DOI: 10.1002/anie.201100334

Bacterial Detection

Sensitive and Selective Bacterial Detection Using Tetracysteine-Tagged Phages in Conjunction with Biarsenical Dye**

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Rapid and sensitive detection of pathogenic bacteria is vital to clinical diagnostics, environmental and food safety, and biodefense. Bacteriophages (or phages for short) are viruses that exclusively infect bacteria, and phage typing (the discrimination of bacterial isolates by testing their vulnerability to a set of phages with different lytic spectra) has long served as an important tool for the identification of pathogenic microorganisms.[1] Typically, one phage can attack only specific strains of targeted bacterial species, and host specificity is usually determined by the outer proteins (receptors) of the bacterium to which a phage attaches during the initial infection process.[2,3] Naturally occurring phages are ubiquitous and extremely numerous and represent a practical source of reagents for bacterial recognition and identification.[4,5]

Various phage-mediated assays have been developed for specific bacterial detection.[6] In addition to the phage amplification assays,[7,8] lytic phages are used for host-cell infection, lysis, and release of uniquely identifiable intracellular components,[9,10] fluorescently labeled phages are employed for direct visualization or measurement of their adsorption or infection of specific bacterial hosts,[11–13] and genetically modified phages such as luciferase[14,15] and green fluorescent protein (GFP)[16,17] reporter phages are applied for the production of progeny phages with discernible reporter signals within their bacterial hosts. Recently, the high fluorescence intensity and photostability of quantum dots (QDs) have been exploited for high-sensitivity bacterial detection using streptavidin-coated QDs combined with in vivo biotinylation of engineered host-specific phages.[18,19]

Although luciferase or GFP can be genetically fused with many proteins, including phage coat proteins, to produce fluorescent chimeras in situ, these fusions are potentially perturbative because of their large sizes. To overcome this problem, Tsien and co-workers developed an innovative method for site-specific fluorescent labeling of recombinant proteins in live cells.[20,21] A tetracysteine (TC; Cys-Cys-Xaa-Xaa-Cys-Cys) peptide tag is genetically incorporated into the target protein of interest where it can be specifically recognized by a membrane-permeant fluorigenic biarsenical dye, such as fluorescein arsenical helix binder (FlAsH). TC residues are significantly smaller than fluorescent proteins and therefore are less likely to disrupt protein function. There are several additional advantages of using biarsenical TC probes for site-specific labeling of intracellular proteins:

1) the TC motif is detectable by biarsenical dye immediately after synthesis, and the requirement of folding or post-translational modification is not needed;
2) biarsenical dyes are membrane-permeant and can be easily loaded into intact cells;
3) many biarsenical analogues of FlAsH with improved photophysical properties have been synthesized, and the addition of new colors to the palette of biarsenical dyes greatly increases assay flexibility.[22–26]

Such in situ labeling has been used as an important tool for cellular function analysis of target proteins.[20,27–32] Herein, we describe the development of a novel method for bacterial detection that integrates the benefits of natural phage specificity and rapid phage replication within bacterial hosts with the high sensitivity of biarsenical labeling of TC-tagged proteins.

Our bacterial detection strategy (TC-phage-FlAsH) is depicted in Figure 1. In the first step, a recombinant ("reagent") phage is constructed by cloning a DNA sequence encoding a TC motif into a phage coat protein gene. A TC tag of 12 amino acids (aa; FLNCCPGCCMEP) is displayed at the tip of the phage's minor coat protein pIII through N-terminus fusion. The 12-aa TC tag was chosen because of its improved affinity and fluorescence compared to 6-aa TC sequences.[33] In the second step, the reagent phages are added to the sample. If bacteria sensitive to these phages are present, the phages will infect and replicate within the bacteria, and each progeny phage will bear a TC tag on the surface protein of pIII. For every bacterium in the sample, a high degree of phage amplification will occur depending on burst size and incubation time. For M13KE, each host cell can produce about 1000 phages within an hour of bacterial infection.[34] In the third step, the cell-membrane-permeant biarsenical dye FlAsH-EDT_2 (EDT = 1,2-ethanedithiol) is incubated with the sample to stain TC-tagged phages, and the result is a significant enhancement in fluorescence of the target bacteria. Upon optimization, experimental conditions with a multiplicity of infection of 10, amplification time of 60 minutes, concentration of FlAsH-EDT_2 reagent of 5 μM, and labeling time of 30 minutes were identified.

