JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 104, No. 1, 55–61. 2007
DOI: 10.1263/jbb.104.55

Evaluation of Cell Wall Binding Domain of *Staphylococcus aureus* Autolysin as Affinity Reagent for Bacteria and Its Application to Bacterial Detection

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Received 26 February 2007/Accepted 21 April 2007

We evaluated the cell wall binding (CWB) domain of *Staphylococcus aureus* autolysin as an affinity reagent for bacteria. A fusion of CWB domain and green fluorescent protein (CWB-GFP) bound to *S. aureus* with a dissociation constant of 15 nM. CWB-GFP bound to a wide range of gram-positive bacteria, but not to most gram-negative bacteria. We suspected that the outer membrane of gram-negative bacteria inhibits the access of CWB-GFP to peptidoglycan layer. Indeed, CWB-GFP bound to gram-negative bacteria when they were treated with benzalkonium chloride. Because CWB-GFP bound to the bacterial peptidoglycan layer, it appeared to be an effective affinity reagent for bacteria and CWB fusion with reporter proteins could be applied to detect bacteria. We also constructed a fusion of CWB and luciferase, which can be used for the rapid detection of bacteria.

[**Key words:** bacterial probe, cell wall binding, affinity reagent, microbe counting]

The detection of bacteria is important in order to monitor hygiene of food and non-food contact surfaces (1), to serve as a warning against biological warfare attack (2), and to evaluate the status of aquatic environment and assess bacterial contribution to material cycles (3). The direct-count technique using epifluorescence microscopy has been widely used for microbe counting (3, 4), although it is tedious and requires special skills for membrane filtration and microscopic manipulation. Recently, an automatic counting system has been developed, which is composed of a membrane unit for separating microbial cells, a focusing-free microscopic device, and an image analysis program (5). In parallel with this mechanical development, various fluorescent probes have been developed for detecting only living cells (6, 7) and in particular bacterium such as *Escherichia coli* O157 (8–10). Recently, the combined use of two different probes has improved the accuracy of detecting only living cells (5). Propidium iodide (PI) and 4′,6-diamidino-2-phenylindole (DAPI) produce fluorescence when combined with DNA. DAPI can penetrate both intact and damaged cell membranes, whereas PI can penetrate the latter. Therefore, the combined use of PI and DAPI enables the enumeration of both viable and dead cells. However, these probes are not specific for bacteria.

Autolysins that bind to bacterial cell surface degrade the peptidoglycan layer. These enzymes are involved in cell wall metabolism such as cell separation of daughter cells after cell division (11–13). The *Staphylococcus aureus* autolysin Atl is initially produced as a 138-kDa protein and has amidase and glucosaminidase domains. Atl undergoes proteolytic processing to generate two major peptidoglycan hydrolases: a 62-kDa *N*-acetylmuramyl-L-alanine amidase and a 51-kDa *N*-acetylglucosaminidase (13, 14). Atl and its processed proteins localize on the cell wall at the septal region of an upcoming cell division site (15). Three repeated sequences, each composed of approximately 150 amino acids, are considered to make up a cell-wall binding (CWB) domain (13). In this report, to evaluate CWB as an affinity reagent for bacteria, we constructed a CWB fusion with green fluorescent protein (GFP). We showed that CWB-GFP binds to a wide range of gram-positive bacteria, but exceptionally not to *Lactobacillus*. CWB-GFP bound to gram-negative bacteria when they were treated with benzalkonium chloride (BC) and to *Lactobacillus* treated with trichloroacetic acid (TCA). Because CWB-GFP bound to the bacterial peptidoglycan layer, it appeared to be an effective affinity reagent for bacteria and CWB fusion with reporter proteins could be applied to detect bacteria. We also demonstrated that a CWB-luciferase fusion can be used for the rapid and sensitive detection of bacteria.

