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Hydrogen sulfide causes excision of a genomic island in *Pseudomonas syringae* pv. *phaseolicola*

H. Neale · N. Deshappriya · D. Arnold · M. E. Wood · M. Whiteman · J. T. Hancock

Abstract Hydrogen sulfide (H₂S) is known to be an important signalling molecule in both animals and plants, despite its toxic nature. In plants it has been seen to control stomatal apertures, so altering the ability of bacteria to invade plant tissues. Bacteria are known to generate H₂S as well as being exposed to plant-generated H₂S. During their interaction with plants pathogenic bacteria are known to undergo alterations to their genomic complement. For example *Pseudomonas syringae* pv. *phaseolicola (Pph)* strain 1302A undergoes loss of a section of DNA known as a genomic island (PPHGI-1) when exposed to the plants resistance response. Loss of PPHGI-1 from *Pph* 1302A enables the pathogen to overcome the plants resistance response and cause disease. Here, with the use of H₂S donor molecules, changes induced in *Pph* 1302A genome, as demonstrated by excision of PPHGI-1, were investigated. *Pph* 1302A cells were found to be resistant to low concentrations of H₂S. However, at sub-lethal H₂S concentrations an increase in the expression of the PPHGI-1 encoded integrase gene (*xerC*), which is responsible for island excision, and a subsequent increase in the presence of the circular form of PPHGI-1 were detected. This suggests that H₂S is able to initiate excision of PPHGI-1 from the *Pph* genome. Therefore, H₂S that may emanate from the plant has an effect on the genome structure of invading bacteria and their ability to cause disease in plants. Modulation of such plant signals may be a way to increase plant defence responses for crops in the future.

Keywords Circular intermediate · Genomic island · Hydrogen sulfide · Plant defence · Plant pathogen interactions · *xerC*

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Introduction

Hydrogen sulfide (H₂S) is found widely in the environment, being generated in soils and marshes (Morse et al. 1987), and produced by human activity (Guidotti 1996). It is also produced by underwater thermal vents (Martin et al. 2008), indicating that organisms have had to tolerate it during millions of years of evolution, during which time this toxic compound has been adopted as a signalling molecule in cells (Wang 2002, 2003). Of particular relevance here, it has been known for some time that H₂S is produced by bacteria (Clarke 1953) and plants (Calderwood and Kopriva 2014).

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H₂S is inherently toxic, for example, being a known inhibitor of Complex IV of mitochondria, hence reducing ATP production (Dorman et al. 2002). However, more recently it has been found to be an important component of cell signalling pathways in both plants and animals, and has been dubbed as a third gasotransmitter (Wang 2002, 2003) in addition to nitric oxide and carbon monoxide. In plants, H₂S is part of important sulfur metabolism suggesting that there are at least low levels in cells all of the time (Calderwood and Kopriva 2014). Dedicated enzymes in plants generate H₂S, such as desulhydrases (Alvarez et al. 2010). The effects in plants include increased germination rates (Dooley et al. 2013), alteration of glutathione levels (de Kok et al. 1985) and mediating the responses to a range of stresses (reviewed by Lisjak et al. 2013). It has been reported that H₂S causes the opening of stomata on plants (Lisjak et al. 2010, 2011), although closure induced by H₂S has also been reported (García-Mata and Lamattina 2010), the difference probably due to the plant growth conditions. Either way, modulation of the opening of stomata will affect the ability of bacteria to invade plants, altering the plant-pathogen dynamics. Therefore, the action of H₂S is important to consider in plant-bacterial interactions.

When bacteria invade a plant it has been found that their genomic complement is not static, but is altered by the conditions governed by the plant (Lovell et al. 2011). Bacteria such as *Pseudomonas syringae* carry mobile genetics elements known as genomic islands (GIs). GIs are large mobilisable fragments of DNA that are part of the flexible bacterial gene pool, which is comprised of transposons, integrons, genomic islets (<10 kb), and GIs (>10 kb: Hacker and Carniel 2001). GIs are usually between 10 and 200 kilobases in length, differ from the genome in their GC content and frequently harbour phage and/or plasmid derived sequences that include transfer genes and integrases responsible for mobilisation (Juhas et al. 2009).

The outcome of invasion of the bacteria on the plant is determined by gene-for-gene relationships (Taylor et al. 1996). In this, if the bacteria expresses an effector protein recognised by the host-plant resistance protein, then the hypersensitive response (HR) is triggered and the plant defends itself against the ingress of the bacteria. On the other hand, if the plant lacks the resistance gene disease may result. Therefore, the presence of this effector gene in the bacteria genome determines if bacteria has the capability of causing disease on certain

plants, or whether the plant mounts a successful resistance response.

