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β-Galactosidase activity in single differentiating bacterial cells

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ABSTRACT *Myxococcus xanthus* **strains containing transcriptional fusions to** *lacZ* **were analyzed and fractionated by differences in their levels of β-galactosidase expression. The fluorogenic substrate for β-galactosidase, fluorescein di-β-galactopyranoside, was introduced into** *M. xanthus* **cells during a rapid decrease in osmolarity of the medium followed by a return to isoosmolarity. Fluorescein, the product of hydrolysis, was retained within the cells and their viability was preserved. Fluorescence increased linearly with time and was proportional to β-galactosidase activity. β-Galactosidase expression in most fusion strains, though beginning at different phases of growth or development, was distributed unimodally amongst cells. However, fusion strain Tn5 lac Ω4473 was shown to be heterogeneous at 9 hr of development. It was possible to separate physically cells that expressed β-galactosidase at a high level from other, still viable, cells with no expression. The approach described here could be adapted to study differentiation in plants and animals as well, where transcriptional fusions and fluorogenic substrates for enzyme probes of gene expression also can be used.**

As organisms develop, they change both temporally and spatially the set of genes they express. Transcriptional fusions to β-galactosidase, for which many analytical tools have been constructed, can be used to detect and to measure these changes in organisms lacking endogenous β-galactosidase activity (1, 2).

Myxococcus xanthus renders both temporal (3) and spatial (4) changes in developmental gene expression readily accessible to experiment. *M. xanthus* is a Gram-negative bacterium that builds multicellular structures, called fruiting bodies, having a species-specific size, shape, and color and within which rod cells differentiate into spherical spores (5, 6). Their developmental pattern of cellular aggregation followed by differentiation parallels the development of cellular slime molds (7) and the differentiation of cartilage and bone in vertebrates (8). The *M. xanthus* program is initiated by starvation, proceeds in a reproducible time sequence, and requires cell movement for its completion (3, 9). A library of strains has been assembled, each containing a transcriptional fusion of *lacZ* to a different developmentally regulated promoter (3, 10).

Fluorescence-activated cell sorting (FACS) has often been used to separate cells that express different antigens. Extending FACS to the analysis and sorting of bacterial cells based on their individual cellular enzyme activities posed several technical challenges. The small size of bacteria required detecting low total levels of enzyme activity. A method using fluorescence would seem to offer the needed sensitivity, and fluorogenic substrates for β-galactosidase are available. Substrate had to be introduced into the cells whereas the hydrolysis product had to be retained, and the procedures for both had to be compatible with cell viability. The linked problems of substrate penetration and product retention had been solved for FACS measurement of β-galactosidase in mammalian and insect cells. Nolan *et al.* (11) and Krasnow *et al.* (12) described FACS assays allowing fluorescence-activated cell analysis and sorting of individual viable mammalian and *Drosophila* embryo cells, respectively, based on their differential β-galactosidase activity acting on a fluorogenic substrate. This assay was shown to detect as few as five β-galactosidase molecules in a single cell (13) and thus ought to have sufficient sensitivity for bacteria.

MATERIALS AND METHODS

Preparation of Cells. Bacteria were grown as described (3, 14, 15), were harvested when they had reached 70–100 Klett photometer units ($3.5–5 \times 10^8$ cells per ml), and were suspended in TPM buffer (10 mM Tris/1 mM potassium phosphate/8 mM $MgSO_4$, pH 7.6). To obtain cells early in the process of fruiting-body development, they were grown as described above, then suspended and incubated in liquid MC7 medium at a concentration of 1000 Klett units (5×10^9 cells per ml). MC7 medium, which lacks nutrients, induces the preaggregation stages of development (15). To examine bacteria in later stages of development (2–16 hr), cells suspended in TPM buffer as above were plated on TPM agar. Bacteria were plated to measure viability on CTT agar (3).

Fluorescence-Activated Cell Analysis and Sorting of Bacteria. Bacteria were analyzed and sorted on a FACStar[Plus] (Becton Dickinson) equipped with an argon ion laser emitting at 488 nm and a nozzle with an 80-μm bore. Fluorescence and side scatter data were collected with logarithmic amplifiers, while forward scatter data were collected with a linear amplifier. *M. xanthus* cells are small (approximately 0.5 μm in diameter, 7 μm in length) and their forward scatter signal is correspondingly small. As a consequence, the forward scatter channel did not resolve cells well enough from background noise to be used as the threshold detector. Instead, side scatter was used to detect cells and to distinguish them from cell fragments and culture debris (16, 17). Even though the forward scatter signal had too much noise for a threshold, once a cell had been detected by its side scatter, the forward scatter measurement was significant and was recorded.

