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Development of a LytE-based high-density surface display system in *Bacillus subtilis*

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Summary

The three N-terminal, tandemly arranged LysM motifs from a *Bacillus subtilis* cell wall hydrolase, LytE, formed a cell wall-binding module. This module, designated $CWBM_{LytE}$, was demonstrated to have tight cell wall-binding capability and could recognize two classes of cell wall binding sites with fivefold difference in affinity. The lower-affinity sites were approximately three times more abundant. Fusion proteins with β-lactamase attached to either the N- or C-terminal end of $CWBM_{LytE}$ showed lower cell wall-binding affinity. The number of the wall-bound fusion proteins was less than that of $CWBM_{LytE}$. These effects were less dramatic with $CWBM_{LytE}$ at the N-terminal end of the fusion. Both $CWBM_{LytE}$ and β-lactamase were essentially functional whether they were at the N- or C-terminal end of the fusion. In the optimal case, 1.2×10^7 molecules could be displayed per cell. As cells overproducing $CWBM_{LytE}$ and its fusions formed filamentous cells (with an average of nine individual cells per filamentous cell), 1.1×10^8 β-lactamase molecules could be displayed per filamentous cell. Overproduced $CWBM_{LytE}$ and its fusions were distributed on the entire cell surface. Surface exposure and accessibility of these proteins were confirmed by immunofluorescence microscopy.

Introduction

The ability to display proteins on the bacterial cell surface has many interesting applications including the development of high-throughput systems to screen for hyperactive/high-affinity variants and the use as diagnostic agents, biosensors and antigen-displaying agents for live vaccine production (Georgiou *et al.*, 1997; Stahl and Uhlen, 1997; Wernerus and Stahl, 2004). *Bacillus subtilis* has many attractive features to serve as a host for protein surface display. As a Gram-positive bacterium, *B. subtilis* does not have the outer membrane. This property makes the surface display system much simpler and easier than in Gram-negative bacteria. As *B. subtilis* is considered as a 'GRAS (generally regarded as safe)' organism and has an excellent safety record in production of food-grade enzymes, it can serve as a whole-cell biocatalyst in food industry (Kabayashi *et al.*, 2000; Kobayashi *et al.*, 2000). Its non-pathogenic nature and the ability to form heat stable spores offer attractions to use this system for the development of spore-based vaccine delivery vehicles and diagnostic tools (Acheson *et al.*, 1997; Isticato *et al.*, 2001; Kim *et al.*, 2005a; Uyen *et al.*, 2007). Furthermore, as a natural soil bacterium, *B. subtilis* is an attractive candidate to address various important environmental issues. The potential applications include functioning as detoxification agents to display enzymes to decompose toxic compounds in contaminated areas and as bioplastic decomposition agents to display depolymerases for bioplastic decomposition.

As cell wall is the outermost surface layer for *B. subtilis*, cell wall-binding modules (CWBM) from the wall-bound proteins would be an attractive tool for surface display of biomolecules. Biochemical and proteomic studies (Foster, 1992; Blackman *et al.*, 1998; Antelmann *et al.*, 2002; Tjalsma *et al.*, 2004) demonstrate that *B. subtilis* at the vegetative phase has a group of cell wall-associated proteins that can be eluted from the cell surface by a high-salt treatment (1.5–5 M LiCl). The majority of these proteins (e.g. LytE) are members of the autolysin family (Lazarevic *et al.*, 1992; Margot *et al.*, 1994; Smith *et al.*, 2000; Yamamoto *et al.*, 2003). Others include a wall-bound protease WprA (Margot and Karamata, 1996), a major wall-associated protein WapA with unknown functions (Foster, 1993) and several less well-characterized wall-associated proteins. Among these proteins, two types of cell wall-binding domains are most common (Desvaux *et al.*, 2006). The first group is designated the type II cell wall-binding domain (pfam 04122) and can be commonly found in amidase (LytC) and its activity modifier (LytB) (Lazarevic *et al.*, 1992). The second group is known as the LysM (lysin motif, pfam 01476) domain which is commonly observed in many bacterial lysins (Ishikawa *et al.*, 1998; Bateman and Bycroft, 2000; Steen *et al.*, 2003;

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Desvaux *et al.*, 2006). To develop an efficient surface display system based on the cell wall-binding domains, systematic studies to characterize the properties of these domains are needed. These properties include the type of the cell wall-binding domains which would be more appropriate for surface display of biomolecules, the affinity of these domains to the cell wall, the types of cell wall binding sites available and the effects for creation of protein fusions on the cell wall binding. In this study, the N-terminal cell wall-binding module ($CWBM_{LytE}$) composed of three tandemly arranged LysM domains (Ishikawa *et al.*, 1998; Margot *et al.*, 1998) from *B. subtilis* LytE (CwlF) was selected as a model system for detailed characterization. It was found to have tighter binding to cell wall in comparison with $CWBM_{LytB}$ which is composed of three N-terminally located type II cell wall-binding domains from LytB (Lazarevic *et al.*, 1992). Two classes of $CWBM_{LytE}$ binding sites (high- and low-affinity sites on a relative scale) were identified. Using β-lactamase as the reporter, effects of fusion of the reporter to $CWBM_{LytE}$ on cell wall binding were examined. The functionality of both $CWBM_{LytE}$ and the reporter enzyme at either end of the fusion and the effects of varying the length of linkers on biological activity and surface accessibility of the fusion proteins were also determined. These studies led to the successful development of a high-density surface display system for biologically active molecules on *B. subtilis* cell surface.

Results

Production and characterization of $CWBM_{LytB}$ and $CWBM_{LytE}$

To determine whether the type II cell wall-binding domain or the LysM-binding domain has stronger binding towards cell wall, $CWBM_{LytB}$ and $CWBM_{LytE}$ were produced via secretion using the *B. subtilis* P43 promoter (Wang and Doi, 1984) for transcription and the levansucrase (SacB) signal sequence (Steinmetz *et al.*, 1985) for secretion. The expected molecular masses for the mature forms of $CWBM_{LytB}$ and $CWBM_{LytE}$ are 35.031 kDa and 19.197 kDa respectively. However, the apparent molecular masses for these modules determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were 38 kDa and 25 kDa respectively (Figs 1 and 2). To address the discrepancy, both wall-bound modules were purified and subjected to both N-terminal sequence determination using the Edman degradation method and molecular mass determination using MALDI-TOF mass spectrometry. Interestingly, the sequence for the first five amino acids at the N-terminal region of $CWBM_{LytB}$ was determined to be GFYKV, not IDPAD as predicted. This implies that an extra proteolytic cleavage was taking place in addition to the expected proteolytic processing by signal peptidase. This cleavage

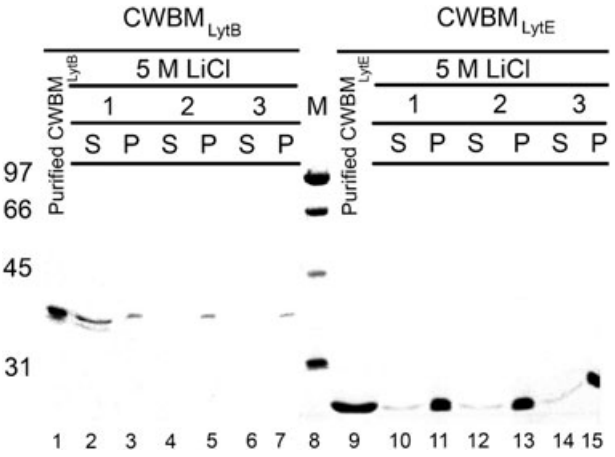

Fig. 1. LiCl elution of $CWBM_{LytB}$ and $CWBM_{LytE}$ from *B. subtilis* cell surface. Cell pellets from WB800[pWB980-LytB] and WB800[pWB980-LytE] overproducing $CWBM_{LytB}$ and $CWBM_{LytE}$, respectively, were used for elution studies. 1, 2 and 3 indicate the number of cycles for LiCl treatment. M represents the molecular mass markers. Their molecular masses expressed in terms of kDa are shown on the left. S and P represent the supernatant and pellet fractions of the LiCl-treated samples.

