Applied and Environmental Microbiology, Mar. 2007, p. 1992–2000 Vol. 73, No. 6
0099-2240/07/$08.00+0 doi:10.1128/AEM.02402-06

Use of High-Affinity Cell Wall-Binding Domains of Bacteriophage Endolysins for Immobilization and Separation of Bacterial Cells[▽]

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Received 12 October 2006/Accepted 21 January 2007

Immobilization and magnetic separation for specific enrichment of microbial cells, such as the pathogen *Listeria monocytogenes*, depends on the availability of suitable affinity molecules. We report here a novel concept for the immobilization and separation of bacterial cells by replacing antibodies with cell wall-binding domains (CBDs) of bacteriophage-encoded peptidoglycan hydrolases (endolysins). These polypeptide modules very specifically recognize and bind to ligands on the gram-positive cell wall with high affinity. With paramagnetic beads coated with recombinant *Listeria* phage endolysin-derived CBD molecules, more than 90% of the viable *L. monocytogenes* cells could be immobilized and recovered from diluted suspensions within 20 to 40 min. Recovery rates were similar for different species and serovars of *Listeria* and were not affected by the presence of other microorganisms. The CBD-based magnetic separation (CBD-MS) procedure was evaluated for capture and detection of *L. monocytogenes* from artificially and naturally contaminated food samples. The CBD separation method was shown to be superior to the established standard procedures; it required less time (48 h versus 96 h) and was the more sensitive method. Furthermore, the generalizability of the CBD-MS approach was demonstrated by using specific phage-encoded CBDs specifically recognizing *Bacillus cereus* and *Clostridium perfringens* cells, respectively. Altogether, CBD polypeptides represent novel and innovative tools for the binding and capture of bacterial cells, with many possible applications in microbiology and diagnostics.

Listeria spp. are non-spore-forming, gram-positive rods which can be isolated from many different environmental sources such as soil, water, plant material, foods, and feces. The genus comprises six species, of which *Listeria monocytogenes* and *L. ivanovii* are potentially pathogenic to humans and animals, respectively. Listeriosis can result in meningitis, meningoencephalitis, septicemia, and generalized infection of the fetus and abortion (30). Many years after the first documented listeriosis epidemic in 1926 (21), contaminated foods have now been recognized as the vehicle for human infections. The organisms are usually present in very low numbers and are accompanied by a numerous and diverse background microflora. Therefore, diagnostic procedures must (i) be able to detect low contamination levels, (ii) yield results in the shortest possible time, (iii) be reliable, and (iv) be inexpensive and easy to use.

In order to increase the number of cells to a detectable level, selective enrichment is an absolute requirement in any protocol for the detection of viable *Listeria* cells. This is usually achieved with the aid of selective enrichment media according to officially adopted protocols (IDF 143A:1995; ISO 11290-1). An additional procedure used to concentrate the target cells is to separate them from food suspension or enrichment cultures. This can be achieved by differential centrifugation (25), size exclusion filtration (2), or selective immobilization. An ideal tool for the latter appeared to be immunomagnetic separation (IMS). However, these antibody-based anti-*Listeria* IMS techniques (4, 6, 9, 26, 28, 29) are hampered by a number of disadvantages, i.e., (i) poor recovery rates—the percentage of cells which could be separated from cell suspensions was 20% or less (9, 29), (ii) inability to detect low contamination levels (22, 29), and (iii) cross-reaction with nontarget bacterial cells (9, 22, 29). Moreover, cross-linking and agglutination of beads were frequently observed. Another class of high-affinity binding molecules are the lectins. However, because they promote strong agglutination and generally lack specificity for a given type of bacterial cell surface, they are unsuitable for separating specific target cells (23, 24) from a diverse microflora.

A new and innovative approach for selective binding and separation of bacterial cells is provided by harnessing the properties of cell wall-binding domains (CBDs) of bacteriophage-encoded peptidoglycan hydrolases (13, 19). This type of enzymes, termed endolysins, generally features a modular architecture, with an N-terminal catalytic domain and a C-terminal CBD. The CBDs from *L. monocytogenes* phage endolysins Ply118 and Ply500 specifically recognize and bind to *Listeria* cells (15). The CBDs feature rapid binding kinetics, very high affinity, and an extraordinary specificity, based upon recognition of carbohydrate components unique on *Listeria* cell wall peptidoglycan (15). The first crystal structure of a *Listeria* phage endolysin was determined very recently (11), and its CBD features a novel and unique fold responsible for recognition of, and binding to, the cognate cell wall ligands. Although the binding is restricted to organisms of the genus *Listeria*, it is not species specific. Instead, it correlates with the different serovar group designations; i.e., CBD118 binds to *Listeria* cells of serovars 1/2, 3, and 7 and CBD500 recognizes cells of serovars 4, 5, and 6 (15). Thus, these two CBD mole-

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▽ Published ahead of print on 2 February 2007.

cules feature nonoverlapping binding ranges and cover the full diversity of different *Listeria* serovars, independent of the species or source.

The aim of this study was to harness the unique properties of the CBDs for attachment and immobilization of *Listeria* cells on solid surfaces. Toward this end, purified recombinant CBD proteins were used to coat paramagnetic beads, which enabled highly efficient capture and recovery of *Listeria* cells from suspensions. The assay permitted fast and sensitive detection of *Listeria* cells from foods, which compared favorably to the standard enrichment procedure. To show that the CBD affinity technology is applicable to other bacterial species as well, we identified CBDs specifically binding to cells of *Bacillus cereus* (16) and *Clostridium perfringens* (33) and demonstrate that they also enable immobilization and recovery of these pathogens.

MATERIALS AND METHODS

Bacteria and culture conditions. *L. monocytogenes* strains EGDe (clinical isolate, serovar 1/2a), WSLC 1001 (ATCC 19112, serovar 1/2c), WSLC 1042 (ATCC 23074, serovar 4b), Scott A (clinical isolate, serovar 4b), and WSLC 1363 (soft cheese isolate, serovar 4b); *L. innocua* WSLC 2012 (ATCC 33091, serovar 6a); and *L. ivanovii* WSLC 3009 (clinical isolate, serovar 5) were grown in 0.5× BHI medium (Oxoid) for 16 to 20 h at 37°C. Cultures were diluted in phosphate-buffered saline (PBS; 120 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) containing 0.1% Tween 20 (PBST) to the required cell densities.

