

## Use of Fluorescein-Di- $\beta$ -D-Galactopyranoside (FDG) and C<sub>12</sub>-FDG as Substrates for $\beta$ -Galactosidase Detection by Flow Cytometry in Animal, Bacterial, and Yeast Cells

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**Fluorescein-di- $\beta$ -D-galactopyranoside (FDG) was found to be a useful substrate for  $\beta$ -galactosidase detection by flow cytometry in gram-negative bacteria, since it entered viable cells and gave a fluorescence emission proportional to the enzymatic activity. C<sub>12</sub>-FDG, a more lipophilic derivative, gave a very poor signal because of the lack of penetration. On the contrary, C<sub>12</sub>-FDG was more sensitive than FDG for  $\beta$ -galactosidase activity determinations in animal cells. In contrast to previous reports, C<sub>12</sub>-FDG did not enter viable yeast cells, so that the use of the substrate required cell permeabilization. Without this treatment, C<sub>12</sub>-FDG penetrates only nonviable yeast cells that may occur in populations expressing  $\beta$ -galactosidase.**

The *lacZ* gene from *Escherichia coli* is very often used as a reporter gene in different types of studies of gene expression regulation such as those designated to determine promoter efficiency, the effects of *trans*-acting factors, and the effects of other regulatory elements in bacterial, yeast, and animal cells (2, 11). Using a chromogenic substrate, such as ONPG (*o*-nitrophenyl- $\beta$ -D-galactopyranoside), one can measure expression of  $\beta$ -galactosidase in cell cultures; but it is not possible to monitor expression in individual cells and to analyze the heterogeneity of expression in cell populations. The use of a fluorogenic substrate, however, makes it possible to determine  $\beta$ -galactosidase activity in a very large number of individual cells by means of flow cytometry. This type of determination can be more informative with regard to the physiology of the cells, since gene expression can be correlated with the stage in the mitotic cycle or the viability under certain conditions. This methodology can also be of special interest from the viewpoint of biotechnology, in which cells transformed with unstable plasmids are very often used. In these cases, plasmid stability and maintenance, effects of gene dosage, and many other parameters relevant for industrial processes could be analyzed.

Nolan et al. (10) developed a technique to analyze  $\beta$ -galactosidase expression in mammalian cells employing fluorescein-di- $\beta$ -D-galactopyranoside (FDG) as a substrate for  $\beta$ -galactosidase, which releases fluorescein, a product that can be detected by a fluorescence-activated cell sorter (FACS), upon hydrolysis. Other fluorogenic substrates have also been described (12, 14), but FDG is the most sensitive and versatile. The emission of fluorescence is proportional to the amount of enzyme in the cells, so the individual expression of enzyme can be precisely determined. This method was improved by Fiering et al. (4), using specific inhibitors for the endogenous mammalian  $\beta$ -galactosidase and modifying assay conditions to achieve optimal substrate penetration (by hypotonic shock) and optimal product retention (by incubation at 4°C). This modification also avoids false positives, which can be generated during the hypotonic treatment because of the permeability of  $\beta$ -galactosidase-negative cells to the fluorescein released as a

reaction product. However, the protocol used for mammalian cells is not adequate for yeast cells. It is necessary to permeabilize yeast cells in order for the substrate to gain access to the enzyme location, and the signal fades rapidly because the product is not retained in the cells. For bacteria, despite the reports about the need for initial permeabilization of the cells for this substrate to penetrate (5, 9), we have recently shown that it is possible to measure  $\beta$ -galactosidase in *E. coli* (LacY<sup>-</sup>) without any permeabilization or cell lysis (1).

Zhang et al. (15) have described a more lipophilic substrate, namely, C<sub>12</sub>-FDG, that apparently penetrates live mammalian and yeast cells without previous permeabilization. The fatty-acid tail of the substrate molecule is believed to allow fast entry through the cell membrane under normal physiological conditions and to result in a better retention of the fluorescent product resulting from  $\beta$ -galactosidase hydrolysis, in cells at 37°C.

In this report, we present results obtained from the use of both substrates, FDG and C<sub>12</sub>-FDG, for  $\beta$ -galactosidase detection in bacterial cells, in permeabilized and nonpermeabilized yeast cells, and in a human cell line. We have checked cell viability by staining with propidium iodide (PI), which occurs only in cells that have lost selective permeability and therefore are nonviable (3).

