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Glycosides of fluorinated *p*-nitrophenol offer improved sensitivity for detection of β-galactosidase and β-glucuronidase in *Escherichia coli* and other Enterobacterales

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A B S T R A C T

We describe the synthesis and evaluation of six halogenated nitrophenyl glycosides for detection of β-galactosidase and β-glucuronidase enzyme activity among Enterobacterales ("coliforms") and *Escherichia coli*, respectively. These were evaluated alongside the established substrates; *o*-nitrophenyl-β-D-galactopyranoside (ONPG), *p*-nitrophenyl-β-D-galactopyranoside (PNPG) and *p*-nitrophenyl-β-D-glucuronide (PNP-GUR). The evaluation was performed using 30 isolates of Enterobacterales including 19 isolates of *E. coli*. Hydrolysis of 2-fluoro-*p*-nitrophenyl-β-D-galactopyranoside (2-fluoro-PNPG) yielded a significantly stronger yellow coloration after a six-hour incubation period compared to hydrolysis of ONPG and PNPG, potentially allowing for a more sensitive detection of Enterobacterales. Similarly, hydrolysis of the novel substrate 2-fluoro-*p*-nitrophenyl-β-D-glucuronide sodium salt (2-fluoro-PNP-GUR Na) by producers of β-glucuronidase also yielded a significantly stronger yellow colouration, potentially allowing for a more sensitive detection of *E. coli*. The yellow chromophore 2-fluoro-PNP retained high colour intensity at reduced pH when compared to *o*-nitrophenol and *p*-nitrophenol. Both substrates potentially offer enhanced sensitivity for the detection of Enterobacterales and *E. coli* in environmental samples as markers of faecal pollution.

o-Nitrophenyl-β-D-galactopyranoside (ONPG) has been used for several decades for detection of β-galactosidase in *Escherichia coli* and other members of the Enterobacterales family.[1,2] These bacteria reside in the gut of animals, including humans, and are commonly termed 'coliforms'. Consequently, they serve as useful 'indicator organisms' to signal the presence of pollution or contamination from faecal material. Detection of β-galactosidase is a useful marker for the presence of such contamination and ONPG has been employed in qualitative and quantitative assays to exclude the presence of coliforms in, for example, water samples.[3–5] ONPG is almost colourless, but when hydrolysed by β-galactosidase, yellow *o*-nitrophenol is liberated indicating the presence of the enzyme. *o*-Nitrophenol appears yellow when in its ionised form (the *o*-nitrophenolate) but is colourless when protonated (e.g. under acidic conditions). Because *o*-nitrophenol has a pKa of 7.2, this means that within the pH range required for optimal growth of Enterobacterales (6.0–7.5),[6] the intensity of the yellow colour may be significantly reduced by some degree of protonation.

Many species of coliforms reside in the gut of a range of animals and some species are naturally found in the environment,[7] so their presence does not have high specificity as a predictive marker of human faecal pollution. The presence of *E. coli* is a more specific indicator of faecal contamination and waterborne pathogens that cause diarrhoea,[8] and it is recommended as such by the World Health Organization.[9] The production of β-glucuronidase is a feature of around 94% of all *E. coli* isolates[10] and this enzyme is generally not produced by other strains of Enterobacterales, except some *Shigella*. Hence chromogenic substrates to detect β-glucuronidase, including those based on nitrophenol, are of potential interest for detecting *E. coli* in environmental samples.[5,11,12]

In this study, we describe the synthesis and evaluation of six chromogenic substrates based on glycosides of halogenated nitrophenol (see Fig. 1) for detection of β-galactosidase and β-glucuronidase produced by Enterobacterales ('coliforms') and *E. coli* respectively, and we compare their performance with standard nitrophenolic substrates.

Nineteen strains of *E. coli* were included in the study. These comprised two strains obtained from the National Collection of Type Cultures (NCTC), Colindale, UK: *E. coli* NCTC 10418 and *E. coli* NCTC 12241 plus 17 isolates of *E. coli* recovered from routine culture of anonymised stool samples obtained from leftover samples referred to the

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Freeman Hospital Microbiology Department for routine culture. Each stool sample was from a distinct patient. Species identification was confirmed by matrix-assisted laser desorption ionisation–time of flight mass spectrometry (MALDI-TOF MS; Bruker, Coventry, UK).

