Journal of Applied Microbiology 2004, **96,** 170–176 doi:10.1046/j.1365-2672.2003.02130.x

Evaluation of novel β-ribosidase substrates for the differentiation of Gram-negative bacteria

L.A. Butterworth[1], J.D. Perry[1], G. Davies[2], M. Burton[2], R.H. Reed[3] and F.K. Gould[1]

[1]Department of Microbiology, Freeman Hospital, Newcastle upon Tyne, UK, [2]Glycosynth, Warrington, UK, and [3]Department of Applied Sciences, Northumbria University, Newcastle upon Tyne, UK

2003/0612: received 14 July 2003, revised 14 September 2003 and accepted 15 September 2003

ABSTRACT

L.A. BUTTERWORTH, J.D. PERRY, G. DAVIES, M. BURTON, R.H. REED AND F.K. GOULD. 2003.

Aims: To synthesize novel substrates for the detection of β-ribosidase and assess their potential for the differentiation of Gram-negative bacteria.

Methods and Results: Two novel chromogenic substrates, 3′,4′-dihydroxyflavone-4′-β-D-ribofuranoside (DHF-riboside) and 5-bromo-4-chloro-3-indolyl-β-D-ribofuranoside (X-riboside) were evaluated along with a known fluorogenic substrate, 4-methylumbelliferyl-β-D-ribofuranoside (4MU-riboside). A total of 543 Gram-negative bacilli were cultured on media containing either DHF-riboside or X-riboside. Hydrolysis of DHF-riboside or X-riboside resulted in the formation of clearly distinguishable black or blue-green colonies, respectively. Hydrolysis of 4MU-riboside was evaluated in a liquid medium in microtiter trays and yielded blue fluorescence on hydrolysis which was measured using fluorimetry. β-Ribosidase activity was widespread with 75% of strains, including 85·6% of Enterobacteriaceae, showing activity with at least one substrate. Genera that demonstrated β-ribosidase activity included *Aeromonas*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Hafnia*, *Klebsiella*, *Morganella*, *Providencia*, *Pseudomonas*, *Salmonella* and *Shigella*. In contrast, strains of *Proteus* spp., *Acinetobacter* spp., *Yersinia enterocolitica*, *Vibrio cholerae* and *Vibrio parahaemolyticus* generally failed to demonstrate β-ribosidase activity.

Conclusions: The novel substrates DHF-riboside and X-riboside are effective for the detection of β-ribosidase in agar-based media and may be useful for the differentiation and identification of Gram-negative bacteria.

Significance and Impact of the Study: This is the first report describing the application and utility of chromogenic substrates for β-ribosidase. These substrates could be applied in chromogenic media for differentiation of Gram-negative bacteria.

Keywords: β-ribosidase, chromogenic substrates, dihydroxyflavone, ribosides, *Yersinia enterocolitica*.

INTRODUCTION

The value of glycosidase substrates as identification markers has long been established in diagnostic microbiology (Kilian and Bülow 1976; Manafi *et al.* 1991). Chromogenic substrates for the detection of glycosidases form the basis of a range of media for the detection of pathogenic bacteria. For example, chromogenic substrates for β-glucosidase are of value for the detection of *Listeria* spp. in food samples (Smith *et al.* 2001) and for the identification of coliforms and enterococci in urine samples (Reisner and Austin 1997). Substrates which detect β-glucuronidase assist in the detection of *Escherichia coli* in food (Venkateswaran *et al.* 1996) and clinical samples (Reisner and Austin 1997) and also help to distinguish *E. coli* O157 from other serotypes (Reinders *et al.* 2000). Substrates for α-galactosidase are useful for the identification of *Salmonella* spp. (Perry *et al.* 1999).

Correspondence to: J.D. Perry, Department of Microbiology, Freeman Hospital, Freeman Road, Newcastle upon Tyne, NE7 7DN, UK (e-mail: jdp@blueyonder.co.uk).

In chromogenic media, glycosidase substrates are often employed in conjunction with other biochemical reactions, such as sugar fermentation or detection of esterase activity, to enable the specific demonstration of target pathogens (Rambach 1990; Perry and Quiring 1997). The increased specificity provided by this approach results in a decrease in the time taken to isolate pathogens and also reduces the time-consuming screening of suspect colonies (Doléans 1994).