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MATERIALS AND METHODS

Plasmid construction A DNA fragment encoding GFP was

amplified with primers G1 and G2 (Table 1) using pGFPuv (Clontech, Palo Alto, CA, USA) as a template and then inserted into the *Hin*dIII and *Not*I sites of plasmid pET21-b(+) (EMD Biosciences, Darmstadt, Germany). Primer G2 was designed to eliminate the *Sac*I site localized in the 3′-region of *gfp* (16). The resulting plasmid was designated pETGFP. A DNA fragment encoding the CWB of *atl* (13) was amplified with primers C1 and C2 (Table 1) using *S. aureus* ATCC6538 chromosomal DNA as a template, then inserted into the *Eco*RI and *Sac*I sites of pETGFP. The resulting plasmid was designated pETCWBGFP. A DNA fragment encoding luciferase was amplified with primers L1 and L2 (Table 1) using luciferase T7 control DNA (Promega, Madison, WI, USA) as a template and then replaced with the *Sal*I and *Not*I fragment of pETCWBGFP. The resulting plasmid was designated pETCWBLuc. Plasmids pETCWBGFP and pETCWBLuc were introduced into *E. coli* Rosetta (DE3) plysS (EMD Biosciences). CWB-GFP and CWB-luciferase (CWB-Luc) proteins with a C-terminal His-tag were expressed in *E. coli* according to the manufacture's instructions. The *E. coli* cells were then collected by centrifugation and disrupted by treatment with lysozyme and ultrasonication. His-tagged proteins were purified by chromatography on a HiTrap Chelating column (Amersham Biosciences, Piscataway, NJ, USA). The fractions containing CWB-GFP and CWB-Luc proteins were eluted with a linear gradient from 0.05 to 0.5 M imidazole in a buffer containing 20 mM phosphate (pH 7.4), 0.5 M NaCl, and 15% glycerol.

Fluorescence activity of CWB-GFP The fluorescence spectra and intensity of purified CWB-GFP and GFP were measured using a FP-6500 fluorescence meter (Jasco, Tokyo). GFP was excited at the highest excitation wavelength (488 nm) (16). One milliliter (approximately 10^9 cells) cultures of *S. aureus* and other bacteria, as listed in Table 2, were collected and washed with buffer A (20 mM Tris–HCl [pH 7.4], 100 mM NaCl). The cells were suspended in 1 ml of buffer A. Fifty microliters of the suspension was mixed with purified CWB-GFP (5 μg), incubated for 1 min at room temperature, and immediately observed under a fluorescence microscope equipped with a 100×UPlanApo objective (BX60; Olympus, Tokyo). A MNIBA filter (470–490 nm) was used for detecting GFP fluorescence. Images were captured using a DP70 cooled charge-coupled device camera (Olympus) and processed using Adobe Photoshop 6.0. Eukaryotic cells, as listed in Table 2, were also used.

TABLE 1. DNA sequence of primers

Primer	DNA sequence
G1	5′-AGAAAAGCTTAGTAAAGGAGAAGAACTTTTCACT-3′
G2	5′-TCATGCGGCCGCAAGCTCATCCATGCCATGTGTA-3′
C1	5′-CATCGAATTCTAAATTAACAGTTGCTGCAAACAA-3′
C2	5′-AGTTGAGCTCGTTAAATCTTTTGCATTTACCCA-3′
L1	5′-CCGGGTCGACATGGAAGACGCCAAAAAC-3′
L2	5′-GTTGCGGCCGCCAATTTGGACTTTCCGCC-3′

The underlined sequences represent additional restriction enzyme sites.

Dissociation constant (K_d) of CWB-GFP for *Bacillus subtilis* and *S. aureus* cells *B. subtilis* and *S. aureus* cells (approximately 1.5×10^7 cells·ml^{-1}) in buffer A were mixed with the indicated amounts of CWB-GFP (69 to 138 nM) and incubated for 3 min at room temperature. The mixture was centrifuged at 20,000×*g* for 3 min, and free CWB-GFP was obtained in the supernatant. The