event led to the loss of 27 amino acids downstream from the expected signal peptidase cleavage site. Consequently, this processed $CWBM_{LytB}$ has the molecular mass of 32.202 kDa which was very close to the molecular mass (32.247 kDa) of this product determined by the MALDI-TOF mass spectrometry analysis (data not shown). In the case of $CWBM_{LytE}$, the levansucrase signal peptide was found to be properly removed and the $CWBM_{LytE}$ product began with the expected IDPAGQ sequence at its N-terminal region. IDPAG sequence was not present in the original LytE sequence and was generated because of the introduction of ClaI and PstI sites downstream of the signal peptidase cleavage site at the gene level. The molecular mass (19.197 kDa) of $CWBM_{LytE}$ determined by MADLI-TOF mass spectrometry was in close agreement with the expected value. Inaccurate molecular mass estimation by SDS-PAGE for $CWBM_{LytE}$ (31% overestimation) is likely caused by the presence of two serine-rich linker sequences in this module. These sequences were predicted to be disordered (Linding *et al.*, 2003; Galzitskaya *et al.*, 2006). As unstructured sequences within proteins have been suggested to bind less SDS (Iakoucheva *et al.*, 2001), this can result in a slower migration of proteins in the electrophoretic run.

$CWBM_{LytE}$ shows tighter cell wall binding

To elute cell wall-binding proteins from *B. subtilis* cell surface, the use of LiCl in the concentration range of 1.5–5 M is a standard practice (Foster, 1992). The levels of wall-bound proteins eluted off from the cell surface by this treatment can provide insights for the strength of the

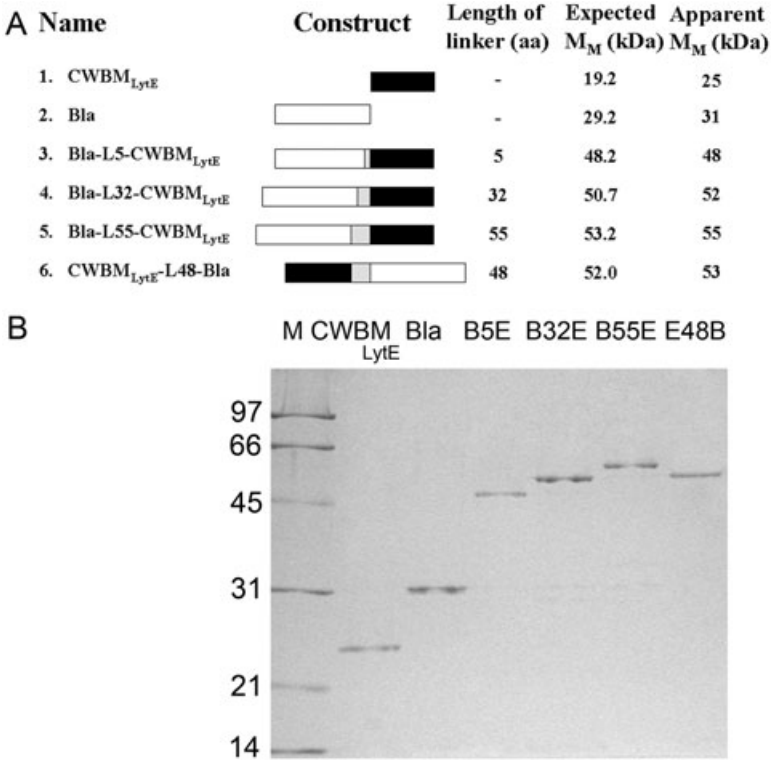

Fig. 2. Construction and properties of $CWBM_{LytE}$ and its fusions.
A. Constructs of $CWBM_{LytE}$ (black), β-lactamase (Bla, white) and their fusions. Linker regions are highlighted in grey. L5, L32, L48 and L55 indicate the presence of 5, 32, 48 and 55 amino acids in the linker regions respectively. Constructs 3 (B5E, B for β-lactamase and E for $CWBM_{LytE}$), 4 (B32E) and 5 (B55E) have the β-lactamase at the N-terminal end. They are the members of the BE series. Construct 6 (E48B) has the β-lactamase at the C-terminal end. M_M indicates molecular mass.
B. Coomassie blue-stained SDS-polyacrylamide gel (12%) profile showing the apparent molecular masses and purities of the purified $CWBM_{LytE}$, β-lactamase and their fusions. M represents the lane loaded with molecular mass markers.

interactions. In this study, 5 M LiCl was used in the elution buffer because of its higher efficiency in eluting these wall-bound proteins off from the cell surface. As shown in Fig. 1, a high percentage of $CWBM_{LytB}$ could be eluted off from the cell surface during the first cycle of LiCl treatment (lane 2). The remaining wall-bound materials were more resistant to the subsequent rounds of LiCl treatment. Only small quantities of $CWBM_{LytB}$ could be eluted during the second and third rounds of LiCl wash. $CWBM_{LytE}$ was even more resistant to the LiCl treatment. Five percent or less of the wall-bound $CWBM_{LytE}$ could be eluted off from the cell surface in each cycle of LiCl wash (lanes 10, 12, 14). Because of the tight binding between $CWBM_{LytE}$ and cell wall, this molecule was selected as the cell wall-anchoring domain to examine its suitability for surface display of proteins on cell surface.

Construction of $CWBM_{LytE}$ fusions

TEM-β-lactamase (Bla) was selected as the reporter in this surface display study because it is an extracellular enzyme that can be exported efficiently in *B. subtilis* and its activity can also be monitored with high sensitivity (Wong and Doi, 1986). With appropriate substrates, activities can be easily determined with less than 100 enzyme molecules (Zlokarnik *et al*., 1998). Two sets of $CWBM_{LytE}$-based fusions were constructed (Fig. 2A). The first set (the BE series) has β-lactamase fused to the N-terminal end of $CWBM_{LytE}$ to generate three fusion proteins: Bla-L5–$CWBM_{LytE}$ (B5E), Bla-L32–$CWBM_{LytE}$ (B32E) and Bla-L55–$CWBM_{LytE}$ (B55E). The length of the linker sequence varies from 5 to 55 amino acids. The second set has one construct which has the β-lactamase domain fused to the C-terminal end of $CWBM_{LytE}$ with a 48-amino-acid linker in between these domains. This fusion protein was designated $CWBM_{LytE}$–L48-Bla (E48B). These linker sequences are rich in glycine, proline and serine. As glycine and proline do not favour the formation of helical and β-sheet structures, their presence promotes the formation of disordered structures in the linker region. Consequently, effects of the linker structures on the folding of the adjacent individual domains can be minimized. Serine is hydrophilic and its presence enhances the solubility of the linker sequence. All these fusion proteins could be produced, eluted from cell wall and purified to high purity for biochemical characterization (Fig. 2B).

TEM-β-lactamase is functional either at the N- or C-terminal end of the fusion

Although TEM-β-lactamase has been used as a reporter enzyme for surface display studies (Strauss and Gotz, 1996; Lattemann *et al*., 2000), it is not sure whether it has comparable specific activity when it is located at either the N- or C-terminal end of the fusion protein. The effect of the presence of linker on β-lactamase activity also has not

Table 1. Specific enzymatic activities of purified β-lactamase, its fusions and $CWBM_{LytE}$.