**FDG is a better substrate for gram-negative cells.** In order to develop an appropriate bacterial gram-negative strain for the determination of  $\beta$ -galactosidase, *E. coli* JM109 was transformed with vector YEp357 carrying an insert of the *Salmonella enteritidis* cryptic plasmid pFM336 (6) that had promoter activity (1). Bacterial cells were grown overnight at 37°C in an orbital shaker at 250 rpm, and about  $2 \times 10^7$  cells, corresponding to 0.1 ml of the culture, were collected by centrifugation and resuspended in 1 ml of sterile distilled water. This sample was divided into two 0.5-ml aliquots; the first was supplemented with 50  $\mu$ l of either an FDG solution (1 mg dissolved in 1 ml of a mixture of 98% water, 1% dimethyl sulfoxide, and 1% ethanol) or a C<sub>12</sub>-FDG solution (0.0427 mg per ml of the same mixture) plus 50  $\mu$ l of PI solution (50  $\mu$ g per ml of distilled water). The second aliquot was supplemented only with PI and used as a negative control. Samples were incubated in the dark at 37°C with shaking at 150 rpm for 30 min. About

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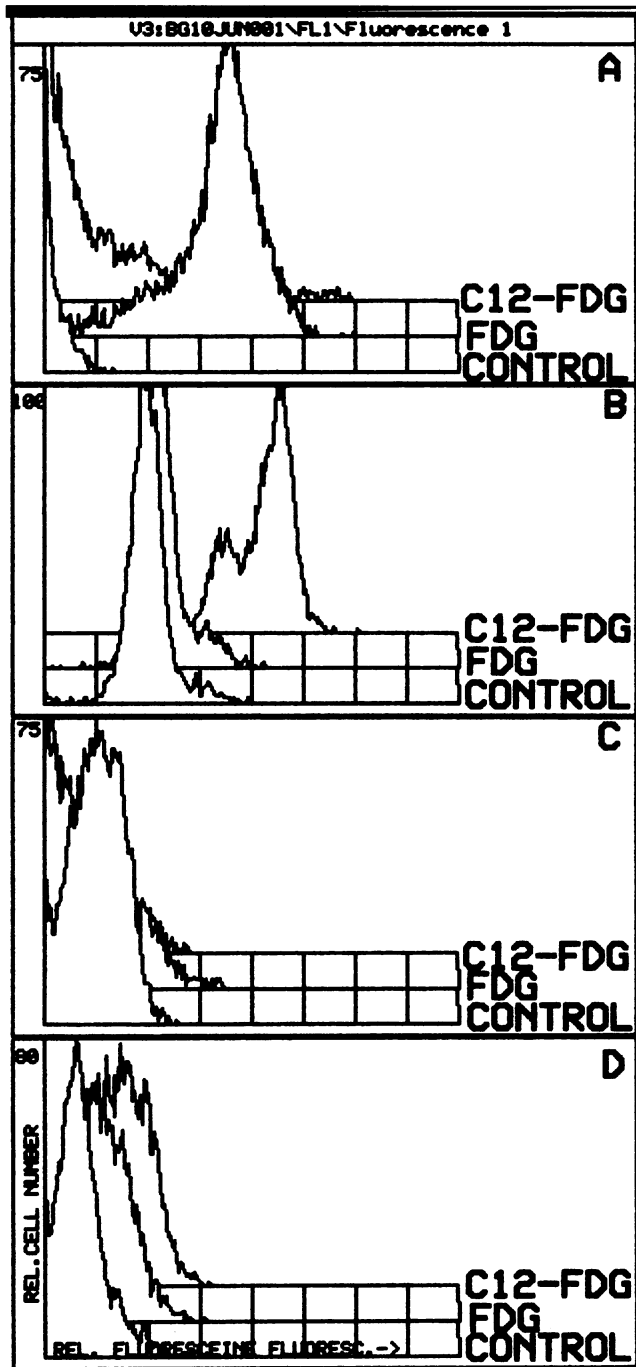


FIG. 1. FACS analysis of the fluorescein signal produced by  $\beta$ -galactosidase hydrolysis of FDG and  $C_{12}$ -FDG in bacterial cells (A), mammalian cells (B), nonpermeabilized yeast cells (C), and permeabilized yeast cells (D). Fluorescence intensity is represented on the abscissa, in arbitrary units, and the relative number of cells is represented on the ordinate.