We also utilised 11 strains from the NCTC representing other species of Enterobacterales, including *Citrobacter freundii* NCTC 9750, *Enterobacter cloacae* NCTC 11936, *Hafnia alvei* NCTC 6578, *Klebsiella aerogenes* NCTC 9777, *Klebsiella pneumoniae* NCTC 13368, *Klebsiella terrigena* NCTC 13038, *Providencia rettgeri* NCTC 7475, *Salmonella* Indiana NCTC 11304, *Serratia marcescens* NCTC 10211, *Shigella sonnei* NCTC 8586 and *Yersinia enterocolitica* NCTC 11176.

Four commercially available standard substrates were kindly provided by Glycosynth, Warrington, UK: *o*-nitrophenyl-β-D-galactopyranoside (ONPG), *p*-nitrophenyl-β-D-galactopyranoside (PNPG), *p*-nitrophenyl-β-D-glucuronide free acid (PNP-GUR), and *p*-nitrophenyl-β-D-glucuronide sodium salt (PNP-GUR Na). The synthesis and structure verification of six halogenated substrates is described in the supplementary material. These included 2-chloro-*p*-nitrophenyl-β-D-galactopyranoside (2-chloro-PNPG), 2-fluoro-*p*-nitrophenyl-β-D-galactopyranoside (2-fluoro-PNPG), 3-fluoro-*p*-nitrophenyl-β-D-galactopyranoside (3-fluoro-PNPG), 2-chloro-*p*-nitrophenyl-β-D-glucuronide sodium salt (2-chloro-PNP-GUR Na), 2-fluoro-*p*-nitrophenyl-β-D-glucuronide sodium salt (2-fluoro-PNP-GUR Na) and 3-fluoro-*p*-nitrophenyl-β-D-glucuronide sodium salt (3-fluoro-PNP-GUR Na).

The release of nitrophenol from chromogenic substrates as a means of detecting enzymatic activity was monitored either visually (for simple qualitative tests) or by measuring absorbance at 405 nm (for quantitative measurement). For the latter, an increase in absorbance at any wavelength will also occur as a result of turbidity due to bacterial growth (irrespective of the presence of any enzyme substrates). In order to measure the nitrophenol produced without interference from bacterial growth, the absorbance increase at 690 nm (attributable solely to bacterial growth) was subtracted from any increase at 405 nm. Also, the readings at both wavelengths obtained at 'time zero' were subtracted from all other readings. These 'adjusted' readings were utilised in Figs. 2–6.

In a preliminary experiment, a subset of eight Enterobacterales was tested with PNPG and three halogenated derivatives. All strains except *P. rettgeri* produced a yellow coloration after overnight incubation attributable to β-galactosidase production. Fig. 2 shows the absorbance generated at 405 nm attributable to hydrolysis of the four substrates over 6 h of incubation. *Y. enterocolitica* was not detectable as a β-galactosidase producer after 6 h incubation with any substrate. For the other six β-galactosidase producers, the strongest signal was observed from the halogenated substrates. In particular, for four of these six species, maximum absorbance was observed with 2-fluoro-PNPG. Moreover, the absorbance generated from 2-fluoro-PNPG was 9-fold higher than that generated from PNPG for these six species.

As a result of the preliminary experiment, PNPG and 2-fluoro-PNPG were evaluated with all 30 isolates of Enterobacterales. ONPG was also included for comparison. After 18 h incubation, all 30 Enterobacterales showed a yellow coloration due to hydrolysis of all three β-galactosidase substrates, except for *P. rettgeri*, which is consistent with the lack of this enzyme in this species. Fig. 3 shows the absorbance generated at 405 nm attributable to hydrolysis of the three substrates over 6 h of incubation for species other than *E. coli*. For nine out of ten strains that produced β-galactosidase, the highest readings were generated from 2-fluoro-PNPG, and for six of these strains the absorbance produced with 2-fluoro-PNPG was over twice that generated from either ONPG or PNPG. The data for *E. coli* are shown in Fig. 4 and show that all strains generated absorbance from 2-fluoro-PNPG that was over twice that produced with PNPG and, on average, almost six times higher (x 5.93) than that produced from ONPG.

