APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1996, p. 3868–3870 Vol. 62, No. 10
0099-2240/96/$04.00+0

Evaluation of Cyclohexenoesculetin-β-D-Galactoside and 8-Hydroxyquinoline-β-D-Galactoside as Substrates for the Detection of β-Galactosidase

A. L. JAMES,[1] J. D. PERRY,[2]* M. FORD,[2] L. ARMSTRONG,[1] AND F. K. GOULD[2]

Department of Chemical and Life Sciences, University of Northumbria,[1] and Department of Microbiology, Freeman Hospital,[2] Newcastle-upon-Tyne, United Kingdom

Received 15 May 1996/Accepted 1 August 1996

We describe the synthesis of two new substrates for the detection of β-galactosidase and evaluate their performance in comparison with that of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Of 171 *Enterobacteriaceae* strains that were able to hydrolyze X-Gal, 166 (97.1%) also hydrolyzed cyclohexenoesculetin-β-D-galactoside whereas only 96 (56.1%) showed evidence of hydrolysis of 8-hydroxyquinoline-β-D-galactoside. No false-positive results were observed with either substrate.

Numerous substrates are available for the detection of β-galactosidase, the most common being *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG), which releases yellow *o*-nitrophenol upon hydrolysis (7). Fluorogenic substrates have also been used with labels such as resorufin, fluorescein, and 4-methylumbelliferone (2, 9). A major limitation of most of these substrates is that the aglycone released by hydrolysis has a tendency to diffuse widely, and they are therefore not suited to incorporation into agar plates.

Because of these limitations, chromogenic substrates which produce nondiffusible products have been developed for incorporation into agar media (8). Such substrates include galactosides of indoxyl and its halogenated derivatives, such as 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (6). Although these substrates are highly effective, their preparation is not straightforward and they are consequently very expensive.

We describe the synthesis of two new substrates for the detection of β-galactosidase based on derivatives of 8-hydroxyquinoline and cyclohexenoesculetin. In each case, the aglycone released by hydrolysis forms a brown-black complex with ferric ions to give a clearly visible nondiffusible product. We present an evaluation of these two substrates, in comparison with X-Gal, for the detection of β-galactosidase within the *Enterobacteriaceae.*

X-Gal was obtained from Sigma Chemical Company Ltd., Poole, United Kingdom. Columbia agar was obtained from Lab M Ltd., Bury, United Kingdom. Chemicals involved in the synthesis of 3,4-cyclohexenoesculetin-7-β-D-galactoside (CHE-Gal) and 8-hydroxyquinoline-β-D-galactoside (8HQ-Gal) were all obtained from Aldrich Chemical Company Ltd., Gillingham, United Kingdom.

CHE-Gal was prepared as follows. 3,4-Cyclohexenoesculetin was prepared by a standard Pechmann reaction involving condensation of ethyl-2-cyclohexanonecarboxylate with 1,2,4-triacetoxybenzene in the presence of 75% (wt/wt) sulfuric acid. The β-galactosidase was prepared by performing a modified Koenigs-Knorr reaction. 3,4-Cyclohexenoesculetin dissolved in an excess of 10% potassium hydroxide solution was treated with an equimolar proportion of α-acetobromoglucose in acetone. The solution was stirred overnight and poured into an ice-water mixture. The solid which separated out was collected, dissolved in dichloromethane, and washed well with water. Evaporation yielded a gray solid, which was recrystallized from hot ethanol to yield the 7-*O*-tetraacetylgalactoside of 3,4-cyclohexenoesculetin.

The dry product was dissolved in methanol and treated with a catalytic amount of sodium methoxide for several hours. The pH of the resulting solution was reduced to around 6.5 by using the ion-exchange resin IR:120 H+. Methanol was removed under reduced pressure until crystallization commenced. The glycoside produced was recrystallized from hot aqueous methanol.

8HQ-Gal was prepared as described above except that 8-hydroxyquinoline was used in place of cyclohexenoesculetin.

CHE-Gal agar was prepared as follows. Columbia agar (41 g), 0.5 g of ferric ammonium citrate, and 0.5 g of CHE-Gal were dissolved by boiling in 1 liter of distilled water. The pH was adjusted to 7.5, and the medium was sterilized by autoclaving for 10 min at 116°C. The medium was then allowed to cool to 55°C before being poured in 20-ml volumes. 8HQ-Gal agar was prepared in an identical fashion except that 0.5 g of 8HQ-Gal was substituted for CHE-Gal. X-Gal agar was also prepared by the same protocol with the exclusion of iron salts, which are not required for the formation of the colored product.

Two hundred fifty strains of *Enterobacteriaceae* collected from a wide range of clinical and environmental samples were identified with the API 20E system (Biomerieux). These strains were cultivated on Columbia agar at 37°C for 24 h. Each strain was then inoculated into physiological saline to produce an inoculum of approximately 10^8 organisms per ml. With a multipoint inoculator (Denley), 1 μl of each suspension was then inoculated onto all three of the test media.

