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ORIGINAL ARTICLE

Evaluation of novel fluorogenic substrates for the detection of glycosidases in *Escherichia coli* and enterococci

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Abstract

Aims: Enzyme substrates based on 4-methylumbelliferone are widely used for the detection of *Escherichia coli* and enterococci in water, by detection of β-glucuronidase and β-glucosidase activity respectively. This study aimed to synthesize and evaluate novel umbelliferone-based substrates with improved sensitivity for these two enzymes.

Methods and Results: A novel β-glucuronide derivative based on 6-chloro-4-methylumbelliferone (CMUG) was synthesized and compared with 4-methyl-umbelliferyl-β-D-glucuronide (MUG) using 42 strains of *E. coli* in a modified membrane lauryl sulfate broth. Over 7 h of incubation, the fluorescence generated from the hydrolysis of CMUG by *E. coli* was over twice that from MUG, and all of the 38 glucuronidase-positive strains generated a higher fluorescence with CMUG compared with MUG. Neither substrate caused inhibition of bacterial growth in any of the tested strains. Four β-glucosidase substrates were also synthesized and evaluated in comparison with 4-methylumbelliferyl-β-D-glucoside (MU-GLU) using 42 strains of enterococci in glucose azide broth. The four substrates comprised β-glucoside derivatives of umbelliferone-3-carboxylic acid and its methyl, ethyl and benzyl esters. Glucosides of the methyl, ethyl and benzyl esters of umbelliferone-3-carboxylic acid, were found to be superior to MU-GLU for the detection of enterococci, especially after 18 h of incubation, while umbelliferone-3-carboxylic acid-β-D-glucoside was inferior. However, the variability in detectable β-glucosidase activity among the different strains of enterococci in short-term assays using the three carboxylate esters (7 h incubation) may compromise their use for rapid detection and enumeration of these faecal indicator bacteria.

Conclusions: The β-glucuronidase substrate CMUG appears to be a more promising detection system than the various β-glucosidase substrates tested.

Significance and Impact of the Study: The novel substrate CMUG showed enhanced sensitivity for the detection of β-glucuronidase-producing bacteria such as *E. coli*, with a clear potential for application in rapid assays for the detection of this indicator organism in natural water and other environmental samples.

Introduction

Enzyme substrates based on the fluorescent compound 4-methylumbelliferone (4-MU; 7-hydroxy-4-methylcoumarin) have been widely exploited in diagnostic microbiology. This is because of their low toxicity, ease of hydrolysis and the intense fluorescence generated by the released 4-MU (James 1994). In particular, glycoside

derivatives of 4-MU are widely used for detection of bacterial enzymes such as β-galactosidase, β-glucuronidase and β-glucosidase (Manafi *et al.* 1991; Dealler 1993). Such assays have been widely applied in the water industry as these enzymes provide convenient markers for the detection of coliforms, *Escherichia coli* and enterococci respectively (Berg and Fiksdal 1988; Edberg *et al.* 1988; Clarke *et al.* 1991; Brenner *et al.* 1993; George *et al.* 2000; Caruso *et al.* 2002; Kramer and Liu 2002; Pope *et al.* 2003). These organisms are recognized as valuable indicators of faecal pollution (Anon 2000, 2002; Edberg *et al.* 2000).

A major limitation of 4-MU is its relatively high pK_a of 7·8, which leads to the partial dissociation of the molecule to its highly fluorescent anion at physiological pH values (Koller and Wolfbeis 1985; Wolfbeis *et al.* 1985). This results in a sub-optimal fluorescent signal in microbiological assays unless alkali is added at the end of an assay to amplify the fluorescence (Manafi *et al.* 1991; Gee *et al.* 1999). Other fluorescent coumarins have been synthesized which have lower pK_a values and consequently higher fluorescence intensities at pH 7 (e.g. Goodwin and Kavanagh 1950; Sherman and Robins 1968; Koller and Wolfbeis 1985; Wolfbeis *et al.* 1985). More recently, Chilvers *et al.* (2001) reported the synthesis of a range of such coumarins that were subsequently derivatized as substrates for β-galactosidase. These substrates demonstrated no detectable bacterial growth inhibition and generated a higher fluorescence yield upon hydrolysis by various coliform bacteria. The present study was designed to synthesize novel coumarinic substrates for the detection of β-glucuronidase and β-glucosidase activity in *E. coli* and enterococci respectively, with a view to evaluating their application as fluorogenic markers in microbiological assays for these bacteria.

Materials and methods

Materials

Unless otherwise stated, all chemicals and solvents were obtained from Sigma-Aldrich Chemical Company Ltd, which was also the source for 4-methylumbelliferyl-β-D-glucuronide (MUG) and 4-methylumbelliferyl-β-D-glucoside (MU-GLU). Bacteriological media and peptones were obtained from Oxoid Ltd, Basingstoke, UK. Bacterial strains were obtained from the National Collection of Type Cultures (NCTC, Health Protection Agency Colindale, London, UK) and the American Type Culture Collection (ATCC strains distributed by LGC Promochem, Teddington, UK). Wild strains were obtained from pathological samples in the Microbiology Department, Freeman Hospital, and identified using standard methods: these wild strains are maintained in the culture collection of the Freeman Hospital. In order to obtain bacterial suspensions with densities equivalent to particular McFarland Standard values, a Densimat (bioMérieux, Basingstoke, UK) was used in all experimental procedures. Bacterial growth was measured using an Anthos 2001 spectrophotometric microtitre plate reader (Labtech International Limited, Uckfield, UK) at an absorption wavelength of 690 nm. Fluorescence was measured using a Labtech Biolite F1 fluorescence microtitre plate reader with excitation and emission filters set at 365 and 440 nm, respectively and expressed in terms of relative fluorescence units (RFU). Sterile, flat-bottomed microtitre trays (Bibby Sterilin Limited, Aberbargoed, UK) were used for all assays.