Here, we describe the evaluation of three substrates for the detection of β-ribosidase activity and assess the taxonomic utility of this enzyme for the differentiation of pathogenic Gram-negative bacteria. The first substrate is 3′,4′-dihydroxyflavone-4′-β-D-ribofuranoside (DHF-riboside). This chromogenic substrate is hydrolysed by β-ribosidase-producing bacteria to release 3′,4′-dihydroxyflavone, which forms an insoluble black chelate with iron. The second chromogenic substrate is 5-bromo-4-chloro-3-indolyl-β-D-ribofuranoside (X-riboside), which is hydrolysed to form an insoluble blue-green complex in air. Finally, these two substrates were compared with the fluorogenic substrate: 4-methylumbelliferyl-β-D-ribofuranoside (4MU-riboside). While the synthesis of 4MU-riboside has been described previously (Schramm *et al.* 1997), there have been no reports of its activity with bacteria. Both X-riboside and 4MU-riboside are based on core molecules that have been extensively used in diagnostic microbiology (Manafi *et al.* 1991). In contrast, 3′,4′-dihydroxyflavone is a novel chromogen; the structure of DHF-riboside and the putative black chelate formed with iron after enzymatic hydrolysis are shown in Fig. 1.

MATERIALS AND METHODS

Equipment and materials

All chemicals were obtained either from Sigma-Aldrich Chemical Company Ltd, Poole, UK, Lancaster Synthesis Ltd, Lancaster, UK, or Ultrafine Ltd, London, UK. Bacteriological media were obtained from Oxoid, Basingstoke, UK. A total of 72 bacterial strains were obtained from the National Collection of Type Cultures (NCTC), Colindale, London, UK. All strains of *Ralstonia* spp. *Pandoraea* spp. and *Burkholderia cepacia* complex were obtained from the