Finally, 10 strains of *Enterobacteriaceae* were chosen for membrane filtration studies. They were *Escherichia coli* NCTC 10418, *Klebsiella pneumoniae* NCTC 10896, *Providencia rettgeri* NCTC 7475, *Enterobacter cloacae* NCTC 11936, *Serratia marcescens* NCTC 10211, *Salmonella typhimurium* NCTC 74, and wild strains of *Citrobacter freundii*, *Klebsiella oxytoca*, *Enterobacter aerogenes*, and *Shigella sonnei*. Suspensions of these 10 strains were prepared as described above and diluted in sterile distilled water by standard methods to produce suspensions containing approximately one organism per ml. Four 100-ml volumes of each suspension were then filtered onto four cel-

* Corresponding author.

TABLE 1. Hydrolysis of different β-galactosidase substrates by strains of *Enterobacteriaceae*

Gram-negative species	No. of strains	% Positive with substrate:		
		X-Gal	CHE-Gal	8HQ-Gal
Citrobacter diversus	5	80	80	0
Citrobacter freundii	11	100	100	55[a]
Enterobacter agglomerans	1	100	100	100
Enterobacter cloacae	20	100	100	55[a]
Escherichia coli	45	100	100	100[a]
Escherichia hermanii	1	100	100	0
Hafnia alvei	7	71	57	14[a]
Klebsiella oxytoca	20	100	100	31
Klebsiella ozaenae	2	100	100	0
Klebsiella pneumoniae	30	100	100	40
Morganella morganii	11	0	0	0
Proteus mirabilis	18	0	0	0
Proteus penneri	4	0	0	0
Proteus vulgaris	5	0	0	0
Providencia rettgeri	3	0	0	0
Providencia stuartii	7	0	0	0
Salmonella sp.	21	0	0	0
Serratia liquefaciens	10	80	80	80
Serratia marcescens	10	80	80	60
Shigella flexneri	2	0	0	0
Shigella sonnei	10	100	100	0
Yersinia enterocolitica	4	100[a]	0	0
Yersinia pseudotuberculosis	3	66	66	0

[a] Weak reaction.

lulose nitrate membrane filters (Sartorius) by a standard filtration method. The filters were placed onto media containing CHE-Gal, 8HQ-Gal, or X-Gal and onto a Columbia agar plate without substrate as a growth control. All plates were incubated at 37°C in air for exactly 18 h. After incubation, the plates were examined for the presence of black or indigoid colonies and colony counts were performed on the membrane filters.

From the results in Table 1 it can be concluded that CHE-Gal, in particular, showed an excellent correlation with X-Gal. The only discrepancy between the results obtained with these two compounds was that X-Gal detected a weak β-galactosidase activity in *Yersinia enterocolitica* whereas no activity was detected in this species with CHE-Gal. In all cases, strains which hydrolyzed CHE-Gal produced a clearly visible black precipitate which remained highly restricted to the bacterial colony. The results for 8HQ-Gal were less conclusive, as many strains which hydrolyzed X-Gal did not produce any discernible reaction with 8HQ-Gal. In addition, many strains which hydrolyzed 8HQ-Gal produced only weak reactions. None of the strains which failed to hydrolyze X-Gal produced any coloration with either of the two test substrates; i.e., there were no false-positive results. The colony types typically produced by *E. coli* with the two test substrates are shown in Fig. 1.

In the membrane filtration study, the following strains produced well-defined black or colored colonies with X-Gal, CHE-Gal, and 8HQ-Gal: *E. coli* NCTC 10418, *K. pneumoniae* NCTC 10896, *E. cloacae* NCTC 11936, *S. marcescens* NCTC 10211, and wild strains of *C. freundii*, *K. oxytoca*, *E. aerogenes*, and *S. sonnei*. On the filters placed on 8HQ-Gal agar, colonies varied in color from light to dark brown, whereas all of these strains produced black colonies on CHE-Gal agar. *S. typhimurium* NCTC 74 and *P. rettgeri* NCTC 7475 did not hydrolyze any of the three substrates. There was no statistical difference between the colony counts on CHE-Gal, X-Gal, 8HQ-Gal, or Columbia agar for any of the organisms tested (data not shown). The colony types produced by *E. cloacae* NCTC 11936 on membrane filters with each of the substrates can be seen in Fig. 2.

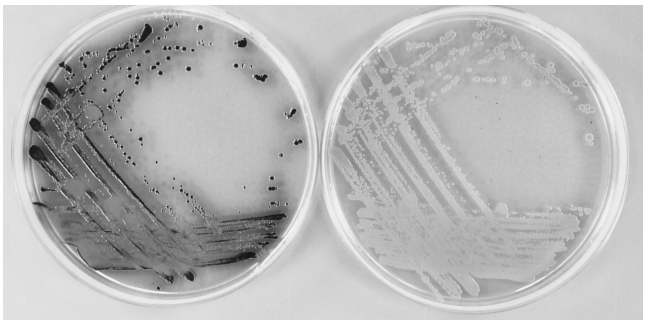

FIG. 1. Hydrolysis of CHE-Gal (left) and 8HQ-Gal (right) by *E. coli* (NCTC 10418).

