+L)

To confirm the positive effect of the *Δpbp1a* mutation on Fab(H+L) secretion, we evaluated the effect of *Δpbp1a* on the wild-type genetic background ATCC13869. Unexpectedly, *Δpbp1a* had no effect on Fab(H+L) secretion from ATCC13869 (Figure 4, lanes 2 and 4). It is known that during YDK010 strain construction, the *cspB* gene encoding the S-layer protein CspB is deleted [40]. We speculated that the *cspB* mutation was somehow involved in allowing Fab(H+L) secretion and investigated Fab secretion in both *ΔcspB* ATCC13869 [41] and the *ΔcspBΔpbp1a* double mutant ATCC13869. The *ΔcspB* single mutation only slightly affected Fab(H+L) secretion (Figure 4, lane 3), but the combined *ΔcspBΔpbp1a* mutation markedly enhanced Fab(H+L) secretion compared with both the single mutants and the wild type (Figure 4, lane 5). Thus, *Δpbp1a* increases Fab(H+L) secretion only in the presence of the *ΔcspB* mutation. In contrast, the *Δpbp1b* single mutation and the *ΔcspBΔpbp1b* double mutation did not affect Fab(H+L) secretion (Figure 4, lanes 6 and 7), coherent with the results from the experiment on YDK010 strain. The *Δpbp1a* mutation increased Fab secretion 5.2 times (11.1 mg/l → 57.6 mg/l) in the

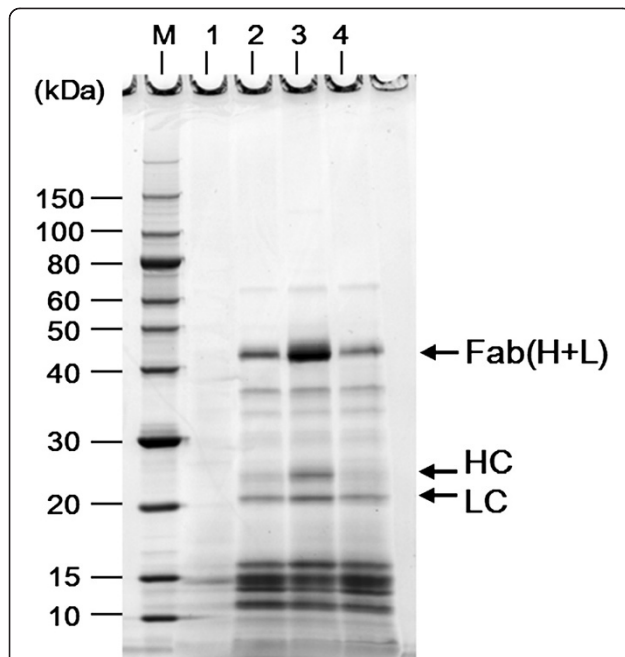**Figure 3 Effect of Δ*pbp1a* or Δ*pbp1b* mutation on Fab secretion by *C. glutamicum* YDK010 strain.** Supernatant proteins were separated by non-reducing SDS-PAGE followed by SYPRO Orange staining. Ten microliters of supernatant mixed with an equal volume of sample buffer were loaded into each lane. Lane 1, YDK010/pPK4 (empty vector), lane 2, YDK010/pPKStrastFabHL; lane 3, YDK010Δpbp1a/pPKStrastFabHL; lane 4, YDK010Δpbp1b/pPKStrastFabHL; M, molecular weight marker. Mutation Δpbp1a but not Δpbp1b markedly increases Fab secretion from YDK010. The data shown represent three independent experiments that yielded similar results.



**Figure 4 Effects of Δ*pbp1a* and Δ*cspB* mutations on Fab secretion by *C. glutamicum* wild-type strain ATCC13869.** Supernatant proteins were separated by nonreducing SDS-PAGE followed by SYPRO Orange staining. Ten microliters of supernatant mixed with an equal volume of sample buffer were loaded into each lane. Lane 1, ATCC13869/pPK4 (empty vector); lane 2, ATCC13869/pPKStrastFabHL; lane 3, ATCC13869ΔcspB/pPKStrastFabHL; lane 4, ATCC13869Δpbp1a/pPKStrastFabHL; lane 5, ATCC13869ΔcspBΔpbp1a/pPKStrastFabHL; lane 6, ATCC13869Δpbp1b/pPKStrastFabHL; lane 7, ATCC13869ΔcspBΔpbp1b/pPKStrastFabHL; M, molecular weight marker. The Δpbp1a mutation increases Fab secretion only in the presence of ΔcspB (ΔcspBΔpbp1a double mutant). The data shown represent three independent experiments that yielded similar results.

YDK010 strain and 3.9 times (8.9 mg/l → 34.9 mg/l) in the ATCC13869Δ*cspB* strain (Table 1). The YDK010 strain probably carries an unknown mutation that also affects the Fab secretion in the YDK010 background.

### Effect of *cspB* and *pbp1a* mutations on the lysozyme sensitivity of *C. glutamicum*

Both CspB and PBP1a are cell wall proteins, and thus, these mutations may affect cell surface integrity. To assess the effects on cell surface integrity, we examined the lysozyme sensitivity of each mutant strain, ATCC13869 WT, Δ*cspB*, Δ*pbp1a*, Δ*cspB*Δ*pbp1a*, Δ*pbp1b*, and Δ*cspB*Δ*pbp1b*, by growth assay in LB liquid medium. All strains exhibited similar growth rates under control conditions (Figure 5a). When 25 μg/ml lysozyme was added to exponentially growing cultures, growth of the wild-type strain was not affected, indicating strong lysozyme resistance (Figure 5b). In contrast, the growth rates of both Δ*cspB* and Δ*pbp1a* single mutants decreased gradually during lysozyme treatment, resulting in lower final growth yields. The Δ*cspB*Δ*pbp1a* double mutant showed higher sensitivity to