Bacillus subtilis (WS 3080), *Pseudomonas fluorescens* (WS 1760), and *Enterococcus faecalis* (WS 1761) were grown in PC (plate count) broth (Merck) at 30°C; *Staphylococcus aureus* (WS 1759), *B. cereus* (HER1399), and *Escherichia coli* (WS 1333) were grown in PC broth at 37°C; *Lactobacillus brevis* (WS 2120) was grown in MRS broth (Oxoid) at 37°C under anaerobic conditions; and *Lactococcus garvieae* (WS1029) was grown in M-17 broth (Oxoid) at 30°C under anaerobic conditions. *C. perfringens* ATCC 3626 was cultivated in TGY medium at 35°C in an anaerobe chamber as previously described (33).

Standard plating procedure for detection of listeriae. Detection of listeriae was performed as described in IDF standard 143A:1995 (for milk and dairy products) and ISO norm 11290-1 (for all other foods). In general, samples of 25 g each were first homogenized in a stomacher blender (Seward, Norfolk, United Kingdom), if applicable. In the IDF procedure, 50 ml of citrate buffer (50 mM sodium citrate, pH 7.4) was used for homogenization and then added to 175 ml of selective *Listeria* ANC enrichment broth (Merck, Darmstadt, Germany). In the ISO protocol, samples are homogenized directly in 225 ml of half-strength Fraser enrichment broth (Merck). After incubation at 30°C for 24 and 48 h, 1 loopful of the enrichment culture was streaked onto selective Oxford *Listeria* agar (Merck). Typical *Listeria* colonies were enumerated after an additional 24 h and 48 h of incubation of the plates at 37°C.

CBD proteins. Recombinant proteins consisting of six-His-tagged N-terminal green fluorescent protein fused to C-terminal CBD118 or CBD500 were produced in *E. coli* and purified as previously described (15). Purified proteins were adjusted to 2.5 mg/ml in PBS buffer and stored at −20°C.

Coating of paramagnetic beads. Ni-nitrilotriacetic acid (NTA) agarose beads (QIAGEN, Hilden, Germany) feature Ni-NTA groups on their surface suitable for binding of six-His tags. Aliquots of 100 μl were coated by mixing with 30 μl of CBD500 for 20 min in a horizontal shaker at 900 rpm. The beads were then separated with a magnetic stand (MPC-S; Dynal) and washed twice with 0.5 ml of buffer A (0.5 M NaCl, 0.05 M Na_2HPO_4, 5 mM imidazole, pH 8.0) and twice with buffer B (buffer A with 25 mM imidazole). Beads were resuspended in PBS (approximately 4.5×10^6 beads/ml) and stored at 4°C.

Lyophilized M-270 Epoxy Dynabeads (Dynal, Oslo, Norway) were suspended in diethyleneglycol-dimethyl ether to a final concentration of 30 mg/ml as recommended by the manufacturer. For coating, 400 μl of beads was washed twice with 800 μl of PBS and resuspended in 100 μl of PBS and 200 μl of 3 M $(NH_4)_2SO_4$ (pH 7.4). A 100-μl volume of CBD118 or CBD500 was then added, and mixtures were incubated in an overhead rotator at 4°C for 16 h at 10 rpm and then incubated at ambient temperature (22°C) for 6 h. Residual epoxy groups were blocked by washing the beads four times with either PBS-BSA buffer (PBS containing 0.1% bovine serum albumin, pH 7.4) or Tris buffer (1.0 M Tris, pH 7.4). The CBD-coated beads (2.0×10^9 beads/ml) were stored at 4°C either in PBS-BSA or in Tris buffer containing 0.02% NaN_3.

The influence of the blocking agent on the binding and recovery by CBD-coated Dynabeads was tested. Separate batches of CBD500- or CBD118-coated beads were blocked with either BSA or Tris, respectively. Different concentrations (1.0×10^7 to 4.0×10^7) of beads were incubated with 10^4 *Listeria* cells for 40 min, and the separation efficiency was determined.

Immobilization, separation, and enumeration of bacterial cells. With the Ni-NTA agarose beads, 100-μl aliquots of *Listeria* cells (10^3 to 10^5 CFU/ml) were mixed with 10 to 40 μl of bead suspension (4.3×10^4 to 1.7×10^5 beads), as determined by counting of the beads in a calibrated microscope grid counting chamber (data not shown). Total volumes were adjusted to 200 μl by addition of PBST. Incubation times were 10, 20, 40, and 60 min. For detachment of cells from Ni-NTA beads, 100 μl of buffer C (0.3 M NaCl, 0.05 M Na_2HPO_4, 0.2 M imidazole, pH 8.0) was added, mixtures were incubated for 10 min, and supernatants containing the liberated cells were removed after magnetic separation. Following incubation of the plates at 37°C for 20 h, cells could be enumerated by colony counting.

With Dynabeads, 5- to 20-μl amounts (1.0×10^7 to 4.0×10^7 beads) were mixed with the bacterial suspensions and the tubes were rotated at 10 rpm for 10 to 40 min. After magnetic separation, beads were resuspended in 100 μl of PBST. Because cells could not be released from beads under mild conditions, the entire mixtures were plated, followed by incubation and enumeration. In order to determine the proportion of cells remaining in the supernatant and/or removed by the washing steps, supernatants were also plated.

Binding specificity of CBD118 and CBD500. Because of their superior characteristics and performance, we carried out all further experiments with CBD-coated Dynabeads only, under the following conditions (hereafter referred to as the CBD standard procedure): 10 μl of coated beads (2×10^7 beads), 100 μl of *Listeria* cells (approximately 10^5 CFU/ml), a 40-min incubation time, and a 200-μl total volume (adjusted with PBST).

Although it has previously been shown that the two CBD species have exclusive, nonoverlapping binding ranges (15), the two different types of CBD-coated beads were tested for cross-reactivity in a recovery assay with *L. monocytogenes* EGDe and Scott A. As a negative control, unspecific binding of *Listeria* cells to uncoated, Tris-blocked Dynabeads was tested.

In order to combine the different binding specificities of the CBD500 and CBD118 proteins, the following two procedures were tested, i.e., (i) beads coated with CBD118 or CBD500 were mixed in a 1:1 ratio, and (ii) CBD proteins were mixed prior to coating (mixed CBD118-CBD500 coating). Performance of the bead preparations was compared in recovery experiments as described above.

Influence of growth medium on bacterial immobilization. To test the potential influence of the growth medium on the binding of CBD proteins to *Listeria* cells, strains EGDe, WSLC 1001, and Scott A were grown in BHI, *Listeria* ANC broth, and half-strength Fraser enrichment broth, followed by subsequent determination of recovery rates as described above.