To measure β-galactosidase activity by fluorescence, fluorescence and scatter data were collected for at least 10,000 cells per sample. By using FACS-DESK programs (11, 18), these data were stored in list mode. To represent the entire population of 10,000 cells at each time point (in a kinetics experiment, for example), the scale value of the 80th percentile of fluorescence was measured and recorded as fluo-

Abbreviations: FDG, fluorescein di-β-galactopyranoside; ONPG, *o*-nitrophenyl β-D-galactopyranoside; PETG, phenylethyl β-D-thiogalactopyranoside; FACS, fluorescence-activated cell sorting.

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rescence units. Percentile is the appropriate statistic because the logarithm of fluorescence was being measured (18). The 80th percentile was used (rather than the 50th) because at low expression levels many expressing cells are poorly resolved from nonexpressing cells. In addition, when the absolute fluorescence is so low that only a few photons are detected (below about 1 fluorescence unit on the logarithmic scale, as in Figs. 1, 2, and 5), output from the photomultiplier tubes becomes unreliable. Use of the 80th percentile provides sufficient fluorescence to overcome these difficulties. As long as a population of cells is hydrolyzing substrate linearly with time, the 80th percentile should provide quantitative measurements of relative enzyme activity just as well as the mean (50th percentile).

Standard Fluorescein Di-β-Galactopyranoside (FDG) Assay Procedure. Exposure to hypertonic conditions followed by hypotonic treatment loaded substrate FDG (or product fluorescein) into *M. xanthus* cells. For loading, 50 μl of cells in TPM was first mixed with 50 μl of 0.2 M NaCl, agitated for 10 sec, and then placed at 4°C for 2 min to allow the cells to equilibrate (at 0.1 M NaCl). Two microliters of the equilibrated mixture was then added to 98 μl of an aqueous solution of 1 mM FDG and 20 μM phenylethyl β-D-thiogalactopyranoside (PETG) at 4°C. This 50-fold dilution rapidly subjected cells to hypotonicity, at which they were held for 1 min. Finally, 1 ml of 4°C TPM (containing 20 μM PETG) was added, restoring isotonicity to maintain cell viability. Fluorescence-activated cell analysis (and sorting if desired) was conducted immediately on the isotonic mixture. PETG was included in the standard assay mixture to slow hydrolysis by β-galactosidase so that the reaction became enzyme-limited, as described below.

RESULTS AND DISCUSSION

Detection of β-Galactosidase Activity in Single Cells. A culture of a Lac$^+$ strain of *M. xanthus*, DK7501, was sorted by FACS after FDG was added to the culture. β-Galactosidase activity encoded by Tn*5 lac* in *M. xanthus* DK7501 cells is confined to the cytoplasm (3, 19). FDG was introduced into the cells by a modification of the osmotic shift procedure of Nolan *et al.* (11) and Krasnow *et al.* (12). Modification was required because *M. xanthus* grows optimally at low (10 mM) salt and fails to grow at 150 mM NaCl (20). Nevertheless, little FDG was hydrolyzed unless the bacteria were first equilibrated with 50 mM or 100 mM NaCl, rapidly diluted to ≈1 mM, and then restored to 10 mM. This treatment generated a population of strongly fluorescent cells (Fig. 1). Initial exposure to 75 mM (data not shown) had the same effect. Comparison of the 50 and 100 mM panels of Fig. 1 with each other (or with 75 mM) illustrates the reproducibility of the fluorescence intensity and particle size distributions (measured by forward scattering shown on the ordinate in the lower panels) of cells subjected to the osmotic changes employed for FDG loading. Initial salt concentrations higher than 100 mM were tested, but these samples loaded less FDG.

