Hydrolysis of aromatic β-glucosides by non-pathogenic bacteria confers a chemical weapon against predators

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Bacteria present in natural environments such as soil have evolved multiple strategies to escape predation. We report that natural isolates of Enterobacteriaceae that actively hydrolyze plant-derived aromatic β-glucosides such as salicin, arbutin and esculin, are able to avoid predation by the bacteriovorous amoeba Dictyostelium discoideum and nematodes of multiple genera belonging to the family Rhabditidae. This advantage can be observed under laboratory culture conditions as well as in the soil environment. The aglycone moiety released by the hydrolysis of β-glucosides is toxic to predators and acts via the dopaminergic receptor Dop-1 in the case of Caenorhabditis elegans. While soil isolates of nematodes belonging to the family Rhabditidae are repelled by the aglycone, laboratory strains and natural isolates of Caenorhabditis sp. are attracted to the compound, mediated by receptors that are independent of Dop-1, leading to their death. The β-glucosides–positive (Bgl++) bacteria that are otherwise non-pathogenic can obtain additional nutrients from the dead predators, thereby switching their role from prey to predator. This study also offers an evolutionary explanation for the retention by bacteria of ‘cryptic’ or ‘silent’ genetic systems such as the bgl operon.

1. Introduction

Bacteria form an integral part of food webs in different ecological niches and face challenges from a range of predators and parasites such as protozoa, nematodes and phages. Predation plays a major role in bacterial mortality in soil, freshwater and marine ecosystems. Fitness of bacteria in their natural habitats hence depends not only on growth and reproduction but also on their ability to defend themselves against natural predators [1]. Bacteria have evolved multiple strategies to combat predators which include increased size, motility [2], secondary metabolite production [3] and biofilm formation [1].

Despite selection pressure to maintain genomes composed predominantly of functional genes, bacteria carry genes that are silent and uninducible under most laboratory growth conditions. Their maintenance in a cryptic state without the accumulation of deleterious mutations is an evolutionary puzzle [4]. The well-studied β-glucoside (bgl) operon, present in E. coli [5] and other Gram-negative bacteria, is one such silent genetic system ([6] and references therein). Upon mutational activation, the bgl operon enables the catabolism of aromatic β-glucosides such as salicin, arbutin and esculin. Composed of an aromatic moiety linked to glucose via a β-glycosidic bond, these compounds are produced by plants as secondary metabolites. Bacteria that show a Bgl++ phenotype can derive energy from the glucose released by the hydrolysis of β-glucosides.

Unlike the members of Enterobacteriaceae present in the gut environment that predominantly show a Bgl− phenotype [7], this study shows that many of their counterparts present in the soil can use the aromatic β-glucosides salicin and arbutin as a carbon source. Characterization of the bgl operon in Klebsiella aerogenes, primarily a soil organism, revealed the loss of the negative elements
in the regulatory region of the operon, leading to a higher basal level of expression of the bgl genes [8]. This niche-specific difference in the pattern of β-glucoside utilization is consistent with the possibility that plant-derived β-glucosides are more likely to be encountered in the soil.

Plant secondary metabolites such as salicin often serve as a defensive tool against herbivores [9]. Whether bacteria that are able to metabolize aromatic β-glucosides and derive energy can also use these compounds for defence against predators, along the same lines as plants, is an intriguing possibility.

The present study was initiated to find a possible link between β-glucoside utilization and defence from predation in members of Enterobacteriaceae. Both laboratory strains and natural isolates of bacteria, comprising β-glucoside positive and negative strains, were selected for the study. Bacteriovorous nematode strains including natural soil nematodes as well as laboratory strains of Caenorhabditis elegans, and the amoeba Dictyostelium discoideum were used as predators, and predator–prey interaction was investigated in the context of β-glucoside metabolism.