Capture from mixed bacterial suspensions. The ability to selectively immobilize and capture different *Listeria* cells (see Table 1) from a mixture of various other bacteria was evaluated. For this purpose, 10^4 *Listeria* cells were mixed with approximately 10^4 CFU each of *B. subtilis*, *E. faecalis*, *S. aureus*, *L. brevis*, *L. garvieae*, *P. fluorescens*, and *E. coli*, resulting in a final ratio of *Listeria* cells to other bacteria of 1:7. Magnetic separation and recovery were carried out as described above. Appropriate dilutions of supernatants and resuspended beads were plated on Oxford agar plates and incubated for 24 h at 37°C.

Comparison of antibody-based IMS (anti-*Listeria* Dynabeads) to CBD-MS. Commercially available anti-*Listeria* Dynabeads were used according to the instructions provided by the manufacturer (Dynal, Oslo, Norway). The strains used to compare their performance to that of CBD-coated beads were *L. monocytogenes* Scott A, EGDe, and WSLC 1001; *L. ivanovii* WSLC 3009; and *L. innocua* WSLC 2012. Recovery experiments were carried out as described above. Because of the strong agglutination tendency of the antibody-coated anti-*Listeria* Dynabeads, immobilization efficiency was also calculated on the basis of the number of cells remaining in the supernatant after contact with the beads.

Artificially contaminated foods. A variety of foods frequently contaminated with listeriae were purchased at local retailers (see Tables 2 and 3). Each sample was divided into several portions of 25 g and packed into sterile plastic bags. One aliquot was then tested for *Listeria* contamination by the standard procedure, and the others were frozen at −80°C (if applicable). Only samples free of listeriae were used in the experiments described below.

Food samples were mixed (in the bags) with 1 ml each of appropriate dilutions of the selected test strain *L. monocytogenes* EGDe or Scott A to obtain initial contamination rates of 0.1, 1.0, 10, and 100 CFU/g. For the negative control, PBS buffer was added. Samples were then stored at 4°C for 24 h in order to simulate

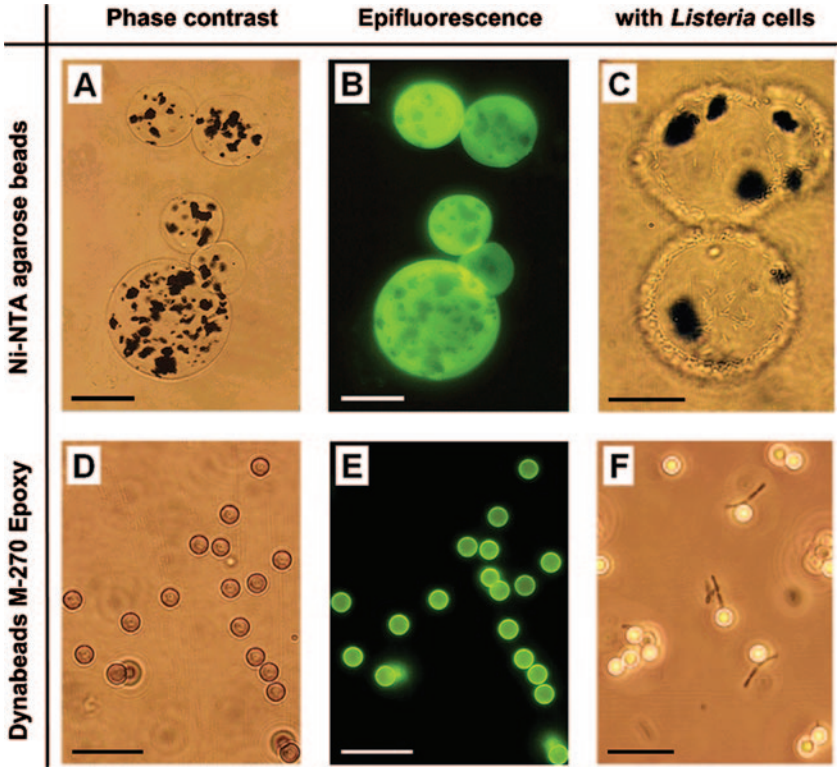

FIG. 1. Magnetic beads coated with GFP-tagged CBD proteins bind and immobilize bacterial cells. Ni-NTA agarose beads coated with CBD500 (A and B) immobilize *L. monocytogenes* Scott A cells onto their surface (C). Coating of M-270 Epoxy Dynabeads with CBD500 (D and E) and binding of *Listeria* cells to their surface (F) are shown. Bars in panels A to C are 20 μm, and those in panels D to F are 10 μm.

more-realistic conditions. After 6, 24, and 48 h of incubation, 100-μl aliquots of enrichment culture were removed and mixed with 10 μl of CBD-coated Dynabeads. To adjust the pH of the mixtures, 90 μl of 2× PBST was added (final volume, 200 μl). Following magnetic capture, supernatants were discarded and beads were resuspended in 100 μl of PBST, plated on Oxford agar, and incubated at 37°C for 24 to 48 h.

Validation of the CBD-MS method with naturally contaminated foods. A diverse range of 275 food samples were purchased from local stores and supermarkets, including meat, poultry, fish, dairy products, and various ready-to-eat deli items. In this experiment, equal amounts of the two different types of CBD-coated Dynabeads (CBD500 and CBD118) were mixed prior to use. Analyses were otherwise performed as described above, including selective enrichment (24 h for CBD-MS, 48 h for the standard method) and plating on Oxford agar. Species identification was carried out by standard biochemical testing and PCR amplification of the *L. monocytogenes*-specific *prfA* gene (31).

Analysis of results. Viable cell counts (bead-cell complexes, cells released from beads, cells in supernatant) were determined by duplicate plating. The number of colonies was assumed to reflect the number of viable cells immobilized on beads (Dynabeads) or the number of cells released from the bead surface (Ni-NTA agarose beads). The recovery rates were calculated on the basis of these counts and the number of residual cells in the supernatant after separation and removal of beads and expressed as percent recovery. All experiments were independently performed in triplicate, results are presented as means, and standard deviations are indicated. Statistical analysis was performed with an unpaired *t* test and an alpha level of 0.05.

