Note

Viable Cell Detection by the Combined Use of Fluorescent Glucose and Fluorescent Glycine

Hideaki MATSUOKA,1,1 Kanenari OISHI,1 Masaaki WATANABE,1 Ikuko KOZONE,1 Mikako SAITO,1 and Shizunobu IGIMI2

1Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan
2National Institute of Health Science, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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The combined use of a fluorescent glucose (2NBDG) and a fluorescent glycine (NBD-Gly) was tried for the detection of viable cells of significant foodborne pathogenic strains in addition to several Escherichia coli strains and coliforms. Thirty-five out of 41 strains showed marked uptake of 2NBDG but 6 strains were not able to take in 2NBDG. Five out of these 6 strains showed NBD-Gly uptake.

Key words: viable cell detection; fluorescent glucose; fluorescent glycine; foodborne pathogenic bacteria

For the confirmation of sterilization of foods, it is necessary to detect viable microorganisms of whatever species and strains. For this purpose, the conventional colony count method is recognized as most reliable, and is in practical use widely. Considering that in many practical cases a small number of cells should be detected rapidly without culture, promising alternatives reported so far are mostly microscopic detection methods using appropriate dyes. In fact, several kinds of dyes have been used in the microscopic distinction of viable cells from dead ones.1-4 The principle of those dye methods is based either on the cell membrane properties or on the intracellular esterase activity. Such a principle is thought to be applicable to many strains in common. In practical samples, however, we often experience pseudo-positive or pseudo-negative results. To overcome such a difficulty, it would be necessary to find a proper combination of multiple dyes rather than to find a particular almighty dye. From this viewpoint, we propose a different strategy for the dye selection. We have focused our attention on assimilable carbon sources, stimulated by a pioneering work about the cell identification system based on the assimilation pattern of 95 carbon sources.5 However species and strains can assimilate at least one of usual carbon sources such as glucose and amino acids. Therefore, it would be easier to select several carbon sources so that viable cells of any strain can assimilate at least one of them. Thus we intended to select proper carbon sources and to modify them with a fluorescent moiety. At first, we synthesized a fluorescent glucose, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-

2NBDG glycolate,5,6 and used it with Escherichia coli, Lactobacillus acidophilus, Saccharomyces cerevisiae, and Candida tropicalis.7 All of these 4 species could take in this fluorescent glucose (2NBDG) rapidly as long as they were living. Then we prepared fluorescent amino acids according to the reference,11 and found a fluorescent glycine, NBD-Gly, to be feasible. Thus, in this study, 2NBDG and NBD-Gly have been used simultaneously to detect viable cells of 41 strains, including 17 foodborne pathogenic microorganisms and several other E. coli strains and coliforms.

2NBDG was prepared, as described previously.4,5 NBD-Gly was newly synthesized. Two ml of a glycine solution (50 mg/ml) and 2 ml of an ethanol solution of NBD-CI (15 mg/ml) were mixed in a 100-ml Erlenmeyer flask. Then, 2 ml of sodium acetic anhydride saturated with ethanol and 40 ml of ethanol were added to the flask. The reaction was done at 75°C in the dark with shaking. After 20 min, the flask was cooled at 4°C to stop the reaction. The reaction mixture was concentrated in vacuo to remove ethanol, and lyophilized. The product was purified with a gel filtration chromatography (Sephadex LH-20). Strains of E. coli (12 strains), coliforms (12 strains), and foodborne pathogenic strains (17 strains) were used as test microorganisms. Each strain was cultured for 6-8 h at 37°C. The culture broth was diluted with PBS to adjust the cell concentration to about 106 cells/ml. To prepare viable cells, 1-ml cell suspensions were centrifuged (5 min, 9000 rpm) and the precipitate was re-suspended in PBS after being washed with 1 ml of PBS. To prepare