We have shown that 8HQ-Gal is able to detect β-galactosidase activity in strains of *Enterobacteriaceae* but its performance is poor in comparison with that of X-Gal. Glycoside derivatives of 8-hydroxyquinoline have been used previously with some success (3–5). The reasons for the poor performance of 8HQ-Gal are unclear, but this may be due to a variety of factors, such as low substrate turnover, poor induction of enzyme activity, or inhibition of optimal cell metabolism. It is well recognized that 8-hydroxyquinoline may be toxic, particularly against gram-positive bacteria (1), and this is also a negative factor when considering the use of such substrates in general-purpose isolation media. It is possible that the use of a gratuitous enzyme inducer, such as isopropyl β-D-thiogalactopyranoside (IPTG), would enhance the performance of 8HQ-Gal; however, this was not necessary for the successful performance of either X-Gal or CHE-Gal. It is noteworthy that both CHE-Gal and 8HQ-Gal yield hydrolysis products which should chelate effectively under anaerobic conditions. This contrasts with the hydrolysis of X-Gal, which yields a colored product only when exposed to oxygen.

CHE-Gal has been shown to produce results comparable to those obtained with X-Gal on both membrane filters and agar media. CHE-Gal therefore provides a useful alternative to existing substrates and could potentially be combined with a fluorogenic compound, such as 4-methylumbelliferyl-β-D-glucuronide, to simultaneously enumerate both *E. coli* and total coliforms on a single membrane filter. CHE-Gal is available from LAB M for further research trials.

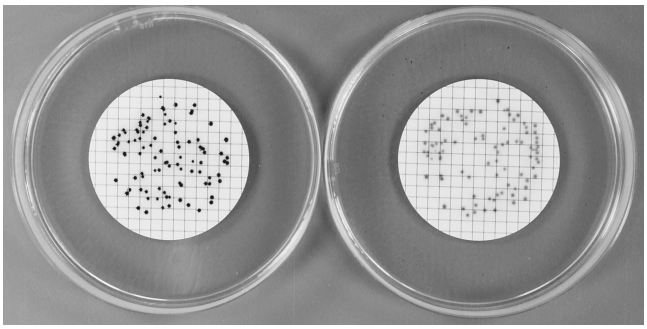

FIG. 2. Detection of β-galactosidase production by *E. cloacae* on a nitrocellulose membrane filter with CHE-Gal (left) and 8HQ-Gal (right).

REFERENCES

1. **Albert, A., M. I. Gibson, and S. D. Rubbo.** 1953. The influence of chemical constitution on anti-bacterial activity. VI. The bactericidal action of 8-hydroxyquinoline (oxine). Br. J. Exp. Pathol. **34:**119–130.
2. **Berg, J. D., and L. Fiksdal.** 1988. Rapid detection of total and fecal coliforms in water by enzymatic hydrolysis of 4-methylumbelliferone-β-D-galactoside. Appl. Environ. Microbiol. **54:**2118–2122.
3. **Dalet, F., and T. Segovia.** 1995. Evaluation of a new agar in Uricult-Trio for rapid detection of *Escherichia coli* in urine. J. Clin. Microbiol. **33:**1395–1398.
4. **James, A. L., and P. Yeoman.** 1987. Detection of specific bacterial enzymes by high contrast metal chelate formation. I. 8-Hydroxyquinoline β-D-glucoside, an alternative to aesculin in the differentiation of members of the family *Enterobacteriaceae*. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **267:**188–193.
5. **James, A. L., and P. Yeoman.** 1988. Detection of specific bacterial enzymes by high contrast metal chelate formation. II. Specific detection of *Escherichia coli* on multipoint inoculated plates using 8-hydroxyquinoline β-D-glucuronide. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **267:**316–321.
6. **Kodaka, H., M. Ishikawa, M. Iwata, F. Kashitani, S. Mizuochi, and K. Yamaguchi.** 1995. Evaluation of new medium with chromogenic substrates for members of the family *Enterobacteriaceae* in urine samples. J. Clin. Microbiol. **33:**199–201.
7. **Lowe, G. H.** 1962. The rapid detection of lactose fermentation in paracolon organisms by the demonstration of β-D-galactosidase. J. Med. Lab. Technol. **19:**21.
8. **Manafi, M., W. Kneifel, and S. Bascomb.** 1991. Fluorogenic and chromogenic substrates used in bacterial diagnostics. Microbiol. Rev. **55:**335–348.
9. **Plovins, A., A. M. Alverez, M. Ibañez, M. Molina, and C. Nombela.** 1994. Use of fluorescein-di-β-D-galactopyranoside (FDG) and C_{12}-FDG as substrates for β-galactosidase detection by flow cytometry in animal, bacterial, and yeast cells. Appl. Environ. Microbiol. **60:**4638–4641.