2. Material and methods

(a) Strains and media

The bacterial strains used in the study are listed in table 1 and the electronic supplementary material, table S1. The D. discoideum wild-type strain NC4 was grown in standard medium (SM) (10 g l⁻¹ glucose, 10 g l⁻¹ peptone, 1 g l⁻¹ yeast extract, 1 g l⁻¹ MgSO₄.7H₂O, 2.25 g l⁻¹ KH₂PO₄, 0.66 g l⁻¹ K₂HPO₄, pH 6.4) with Klebsiella aerogenes. The axenic strain Ax2 was grown in SM medium with Klebsiella or axenically in HL5 medium (15.4 g l⁻¹ glucose, 14.3 g l⁻¹ Difco proteose peptone, 7.15 g l⁻¹ Difco yeast extract, 0.49 g l⁻¹ KH₂PO₄, 0.507 g l⁻¹ Na₂HPO₄, pH 6.4). SM/5 medium was prepared as described by Sussman [14]. The C. elegans wild-type strain N2 was grown in nematode growth medium (NGM) plates seeded with E.coli OP50 as described in WormBook available online (www.wormbook.org).

(b) Dictyostelium discoideum viability assay

The protocol for amoeba–bacteria co-culture experiment to monitor viability of D. discoideum was adopted from Sussman [14]. Briefly, bacterial cultures were grown in Luria broth to OD₆₀₀ ≈ 1.0, washed with KK2 buffer (2.25 g l⁻¹ KH₂PO₄ and 0.66 g l⁻¹ K₂HPO₄, pH 6.4) and resuspended in KK2 buffer. The washed culture (100 µl) was added to 10 ml SM/5 medium without glucose. Amoebae were grown either in HL5 (for Ax2) or in SM agar + bacteria (for NC4), washed with KK2, resuspended in the buffer and counted using a haemocytometer. Approximately, 10⁷ amoebae were added to the SM/5 medium containing bacterial cells followed by addition of specific sugars. The flashes were then incubated at 22°C with moderate shaking. Viability of the amoebae was monitored at different time intervals by plating aliquots of equal volumes from the cultures on SM agar plates seeded with the laboratory strain of Klebsiella used as the normal feed for the amoebae. Viable amoebae feed on Klebsiella and form clearings in the bacterial lawn known as plaques. The number of plaques formed at different time points was plotted against time.

Viability of amoebae (as plaques on bacterial lawn) was also checked by plating them on SM plates (without glucose) along with overnight-grown bacteria (both β-glucoside positive and negative members) in the presence or absence of β-glucosides.

(c) Chemotaxis assay

Caenorhabditis elegans chemotaxis assays were performed as described by Zhang et al. [15]. Briefly, Bgl⁺ and Bgl⁻ bacteria were grown in lysogeny broth for 24 h in the presence of glucose or β-glucoside. Approximately 25 µl of the culture (test sample and control) was spotted 6 cm apart on behavioural plates (1.6% agar, 5 mM potassium phosphate, pH 6, 1 mM CaCl₂, 1 mM MgSO₄). The plates were dried for 5 h at room temperature, and 1 µl of 1 M sodium azide (anaesthetic) was added to both the spots a few minutes before the assay to fix the worms.

<table>
<thead>
<tr>
<th>Strains of bacteria, nematodes and amoebae used in the study².</th>
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<tbody>
<tr>
<td><strong>E. coli strains</strong></td>
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<tr>
<td>OP50                        Bgl⁺</td>
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<tr>
<td><strong>Shigella sonnei strains</strong></td>
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<tr>
<td>AK1                        Bgl⁺</td>
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<tr>
<td>AK102                      Bgl⁺/mutant of AK1</td>
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<tr>
<td>AK102Δbgl:kan              Bgl⁺</td>
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<tr>
<td>natural isolates of Enterobacteriaceae</td>
</tr>
<tr>
<td>50a                       Bgl⁺/soil isolate this work</td>
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<tr>
<td>RS-Bgl⁺                   Bgl⁺/soil isolate this work</td>
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<tr>
<td>RS-Bgl⁻                   Bgl⁻/soil isolate this work</td>
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<td><strong>Klebsiella aerogenes</strong></td>
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|                            Bgl⁺                  | See http://dictybase.org/.
| **Dictyostelium discoideum strains**                         |
| NC4                       wild-type             | Th. M. Konijn, University of Leiden |
| Ax2                       axenic mutant of NC4 | [13] and B. Wurster, University of Konstanz |
| **Caenorhabditis elegans strain**                            |
| N2                        bacteriovorous      | [10] |

² Additional strains used in specific experiments are indicated in the electronic supplementary material.
entering the spots. One-day-old adult worms (40–100) were washed with M9 buffer and placed in the behavioural plates equidistant from both bacterial spots. After 1 h, the number of worms in both bacterial spots was counted. The chemotaxis index, which is indicative of the preference of the nematode in terms of the bacterial cultures spotted, was calculated as:

\[
\text{chemotaxis index} = \frac{\text{number of worms in test spot} - \text{number of worms in control spot}}{\text{total number of worms}}.
\]

Chemotaxis assays were also performed using a 32 mM solution of saligenin in ethanol, using the solvent as a control.