1 To whom correspondence should be addressed. Tel: +81-42-388-7029; Fax: +81-42-387-1503; E-mail: bio-func@cc.tuat.ac.jp
dead cells, the precipitate was suspended in 1 ml of ethanol. After the ethanol treatment for 30 min, the cells were washed and re-suspended in PBS. 2NBDG (100 μM) was added to both cell suspensions, respectively. These two suspensions were incubated for a time period from 1 to 60 min at 37°C. To stop the reaction, the cell suspensions were mixed with 100 μl of formalin and left to stand for 1 min. The cells were collected by centrifugation, washed with PBS, and re-suspended in PBS. The uptake of 2NBDG was measured from the fluorescent intensity of the cell suspension that was measured with a fluorescent spectrophotometer at 550 nm with excitation at 475 nm. For the microscopic observation, 2NBDG (100 μM) or NBD-Gly (1 mM) was added to a one-ml test sample, and incubated at 37°C for 1 min. Formalin was added to the cell suspension. The cell suspension was passed through a membrane filter with a diameter of 25 mm and a pore size of 0.22 μm. After it was washed with PBS, the filter was placed on a slide glass and 5 μl of olive oil was put on it. Then the membrane filter was covered with a cover glass. The number of all cells on the membrane filter was counted with a fluorescent microscope.

Figure 1 shows typical patterns of the time course of 2NBDG uptake. The pattern depends upon the uptake rate of 2NBDG ($V_{IN}$) and the rate of its decomposition into a non-fluorescent fragment ($V_{DECOM}$). In the case of $S$. Typhimurium PT49, the fluorescence intensity increased markedly and then decreased immediately. This indicates that both $V_{IN}$ and $V_{DECOM}$ are so high that the 2NBDG in the bulk solution has been exhausted within 30 min. In the case of Pseudomonas aeruginosa TU17, $V_{IN}$ was as high as that of $S$. Typhimurium PT49 but $V_{DECOM}$ was low. Consequently 2NBDG seemed to accumulate in the cell. In the case of $E$. coli K-12, $V_{IN}$ and $V_{DECOM}$ were lower than those of $S$. Typhimurium PT49. $E$. coli O91 and Listeria monocytogenes Y7 showed much lower $V_{IN}$ and $V_{DECOM}$. On the other hand, $E$. coli AW539 and Aeromonas hydrophila showed only slight fluorescence at 1 min and no appreciable change of the fluorescence after that. This suggests no uptake of 2NBDG. Scanning these patterns, we have concluded that it is possible to judge within 1 min whether each strain can take in 2NBDG. Therefore the 2NBDG uptake time has been decided as 1 min in the following experiments.

The fluorescent intensities of all strains were measured (data not shown). The 2NBDG uptake by the viable cells was clearly observed with 35 out of 41 strains. Six strains, $E$. coli ATCC 8739, $E$. coli AW539, $A$. hydrophila JCM 1027, Vibrio mimicus 10393, $P$. shigelloides NP321, and Bacillus cereus JCM 2152, showed only slight or no fluorescence. The dead cells showed slight fluorescence that was thought to be due to the adsorption on the outer cell surface.

Figure 2 shows the results obtained with test samples containing a small number (10-100) of cells of $E$. coli K-12. The square of the correlation coefficient and the gradient of a least-square line were 0.972 and 1.098, respectively. Therefore we concluded that the experimental conditions for optical measurement were adjusted properly.

From the fluorescence intensity that respective single-cells showed, it was judged whether the respective strains could take in 2NBDG. Figure 3 (A-D, a-d) shows examples of fluorescent cells. Among the 12 coliform bacteria, all strains became fluorescent. On the other hand, among 12 $E$. coli strains, $E$. coli
ATCC 8739 and E. coli AW539 could not emit sufficient fluorescence and, among 17 foodborne pathogenic microorganisms, 4 strains (A. hydrophila JCM 1027, V. mimicus 10393, P. shigelloides NP321, and B. cereus JCM 2152) did not show sufficient fluorescence. In total, 6 out of 41 strains could not take in 2NBDG. These results agreed well with the results obtained with a fluorescent spectrophotometer using cell suspensions. To 6 strains that showed no uptake of 2NBDG, NBD-Gly was applied. Viable cells of 5 strains showed marked uptake of NBD-Gly. Figure 3 (E, e) shows a microscopic image of E. coli AW539. The strain that did not take 2NBDG and NBD-Gly was only 1 strain, E. coli ATCC 8739. Therefore viable cells of 40 out of 41 strains could be detected by only 2 fluorescent derivatives of glucose and amino acid.

This method does not require long-time culture and therefore is applicable to cells that grow slowly or practically cannot be cultured. The incubation time was as short as 1 min, so that total time can be markedly shortened. These results imply a promising property of our strategy focused on the fluorescent derivatives of carbon sources. The next target is the development of a 3rd fluorescent carbon source to cover much more species and strains by combined use.

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