Pseudomonas syringae pv. *phaseolicola* (*Pph*) causes halo blight disease on common bean. The symptoms are characterised by the appearance of water-soaked lesions on leaves, pods and stems. These lesions develop into greenish-yellow haloes on leaves. Seeds may also be affected, sometimes showing wrinkled or buttery-yellow patches on the seed coat.

*Pph*1302A contains a 106 kb GI, PPHGI-1, on which is encoded an effector gene, *avrPphB* (also known as *hopAR1*) (Pitman et al. 2005). If the bacteria containing PPHGI-1 infects the bean *P. vulgaris* cultivar Tendergreen, which contains the *R3* resistance gene then the HR in the plant is triggered. However on exposure to the HR, PPHGI-1 can be excised from the bacterial chromosome, forms a circular molecule and is then lost from the bacterial cell. This results in the loss of the effector gene *avrPphB*, so enabling the resultant bacteria to cause disease in plants in which a HR would have resulted before. Pitman et al. (2005) further demonstrated that the integrase gene *xerC* (PPHGI-1 ORF.100) is required for GI excision and that *xer*C transcriptional expression is greatly increased (50 times) in leaves undergoing the HR compared to in vitro conditions. Furthermore, Lovell et al. (2009, 2011) demonstrated that PPHGI-1 can be transferred between strains of *Pph* via natural transformation and is able to integrate into the recipient strains chromosome.

Using PPHGI-1 as an example shows that factors produced by the plant host can have a profound effect on the way the bacteria respond to their environment and cause disease in the plant. Furthermore, the intra-plant environment the bacteria encounters on invasion is not static (O'Leary et al. 2016). The apoplast, the space between the cells the bacteria will first invade, is rich in a variety of components. For example, the abundance of nutrients in the apoplast has been studied (Rico and Preston 2008) as well as the protein complement (Petriccione et al. 2014). However, the constituents of the apoplast are not fixed. It was recently shown that leaf apoplastic fluid is altered during the first 6 h following the initiation of a bacteria-plant interaction (O'Leary et al. 2016), while the presence of proteins can alter over a period of days (Petriccione et al. 2014). The complement of the apoplastic fluid may contain many signalling molecules such as reactive oxygen species (ROS) and gasotransmitters. Of importance here is that the hydrogen sulfide (H₂S) produced by plants is not

confined to the cells but can be measured when released (Wilson et al. 1978), and therefore there is likely exposure of invading bacteria to plant-derived H_2S.

Here, we investigate whether exposure to H_2S has an effect on the *Pph* 1302A genome by exposing the bacteria to H_2S donor molecules and measuring the expression of the integrase *xerC* and the subsequent formation of the PPHGI-1 circular intermediate, produced following excision of the GI from the chromosome. It was found that sub-lethal concentrations of H_2S donors led to an increase in PPHGI-1 excision. Such data will be informative of whether such signalling molecules from the plant can influence the onset or progression of bacterial disease in plants.

Materials and methods

Bacterial growth conditions

Pph strain 1302A was cultured at 25 °C for 48 h on King's B (KB) agar plates (Difco, UK). Broth cultures were grown in Luria Bertani media (LB, Difco, UK) overnight at 25 °C.

Growth assays

For in vitro growth rates, three replicates of *Pph* 1302A were grown overnight in LB broth and diluted to 8×10^8 CFU/ml (OD_{600} 1.0). Cells (1 ml) were sub-cultured into 9 ml fresh LB broth and the optical density measured and recorded. Cultures were incubated at 25 °C with shaking (10×g). After 1 h the hydrogen sulfide donors, i.e. sodium hydrosulfide (NaSH), sodium sulfide (Na$_2$S) (both from Sigma Aldrich, UK) and AP39

[10-oxo-10-(4-(3-thioxo-3H-1,2-dithiol-5yl)phenoxy)decyl) triphenylphosphonium bromide] (Le Trionnaire et al. 2014) were added at final concentrations of 100 μM, 200 μM, 500 μM, 1 mM, 10 mM, 100 mM as appropriate. Optical densities were measured and recorded every hour for a further 8 h.