BSA	Bla	B5E	B32E	B55E	E48B	$CWBM_{LytE}$
–			Specific activities ($\times 10^9$ units $mole^{-1}$)			
	3.82 ± 0.20	2.36 ± 0.09	2.71 ± 0.28	3.49 ± 0.24	3.29 ± 0.18	ND
			Ratio (relative to the specific activity of Bla)			
	1	0.62	0.71	0.91	0.86	ND
+			Specific activities ($\times 10^9$ units $mole^{-1}$)			
	6.02 ± 0.53	5.49 ± 0.51	5.67 ± 0.49	5.87 ± 0.36	5.58 ± 0.44	ND
			Ratio (relative to the specific activity of Bla)			
	1	0.91	0.94	0.97	0.93	ND

One unit of β-lactamase activity is defined as hydrolysis of 1 μmole of substrate per min at 37°C. + and – indicate the presence and absence of BSA in the assay.
ND, not detectable.

been thoroughly examined. Two reports (Laraki *et al.*, 1999; Kimura *et al.*, 2004) illustrate the importance of inclusion of bovine serum albumin (BSA) in the β-lactamase activity assays to get accurate and reproducible result. These enzymes are the IMP-1-type metallo-β-lactamase from *Pseudomonas aeruginosa* and the CTX-M-19-type β-lactamase from *Klebsiella pneumoniae*. Therefore, effect of BSA on TEM-β-lactamase activity has to be studied. By examining the specific activities of β-lactamase in purified B5E, B32E and B55E, increasing the length of the linker was found to improve the specific activity of the reporter enzyme in the fusion protein (Table 1). This was true when the assay was performed in the absence of BSA. Under this condition, both B55E and E48B showed good specific activity (86–91% of the control β-lactamase specific activity). In contrast, when activity assays were performed in the presence of BSA, all lactamase fusions showed excellent specific activities (91–97% of the control β-lactamase specific activity) whether the β-lactamase moiety was at the N- or C-terminal end.

Two classes of $CWBM_{LytE}$ binding sites in cell wall

To examine the interactions between cell wall and $CWBM_{LytE}$ and its fusions, purified proteins from low to high quantities were mixed with purified cell wall. Wall-bound protein complexes were separated from the unbound proteins by centrifugation. Data were analysed using Scatchard plots (Smith and Sestili, 1980) to gain information concerning the binding affinity and the number of cell wall binding sites (B_{max}) recognized by $CWBM_{LytE}$. Interestingly, $CWBM_{LytE}$ and its fusions consistently recognized two classes of binding sites in cell wall (Fig. 3 and Table 2). In the case of $CWBM_{LytE}$, the two classes of binding sites had an affinity difference of approximately five times. $CWBM_{LytE}$ bound to the high-affinity sites with a K_d of 21 nM. Fusion of β-lactamase to either N- or C-terminus of $CWBM_{LytE}$ reduced the binding affinity for both classes of binding sites in cell wall. The B5E fusion which showed the most dramatic reduction in binding affinity had 1.6 and 2.4 times reduction for high- and low-affinity classes respectively. The relative binding affinity of $CWBM_{LytE}$ and its fusions to cell wall followed the order of $CWBM_{LytE}$ > E48B > B55E. For β-lactamase fused to the N-terminal end of $CWBM_{LytE}$, varying the length of the linker sequence from 5 to 55 amino acids did not significantly affect the binding affinities. This is particularly true for the high-affinity sites. Fusion of β-lactamase to $CWBM_{LytE}$ reduced the total number of cell

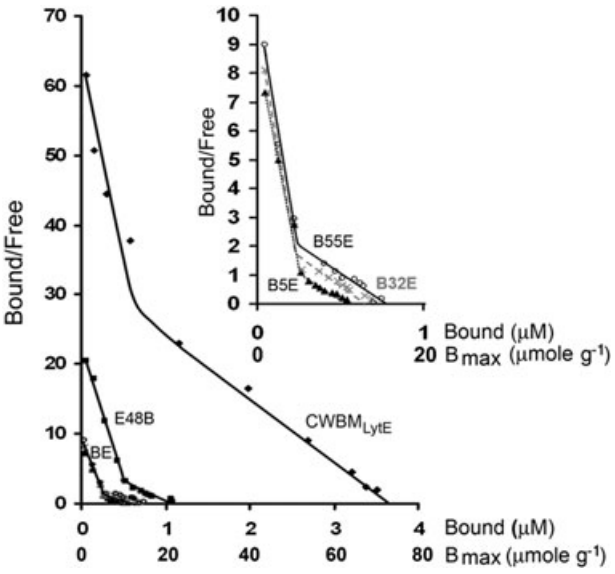

Fig. 3. Binding of purified $CWBM_{LytE}$ and its fusions to purified *B. subtilis* cell wall as analysed by Scatchard plots. The slopes and the intercepts on the *x*-axis reflect the binding constant and the maximal number of binding sites (B_{max}, expressed in terms of μmole of bound protein per gram of purified cell wall) respectively. Closed diamond, closed square and the mixed symbols (closed triangle, grey cross and open circle) are for $CWBM_{LytE}$, E48B and the three members of the BE (B5E, B32E and B55E) series respectively. In the inset, the region which corresponds to the lower left-hand corner in the larger panel is magnified to show the binding properties of B5E (closed triangle), B32E (grey cross) and B55E (open circle) respectively. Three independent experiments were carried out. The standard errors were in the range of 10% or less.

Table 2. Cell wall-binding properties of $CWBM_{LytE}$ and its fusions.

		Construct				
Binding site	Property	$CWBM_{LytE}$	B5E	B32E	B55E	E48B
High affinity	K_d (nM)	20.7 ± 2.0	32.2 ± 2.5	31.4 ± 2.6	30.8 ± 2.4	26.4 ± 2.2
	B_{max} (μmole g^{-1})	22	6.1	6.2	6.3	12
Low affinity	K_d (nM)	108 ± 8	262 ± 15	235 ± 18	273 ± 21	210 ± 14
	B_{max} (μmole g^{-1})	74	10.8	13.1	15.6	22.5
Total number		96	17	19	22	35

B_{max}, maximal number of binding sites per gram of cell wall.

wall binding sites which ranged from 17 (for B5E) to 35 (for E48B) μmole g^{-1} of cell wall. E48B, which has the highest number of cell wall binding sites among the fusions, had a 2.7-fold decrease in the total number of cell wall binding sites. It was even more dramatic for B5E which showed an approximately sixfold reduction. On average, the number of the high-affinity binding sites in cell wall was two to three times less than the number of low-affinity sites for these fusion proteins (Table 2).

Development of quantification methods for the $CWBM_{LytE}$-based cell wall-binding system and the distribution of B55E in different cellular locations

To quantify the number of binding sites per cell for the $CWBM_{LytE}$-based cell wall-binding system, the traditional LiCl treatment was relatively ineffective (Fig. 1). As all the constructs behaved similarly, B55E was used as an example to illustrate the approaches for the establishment of other methods for quantification of these wall-bound proteins. These alternate methods will also allow one to determine the distribution of these fusion proteins in different cellular locations. Addition of the sample loading buffer used in SDS-PAGE to the B55E-producing cells together with a 10 min boiling treatment was found to effectively elute B55E off from the cell surface as demonstrated by Western blot with the $CWBM_{LytE}$-specific antibodies (Fig. 4, lane 3). To determine whether the majority of B55E could be eluted off from the cell surface by this method, the same amounts of cells were first treated with lysozyme to generate protoplasts. The supernatant (lane 4) which contained the released wall-bound proteins was separated from the protoplasts which were subsequently lysed by French press to generate the soluble and pellet protoplast fractions (lanes 5 and 6). The supernatant fraction showed comparable intensity for the B55E protein band in comparison with the boiling method in the presence of SDS (lane 4 versus lane 3). A low level of cell lysis occurred in the lysozyme treatment as the supernatant contained low levels of the B55E precursor. Analysis of the soluble and pellet protoplast fractions demonstrated that there were high levels of B55E precursor in the protoplasts (lanes 5 and 6) with the majority of the precursor in the form of inclusion bodies. Most important, no mature form of B55E was detected in either the soluble or insoluble protoplast fraction. With all the information, a rapid and valid quantification was developed by treating the B55E producing cells with lysozyme followed by French press (lanes 7 and 8). In this case, the lysozyme-treated supernatant was not separated from the protoplast before the French press treatment. The mature form of B55E in the soluble total cell lysate represented the wall-bound B55E proteins (the lower band in lane 7). It showed comparable intensity as those observed in lanes 3 and 4. The B55E proteins were produced in excess with