Methods

Synthesis of 6-chloro-4-methylumbelliferone-β-D-glucuronide

The core molecule (6-chloro-4-methylumbelliferone) was synthesized as described previously (Chilvers *et al.* 2001). The glucuronide derivative was then prepared as follows: recrystallized 6-chloro-4-methylumbelliferone (2·1 g, 10 mmol) was powdered and suspended in 40 ml of dichloromethane containing dried, powdered 0·4 nm molecular sieves. A 3-ml portion of 2,4,6-collidine was added to the stirred solution to dissolve the coumarin. After 1 h, 3·5 g of dry silver carbonate was added, followed, after a 30-min interval by methyl 1-bromo-2,3,4-triacetyl-α-glucopyranoside uronate (5·95 g, 15 mmol). The reaction mixture was stirred in the dark at ambient temperature (10–15°C) for 3 days and progress was followed by thin layer chromatography (TLC) (silica gel/ phase solvent ethyl acetate). This mixture was then filtered on a sintered-glass funnel through a bed of coarse silica gel and the product was eluted to completion using 100 ml of dichloromethane. A second filtration through a short column of silica gel gave a pale yellow solution. This was washed with water (1 × 50 ml) to remove soluble silver salts and subsequently with cold 0·5 mol l^{-1} HCl (3 × 50 ml), to remove collidine. The dichloromethane layer was finally washed with sodium bicarbonate (2 × 30 ml) and water, and dried over anhydrous magnesium sulfate.

After filtration dichloromethane was removed under reduced pressure and to the residual solution was added methanol (50 ml) at 30–40°C with swirling. A mass of small crystals of the product soon separated. After swirling these were removed by vacuum filtration, washed with a little methanol and air dried to give 2·6 g of the protected glycoside. The product was homogenous by TLC and was deprotected directly by deacetylation.

To obviate possible hydrolysis/methanolysis of the glycosidic bond, potassium cyanide was initially employed as the methanolysis catalyst. The protected glucuronide was dissolved in 50 ml of dry dichloromethane and an equal volume of methanol added. To the stirred solution was added a suspension of 0·1 g of dried potassium cyanide dissolved in 10 ml methanol. The reaction mixture was stirred overnight, and then set aside at 30°C for 60 h. A viscous gel formed and TLC indicated almost total deacetylation. Solvent was removed under reduced pressure and the resulting powder was agitated with methanol to obtain a filterable suspension. After vacuum filtration the product was air dried. The deacetylated product (1·8 g) was dissolved in 25 ml of 1 : 1 methanol/acetonitrile. To the stirred solution was added 0·5 mol l^{-1} aqueous lithium hydroxide in sufficient amount to raise the pH to 12·0. Stirring was continued for 4 h with addition of extra alkali to maintain the pH at 12 during the period of hydrolysis. To the alkaline solution were added portions of washed amberlite IR120 (H^+) with stirring until the pH fell to around 8·0. The filtrate and resin washings were combined and evaporated under reduced pressure to give a pale fawn solid which after washing with 50 ml of diethyl ether was removed by vacuum filtration and dried *in vacuo*. TLC indicated only baseline material and no original core molecule. The yield was 1·42 g.

Synthesis of coumarinic glucosides

Four coumarins were synthesized for subsequent derivatization as β-glucosides. Methyl umbelliferone-3-carboxylate, ethyl umbelliferone-3-carboxylate and benzyl umbelliferone-3-carboxylate were synthesized by a Knoevenagel condensation of 2,4-dihydroxybezaldehyde with dimethyl malonate, diethyl malonate or dibenzyl malonate respectively, and umbelliferone-3-carboxylic acid was prepared by hydrolysis of ethyl umbelliferone-3-carboxylate, all as described by Chilvers *et al.* (2001). Glycosidation of all four coumarins was performed as described by Chilvers *et al.* (2001) using α-acetobromo-D-glucose in place of α-acetobromo-D-galactose. Figure 1 and Table 1 illustrate the structures of all of the coumarins used in this study.

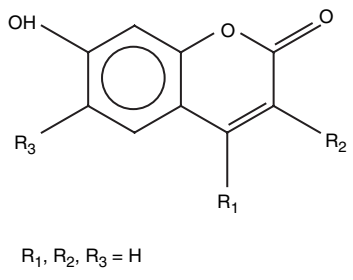

Figure 1 Structure of umbelliferone (7-hydroxycoumarin).