TABLE 2. Binding of CWB-GFP to various strains

Strain	Without treatment	BC[a]	TCA[b]	Reference
Gram-positive				
Bacillus subtilis	+	+	+	168S (15)
Bifidobacterium longum	+	+	+	JCM1210
Corynebacterium glutamicum	+	+	+	ATCC13032
Dactylosprangium vinaceum	+	+	+	HUT 6560
Lactobacillus acidophilus	−	−	+	JCM1028
Lactobacillus plantarum	−	−	+	JCM1055
Micromonospora inyoensis	+	+	+	HUT 6602
Rhodococcus opacus	+	+	+	JCM 9703
Streptomyces californicus	+	+	+	HUT 6049
Staphylococcus aureus	+	+	+	ATCC6538
Bacillus subtilis spore	−	+	+	168S (15)
Bacillus thuringiensis spore	+	+	+	NBRC101235
Gram-negative				
Acinetobacter johnsonii	+	+	+	ATCC17909
Agrobacterium tumefaciens	−	+	+	LBA4404 (21)
Enterobacter aerogenes	−	+	+	ATCC9621
Escherichia coli	−	+	+	MG1655
Pseudomonas aeruginosa	−	+	+	PAO1 (22)
Pseudomonas putida	−	+	+	PRS2000 (23)
Rhodovulum sp.	−	+	+	HUT 8103
Serratia marcescens	−	+	+	ATCC 14756
Eukaryote				
Saccharomyces cerevisiae	−	−	−	ATCC204508
Schizosaccharomyces pombe	−	−	−	HUT 7157
Candida albicans	−	−	−	HUT7501
Debaryomyces hansenii	−	−	−	HUT 7024
Aspergillus niger	−	−	−	HUT2014
Penicillium expansum	−	−	−	HUT 4122
Sheep blood cells	−	−	−	Japan Lamb Co.

[a] Benzalkonium chloride-treated cells.
[b] Trichloroacetic acid-treated cells.

ratio of bound to free CWB-GFP was plotted as a function of the concentration of bound CWB-GFP. The K_d and maximum binding constants of CWB-GFP of bacterial cells were determined by Scatchard analysis.

Treatment of gram-negative strains with benzalkonium chloride (BC) to remove cell surface constituents that hinder binding sites of CWB-GFP Cultures of gram-negative organisms, as listed in Table 2, were collected and washed with buffer A. The cells were suspended in 50 μl of buffer A containing 0.05% BC (Wako, Osaka), and allowed to stand for 2 min at room temperature. The suspension was mixed with purified CWB-GFP (5 μg), incubated for 1 min at room temperature, and then observed by fluorescence microscopy.

Treatment of *Lactobacillus* strains with trichloroacetic acid (TCA) to remove cell surface constituents that hinder binding sites of CWB-GFP Cultures of *Lactobacillus acidophilus* and *L. plantarum* cells were collected and washed with buffer A. The cells were then mixed with 0.1% TCA and heated at 90°C for 10 min to remove cell surface constituents that hinder the binding sites of a CWB protein (17). Next, the cells were mixed with CWB-GFP (5 μg), incubated for 1 min at room temperature, and observed under a fluorescence microscope. Peptidoglycan layers purified from *L. acidophilus* and *Enterococcus faecalis* were purchased from Wako Chemical, and that from *S. aureus* was purchased from Sigma (St. Louis, MO, USA).

Preparation of *Bacillus* spore Spores of *B. subtilis* and *B. thuringiensis* were prepared using Schaeffer medium (12). Spores were collected and washed with buffer A. The spores were treated with BC and TCA as described above.

Automatic counting system Bioplorer (Matsushita Ecology Systems, Aichi) was used for the nonculture method of determining the numbers of microbial cells. Bioplorer is composed of a membrane unit for separating microbial cells, a focusing-free microscopic device, and an image analysis program (5). *B. subtilis* cells were mixed with CWB-GFP and then applied to the membrane unit. Fluorescent images from 30 sections of the membrane are then recorded and the total numbers of cells were determined automatically. Staining and the detection of *B. subtilis* with DAPI and PI were performed as described in the instructions in the use of the Bioplorer.