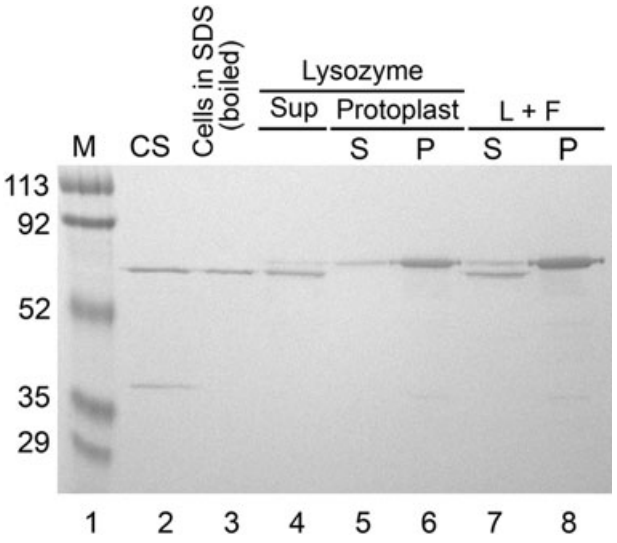

Fig. 4. Distribution of B55E in different cellular compartments and the establishment of methods to quantify wall-bound B55E. Samples were analysed by Western blotting and probed with anti-$CWBM_{LytE}$ antiserum. CS, S and P represent culture supernatant, soluble and pellet fractions respectively. Sup indicates the supernatant fraction derived from the lysozyme-treated cell sample. L + F represent the formation of protoplast by the lysozyme treatment followed by the use of French press for cell lysis. M is the lane loaded with the pre-stained protein molecular mass markers. The molecular masses of these proteins are expressed in terms of kDa as shown on the left. Equivalent amount of samples was loaded for comparison. The 37-kDa protein band observed in lane 2 is likely to be a degradation production of B55E.

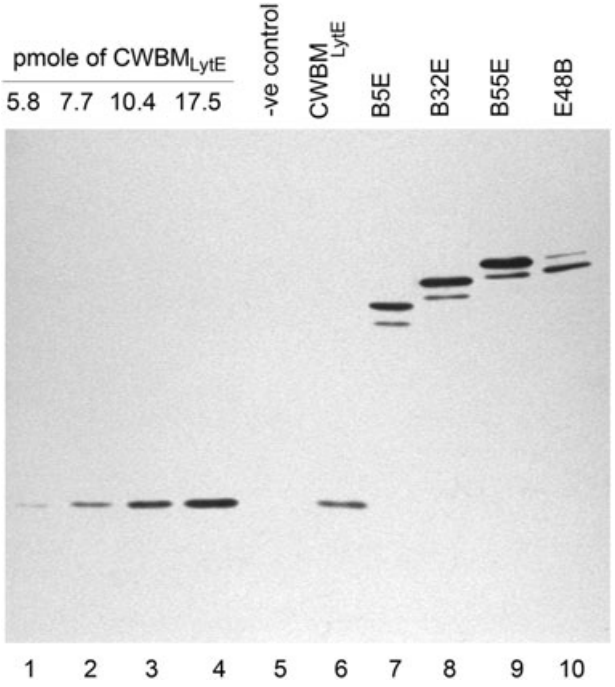

Fig. 5. Quantification of wall-bound CWBM$_{LytE}$ and its fusion proteins by Western blot probed with the anti-CWBM$_{LytE}$ serum. The protein samples were resolved using a 6–15% polyacrylamide gradient gel in the presence of SDS before blotting. '–ve control' represents sample from WB800[pWB980] which has the expression vector without any insert. The lower bands observed in lanes 7–10 represent the mature form of fusion proteins. They were used in the quantification study. A total of 4.5×10^4 cells were loaded in each lane for lanes 7–10. A total of 1.8×10^4 cells (2.5-fold less) were loaded in lane 6 to ensure that the band intensity could be within the linear range of the calibration curve of CWBM$_{LytE}$.

some of them in the culture supernatant (lane 2). Comparison of band intensities in lanes 2, 7 and 8 suggested that the distribution of B55E in culture supernatant, cell wall and intracellular fraction was 16%, 14% and 70% respectively.

The CWBM$_{LytE}$-based cell wall-binding system can display up to 10^7 molecules per cell

The number of cell wall binding sites per cell was estimated using Western blot (Fig. 5). Using the standard curve generated by known amounts of purified CWBM$_{LytE}$, the band intensity of the mature form of CWBM$_{LytE}$ and its fusions could be quantified. The levels of wall-bound CWBM$_{LytE}$ and E48B were higher than those of the BE fusion series (Table 3). This correlated well with the absence (for CWBM$_{LytE}$) or presence (for E48B) of low levels of precursor molecules in the production hosts for these two constructs. In contrast, the BE fusion series had high levels of precursors which accumulated mainly in the form of inclusion bodies in the cell. As the cell number in the culture was determined by cell-plating method, it was essential to determine the number of cells forming each colony. As shown in Fig. 6, *B. subtilis* cells displaying CWBM$_{LytE}$ and its fusions tended to cluster together to form filamentous cells. On average, each filamentous cell was composed of nine individual bacterial cells (data not shown). After normalization of the presence of multiple cells in each filamentous cell, each regular *B. subtilis* cell could display 3×10^7 CWBM$_{LytE}$, 1.2×10^7 E48B and an average of 6×10^6 molecules from the BE fusion series (Table 3).

CWBM$_{LytE}$ and its fusions bind to the B. subtilis *cell surface*

Binding of CWBM$_{LytE}$ and its fusions to cell surface and the surface accessibility of the exposed molecules would be best demonstrated by immunofluorescent staining using CWBM$_{LytE}$- and β-lactamase-specific antibodies. The effective pore radii of the *B. subtilis* peptidoglycan layers have been estimated to be 2.1–2.5 nm (Hughes *et al.*, 1975; Demchick and Koch, 1996). Globular proteins with their molecular weight less than 50 000 are suggested to be able to penetrate through the peptidoglycan layers. As immunoglobulin G is a glycoprotein with the molecular weight > 150 000, it should only react with the surface exposed wall-bound proteins. For the fluorescence microscopic studies (Fig. 6A), *B. subtilis* WB800[pWB980] (control) and the strains overproducing CWBM$_{LytE}$ and E48B were stained with fluorescent dyes specific for DNA (blue coloured), hydrophobic molecules (membrane and protein staining, green coloured) and antibodies against either CWBM$_{LytE}$ or β-lactamase (probed with fluorescent dye-conjugated secondary antibodies, red coloured).

Table 3. Number of cell wall binding sites on *B. subtilis* cell surface.

Cell type	CWBM$_{LytE}$	B5E	B32E	B55E	E48B	Bla
			Number of binding sites			
Colony-forming unit (filamentous cell)	26×10^7	4.0×10^7	5.2×10^7	7.3×10^7	11×10^7	ND
Single cell[a]	2.9×10^7	0.44×10^7	0.58×10^7	0.81×10^7	1.2×10^7	ND
			Ratio			
	1	0.15	0.20	0.28	0.42	ND

a. On average, nine single cells are clustered to form a filamentous cell or a colony-forming unit.
ND, not detected.