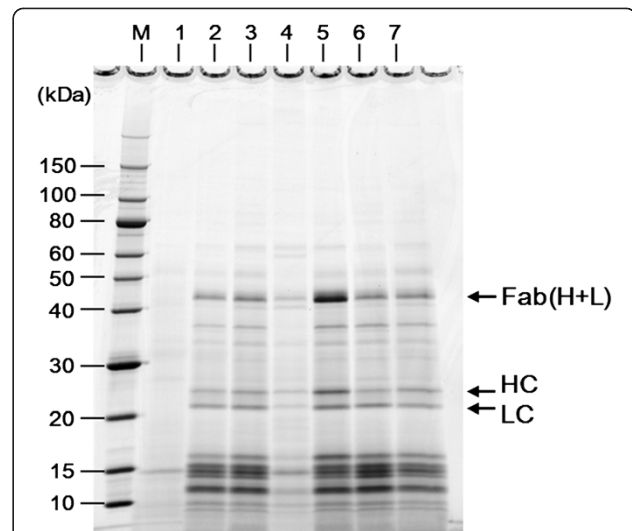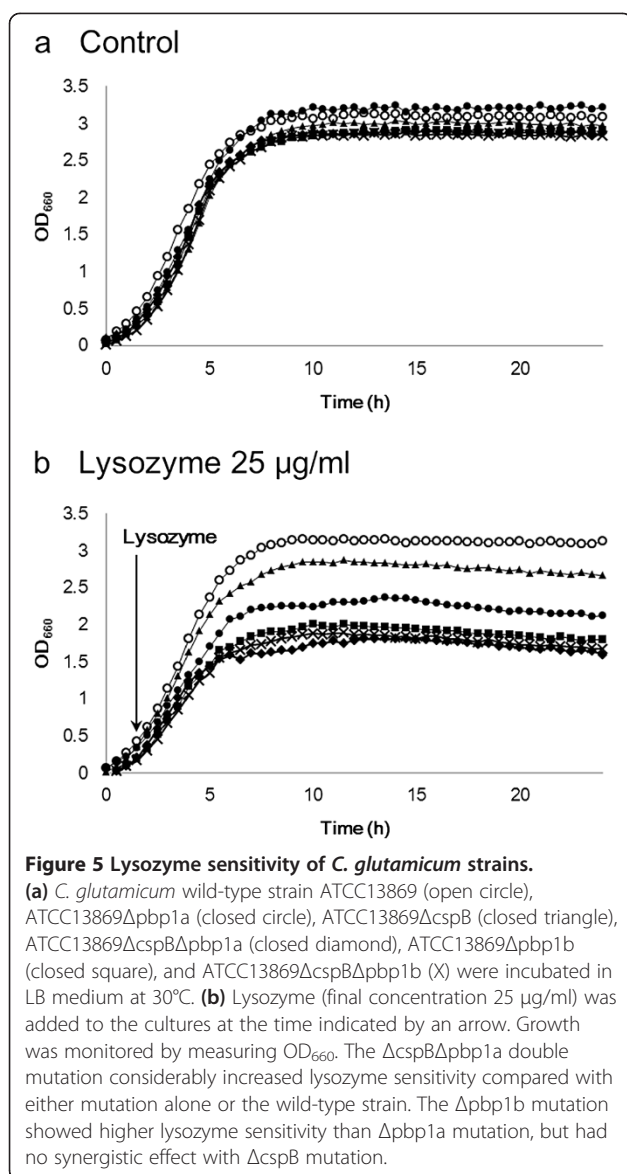lysozyme than did the Δ*pbp1a* and Δ*cspB* single mutants (Figure 5b). These results suggest that the Δ*cspB* and Δ*pbp1a* mutations affect cell surface integrity and that the two mutations act together to disrupt cell surface integrity and enhance lysozyme sensitivity. In contrast, the Δ*pbp1b* single mutant showed higher lysozyme sensitivity than the Δ*pbp1a* mutant, which was comparable to the Δ*cspB*Δ*pbp1b* double mutant. However, no

**Table 1 Fab production yields by test tube culture**

| Strain | Fab production yield (mg/l) |
|---|---|
| YDK010 | 11.1 ± 0.3 |
| YDK010Δpbp1a | 57.6 ± 2.3 |
| YDK010Δpbp1b | 10.6 ± 0.5 |
| ATCC13869 | 6.4 ± 0.4 |
| ATCC13869ΔcspB | 8.9 ± 0.8 |
| ATCC13869Δpbp1a | 2.2 ± 0.4 |
| ATCC13869ΔcspBΔpbp1a | 34.9 ± 1.8 |
| ATCC13869Δpbp1b | 8.1 ± 1.1 |
| ATCC13869ΔcspBΔpbp1b | 7.9 ± 1.3 |

The amount of secreted Fab in culture supernatant was quantified as described in Methods. Yields represent the average of measurements from three separate test tube cultures.

**Figure 5 Lysozyme sensitivity of *C. glutamicum* strains.**
**(a)** *C. glutamicum* wild-type strain ATCC13869 (open circle),
ATCC13869Δpbp1a (closed circle), ATCC13869ΔcspB (closed triangle),
ATCC13869ΔcspBΔpbp1a (closed diamond), ATCC13869Δpbp1b
(closed square), and ATCC13869ΔcspBΔpbp1b (X) were incubated in
LB medium at 30℃. **(b)** Lysozyme (final concentration 25 μg/ml) was
added to the cultures at the time indicated by an arrow. Growth
was monitored by measuring $OD_{660}$. The ΔcspBΔpbp1a double
mutation considerably increased lysozyme sensitivity compared with
either mutation alone or the wild-type strain. The Δpbp1b mutation
showed higher lysozyme sensitivity than Δpbp1a mutation, but had
no synergistic effect with ΔcspB mutation.

synergistic effect was observed between *ΔcspB* and
*Δpbp1b* mutations.