(d) Isolation of bacteria, nematodes and amoebae from soil

All soil samples were collected from the campus of the Indian Institute of Science, Bangalore, India, from areas with dense vegetation. The collection was done in the month of May, 2012. Enterobacteriaceae from soil were isolated by serial dilution and plated on an Eosin Methylene Blue (EMB) agar plate. Individual colonies from the EMB agar plate were then patched on MacConkey agar + β-glucose plates to analyse its status of β-glucose utilization. Nematodes were isolated by a centrifugal floatation method as described in WormBook using 1.38 M MgSO₄·7H₂O solution. For the isolation of amoebae, approximately 0.5 g of soil was suspended in 1 ml KK2 buffer, mixed properly and 100 μl of the mixture was spread on SM/5 plates. Plates were then incubated at 22°C, and fruiting bodies of amoebae (cellular slime moulds) were observed after 4–5 days.

(e) Saligenin avoidance assay

This is a modification of C. elegans lawn avoidance assay ([16], electronic supplementary material, methods). Two conditions were created for the behavioural assays. Under the NGM condition, NGM agar plates (6 cm size) containing 28 mM saligenin were prepared. A hole of diameter 2.5 cm was made in the centre and was filled with NGM agar without saligenin. Twenty adult worms were added at the centre. The number of worms in the inner circle was counted after 3 h and plotted as percentage occupancy with respect to the total number of worms added. For the Saligenin condition, NGM agar without saligenin was used to prepare the plates, and NGM saligenin agar was used to fill the hole in the centre.

(f) Nematode viability assay in soil

Autoclaved soil (approx. 10 g) was placed on 6 cm plates. Approximately 100 adult N2 or Oscheitus tipulae worms were inoculated separately in the soil along with bacteria (10⁶ cells of Bgl⁻ or Bgl⁺) with or without addition of salicin (approx. 1% final concentration). Both RS-Bgl⁻ and RS-Bgl⁺ strains used are Enterobacteriaceae members isolated from soil. The Shigella sonnei strains AK1 (Bgl⁻) and AK102 (Bgl⁺) were used as controls. An equal volume of water was added in all the conditions to maintain the moisture. All soil microcosms were processed after 10 days. Nematodes were extracted as described earlier and their numbers were plotted.

3. Results

(a) Bacteria actively hydrolyzing aromatic β-glucosides are toxic to predators

Members of Enterobacteriaceae comprising both β-glucoside degraders and non-degraders (isolated from different niches) were selected to test the effect of catabolism of the aromatic β-glucosides salicin, arbutin and esculin on their predators D. discoideum strains NC4 (wild-type) and Ax2 (an axenic derivative of NC4), and C. elegans (strain N2; table 1).

The effect of salicin metabolism on D. discoideum was monitored by amoeba–bacteria co-culture experiments as described previously ([14] and see §2). NC4 amoebae showed loss of viability when they were grown in the presence of Bgl⁺ bacteria and 35 mM salicin (the concentration of salicin which when used as the sole carbon source allows growth of bacteria; figure 1; electronic supplementary material, figure S1a). Bgl⁻ bacteria along with the same concentration of salicin or Bgl⁺ bacteria growing on glucose were not toxic to the amoebae. No increase in cell count was observed in the presence of salicin alone, as expected, owing to the absence of bacteria as food. Disruption of the bgl operon in the Bgl⁻ strain led to the loss of growth inhibition of NC4 indicating the requirement for salicin catabolism, mediated by the bgl genes, for the toxicity observed. The axenic strain Ax2 showed a similar growth inhibition in the presence of Bgl⁻ bacteria + salicin (see the electronic supplementary material, table S2) and Bgl⁺ bacteria + arbutin (see the electronic supplementary material, figure S1b). Growth arrest of amoebae was also observed when the Bgl⁺ K. aerogenes strain, which is used as the normal food for amoebae in the laboratory, was grown in the presence of a lower concentration of 14 mM salicin or a combination of arbutin, salicin and esculin at concentrations of 3 mM each (see the electronic supplementary material, table S3). These observations indicate that the loss of viability of amoebae is correlated with the simultaneous presence of aromatic β-glucosides and Bgl⁻ bacteria.