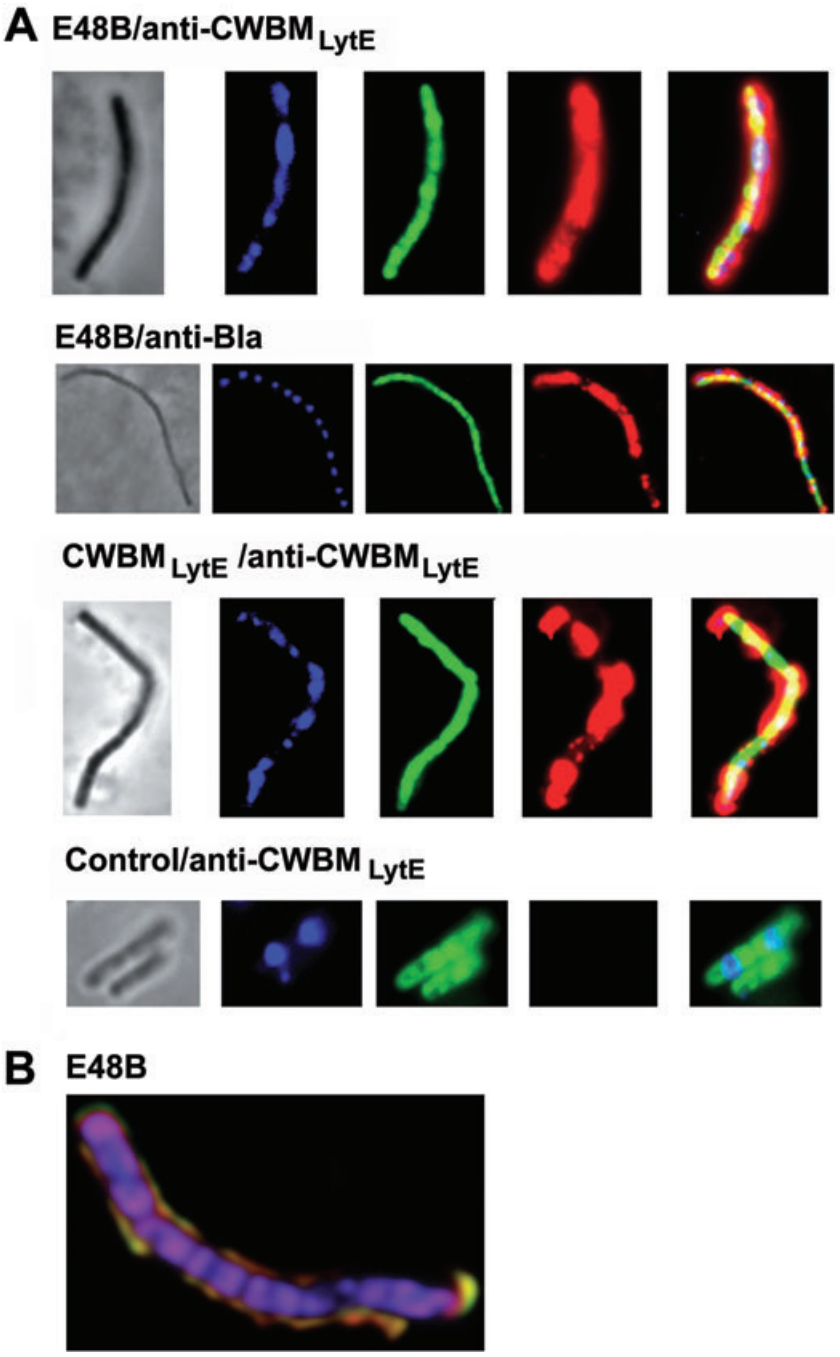

Fig. 6. Surface display of $CWBM_{LytE}$ and E48B on *B. subtilis* cell surface as studied by immunofluorescence microscopy.
A. Localization studies using the fluorescence microscope. *Bacillus subtilis* cells WB800[pWB980] (control), WB800[pWB980-LytE] and WB800[pWB980-LytE-L48-Bla] carrying the control plasmid and plasmids for overproduction of $CWBM_{LytE}$ and E48B were used in the study. Blue: DNA stained with Hoechst 33258; Green: hydrophobic molecules stained with dihexaoxacarbocyanine iodide ($DiOC_6$); Red: anti-$CWBM_{LytE}$ or anti-Bla antiserum. The last panels on the right represent composite images generated by merging of the images showing DNA, hydrophobic molecule and cell wall-binding protein stains. Yellow: overlapping of red- and green-coloured regions.
B. Localization study of E48B producing *B. subtilis* using the confocal fluorescence microscope. Blue: DNA, Pink: hydrophobic molecules, Red: cell wall stained with Alexa Fluor 660-labelled wheat germ agglutinin; Green: anti-Bla antiserum; and Yellow: overlapping of red- and green-coloured regions.

E48B was selected for the study because it had the highest levels of wall-bound fusion proteins. Although the control strain should produce the full-length wild-type LytE proteins, the level of surface exposed LytE was below detection limit. In contrast, the $CWBM_{LytE}$ overproduction strain was stained very well with the $CWBM_{LytE}$-specific antibodies. Superimposing images generated by staining with DNA, hydrophobic molecules and $CWBM_{LytE}$ specific agents suggested that $CWBM_{LytE}$ was located on the cell surface. The same was true for the E48B strain overproducing the $CWBM_{LytE}$ fusion which could be stained by both the $CWBM_{LytE}$- and β-lactamase-specific antibodies. To further examine the association of E48B with the peptidoglycan layer, a confocal fluorescence microscope that could handle four different fluorescent dyes was used in the study (Fig. 6B). E48B was stained blue for DNA, pink for hydrophobic molecules, red for Alexa 660-conjugated wheat germ agglutinin which binds specifically to *N*-acetylglucosamine present in the peptidoglycan layers and green for antibodies specific for lactamase. It was

interesting to observe the presence of some yellow-coloured zones in certain regions of the bacterial cell surface. These yellow-coloured zones were generated because of the overlap between the red- and green-coloured zones. Their presence suggested the colocalization of E48B with the peptidoglycan. E48B, if not all, should also be surface accessible to antibodies.

Effect of varying the length of the linker between β-lactamase and CWBM$_{LytE}$ on both the enzymatic activity and the amount of wall-bound CWBM$_{LytE}$-based fusion proteins

The length of the linker can affect the activity of the displayed β-lactamase and the amount of the wall-bound fusion proteins. It has previously been reported that folding of TEM-β-lactamase can be interfered within the cell wall environment (Strauss and Gotz, 1996). This leads to a 20–40% reduction in activity. To examine the cell wall effect, wall-bound β-lactamase activities were determined using both intact cells and the cell wall fractions. The cell wall fractions were the supernatant fractions containing both the cell wall and the wall-bound proteins released from intact cell surface by the lysozyme treatment. Wall-bound β-lactamase in these fractions presumably could be properly folded as cell wall was extensively degraded by lysozyme and should not function as a physical barrier to interfere with the folding of β-lactamase. As shown in Fig. 7, although minor differences in the β-lactamase activities from both the cell wall fractions and the corresponding intact cells producing B5E, B32E and B55E were observed, these differences were not statistically significant. Inability to observe the cell wall interference effect could partially be contributed by assaying the β-lactamase activities in the presence of BSA which can enhance the β-lactamase activity. In terms of the amounts of the wall-bound enzymes as reflected by the β-lactamase activity, the three members of the BE fusion series (B5E, B32E and B55E) showed comparable activities. The E48B construct showed a 2.4-fold higher activity in reference to the activities from the BE fusion constructs. This observation was consistent with the observation that the amount of surface-displayed E48B was two to three times higher than the amounts of the surface-displayed molecules from the BE fusion series as determined by Western blot (Fig. 5 and Table 3).