**Antigen-binding activity of Fab secreted by *C. glutamicum***
To determine whether the secreted Fab has a correct
antigen-binding activity, it was partially purified from
culture supernatant by protein G affinity column, and
binding activity to its antigen (human HER2/ErbB2) was
evaluated by surface plasmon resonance assay as described
in Methods. As shown in Figure 6, the Fab secreted by
*C. glutamicum* had binding affinity to the antigen. The
dissociation constant ($K_D$) value of this partially purified
Fab was calculated to be 0.36 nM, a value comparable to
that in a previous report by Khalili, et al. [42]. This con-
firmed that the secreted HC and LC assembled each other
and formed a complete Fab structure in the supernatant.

## Discussion
In this study, we attempted to produce the clinically
important antibody Fab fragment, a heterodimeric mo-
lecule containing an intermolecular disulfide bond, using
the CORYNEX® system. As shown in Figure 2, the re-
combinant Fab(H+L) was secreted into the culture
medium by the industrial strain YDK010, in addition to
the monomeric HC and LC. Sequencing of the N-
terminal amino acids revealed that the signal peptide de-
rived from *C. ammoniagenes* CspA was properly cleaved
during secretion. It was also shown that an intermolecu-
lar disulfide bond was formed between HC and LC. The
secreted and partially purified Fab showed high binding
activity to its antigen HER2/ ErbB2 (Figure 6). This is
the first Fab expression experiment performed in *C. glu-
tamicum* by the conventional CORYNEX® system using
the *cspB* promoter and the Sec-dependent CspA signal
peptide. Recently, secretion of antibody single-chain
variable fragment (scFv) in *C. glutamicum* was also re-
ported [43-45]. Yim et al. reported the secretion of scFv
in the *C. glutamicum* ATCC13032 strain, with approxi-
mately 18 mg/l in flask cultivation and 68 mg/l in a 5-L
bioreactor, using a fully synthetic H36 promoter and the
Sec-dependent PorB signal peptide [45]. scFv is a mono-
meric molecule, whereas Fab is a heterodimer containing
an intermolecular disulfide bond. The present report
thus reveals the ability of *C. glutamicum* to secrete a
recombinant heteromultimeric protein in active form.
Production of recombinant antibody fragments using
microbial expression systems has also been reported,
with yields of several dozen mg/l [3-13]. In our study,
*C. glutamicum* showed the ability to secrete recom-
binant antibody fragment at equal or even higher levels
than other expression systems. Moreover, this secretion
system has a great advantage for protein purification be-
cause *C. glutamicum* produces only small amounts of
endogenous extracellular proteins. However, accumu-
lation of the secreted Fab in the YDK010 strain was still
low. To identify the bottleneck in Fab production in
*C. glutamicum*, we investigated the effects of specific cell
wall-related genes and succeeded in developing a strain
with improved secretion ability by mutating *cspB* and
*pbp1a*.

We first screened the nonessential PBPs in the industrial
strain YDK010 (originally derived from the wild-type
ATCC13869 strain) and found that the *Δpbp1a* deletion
markedly improved Fab secretion (Figure 3). However, this
effect was not observed on the wild-type background. Fur-
ther genetic analysis revealed that a mutation in the *cspB*
gene was also involved in the improvement of Fab secre-
tion. A reconstituted double mutant *ΔcspBΔpbp1a* on the
wild-type ATCC13869 background, but neither of the
constituent mutations alone, showed improved Fab secre-
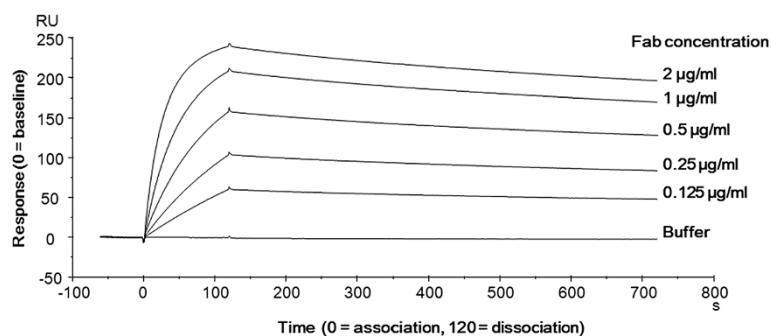tion (Figure 4). Both *cspB* and *pbp1a* are cell wall-related

**Figure 6 Antigen-binding activity of Fab secreted by *C. glutamicum*.** The secreted Fab was partially purified and assayed for antigen-binding activity by Biacore X100. A CM5 sensor chip immobilized with the extracellular domain of recombinant human HER2/ErbB2 (577 RU) was used to obtain the binding sensorgram of Fab.

genes; PBP1a is involved in PG synthesis, and CspB forms the cell wall S-layer. These results suggest that there are at least two crucial permeability barriers to Fab secretion in the cell surface structure of *C. glutamicum*.

Because these two genes are both cell wall-related genes, mutations in them may affect the integrity of the cell surface structure. Indeed, a lysozyme sensitivity test supported this idea. Both the *ΔcspB* and *Δpbp1a* single mutant strains showed higher lysozyme sensitivity than the wild type, whereas the *ΔcspBΔpbp1a* double mutant strain showed far greater sensitivity than either single mutant strain (Figure 5). These results indicate that these mutations affect cell surface integrity. Lysozyme sensitivity could be an effective parameter for screening mutations (such as *cspB* and *pbp1a*) that affect, either directly or in combination with other mutations, protein secretion in *C. glutamicum*.