Bioluminescent activity of CWB-Luc Various concentrations (from 0.01 to 1 nM) of CWB-Luc fusion protein or firefly luciferase (18) were mixed with 50 μl of a luminescence buffer (30 mM Tris–HCl [pH 7.4], 4 mM $MgCl_2$, 2 mM ATP, and 1 mM luciferin). ATP, luciferin, and wild-type luciferase (firefly) were purchased from Sigma. For the binding assay, various numbers of *B. subtilis* cells suspended in 100 μl of buffer A were mixed with 1 μg of CWB-GFP. After incubation for 3 min, *B. subtilis* was precipitated by centrifugation for 3 min at 20,000×*g*. The cell pellet was washed with buffer A, suspended in 50 μl of the luminescence buffer, and luminescence was measured using Lumitester C-100 (Kikkoman, Chiba). Cell numbers were counted as colony forming units.

Bacterial strains and eukaryotic cells Bacterial, fungal, and yeast strains, as listed in Table 2, were obtained from American Type Culture Collection (Manassas, VA, USA), RIKEN Bioresource Center (Tsukuba), National Institute of Technology and Evaluation (Tokyo), and Hiroshima University Culture Collection (Hiroshima). Sheep blood was obtained from Japan Lamb Co. (Fukuyama). All bacteria were cultured in 2xYT media containing 16 $g \cdot l^{-1}$ tryptone, 10 $g \cdot l^{-1}$ yeast extract, and 5 $g \cdot l^{-1}$ NaCl. *B. subtilis*, *B. longum*, *C. glutamicum*, *E. coli*, *L. acidophilus*, *L. plantarum*, and *S. aureus* were cultured at 37°C, whereas other bacteria were cultured at 28°C.

RESULTS AND DISCUSSION

Construction of CWB-GFP We obtained a DNA fragment encoding the putative CWB domain of *S. aureus* autolysin, Atl. To characterize the CWB protein as an affinity reagent for bacteria, we constructed a CWB–GFP fusion. Because the molecular masses of CWB and GFP are 53 and 30 kDa (13, 16), respectively, that of the fusion protein was estimated to be 83 kDa. SDS–PAGE revealed the presence of an 83-kDa protein in both the crude cell lysate of the recombinant *E. coli* and the fractions purified by His-Trap column chromatography (data not shown). Based on spectral analysis, CWB-GFP had similar excitation and emission spectra as GFP but 20% lower fluorescence intensity than GFP when excited at 488 nm (data not shown).

CWB-GFP bound to *S. aureus* A suspension of *S. aureus* was mixed with a purified CWB-GFP and then observed by fluorescence microscopy. CWB-GFP bound to the entire cell surface of *S. aureus* (Fig. 1). The fluorescence intensity was as strong as that obtained using conventional methods such as DAPI staining (data not shown). A plot of CWB-GFP (bound) per CWB-GFP (free) versus CWB-GFP (bound) (Scatchard plot) yields a straight line of slope ($-1 \cdot Kd^{-1}$, Kd=15 nM) whose intercept on the X-axis is the maximum of CWB-GFP (bound) (40 nM). In this experiment, 1.5×10^7 *S. aureus* cells $\cdot$ ml^{-1} was used, suggesting that 1.6×10^6 molecules of CWB-GFP would bind to a single *S. aureus* cell (Fig. 2).

CWB-GFP bound to most gram-positive bacteria CWB-GFP bound not only to *S. aureus*, but also to *Corynebacterium glutamicum* and *B. subtilis* (Fig. 1). It seemed likely that CWB-GFP binds to *B. subtilis* more strongly than to *S. aureus* (Fig. 1). Indeed, CWB-GFP bound to *B. subtilis* with a K_d of 6.5 nM and its maximum binding constant was 2.2×10^6 molecules of CWB-GFP per cell (Fig. 2), indicating that it bound to *B. subtilis* with a higher affinity than to *S. aureus*. The difference in the cell wall components may affect the binding of CWB-GFP to peptidoglycan as described below.