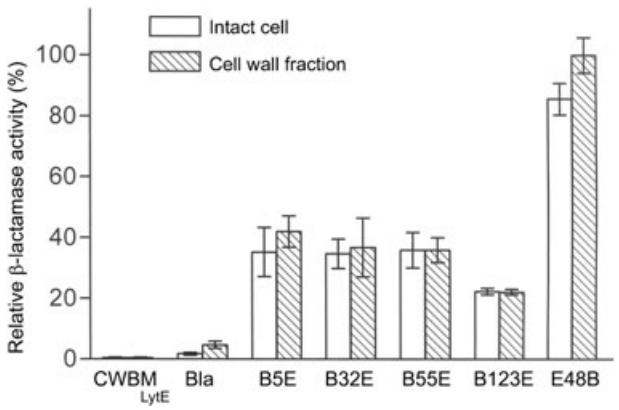

Fig. 7. Relative β-lactamase activities of wall-bound fusions on the surface of intact cells and in the cell wall fraction. Cell wall fraction is defined as the supernatant fraction generated after centrifugal removal of protoplasts from the intact cell samples that have been treated with lysozyme. β-Lactamase activity was determined in the presence of BSA. The activity from the cell wall fraction of E48B was set as 100%. Three independent experiments were carried. CWBM$_{LytE}$: cell wall-binding module from LytE. Bla: secreted TEM-β-lactamase. This is the mature form of β-lactamase in the culture supernatant of WB800[pWB980-Bla]. Secretion of this protein is directed by the *B. subtilis* SacB signal peptide.

Discussion

An ideal bacterial surface display system should have the ability to display functional biomolecules at a high cell surface density in a surface-accessible manner. The cell wall-binding module needs to be able to bind tightly to the cell wall. The CWBM$_{LytE}$-based system in *B. subtilis* has all the desirable features to be an attractive surface display system. Formation of filamentous cells allows the display of 7×10^7 and 1×10^8 B55E and E48B molecules respectively (Table 3). This corresponded to the display of 8×10^6 of B55E and 1×10^7 of E48B per individual cell within the filamentous cell. The numbers of molecules displayed per individual cell are approximately six to eight times more than those reported using the LytC-based display system which can display 1.2×10^6 lipase molecules (Kabayashi *et al.*, 2000). Formation of these filamentous cells is not unexpected as LytE plays a role in cell separation during division (Ishikawa *et al.*, 1998). Overproduction of CWBM$_{LytE}$ fusion proteins can potentially compete with LytE and other LysM domain-carrying autolysins (e.g. LytF) for binding sites at the septa.

CWBM$_{LytE}$, composed of three LysM domains, has long been suggested to be the cell wall-binding domain (Ishikawa *et al.*, 1998; Margot *et al.*, 1998). This assumption was confirmed in this study. Interactions of LysM motifs derived from *Listeria*, *Lactococcus* and *Lactobacillus* with cell wall have been studied (Loessner *et al.*, 2002; Steen *et al.*, 2003; Turner *et al.*, 2004). However, this study is the first to demonstrate that a LysM-based module (i.e. CWBM$_{LytE}$) could recognize two different classes of cell wall binding sites. Although the exact chemical nature of these different binding sites is unknown, presence of lipoteichoic acid has been suggested to negatively affect interactions between LysM motifs and cell wall (Steen *et al.*, 2003). Presence of the cell wall-associated materials (e.g. teichoic acid and teichuronic acid) in *B. subtilis*

can potentially generate heterogeneity in cell wall binding sites recognized by $CWBM_{LytE}$ (Foster and Popham, 2002).

In contrast to LytC which distributes uniformly on the cell surface, localization studies of LytE expressed from the single-copy chromosomal gene demonstrates that LytE localizes at the septa and the two poles of *B. subtilis* (Yamamoto *et al.*, 2003). This raises a major concern if $CWBM_{LytE}$ fusion proteins can only be displayed at the septa and cell poles. However, our studies (Fig. 6) illustrated that the $CWBM_{LytE}$ fusion proteins, when overproduced, could be distributed on the entire cell surface. It is tempting to speculate that the high-affinity sites may be preferentially localized to the septa and cell poles while the lower-affinity binding sites may be more abundant in regions other than the septa and cell poles. Alternatively, other LytE sequence located outside of $CWBM_{LytE}$ may be required for proper targeting of LytE to septa and poles.

The use of the binding domains that bind to cell wall non-covalently for surface display of biomolecules has been well studied (Strauss and Gotz, 1996; Acheson *et al.*, 1997; Kabayashi *et al.*, 2000; Turner *et al.*, 2004). However, the effects of addition of the reporter molecule to the cell wall-binding domain on the cell wall-binding affinity and the number of the wall-bound fusion proteins have not been systematically examined. In reference to $CWBM_{LytE}$ alone, addition of extra domains such as β-lactamase to $CWBM_{LytE}$ results in lowering the number of the $CWBM_{LytE}$ fusions that can be displayed on cell surface (Tables 2 and 3). This can potentially be attributed to the reduction of the cell wall-binding affinity in these fusions and the reduction of the production yield because of the formation of inclusion bodies for these fusion proteins. Interestingly, fusion of β-lactamase to the C-terminal end of $CWBM_{LytE}$ (E48B) has less dramatic effects on both the cell wall-binding affinity reduction and the formation of inclusion bodies. This leads to an increase of E48B on the cell surface by 1.5 times in comparison with B55E. In LytE, the $CWBM_{LytE}$ domain is naturally at the N-terminal end (Ishikawa *et al.*, 1998; Margot *et al.*, 1998). Our study indicated that $CWBM_{LytE}$ at the N-terminal end of a fusion functioned better. However, it was still an effective cell wall-binding domain when it was located at the C-terminal end of the fusion.

One of the important features for achieving high density surface display in this system is the ability to produce excess amounts of $CWBM_{LytE}$ and its fusion proteins from the production strains. As a consequence, free forms of these proteins could be detected in the culture media (Fig. 4, lane 2). These proteins were believed to be in equilibrium with those bound to cell wall and were able to maintain a high level of wall-bound $CWBM_{LytE}$ fusion proteins on the cell surface. $CWBM_{LytE}$ and their fusions in the culture media were found to be biologically active as addition of purified cell wall to the culture media could capture these proteins (data not shown). Presence of excess recombinant LysM-based cell wall-binding domains in culture media with *Lactobacillus fermentum* and *Lactococcus lactis* as the production hosts have also been observed (Turner *et al.*, 2004; Bosma *et al.*, 2006). In fact, based on this feature, a novel surface display system to display biomolecules on non-living and non-genetically modified Gram-positive cells has recently been developed (Bosma *et al.*, 2006).

A linker was introduced between $CWBM_{LytE}$ and β-lactamase for two reasons. First, the linker can potentially allow each domain to fold independently without interference from each other. Second, the linker sequence can serve as a spacer to project the reporter enzyme to the cell surface (Strauss and Gotz, 1996; Nguyen and Schumann, 2006). The thickness of the *B. subtilis* cell wall has recently been determined to be 33 nm (Matias and Beveridge, 2005). If a glycine-rich linker sequence adopts the configuration as polyglycine with a translation rise between each residue in the range of 0.31–0.34 nm (Ramachandran and Sasisekharan, 1968), the linker needs to be 97–106 amino acids in length to fully penetrate through the peptidoglycan layer. Indeed, surface display in *B. subtilis* (Nguyen and Schumann, 2006) using the sortase-mediated covalent anchoring system suggests that the optimal length of linker is 123 amino acids in length. In our case, no linker length dependence on enzymatic activities could be observed for the B5E, B32E and B55E fusions (Fig. 7). As the longest linker in these fusions was 55 amino acids in length, an extra construct, B123E with a 123-amino-acid linker, was made. It behaved in the same manner as the B55E construct except that its production yield was less as reflected by its activity (Fig. 7) and Western blot (data not shown). The discrepancy of the optimal linker length in the *B. subtilis* surface display systems can be attributed to the use of different reporter enzymes in these studies. In the sortase-mediated display system, α-amylase was used as the reporter enzyme. As its substrate, amylose or starch, is a polysaccharide and is bulky in structure, it cannot efficiently penetrate through the bacterial cell wall. Displaying α-amylase through a 33-nm-thick cell wall would require a long linker (97 amino acids or more). In contrast, the β-lactamase substrate (PADAC) used in our assays has a molecular weight of 562.6 and should be freely diffusible into cell wall. As long as folding of the displayed β-lactamase is not interfered by the cell wall, short linker sequences are sufficient.