Cell-to-cell variation in uptake of FDG would limit the precision of β-galactosidase activity measurements based on fluorescence intensity. Rather than study the uptake of the nonfluorescent FDG, fluorescein, whose chemical properties resemble those of FDG, was used instead. Cells were loaded with 10 μM fluorescein by the same procedure as for FDG, held for 45 min, and then sorted by FACS (Fig. 2). A single narrow peak was obtained, showing uptake and retention for at least 45 min, a time sufficient for a typical FACS analysis. The ratio of peak half-width to mean fluorescence intensity was taken as a normalized measure of variation. For the distribution shown in Fig. 2, this ratio was 0.5 (half-width of 5 for a mean of 10), the same as the ratio for the autofluorescence variation of a population of Lac$^-$ (DK1622) cells which showed a half-width of 0.5 for a mean of 1 (data not shown). The autofluorescence of a Lac$^-$ strain is about 1 fluorescence unit; fluorescent pigments have been reported in *M. xanthus* (21). This similarity between uptake variation and autofluorescence variation implies that the observed uptake variation can be explained by the variation in length among asynchronous cells in different cell-cycle stages (22). It follows that variation in fluorescein uptake is insignificant relative to the variation in cell length, and hence cell volume. To the extent that uptake of FDG resembles that of fluorescein, substrate uptake was concluded to be sufficiently uniform from cell to cell to provide reliable fluorescence measurements of β-galactosidase activity in individual cells. Statistical accuracy was obtained by examining 10,000 cells per sample.

A mixture of equal numbers of Lac$^-$ (DK1622) and Lac$^+$ (DK7501) cells was well resolved by FACS into two approximately equal populations of strongly and weakly fluorescent cells. The strongly fluorescent peak in that output (not shown) had the same intensity as FDG-loaded DK7501, about 50 fluorescence units (Fig. 1). The weakly fluorescent peak

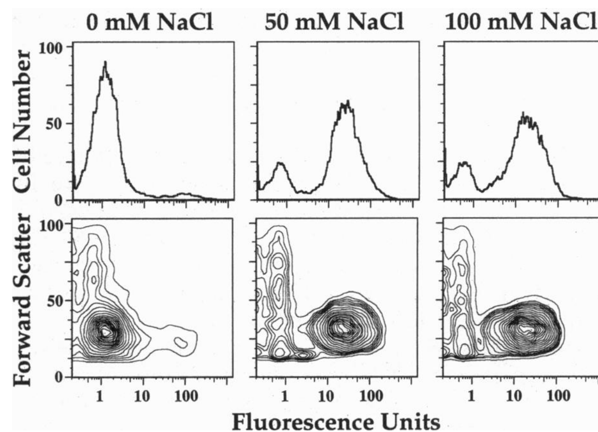

FIG. 1. FDG hydrolysis depends on exposure of Lac$^+$ *M. xanthus* cells to supernormal concentrations of NaCl. (*Upper*) Logarithm of fluorescence intensity (in arbitrary units) versus cell number. (*Lower*) Contour plots of fluorescence intensity versus forward scatter, which measures cell size and shape. Contours represent the relative numbers of cells; 5% of the population lies between any adjacent pair of contour lines (15). After the cells were equilibrated at 32°C in TPM plus 0, 50, or 100 mM NaCl for 2 min, the cell suspension was rapidly diluted 50-fold into a solution of 1 mM FDG at 32°C. After 1 min, this mixture was diluted 20-fold into TPM at 4°C and analyzed by FACS.

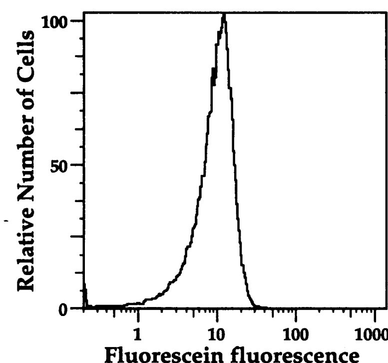

FIG. 2. Penetration and retention of fluorescein. Cells equilibrated at 32°C in TPM plus 0.1 M NaCl were diluted 50-fold into 10 μM fluorescein. After 15 min at 32°C and 30 min at 4°C, the mixture was immediately diluted 20-fold into TPM at 4°C and analyzed by FACS.

had the (autofluorescence) intensity of strain DK1622 run alone or DK7501 before FDG had been loaded. In this 1:1 mixture there was no indication of fluorescein exchange between cells, confirming retention of the FDG hydrolysis product by the cells.

FDG loading and FACS handling were found to have little effect on cell viability. To assess their capacity to form colonies, treated and sorted *M. xanthus* cells were plated on CTT agar and incubated for 6 days (3). Survival was 80 ± 20% (mean ± SD for 5 experiments) of the objects recognized as cells by their light-scattering intensities, using the threshold described in *Materials and Methods*.