5,000 cells from each sample were analyzed in a FACSscan flow cytometer (laser power, 25 mW; wavelength, 488 nm) (Becton Dickinson, San Jose, Calif.). The trigger parameter was set with forward-side scatter (FSC). The fluorescent signals emitted by fluorescein (530 nm) and PI (630 nm) were distin-

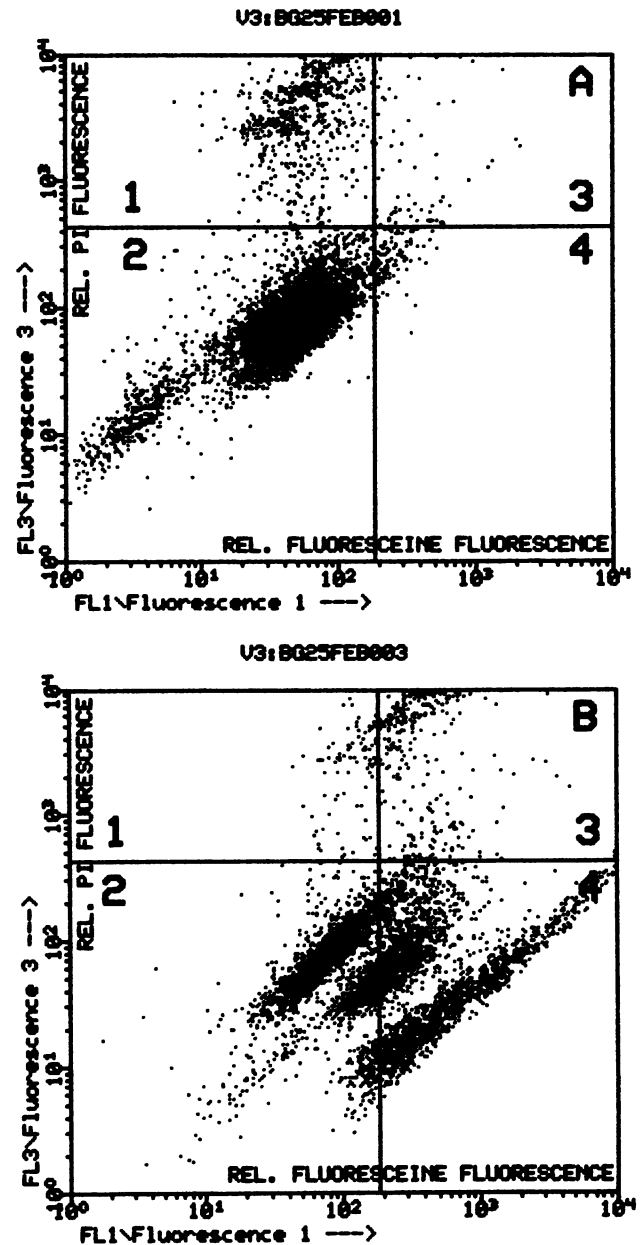


FIG. 2. FACS analysis demonstration that  $C_{12}$ -FDG enters both viable and nonviable mammalian cells. FL1, fluorescence due to fluorescein released by  $\beta$ -galactosidase ( $\beta$ -Gal) hydrolysis of  $C_{12}$ -FDG; FL3, fluorescence due to PI bound to nucleic acids in nonviable cells. The quadrants define four populations: 1,  $\beta$ -Gal-negative dead cells; 2,  $\beta$ -Gal-negative live cells; 3,  $\beta$ -Gal-positive dead cells; and 4,  $\beta$ -Gal-positive live cells. Each point represents one cell. (A) Cells incubated only with PI; (B) cells incubated with  $C_{12}$ -FDG and PI. Viable and nonviable cells displayed fluorescein signal.

guished by means of a dichroic mirror (Becton Dickinson DM560) and acquired in log mode. No gating was done; therefore, all cells displaying fluorescence higher than the FSC channel threshold were acquired. The lack of viability of PI-stained bacteria was confirmed by sorting stained cells and inoculating them in nutrient medium (1).