The feasibility of the proposed TC-phage-FlAsH approach for bacterial detection was initially examined by

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[**] This work was supported by the National Natural Science Foundation of China (20675070, 20975087, 90913015, 21027010), the Program for New Century Excellent Talents in University (NCET-07-0729), Research Funds for the Doctoral Program of Higher Education of China (20090121120008, 20090121110009), and the NFFTBS (No. J1030415), for which we are most grateful.

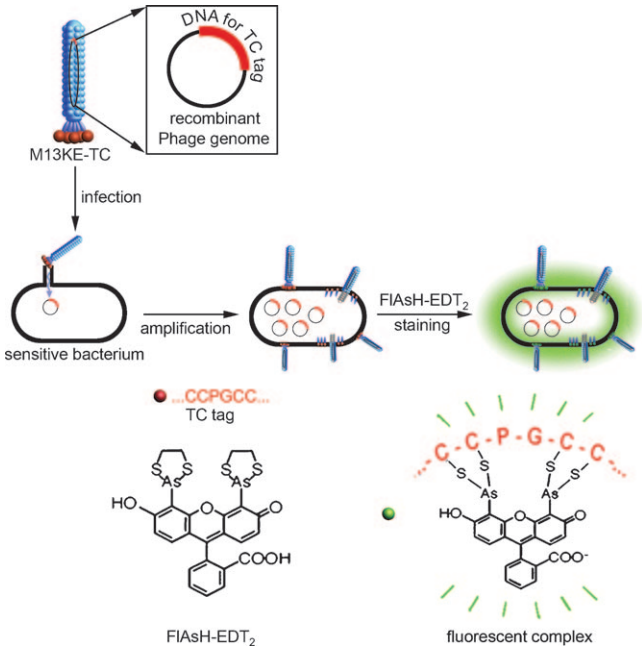

Figure 1. The TC-phage-FlAsH strategy for bacterial detection. Filamentous bacteriophage M13KE and its host cell *Escherichia coli* ER2738 are employed as a model system. DNA that encodes for the TC tag is inserted into the coat protein pIII gene to facilitate fused expression of a TC tag on each copy of pIII at one distal end of the filamentous virion. The recombinant TC-tagged phage is used to inoculate the bacterial sample. After recognition and binding, phage DNA is injected into the host cell's cytoplasm, where it directs the production of progeny phages. Phage replication in the infected host yields a large number of progeny phages with capsids displaying TC tags that can be fluorescently labeled by the membrane-permeant biarsenical dye FlAsH-EDT$_2$ to illuminate the bacterium.

flow cytometry. Figure 2 a–c shows dot plots of side scattering versus fluorescence obtained for *E. coli* ER2738 without phage (blank control), incubated with wild-type M13KE (negative control), and incubated with M13KE-TC phage, respectively. The comparable scattering signals for all three samples indicate that host cells were susceptible to M13KE infection, and TC motif expression on the coat protein pIII of M13KE followed by FlAsH staining did not induce host-cell aggregation. In contrast to the minimal background fluorescence detected for both the blank and negative controls, a significant enhancement in fluorescence was observed for M13KE-TC-infected cells. A threshold of 160 arbitrary units was used on the fluorescence channel to facilitate discrimination. The percentages of populations that produced fluorescence intensity above this threshold were 1.95, 7.15, and 89.4% for host cells without phage, infected with M13KE, and infected with M13KE-TC, respectively. The fluorescence histograms for these three samples are displayed in Figure 2 d, in which differences can be easily discerned. The median fluorescence intensities were 11, 45, and 515, respectively.