Quantitation of β-Galactosidase Activity of Single Cells. For kinetic experiments, data were collected from 10^4 cells, and the number of fluorescence units was calculated for each time point as described in *Materials and Methods*. In initial experiments, the fluorescence of FDG-loaded Lac$^+$ cells increased rapidly for only a few seconds before leveling off. It thus appeared that all the intracellular FDG was hydrolyzed within seconds, causing the reaction to be substrate-, not enzyme-limited. This was not unexpected, because a limited amount of FDG would be loaded by an osmotic pulse of 1 min, considering that an *M. xanthus* cell has a volume of about 5×10^{-12} cm^3. To achieve the desired enzyme-limited conditions, catalysis was slowed by addition of the competitive inhibitor PETG (13, 23) and by performing the reaction at 4°C. A K_i of 2.5 μM for PETG had been determined in other experiments (M.R., unpublished work), and an appropriate PETG concentration was found by adding either 20, 100, or 1000 μM PETG during (1 mM) FDG loading. At 20 and 100 μM PETG, a significant level of fluorescence developed at an approximately constant rate; to maintain sensitivity, the concentration of 20 μM PETG was adopted. Fig. 3 shows the kinetics of appearance of fluorescence, using 20 μM PETG, for the three *lacZ* fusion strains DK7501, DK7545, and DK7527; samples of these three strains were taken every half minute between 1.75 and 10 min of enzymatic reaction. Fluorescence developed at a constant rate for 8 min for each of the three strains, indicating that an enzyme-limited condition had been achieved.

The approximately linear fluorescence increase of Fig. 3 implies that, under these conditions, the rate of fluorescence accumulation should be proportional to the intracellular (inhibited) β-galactosidase activity. Since β-galactosidase is stable in *M. xanthus* cells (10), the activity is tentatively assumed to be proportional to the level of enzyme protein. To

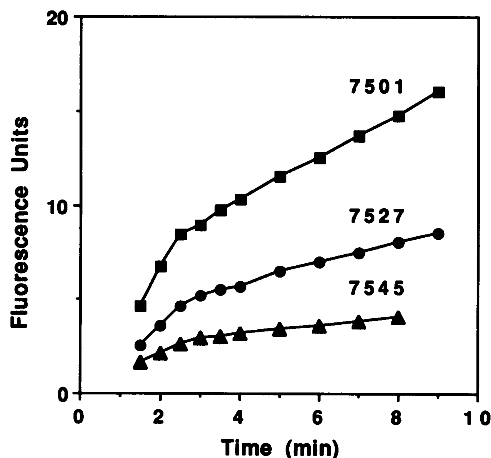

FIG. 3. Effect of PETG on the accumulation of fluorescence in *lacZ* fusion strains DK7501, DK7527, and DK7545. FDG (1 mM) and PETG (20 μM) were loaded at 4°C, and the 80th percentile of fluorescence of 10^4 cells was measured as a function of hydrolysis time.

test whether the intracellular rate of fluorescence accumulation is an accurate measure of β-galactosidase activity, the rate of FDG hydrolysis measured by FACS was compared with *o*-nitrophenyl β-D-galactopyranoside (ONPG) hydrolysis in a sonic extract made from a mass culture suspension of the same strain under the same conditions. Such comparisons were made with the three strains and experiments of Fig. 3; the relative rates of fluorescence accumulation were DK7545 < DK7527 < DK7501. This is the same rank order as the rates of ONPG hydrolysis for sonic extracts of the same strains, which were 52, 83, and 231 Miller units, respectively.