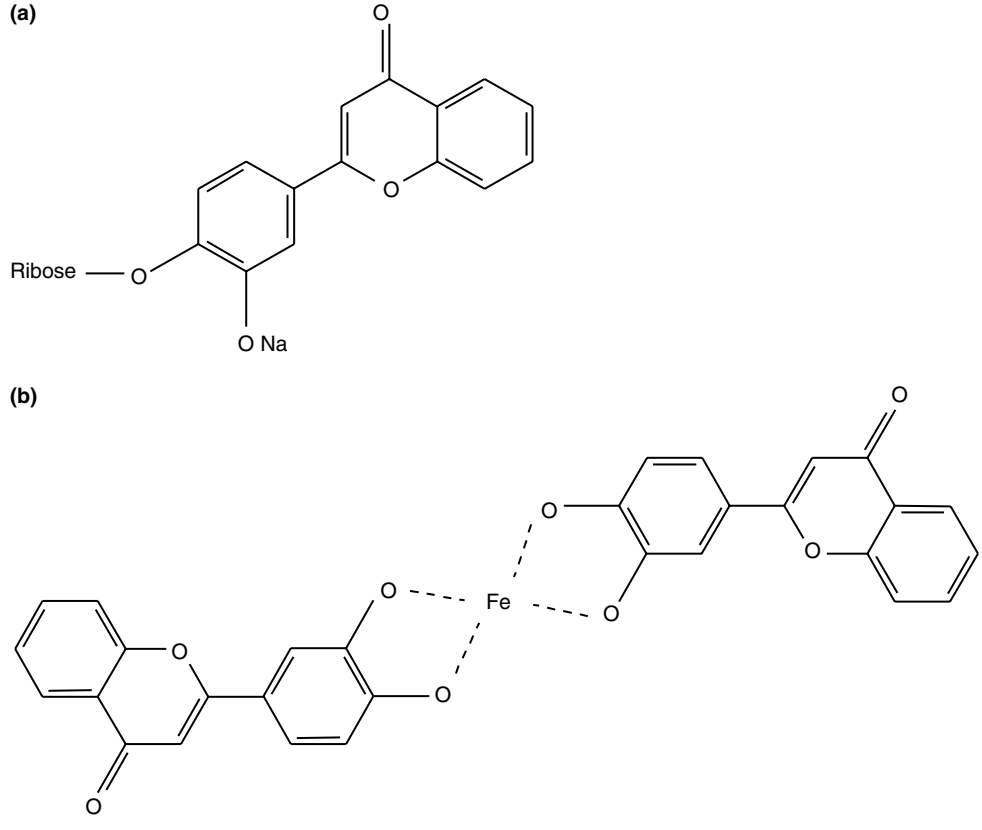

Fig. 1 shows (a) the structure of 3′,4′-dihydroxyflavone-4′-β-D-ribofuranoside sodium salt and (b) the putative black chelate formed with iron after enzymatic hydrolysis

Belgian Co-ordinated Collections of Micro-organisms (BCCM^{TM}/LMG), Gent, Belgium. 410 wild strains were isolated from pathological samples in the Microbiology Department, Freeman Hospital. All wild strains were characterized using API 20E or API 20NE (bioMérieux, Basingstoke, UK), with supplementary biochemical and serological tests, if required. Each strain was stored as lenticules at −20°C as previously described (Codd *et al.* 1998). Bacterial suspensions were standardized using a Densimat (bioMérieux). Fluorescence was measured using a Labtech Biolite F1 fluorescence microtitre plate reader (Labtech International Ltd, Uckfield, UK) with excitation and emission filters at 365 nm and 440 nm, respectively. Sterile, flat-bottomed microtitre trays (Bibby Sterilin Limited, Aberbargoed, UK) were used throughout. Double-strength phosphate buffer was prepared by dissolving 5·92 g of sodium dihydrogen phosphate plus 23 g of disodium hydrogen phosphate in 1 l of deionized water, adjusted to pH 7·4 and sterilized at 116°C for 20 min.

Synthesis of 3′,4′-dihydroxyflavone-4′-β-D-ribofuranoside sodium salt

A 50-ml round-bottomed flask (magnetic stirrer) charged with 3′,4′-dihydroxyflavone (500 mg), β-D-ribofuranose tetraacetate (640 mg), 3-Å molecular sieves (5·2 g) and dry dichloromethane (20 ml) was stirred for 15 min, then boron trifluoride diethyl etherate catalyst (2·0 ml) was added. After a further 20 min, the reaction mixture was poured into saturated sodium bicarbonate (150 ml). The organic layer was diluted with dry dichloromethane (30 ml), separated from the aqueous layer and then washed with saturated sodium bicarbonate (11 × 50 ml). After drying the organic phase with magnesium sulphate for 1 h, evaporation to dryness yielded the glycoside triacetate as a dark gum (390 mg). The acetates were removed by dissolving the gum (315 mg) in a sodium methoxide solution [methanol (5 ml), sodium (50 mg)]. After 16 h, the solution was concentrated to *ca* 2 ml by evaporation. Addition of diethyl ether (10 ml) precipitated the product. It was collected by vacuum filtration, washed (diethyl ether 4 × 5 ml) and immediately transferred to a desiccator and dried over phosphorous pentoxide under vacuum for 2 h. The product was a yellow powder (201 mg).

