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Development of a Sensitive Chemiluminometric Assay for the Detection of β-Galactosidase in Permeabilized Coliform Bacteria and Comparison with Fluorometry and Colorimetry

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We developed a chemiluminometric assay of β-galactosidase in coliform bacteria, using a phenylgalactose-substituted 1,2-dioxetane derivative as a substrate. Permeabilization of cells is required to ensure the efficient cellular uptake of this compound. By this method, one coliform seeded in 100 ml of sterile water can be detected after a 6- to 9-h propagation phase followed by a 45-min enzyme assay in the presence of polymyxin B. Compared with fluorometry and colorimetry, chemiluminometry afforded 4- and 1,000-fold increases in sensitivity and 1- and 6-h increases in the speed of detection, respectively.

The assay of bacterial β-galactosidase is of interest in clinical, environmental, food, and molecular microbiology. The many batteries of biochemical tests for the identification of bacterial isolates include the ONPG (*o*-nitrophenyl-β-D-galactopyranoside) test to demonstrate this enzyme (18). In drinking water microbiology, several commercial presence-absence broth tests for coliforms, e.g., Colilert, Colisure, and Colifast, as well as agar counting methods, e.g., Chromagar, Chromocult, Microsure *Escherichia coli*, and some confirmatory tests, e.g., Colifirm, rely on the direct detection of β-galactosidase. Conversely, the inhibition of its biosynthesis and activity by toxicants forms the basis of a microbiotest for toxicity screening of environmental samples (7, 17, 19, 25). Rapid enzymatic tests are also emerging in food microbiology (12), but so far the assay of β-galactosidase has found limited application, except for identification purposes. Finally, in studies of the regulation of gene transcription in both prokaryotic and eukaryotic cells, β-galactosidase is monitored as the expression product of the *lacZ* reporter gene (9, 16, 20, 21).

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Chromogenic substrates, e.g., ONPG and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), as well as fluorogenic ones, e.g., MU-Gal (4-methylumbelliferyl-β-D-galactopyranoside), are commonly used to assay β-galactosidase in bacteria. The former class of compounds requires visual or spectrophotometric detection of a colored reaction product (ONP or indigo) and, hence, affords limited sensitivity. This limitation is of no concern in a confirmatory ONPG test of bacterial isolates because the abundance of available cells permits a rapid cleavage of the substrate. However, when a small number of bacteria have to be detected in a sample, e.g., coliforms in drinking water by using the minerals modified ONPG-MUG (methylumbelliferyl-β-glucuronide) test (8), approximately 24 h is needed to allow sufficient bacterial propagation and enzymatic hydrolysis before the yellow color of the ONP becomes visually detectable (2). Spectrophotometric end point detection may reduce this incubation period to 16 to 18 h (28). Application for fluorogenic MU-Gal has been found in a method to detect fecal coliforms in water (5) and in a toxicity test with *E. coli* (19). The use of another, supposedly superior fluorogenic substrate, fluorescein-di-galactopyranoside, in bacteriology has been restricted to the detection of β-galactosidase in single cells by epifluorescence microscopy or flow cytometry (1, 20, 23, 26).

A further substantial gain in sensitivity would theoretically result from the use of a chemiluminogenic substrate for β-galactosidase. 3-{4-Methoxyspiro[1,2-dioxetane-3,2′-tricyclo(3.3.1.1^{3,7})decan]-yl}phenyl-β-D-galactopyranoside (AMPGD), a phenylgalactose-substituted 1,2-dioxetane compound described by Bronstein et al. (6) and Schaap et al. (27), has been used in connection with gene reporter assays in eukaryotic cells (3, 16). This substrate reportedly affords 500 and 500,000 times higher sensitivity than MU-Gal and ONPG, respectively, yielding a detection limit of as little as 2 fg of β-galactosidase (16). This quantity corresponds to approximately 1 induced or 10^3 to 10^4 uninduced *E. coli* cells (15). Hence, AMPGD would be an extremely powerful substrate for the sensitive assay of bacterial β-galactosidase in samples containing small numbers of cells.