After 4 h incubation, the average absorbance attributable to

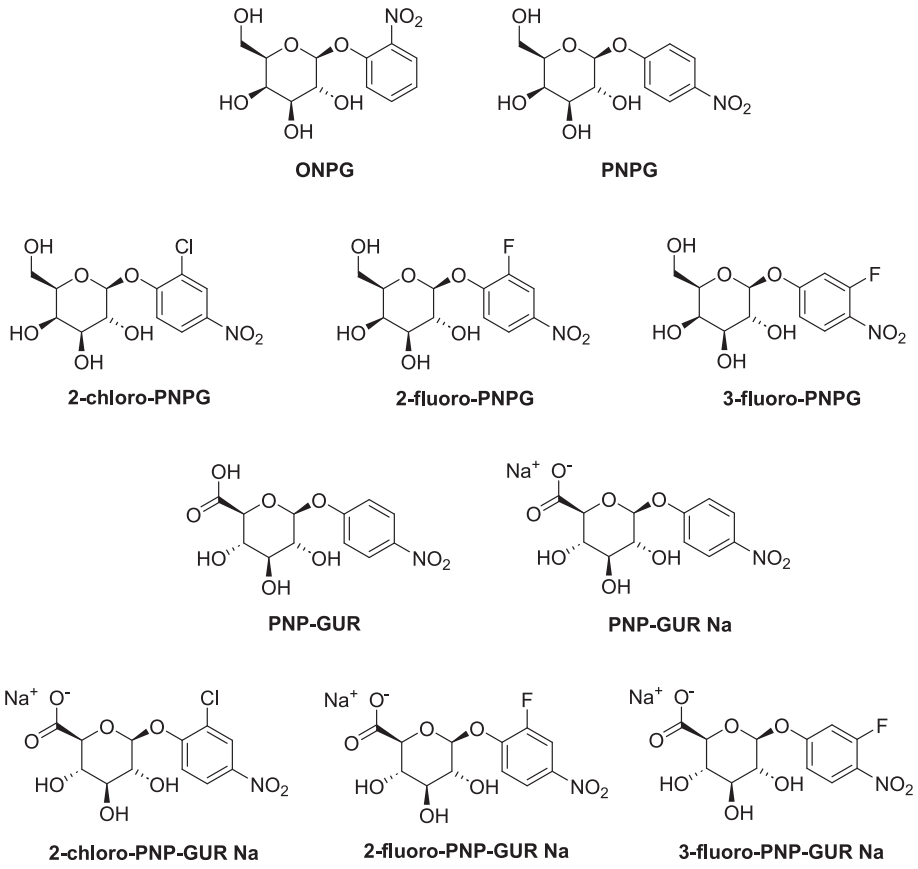

Fig. 1. Structures of chromogenic substrates based on nitrophenol.