Table 1 Umbelliferone derivatives derivatized to form glycosides

Derivative	R_1	R_2	R_3
4-Methylumbelliferone	CH_3	H	H
Umbelliferone-3-carboxylic acid	H	COOH	H
Methyl umbelliferone-3-carboxylate	H	$COOCH_3$	H
Ethyl umbelliferone-3-carboxylate	H	$COOC_2H_5$	H
Benzyl umbelliferone-3-carboxylate	H	$COOCH_2C_6H_5$	H
6-Chloro-4-methylumbelliferone	CH_3	H	Cl

Evaluation of 6-chloro-4-methylumbelliferyl-β-D-glucuronide (CMUG)

This substrate was evaluated in comparison with 4-methylumbelliferyl-β-D-glucuronide (MUG) with 42 *E. coli* strains. The four strains from the National Collection of Type Cultures (NCTC), Colindale, UK, comprised NCTC 11560, 9001, 10418 and 12079 while the 38 wild strains had been previously recovered from pathological samples and formally identified as *E. coli* using standard biochemical tests. *Providencia rettgeri* (NCTC 7475), *Klebsiella pneumoniae* (NCTC 10896), *Enterobacter cloacae* (NCTC 11936), *Proteus mirabilis* (NCTC 10975), *Pseudomonas aeruginosa* (NCTC 10662), *Serratia marcescens* (NCTC 10211), *Citrobacter freundii* (NCTC 9750) and *Hafnia alvei* (NCTC 8105) were also tested as β-glucuronidase-negative controls.

Assays were performed in a modification of the UK reference method (Anon 2002) membrane lauryl sulfate broth (mMLSB) that excluded lactose and phenol red. The composition of the medium was: proteose peptone (39 g), yeast extract (6 g) and sodium lauryl sulfate (1 g) per litre of deionized water. Once the ingredients were dissolved, the pH was adjusted to pH 7·4 and the medium sterilized by filtration.

An amount equivalent to 0·01 mmol of each glucuronide substrate was weighed out and dissolved in 10 ml of mMLSB. Bacteria were harvested from Columbia agar after 18 h of incubation at 37°C and suspended in mMLSB to a density equivalent to a McFarland Standard of 1·0 and then diluted (1 : 300) in mMLSB to give a suspension density of approx. 10^6 CFU ml^{-1}. Plate counts were performed to confirm bacterial numbers.

A 50-μl aliquot of each bacterial suspension was then added to an equal volume of substrate solution in mMLSB within a microtitre well. All tests were performed in triplicate and appropriate substrate-free and organism-free control wells were also included. Trays were incubated with shaking at 37°C for 7 h, and absorbance (690 nm) and fluorescence (365/440 nm) were measured

every 30 min. Trays were then incubated overnight at 37°C without shaking, to record an 18-h reading of both fluorescence and absorbance. All data are expressed as mean values based on triplicate assays.

Evaluation of coumarinic glucosides

The four glucoside substrates were evaluated in comparison with MU-GLU with 42 strains of enterococci. The strains obtained from National Culture Collections comprised *Enterococcus faecalis* (NCTC 775, 5957, 8132, 12697, 10927), *Enterococcus faecium* (NCTC 7171, 12952), *Enterococcus casseliflavus* (NCTC 12361), *Enterococcus durans* (NCTC 8307), *Enterococcus hirae* (ATCC 10541), *Enterococcus gallinarum* (NCTC 11428), *Enterococcus mundti* (NCTC 12363), *Enterococcus raffinosus* (NCTC 13192). The wild strains comprised 20 strains of *E. faecalis* and nine strains of *E. faecium* from pathological samples, formally identified to species level using conventional biochemical tests (all wild strains are maintained within the culture collection of the Microbiology Department, Freeman Hospital). *Streptococcus oralis* (NCTC 11427), *Streptococcus agalactiae* (NCTC 8181), *Streptococcus gordonii* (NCTC 7865), *Streptococcus salivarius* (NCTC 8618) and *Streptococcus sanguis* (NCTC 7863) were included as β-glucosidase-negative controls.

Assays were performed in UK reference method (Anon 1994) glucose azide broth (GAB) comprising beef extract (9 g), tryptone (3 g), glucose (15 g), sodium chloride (15 g) and sodium azide (0·4 g) per litre of deionized water. Once the ingredients were dissolved, the pH was adjusted to pH 7·2 and the medium sterilized by filtration. An amount equivalent to 0·01 mmol of each substrate was weighed out and dissolved in 10 ml of GAB. Bacterial strains were harvested from Columbia blood agar after 18 h incubation at 37°C and suspended in GAB to a density equivalent to a McFarland Standard of 1·0 and then diluted (1 : 30) in GAB to a suspension density of approx. 10^7 CFU ml^{-1}. Plate counts were performed to confirm bacterial numbers. Assays were performed in triplicate and reported as mean values, exactly as for *E. coli*.

Results

All strains of *E. coli* showed growth in mMLSB over the 18-h incubation period apart from *E. coli* NCTC 10418, which was inhibited by this selective medium and therefore did not demonstrate β-glucuronidase activity. Of the remaining 41 strains growing in mMLSB, neither CMUG nor MUG produced any measurable inhibitory effect on growth. Figure 2a shows a representative example, for growth (absorbance) of *E. coli* strain 34: after an initial lag period of around 2 h, all three curves showed broadly