CBD proteins from *Bacillus* and *Clostridium* phage endolysins. The CBDs from endolysins Ply21 (16) and Ply3626 (33) were identified by bioinformatics as previously described (15). Briefly, C-terminal *ply* gene fragments corresponding to amino acid residues K_{136} to K_{263} of Ply21 and K_{197} to I_{347} of Ply3626, respectively, were amplified and inserted into pHGFP. The HGFP-CBD21 and HGFP-CBD3626 fusion proteins were produced in *E. coli*, purified by immobilized metal-chelating chromatography, and tested for the ability to fluorescently decorate cell walls of *B. cereus* and *C. perfringens* as described previously (15). The organisms were cultured as specified in the paragraph on bacteria and culture conditions, and immobilization of bacterial cells on CBD-coated Dynabeads, magnetic separation, and determination of recovery rates were performed as described above for *Listeria* cells.

RESULTS

Coating of paramagnetic beads with CBD proteins. The two different types of beads tested here required different coating procedures. (i) The N-terminal six-His tags on the recombinant CBD fusion proteins were attached to affinity ligands on the Ni-NTA magnetic agarose beads, and (ii) primary amino groups were covalently linked to preactivated epoxy groups on the surface of the hydrophilic, polymer-coated Dynabeads. Fluorescence microscopy revealed that both procedures resulted in bead surfaces very evenly coated with the green fluorescent protein (GFP)-CBD hybrids (Fig. 1). Both types of beads were capable of immobilizing *Listeria* cells by means of the CBD coatings (Fig. 1C and F). After sufficient contact, beads with the attached cells could be separated from liquid suspensions by magnetic forces.

With respect to the CBD-coated Ni-NTA-agarose beads, a significant proportion of *L. monocytogenes* Scott A cells could be magnetically separated from bacterial suspensions. The individual recovery rates obtained with variable numbers of coated beads, incubation times, and cell concentrations are presented in Fig. 2. The best results with this type of beads (Ni-NTA agarose) were obtained with a bead concentration of $\sim 2 \times 10^5$ per 200-μl assay volume and an incubation time of 40 min, independent of the cell concentration.

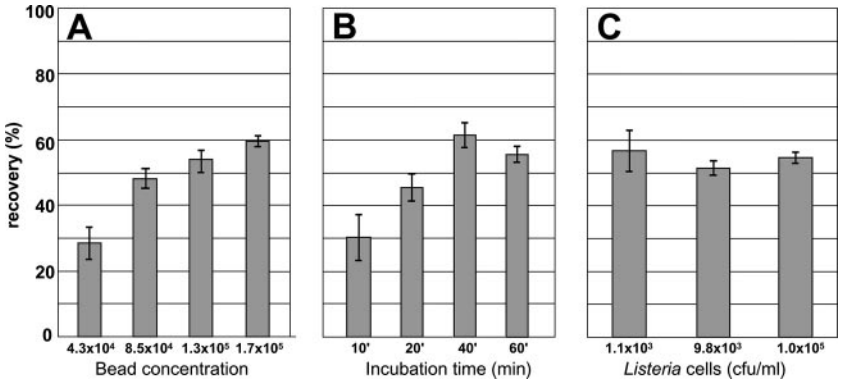

FIG. 2. Performance of CBD500-coated Ni-NTA magnetic agarose beads for separation of *L. monocytogenes* Scott A. Three variables were tested for their effects on recovery rates, i.e., (A) different bead concentrations (indicated as the absolute number used in the 200-μl assay volume (with 10^4 cells, 40-min incubation), (B) different incubation times (with 10^4 cells, 1.7×10^5 beads), and (C) different cell concentrations (with 1.7×10^5 beads, 40-min incubation).

It should be noted here that because our initial experiments clearly showed the superior performance of Dynabeads over agarose beads, all of the further experiments carried out and described below were performed with coated Dynabeads only.

Blocking agent influences performance of CBD-coated beads. The different substances tested for saturation and blocking of the residual epoxy groups on activated M-270 Dynabeads (after coating with CBD proteins) influenced the efficiency of cell binding and recovery (Fig. 3A). When BSA was used as the blocking agent, *L. monocytogenes* EGDe recovery rates were dependent on the number of beads used (57 to 91%). With Tris-blocked CBD beads, recovery rates were much higher (94 to 98%) and, at the concentrations used, appeared to be largely independent of the bead concentration. These results suggested a possible steric interference of the (relatively large) BSA molecule with the interaction of the CBD protein and its ligand on the *Listeria* cell surface. Therefore, Tris was used as the blocking substance in all experiments.

Binding specificity of CBD-coated beads. The stringent specificity of the CBD118 and CBD500 proteins for serovar-correlated recognition of and binding with nanomolar affinity to cell wall-associated carbohydrates has been previously demonstrated (15). Here, we found that these binding properties of these proteins were apparently not influenced by their coating of the magnetic bead surfaces (Fig. 3B), which agrees well with our previous findings that the CBD molecules perform very well when immobilized on the surface of sensor chips used for real-time affinity measurements (15). Regarding specificity, 96% of *L. monocytogenes* EGDe (serovar 1/2a) cells could be immobilized on CBD118-coated beads but only 5.9% of these cells could be recovered with CBD500-coated beads. Similar results were obtained when *L. monocytogenes* Scott A (serovar 4b) was incubated with CBD500- and CBD118-coated beads (94.2% versus 10.4%, respectively). It should be noted that, in naturally contaminated samples with an unknown microbial load, a mixed (pooled) preparation of the two different types of CBD beads (CBD118 and CBD500) is required in order to immobilize the potentially present *Listeria* cells of all common serovar groups. The differently coated beads do not interfere with each other; i.e., the separation efficiency of a pooled 1:1 mixture of CBD118 and CBD500 beads was indistinguishable from the results obtained when the different beads were used in separate assays (Fig. 3B). However, attempts to produce beads with a mixed coating consisting of both CBDs were unsuccessful; fluorescence microscopy indicated that the pro-

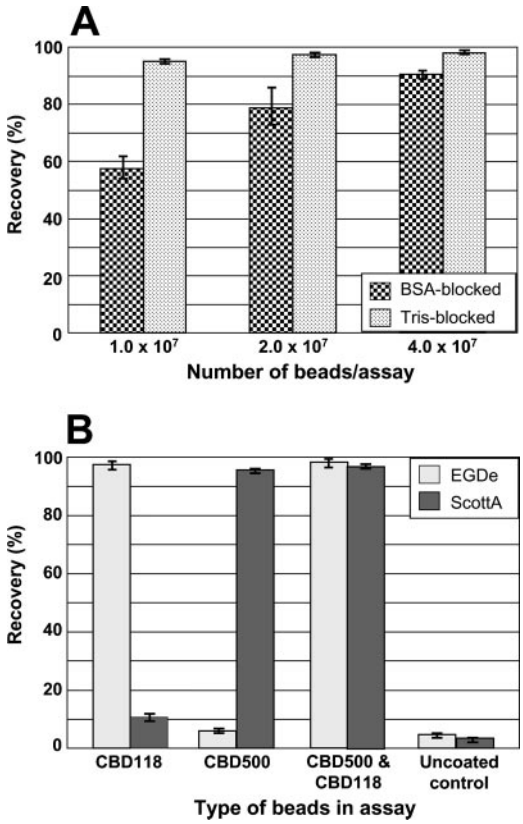

FIG. 3. Influence of blocking agent and CBD type on binding and recovery with CBD-coated Dynabeads. (A) Immobilization of *L. monocytogenes* EGDe with CBD118-coated beads blocked with either BSA or Tris. (B) Separation of *L. monocytogenes* EGDe or Scott A after contact with beads coated with CBD118, CBD500, or a 1:1 mixture of both bead types or with uncoated, Tris-blocked control beads, respectively.