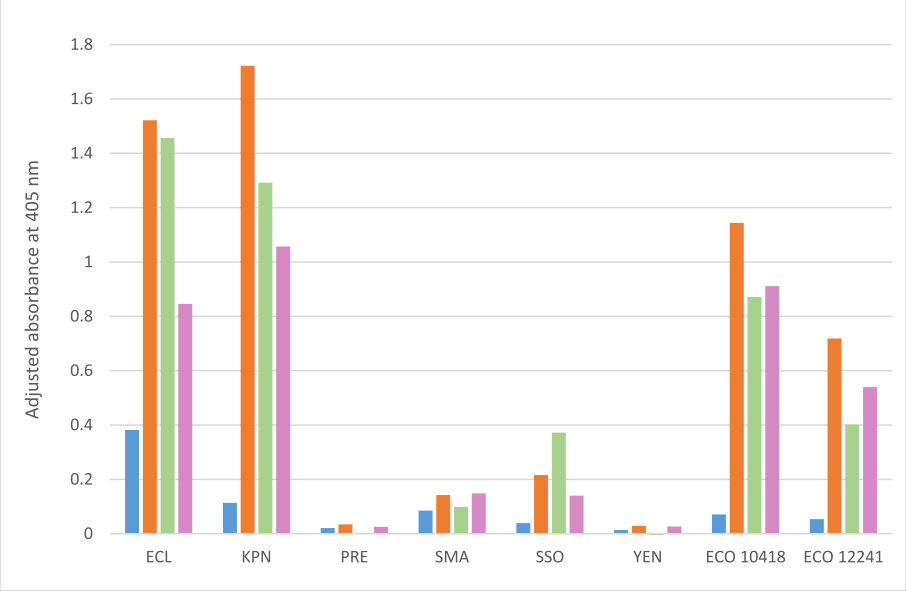

Fig. 2. Average increases in absorbance at 405 nm by eight strains of Enterobacterales after 6 h incubation in the presence of four nitrophenolic substrates for β-galactosidase (PNPG, blue bars; 2-fluoro-PNPG, orange bars; 2-chloro-PNPG, green bars, and 3-fluoro-PNPG, pink bars). The strains include, *E. cloacae* (ECL), *K. pneumoniae* (KPN), *P. rettgeri* (PRE), *S. marcescens* (SMA), *S. sonnei* (SSO), *Y. enterocolitica* (YEN), *E. coli* (ECO) NCTC 10418 and *E. coli* (ECO) NCTC 12241. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

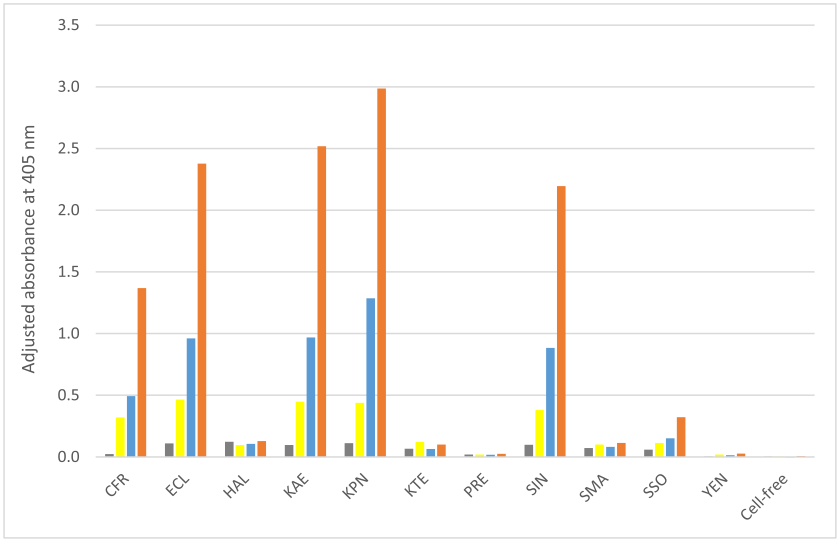

Fig. 3. Average increases in absorbance at 405 nm by eleven strains of Enterobacterales after 6 h incubation in the presence of three nitrophenolic substrates for β-galactosidase (ONPG, yellow bars; PNPG, blue bars; 2-fluoro-PNPG, orange bars, and substrate-free control, grey bars). The strains include *C. freundii* (CFR), *E. cloacae* (ECL), *H. alvei* (HAL), *K. aerogenes* (KAE), *K. pneumoniae* (KPN), *K. terrigena* (KTE), *P. rettgeri* (PRE), *S. Indiana* (SIN), *S. marcescens* (SMA), *S. sonnei* (SSO) and *Y. enterocolitica* (YEN). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hydrolysis of 2-fluoro-PNPG by all 29 Enterobacterales that produced β-galactosidase was significantly higher than that from ONPG ($Q = 5.75$; $p = 0.00032$). In pairwise comparisons after 6 h incubation, the average absorbance with 2-fluoro-PNPG was significantly higher than both ONPG ($Q = 12.67$; $p < 0.00001$) and PNPG ($Q = 9.18$ $p < 0.00001$). The higher sensitivity of 2-fluoro-PNPG has the potential to offer a faster detection of Enterobacterales than the other two substrates. For example, using an arbitrary cut-off of an increase in absorbance of 0.05 to define positivity after 4 h incubation resulted in 15/29 isolates detected using 2-fluoro-PNPG, 8/29 using PNPG, and only 1/29 positive isolates using ONPG.