Gene Expression in Differentiating Cells. Changes in β-galactosidase activity were measured in cells that were beginning their development and differentiation. DK4521 carries a *lacZ* transcriptional fusion (Ω4521) that expresses very little β-galactosidase during growth, then begins high-level expression of the enzyme a few hours after development is initiated by nutrient limitation (3, 15). In this strain, *lacZ* is fused to an early, development-specific promoter. Fig. 4*A* shows the 80th percentile fluorescence of the population for cells that had been developing for 1, 2, 3, or 5 hr as a function of the enzymatic reaction time. In each population the fluorescence increased linearly with time. The rates of fluorescence increase for the first 3 min of reaction measured from these curves are shown in Fig. 4*B*. These fluorescence increases track the values of β-galactosidase activity measured by ONPG hydrolysis on the mass cultures before FACS (Fig. 4*C*). Fluorescence increases also detected the first cells to produce β-galactosidase after development was induced by starvation. Fig. 4*D* quantifies the correlation between fluorescence from populations of single cells and ONPG hydrolysis from corresponding mass cultures. Similar results were also obtained in five independent experiments. Also plotted in Fig. 4*D*, as open circles, are the corresponding data for strains DK7501, DK7545, and DK7527. The regression line in Fig. 4*D* does not pass through the origin because there is a small amount of *o*-nitrophenol production (from ONPG) in cell extracts present before development starts (Fig. 4*C*; refs. 3 and 15). It appears that fluorescence has a lower background than ONPG hydrolysis; compare the 1- and 1.5-hr samples in Fig. 4 *B* and *C*. Since fluorescence is given in arbitrary units, the β-galactosidase activities are relative values. Nevertheless, the relative specific activities for all four strains are proportional to the (ONPG) β-galactosidase specific activities. The correlation between β-galactosidase activity levels in FACS with activity levels determined by colorimetric (ONPG) assay demonstrates that FACS-based analysis is a valid and concordant supplement to colorimetric assays on the mass cultures.

β-Galactosidase activity was also measured in fusion strains which—like fruiting-body development itself—require, in addition to starvation of cells at a high density, an agar (or other) surface for enzyme expression. Cells in the course of fruiting-body development were scraped from the agar surface, suspended in TPM buffer, loaded with FDG, and analyzed by FACS. The rate of fluorescence accumulation was measured in three strains that have *lacZ* fusions to different developmentally regulated promoters: DK4473 has a promoter that becomes active before 8 hr; DK4499 has a promoter that begins to be active at 7 hr; and DK4506 has one that becomes active at 14 hr (3). Again the rate of fluorescence increase correlated with ONPG hydrolysis, for all three strains. In addition, the relative time at which a discrete population of highly fluorescent cells first appeared in the FACS assay corresponded to the time at which β-galactosidase initially increased above basal levels as assayed by ONPG hydrolysis. These results further support the reliability of the FACS measurements as a concordant supplement to the ONPG hydrolysis assay even for cells developing on agar. Significantly, the cell-to-cell distribution of β-galacto-

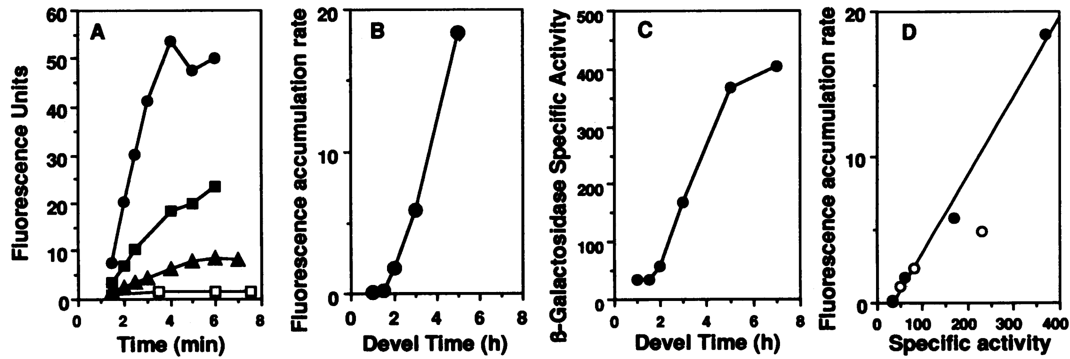

FIG. 4. β-Galactosidase activity in DK4521 cells during the early stages of development in liquid culture. (*A*) Fluorescence from FDG as a function of hydrolysis time. Each curve represents measurements made on cells harvested at a particular time after starvation had initiated development: □, 1 hr; ▲, 2 hr; ■, 3 hr; ●, 5 hr. (*B*) Rate of fluorescence accumulation for the first 3 min of reaction (slopes from *A*) as a function of the time of development. (*C*) Specific activities of β-galactosidase (nmol of *o*-nitrophenol per minute per milligram of protein) in the same cells as determined by ONPG hydrolysis. (*D*) Plot of β-galactosidase activity measured by FDG hydrolysis in single cells (*y* axis; values from *B*) against ONPG hydrolysis from mass cultures (*x* axis) for corresponding developmental times. ●, Values from *C*; ○, corresponding activity measurements for strains DK7501, DK7527, and DK7545, taken from Fig. 3.

sidase activity was unimodal in the developing strains DK4521, DK4499, and DK4506, implying that the enzyme was accumulating in roughly similar fashion in all the cells of each of these strains (data not shown).