CspB is a structural protein of the S-layer that forms solid two-dimensional paracrystalline arrays surrounding the entire cell [37]. It is likely that the S-layer interferes with the release of Fab into the extracellular space. Genome sequence data suggest that *C. glutamicum* has at least nine PBPs [30]. Mutants of *pbp1a* and *pbp1b*, encoding class A HMW-PBPs, exhibited a similar morphological phenotype [30], although these proteins have distinct binding partners [31]. PBP1a interacts with the cell division protein DivIVA, whereas PBP1b interacts with the morphogenic protein RodA [31]. PBP1b also interacts with PBP2a and PBP2b, though PBP1a does not interact directly with any other HMW-PBP [31]. Thus, PBP1a may have a distinct function among HMW-PBPs in *C. glutamicum*, possibly related to protein secretion.

*C. glutamicum* has a (coryno)mycolate hydrophobic layer between the PG-arabinogalactan layer and S-layer thought to function as a permeability barrier to antibiotics and host defense molecules [26-29]. It is still unclear how secreted proteins pass through this hydrophobic layer. The mycolate layer is composed of free trehalose mycolates and mycolic acid covalently bound to arabinogalactan,

which is in turn attached to the PG layer [28,29]. Loss of PBP1a may affect formation of the mycolate layer by modifying the PG-arabinogalactan layer.

We also examined the secretion of proteins other than Fab, but the *ΔcspBΔpbp1a* double mutation was only effective for Fab as far as tested (data not shown). This suggests that the bottleneck in protein production is different in each case.

In addition to Fab and constituent monomers, protein bands at approximately 34–37 kDa were detected in the culture supernatant of Fab producer strains. It is clear that these were the degradation products of the secreted Fab, because they were specifically detected by Western blotting with anti-IgG (Figure 2b). Protein bands of approximately 12–16 kDa were also detected by SYPRO Orange staining and did not react with anti-IgG. These could be degradation products of Fab, because they were not detected in the culture supernatant of the strain carrying the empty vector pPK4 (Figure 2a). The protease (s) responsible for Fab degradation are unknown. Identification and deletion of such protease(s) is expected to further improve Fab production by the CORYNEX® system.

## Conclusions

We have shown that a combination of *ΔcspB* and *Δpbp1a* mutations improves recombinant Fab secretion from *C. glutamicum*, suggesting that there are at least two permeability barriers to Fab secretion in *C. glutamicum*: the PG layer and the S-layer (Figure 7). Cell wall-associated genes are thus promising targets for further improvement in recombinant protein secretion by *C. glutamicum*. Moreover, lysozyme sensitivity could be an effective parameter for screening mutations that affect protein secretion.

## Methods
### Bacterial strains, plasmids, and culture media
The bacterial strains and plasmids used in this study are listed in Additional file 2: Table S1. *E. coli* JM109 was
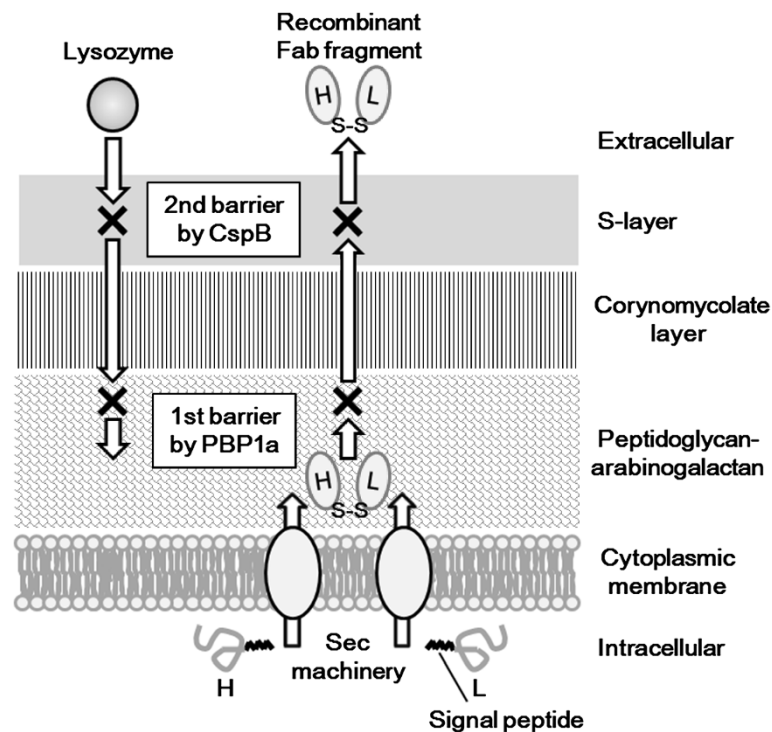
**Figure 7 Cell wall-permeability barriers to the recombinant Fab(H+L) secretion by *C. glutamicum*.** HC and LC of the Fab fragment are secreted through the cytoplasmic membrane by the Sec machinery and form a heterodimer with an intermolecular disulfide bond (S–S) in the extracellular space. There are at least two crucial permeability barriers (X) interrupting Fab secretion; the PG-arabinogalactan layer synthesized by PBP1a and the S-layer composed of CspB. These two structures also function as permeability barriers to lysozyme.