In a preliminary experiment, a subset of eight *E. coli* was tested with PNP-GUR and three halogenated derivatives. All strains except EC6 and EC7 produced a yellow coloration after overnight incubation attributable to β-glucuronidase production. Fig. 5 shows the absorbance generated at 405 nm attributable to hydrolysis of the four substrates over 6 h of incubation. For five out of six strains that produced β-glucuronidase, the highest readings were generated from 2-fluoro-PNP-GUR Na, and this compound was selected for further testing. In contrast, PNP-GUR (a free acid) was noticeable by its poor performance. This was attributed to the lower pH of the medium when compared to that of the three halo-PNP-glucuronides, which were present in their sodium salt forms. PNP-GUR in the form of its free acid is the most readily available product of commerce and the one overwhelmingly encountered in research. However, in order to provide a better comparison with the performance of 2-fluoro-PNP-GUR Na, its sodium salt

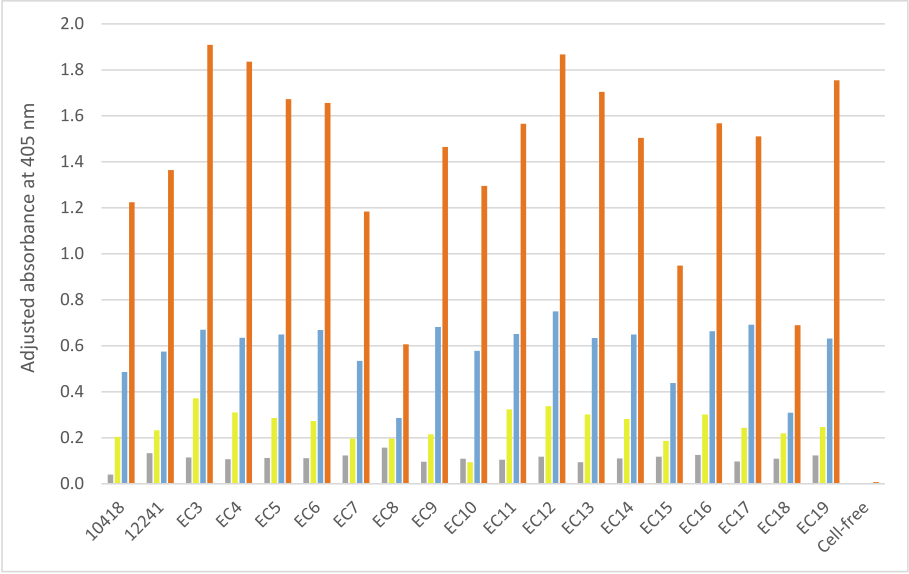

Fig. 4. Average increases in absorbance at 405 nm by 19 isolates of *E. coli* after 6 h incubation in the presence of three nitrophenolic substrates for β-galactosidase (ONPG, yellow bars; PNPG, blue bars; 2-fluoro-PNPG, orange bars, and substrate-free control, grey bars). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