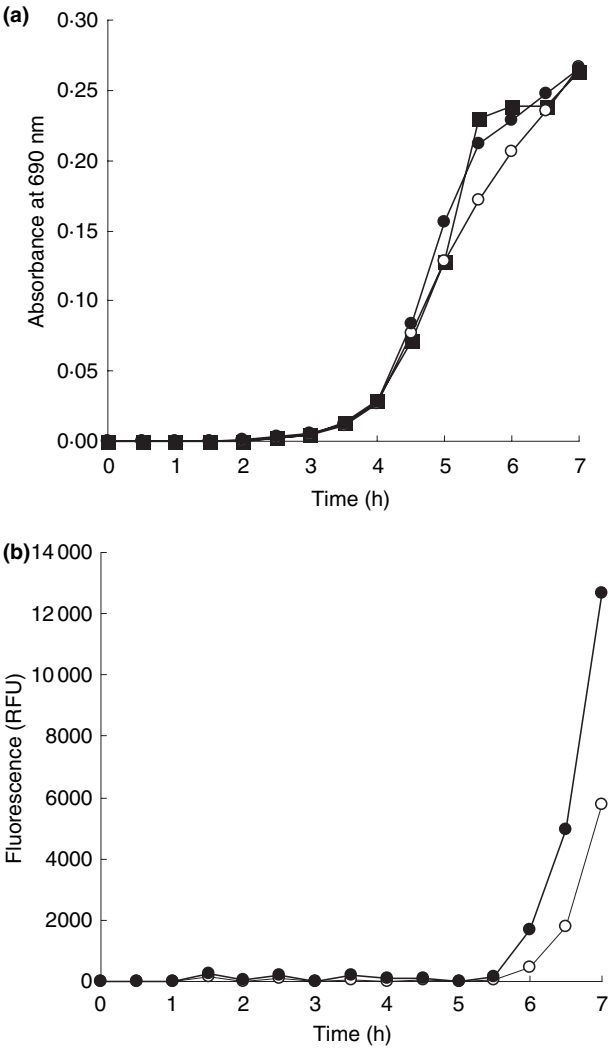

Figure 2 Absorbance and fluorescence increases in *Escherichia coli* strain 34 in membrane lauryl sulfate broth medium with and without fluorogenic substrates: (a) absorbance at 690 nm over a 7-h incubation period; (b) equivalent data for fluorescence (relative fluorescence units, RFU). (●) 6-chloro-4-methylumbelliferyl-β-D-glucuronide; (○) 4-methylumbelliferyl-β-D-glucuronide; (■) substrate-free control.

similar absorbance increases up to 7 h. Figure 2b shows the development of fluorescence due to β-glucuronidase activity in this strain; both CMUG and MUG showed no substantial fluorescence until 6 h, with higher values for CMUG between 6 and 7 h. Similar results were obtained with the other β-glucuronidase-positive strains (data not shown), demonstrating that initial growth was followed by detectable enzyme activity only several hours later.

Three of the strains of *E. coli* grew well but failed to show hydrolysis activity with either substrate within 18 h of incubation, confirming their lack of detectable β-glucuronidase enzyme activity. These included two of 38 (7·9 %) wild strains (12 and 40) and *E. coli* NCTC 12079.

The latter is a strain of *E. coli* serotype O157 in which β-glucuronidase is typically absent (Thompson *et al.* 1990). Strains of *E. coli* that fail to demonstrate β-glucuronidase are not uncommon and these findings are consistent with those of Martins *et al.* (1993) who examined 435 strains and demonstrated that 7·6% of strains failed to demonstrate enzyme activity in MUG-based media.

All eight strains of the non-*E. coli* species tested showed no measurable increase in fluorescence over the 18-h incubation period with both substrates, indicating an absence of β-glucuronidase activity in these bacteria. Of the remaining 38 *E. coli* strains a total of 33 exhibited β-glucuronidase activity with both substrates at 7 h while all 38 strains showed activity at 18 h. Of the 33 strains showing positive activity for β-glucuronidase at 7 h, all gave a higher fluorescence with CMUG compared with MUG (Fig. 3a). While there was a broad spread of fluorescence values at 7 h between the different strains, the fluorescence increase due to CMUG was generally around twice that of MUG, at an overall average of 11 995 RFU (SD = 6070) and 5389 RFU (SD = 2678) respectively. A paired *t*-test ($n = 33$) on the data shown in Fig. 3a gave $t = 10{\cdot}85$ and $P = 2 \times 10^{-12}$ demonstrating a highly significant difference between the results for the two substrates at 7 h.

The trend was less clear-cut at 18 h (Fig. 3b); thus, while the majority of the 38 strains gave a slightly higher fluorescence with CMUG, a few strains showed the opposite response (Fig. 3b). Overall, while there was less variability in the fluorescence intensities obtained with individual strains at 18 h compared with 7 h and the average values for both substrates were broadly similar, at 22 535 RFU for CMUG and 21 310 RFU for MUG, a paired *t*-test ($n = 38$) still demonstrated a statistically significant difference, though with a smaller *t* value (4·64) and *P* value ($4{\cdot}3 \times 10^{-5}$) than at 7 h. Taken together, these results indicate that CMUG appears to be a promising substrate for the detection of β-glucuronidase-producing *E. coli*, especially in short-term assays, where it outperforms MUG (Fig. 3a).

The results obtained using the four novel β-glucosidase substrates were more complex to interpret than those for β-glucuronidase. Thus all 42 test strains of enterococci grew in GAB and there was no evidence of any growth inhibition attributable to the presence of fluorogenic substrates. Figure 4a shows a representative data set for growth (absorbance) of *E. faecalis* strain EFA6. While growth was detectable slightly earlier in the presence of the four novel substrates than with MU-GLU or the control, a similar overall trend was observed with no evidence of growth inhibition in the presence of any of the β-glucosidase substrates. Equivalent data for the development of fluorescence in *E. faecalis* strain EFA6 are shown in Fig. 4b. The three carboxylate ester substrates developed strong positive fluorescence signals between 4 and 7 h, in contrast to umbelliferone-3-carboxylic acid-β-D-glucoside and MU-GLU which showed no evidence of positive fluorescence during the initial 7-h period.