The growth inhibition seen in these studies translated to lethality if the exposure of the amoeba to Bgl⁺ bacteria actively hydrolyzing β-glucosides crossed a threshold in terms of time of exposure. They could be rescued from lethality if they were transferred from the toxic environment to normal growth conditions within the threshold of exposure (see below).

The effect of Bgl⁻ bacteria on C. elegans was monitored by a killing assay. Bgl⁻ bacteria were grown for 36 h at 37°C in NGM without glucose but supplemented with 35 mM salicin. One-day-old adult N2 worms were transferred to the bacterial lawn and their viability was monitored. A majority of the nematodes died within a few days on plates containing salicin and Bgl⁻ bacteria (figure 2). Lethality was also observed when eggs were exposed to Bgl⁻ bacteria growing on salicin (see the electronic supplementary material, table S4). In the control plates, i.e. Bgl⁻ bacteria growing on glucose and Bgl⁺ bacteria grown in the presence of salicin, the viability of the worms was unaltered and they showed normal growth and development.

(b) Lethality is due to the aglycone formed during β-glucoside degradation

The observed killing of the amoebae and nematodes could be because Bgl⁻ bacteria are modified and made indestable...
in the presence of salicin, thereby leading to starvation of the predators. Alternatively, lethality could be due to a by-product of salicin hydrolysis. To distinguish between these two possibilities, Bgl$^{\text{+}}$ bacteria were grown in M9 minimal medium containing 35 mM salicin, washed and plated with D. discoideum NC4/Ax2 cells in SM medium. Formation of plaques and subsequent normal development of the amoebae indicated that lethality is not due to modification of the Bgl$^{\text{+}}$ bacteria by growth in the presence of salicin. Similarly C. elegans could also feed on washed Bgl$^{\text{+}}$ bacteria that were pre-grown in minimal salicin medium. These results suggest that death associated with Bgl$^{\text{+}}$ bacteria grown in the presence of salicin is related to the presence of a toxic by-product of salicin hydrolysis. This was confirmed by the observation that the concentrated supernatant obtained from Bgl$^{\text{+}}$ strains grown in the presence of salicin could induce growth inhibition of predators. When present at a final concentration of 0.4 per cent on NGM plates seeded with OP50, one-day-old N2 worms succumbed in around 6 days (see the electronic supplementary material, figure S2). A similar concentration of the supernatant also inhibited the growth of AxA2 cells (see the electronic supplementary material, table S2, last row).

In an attempt to identify the product of saligenin degradation by Bgl$^{\text{+}}$ bacteria, an organic extract of the supernatant of Bgl$^{\text{+}}$ bacterial culture grown in M9 salicin (35 mM) medium was fractionated by thin layer chromatography, and also subjected to NMR analysis (see the electronic supplementary material, methods). Saligenin (2-hydroxybenzyl alcohol) was found to be the major product of salicin hydrolysis (see the electronic supplementary material, figures S3 and S4). Supernatants of Bgl$^{\text{+}}$ and Bgl$^{\text{−}}$ bacterial cultures grown in the presence of salicin were also subjected to saligenin assay [5] to confirm the presence of the aglycone. When compared with a standard curve obtained by plotting saligenin concentrations against OD$_{509}$, 50a, AK102, AK1 and OP50 supernatants showed saligenin concentrations of 28, 25, 0.1 and 0.1 mM, respectively.

To test if saligenin had a direct effect on viability, AxA2 cells were incubated for 12 h in HL5 growth medium containing different concentrations of the compound, and viability of the amoebae was determined as described above. The amoebae exposed to saligenin showed reduced viability with a minimum inhibitory concentration of 24 mM. Similarly, when one-day-old adult N2 worms were placed on NGM bacterial plates with different concentrations of saligenin, lethality was observed at concentrations of 28 mM and above (figure 3).