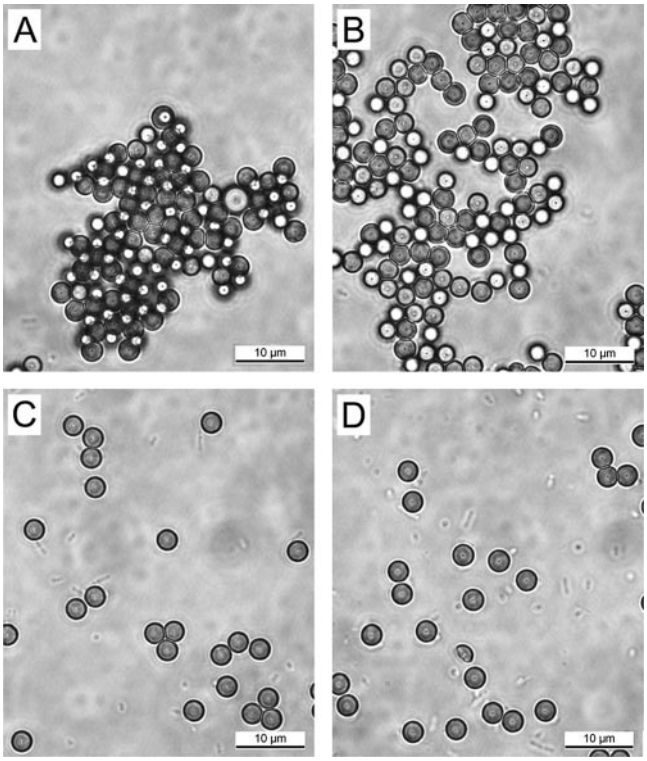

FIG. 4. CBD-coated beads do not lead to agglutination of cell-bead mixtures. Light microscopy images show the cross-linked agglomerates formed after incubation of anti-*Listeria* Dynabeads with cell suspensions of both *L. monocytogenes* Scott A (panel A) and EGDe (panel B), respectively. In contrast, beads coated with CBD500 (panel C) or CBD118 (panel D) did not show significant agglutination after incubation with Scott A and EGDe cells, respectively.

cedure did not yield an even distribution of the CBD proteins on the bead surfaces.

The effect of an additional washing step on the recovery of *L. monocytogenes* EGDe with CBD118-coated beads was insignificant; wash fractions contained only 0.6 to 3.7% of the total cells.

CBD-coated Dynabeads show minimal agglutination. A severe problem often seen with antibody-coated beads is visible agglutination of the beads due to cross-linkage effects. This becomes especially relevant in magnetic separation assays where surface plating is used to determine viable counts. We observed strong agglutination with anti-*Listeria* Dynabeads (Fig. 4A and B), whereas the CBD-coated beads showed little tendency to agglutinate (Fig. 4C and D).

Agglutination was also thought to be responsible for the poor performance of antibody-coated beads in recovery. In our experiments, recovery rates with anti-*Listeria* Dynabeads (three independent trials with three different production batches) were 3.0% (*L. monocytogenes* Scott A), 4.1% (*L. innocua* WSLC 2012), 4.7% (*L. ivanovii* WSLC 3009), 8.8% (*L. monocytogenes* WSLC 1001), and 13.5% (*L. monocytogenes* EGDe). However, the viable counts obtained by plating the supernatants after magnetic separation suggested that most of the cells were still in suspension (the supernatant) and therefore were not immobilized in the large bead-cell complexes (results not shown).

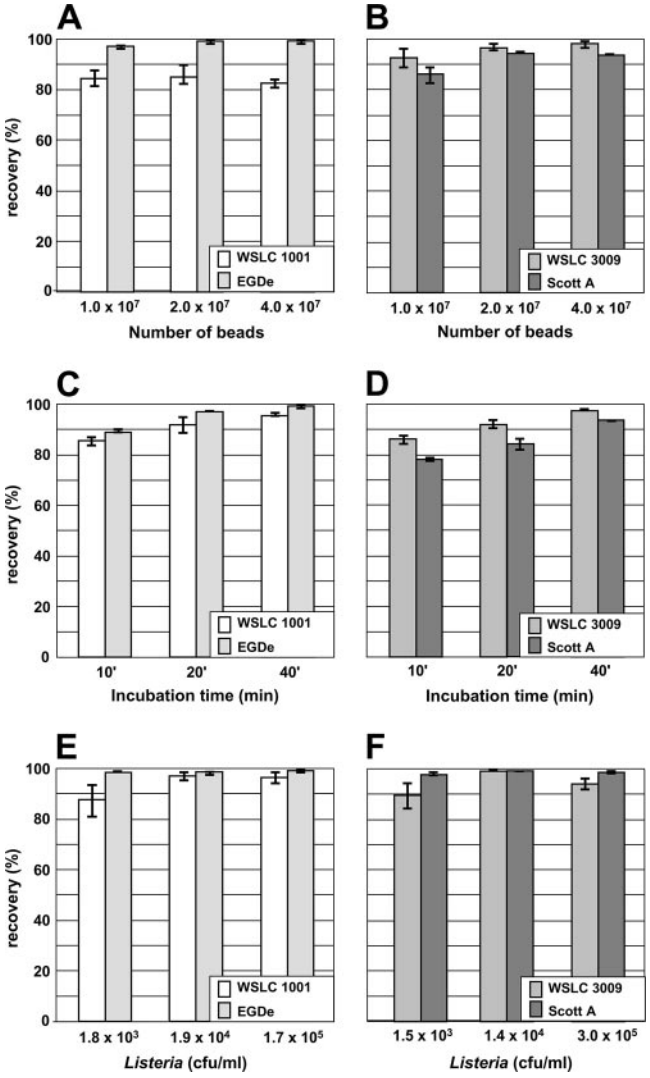

FIG. 5. Optimization of parameters for separation and recovery of *Listeria* cells with CBD-coated Dynabeads. CBD118 beads were tested with *L. monocytogenes* WSLC 1001 and EGDe, and CBD500 beads were used with *L. monocytogenes* Scott A and *L. ivanovii* WSLC 3009. Three variables were tested, i.e., different bead concentrations, indicated as the absolute number used in the 200-μl assay volume (with 10^4 cells, 40-min incubation time) (panels A and B), different incubation times (10^4 cells, 2×10^7 beads) (panels C and D), and different cell concentrations (2×10^7 beads, 40-min incubation) (panels E and F).