Stabilization of the wall-bound proteins from *B. subtilis* has been reported with WB700, a seven-extracellular-protease-deficient strain, and 168WA, a cell wall protease (WprA)-deficient *B. subtilis* strain as the hosts (Kabayashi *et al.*, 2000; Antelmann *et al.*, 2002). WB800 (Wu *et al.*,

2002), an eight-protease-deficient strain created by inactivation of *wprA* in the genetic background of WB700, is essential to achieve a high-density surface display of biomolecules. In addition to various systems developed for *B. subtilis* to display biomolecules on the surface of vegetative cells (Acheson *et al.*, 1997; Kabayashi *et al.*, 2000; Kim *et al.*, 2005b; Nguyen and Schumann, 2006) and spores (Isticato *et al.*, 2001; Kim *et al.*, 2005a; Uyen *et al.*, 2007), the combination of the $CWBM_{LytE}$-based surface display system with WB800 offers an attractive alternative for functional display of biomolecules on *B. subtilis* cell surface at high surface density. Because of the filamentous nature of these cells, this display system may be ideal to display depolymerization enzymes such as bioplastic hydrolases which act on long polymeric substrates. Anchoring cells at one end of the filament can potentially promote the attachment of cells in the other end of the filament to the polymeric substrate surface in a cooperative manner.

Experimental procedures

Bacteria and growth condition

Bacillus subtilis WB800 (Wu *et al.*, 2002) was used as the expression host for cloning and protein expression. The cells were cultured in super-rich medium (Halling *et al.*, 1977) for 8 h and harvested for various analyses.

Construction of expression vectors

To construct pWB980-LytE for production of the LytE-based cell wall-binding module ($CWBM_{LytE}$), a 575 bp DNA fragment was amplified by PCR using *B. subtilis* 168 chromosomal DNA as template and the following pair of primers 5′-CTACCGCACTGCTGGCAGGAGGC GCAACTCAAGCTT TTGCAATCGATCCTGCAGGACAAAGCATTAAGGTGAAAA AAGG-3′ and 5′-GAGTCGACGATTATGAGCTGC TTGTGC TTGTTCC-3′. The amplified fragment was digested with BtsI and SalI, and inserted into the BtsI–SalI sites in pWB980 to generate pWB980-LytE. To construct pWB980-LytB, the 963 bp sequence encoding the cell wall-binding module from *lytB* ($CWBM_{LytB}$) was amplified from *B. subtilis* chromosomal DNA with the following two primers: 5′-GATCCTGCAGAC TCAAACATCTCAGTTAAATTGTTAA ATTATATTG-3′ and 5′-GATGCGTCGACGATTATTCATCAGCAAGTGAATTCTC-3′. The amplified PCR product was cut by PstI and SalI and was applied to replace the PstI–SalI *lytE* fragment in pWB980-LytE to generate pWB980-LytB. To develop an expression vector for secretory production of TEM-β-lactamase, a 788 bp DNA fragment encoding the mature form of TEM-β-lactamase was amplified by PCR using pBlueScript (Strategene) as template with the following two primers 5′-GAAAGCTTTT GCACACCCAGAAACGCTGGTGAAAG and 5′-GTGGATC CTTATCCTGCAGGATCGATCCAATGCTTAATCAGTGAGG. The amplified fragment was digested with HindIII and BamHI, and inserted to the HindIII–BamHI sites in pWB980 to generate pWB980-Bla. This version of β-lactamase has five extra amino acids (IDPAG) at the C-terminal end because of the addition of a ClaI site and a PstI at the 3′ end of the *bla* gene. Plasmid pWB980-Bla-L5-LytE was designed for the production of the β-lactamase–LytE cell wall-binding domain fusion (B5E) with the above-mentioned five amino acids (IDPAG) as the linker. This plasmid was constructed by insertion of a PstI–SphI fragment encoding $CWBM_{LytE}$ from pWB980-LytE into the PstI–SphI sites in pWB980-Bla. To construct pWB980-Bla-L32-LytE, which encodes a fusion protein containing a 32-amino-acid linker linking β-lactamase and $CWBM_{LytE}$ (B32E), a 96 bp PstI linker encoding a 32-amino-acid-long sequence was generated by annealing the two primers (5′-GATCCTGCAGGAACTAGTCCGTCGACACCT GAGGGACCTAGTACTCCTTCGAATCCGAG-3′ and 5′-GT CCTGCAGGTTGATCAGGCCCGGGAGATGG TGTGCTCG GATTCGAAGGAGTACTAG-3′) followed by 30 rounds of PCR reaction. This DNA product after PstI digestion was inserted into the PstI site in pWB980-Bla-L5-LytE to generate pWB980-Bla-L32-LytE. To extend the length of linker from 32 amino acids to 55 amino acids, a 94 bp DNA fragment was generated by primer annealing (5′-CCATCTCCCGGGCCTG CATCTTCAGGCTCTTCATCATCGTCGTCTT CTTC GTCA AATGCAAGC 3′-and 5′-CAGGTTGATCAGGTGTAGTC GTCGTGCCGGAGCTTGC ATTTGACGAAGAAGAC-3′) followed by PCR reaction as described above. The amplified fragment was digested by AvaI and BclI, and inserted into the AvaI–BclI sites in pWB980-Bla-L32-LytE to generate pWB980-Bla-L55-LytE, which encodes a fusion protein with a 55-amino-acid linker (B55E).

Construction of pWB980-LytE-L48-Bla for production of E48B involved a two-step process. In the first step, an intermediate plasmid (pWB980-LytE-L) carrying $CWBM_{LytE}$ and part of the synthetic linker sequence was constructed by PCR amplification with pWB980-LytE as template and the two primers (5′-GATCCTGCAGGACAA AGCATTAAGGTG-3′ and 5′-CTGTCGACGGATCCGATGTAGATGACGTTTTGCT GCTTGAAGACGATGAAGATGAAG CAGGTTTTGAGCTGC TTGTGCTTGTTCC-3′). The amplified 597 bp DNA fragment was digested with PstI and SalI and inserted to PstI/SalI-digested pWB980-LytE vector to generate pWB980-LytE-L. The *bla* gene carrying the part of the linker sequence at the 5′ end was generated by PCR amplification using pWB980-Bla as template with two primers (5′-GATCCGTCGACA CCTGAGGGACCTAGTACTCCTTCGAATCCGAGCACACC ATCTCCC GGGCCTGATCAACCACACCCAGAAACGCT GGTGAAAG-3′ and 5′-GAAGCTAGCTT GGCATGCTATTA CCAATGCTTAATCAGTGAGGCACCTATCTCAG-3′). The amplified product was digested with SalI and NheI and was inserted to SalI/NheI-digested pWB980-LytE-L to generate pWB980-LytE-L48-Bla. All cloned DNA sequences were confirmed to be correct by DNA sequencing.

Purification of recombinant β-lactamase and cell wall-binding proteins

To purify β-lactamase, 200 ml of culture supernatant from WB800[pWB980-Bla] that had been cultivated for 8 h was applied to an anion exchanger column (20 cm in length × 3 cm in diameter) packed with MacroPrep Q (BioRad). After washing with 1 l of TSC buffer (20 mM Tris HCl, pH 8.0, 10 mM NaCl), the bound β-lactamase was

eluted with a NaCl gradient from 10 to 300 mM in TSC buffer. The pooled β-lactamase fractions were concentrated using the Ultrafree column (Millipore) prior to applying to a BioPrep SE100/17 gel filtration column (BioRad) equilibrated with the SPSC-1 buffer (20 mM sodium phosphate, pH 8.0, 0.15 M sodium chloride). To purify $CWBM_{LytE}$ and its fusions, the protein-producing cells were cultivated for 8 h in super-rich medium (200 ml each) and harvested for purification purpose. $CWBM_{LytE}$ and its fusions were eluted off from cell surface using 5 M LiCl in 25 mM Tris HCl, pH 7.2, diluted 20 times with SPSC-1 buffer and applied to a cation exchanger column packed with MacroPrep High S matrix (BioRad). After washing with the loading buffer, $CWBM_{LytE}$ and its fusions were eluted using a NaCl gradient from 0 to 1.5 M in SPSC-1 buffer. Fractions containing $CWBM_{LytE}$ and its fusions were pooled and applied to a BioPrep SE100/17 gel filtration column in SPSC-2 buffer (same as SPSC-1, except that 0.5 M NaCl instead of 0.15 M NaCl was used to minimize non-specific electrostatic interactions between proteins and the matrix). To purify $CWBM_{LytE}$ and its fusions in the culture supernatant (i.e. the non-wall-bound population), these samples were applied directly to the MacroPrep High S column as described above.