To determine whether the toxicity associated with saligenin is reversible, adult worms exposed to 28 mM saligenin at different lengths of time were transferred to fresh NGM plates containing OP50. The sick worms exposed to saligenin for 3–4 days recovered, grew and reproduced normally when shifted to non-toxic condition. Longer exposure to saligenin resulted in rapid and irreversible decrease in viability as shown before (figure 3). Similarly, amoebae could be...
rescued after up to 6 h of exposure to 24 mM saligenin by transferring them on to SM plates seeded with bacteria. Thus, the lethal effect of saligenin is reversible within specific thresholds of exposure. Exposure beyond this threshold resulted in permanent loss of viability.

(c) Effect of β-glucoside utilization on predator behaviour

Caenorhabditis elegans is known to exhibit a variety of behavioural responses towards bacteria and chemicals present in the environment [15–17] which help them avoid toxic food or chemicals. To determine whether saligenin metabolism influences the behaviour of C. elegans, chemotaxis assays were performed as described by Zhang et al. (15) and see §2). Specifically, the assay involves monitoring the preference of C. elegans towards bacteria or Bgl\(^{+}\) bacteria when they were grown in salicin medium over Bgl\(^{-}\) bacteria or Bgl\(^{+}\) bacteria grown on glucose (figure 4). Similar results were obtained with arbutin when used in the assay instead of salicin (see the electronic supplementary material, figure S5a). Caenorhabditis elegans was also attracted towards saligenin (dissolved in ethanol at a final concentration of 32 mM) when used in the chemotaxis assay with the solvent as control, indicating that saligenin can act as a chemo-attractant (see the electronic supplementary material, figure S5a). To complement these observations, a lawn avoidance assay was conducted (16) and the electronic supplementary material, methods, which measures bacterial lawn occupancy of C. elegans under different conditions. Caenorhabditis elegans spent more time on plates containing the bacterial strain 50a (Bgl\(^{+}\)) grown on salicin even though it was toxic to them and the occupancy was equivalent to that for the control strain OP50 (Bgl\(^{-}\)). However, they mostly avoided the bacterial lawn of 50a grown on glucose (see the electronic supplementary material, figure S5b). Thus, bacteria that metabolize aromatic β-glucosides are able to attract the nematodes allowing for a greater exposure to the toxic aglycones.

(d) Mechanism of saligenin toxicity

Previous reports have demonstrated local anaesthetic and adrenergic activity of saligenin and its derivatives [18–20]. A search for DNA sequences orthologous to the mouse and rat adrenoceptors in WormBase showed sequences with significant similarity to those encoding multiple receptors in C. elegans such as Dop-1, Ser-5, Ser-7 and Tyra-3. Saligenin toxicity in the nematodes could be equivalent to an overdose of a local anaesthetic and might be mediated by orthologues of adrenergic receptors. In such a case, loss of the receptor is expected to rescue the worms from saligenin toxicity. To address this, viability of various receptor mutants in the presence of saligenin was determined with N2 as the control. Partial rescue of lethality was observed with the dop-1 (dopamine receptor) mutant, suggesting that saligenin might act via dopamine receptors in C. elegans (figure 5). The ser5 mutant appeared to be more sensitive than wild-type for reasons unknown. All mutants showed a comparable lifespan when kept in control plates without saligenin. The dop-1 mutant was also resistant to the anaesthetic action of saligenin. When suspended in 40 mM saligenin for 20 min, only 20 per cent of the dop-1 mutant stopped the thrashing movement as against 90 per cent observed with the wild-type (see the electronic supplementary material, figure S5c). This is consistent with the possibility that saligenin acts via the Dop-1 receptor. However, the mutant did not show any alteration in its chemotactic behaviour towards saligenin, suggesting that the behavioural response observed involves a different receptor and pathway independent of the Dop-1 receptor.

(e) Coexistence of nematodes and amoebae with both Bgl\(^{+}\) and Bgl\(^{-}\) bacteria in the soil

The experiments described so far were carried out with bacterial strains, amoebae and nematodes maintained in the laboratory. In an attempt to gain insights on the ecological significance of the observations, we examined the distribution of

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**Figure 4.** Effect of salicin metabolism on worm behaviour. Chemotaxis assay of N2. The Bgl\(^{+}\) bacterial strains AK102 and 50a were used as the test strains and the Bgl\(^{-}\) strains AK1 and OP50 as the control strains to calculate the chemotaxis index that gives an indication of the degree of attraction or repulsion towards the compound tested. In the case of AK102Sal (AK102 + saligenin) versus AK102Glu (AK102 + glucose), AK102Sal was used as the test condition. \(n = 5\). See §2 for details. (Online version in colour.)