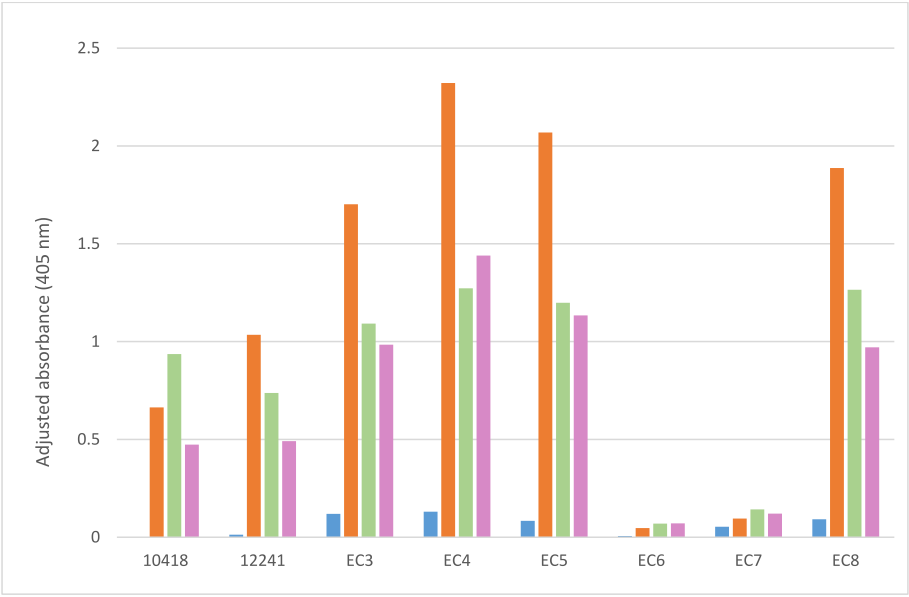

Fig. 5. Average increases in absorbance at 405 nm by eight strains of *E. coli* after 6 h incubation in the presence of four nitrophenolic substrates for β-glucuronidase (PNP-GUR, blue bars; 2-fluoro-PNP-GUR Na, orange bars; 2-chloro-PNP-GUR Na, green bars, and 3-fluoro-PNP-GUR Na, pink bars). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

form, PNP-GUR Na, was used for subsequent additional testing.

As a result of the preliminary experiment with halogenated glucuronides, PNP-GUR Na and 2-fluoro-PNP-GUR Na were evaluated with all 30 isolates of Enterobacterales. After 18 h incubation, 16/19 isolates of *E. coli* showed a visible yellow coloration due to hydrolysis of both β-glucuronidase substrates and *S. sonnei* produced a weak yellow coloration. The remaining 13 Enterobacterales (including three *E. coli* isolates) remained colourless with both substrates, indicating lack of β-glucuronidase activity. Fig. 6 shows the absorbance generated at 405 nm attributable to hydrolysis of both substrates after 6 h incubation by the 17 isolates that produced β-glucuronidase and three *E. coli* isolates lacking β-glucuronidase (EC6, EC7, and EC10). For the isolates with β-glucuronidase activity, the average absorbance was higher after 6 h when tested with 2-fluoro-PNP-GUR Na ($p < 0.00001$), however there was no statistical difference between the two substrates after 4 h incubation ($p = 0.67$). The 10 other isolates of Enterobacterales without β-glucuronidase not included in Fig. 6 had absorbance increases of <0.24 with both substrates over the same incubation period.

Additionally, fig. S1 (provided in the supplementary information) shows the average growth rate of the 30 isolates of Enterobacterales with three chromogenic substrates for β-galactosidase (ONPG, PNPG, and 2-fluoro-PNPG), while fig. S2 shows the average growth rate of these microorganisms with two chromogenic substrates for β-glucuronidase (PNP-GUR Na and 2-fluoro-PNP-GUR). Growth in the presence of ONPG was equivalent to that of the substrate-free control. However, there was evidence of a slight attenuation of growth rate when