grown in Luria–Bertani (LB) broth [46] and used for plasmid construction. *C. glutamicum* wild-type strain ATCC13869 and its derivative YDK010, an industrial strain for protein secretion [40], were used. *C. glutamicum* strains were grown at 30°C in modified CM2G medium [18] consisting of 10 g/l polypeptone, 10 g/l yeast extract, 5 g/l glucose, 5 g/l NaCl, and 0.2 g/l DL-methionine (pH 7.2), or modified CMDex medium [41] consisting of 5 g/l glucose, 10 g/l polypeptone, 10 g/l yeast extract, 1 g/l $KH_2PO_4$, 0.4 g/l $MgSO_4 \cdot 7H_2O$, 3 g/l urea, 0.01 g/l $FeSO_4 \cdot 7H_2O$, 0.01 g/l $MnSO_4 \cdot 5H_2O$, 1.2 g/l (as total nitrogen) soybean hydrolysate, and 10 μg/l biotin (pH 7.5). For antibody Fab fragment secretion, *C. glutamicum* strains were cultured in 3 ml of liquid CM2G medium at 30°C overnight and 0.2 ml of the cultures were inoculated in 4 ml of modified liquid MMTG medium [18] consisting of 120 g/l glucose, 3 g/l $MgSO_4 \cdot 7H_2O$, 30 g/l $(NH_4)_2SO_4$, 1.5 g/l $KH_2PO_4$, 0.03 g/l $FeSO_4 \cdot 7H_2O$, 0.03 g/l $MnSO_4 \cdot 5H_2O$, 0.45 mg/l thiamine hydrochloride, 0.45 mg/l biotin, 0.15 mg/l DL-methionine, 0.2 g/l (as total nitrogen) soybean hydrolysate, and 50 g/l $CaCO_3$ (pH 7.0) in a test tube, and then cultured at 30°C for 96 h. Kanamycin (25 μg/ml)

was added to the culture medium as required. To prepare agar plates, agar (20 g/l) was added to the growth media.

### Construction of a plasmid for Fab secretion

The DNA sequences encoding the variable regions of the HC and LCs of anti-HER2 (GenBank accession numbers, AY513484 and AY513485, respectively) and each constant region were designed by incorporating the *C. glutamicum* codon bias (GenScript, Piscataway, NJ, USA). DNA fragments containing the promoter of *cspB* from *C. glutamicum* and the signal sequence of CspA from *C. ammoniagenes* were fused to the HC or LC genes by GenScript to produce the tandem expression cassette (Figure 1 and Additional file 1: Figure S1). The synthesized construct was digested with *Bam*HI or *Xba*I, respectively, and inserted into the *Bam*HI and *Xba*I sites of pPK4 [18] to obtain pPKStrastFabHL. The cloned fragments were sequenced to confirm the intended construction.

### Protein analysis

The culture supernatant was obtained by centrifugation and 10 μl of supernatant was mixed with an equal

volume of SDS sample buffer (Bio-Rad, Hercules, CA, USA) without reducing agent. Proteins were separated by 10%–20% gradient polyacrylamide gel electrophoresis (PAGE) as described by Laemmli [47] under non-reducing conditions, and the gels were stained with SYPRO Orange (Life Technologies, Carlsbad, CA, USA). Estimation of Fab production yield was done by quantifying the intensity of protein bands in gels using Multi Gauge software (Fujifilm, Tokyo, Japan). A standard curve was generated using a standard protein solution of known concentration run in the same gel. For determining the N-terminal amino acid sequence, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting after separation by sodium dodecyl sulfate (SDS)-PAGE, and the protein bands were directly applied to a gas-phase protein sequencer (model PSQ; Shimadzu, Kyoto, Japan) equipped with an in-line amino acid analyzer (model RF-550; Shimadzu), as described previously [48]. Western blotting analysis was performed with alkaline phosphatase (AP) conjugated anti-human IgG (H+L) antibody (Rockland Immunochemicals, Gilbertsville, PA, USA) and an AP conjugate substrate kit (Bio-Rad, Hercules, CA, USA).

## Antigen-binding activity analysis of Fab

Culture supernatant was collected by centrifugation and filtered with a 0.22 μm Millex-GV syringe filter unit (Merck Millipore, Billerica, MA, USA). 20 ml of the filtrated supernatant was injected directly onto 1 ml of HiTrap Protein G affinity column (GE Healthcare UK. Ltd., Buckinghamshire, England) which was pre-equilibrated with 20 mM Tris–HCl (pH 7.5). Subsequently, Fab trapped in the column was eluted with 3 ml of 0.1 M Glycine-HCl (pH 2.7), and 20 μl of 2 M Tris–HCl (pH 8.5) was added immediately to bring to physiological pH. Biacore X100 (GE Healthcare UK. Ltd., Buckinghamshire, England) was used for surface plasmon resonance analysis. The extracellular domain of recombinant human HER2/ErbB2 (Sino Biological Inc., Beijing, China) was diluted in 10 mM sodium phosphate buffer (pH 6.0) and immobilized on a CM5 sensor chip (GE Healthcare UK. Ltd., Buckinghamshire, England) to achieve 577 resonance units (RU) by amine coupling according to the manufacturer's instructions. Various concentrations of samples were injected into the flow cell diluted with HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20). All kinetic measurements were conducted at 25°C at a flow rate of 30 μl/min with an association time of 120 s and dissociation time of 600 s. Chip regeneration was accomplished by exposing the chip to 10 mM Glycine-HCl (pH 1.5) for 120 s. Data were calculated using Biacore X100 evaluation software (GE Healthcare UK. Ltd., Buckinghamshire, England) by fitting the data to a 1:1 binding model.