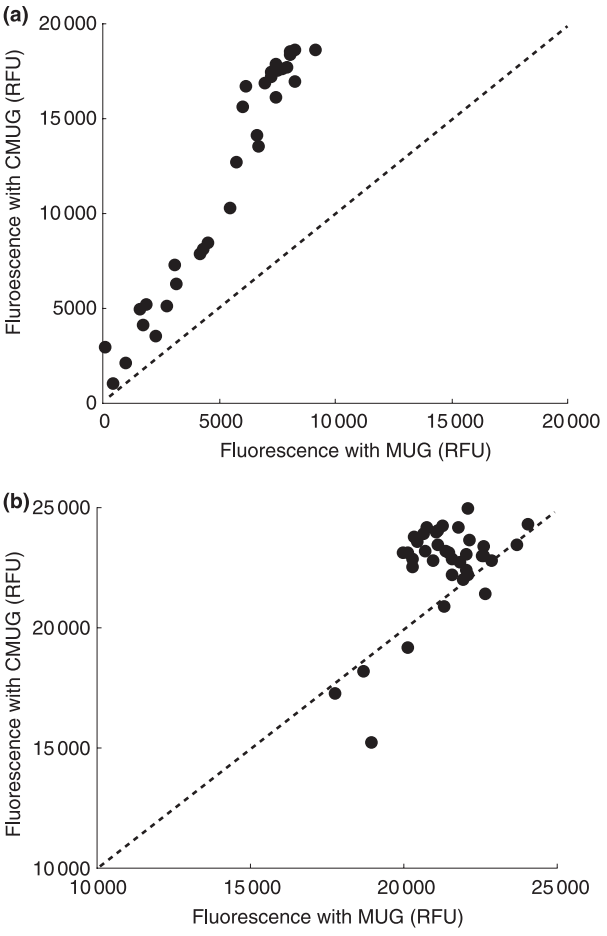

Figure 3 Comparative fluorescence (relative fluorescence units, RFU) of two β-glucuronidase substrates for 38 strains of *Escherichia coli* in membrane lauryl sulfate broth medium: (a) data for 6-chloro-4-methylumbelliferyl-β-D-glucuronide, CMUG (0·5 mmol l^{-1}), and 4-methylumbelliferyl-β-D-glucuronide, MUG (0·5 mmol l^{-1}), following 7-h incubation; (b) data following 18-h incubation. Each dotted diagonal shows the line of equivalence, predicted where both substrates produce the same response.

When the data set for all 42 strains was examined in detail, a total of 31 strains were found to be positive for β-glucosidase activity at 7 h with one or more of the substrates, with the remaining 11 strains (all *E. faecium*) gave no detectable fluorescence with any substrate at 7 h. Figure 5a shows the data for benzyl umbelliferone-3-carboxylate-β-D-glucoside in comparison with MU-GLU, while equivalent plots for ethyl umbelliferone-3-carboxylate-β-D-glucoside, methyl umbelliferone-3-carboxylate-β-D-glucoside and umbelliferone-3-carboxylic acid-β-D-glucoside are shown in Fig. 5b–d respectively.

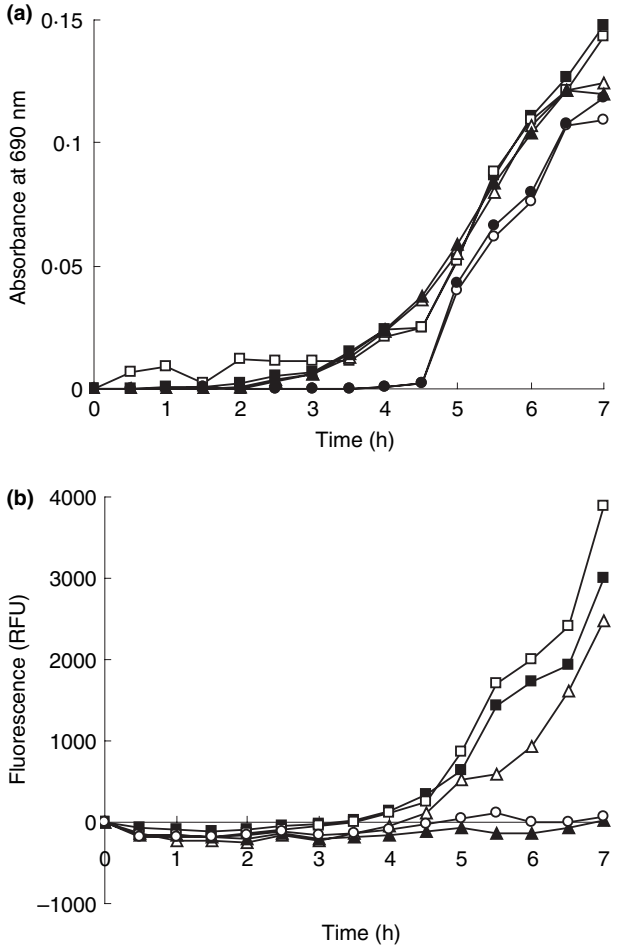

Figure 4 Absorbance and fluorescence increases in *Enterococcus faecalis* strain EFA6 in glucose azide broth medium with and without fluorogenic substrates: (a) absorbance at 690 nm over a 7-h incubation period; (b) equivalent data for fluorescence (relative fluorescence units, RFU). (■), Benzyl umbelliferone-3-carboxylate-β-D-glucoside; (□), ethyl umbelliferone-3-carboxylate-β-D-glucoside; (△), methyl umbelliferone-3-carboxylate-β-D-glucoside; (▲), umbelliferone-3-carboxylic acid-β-D-glucoside; (○), 4-methylumbelliferyl-β-D-glucoside; (●), substrate-free control.