The purpose of this study was to evaluate the performance of a chemiluminometric assay of β-galactosidase in selected strains of coliform bacteria, in order to test the potential of this approach for possible application to drinking water or foods.

Bacterial strains. *E. coli* 2, *Enterobacter cloacae* 33, and *Citrobacter freundii* 27 were isolated from natural well waters. Identification was done by using the API 20E test (bioMérieux, Marcy l'Etoile, France). *Klebsiella pneumoniae* 36 and 17 strains of *Aeromonas hydrophila*, *Aeromonas sobriae*, and *Aeromonas caviae* were kindly donated by D. van der Kooij (Kiwa, Nieuwegein, The Netherlands). Daily subcultures in tryptic soy broth (TSB) without dextrose (Difco Laboratories, Detroit, Mich.) but supplemented with 0.001% isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, St. Louis, Mo.) (13) were made from stock cultures on tryptic soy agar (TSA; Oxoid, Ltd., Basingstoke, United Kingdom) and grown overnight at 35°C. IPTG was included to induce β-galactosidase (13), which was essential to achieve optimal sensitivity. When *E. coli* was grown overnight in the absence of IPTG, a 100- to 1,000-fold-higher limit of detectability was obtained.

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TABLE 1. Effects of different membrane permeabilizers[a] on chemiluminometric detection (S/N ratio) of serial dilutions of *E. coli* 2 in low-nutrient medium

Sample (CFU/ml)	S/N ratio with[a]:							
	Polymyxin	Polymyxin + lysozyme	6-Thiatetracycline	Colistin	PMBN	Toluene	Cetrimide	No permeabilizer
10^1	1.0	0.9	0.9	0.8	0.8	0.9	0.6	0.8
10^2	1.6	1.1	0.8	0.9	0.8	1.0	0.6	0.9
10^3	5.2	4.1	0.9	2.0	1.4	2.9	0.6	0.9
10^4	40.0	32.0	1.1	12.6	6.4	20.5	0.6	1.3
10^5	395	298	6.2	121	57.0	213	2.1	6.1
10^6	3,764	3,025	62.0	1,237	658	2,455	17.0	61.0
10^7	11,835	10,508	416	4,511	4,924	10,363	262	540

[a] The concentrations (per milliliter) in water were 100 μg of polymyxin B sulfate (Sigma), 100 and 250 μg of polymyxin B sulfate and lysozyme, respectively (a mixture) (reference substance; International Pharmaceutical Federation (FIP), Laboratory of Biochemistry and Physical Pharmacy, University of Ghent, Ghent, Belgium), 150 μg of 6-thiatetracycline (E. Merck AG), 100 μg of colistin, 300 μg of PMBN (polymyxin B nonapeptide; Boehringer, Mannheim, Germany), and 10 mg of cetrimide.

Medium and growth conditions. A low-nutrient growth medium, purposely developed for the method to minimize the effects of the luminescent background and light quenching (31) contained (per liter) tryptone (Oxoid, Ltd.), 1 g; IPTG, 0.01 g; sodium dodecyl sulfate (SDS; E. Merck AG, Darmstadt, Germany), 0.05 g; and NaCl, 5 g, in 0.05 M potassium phosphate buffer (pH 7.3). The addition of SDS and sodium chloride slightly enhanced the signal-to-background ratio, the former probably by activating the enzyme (5).

Serial dilutions of overnight cultures were made in the low-nutrient medium. In case a propagation phase preceded the actual enzyme assay, preincubation of the samples was carried out at 35°C for 4 to 6 h.