## Construction of *pbp1a* and *pbp1b* deletion mutants of *C. glutamicum*

*C. glutamicum* disruptants were constructed as described previously [41]. Plasmid pBS5T [49], which carries a temperature-sensitive replication origin and the *Bacillus subtilis sacB* gene, was used as a suicide vector [50]. To construct a *pbp1a* disruptant, two successive rounds of PCR were performed. In the first-round, a 1.0-kb upstream region of the *pbp1a* gene was PCR-amplified from ATCC13869 chromosomal DNA template using the primers 5′-GTCGGATCCGCCCCCCTGAGCCAAATATTC-3′ and 5′-TTTCTAGCGGAAGAACTGGTTGATGGCGTCGAGCTTTGTCAGAGA-ATTCGTGGT-3′, and a 1.0-kb downstream region of the *pbp1a* gene was PCR-amplified using primers 5′-GTGTCCACCACGAATTCTCTGACAAAGC-TCGACGCCATCAACCAGTTCTTCC-3′ and 5′-AGTATCTAGATTCGAGTCGCTT-TTGGTTGGC-3′. Second-round PCRs were performed on the first-round PCR products using the primers 5′-GTCGGATCCGCCCCCCTGAGCCAAATATTC-3′ and 5′- AGTATCTAGATTCGAGTCGCTTTTGGTTGGC-3′. The amplified fragments were digested with BamHI and XbaI and inserted into the BamHI–XbaI site of pBS5T to obtain pBS5TΔpbp1a. Similarly, to construct a *pbp1b* disruptant, a 1.3-kb upstream region of the *pbp1b* gene was PCR-amplified from ATCC13869 chromosomal DNA template using the primers 5′-CGGCGAACTCAAAAACAGCAT-3′ and 5′-GGATAGTCAGCCCCGGCAGGATCCTTTTGCCACTGCTCTTTTTG –3′, and a 1.1-kb downstream region of the *pbp1b* gene was PCR-amplified using primers 5′- CAAAAAGAGCAGTGGCAAAAGGATCCTGCCGGGGCTGACTATC-3′ and 5′- CCAAACAACCCGAAGCTCAAC-3′ as first-round PCRs. Second-round PCRs were performed on the first-round PCR products using primers 5′-CGGCGAACTCA-AAAACAGCAT-3′ and 5′-CCAAACAACCCGAAGCTCAAC-3′. Amplified fragments were digested with PstI and SalI, and the resulting 2.2-kb fragment was inserted into the PstI–SalI site of pBS5T to give pBS5TΔpbp1b. Vector pBS5TΔpbp1a or pBS5TΔpbp1b was introduced into *C. glutamicum* by electroporation, and kanamycin-resistant transformants were selected at 34°C. Because pBS5T does not replicate at 34°C, only single-crossover chromosomal integrants grew on kanamycin-containing CMDex plates at 34°C. One of the kanamycin-resistant transformants was grown in CMDex medium without kanamycin overnight, and the cells were spread on sucrose-containing CMDex agar plates (10% sucrose). Cells carrying the *sacB* gene do not grow in the presence of sucrose, and thus, only cells in which the *sacB* gene was excised from the chromosome by a second homologous recombination event grew on the sucrose-containing plates. The resulting sucrose-resistant recombinants presumably had the wild-type or the deleted

pbp1a or pbp1b gene, depending on the recombination points. The desired disruptants were selected by PCR. The pbp1a or pbp1b deletion mutants of *C. glutamicum* were designated as YDK010Δpbp1a, YDK010Δpbp1b, ATCC 13869Δpbp1a, ATCC13869Δpbp1b, ATCC13869ΔcspBΔpbp1a, and ATCC13869ΔcspBΔpbp1b, respectively.

### Lysozyme sensitivity test of *C. glutamicum*

Lysozyme sensitivities of *C. glutamicum* ATCC13869, ΔcspB, Δpbp1a, ΔcspBΔpbp1a, Δpbp1b, and ΔcspBΔpbp1b mutants were evaluated by growth assay in LB liquid medium or plate assay, as described previously [51].

### Additional files

**Additional file 1: Figure S1.** Nucleotide sequence of the co-expression cassette of Fab(H+L) in pPKStrastFabHL, with the amino acid sequences given below. The sequence is presented in the 5′ to 3′ direction. The putative ribosome-binding site (RBS), the amino acid sequence of the CspA signal peptide, and restriction enzyme sites are boxed, underlined, and described in lower case, respectively. The sequences of HC and LC gene of the anti-HER2 Fab fragment are described in boldface.

**Additional file 2: Table S1.** Bacterial strains and plasmids used in this study.

### Abbreviations

AP: Alkaline phosphatase; ATCC: American-type culture collection; CORYNEX®: *Corynebacterium glutamicum* protein expression system; Csp: Cell surface protein; Da: Dalton; Fab: Fragment antigen-binding; HC: Heavy chain; HMW: High molecular weight; IgG: Immunoglobulin G; LC: Light chain; LMW: Low molecular weight; OD: Optical density; PBP: Penicillin-binding protein; PCR: Polymerase chain reaction; PG: Peptidoglycan; PVDF: Polyvinylidene difluoride; RU: Resonance unit; S-layer: Surface layer; scFv: Single-chain variable fragment; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; Strain name/plasmid name: Denotes a plasmid-carrying strain.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

YM designed and performed the Fab secretion and analyses, constructed the mutant strains, coordinated the work, and drafted the manuscript. HI designed the mutant strains and coordinated the work. YK and NMT performed the lysozyme sensitivity test. EAK and YAVY designed and constructed the mutant strains. MD assayed the antigen-binding activity of Fab. YK and MW supervised the work and reviewed the final manuscript. All authors read and approved the final manuscript.