The above results demonstrate the following four conclusions:

1) the intrinsic expression of TC-rich proteins is low in both the host bacterial strain and the wild-type M13KE phage;
2) fusion of a TC tag to the pIII coat protein of M13KE does not disrupt its binding to host-cell surface receptors and subsequent delivery of genetic material into the cell;
3) infected phages can replicate rapidly in bacteria with every progeny phage expressing TC tags;
4) TC motif expression on coat proteins of phages amplified within host cells enables specific bacterial detection upon FlAsH staining.

The feasibility of using the TC-phage-FlAsH strategy for bacterial detection was verified by fluorescence microscopy. Figure 3 shows the bright-field and fluorescence micrographs of *E. coli* ER2738 cells infected with M13KE and M13KE-TC (Figure 3 a and b, respectively). In agreement with data obtained by flow cytometry, the microscopy results showed almost no fluorescence signal for bacteria treated with the wild-type phage M13KE. Bright fluorescence was observed for cells infected with M13KE-TC phages, and single bacterial cells were readily identified (Figure 3 b). It was noted that the fluorescence signal remained detectable even after 24 hours of sample storage.

The specificity of the proposed TC-phage-FlAsH approach for bacterial detection was assessed by analyzing eight types of M13KE-insensitive bacteria in parallel with the host strain *E. coli* ER2738. The eight control bacteria were *E. coli* ER2566, *E. coli* BL21, *Micrococcus lysodeikticus*, *Vibrio harveyi*, *Vibrio alginolyticus*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella* sp. After phage inoculation, proliferation, and FlAsH staining, these bacterial samples were analyzed by flow cytometry. The median fluorescence intensities are plotted in Figure 4. Only host bacterial strain *E. coli* ER2738 exhibited strong fluorescence, whereas

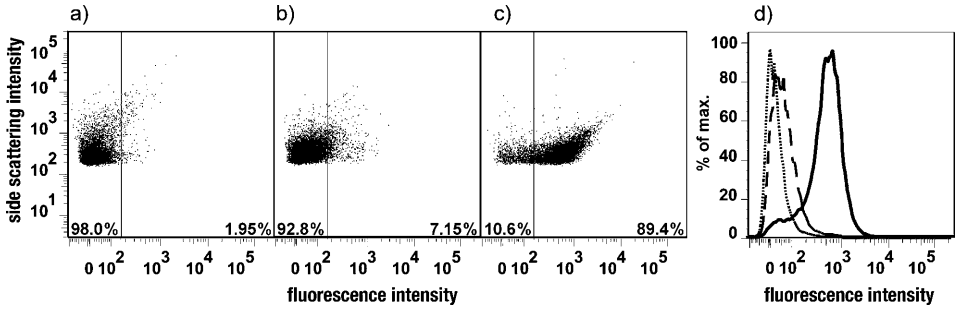

Figure 2. Bacterial detection with the TC-phage-FlAsH strategy using flow cytometry. Side scattering versus fluorescence intensity graphs for *E. coli* ER2738 a) without phage (blank control), b) inoculated with wild-type M13KE phage (negative control), and c) inoculated with TC-tagged recombinant phage M13KE-TC. Solid lines on the dot plots indicate the threshold. d) Bacterial fluorescence histograms plotted for *E. coli* ER2738 alone (•••••), incubated with wild-type M13KE phage (-----), and incubated with M13KE-TC phage (——).

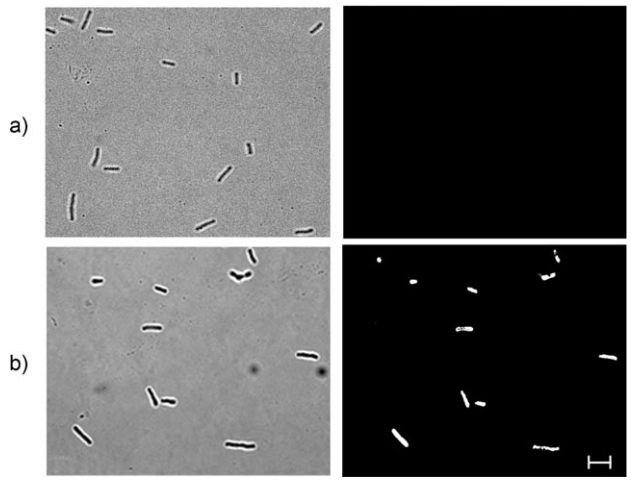

Figure 3. Bright-field (left) and fluorescence microscopic images (right) of *E. coli* ER2738 inoculated with wild-type M13KE phage (a) and TC-tagged recombinant phage M13KE-TC (b). Scale bar: 4 μm.

very low fluorescence signals were detected for all other bacterial samples, even for strains *E. coli* ER2566 and *E. coli* BL21 that belong to the same species. These results demonstrate that the TC-phage-FlAsH approach can specifically distinguish host cells from other bacteria.