However, a qualitatively different result was obtained for DK4473. The Tn*5 lac* insertion in DK4473 reports the activity of a gene, *devR*, which behaves like an essential regulatory switch in *Myxococcus* fruiting-body development (L. Thony-Meyer and D.K., unpublished work). FACS profiles for DK4473 at developmental time points of 0, 2, 4, 6, 8, and 10 hr are presented in Fig. 5 *Left*. Although some β-galactosidase expression (above background) is evident in the 0-hr sample, at 2 hr a distinct population of expressing cells is evident as a leading peak (see 2-hr *Inset*). At 4 hr the leading peak has increased somewhat in size. There is little change in the apparent number of cells in the leading peak thereafter, but its mode continues to shift to higher fluorescence intensities, at least up to 8 hr. These profiles suggest the existence of a subpopulation of cells that express β-galactosidase distinct from a second subpopulation of nonexpressing cells.

If the difference between these two subpopulations were due to differences in FDG uptake, that difference should also have been evident in the 2-, 4-, and 6-hr cultures of DK4499, DK4506, and DK4521, but those populations were unimodal. To investigate the existence of a distinct subpopulation of cells expressing high enzyme levels, the peak of expressing cells in the DK4473 sample taken at 9 hr of development (highlighted in Fig. 5 *Right*) was isolated by FACS and then reanalyzed. Fig. 5 *Right* shows the FACS fluorescence distributions before and after sorting. After sorting, the cells retained their high activity state. No cells expressing low levels were detected in the sorted subpopulation. These results support the view that the two cell populations, one expressing a high level of β-galactosidase and the other virtually none, correspond to two different regulatory states.

The discrimination of FACS is potentially more refined than a selection based (for example) on drug resistance,

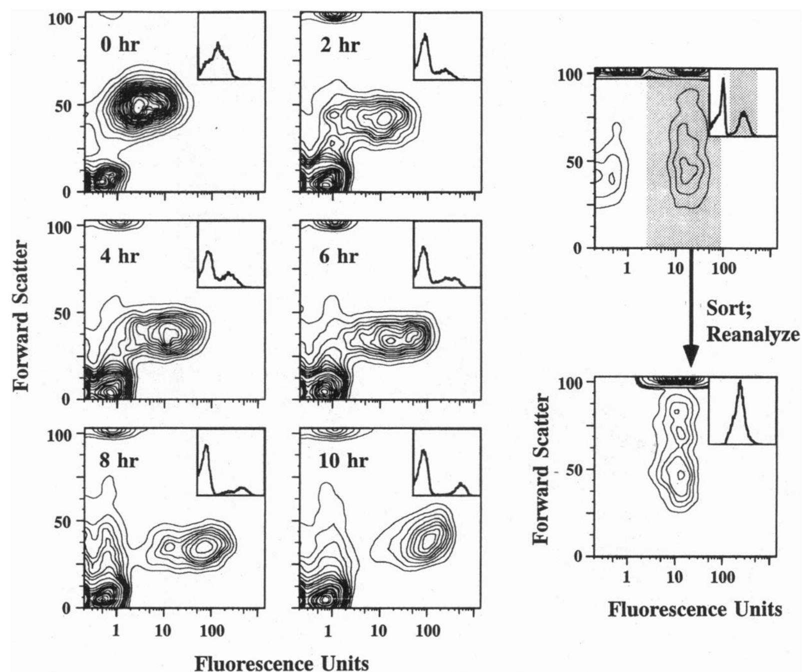

FIG. 5. Cell-to-cell heterogeneity of β-galactosidase activity in developing DK4473. (*Left*) FACS distributions of cells at 0, 2, 4, 6, 8, and 10 hr of development are shown as contour plots of forward scatter versus fluorescence intensity. *Insets* show the distribution of fluorescence intensity versus the number of cells. (*Right*) The entire 9-hr population compared with the sorted population of β-galactosidase-positive cells.

because sorting can be triggered by any window of enzyme activity as measured by fluorescence. Thus it can allow a fast, efficient enrichment and/or screening of genotypically identical cells in different (developmental) regulatory states.

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