Synthesis of X-β-D-ribofuranoside

In a 500 ml flask with magnetic stirrer, a mixture of 2, 3,5-tri-O-acetyl-α/β-D-ribofuranosyl trichloroacetimide (145·0 g) (Chiu-Machado *et al.* 1995), 3-Å molecular sieves (2·0 g), dry dichloromethane (300 ml) and dry 1-acetyl-5-bromo-4-chloroindoxyl (82·9 g) (Holt and Sadler 1958) was stirred for 10 min then trimethyl-silyl-triflate (8 ml) was added in one portion. After 1 h, the mixture was poured into dry dichloromethane (3 l) and washed with 1 M sodium hydroxide (4 × 2 l). The organic layer was separated, filtered through celite, concentrated to *ca* 1 l, then washed further sequentially with 1 M sodium hydroxide (6 × 1 l) and deionized water (6 × 1 l). After drying with magnesium sulphate, the organic layer was filtered through celite and concentrated to afford a dark brown solid (87·4g). This was purified by flash chromatography on Silica Gel C60 (600g) using 60/80 petroleum ether/ethyl acetate/triethylamine in the ratio 1 : 1 : 0·05 v/v/v as the eluting solvent. This gave a brown oil (55 g) which was taken up in warm methanol (30 ml) and stored at ambient temperature for 16 h. The precipitate that formed was removed by filtration and discarded. Concentration of the filtrate at 40°C *in vacuo* afforded the product peracetate as an oil (33·6 g). The acetates were removed by treating the oil (30 g) with sodium methoxide solution [methanol (205 ml), sodium (0·25 g)] for 1·5 h. After concentrating to dryness and triturating with acetone (500 ml), the grey solid that formed was removed by filtration and discarded. The filtrate was concentrated to dryness and triturated with methanol (80 ml). The precipitated product (2·57 g) was recovered by filtration. After concentrating the filtrate from this, further trituration with methanol (30 ml) afforded a second crop (4·04 g). The combined crops were washed with acetone (20 ml). This gave the title compound as a white solid (*ca* 2·5 g). The synthesis of 4MU-β-D-ribofuranoside has been reported previously (Schramm *et al.* 1997).

Determination of optimal substrate concentration

In an initial evaluation to determine the optimal substrate concentration, DHF-riboside was incorporated into Columbia agar at a range of concentrations from 100–800 mg l^{-1}. Each formulation was supplemented with 500 mg l^{-1} of ferric ammonium citrate. After autoclaving, plates were poured and inoculated with NCTC strains representing 10 different bacterial species. The optimal substrate concentration was assessed visually and was found to be 400 mg l^{-1}, as this allowed clear recognition of β-ribosidase producing colonies with minimal background colouration of the agar medium. At this concentration, DHF-riboside caused the agar medium to appear pale yellow.

The optimal concentration of X-riboside was established as described for DHF-riboside using a concentration ranging from 50 to 300 mg l^{-1} without ferric ammonium citrate. The optimal concentration was found to be 80 mg l^{-1} and none of the substrate concentrations tested had any impact on the background colour of the agar medium, with positive strains producing blue-green colonies.

Preparation of chromogenic media

A test medium containing DHF-riboside was prepared as follows: 400 mg of DHF-riboside, 40 g of Columbia agar and 500 mg of ferric ammonium citrate were measured and added to 1 l of deionized water. This was sterilized by autoclaving at 116°C for 20 min, cooled to 50°C and then dispensed in 20-ml volumes into Petri plates. The plates were lightly dried to remove surface moisture, then stored at 4°C for a maximum of 1 month. A test medium containing 80 mg l^{-1} X-riboside was prepared exactly as described above in Columbia agar without ferric ammonium citrate. Control media were prepared consisting of Columbia agar with and without 500 mg l^{-1} of ferric ammonium citrate.

A solution of 4MU-riboside was prepared by dissolving 10 mg in 10 ml of double-strength phosphate buffer and sterilizing by filtration. This was then dispensed in 50-μl aliquots into the wells of a sterile microtitre tray.

Inoculation of test media

Inocula of test strains were prepared as follows: a lenticule of each strain was placed on the surface of an individual Columbia agar plate (supplemented with 5% horse blood), left for 10 min to re-hydrate and the resulting drop spread for single colonies. All plates were incubated for 24 h at 37°C in aerobic conditions. Colonies from each bacterial strain were then suspended in an aliquot of sterile deionized water and corrected to a density equivalent to McFarland standard 1 (3×10^8 CFU ml^{-1}). A 50-μl volume of each bacterial suspension was then added to individual wells containing 4MU-riboside solution, and the contents were mixed gently. The initial fluorescence was measured and the trays were then incubated aerobically in a shaker incubator at 37°C. Fluorescence was measured again after 18 h of incubation. After the final reading (18 h) the data were accumulated, processed and zeroed to calculate the overall increase in fluorescence for each bacterial strain. Strains were denoted as being 'positive' for β-ribosidase activity if the increase in fluorescence was ≥5000 relative fluorescence units.