Because bacteria usually exist as mixed populations in natural environments, the interference of coexisting nonsensitive bacteria in the detection of target bacteria was examined. A cocktail was prepared by combining equal proportions of *E. coli* BL21, *V. alginolyticus*, *P. aeruginosa*, *Staphylococcus aureus*, and *Salmonella* sp. cells, in which phage M13KE cannot propagate. Then, a series of bacterial samples containing different percentages of *E. coli* ER2738 cells (0 to 100% of the total cell number), but a constant total number of 2×10^6 cells, was prepared by mixing *E. coli* ER2738 with the cocktail solution. The mixed samples were inoculated with M13KE-TC and stained with FlAsH-EDT_2. The results obtained using flow cytometry are shown in Figure 5. Clearly, the mixture of these nontarget bacteria yielded very weak fluorescence, whereas target cells exhibited relatively strong signals. *E. coli* ER2738 cells at a relative abundance as low as 10% were readily detected in the cell mixture. The number of high-fluorescence bacteria that were detected corresponded well with the number of target *E. coli* ER2738 cells in the sample. When the target cells were 10, 25, 50, and 100% of the total cell number, the detected percentages of population falling in the region above the threshold of 160 were 21.5, 33.3, 55.4, and 86.2%, respectively. The linear correlation coefficient between the mixed and detected percentages was 0.991, which suggests that the TC-phage-FlAsH approach is suitable for quantitative analysis of target bacteria in a mixture.

The general applicability of using the TC-phage-FLAsH strategy for sensitive and specific bacterial detection was further validated using T7 phage. To construct the recombinant phage T7-TC, we cloned a DNA sequence encoding the 12-aa TC tag into the 10B capsid protein via the T7Select10-3b vector provided by the T7Select Cloning Kit (Novagen). According to the manufacturer's datasheet, each T7 phage can express 5–15 copies of the TC tag. Besides the *E. coli* BLT5403 host strain provided by the kit, eight other bacterial strains were also tested with T7-TC phage in parallel.

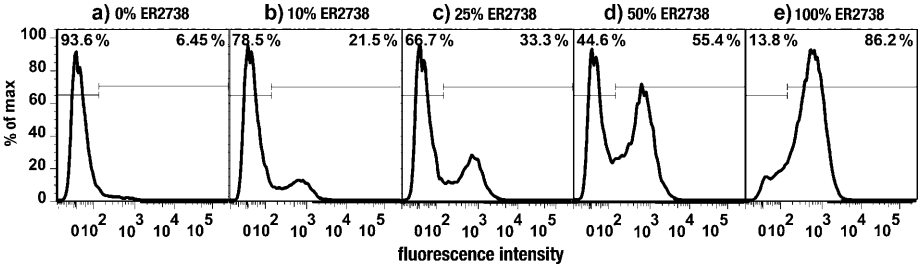

Figure 5. Detection of target bacteria in a mixed sample using the TC-phage-FlAsH strategy.

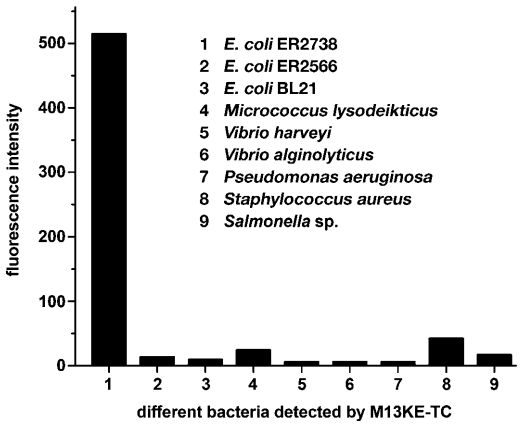

Figure 4. Flow cytometric analysis of eight different M13KE-insensitive bacteria and the host strain *E. coli* ER2738 using the TC-phage-FlAsH strategy.