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### References

1. Reichert JM, Rosensweig CJ, Faden LB, Dewitz MC: **Monoclonal antibody successes in the clinic.** *Nat Biotechnol* 2005, **23**:1073–1078.
2. Holliger P, Hudson PJ: **Engineered antibody fragments and the rise of single domains.** *Nat Biotechnol* 2005, **23**:1126–1136.
3. Levy R, Weiss R, Chen G, Iverson BL, Georgiou G: **Production of correctly folded fab antibody fragment in the cytoplasm of *Escherichia coli* trxB gor mutants via the coexpression of molecular chaperones.** *Protein Expr Purif* 2001, **23**:338–347.
4. Venturi M, Seifert C, Hunte C: **High level production of functional antibody fab fragments in an oxidizing bacterial cytoplasm.** *J Mol Biol* 2002, **315**:1–8.
5. Quintero-Hernandez V, Juarez-Gonzalez VR, Ortiz-Leon M, Sanchez R, Possani LD, Becerril B: **The change of the scFv into the Fab format improves the stability and in vivo toxin neutralization capacity of recombinant antibodies.** *Mol Immunol* 2007, **44**:1307–1315.
6. Leonard B, Sharma V, Smider V: **Co-expression of antibody fab heavy and light chain genes from separate evolved compatible replicons in *E. coli*.** *J Immunol Methods* 2006, **317**:56–63.
7. Masri SA, Rast H, Hu WG, Nagata LP, Chau D, Jager S, Mah D: **Cloning and expression in *E. coli* of a functional Fab fragment obtained from single human lymphocyte against anthrax toxin.** *Mol Immunol* 2007, **44**:2101–2106.
8. Ukkonen K, Veijola J, Vasala A, Neubauer P: **Effect of culture medium, host strain and oxygen transfer on recombinant Fab antibody fragment yield and leakage to medium in shaken *E. coli* cultures.** *Microb Cell Fact* 2013, **12**:73.
9. Lange S, Schmitt J, Schmid RD: **High-yield expression of the recombinant, atrazine-specific Fab fragment K411B by the methylotrophic yeast *Pichia pastoris*.** *J Immunol Methods* 2001, **255**:103–114.
10. Ning D, Junjian X, Qing Z, Sheng X, Wenyin C, Guirong R, Xunzhang W: **Production of recombinant humanized anti-HBsAg Fab fragment from *Pichia pastoris* by fermentation.** *J Biochem Mol Biol* 2005, **38**:294–299.
11. Gasser B, Maurer M, Gach J, Kunert R, Mattanovich D: **Engineering of *Pichia pastoris* for Improved Production of Antibody Fragments.** *Biotechnol Bioeng* 2006, **94**:353–361.
12. Burtet RT, Santos-Silva MA, Buss GA, Moraes LM, Maranháo AQ, Brigido MM: **Production of a recombinant Fab in *Pichia pastoris* from a monocistronic expression vector.** *J Biochem* 2007, **142**:665–669.
13. Weaver-Feldhaus JM, Lou J, Coleman JR, Siegel RW, Marks JD, Feldhaus MJ: **Yeast mating for combinatorial Fab library generation and surface display.** *FEBS Lett* 2004, **564**:24–34.
14. Ikeda M, Nakagawa S: **The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes.** *Appl Microbiol Biotechnol* 2003, **62**:99–109.
15. Kalinowski J, Bathe B, Bartels D, Bischoff N, Bott M, Burkovski A, Dusch N, Eggeling L, Eikmanns BJ, Gaigalat L, Goesmann A, Hartmann M, Huthmacher K, Krämer R, Linke B, McHardy AC, Meyer F, Möckel B, Pfefferle W, Pühler A, Rey DA, Rückert C, Rupp O, Sahm H, Wendisch VF, Wiegräbe I, Tauch A: **The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins.** *J Biotechnol* 2003, **104**:5–25.
16. Hermann T: **Industrial production of amino acids by coryneform bacteria.** *J Biotechnol* 2003, **104**:155–172.
17. Krämer R: **Secretion of amino acids by bacteria: physiology and mechanism.** *FEMS Microbiol Rev* 1994, **12**:75–94.
18. Kikuchi Y, Date M, Yokoyama K, Umezawa Y, Matsui H: **Secretion of active-form *Streptoverticillium mobaraense* transglutaminase by *Corynebacterium glutamicum*: processing of the pro-transglutaminase by a cosecreted subtilisin-like protease from *Streptomyces albogriseolus*.** *Appl Environ Microbiol* 2003, **69**:358–366.
19. Date M, Yokoyama K, Umezawa Y, Matsui H, Kikuchi Y: **Production of native-type *Streptoverticillium mobaraense* transglutaminase in *Corynebacterium glutamicum*.** *Appl Environ Microbiol* 2003, **69**:3011–3014.
20. Date M, Yokoyama K, Umezawa Y, Matsui H, Kikuchi Y: **High level expression of *Streptomyces mobaraensis* transglutaminase in *Corynebacterium glutamicum* using a chimeric pro-region from *Streptomyces cinnamoneus* transglutaminase.** *J Biotechnol* 2004, **110**:219–226.
21. Date M, Yokoyama K, Umezawa Y, Matsui H, Kikuchi Y: **Secretion of human epidermal growth factor by *Corynebacterium glutamicum*.** *Lett Appl Microbiol* 2006, **42**:66–70.

22. Kikuchi Y, Date M, Itaya H, Matsui K, Wu LF: **Functional analysis of the twin-arginine translocation pathway in** *Corynebacterium glutamicum* **ATCC 13869.** *Appl Environ Microbiol* 2006, **72**:7183–7192.

23. Kikuchi Y, Itaya H, Date M, Matsui K, Wu LF: **Production of** *Chryseobacterium proteolyticum* **protein-glutaminase using the twin-arginine translocation pathway in** *Corynebacterium glutamicum.* *Appl Microbiol Biotechnol* 2008, **78**:67–74.

24. Kikuchi Y, Itaya H, Date M, Matsui K, Wu LF: **TatABC overexpression improves** *Corynebacterium glutamicum* **Tat-dependent protein secretion.** *Appl Environ Microbiol* 2009, **75**:603–607.

25. Schindler M, Assaf Y, Sharon N, Chipman DM: **Mechanism of lysozyme catalysis: role of ground-state strain in subsite D in hen egg-white and human lysozymes.** *Biochemistry* 1977, **16**:423–431.

26. Draper P: **The outer parts of the mycobacterial envelope as permeability barriers.** *Front Biosci* 1998, **3**:D1253–D1261.

27. Jarlier V, Nikaido H: **Permeability barrier to hydrophilic solutes in** *Mycobacterium chelonei.* *J Bacteriol* 1990, **172**:1418–1423.