Enzyme assay. In the standard two-stage procedure, the growth step was separate from the enzyme assay to permit the use of a membrane permeabilizer in the latter. To this end, 400-μl aliquots were taken from the growth mixture after preincubation, combined with stock solutions (50 μl of each) of polymyxin B sulfate (concentration, 100 μg/ml; Sigma) and the enzyme substrate, and incubated at 35°C for 45 min. When toluene was used, 2 ml of the growth mixture was mixed with 0.1 ml of toluene and incubated for 10 min at 35°C, prior to the actual enzyme assay. Substrates tested included chemiluminogenic Cl-AMPGD (a chloro derivative of AMPGD, available as Galacton; Tropix, Bedford, Mass.), diluted in 0.05 M sodium phosphate buffer (pH 6.5) at a concentration of 275 μM; fluorogenic MU-Gal (Melford Laboratories Ltd., Chelsworth, United Kingdom) dissolved in dimethyl sulfoxide (DMSO)–0.1 M sodium phosphate buffer (pH 7.3) (10:90 [vol/vol]) at a concentration of 0.5 mg/ml; and chromogenic ONPG (Melford) dissolved in 0.1 M sodium phosphate buffer (pH 7.3) at a concentration of 15 mg/ml. In addition, each stock solution contained 10 mM $MgCl_2 \cdot 6H_2O$ as a cofactor for the enzyme.

For comparative purposes, one-stage procedures were also tested in which the enzyme substrate was incorporated in the low-nutrient medium from the start at a concentration of 27.5 μM Cl-AMPGD, 0.05 mg of MU-Gal per ml, or 1.5 mg of ONPG per ml. MU-Gal was added from a stock solution containing 4.5 mg/ml in DMSO, whereas the other compounds were added directly to the medium. To assay the enzyme activity, 500-μl aliquots of the growth mixtures were subjected directly to the chemiluminometric, fluorometric, or colorimetric measurement.

Instrumental measurements. After the enzymatic cleavage of Cl-AMPGD, chemiluminescent light emission was triggered by adding 100 μl of the alkaline enhancer (Emerald [Tropix], 1.6 μg/ml in 0.5 M aqueous piperidine) to the assay mixture, by using the pump which is part of the luminometer (AutoLumat LB 953; EG&G Berthold, Bad Wildbad, Germany). Chemiluminescence was measured over a 10-s period with a delay time of 2 s. Fluorescence was measured (λ excitation, 365 nm; λ emission, 465 nm) with a model SFM25 fluorometer (Kontron AG, Zürich, Switzerland) after alkalinization with 50 μl of 10 M NaOH. In the colorimetric assay of β-galactosidase, absorbance was determined in a Uvikon 940 spectrophotometer (Kontron AG) at 410 nm after the addition of 250 μl of 1 M sodium carbonate.

In chemiluminometry, the responses obtained for the samples (signal, S) and the blanks (background, N), expressed in relative light units (RLUs), were divided to yield signal-to-background (S/N) ratios, according to the method of Bronstein et al. (6). For fluorometry and colorimetry, the response of the blank (N), expressed in relative fluorescence units (RFUs) or absorbance units (AUs), respectively, was subtracted from that of the sample (S), to give the S-minus-N value.

In fluorometry and colorimetry the detection threshold, i.e., the minimally required difference between S and N that indicates a positive response, was estimated by determining the fluctuation of the signal for a blank sample and multiplying this value arbitrarily by a factor of 5. This resulted in a threshold S-minus-N value of 40 ± 14 RFU ($n = 7$) and 0.05 ± 0.009 AU ($n = 6$), respectively.

Likewise, in chemiluminometry the detection threshold, i.e., the minimally required S/N ratio that indicates a positive response, was estimated by adding the fluctuation of the signal for a blank sample, multiplied by 5, to the average blank sample value. This figure was then divided by the average blank value itself to yield the threshold S/N ratio. Thus, a value of 2.1 ± 0.5 ($n = 7$) was set as the threshold S/N ratio. For example, with a signal fluctuation value of 633 and an average blank sample value of 3,085, the threshold S/N ratio would be 2.