Superior separation and recovery of *Listeria* cells with CBD-coated beads. A series of experiments was carried out in order to determine the optimum parameters for recovery of *Listeria* cells with CBD-coated Dynabeads (Fig. 5). The variables tested were (i) type of CBD coating, (ii) *Listeria* strains, (iii) bead numbers, (iv) incubations times, and (v) cell concentrations. In conclusion, addition of 2.0×10^7 beads and an incubation time of 40 min produced the highest separation efficiency with both of the strains tested.

Influence of medium and microflora on separation efficiency. For the application of CBD-MS to selective enrichment cultures, it was necessary to examine the potential influence of intrinsic factors on recovery. Our results indicated only a slight

TABLE 1. Efficiencies of recovery of different *Listeria* species and serovars from mixed bacterial suspensions[a]

Strain (serovar)	CBD type on beads	Rate of recovery (mean ± SD)[b]
L. monocytogenes		
EGDe (1/2a)	CBD118	97.2 ± 0.9
WSLC 1001 (1/2c)	CBD118	94.9 ± 1.8
Scott A (4b)	CBD500	96.2 ± 0.8
WSLC 1042 (4b)	CBD500	96.6 ± 1.4
WSLC 1363 (4b)	CBD500	93.8 ± 0.9
L. ivanovii WSLC 3009 (5)	CBD500	92.2 ± 1.8
L. innocua WSLC 2012 (6b)	CBD500	98.6 ± 0.9

[a] See Materials and Methods for details.
[b] Percent viable cells (measured as CFU) which were captured on the beads and could be recovered from suspension.

effect of the growth medium on the binding and separation efficiency of *L. monocytogenes* cells. Recovery rates decreased by approximately 6% when cells were grown and suspended in selective growth medium (ANC broth or Fraser broth), compared to cells grown in BHI (data not shown). Results obtained in the presence of a heterogeneous microbial community far outnumbering the *Listeria* cells (Table 1) were in the same percent ranges as those obtained with pure cultures (Fig. 5E and F).

CBD-MS effectively separates *Listeria* cells from contaminated foods. The performance of CBD-MS for detection of *L. monocytogenes* EGDe and Scott A in different spiked foods was compared to that of the official standard plating method.

After a shortened selective enrichment of only 6 h, detection of *Listeria* EGDe at an initial contamination level of 1.0 CFU/g was possible in turkey breast and minced meat; a level of 10 CFU/g could be detected in lettuce, smoked salmon, minced meat, turkey breast, and milk; and 100 CFU/g were detected in all foods (Table 2). Essentially identical results were obtained when *L. monocytogenes* strain Scott A was used (Table 3).

Following enrichment for 24 h, the CBD-MS assay permitted detection of *Listeria* EGDe at an initial contamination level of 0.1 CFU/g in all foods, except for soft cheese, and higher initial contaminations were detected in all of the foods tested. Again, results obtained for strain Scott A were highly similar; 0.1 CFU/g yielded positive results in all of the foods tested except minced meat, where 1.0 CFU/g was required. Equivalent results were achieved when the enrichment was performed for 48 h, although CBD-MS still proved to be more sensitive in the detection of lower contamination levels in several foods. It was noted that, in some cases, the longer 48-h enrichment in selective enrichment medium actually yielded fewer positive results than shorter incubation periods (e.g., EGDe cells in turkey breast meat and Scott A cells in camembert).

Overall, the results obtained with the CBD-MS assay were superior to those obtained with the standard plating procedure (longer incubation times, less sensitivity).

Validation of the CBD-MS method with naturally contaminated foods. In order to confirm the results from the model experiments and to practically validate the performance of CBD-MS, a total of 275 potentially naturally contaminated food samples was tested for listeriae by both methods (Table 4). Overall, 42 samples gave positive results with the standard plating method (26 were *L. monocytogenes*) whereas 45 samples were *Listeria* positive by the CBD-MS method (28 were *L. monocytogenes*). The CBD-MS procedure performed better; it identified all of the samples which tested positive by the standard method and detected listeriae in three additional samples. Besides this increase in sensitivity, it required only half the time (48 h) compared to the standard protocol (96 h).

TABLE 2. Comparison of standard plating and magnetic separation with CBD118-coated beads for detection of *L. monocytogenes* EGDe in artificially contaminated food samples

Food sample and initial contamination (CFU/g)	Result obtained with following conditions for selective enrichment culture[a]					
	6 h		24 h		48 h	
	IDF/ISO[b]	CBD-MS	IDF/ISO	CBD-MS	IDF/ISO	CBD-MS
Sliced iceberg lettuce						
0	−	−	−	−	−	−
0.1	−	−	+++	+++	+++	+++
1	(+)	−	+++	+++	+++	+++
10	−	+	+++	+++	+++	+++
100	−	++	+++	+++	+++	+++
Camembert soft cheese						
0	−	−	−	−	−	−
0.1	−	−	−	−	−	+
1	−	−	++	+++	++	++
10	−	−	+++	+++	+++	+++
100	+	++	+++	+++	+++	+++
Red smear cheese						
0	−	−	−	−	−	−
0.1	−	−	−	+	−	+
1	−	(+)	−	++	−	+
10	−	−	+++	+++	+++	+++
100	−	+	+++	+++	+++	+++
Smoked salmon						
0	−	−	−	−	−	−
0.1	−	−	+	++	++	++
1	−	−	++	++	++	+++
10	−	+	+++	+++	+++	+++
100	+	++	+++	+++	+++	+++
Minced meat						
0	−	−	−	−	−	−
0.1	−	+	+	+	+++	+++
1	−	+	+	+++	+++	+++
10	−	+	+++	+++	+++	+++
100	−	++	+++	+++	+++	+++
Turkey breast						
0	−	−	−	−	−	−
0.1	−	+	+	++	−	+
1	−	−	+++	+++	++	+++
10	−	+	+++	+++	+++	+++
100	+	+	+++	+++	+++	+++
Milk (pasteurized)						
0	−	−	−	−	−	−
0.1	−	−	+++	+++	+++	+++
1	−	−	+++	+++	+++	+++
10	−	+	+++	+++	+++	+++
100	+	++	+++	+++	+++	+++

[a] The number of plus signs indicates the number of typical *Listeria* colonies on Oxford agar as follows: (+), presence of a single colony on one plate; +, 1 to 10 colonies; ++, 10 to 50 colonies; +++, more than 50 colonies.
[b] See Materials and Methods for details.