Using a multi-point inoculator (Mast Laboratories, Bootle, UK), a 1-μl volume of each bacterial suspension was inoculated onto the surface of both of the test media and both of the control media. All media were then incubated aerobically for 18 h at 37°C. After incubation, the growth of each strain was examined visually and scored as either positive or negative for β-ribosidase activity, based on colour development. On the medium containing X-riboside, strains were regarded as positive if they generated a blue-green colouration over and above any natural colouration produced on the control medium (Columbia agar). On the medium containing DHF-riboside, strains which produced a black or brown colouration over and above that generated on the control medium (Columbia agar supplemented with ferric ammonium citrate) were also regarded as positive. All strains were tested with the three substrates on three separate occasions, to ensure reproducibility of results.

RESULTS

Figure 2 shows the appearance of colonies of *E. coli* (NCTC 10418) on Columbia agar with DHF-riboside; the colour development is a result of hydrolysis of the chromogenic substrate and its subsequent chelation in the presence of iron. Strains producing β-ribosidase typically grew as black colonies with no diffusion of colour into the surrounding medium. These black colonies were easily differentiated from β-ribosidase negative colonies, which were cream or colourless. Fig. 3 shows the appearance of colonies of *E. coli* (NCTC 10418) on Columbia agar supplemented with X-riboside. Colonies were blue-green, with no diffusion of the chromogen. β-ribosidase negative colonies were cream or colourless.

Table 1 shows the proportion of each species that were positive for β-ribosidase activity with each of the three substrates tested. The fluorogenic substrate 4MU-riboside was hydrolysed by 74% of all strains compared with 72 and 53% for DHF-riboside and X-riboside, respectively. The lower sensitivity of X-riboside was mainly because of its poor activity with nonfermentative Gram-negative rods as only 1·2% of strains expressed activity with X-riboside compared with 55% of strains that expressed activity with the other β-riboside substrates.

Among the Enterobacteriaceae, most strains (86%) were positive for β-ribosidase activity and there was generally good correlation between the activities of the three substrates. A notable exception was *Enterobacter aerogenes*, as all 12 test strains hydrolysed 4MU-riboside and DHF-riboside but only one strain (NCTC 9777) formed green colonies with X-riboside.

DISCUSSION

This is the first report of the use of β-ribosidase activity for the differentiation of bacteria and the demonstration of such activity in bacterial colonies. Although most strains showed consistent results with the three substrates, there were inconsistencies with some species. Most notably, nonfermentative Gram-negative rods showed poor activity with X-riboside. The reasons for the differences between the activities of different substrates are unclear but may be due to different rates of substrate uptake through the cell envelope or differences in the substrate affinity of the β-ribosidase enzyme within different species. It would be of

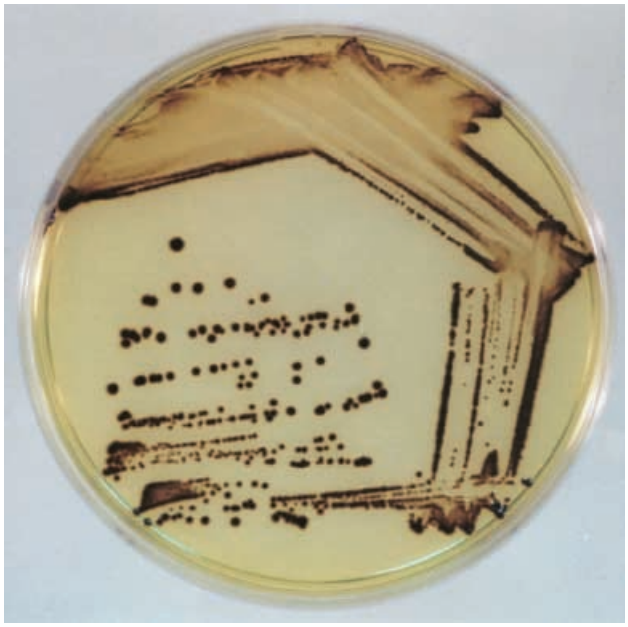

Fig. 2 Black colonies of *Escherichia coli* (NCTC 10418) on Columbia agar with DHF-riboside and iron