Permeability of coliform bacterial cells for Cl-AMPGD. When in a preliminary experiment β-galactosidase was assayed by chemiluminometry in serial dilutions of *E. coli* 2 overnight cultures, the detection limit (10^4 to 10^5 CFU/ml) was several orders of magnitude higher than that predicted on the basis of the results of Jain and Magrath's study (16). The major factor accounting for this discrepancy was the relative impermeability of the bacteria for the substrate or, alternatively, the partial intracellular retention of the enzymatic cleavage product. It is well-known that the uptake of other substrates for β-galactosidase, notably ONPG by *E. coli*, can be substantially improved by pretreating the cells with toluene (14). As shown in Table 1,

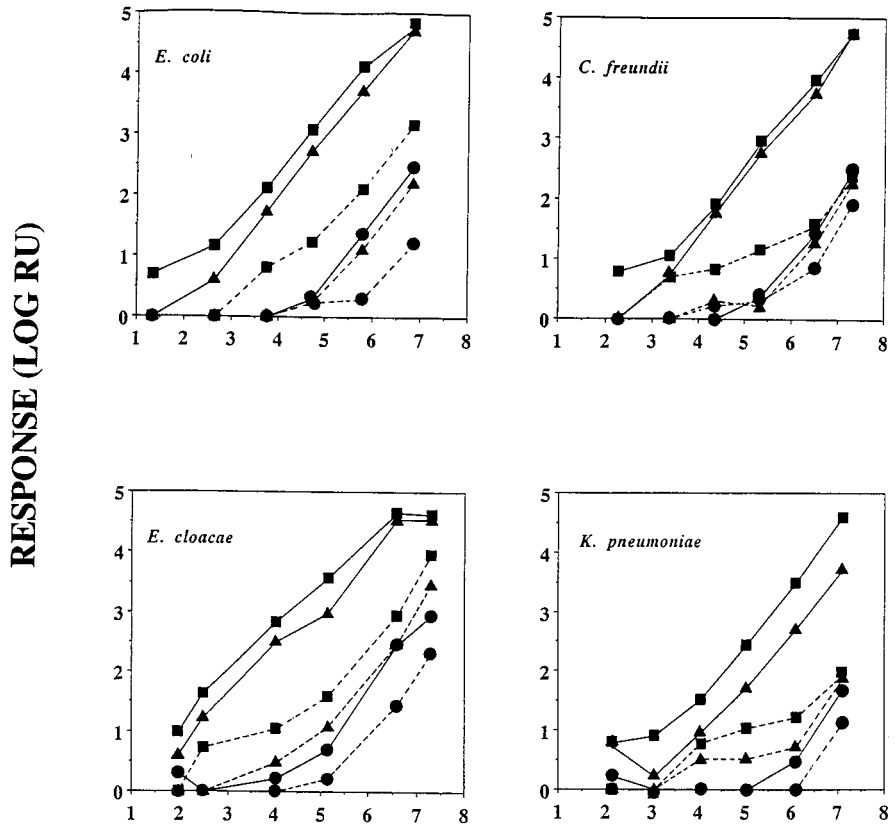

FIG. 1. Detection of *E. coli*, *C. freundii*, *E. cloacae*, and *K. pneumoniae* after dilution and preincubation in the low-nutrient growth medium by one-step (dotted lines) and the two-step (solid lines) procedures with chemiluminometric (■), fluorometric (▲), and colorimetric (●) detection. Responses are expressed as relative units (RU; 10 RU, the detection threshold for chemiluminometry, fluorometry, or colorimetry, i.e., an S/N ratio of 2 or an S-minus-N value of 40 or 0.05, respectively). Each point represents the mean of three individual experiments. The average variabilities of the responses (expressed as the means of the coefficients of variation obtained for the six datum points and the four strains) were 40.0% ± 26.0% (two-step chemiluminometry), 50.2% ± 39.9% (two-step fluorometry), 29.5% ± 33.8% (two-step colorimetry), 35.6% ± 31.1% (one-step chemiluminometry), 37.9% ± 36.3% (one-step fluorometry), and 24.6% ± 33.8% (one-step colorimetry). The mean of the coefficient of variation for the corresponding number of cells was 47.3% ± 25.9%.