TABLE 3. Comparison of standard plating and magnetic separation with CBD500-coated beads for detection of *L. monocytogenes* Scott A in artificially contaminated food samples

Food sample and initial contamination (CFU/g)	Result obtained with following conditions for selective enrichment culture[a]					
	6 h		24 h		48 h	
	IDF/ISO[b]	CBD-MS	IDF/ISO	CBD-MS	IDF/ISO	CBD-MS
Sliced iceberg lettuce						
0	−	−	−	−	−	−
0.1	−	−	++	+++	+++	+++
1	−	−	+++	+++	+++	+++
10	−	+	+++	+++	+++	+++
100	−	+	+++	+++	+++	+++
Camembert soft cheese						
0	−	−	−	−	−	−
0.1	−	−	+	+++	+	+
1	−	+	+++	+++	+	−
10	+	+	+++	+++	++	++
100	+	++	+++	+++	+++	+++
Red smear cheese						
0	−	−	−	−	−	−
0.1	−	−	+	+++	+	+
1	−	−	+++	+++	+++	+++
10	−	+	+++	+++	+++	+++
100	−	+	+++	+++	+++	+++
Smoked salmon						
0	−	−	−	−	−	−
0.1	−	−	−	+	+	++
1	−	−	+	+	++	+++
10	−	+	+++	+++	+++	+++
100	+	++	+++	+++	+++	+++
Minced meat						
0	−	−	−	−	−	−
0.1	−	−	−	−	−	−
1	−	−	−	+	++	+++
10	−	−	−	++	+++	+++
100	+	++	++	+++	+++	+++
Turkey breast						
0	−	−	−	−	−	−
0.1	−	−	−	+	+	++
1	−	−	−	+	+	++
10	−	−	+	++	++	+++
100	−	+	++	+++	+++	+++
Milk (pasteurized)						
0	−	−	−	−	−	−
0.1	−	−	−	+	−	+
1	−	−	+	+	−	+
10	+	+	+	+++	+	++
100	+	+++	+++	+++	+++	+++

[a] The number of plus signs indicates the number of typical *Listeria* colonies on Oxford agar as follows: +, 1 to 10 colonies; ++, 10 to 50 colonies; +++, more than 50 colonies.
[b] See Materials and Methods for details.

CBD proteins from *Bacillus* and *Clostridium* phages. In order to provide proof of concept for a wide generalizability of the CBD approach, we also identified, cloned, and produced in *E. coli* endolysin-derived CBD proteins from phages infecting other gram-positive food-borne pathogens (*B. cereus* and *C. perfringens*). The brief results reported here (Fig. 6) show the binding of the different CBD molecules to the cell surfaces of these organisms and clearly demonstrate the suitability of the phage-encoded binding domains for immobilization and recovery of cells of these pathogens. In general, both CBD21 (for *B. cereus*) and CBD3626 (for *C. perfringens*) showed binding and immobilization properties similar to those of the *Listeria* CBD reagents.

TABLE 4. Validation of CBD-MS plating with naturally contaminated food samples

Food type or parameter	No. of samples positive/no. tested (% positive)[a]	
	CBD-MS plating	Standard plating method
Meat and meat products	6/26 (23)	5/26 (19)
Poultry	9/13 (69)	9/13 (69)
Fish and seafood	25/92 (27)	23/92 (25)
Dairy products	4/110 (4)	4/110 (4)
Delicatessen salads	1/34 (3)	1/34 (3)
Total positive (*Listeria* spp.)	45/275 (16)	42/275 (15)
Positive for *L. monocytogenes*	28/275 (10)	26/275 (9)
Relative performance (%)[b]	100	93
Total time required (h)	48[c]	96[d]

[a] Values were rounded to the next whole number.
[b] Relative performance was set at 100% for the system which detected the most positive samples.
[c] Selective enrichment for 22 h, CBD-MS, and plate incubation for 24 h.
[d] International IDF standard/ISO norm (selective enrichment for 48 h and plate incubation for 48 h).

DISCUSSION

One of the critical steps in the detection of microbial pathogens such as listeriae is elevation of the number of target cells to a detectable level. In the case of viable organisms, this is usually achieved by selective enrichment. In the first attempt to use IMS for listeriae (26), cultures had to be grown at 25°C because antiflagellar antibodies were used. Other anti-*Listeria* antibodies recognize only selected serovars (10, 12) or secreted proteins (3, 32) and are therefore not useful for IMS. Because of the lack of specificity (4, 9, 29) and poor separation efficiency and sensitivity (5, 7, 8, 9, 29), it appears as if antibodies may not be the best-suited tool for *Listeria* immobilization and separation.

Other researchers used intact, immobilized bacteriophages for magnetic separation (1, 27). With salmonellae, this approach yielded a recovery of only 19.3% of the cells (27). The other drawback of the method was that the cells were actually infected by the immobilized phages and eventually lysed. Thus, their genetic material was degraded and subsequent plating and/or biochemical or genetic analysis was not possible.