**Figure 5.** Effect of saligenin on the viability of receptor mutants of N2. Survival of receptor mutants (plotted as percentage live worms versus time) was monitored on NGM plates containing 28 mM saligenin and OP50. Approximately 20 one-day-old adults were used in each experiment. \(n = 3\).
bacteria, amoeba and nematodes in the soil from a large number of soil samples (see §2 for details). Bgl$^+$ and Bgl$^-$ bacteria could be detected with comparable frequencies (359 out of 457) in 48 soil samples (see the electronic supplementary material, table S5). Both Bgl$^+$ and Bgl$^-$ bacteria independently coexist with both predators in 17 out of 31 soil samples (see the electronic supplementary material, figure S6). In 11 out of 31 samples, amoebae and nematodes were found to coexist with both Bgl$^+$ and Bgl$^-$ bacteria, consistent with the universal coexistence of predator and prey in the same environment.

(f) Saligenin is toxic to natural isolates of nematodes

In an attempt to extend the observations with laboratory strains to natural isolates, the toxicity studies carried out with C. elegans were repeated with soil nematodes Mesorhabditis sp., O. tipulae and Rhabditis rainai (see the electronic supplementary material, table S6). NGM agar plates with or without 28 mM saligenin were spotted with the E coli strain OP50 and were used as test and control plates, respectively, for the viability assay. Adult worms of natural isolates were added to the plates and their viability was monitored on successive days.

Figure 6. Effect of saligenin on the viability of soil nematodes. Survival of soil nematodes (plotted as percentage live worms versus time) was monitored on NGM plates containing 28 mM saligenin and OP50. Approximately 20 adult nematodes were used in each experiment. $n = 3$. % live worms

Figure 7. Effect of saligenin on the behaviour of soil nematodes. Avoidance assays with soil nematodes were performed as described in §2 and plotted as % occupancy which is inversely related to avoidance. Approximately 20 adults were used for each experiment. $n = 5$. Error bars indicate s.d.

(h) Salicin metabolism by Bgl$^+$ bacteria is toxic to nematodes in soil

In an attempt to simulate the soil environment in the laboratory, salicin was externally added to soil that was seeded with bacteria and nematodes, and the effect of its metabolism on nematodes was analysed in situ (§2). Salicin utilization by Bgl$^+$ bacteria in soil was toxic to nematodes indicated by a decline in nematode count, whereas the count increased in the presence of Bgl$^-$ bacteria (figure 8 and electronic supplementary material, figure S12). Growth of nematodes was also inhibited owing to starvation when no bacteria were added to the soil. These studies indicate that saligenin toxicity can manifest in the soil environment.

4. Discussion

Bacteria are known to adopt multiple strategies to combat predation under different environmental conditions. The results reported here shed light on one such strategy—release of toxic aromatic compounds by the hydrolysis of $\beta$-glucosides by members of Enterobacteriaceae. In contrast to the

(g) Soil nematodes avoid saligenin

It was observed during viability assays that the soil isolates tend to move out of the plates containing saligenin. However, this behaviour was not observed in control plates. This prompted us to monitor the avoidance behaviour of soil nematodes to saligenin (§2). All soil nematodes of genera Oscheius and Mesorhabditis robustly avoid saligenin (figure 7). For instance members of Mesorhabditis could sense saligenin concentrations as low as 4 mM and avoid it (see the electronic supplementary material, figure S9). Even though the plots show some occupancy after 3 h, avoidance behaviour could be seen as early as 15 min. However, no avoidance was observed against hydroquinone and salicin (see the electronic supplementary material, figure S10).

