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

Abo Bakr F. Ahmed,[1] Kanako Noguchi,[1] Yasuo Asami,[1] Kazutaka Nomura,[1] Hiroya Fujii,[2] Minoru Sakata,[2] Akihiko Tokita,[2] Kenichi Noda,[1] and Akio Kuroda[1]*

Department of Molecular Biotechnology, Hiroshima University, 1-3-1 Kagamiyama, Higashihiroshima, Hiroshima 739-8530, Japan[1] *and Bussan Nanotech Institute, 2-1 Kouyadai, Tsukuba, Ibaraki 305-0074, Japan*[2]

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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.

* Corresponding author. e-mail: akuroda@hiroshima-u.ac.jp
phone: +81-(0)82-424-7758 fax: +81-(0)82-424-7047
The first two authors contributed equally to this work.

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.

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 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.

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.

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