On the other hand, CWB-GFP did not bind to gram-negative bacteria such as *E. coli*, *A. tumefaciens*, and *Pseudomonas aeruginosa* (Fig. 1). CWB-GFP did not bind to the eukaryote *Saccharomyces cerevisiae*, several other strains of yeast and fungi, and sheep blood cells (Table 2).

We next examined whether CWB-GFP binds to only gram-positive bacteria. CWB-GFP bound to most gram-positive bacteria, including *Bifidobacterium longum*, *Dactylosprangium vinaceum*, *Micromonospora inyonensis*, *Rhodococcus opacus*, and *Streptomyces californicus*, but it did not bind to *L. acidophilus* and *L. plantarum* (Table 2). CWB-GFP did not bind to other gram-negative bacteria including *E. aerogenes*, *P. putida*, and *Rhodovulum* sp., and *S. marcescens* (Table 2); however, it bound to *Acinetobacter johnsonii* and gram-positive bacteria (Table 2). We confirmed that *A. johnsonii* was not stained using the conventional Gram's method, which differentiates bacterial species into gram-positive and gram-negative groups, depending on the thickness of the cell wall. CWB-GFP bound to thin and thick cell walls, implying that CWB-GFP binds to only the cell wall surface.

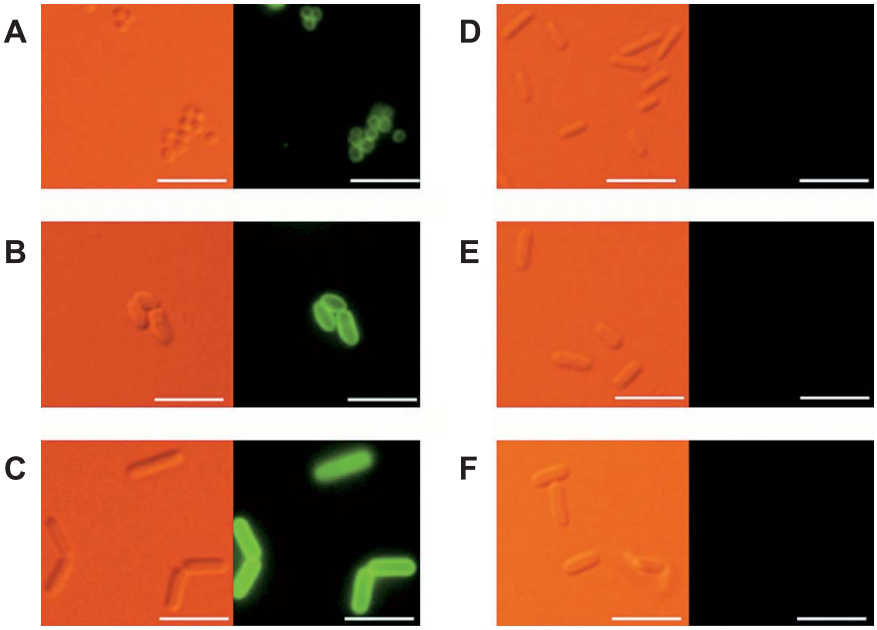

FIG. 1. Fluorescence image of CWB-GFP that bound to gram-positive bacteria. Growing bacteria (10^9 cells·ml^{-1}) were mixed with purified CWB-GFP and then observed under a fluorescence microscope. (A) *S. aureus*, (B) *C. glutamicum*, (C) *B. subtilis*, (D) *E. coli*, (E) *A. tumefaciens*, and (F) *P. aeruginosa* were used. Phase-contrast (left) and GFP (right) images are shown. Bars represent 5 μm.