FDG was able to enter live *E. coli* cells, causing fluorescence

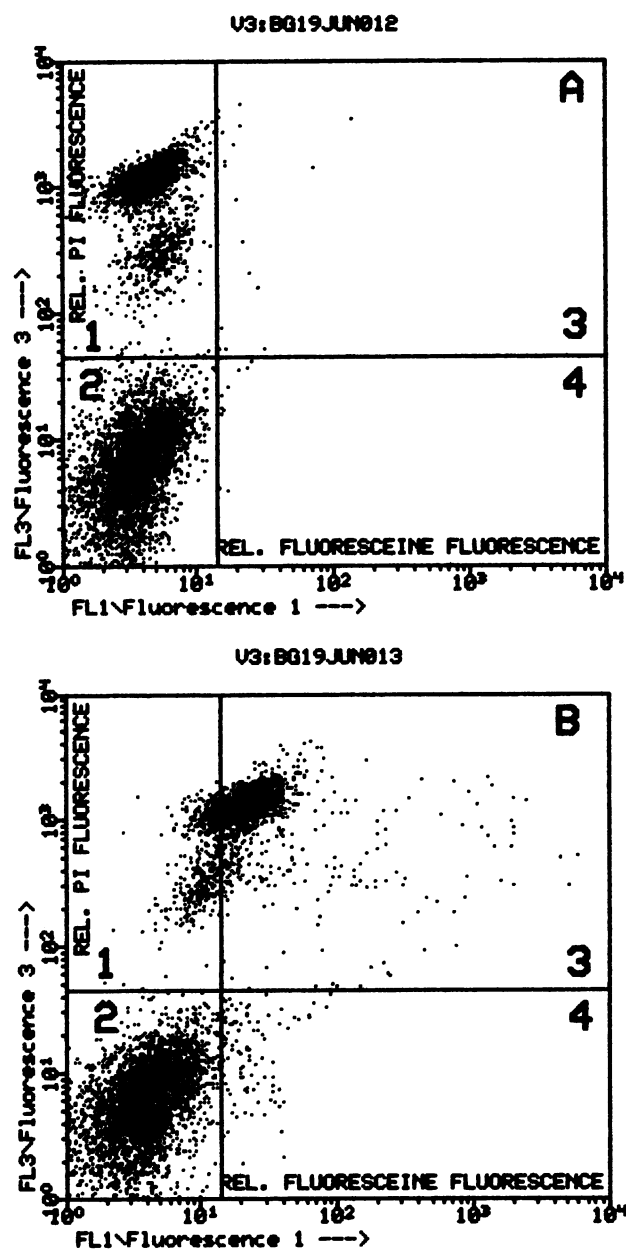


FIG. 3. FACS analysis demonstration that FDG enters only non-viable yeast cells. FL1 and FL3 and quadrants are defined in the legend to Fig. 2. (A) Yeast cells incubated only with PI; the two populations, namely, viable and nonviable cells, can be distinguished. (B) Yeast cells incubated with FDG and PI; only the nonviable population showed fluorescence due to fluorescein.

emission proportional to the amount of  $\beta$ -galactosidase (Fig. 1A). This substrate has been used in FACS determination of fluorescence (1, 9), in fluorescence microscopy studies, and in fixed bacterial cells to measure the fluorescent signal with a fluorimeter (5). In contrast,  $C_{12}$ -FDG gave only a weak fluorescent signal in these cells (Fig. 1A), which could be confirmed as corresponding to PI-positive, and therefore nonviable, cells (data not shown). Thus,  $C_{12}$ -FDG does not penetrate into *E. coli* cells unless they are damaged or dead, as indicated by PI staining. Our interpretation is that the outer

membrane of gram-negative bacterial cells functions as a barrier for the lipophilic molecule  $C_{12}$ -FDG. The lipopolysaccharide-phospholipid bilayer usually has a very low permeability towards lipophilic or hydrophobic solutes (8). Therefore, FDG is a better substrate than  $C_{12}$ -FDG for gram-negative bacteria.

**$C_{12}$ -FDG is an adequate substrate for animal cells.** Endogenous  $\beta$ -galactosidase activity was measured in a human lymphoblastoid cell line, 45.1, according to the method of Zhang et al. (15). Cells were grown in RPMI with 10% calf serum in an incubator adjusted to 37°C, 5% CO<sub>2</sub>, and a humid atmosphere. A total of 10<sup>6</sup> cells was resuspended in 1 ml of phosphate-buffered saline and divided into two aliquots to be processed as indicated for bacterial cells. No hypotonic shock or chilling of the cells at 4°C was employed, in contrast to the procedure with FDG as a substrate, resulting in a much higher viability of the cells and a shorter reaction time.

Our results clearly indicated that  $C_{12}$ -FDG was a much better substrate, giving a signal that was 100 times higher than the one obtained with FDG (Fig. 1B). This observation essentially confirmed those of Zhang et al. (15), but these authors did not carry out a direct test by PI staining to confirm that the substrate did not affect cell viability. As shown in Fig. 2, our results also demonstrated that cells active on the substrate were PI negative, and therefore viable.