The three carboxylate esters (Fig. 5a–c) showed a similar overall pattern, with most strains showing a greater fluorescence with the novel substrate, compared with MU-GLU. However, there was a far greater variability in the level of detectable fluorescence than was observed for *E. coli*/β-glucuronidase (cf. Fig. 3a); thus while a few enterococci gave strong fluorescence at 7 h with all substrates, many of the strains gave far lower fluorescence values, including several strains which gave low positive fluorescence values with the carboxylate substrates but not with MU-GLU, with the latter substrate generating a positive fluorescence value for a minority of strains at 7 h. The average level of fluorescence generated after 7 h was 3725 for benzyl umbelliferone-3-carboxylate-β-D-glucoside (27 strains positive at 7 h), 2833 for ethyl umbelliferone-3-carboxylate-β-D-glucoside (27 strains positive at 7 h) and 2299 for methyl umbelliferone-3-carboxylate-β-D-glucoside (25 strains positive at 7 h), compared with 294 for MU-GLU (only eight strains positive at 7 h). These average values have been compared using a paired *t*-test ($n = 31$), giving the following results: $t = 2{\cdot}20$ ($P = 0{\cdot}035$) for benzyl umbelliferone-3-carboxylate-β-D-glucoside; $t = 3{\cdot}10$ ($P = 0{\cdot}0042$) for ethyl umbelliferone-3-carboxylate-β-D-glucoside; and $t = 2{\cdot}52$ ($P = 0{\cdot}017$) for methyl umbelliferone-3-carboxylate-β-D-glucoside, showing that all three novel carboxylate substrates gave a statistically higher value compared with MU-GLU. Umbelliferone-3-carboxylic acid-β-D-glucoside appeared to be less effective that MU-GLU for the detection of β-glucosidase activity (Fig. 5d), with only five strains positive at 7 h and giving an overall average fluorescence increase of only 37 RFU, compared with 294 RFU for MU-GLU. However, a paired *t*-test on this data set ($n = 31$) gave $t = 1{\cdot}27$ ($P = 0{\cdot}21$), showing that the difference between these two substrates was not statistically significant. Nevertheless, Fig. 5d suggests that umbelliferone-3-carboxylic acid-β-D-glucoside may be less effective than MU-GLU in those few strains showing strong β-glucosidase activity at 7 h, indicating that this substrate may be less useful than the carboxylate substrates in short-term assays.

At 18 h all 42 strains of enterococci showed evidence of β-glucosidase activity with the control substrate MU-GLU (Fig. 6). However, the average fluorescence increase of 481 RFU was not high especially when compared with some of the other substrates, especially the carboxylate substrates benzyl umbelliferone-3-carboxylate-β-D-glucoside (Fig. 6a), giving a mean fluorescence of 4931 RFU ($n = 42$), $t = 2{\cdot}78$ ($P = 0{\cdot}008$); ethyl umbelliferone-3-carboxylate-β-D-glucoside (Fig. 6b), with a mean fluorescence of 4121 RFU ($n = 42$), and $t = 3{\cdot}60$ ($P = 0{\cdot}0008$) and methyl umbelliferone-3-carboxylate-β-D-glucoside (Fig. 6c), with a mean fluorescence of 3928 RFU ($n = 42$), $t = 3{\cdot}19$ ($P = 0{\cdot}003$). These results demonstrate that the fluorescence obtained with all three carboxylate substrates was significantly higher than that for MU-GLU. However, a similar broad range of fluorescence values was observed at 18 h as at 7 h (cf. Figs 5a–c and 6a–c).

Figure 6d shows the data for fluorescence due to umbelliferone-3-carboxylic acid-β-D-glucoside compared with MU-GLU at 18 h. Overall, the two substrates gave broadly equivalent values for most of the strains, with an overall average fluorescence of 569 for umbelliferone-3-carboxylic acid-β-D-glucoside and 481 RFU for MU-GLU, giving $t = 0{\cdot}999$ ($n = 42$) and $P = 0{\cdot}324$ (no significant difference).

All five strains of *Streptococcus* spp. tested produced a slight overall decrease in fluorescence over the 18-h incu-