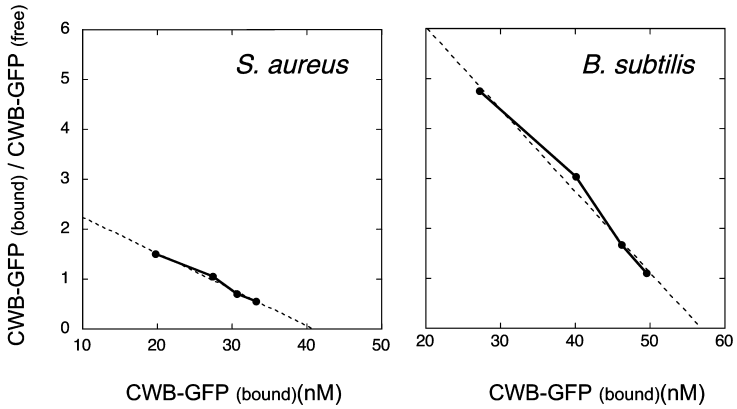

FIG. 2. Scatchard analysis of CWB-GFP binding to *S. aureus* and *B. subtilis*. *B. subtilis* and *S. aureus* (approximately 1.5×10^7 cells·ml^{-1}) were mixed with CWB-GFP. The mixture was centrifuged, and CWB-GFP (free) was obtained in the supernatant. The precipitated CWB-GFP, which bound to cells, was defined as CWB-GFP (bound). The ratio of bound to free CWB-GFP was plotted as a function of the concentration of the bound form of CWB-GFP.

CWB-GFP strongly bound to BC-treated gram-negative bacteria similarly to gram-positive bacteria We suspected that the outer membranes of gram-negative bacteria except *A. johnsonii* inhibited the access of CWB-GFP to the peptidoglycan layer. BC is a nitrogenous cationic surface-acting agent that is used as an antiseptic. The bactericidal action of BC is thought to be due to the disruption of cellular membrane bilayers. Suspensions of *E. aerogenes*, *P. putida*, and *S. marcescens* were mixed with 0.05% BC and CWB-GFP, and then observed by fluorescence microscopy. CWB-GFP bound to these bacteria in the presence of 0.05% BC (Fig. 3). The treatment with BC also allowed CWB-GFP to bind to other gram-negative bacteria including *A. tumefaciens*, *P. aeruginosa*, and *Rhodovulum* sp. (Table 2).

TCA treatment allowed CWB-GFP to bind to *Lactobacillus* Although CWB-GFP did not bind to intact *L. acidophilus*, it bound to the purified peptidoglycan layer of *L. acidophilus*, *E. faecalis* and *S. aureus* (data not shown). Therefore, we also speculated that some components inhibit the access of CWB-GFP to the peptidoglycan layer in *Lactobacillus*. Treatment with BC, however, was not effective in *Lactobacillus* (Table 2). As teichoic acid of *L. plantarum* is highly substituted with D-alanyl esters (19), the removal of D-alanyl substitutions from teichoic acid has been reported to render the cell wall with a higher capacity to bind cationic peptidoglycan hydrolases (19). It is known that treatment of cell walls with TCA removes peptidoglycan-associated polymers such as teichoic acids (17). We therefore

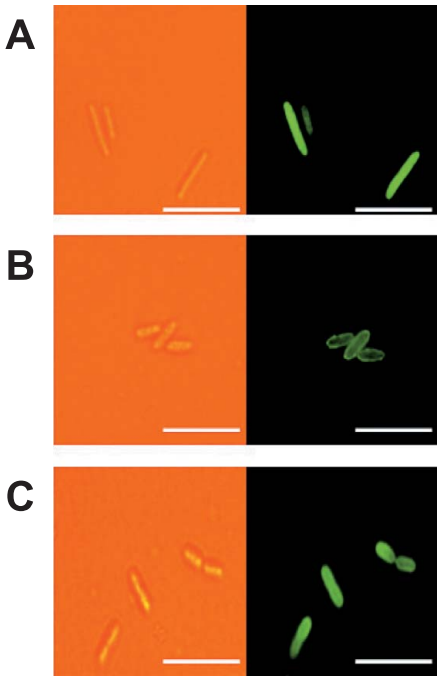

FIG. 3. Treatment of BC allows CWB-GFP to bind to gram-negative bacteria. Growing bacteria were treated with BC and then mixed with CWB-GFP. (A) *E. aerogenes*, (B) *P. putida*, and (C) *S. marcescens* were observed with a fluorescence microscope. Bars represent 5 μm.

treated *Lactobacillus* with 0.1% TCA. We found that treatment with TCA allowed CWB-GFP to bind to *Lactobacillus* (Fig. 4). CWB-GFP also bound to TCA-treated Gram-negative bacteria (Table 2).