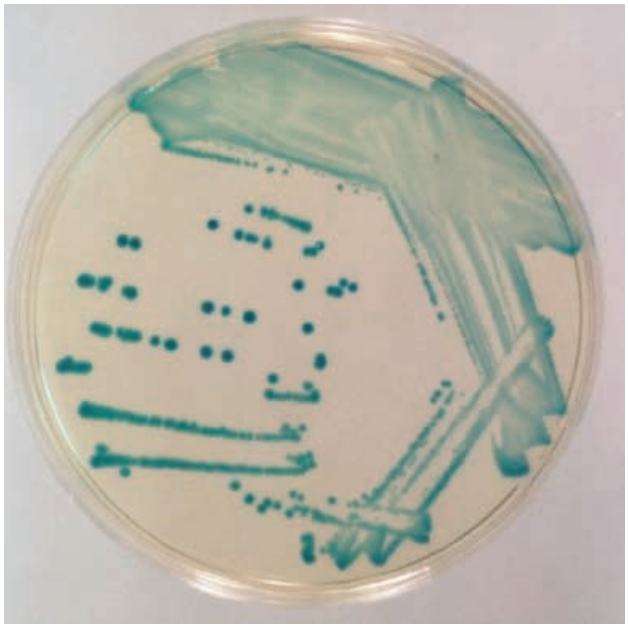

Fig. 3 Blue-green colonies of *Escherichia coli* (NCTC 10418) on Columbia agar with X-riboside

interest to examine the activity of these substrates with purified β-ribosidase from different species.

There are a number of potential applications of these substrates in diagnostic microbiology. All test strains of *Salm.* spp. (35 strains) and *Shigella* spp. (31 strains) were uniformly positive for β-ribosidase activity when assayed using 4MU-riboside and DHF-riboside which may be of diagnostic value in conjunction with other biochemical markers. One strain of Salm. (*Salm. choleraesuis* NCTC 10653) failed to generate any colouration when tested with X-riboside. None of the glycosidases commonly sought in diagnostic microbiology is uniformly produced by species of *Shigella*, and this has been a limiting factor in designing suitable diagnostic methods for their detection. Hydrolysis of X-riboside could enable the distinction between *Shigella* spp. and *Proteus* spp which are difficult to differentiate on many microbiological media. X-riboside could also be used for differentiation of *Proteus* spp from the closely related *Providencia* spp.

Application of chromogenic ribosides could be of particular value for the detection of *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Yersinia enterocolitica* as, in contrast to most other Gram-negative bacteria, these strains failed to generate any colouration with either X-riboside or DHF-riboside. The nine strains of *V. cholerae* and four strains of *V. parahaemolyticus* tested were all obtained from NCTC and were from diverse origins. None of these strains was able to hydrolyse either of the chromogenic substrates. It is possible that these substrates could provide a means of differentiating these pathogenic vibrios from most Enterobacteriaceae and also the closely related *Aeromonas* spp. Both chromogenic ribosides were hydrolysed by *Vibrio alginolyticus* (NCTC 12160), *Vibrio anguillarum* (NCTC 12159),*Vibrio fluvialis* (NCTC 11327) and *Vibrio metschnikovii* (NCTC 8443).

Yersinia enterocolitica is an uncommon but important cause of enteritis in humans. In this study, 27 strains, including 10 NCTC strains from diverse origins, failed to hydrolyse either of the chromogenic substrates, although one strain (NCTC 10598) hydrolysed 4MU-riboside. Cefsulodin-irgasan-novobiocin (CIN) agar is an effective selective medium on which *Y. enterocolitica* generates red mannitol-fermenting colonies (Schiemann 1979). Its specificity is limited however because other genera such as *Serratia*, *Citrobacter*, *Aeromonas*, and *Providencia* are also able to generate similar colonies on CIN agar. However, all of these genera show β-ribosidase activity, which may provide a convenient means for their differentiation from *Y. enterocolitica* by inclusion of a chromogenic ribosidase substrate into CIN agar. This could also allow the differentiation of *Y. enterocolitica* from non-pathogenic *Yersinia* species such as *Yersinia frederiksenii* and *Yersinia intermedia*.