**$\beta$ -Galactosidase measurement in yeast cells.** Zhang et al. (15) also reported data on the use of  $C_{12}$ -FDG for  $\beta$ -galactosidase determinations on yeast cells transformed with plasmids carrying the *lacZ* gene under the control of modified promoters that determined changes in the level of expression. In that study, activity measurements were based on the intensity of fluorescence emitted by the nonpermeabilized yeast cells, because of the action of the enzyme on the fluorogenic substrate. By a combination of phase-contrast and fluorescence microscopy, *lacZ* cells were distinguished from those that did not carry the gene. These studies did not involve cell permeabilization. Zhang et al. assumed that fluorescent cells were viable, probably on the basis of their refringence under the phase-contrast microscope, without further checking of cell viability by growth or vital staining. In our laboratory, it was shown that phase-contrast refringence is not a valid criterion for cell viability (3); therefore, it was possible that the cells displaying a fluorescent signal had lost selective permeability. In fact, we observed that cells expressing  $\beta$ -galactosidase lysed to a significant extent when grown for a long period of time in minimal medium (data not shown).

We determined  $\beta$ -galactosidase activities in *Saccharomyces cerevisiae* TD-28 (*MATa ura3 $\Delta$ 52 inos1-131 can<sup>r</sup>*) transformed with plasmid YEP357BB, which carried the *lacZ* gene under the control of the promoter of *SLT2*, a gene of *S. cerevisiae* expressed at low levels (13). In our procedure, 50 ml of minimal medium in 250-ml flasks was inoculated with a preinoculum, grown for 12 h to an optical density at 600 nm of 0.4, and grown at 28°C in an orbital shaker at 200 rpm to a final optical density at 600 nm of 1.6. Samples (50  $\mu$ l) of culture were collected by centrifugation and resuspended in 100  $\mu$ l of Z buffer (0.06 M Na<sub>2</sub>HPO<sub>4</sub>, 0.04 M NaH<sub>2</sub>PO<sub>4</sub>, 0.01 M KCl, 0.001 M MgSO<sub>4</sub>, and 0.27% mercaptoethanol). Samples were divided into two aliquots and processed for  $\beta$ -galactosidase determination as indicated for bacterial cells. When cell permeabilization was performed, before activity measurement we added 1 ml of cold 70% ethanol to cells collected by centrifugation for 5 min under strong shaking followed by centrifugation and resuspension in Z buffer. Ethanol permeabilization was checked by PI staining and FACS analysis. Other permeabilizing agents, such as the mixture toluene-methanol-Triton

X-100, isopropanol, and acetone, were also tested, with ethanol giving 100% PI-positive cells, whereas the others usually gave around 70% permeabilization. Furthermore, alterations in cell shape, as observed by phase-contrast microscopy, were minor with ethanol but more significant with other permeabilizers.

$\beta$ -Galactosidase activity determinations were carried out with both substrates  $C_{12}$ -FDG and FDG (Fig. 1C and D). The fluorescent signal in permeabilized cells was significantly stronger with  $C_{12}$ -FDG (Fig. 1D) than with FDG. Because the cells were permeabilized, these results cannot be explained in terms of better penetration by the former substrate; probably, the product of  $C_{12}$ -FDG hydrolysis is retained better within the cellular membranes. Furthermore, very few cells displayed a fluorescent signal in nonpermeabilized suspensions (Fig. 1C), suggesting that none of the substrates could penetrate viable cells. This result was confirmed by the observation that  $\beta$ -galactosidase-positive cells were also PI positive (Fig. 3) and therefore were cells which had lost selective permeability. The fluorescent signal in nonpermeabilized cells was enhanced and made more homogeneous upon permeabilization.

The sensitivity of the  $C_{12}$ -FDG method for flow cytometry determination of  $\beta$ -galactosidase activity was clearly higher than the sensitivity obtained with the chromogenic substrate ONPG. As shown here, when the expression controlled by a weak promoter such as the *SLT2* promoter was tested, the former procedure was carried out with samples of 50  $\mu$ l of culture whereas the latter required the use of approximately 10 ml of culture when the cells were permeabilized or 5 ml when cells were homogenized by mechanical breakage (data not shown).

We conclude that it is necessary to measure  $\beta$ -galactosidase activity in yeast cells by means of flow cytometry with previous permeabilization of the cells in order to obtain a clearly detectable fluorescent signal and a reproducible measurement of enzymatic activity. In our investigation,  $C_{12}$ -FDG proved to be an excellent substrate for assaying the expression of a  $\beta$ -galactosidase reporter system in yeast cells. The protocol for use of this substrate was simpler than that employed for other fluorogenic substrates, such as  $\alpha$ -naphthol- $\beta$ -D-galactopyranoside (12) and resofurin- $\beta$ -D-galactopyranoside (14), and chromogenic substrates, such as ONPG (7). Moreover,  $C_{12}$ -FDG allowed more sensitivity and resolution than the other substrates, including FDG.

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