CWB-GFP bound to spores We next examined whether CWB-GFP binds to the spores of *Bacillus thuringiensis*, a close relative of *Bacillus anthrax*. CWB-GFP bound to *B. thuringiensis* spores (Fig. 5). In contrast, CWB-GFP did not bind to *B. subtilis* spores but bound to the spore when they were treated with BC or TCA (Table 2).

Binding was observed in a wide range of bacteria, which have different types of peptidoglycan, suggesting that the CWB domain recognized a conserved domain of peptidoglycans. *S. aureus*, *B. subtilis*, and *L. acidophilus* peptidoglycans belong to the A-type peptidoglycan (17). Although there are differences in the composition of the peptide part, A-type peptidoglycans all have L-Ala as the first amino acid, which attaches the peptide to the glycan chain. Besides that, the cross-link in this type of peptidoglycan is always between the fourth (D-Ala) and third amino acids (always a diaminoacid, such as meso-diaminopimelic acid (*e.g.*, *B. subtilis*) or L-Lys (*e.g.*, *S. aureus*) (17). *C. glutamicum* and *R. opacus* peptidoglycans belong to B-type peptidoglycan; the first amino acid residue is not L-Ala but L-Gly. Cross-linking of peptidoglycan is achieved by the formation of a bond between D-Ala in the fourth position and the second amino acid (D-Glu) in the other peptide chain (17). The only moiety that A-type and B-type peptidoglycans have in common is the *N*-acetylglucosamine-*N*-acetylmureine glycan polymer (17). In spore peptidoglycans, many peptidoglycan peptides are removed. Although we have not experimentally determined CWB binding sites in peptidoglycans, we believe that CWB possibly binds to a glycan polymer.

Rapid detection of *B. subtilis* using CWB-Luc fusion Our results imply that CWB can be used as an affinity reagent for bacteria. Luciferases are unique enzymes because they can generate visible light as an end-product of catalysis and therefore can be used in an ATP-based bioluminescence assay (1, 20). We constructed a fusion gene of CWB and firefly luciferase (CWB-Luc) and purified it from the crude lysate of the recombinant *E. coli*. The luminescence due to CWB-Luc was proportional to the protein concentration, but the intensity was approximately one-fourth that of the wild-type luciferase (data not shown). To demonstrate the ability of this fusion protein to detect bacteria with high sensitivity, we mixed it with a vegetative culture of *B. subtilis* and then removed the bound fraction by centrifugation. The amount of precipitated CWB-Luc was proportional to the number of *B. subtilis* cells (Fig. 6). Our results suggest that it is possible to detect as few as several hundreds of *B. subtilis* cells in a 50 μl sample within 10 min.

Rapid and sensitive determination of bacteria is extremely important in biotechnology, medical diagnosis, brewage fermentation processes, and the current fight against bioterrorism (2). Because CWB-GFP does not bind to blood cells,