In the presence of iron, 3′,4′-dihydroxyflavone appears to be a useful, nondiffusible chromogen. It may be derivatized with carbohydrates other than ribofuranose to provide other chromogenic glycosidase substrates. Other chromogens that form black chelates in the presence of iron salts and which have been derivatized to form effective enzyme substrates are 3,4-cyclohexenoesculetin and 8-hydroxyquinoline (James *et al.* 1996; Reinders *et al.* 2000). These

Table 1 Hydrolysis of three riboside substrates by strains of Gram-negative bacteria

Organism	No. of strains	No. (%) of strains positive with each substrate		
		4MU-riboside	DHF-riboside	X-riboside
Enterobacteriaceae				
Citrobacter diversus	9	9 (100)	9 (100)	9 (100)
Citrobacter freundii	18	18 (100)	18 (100)	18 (100)
Enterobacter aerogenes	12	12 (100)	12 (100)	1 (8)
Enterobacter cloacae	23	22 (96)	23 (100)	23 (100)
Escherichia coli	55	55 (100)	55 (100)	55 (100)
Hafnia alvei	10	10 (100)	10 (100)	10 (100)
Klebsiella oxytoca	13	13 (100)	13 (100)	13 (100)
Klebsiella ozaenae	6	6 (100)	6 (100)	5 (83)
Klebsiella pneumoniae	26	26 (100)	26 (100)	26 (100)
Morganella morganii	12	10 (83)	11 (92)	11 (92)
Proteus mirabilis	16	0 (0)	0 (0)	0 (0)
Proteus penneri	2	0 (0)	0 (0)	0 (0)
Proteus vulgaris	4	1 (25)	3 (75)	0 (0)
Providencia rettgeri	4	4 (100)	4 (100)	4 (100)
Providencia alcalifaciens	2	2 (100)	2 (100)	2 (100)
Providencia stuartii	11	11 (100)	11 (100)	11 (100)
Salmonella spp.	35	35 (100)	35 (100)	34 (97)
Serratia liquefaciens	9	9 (100)	9 (100)	9 (100)
Serratia marcescens	11	11 (100)	11 (100)	11 (100)
Shigella boydii	5	5 (100)	5 (100)	5 (100)
Shigella dysenteriae	6	6 (100)	6 (100)	6 (100)
Shigella flexneri	5	5 (100)	5 (100)	5 (100)
Shigella sonnei	15	15 (100)	15 (100)	15 (100)
Yersinia enterocolitica	27	1 (4)	0 (0)	0 (0)
Yersinia frederiksenii	5	5 (100)	5 (100)	5 (100)
Yersinia intermedia	1	1 (100)	1 (100)	1 (100)
Yersinia kristensenii	2	0 (0)	0 (0)	0 (0)
Yersinia pseudotuberculosis	3	1 (33)	0 (0)	0 (0)
Vibrionaceae				
Aeromonas caviae	2	2 (100)	2 (100)	2 (100)
Aeromonas hydrophila	1	1 (100)	1 (100)	1 (100)
Aeromonas sobria	1	1 (100)	1 (100)	1 (100)
Plesiomonas shigelloides	1	1 (100)	1 (100)	1 (100)
Vibrio cholerae	9	4 (44)	0 (0)	0 (0)
Vibrio parahaemolyticus	4	0 (0)	0 (0)	0 (0)
Vibrio (other species)	17	10 (59)	4 (24)	4 (24)
Nonfermentative strains				
Acinetobacter spp.	26	2 (8)	0 (0)	0 (0)
Burkholderia cepacia	34	13 (38)	18 (53)	1 (3)
Pseudomonas aeruginosa	74	72 (97)	70 (95)	0 (0)
Ralstonia spp.	19	1 (5)	0 (0)	1 (5)
Stenotrophomonas maltophilia	2	2 (100)	2 (100)	0 (0)
Pandoraea spp.	8	0 (0)	0 (0)	0 (0)
Total no. of strains (% positive)	543	402 (74)	394 (72)	290 (53)

substrates have the advantage that black colonies are very easy to detect in mixed cultures and the black nondiffusible chelates formed can be used to mask other chromogenic reactions. This feature has been exploited in the design of chromogenic media (Perry *et al.* 1999).