which illustrates the effects of different membrane permeabilizers on the chemiluminometric response, this chemical exerted a similar effect toward Cl-AMPGD, as it decreased the detection limit of *E. coli* by a factor of 100 (10^2 to 10^3 CFU/ml). However, practical considerations limit the routine application of toluene, including its toxicity and its caustic action on the plastic cuvettes of the luminometer. In addition, toluene reportedly inhibits β-galactosidase to some extent (24). Polymyxin B, at a concentration of 10 μg/ml, does not have those drawbacks and proved superior to toluene, yielding approximately twofold-higher S/N ratios. This antibiotic disrupts the outer and cytoplasmic membranes of gram-negative bacteria (10) but, according to Teuber (29), does not release β-galactosidase to a significant extent. In our study, after 45 min of contact between *E. coli* and polymyxin B, about 25% of the enzymatic activity was extracellular, as evidenced by the chemiluminescence response obtained after removal of the cells in a Millex GV 20 unit (Millipore, Bedford, Mass.). The combined action of polymyxin B and lysozyme released a considerably larger amount of enzyme (80%), but this proved of no further

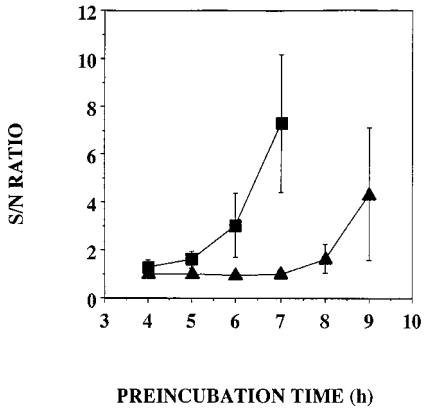

FIG. 2. Sensitivity of chemiluminometric assay for detection of *E. coli* (■) and *K. pneumoniae* (▲) seeded in sterile distilled water (1 CFU/100 ml), after concentration and preincubation in the low-nutrient growth medium. Each point represents the mean ± standard deviation for three individual experiments.

benefit to the chemiluminescence S/N ratio, suggesting that there was free access of Cl-AMPGD to the mostly intracellular enzyme after permeabilization with polymyxin B only. The reproducibility of polymyxin B treatment was satisfactory (the coefficient of variation for the response obtained by repeatedly assaying a 10^{-5} dilution of an *E. coli* overnight culture was 15% [$n = 12$]). Why colistin (polymyxin E), a close structural analog of polymyxin B with a similar mechanism of action, was less effective when applied in the same concentration remains unclear. It cannot be excluded that 6-thiatetracycline (22) and cetrimide also permeabilized the cells, but these compounds may have interfered somehow with the mechanisms of chemiluminescent light emission, so that their effect could have been obscured. The results obtained with polymyxin B nonapeptide, a specific destabilizer of the outer membrane that leaves the cytoplasmic membrane intact (30), suggests that mainly the latter forms the barrier to the transport of Cl-AMPGD into the cell.

Comparison of sensitivities of chemiluminometry, fluorometry, and colorimetry in one-stage and two-stage methods. The superiority of chemiluminometry over fluorometry and colorimetry for the sensitive detection of β-galactosidase in coliforms, in both a one-stage and a two-stage method with polymyxin B, is illustrated in Fig. 1. Compared with fluorometry and colorimetry, chemiluminometry allowed the detection of a smaller number of cells, the increase in sensitivity being 4- and 1,000-fold, respectively, in the two-stage procedure and 20- and 100-fold, respectively, in the one-stage procedure.

The addition of polymyxin B enhanced the sensitivity by factors of 60 (chemiluminometry), 400 (fluorometry), and 6 (colorimetry) with reference to the corresponding one-stage procedures.

Furthermore, blank sample values in the one-stage procedures significantly increased in the course of prolonged incubation, possibly because of autohydrolysis of the substrates, unlike the results with the two-stage procedures, where the contact time was only 45 min. This effect was most evident with Cl-AMPGD, yielding blank sample RLU values after 9 to 10 h of incubation that were as much as threefold higher than those in the two-stage procedure.