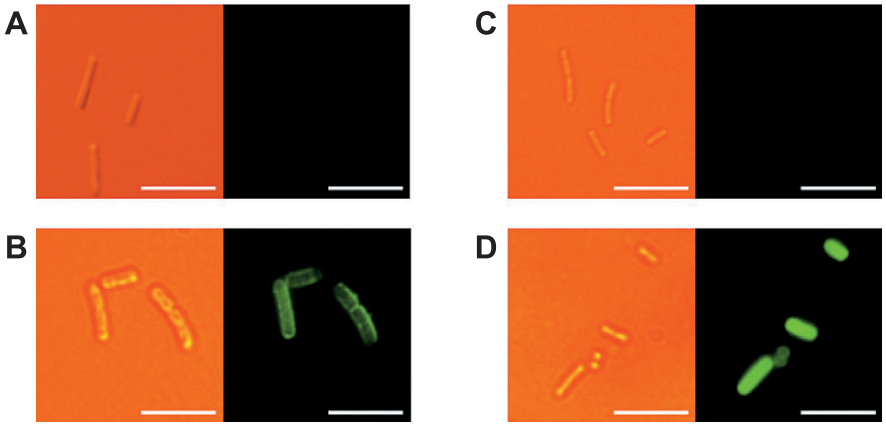

FIG. 4. Treatment with TCA allows CWB-GFP to bind to *Lactobacillus* spp. (A, B) *L. plantarum*. (C, D) *L. acidophilum*. Bacteria were mixed with CWB-GFP without (A, C) or with (B, D) TCA treatment and then observed by fluorescence microscopy. Bars represent 5 μm.

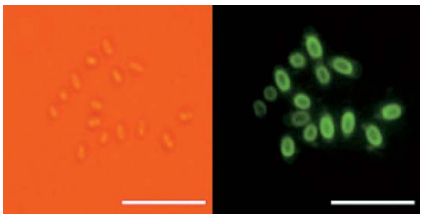

FIG. 5. CWB-GFP bound to *Bacillus thuringiensis* spores. Bars represent 5 μm.

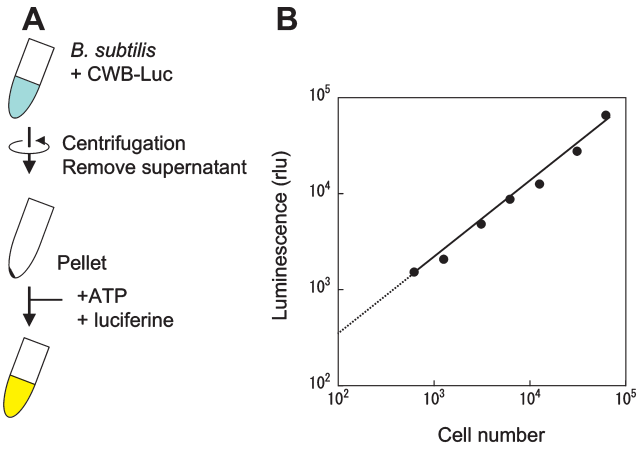

FIG. 6. Detection assay of *B. subtilis* using CWB-Luc. (A) Schematic of assay using CWB-Luc. *B. subtilis* cells were mixed with CWB-Luc and then collected by centrifugation. After the supernatant containing free CWB-Luc was removed, ATP and luciferine were added to the precipitant. Relative luminescence unit (rlu) was measured using a luminometer. (B) The precipitated CWB-Luc showed a luminescence intensity proportional to the number of cells in a 50 μl sample.

it may be easy to detect bacterial infection by fluorescent microscopy. Only *S. aureus*, but not blood cells, showed fluorescence when both of them were treated with CWB-GFP, indicating that this system can be applied to medical diagnosis. On the other hand, Shimakita *et al*. demonstrated that the automatic counting system, Bioplorer, be used to enumerate viable microbial cells in food samples using DAPI and PI as fluorescence probes (5). This demonstration promoted us to apply CWB-GFP for counting bacterial cells using Bioplorer. The *B. subtilis* count obtained using DAPI was almost the same as that obtained using CWB-GFP as a probe (data not shown). Although further investigation is necessary, this result indicates that CWB-GFP could be used as a fluorescent probe for bacteria using the autocounting system.

In summary, CWB-GFP bound to a wide range of gram-positive bacteria except for those belonging to the genus *Lactobacillus*. However, this fusion protein bound to different species of *Lactobacillus* when they were treated with TCA and to gram-negative bacteria when they were treated with BC. Thus, we conclude that CWB is useful as an affinity reagent for detecting bacteria. To our knowledge, this is the first study wherein an affinity protein was developed and genetically modified with reporter proteins such as GFP and luciferase for detecting a wide range of bacteria.

ACKNOWLEDGMENTS

This work was supported by a grant from Hiroshima Prefectural Institute of Industrial Science and Technology and in part by the Special Coordination Funds from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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