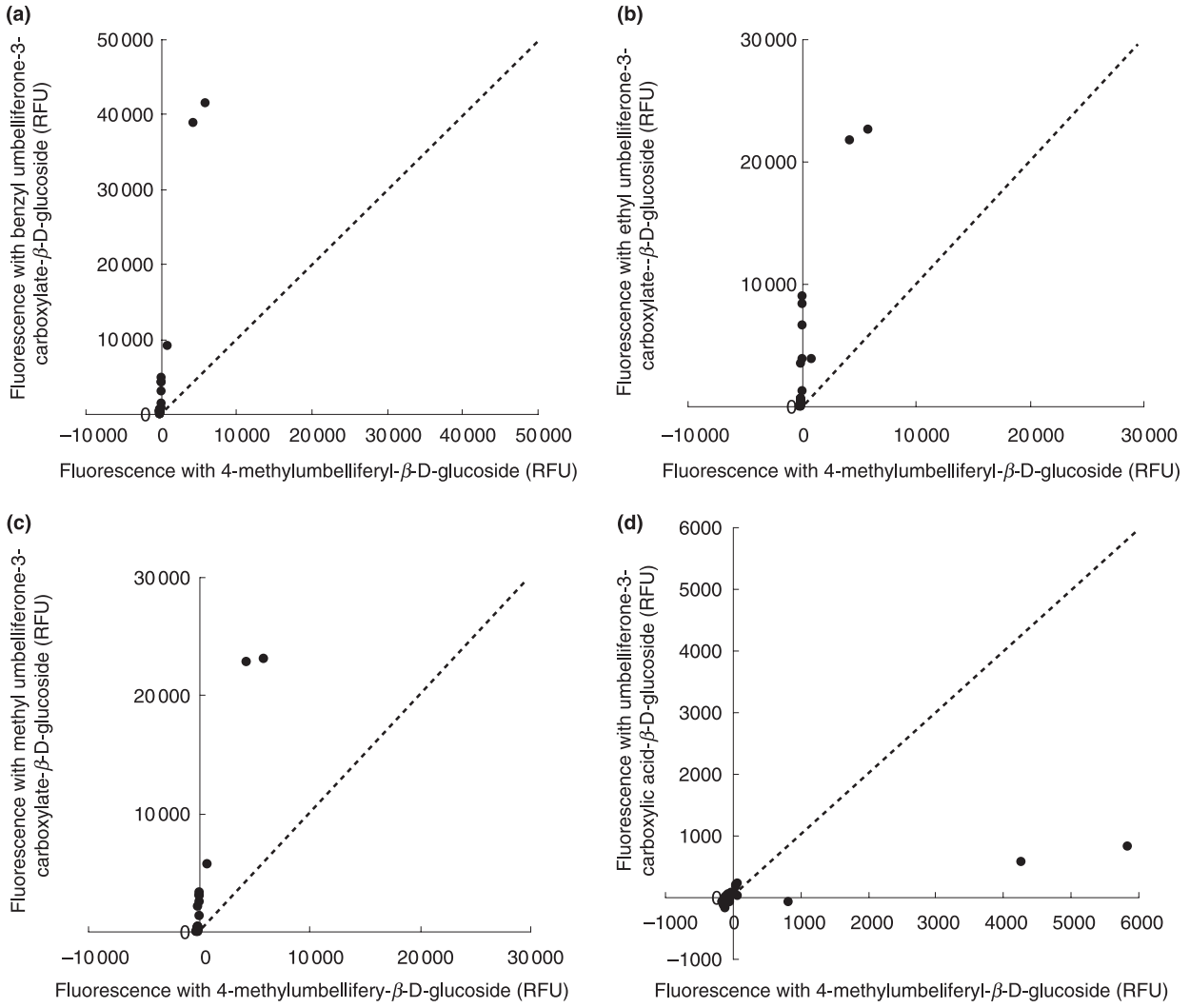

Figure 5 Comparative fluorescence (relative fluorescence units, RFU) of four novel β-glucuronidase substrates compared with 4-methylumbelliferyl-β-D-glucuronide for 31 strains of enterococci in glucose azide broth medium (7 h incubation): (a) data for benzyl umbelliferone-3-carboxylate-β-D-glucoside (0·5 mmol l^{-1}) against 4-methylumbelliferyl-β-D-glucuronide (0·5 mmol l^{-1}); (b) equivalent data for ethyl umbelliferone-3-carboxylate-β-D-glucoside; (c) methyl umbelliferone-3-carboxylate-β-D-glucoside; and (d) umbelliferone-3-carboxylic acid-β-D-glucoside. Each dotted diagonal shows the line of equivalence, predicted where both substrates produce the same response.

bation period (because of quenching) with all substrates, indicating an absence of β-glucosidase activity.

Discussion

Glycosides of 4-MU remain the most widely used fluorogenic substrates in diagnostic microbiology. Such substrates have provided useful tools for the rapid detection and quantification of faecal indicator bacteria both by membrane filtration techniques (e.g. Berg and Fiksdal 1988; Brenner *et al.* 1993), and by 'most probable number' methodologies (e.g. Kramer and Liu 2002). However, the limitations of 4-MU are well-recognized (Manafi *et al.* 1991) and several studies have described the synthesis of novel umbelliferones with lower pK_a values (Goodwin and Kavanagh 1950; Sherman and Robins 1968; Koller and Wolfbeis 1985; Wolfbeis *et al.* 1985). Substrates based on such fluorophores have been shown to be particularly advantageous for the assay of enzymes that have a non-alkaline pH optimum (Tsvetkova *et al.* 1996; Gee *et al.* 1999). Chilvers *et al.* (2001) described a range of coumarins with pK_a values lower than that of 4-MU that were derivatized to form effective substrates for the detection of bacterial β-galactosidase in coliform bacteria. Several of the coumarin molecules described in that study have now been used to synthesize substrates for the detection of