Interestingly, none of the wild isolates of Caenorhabditis avoided saligenin. Instead, they showed a positive chemotaxis towards saligenin similar to N2 in a chemotaxis assay performed as described before (see the electronic supplementary material, figure S11). Hence, members of Enterobacteriaceae exhibit two strategies against their predators via $\beta$-glucoside metabolism; i.e. repulsion of Oscheius and Mesorhabditis species and attraction followed by killing of Caenorhabditis species.
to the general mechanism of self defence. Bacterial species have the capacity to break down aromatic metabolites mediated by a micro-organism. As many soil-dwelling protozoa [22], the results presented in our report are novel as reported the inhibitory action of plant glucosides against predatory agents of mortality of predators. Previous studies have shown in N2. However, this behaviour is short-term and transient in soil and are readily converted to other forms compared with controls (see the electronic supplementary material, figure S13), indicating that bacteria can derive nutrients from the predators they have killed. Thus, despite being non-pathogenic, the ability to metabolize aromatic β-glucosides can transform the role of bacteria from being a prey to predator.

Insensitivity of the dop-1 mutants of C. elegans to saligenin suggests that it acts via the Dop 1 receptor. Many adrenergic agonists like salbutamol and salmeterol bind to their receptors via their saligenin head [23,24]. It has also been observed that the residues responsible for the binding of saligenin to the human adrenergic receptor are conserved in the dop-1 receptor of C. elegans. Saligenin toxicity in the case of D. discoideum is likely to be mediated by a different mechanism, as a genome-wide search did not reveal any orthologues of rat/mouse adrenoceptor sequences.

The sensitivity seen on agar medium could also be observed when the nematodes were exposed to salicin-degrading bacteria in the soil along with salicin, affirming that the observation we are reporting is relevant in the natural context.

In addition to the metabolic advantage gained from the hydrolysis of β-glucosides, the ability to repel or lure predators to their death and thereby gain a nutritional advantage is likely to be a strong selective force to retain the bgl genes in the genome. Expression of the E. coli bgl operon showed a modest increase in the presence of predator/predator culture supernatant, indicating an active response of the bacterium to unknown signals from the predator (see the electronic supplementary material, figure S14). Toxicity genes in bacteria have been shown to respond in the same manner to signals from the predator, which in turn results in the killing of the predator [25].

The toxicity associated with saligenin that we have observed requires a reasonably high concentration of the aglycone. Are such concentrations physiologically relevant? Our attempts to detect β-glucosides in soil were not successful. This could be attributed to the fact that most of the β-glucosides are transient in soil and are readily converted to other forms by biotic or abiotic factors [26]. Compared with other secondary metabolites, β-glucosides are quite abundant in plants, especially in Salicaceae members [9,27,28], and are more likely to be encountered in the soil (derived from decomposing vegetation) than in the animal gut. This is consistent with the niche-specific difference in the pattern of β-glucoside utilization by different members of Enterobacteriaceae mentioned before. Our observations also indicate that in situations where multiple β-glucosides are present in the same environment, the concentrations of individual β-glucosides can be as low as 3 mM (see the electronic supplementary material, table S3).

The distribution of predator and prey analysed in this study showed coexistence of predator and prey in many soil samples. A model based on the interspecies interactions observed is depicted in figure 9. The stable coexistence of predator and prey over a long time range could be related to the oscillatory output [30] expected from the model. As evasion of predators is related to the ability to hydrolyze aromatic β-glucosides that rests with Bgl+ strains, this leads to the interesting questions as to who dies and who benefits in mixed populations that have both Bgl+ and Bgl− strains.

**Figure 8.** Effect of salicin metabolism on nematodes in the soil environment. Soil microcosm experiments were performed as described in §2. Briefly, approximately 10⁸ cells of either RS-Bgl+ or RS-Bgl− bacteria (soil isolates) were mixed with sterile soil along with approximately 100 nematodes (either N2 or O. tipulae). The number of adult nematodes extracted after 10 days were plotted in each case. n = 3. Error bars represent s.d. (Online version in colour.)

antipredatory actions of pathogenic bacteria that have evolved specific tools such as extracellular proteases targeted against predators [21], our studies involved non-pathogenic bacteria that are armed only with genes that enable the uptake and hydrolysis of aromatic β-glucosides. The energy investment is minimal for the bacterium and a part of it could be derived from the glucose generated during the hydrolysis of β-glucosides. Hence, this strategy appears to confer dual advantage for the bacteria as it provides escape from predation in addition to a carbon source for growth. The aglycone molecules produced in the course of hydrolysis of aromatic β-glucosides were found to be the causative agents of mortality of predators. Previous studies have reported the inhibitory action of plant glucosides against protozoa [22]. The results presented in our report are novel as the generation of the toxic aglycone involves catabolic activity mediated by a micro-organism. As many soil-dwelling bacterial species have the capacity to break down aromatic β-glucosides, typified by K. aerogenes [8], this is likely to be a general mechanism of self defence.