Figure 6a,b shows the bright-field and fluorescence micrographs, respectively, of *E. coli* BLT5403 cells infected with T7-TC. Similar to the results obtained with M13KE-TC-infected *E. coli* ER2738, the T7 host bacteria *E. coli* BLT5403 exhibit bright fluorescence and single bacterial cells can be clearly identified by fluorescence microscopy. The median fluorescence intensities of the flow cytometric data for all nine bacterial strains tested are plotted in Figure 6c. As bacteriophage T7 is a phage capable of infecting most strains of *E. coli*, strong fluorescence signals were observed for all three *E. coli* strains. Interestingly, *E. coli* ER2566 yielded a slightly higher signal than *E. coli* BLT5403 host strain, while the signal generated by *E. coli* BL21 was only 70% of that of *E. coli* BLT5403. Note that although much weaker fluorescence signals were detected for all non-*E. coli* bacteria as expected, the signal of *S. aureus* was somewhat high with unknown reason. These results demonstrate that the proposed TC-phage-FlAsH approach is not only suitable for

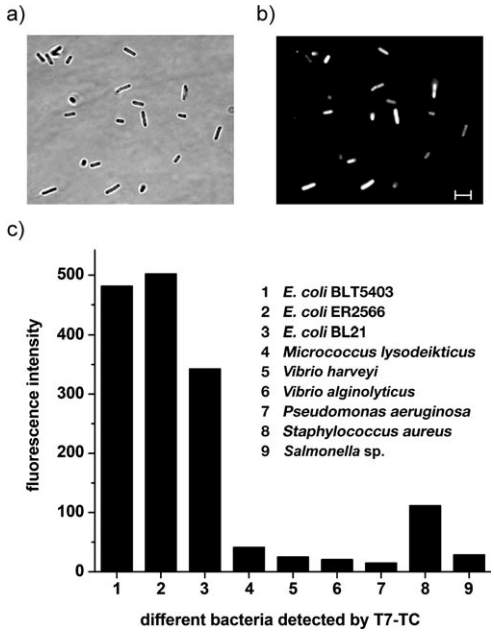

Figure 6. Bacterial detection with the TC-phage-FlAsH strategy using T7-TC. a) Bright-field and b) fluorescence microscopic images of *E. coli* BLT5403 inoculated with TC-tagged recombinant phage T7-TC. c) Flow cytometric analysis of eight different bacteria and the host strain *E. coli* BLT5403 using T7-TC. Scale bar: 4 μm.

sensitive and specific bacterial detection, but also applicable to both temperate (M13) and virulent (T7) phages.

In summary, by utilizing a genetically engineered TC-tagged phage as a recognition and amplification element, we have developed a sensitive and rapid assay method for the specific detection of bacteria. Using both M13 and T7 phages as the model system, we demonstrate that a TC motif can be site-specifically stained by biarsenical dye within a bacterial cell, and the labeled bacteria are readily detectable by flow cytometry and fluorescence microscopy. The coexistence of insensitive cells did not influence the specificity and affinity of phage infection of host cells. Phages are robust reagents in the environment and can be cost-effectively mass-produced. With innumerous types of phages available, each capable of infecting strains or a subgroup of strains within the targeted bacterial species, the present method holds great potential for the efficient detection of bacterial cells. Moreover, TC tags could be integrated into (single display) or combined with (double display) target-recognizing peptides identified by the well-established phage display technique to construct bifunctional (capable of recognition and generation of a readable output) target-specific phage particles for chemical and biological sensing. It is anticipated that the phage assay described here could also be used for antibiotic susceptibility testing; in the presence of antibiotics, bacteria that are drug-resistant retain their viability, which facilitates phage infection, replication, and fluorescent staining.