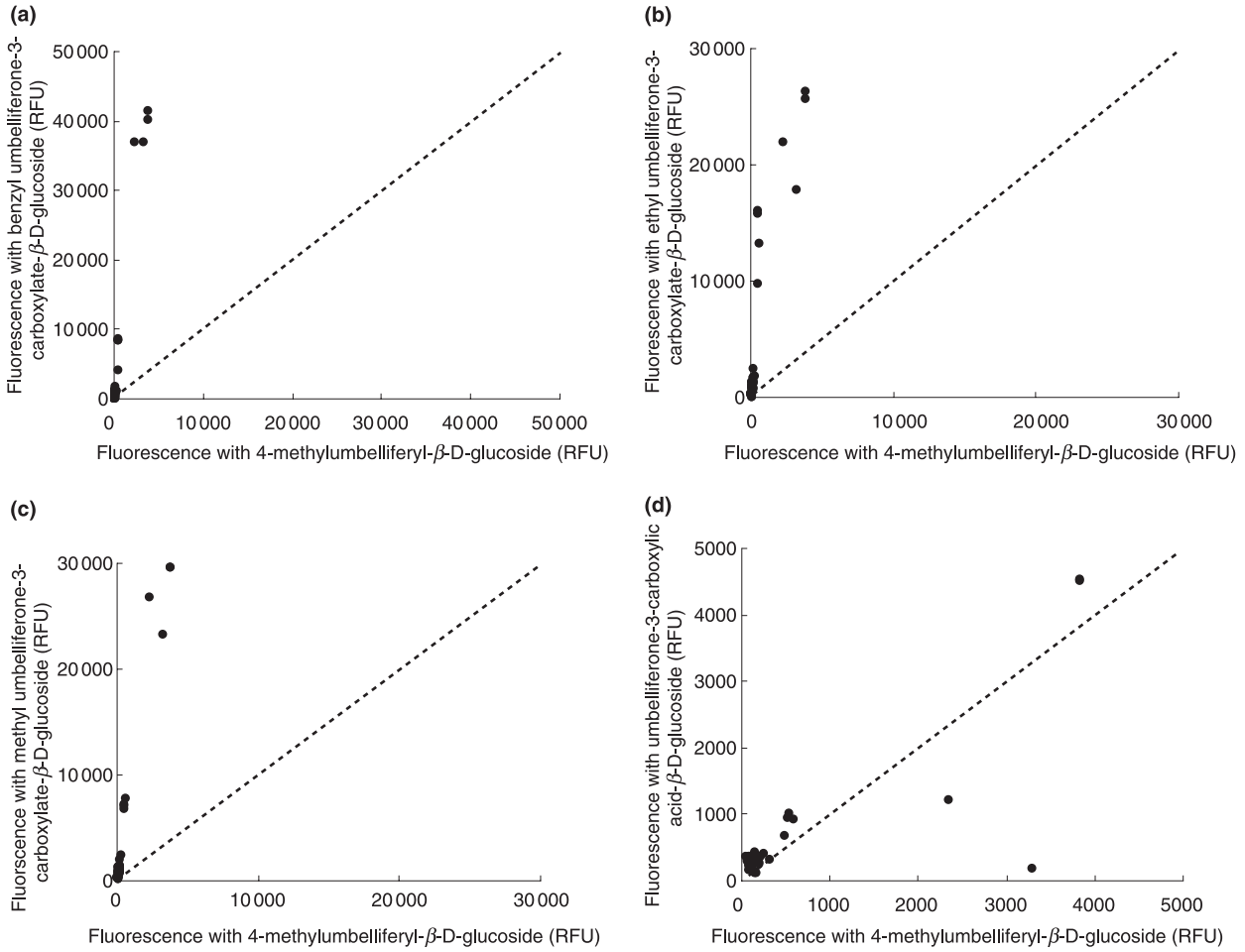

Figure 6 Comparative fluorescence (relative fluorescence units, RFU) of four novel β-glucuronidase substrates compared with 4-methylumbelliferyl-β-D-glucuronide for 42 strains of enterococci in glucose azide broth medium (18 h incubation): (a) data for benzyl umbelliferone-3-carboxylate-β-D-glucoside (0·5 mmol l^{-1}) against 4-methylumbelliferyl-β-D-glucuronide (0·5 mmol l^{-1}); (b) equivalent data for ethyl umbelliferone-3-carboxylate-β-D-glucoside; (c) methyl umbelliferone-3-carboxylate-β-D-glucoside; and (d) umbelliferone-3-carboxylic acid-β-D-glucoside. Each dotted diagonal shows the line of equivalence, predicted where both substrates produce the same response.

β-glucuronidase and β-glucosidase activity in *E. coli* and enterococci respectively.

In the present study, CMUG proved to be effective for the detection of β-glucuronidase-producing *E. coli*. The average increase in fluorescence generated by such strains from CMUG was over twice that generated from MUG after an incubation period of 7 h. The likely explanation for this observation is the higher fluorescence of the released 6-chloro-4-methylumbelliferone at pH 7·4 due to its lower pK_a (Chilvers *et al.* 2001). As CMUG offers the potential for increased sensitivity in the detection of faecal indicator organisms in natural waters, the next step will be to test this substrate in rapid assay most probable number format for the detection of *E. coli*.

We have also described the synthesis and evaluation of coumarinic glucosides that have enhanced activity for the detection of β-glucosidase-producing enterococci. While some of these novel glucoside derivatives were found to generate levels of fluorescence over 10 times higher than those produced using MU-GLU when tested in GAB over an incubation period of 7 h, the variability in β-glucosidase activity observed for different strains of enterococci (Fig. 5) and in particular the poor response noted at 7 h for all tested strains of *E. faecium* may compromise their use in short-term assays, as a substantial biomass is clearly required to achieve a detectable response with many strains of enterococci (e.g. Fig. 6). This observation is consistent with the findings of Adcock and Saint (2001), who reported that broth-based β-glucosidase assays may underestimate the true number of enterococci in natural waters, even after 24 h incubation.

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