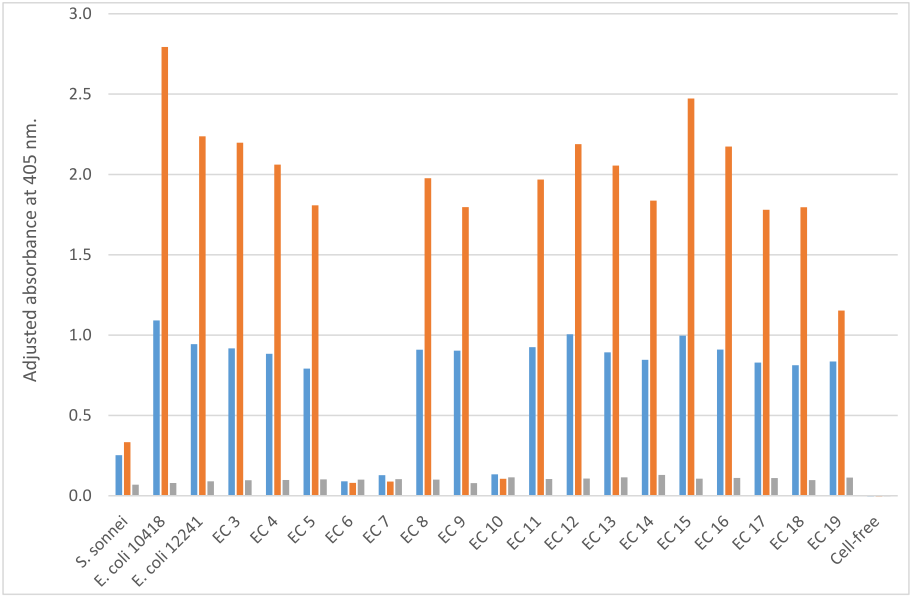

Fig. 6. Average increases in absorbance at 405 nm by *S. sonnei* and nineteen isolates of *E. coli* after 6 h incubation in the presence of two nitrophenolic substrates for β-glucuronidase (PNP-GUR Na, blue bars; 2-fluoro-PNP-GUR Na, orange bars, and substrate-free control, grey bars). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

glycosides of either PNP or 2-fluoro-PNP were present.

Subculture of broth from all wells after 18 h incubation yielded a pure growth of the expected species for 27/30 Enterobacterales when incubated with 2-fluoro-PNPG. In three cases, *E. coli* NCTC 10418, *E. cloacae* NCTC 11936 and *K. pneumoniae* NCTC 13368, no growth was recovered from the wells after subculture despite high readings at 690 nm indicating growth, and a strong yellow coloration attributable to β-galactosidase activity. Expected growth was recovered for all strains when subcultured from substrate-free wells, and all wells containing ONPG, PNPG, PNP-GUR Na, and 2-fluoro-PNP-GUR Na.

Fig. 7 shows that above pH 6, ONP had a significantly lower absorbance than either of the other two chromophores when solutions of 0.09 mM were compared. At pH 8.0, the absorbance of PNP and 2-fluoro-PNP were comparable but the absorbance of PNP declined sharply in relation to decreasing pH. This resulted in an absorbance of 2-fluoro-PNP that was more than double that of PNP at pH 6.5.

Chromogenic enzyme substrates based on nitrophenol are still widely used in diagnostic microbiology, particularly for the detection of Enterobacterales ('coliforms') in water samples. This is exemplified by the defined substrate technology that employs a combination of ONPG and the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) to achieve the simultaneous enumeration of coliforms and *E. coli*.[13] Such methods are cost-effective, easy to use, and lend themselves to automation.[14] One disadvantage of ONP as a chromogen is its relative lack of colour intensity, and for PNP there is a sharp reduction in colour as pH decreases. When coliforms hydrolyse glycosides such as ONPG or PNP-GUR they release galactose and glucuronic acid respectively that can subsequently be fermented to generate organic acids, thus potentially lowering the pH. We have shown in this study how two substrates based on 2-fluoro-PNP are hydrolysed to generate a chromophore with stronger colour intensity than that given by ONP or PNP. At least part of the reason for this higher colour intensity is likely to be the relative stability of the colour as pH decreases, due to the lower pKa of 2-fluoro-PNP (6.03) compared with PNP (7.15). Other factors might