Experimental Section

The TC-FlAsH In-Cell Tetracysteine Tag Detection Kit was purchased from Molecular Probes of Invitrogen, Inc. (Eugene, OR, USA). Oligonucleotides were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The wash buffer, Hanks balanced saline solution (HBSS), was purchased from Sangon Biotech. Enzymes used for molecular cloning were obtained from New England BioLabs (NEB, Ipswich, MA, USA). The Ph.D. Peptide Display Cloning System from NEB was used for the construction of recombinant M13KE-TC phage. The T7Select Cloning Kit with T7Select10-3b vector was purchased from Novagen and was used for the construction of recombinant T7-TC phage. To introduce the TC sequence FLNCCPGCCMEP into M13KE phage particles, oligonucleotide primers that are complementary to each other and contain overhanging nucleotides were synthesized with the following sequences:

P1, 5′-GTACCTTTCTATTCTCACTCTTTCCTGAACTGTTGTCCCGGCTGCTGCATGGAGCCTTC-3′;

P2, 5′-GGCCGAAGGCTCCATGCAGCAGCCGGGACAACAGTTCAGGAAAGAGTGAGAATAGAAAG-3′.

For T7 phage, the primers' sequences were:

Q1, 5′-AATTCTTTCCTGAACTGTTGTCCCGGCTGCTGCATGGAGCCTTAAA-3′;

Q2, 5′-AGCTTTAAGGCTCCATGCAGCAGCCGGGACAACAGTTCAGGAAAG-3′.

P1 and P2 (Q1 and Q2) were annealed to each other by heating at 95 °C for 5 min at a final concentration of 1 pmol μL^{-1} in T4 DNA ligase buffer (NEB) followed by cooling to room temperature. The product of P1 and P2 was inserted into M13KE arms at the *Acc65*I and *Eag*I restriction sites (N terminus of minor coat protein pIII), which resulted in the TC-tagged recombinant phage M13KE-TC. The colonies were verified by PCR and sequencing the N terminus of pIII protein. The product of Q1 and Q2 was inserted into T7 arms at the *Ecor*I and *Hind*III restriction sites (C terminus of the 10B capsid protein), which resulted in the TC-tagged recombinant phage T7-TC. The colonies were verified by sequencing the C terminus of the 10B capsid protein. Phage titers were ascertained by plaque assay.

Bacterial cultures in the logarithmic growth phase were inoculated with M13KE-TC phage particles at a multiplicity of infection of 10. The mixtures were incubated at 37 °C for 60 min with vigorous shaking, and then the cells were centrifuged and washed with HBSS. The bacterial cells were resuspended in FlAsH-EDT_2 (25 μL, 5 μM) while being kept in the dark and incubated for 30 min at room temperature. After incubation, excess reagent was removed by three dithiol washes with 2,3-dimercaptopropanol (BAL; 100 μL) and the samples were then resuspended in HBSS (400 μL) for flow cytometric analysis. A Becton Dickinson FACSAria flow cytometer was used with a single 488 nm excitation laser and a 530/30 nm bandpass detector. A total of 10000 events were collected for each sample. For fluorescence microscopy analysis, the rinsed cells were resuspended in HBSS (20 μL) and images were obtained with an Axio Imager A1 fluorescence microscope (Carl Zeiss) using an Alpha Apochromatic Plan oil-immersion objective lens (100×).

The experimental procedure was adjusted for T7-TC, which is a virulent phage. Bacterial cultures in the logarithmic growth phase were washed with and resuspended in HBSS, and then inoculated with T7-TC phage particles at a multiplicity of infection of 75. FlAsH-EDT_2 with a final concentration of 5 μM was added at the time of phage inoculation. The mixtures were kept in the dark and incubated at 37 °C for 30 min with vigorous shaking. After incubation, excess reagent was removed by two dithiol washes with BAL (100 μL) and the samples were then resuspended in HBSS for flow cytometric and fluorescence microscopic analysis as M13KE-TC.

Received: January 14, 2011
Revised: March 14, 2011
Published online: May 10, 2011

Keywords: bacteria · bacteriophages · dyes/pigments · fluorescence · tetracysteine

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