In conclusion, all three β-ribosidase substrates showed widespread activity with Gram-negative bacteria. Both chromogenic ribosides were readily hydrolysed to yield brightly coloured complexes that remained restricted to bacterial colonies, allowing clear distinction between

β-ribosidase positive and negative strains. Furthermore, there was no detectable inhibition of bacterial growth resulting from the addition of these substrates to agar-based media. While a number of potential applications of these substrates have been highlighted, other applications, perhaps outside clinical microbiology, may yet be recognized.

REFERENCES

Chiu-Machado, I., Castro-Palomino, J.C., Madrazo-Alonso, O., Lopetegui-Palacios, C. and Verez-Bencomo, V. (1995) Synthesis of ribofuranosides by catalysis with lewis acids. Glycosidation versus transacylation. *Journal of Carbohydrate Chemistry* **14**, 551–556.

Codd, A.A., Richardson, I.R. and Andrews, N. (1998) Lenticules for the control of quantitative methods in food microbiology. *Journal of Applied Microbiology* **85**, 913–917.

Doléans, F. (1994) A new approach in bacteriology with chromogenic media. *Microbiologia* **10**, 195–202.

Holt, S.J. and Sadler, P.W. (1958) Studies in enzyme cytochemistry II. Synthesis of indigogenic substrates for esterases. *Proceedings of the Royal Society of London. Series B. Biological Sciences.* **148**, 481–494.

James, A.L., Perry, J.D., Ford, M., Armstrong, L. and Gould F.K. (1996) Evaluation of cyclohexenoesculetin-beta-D-galactoside and 8-hydroxyquinoline-beta-D-galactoside as substrates for the detection of beta-galactosidase. *Applied and Environmental Microbiology* **62**, 3868–3870.

Kilian, M. and Bülow, P. (1976) Rapid diagnosis of Enterobacteriaceae I. Detection of bacterial glycosidases. *Acta Pathologica, Microbiologica, et Immunologica Scandinavica* Sect B **84**, 245–251.

Manafi, M., Kneifel, W. and Bascomb, S. (1991) Fluorogenic and chromogenic substrates used in bacterial diagnostics. *Microbiology Reviews* **55**, 335–348.

Perry, D.F. and Quiring, C. (1997) Fundamental aspects of enzyme/chromogenic substrate interactions in agar media formulations for esterase and glycosidase detection in *Salmonella*. In *Salmonella and Salmonellosis* '97 Proceedings pp. 63–70, France: Ploufragan.

Perry, J.D., Ford, M., Taylor, J., Jones, A.L., Freeman, R. and Gould, F.K. (1999) ABC medium, a new chromogenic agar for selective isolation of *Salmonella* spp. *Journal of Clinical Microbiology* **37**, 766–768.

Rambach, A. (1990) New plate medium for facilitated differentiation of *Salmonella* spp. from *Proteus* spp. and other enteric bacteria. *Applied and Environmental Microbiology* **56**, 301–303.

Reinders, R.D., Bijker, P.G., Huis In't Veld, J.H. and Van Knapen, F. (2000) Use of 8-hydroxyquinoline-beta-D-glucuronide for presumptive identification of Shiga toxin-producing *Escherichia coli* O157. *Letters in Applied Microbiology* **30**, 411–414.

Reisner, B.S. and Austin, E.F. (1997) Evaluation of CPS ID2 chromogenic agar as a single medium for urine culture. *Diagnostic Microbiology and Infectious Disease* **28**, 113–117.

Schiemann, D.A. (1979) Synthesis of a selective agar medium for *Yersinia enterocolitica*. *Canadian Journal of Microbiology* **25**, 1298-1304.

Schramm, V.L., Furneaux, R.H., Tyler, P.C. and Clinch, K. (1997) Enzyme detection/assay methods and substrates. Patent Cooperation Treaty International Publication Number WO 97/31008.

Smith, P.A., Mellors, D., Holroyd, A. and Gray, C. (2001) A new chromogenic medium for the isolation of *Listeria* spp. *Letters in Applied Microbiology* **32**, 78–82.

Venkateswaran, K., Murakoshi, A. and Satake, M. (1996) Comparison of commercially available kits with standard methods for the detection of coliforms and *Escherichia coli* in foods. *Applied and Environmental Microbiology* **62**, 2236–2243.