The response to saligenin by soil nematodes belonging to the *Mesorhabditis* genus and *O. tipulae* is distinctly different from that exhibited by *Caenorhabditis* species. While the former avoid the compound, the latter are attracted by the compound with lethal consequences. Aversive learning behaviour against toxic chemicals owing to repeated exposure has been observed that the residues responsible for the binding of saligenin to the human adrenergic receptor are conserved in the dop-1 receptor of C. elegans. Saligenin toxicity in the case of D. discoideum is likely to be mediated by a different mechanism, as a genome-wide search did not reveal any orthologues of rat/mouse adrenoceptor sequences.

The sensitivity seen on agar medium could also be observed when the nematodes were exposed to salicin-degrading bacteria in the soil along with salicin, affirming that the observation we are reporting is relevant in the natural context.

In addition to the metabolic advantage gained from the hydrolysis of β-glucosides, the ability to repel or lure predators to their death and thereby gain a nutritional advantage is likely to be a strong selective force to retain the bgl genes in the genome. Expression of the E. coli bgl operon showed a modest increase in the presence of predator/predator culture supernatant, indicating an active response of the bacterium to unknown signals from the predator (see the electronic supplementary material, figure S14). Toxicity genes in bacteria have been shown to respond in the same manner to signals from the predator, which in turn results in the killing of the predator [25].

The toxicity associated with saligenin that we have observed requires a reasonably high concentration of the aglycone. Are such concentrations physiologically relevant? Our attempts to detect β-glucosides in soil were not successful. This could be attributed to the fact that most of the β-glucosides are transient in soil and are readily converted to other forms by biotic or abiotic factors [26]. Compared with other secondary metabolites, β-glucosides are quite abundant in plants, especially in Salicaceae members [9,27,28], and are more likely to be encountered in the soil (derived from decomposing vegetation) than in the animal gut. This is consistent with the niche-specific difference in the pattern of β-glucoside utilization by different members of Enterobacteriaceae mentioned before. Our observations also indicate that in situations where multiple β-glucosides are present in the same environment, the concentrations of individual β-glucosides can be as low as 3 mM (see the electronic supplementary material, table S3).

The distribution of predator and prey analysed in this study showed coexistence of predator and prey in many soil samples. A model based on the interspecies interactions observed is depicted in figure 9. The stable coexistence of predator and prey over a long time range could be related to the oscillatory output [30] expected from the model. As evasion of predators is related to the ability to hydrolyze aromatic β-glucosides that rests with Bgl+ strains, this leads to the interesting questions as to who dies and who benefits in mixed populations that have both Bgl+ and Bgl− strains.
Bgl⁻ bacteria might derive an advantage from Bgl⁺ bacteria in terms of protection from predators and hence may tend to occupy the same niche with Bgl⁺ bacteria whenever predators are present. Our observation that in 11 out of 31 soil samples, Bgl⁻ and Bgl⁺ strains coexist with predators supports this possibility. However, further experiments are necessary to validate this observation.

These studies also suggest possible strategies for the biocontrol of parasitic amoebae and nematodes by using aglycones from plant-derived aromatic β-glucosides.

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Figure 9. A model to explain the coexistence of predator and prey in the context of β-glucoside metabolism. (1) Nematodes inhibit amoeba growth, (2) nematodes grow on amoebae, (3) amoebae repel nematodes above a certain density [29], (4) amoebae inhibit bacterial growth, (5) amoebae grow on bacteria, (6) Bgl⁻ bacteria can inhibit amoeba growth in the presence of β-glucosides, (7) bacteria can grow on dead amoebae, (8) nematodes inhibit bacterial growth, (9) nematodes grow on bacteria, (10) Bgl⁻ bacteria can inhibit nematode growth in the presence of β-glucosides, (11) bacteria can grow on dead nematodes. (Online version in colour.)
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