28. Puech V, Chami M, Lemassu A, Lanéelle MA, Schiffler B, Gounon P, Bayan N, Benz R, Daffé M: **Structure of the cell envelope of corynebacteria: importance of the non-covalently bound lipids in the formation of the cell wall permeability barrier and fracture plane.** *Microbiology* 2001, **147**:1365–1382.

29. Eggeling L, Sahm H: **The cell wall barrier of** *Corynebacterium glutamicum* **and amino acid efflux.** *J Biosci Bioeng* 2001, **92**:201–213.

30. Valbuena N, Letek M, Ordóñez E, Ayala J, Daniel RA, Gil JA, Mateos LM: **Characterization of HMW-PBPs from the rod-shaped actinomycete** *Corynebacterium glutamicum*: **peptidoglycan synthesis in cells lacking actin-like cytoskeletal structures.** *Mol Microbiol* 2007, **66**:643–657.

31. Letek M, Fiuza M, Ordóñez E, Villadangos AF, Ramos A, Mateos LM, Gil JA: **Cell growth and cell division in the rod-shaped actinomycete** *Corynebacterium glutamicum.* *Antonie van Leeuwenhoek* 2008, **94**:99–109.

32. Joliff G, Mathieu L, Hahn V, Bayan N, Duchiron F, Renaud M, Schechter E, Leblon G: **Cloning and nucleotide sequence of the** *csp1* **gene encoding PS1, one of the two major secreted proteins of** *Corynebacterium glutamicum*: **the deduced N-terminal region of PS1 is similar to the** *Mycobacterium* **antigen 85 complex.** *Mol Microbiol* 1992, **6**:2349–2362.

33. Hansmeier N, Bartels FW, Ros R, Anselmetti D, Tauch A, Pühler A, Kalinowski J: **Classification of hyper-variable** *Corynebacterium glutamicum* **surface-layer proteins by sequence analyses and atomic force microscopy.** *J Biotechnol* 2004, **112**:177–193.

34. Payret JL, Bayan N, Joliff G, Gulik-Krzywicki T, Mathieu L, Schechter E, Leblon G: **Characterization of the** *cspB* **gene encoding PS2, an ordered surface-layer protein in** *Corynebacterium glutamicum.* *Mol Microbiol* 1993, **9**:97–109.

35. Blaser MJ, Smith PF, Hopkins JA, Heinzer I, Bryner JH, Wang WL: **Pathogenesis of** *Campylobacter fetus* **infections: serum resistance associated with high-molecular-weight surface proteins.** *Infect Dis* 1987, **155**:696–706.

36. Merino S, Albertí S, Tomás JM: *Aeromonas salmonicida* **resistance to complement-mediated killing.** *Infect Immun* 1994, **62**:5483–5490.

37. Schneitz C, Nuotio L, Lounatma K: **Adhesion of** *Lactobacillus acidophilus* **to avian intestinal epithelial cells mediated by the crystalline bacterial cell surface layer (S-layer).** *J Appl Bacteriol* 1993, **74**:290–294.

38. Sála M, Sleytr UB: **S-Layer proteins.** *J Bacteriol* 2000, **182**:859–868.

39. Valbuena N, Letek M, Ramos A, Ayala J, Nakunst D, Kalinowski J, Mateos LM, Gil JA: **Morphological changes and proteome response of** *Corynebacterium glutamicum* **to a partial depletion of FtsI.** *Microbiology* 2006, **152**:2491–2503.

40. Kikuchi Y, Date M, Umezawa Y, Yokoyama K, Heima H, Matsui H: **Method for the secretion and production protein.** *Int Pat Cooperat Treaty Pat* 2002, WO2002/081694.

41. Takeshita R, Ito H, Wachi M: **A role of the** *cspA* **gene encoding a mycolyltransferase in the growth under alkaline conditions of** *Corynebacterium glutamicum.* *Biosci Biotechnol Biochem* 2010, **74**:1617–1623.

42. Khalili H, Godwin A, Choi JW, Lever R, Brocchini S: **Comparative binding of disulfide-bridged PEG-Fabs.** *Bioconjug Chem* 2012, **23**:2262–2277.

43. An SJ, Yim SS, Jeong KJ: **Development of a secretion system for the production of heterologous proteins in** *Corynebacterium glutamicum* **using PorB signal peptide.** *Protein Expr Purif* 2013, **89**:251–257.

44. Yim SS, An SJ, Kang M, Lee J, Jeong KJ: **Isolation of fully synthetic promoters for high-level gene expression in** *Corynebacterium glutamicum.* *Biotechnol Bioeng* 2013, **110**:2959–2971.

45. Yim SS, An SJ, Choi JW, Ryu AJ, Jeong KJ: **High-level secretory production of recombinant single-chain variable fragment (scFv) in** *Corynebacterium glutamicum.* *Appl Microbiol Biotechnol* 2014, **98**:273–284.

46. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor: Cold Spring Harbor Press; 1989.

47. Laemmli UK: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4.** *Nature* 1970, **227**:680–685.

48. Kikuchi Y, Kojima H, Tanaka T, Takatsuka Y, Kamio Y: **Characterization of a second lysine decarboxylase isolated from** *Escherichia coli.* *J Bacteriol* 1997, **179**:4486–4492.

49. Chinen A, Kozlov YI, Hara Y, Izui H, Yasueda H: **Innovative metabolic pathway design for efficient l-glutamate production by suppressing $CO_2$ emission.** *J Biosci Bioeng* 2007, **103**:262–269.

50. Quandt J, Hynes MF: **Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria.** *Gene* 1993, **127**:15–21.

51. Hirasawa T, Wachi M, Nagai K: **A mutation in the** *Corynebacterium glutamicum ltsA* **gene causes susceptibility to lysozyme, temperature-sensitive growth, and L-glutamate production.** *J Bacteriol* 2000, **182**:2696–2701.