We demonstrate here that CBDs from bacteriophage endolysins are suitable for the immobilization and capture required for diagnostic procedures. CBD proteins can be inexpensively produced and offer rapid, specific, and high-affinity binding to bacterial cell surfaces (15). The chimeric protein constructs used in this study consist of a His-tagged GFP and a variable CBD and offer several advantages. (i) The affinity tag is useful for simple one-step purification of recombinant proteins from *E. coli* lysates, (ii) the GFP reporter permits visualization of the coating efficiency, and (iii) the GFP moiety (29.7 kDa) replaces (in a spatial sense) the deleted enzymatically active domain and may therefore act as a required spacer (11), en-

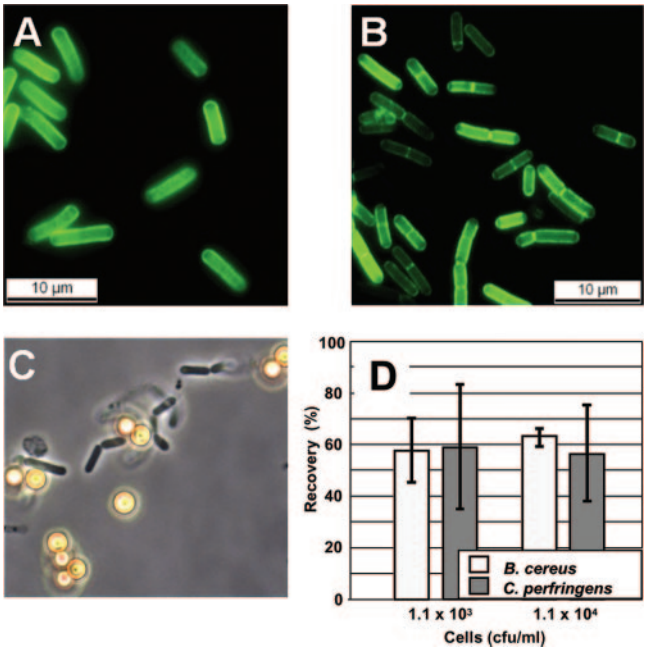

FIG. 6. CBD proteins from endolysins of *B. cereus* and *C. perfringens* bacteriophages for labeling and immobilization of host target bacteria. Panels: A, *B. cereus* cells fluorescently decorated by GFP-tagged CBD21; B, *C. perfringens* cells labeled by GFP-CBD3626 (see text for details); C, *C. perfringens* cells immobilized on CBD3626-coated Dynabeads; D, rates of immobilization and recovery of cells of the two organisms with Dynabeads coated with the respective CBD molecules.

abling interaction of the surface-bound CBD moiety (15 and 17 kDa for CBD118 and CBD500, respectively) with the bacterial cells.

We have evaluated different types of magnetic beads for their applicability with CBD proteins. The agarose beads tested are heterogeneous and relatively large (diameter, 20 to 70 μm), featuring Ni-NTA ligands for affinity coating. Their advantage is the convenient coating procedure, but the disadvantages (high sedimentation rate, low surface/volume ratio, unstable CBD binding) precluded further use for isolation of whole bacterial cells in low concentrations. M-270 Dynabeads are small, hydrophilic, polystyrene-coated particles whose specific surface chemistry offers covalent protein immobilization. Their particular advantages are a low sedimentation rate, high stability, and a high surface/volume ratio. The surface area provided by the different types of beads tested in this study was initially considered to potentially have a major influence on the recovery rates. With respect to the numbers of beads used in our optimization trials, this was calculated as follows. Forty microliters of Ni-NTA agarose bead suspension contains 1.72×10^5 beads with an average radius (r) of 25 μm. The surface area of a single bead ($4\pi r^2$) is therefore 7,854 μm^2, and the surface area of 40 μl of agarose beads is 13.5 cm^2. In contrast, 10 μl of M-270 Dynabeads contains 2×10^7 beads with $r = 1.4$ μm. The surface area of a single bead is 24.6 μm^2, and a 10-μl aliquot of Dynabeads has a total surface area of 4.92 cm^2. Therefore, Dynabeads (in the concentration used) feature only about one-third of the total CBD-coated surface area compared to agarose beads. This suggests that the plain surface area is not the only determining factor for immobilization efficiency and recovery of suspended target cells. Other important bead properties are the more equal distribution and much lower sedimentation rate of Dynabeads (increased availability of the coated surface) and the stability of the CBD coating (His tag-based affinity binding to agarose beads versus covalent binding to Dynabeads).

Our aim was to develop a novel separation technique able to quantitatively isolate low concentrations of *Listeria* cells (and those of other bacteria) from small volumes of selective enrichment culture. After optimization of several parameters, recovery rates of 86 to 99% could be obtained for cells of different *Listeria* strains, even in the presence of an excess microbial background. This specificity is mediated by the highly specific recognition properties of the CBDs (15, 20) and represents a clear advantage over antibodies, which frequently show cross-reactions with other cells (9, 22, 29). Important findings were that the target specificity of CBD118 and CBD500 is maintained when the proteins are immobilized on the bead surfaces and that the specific interaction between beads and cells also worked in mixed preparations of CBD118- and CBD500-coated beads.

The CBD bead assay was extensively tested with artificially contaminated foods. As previously reported for IMS (26) and other detection methods (17), the complex and specific matrices and microbial backgrounds of different foods significantly influence detection limits, and this is why foods represent one of the most difficult substrates for microbial diagnostics. It is noteworthy that the CBD-MS assay permitted detection of 100 CFU/g (legal or suggested limit for *L. monocytogenes* in specific foods in some countries) in all of the foods tested after only 6 h of pre-enrichment and that the lowest contamination level (0.1 CFU/g) could be detected in all of the foods except one after only 24 h of selective enrichment culture. This compares favorably to standard surface plating, which required 48 h (96 h by the official procedures) and was also less sensitive.

In order to combine the sensitivity of CBD-MS with species specificity and an even more rapid assay readout, we have aimed to integrated CBD-MS separation into protocols for real-time PCR detection (U. Bruns et al., unpublished data). A specific assay to detect only the viable cells is offered by combination of CBD-MS with reporter phage-based detection (17, 18), with very promising results (unpublished data). Yet another possible application of CBDs is their use in enzyme-linked immunosorbent assay-like formats.

Besides application to *L. monocytogenes*, we have also demonstrated that endolysin-encoded CBDs from phages infecting *B. cereus* (16) and *C. perfringens* (33) can specifically bind and immobilize these important pathogens. Although there clearly is much more work needed for optimizing the application of these and other CBD molecules (14), it is conceivable that the concept of using bacteriophage-based specific affinity reagents is also applicable to a wide range of bacteria.

ACKNOWLEDGMENTS

This work was supported by the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn, Germany), the AiF, and the Ministry of Economics and Labor, Berlin, Germany (project AIF-FV 13433 N). R.L. was supported by fellowship 388/2004 from the BNF Qualification

Program (Bern, Switzerland), and M.S. was funded by a life science project from the Wiener Wissenschafts-, Forschungs-, und Technologie Fonds (WWTF), Vienna, Austria.

We thank Siegfried Scherer for supporting J.W.K. during his time at TU Munich, Monique Herensperger and Mèlanie Mermod for excellent technical assistance, and Steven Hagens for critical reading of the manuscript. Siobain Duffy contributed to preliminary experiments with Ni-NTA beads. We also thank Profos AG, Regensburg, Germany, for providing a plasmid and information on CBD3626.

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