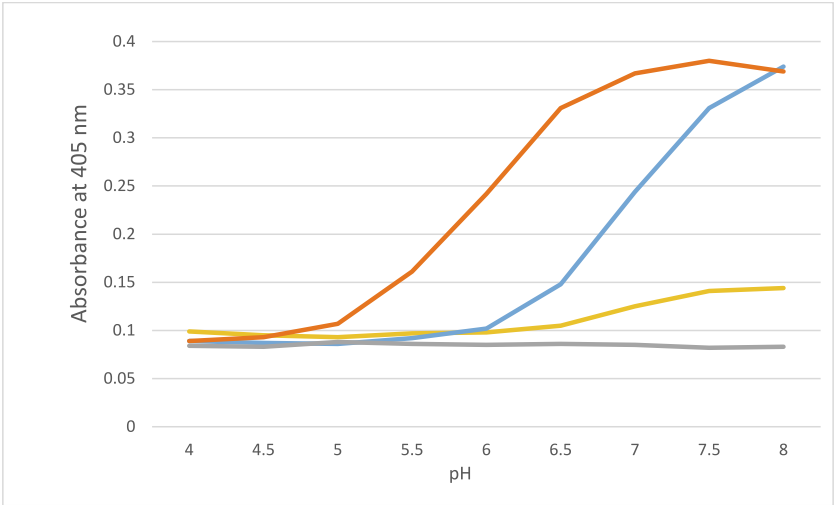

Fig. 7. Average absorbance at 405 nm of ONP (yellow line), PNP (blue line), and 2-fluoro-PNP (orange line) at a range of different pH values compared to peptone water without additive (grey line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

also be relevant including the relative rate of hydrolysis of the different substrates by bacterial glycosidases. We have shown that 2-fluoro-PNPG may allow for the earlier detection of coliforms, when compared with the traditional substrates ONPG and PNPG.

One possible drawback of the use of 2-fluoro-PNPG for detection of β-galactosidase was the fact that 3 out of 30 Enterobacterales appeared to undergo an 'auto-sterilisation' where no viable bacteria could be recovered after 18 h incubation. We hypothesize this may be due to an accumulation of 2-fluoro-PNP exerting a toxic effect against isolates that have strong β-galactosidase activity and/or enhanced susceptibility to 2-fluoro-PNP. It is not clear why this phenomenon was not observed when testing 2-fluoro-PNP-GUR Na. β-Galactosides of halogenated phenols have been used previously as 'suicide substrates' to selectively inhibit strong producers of β-galactosidase, such as *E. coli*, and allow the growth of target pathogens such as *Shigella* and *Salmonella*, in which β-galactosidase activity is weak or absent. For example, Park et al., described the use of 4-chloro-2-cyclopentylphenyl-β-D-galactopyranoside to selectively inhibit *E. coli* and allow the growth of *Shigella* species.[15] More recently, triclosan-β-D-galactopyranoside has been shown to have strong antibacterial activity against a range of Enterobacterales.[16] The issue of auto-sterilisation with 2-fluoro-PNPG with a small minority of β-galactosidase-producing isolates is only a problem if confirmatory tests (requiring subculture) are required on tubes or wells showing positive results.

We have described the synthesis and evaluation of halogenated nitrophenyl glycosides for the detection of coliforms and *E. coli* that show some utility in diagnostic microbiology. There are no previous reports of these substrates for detection of microorganisms, furthermore, the fluorinated derivatives of PNP-glucuronide sodium salts are entirely novel. Further work is required with actual environmental samples, alongside standard methods, to demonstrate any 'real-world' advantage of such substrates.

CRediT authorship contribution statement

Michael Burton: Writing – review & editing, Funding acquisition, Conceptualization. **Chris Cains:** Investigation. **Danielle J.C. Fenwick:** Investigation. **Amy Foster:** Investigation. **Clair L. Preece:** Investigation. **Sidrah Saleem:** Investigation. **Stephen P. Stanforth:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Conceptualization. **Hayley J. Turner:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization. **Graeme Turnbull:** Writing – review & editing, Writing – original draft, Supervision, Methodology. **John D. Perry:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Graeme Turnbull reports financial support and writing assistance were provided by Glycosynth Ltd. The work presented in this manuscript was conducted during PhD studentships funded wholly by Glycosynth Ltd. and carried out at Glycosynth Ltd., Northumbria University, and Freeman Hospital. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2026.130573.

Data availability

All experimental data is provided in the manuscript and associated supplementary information.

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