N-terminal protein sequence determination and MALDI-TOF mass spectrometry

The first five residues in the N-terminal region of the purified $CWBM_{LytB}$ and $CWBM_{LytE}$ were determined as previously described (Ng *et al*., 1992) using the Edman degradation method at the Protein core facility, University of British Columbia. Protein mass spectrometry analyses were performed at the Southern Alberta Mass Spectrometry (SAMS) Proteomics Research Centre, University of Calgary. Purified and concentrated samples were applied to a Voyager-DE STR MALDI-TOF Biospectrometry Workstation (Applied Biosystems, USA) using sinapic acid as the matrix. Whole-protein spectra were recorded in a linear mode.

Binding of $CWBM_{LytE}$ and its fusions to purified cell wall

Binding affinities of $CWBM_{LytE}$ and its fusions to purified bacterial cell wall and the number of binding sites per gram of purified cell wall were estimated using Scatchard plots. In these assays, each assay tube contained 2.5 μg of purified bacterial wall and different concentrations (0.05–5 μM) of cell wall-binding proteins. The binding buffer was PBS, pH 7.4, and the final volume was 25 μl per tube. The binding reactions were preceded at 4°C with gentle rotation (10 r.p.m.) for 15 min. The wall-bound proteins were separated from the unbound fraction by centrifugation at 12 000 *g* for 5 min. Both the free and wall-bound proteins were analysed in a gradient gel (6–10%) via SDS-PAGE. The image of the Coomassie blue-stained gel was taken using the BioRad Gel Doc 2000 system and the band intensity of the digitized image was quantified with a Fuji bioimaging analyser system (BAS 1000 Fuji Photo Film) and the MacBAS software.

Determination of the number of the wall-bound proteins per cell

The number of wall-bound $CWBM_{LytE}$ and its fusions on each bacterial surface was estimated by Western blotting probed with antibodies specifically against $CWBM_{LytE}$. Known amounts of bacterial cells were lysozyme treated and lysed using French press. Protein samples were separated on a 6–15% gradient gel via SDS-PAGE. In the same gel, different amounts of purified $CWBM_{LytE}$ were loaded for preparation of a calibration curve for quantification. The cell number per millilitre of culture was determined by colony counting on agar plates with serial dilution. As cells overproducing proteins carrying the cell wall-binding domains tended to form filamentous cells with several cells clustered together, the average cell number per filamentous cell was determined by counting the number of the stained DNA regions using the DNA-specific fluorescent dye (Hoechst 33258) under the fluorescence microscope. Each DNA-stained region was assumed to represent a single cell. The average cell number per filamentous cell was also determined by counting individual cells under the phase contrast microscope after the filamentous cells on the glass slides had been treated with lysozyme for 1 h.

Immunolocalization of recombinant $CWBM_{LytE}$ and E48B on cell surface

Localization of wall-bound $CWBM_{LytE}$ and E48B on bacterial surface was examined using both regular fluorescence and confocal fluorescence microscopes. Cells were cultivated for 8 h, washed twice with PBS and fixed with 4% paraformaldehyde for 30 min at 4°C. The cell samples were then first incubated with a primary antibody cocktail which contained primary antibodies (mouse antibodies against $CWBM_{LytE}$ or rabbit antibodies against β-lactamase), Hoechst 33258 (a fluorescent dye for DNA) and dihexaoxacarbocyanine iodide ($DiOC_6$ for membrane and hydrophobic molecules) in PBS with 3% BSA for 2 h. After washing twice in PBS, the samples were incubated with a second antibody cocktail containing secondary antibodies (goat anti-mouse Alexa Fluor 568-conjugated or anti-rabbit Alexa Fluor 488-conjugated immunoglobulin G antibodies), Hoechst 33258 and $DiOC_6$ in PBS with 3% BSA for another 2 h. In the case of confocal microscopic studies, Alexa Fluor 660-labelled wheat germ agglutinin was included in the secondary antibody cocktail for cell wall staining. After washing with PBS twice, cells were immobilized on the poly-lysine-treated coverslips and immersed in SlowFade antifade reagent to prevent photobleaching. All fluorescent dyes were purchased from Molecular Probes, USA. For general fluorescence studies, the Leica DMRB microscope (100X/1.30 objective) equipped with the digital CCD camera from Princeton Instruments (Roper) was used. For confocal microscope, the Leica DM RXA2 system equipped with the cooled scientific CCD camera from Princeton Instruments at the Microscopy and Imaging Facility, University of Calgary, was used.

Enzymatic assays

The activities of β-lactamase and its fusions were assayed as previously described (Wong and Doi, 1986) with 7-(thienyl-2-acetamido)-3-[2-(4-N, N-dimethyl-amino-phenylazo) pyridinium-methyl]-3-cephem-4-carboxylic acid (PADAC) as the substrate. Bovine serum albumin (final concentration,

20 μg ml^{-1}) was added to stabilize β-lactamase (Laraki *et al*., 1999; Kimura *et al*., 2004). To compare β-lactamase activities from intact cells and the cell wall fractions of the lysozyme treated cells, the culture samples were prepared as follows: *B. subtilis* strains were cultured in super-rich medium containing kanamycin (10 μg ml^{-1}) for 9 h at 30°C as they maintained peak levels of surface-displayed fusion proteins around this time point. Cells from 1.5 ml of culture were collected (12 000 *g* for 5 min), washed once with equal volume of SET buffer (20% sucrose, 50 mM Tris HCl, pH 7.6, 50 mM EDTA) and then re-suspended in 1.5 ml of SET buffer containing 0.1 mM PMSF and 20 μg ml^{-1} BSA. Cells (300 μl each) were then transferred to two new microcentrifuge tubes respectively. Fifty microlitres of SET buffer containing 20 mg ml^{-1} lysozyme (final concentration of 1 mg ml^{-1}) was added to one tube to prepare protoplasts. Equal volume of SET buffer was added to the other tube which served as the whole-cell sample. Both tubes were incubated at 37°C for 60 min. Protoplast formation in lysozyme-added samples was monitored under the microscope. Supernatants prepared from the lysozyme-treated cells were generated by centrifugation to remove the protoplasts and were designated cell wall fractions. The pelleted protoplasts were re-suspended in 350 μl of lysis buffer (50 mM Tris HCl, pH 8.0, 5 mM $MgSO_4$) and lysed by sonication. Release of intracellular proteins because of cell lysis during protoplast preparation was corrected by determining the degree of cell lysis using glucose 6-phosphate dehydrogenase (G6PDH) as the cytoplasmic marker. Assays for G6PDH activity were performed as described (Merchante *et al*., 1995). All these assays were analysed in triplicate.

Other methods

Cell wall from a stationary-phase *B. subtilis* culture was isolated as described by the published procedures (Kuroda and Sekiguchi, 1990). Purified proteins were quantified by measuring the absorbance at 280 nm under denaturing conditions as previously described (Gill and Von Hippel, 1989). Purified $CWBM_{LytE}$ was used as antigen to generate polyclonal antibodies in mice. Rabbit polyclonal antibodies against TEM-β-lactamase were prepared according to the published procedures (Wong and Doi, 1986). Both *B. subtilis* plasmid isolation and transformation were performed as described (Wu *et al*., 2002).

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