Chemiluminometric detection of coliforms in water samples. One potential application of the chemiluminometric assay of β-galactosidase is the detection of coliforms in water. As part of a preliminary assessment of the performance of the method for this purpose, samples of sterile distilled water were seeded with *E. coli* and *K. pneumoniae* and subjected to a concentration step prior to preincubation and enzyme assay. Seeded sterile distilled water (100 ml) was filtered through a 47-mm-diameter (0.22-μm-pore size) Durapore membrane filter (Millipore). Simultaneously, a blank sample, i.e., sterile distilled water, was processed in the same way. Of several types of membrane filters, polyvinylidene difluoride consistently yielded the highest response. After filtration, the membrane filters were placed in screw-cap sterile 100-ml cups (Gosselin, Hazebrouck, France). A 5-ml portion of the low-nutrient medium was added to each cup. The cups were vortexed for 2 min; subjected to sonication (Branson Ultrasonics Corp., Danbury, Conn.) for 10 min, which slightly enhanced the S/N ratio; and preincubated for variable times at 35°C in an incubator. IPTG was essential to optimally induce β-galactosidase (13). When *E. coli* 2 was preincubated for 4 h in low-nutrient medium without IPTG, the number of cells that could be detected was 100 times higher than that with preincubation in its presence.

As shown in Fig. 2, 1 CFU of each species per 100 ml could be detected after a preincubation time of 6 to 9 h. To examine the possible interference of other β-galactosidase-containing bacteria such as *Aeromonas* spp., the chemiluminometric procedure was applied to serial dilutions of 17 strains of *A. hydrophila*, *A. sobriae*, and *A. caviae* in the low-nutrient medium. None of the isolates gave a positive response at concentrations below 10^4 to 10^5 CFU/ml. The relatively lower level of response obtained compared with that of coliforms may be ascribed to a reduced level of β-galactosidase activity and/or less efficient uptake of the substrate. Compared with fluorometry and colorimetry, chemiluminometry enables the detection of a given number of coliforms more quickly, the difference being 1 and 6 h, respectively, for 50 CFU/100 ml (Fig. 3). Although the corresponding detection times for naturally occurring, possibly stressed or injured coliforms in water remain to be ascertained, this result suggests a potential for the two-stage chemiluminometric procedure that significantly supersedes that of the most rapid existing enzymatic methods for coliforms (2, 5). Therefore, if further optimization of the medium composition, the enzyme assay, and the sample preconcentration could reduce the duration of the test to less than 8 h, which appears to lie within reach, the advantage of speed could outweigh the disadvantage of the method's relative complexity. Even with its present degree of sophistication, chemiluminometric enzyme detection remains simpler to perform than PCR, currently the only experimental approach that has the potential to detect one coliform per 100 ml in less than a working day (4). However, PCR also detects dead cells, which brings into question its applicability for the detection of coliforms in treated drinking water (11). Although the chemiluminogenic substrate is expensive, its contribution to the total cost of a test would be only $0.70, because of the low concentration used. The stability of the substrate (up to 9 months when stored at 2 to 8°C) is sufficient for practical purposes. The application of the chemiluminometric procedure could be justified in emergencies, e.g., breakdowns in water supply or construction works or disasters, etc., rather than in daily routine water testing. For the procedure to be acceptable for this purpose, however, it will have to be thoroughly validated.

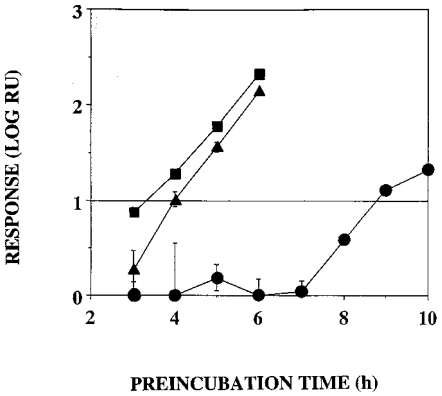

FIG. 3. Comparison of two-step procedure with chemiluminometric (■), fluorometric (▲), and colorimetric (●) detection of *E. coli* seeded in sterile distilled water (50 CFU/100 ml), after concentration and preincubation in the low-nutrient growth medium. Responses are expressed as relative units, as explained in the legend to Fig. 1. Each point represents the mean ± standard deviation for three measurements of 400-μl aliquots taken from the same preincubation mixture.

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