qPCR

qPCR was used to quantify the expression levels of *xerC* and the amount of circular intermediate produced in vitro, using the primers as listed in Table 1. Samples were taken at 2 h and 5 h post inoculation with the hydrogen sulfide donors. Gene expression was stopped using RNA protect reagent (Qiagen, UK) or DNA lysis solution (Gentra Systems, UK). For quantification of *xerC* expression, RNA was extracted using the RNAeasy kit (Qiagen, UK) followed by a second DNase step of 15 min at 37 °C (Promega, UK). cDNA was synthesised using the TaqMan reverse transcription kit (Qiagen, UK). For circular intermediate quantification, DNA was extracted using the Puregene total DNA extraction kit (Gentra Systems, UK). qPCR was carried out on an ABI 7300 Real-Time PCR System (Applied Biosystems), calibrated with a 7300 Real-Time PCR Systems Spectral Calibration kit (Applied Biosystems). Probes (Table 1) were labelled with 3′ FAM and 5′ TAMRA TaqMan dyes. The reaction volume of 25 μl was composed of 12.5 μl TaqMan PCR mastermix (Applied Biosystems), 2 μl each primer (10 μM), 2 μl probe (5 μM) and 6.5 μl RNase free water. Standard qPCR cycling conditions were 50 °C for 2 min, 95 °C for 10 min and 40× cycles of 95 °C for 15 s, followed by 60 °C for 1 min.

Table 1 PCR primers used for in this study. * Probes labelled with 5′ FAM and 3′ TAMRA TaqMan dyes

Primer name	Description	Sequence
acpP	Housekeeping gene	Forward TTGGCGTCAAATCAGAAGAG
		Reverse GCTTCTTCGTCAGGGATTTC
		Probe *ACCTGGGCGCTGACTCCCTG*
xerC	PPHGI-1 bound *xerC* integrase	Forward CGACGATACGGCCTCCAA
		Reverse AAAGGTGCGGTCGACATCA
		Probe *CCCCCTATAGCGGAGCGTCTGGAA*
QCI	Circular intermediate	Forward CATGGGCCTTCCAGATTTTC
		Reverse CTGCGGTTTGGGATACTGAAC
		Probe *CGTAACGCTGAGGCAGGCCCC*

The X-fold change in gene expression was calculated using untreated WT as calibrator and *acpP* as the internal control. Statistically significant differences were determined by ANOVA and Student's t test ($p < 0.05$).

Results

Three separate donor molecules were used to supply H_2S to *Pph* 1302A and the growth of the bacteria monitored for 8 h. One of the mostly commonly used donors for the supply for H_2S is NaSH (Zhao et al. 2014). The addition of NaSH to *Pph* 1302A had little effect on growth rate up to a concentration of 1 mM (Fig. 1a). Treatment with 10 mM showed a slight decrease in the growth rate of the cells while 100 mM was toxic, completely inhibiting growth. Another H_2S donor with similar kinetics for the release of the gas is Na_2S. Treatment with this donor had more of a negative effect on cell growth. Some reduction in growth was seen even at a concentration of 10 μM, with a concentration dependent effect seen up to 10 mM, at which total growth inhibition was seen (Fig. 1b). A new H_2S donor molecule more recently reported is AP39 (Le Trionnaire et al. 2014; Szczesny et al. 2014). AP39 had little effect on bacterial growth up to 1 mM but at 10 mM growth was completely stopped (Fig. 1c).

One of the responses of bacteria to plant defences is a change in their genetic complement (Arnold et al. 2011), which in the case of *Pph* 1302A includes the loss of PPHGI-1 (Pitman et al. 2005). Markers of this event include the expression of the integrase *xerC* and the subsequent formation of the circular intermediate. Therefore, both of these markers were assessed following treatment of *Pph* 1302A with H_2S donors.

As a measure of the effect of H_2S on *Pph* 1302A the expression of the integrase *xerC* was measured in vitro following a 2 h treatment with H_2S donors. Treatment with NaSH gave a concentration dependent increase in *xerC* expression up to 1 mM (Fig. 2a). Little effect was seen at the lowest concentration tested but there was a rise in expression of *xerC* approximately 1.6 fold following treatment with 100 μM NaSH, a concentration which did not affect cell growth

(Fig. 1a). Treatment with concentrations of 10 mM or above showed a large reduction in *xerC* expression (Fig. 2a). Similar results were seen when using Na_2S as the H_2S source (Fig. 2b), probably due to the increased toxicity of H_2S at these concentrations.

With AP39, there was over a 3 fold increase in *xerC* expression on treatment with 100 μM donor after 2 h (Fig. 2c), a concentration at which this H_2S donor had little effect on bacterial growth (Fig. 1c). This rose to nearly 5-fold with 500 μM donor, but higher concentrations showed a reduction of *xerC* expression compared to the maximal seen, again perhaps because of the detrimental effects of high concentrations of H_2S which would be present.

XerC expression leads to the excision of PPHGI from the bacterial chromosome and the subsequent formation of a circular intermediate molecule (Pitman et al. 2005). To confirm further the effects of H_2S donors on the *Pph* 1302A genome, circular intermediate formation was measured. After 2 h the production of circular intermediate increased on H_2S exposure, using NaSH as a source of the gas (Fig. 3a). Importantly, at concentrations at which growth rates were not affected, for example 500 μM NaSH, the formation of the circulate intermediate was increased by more than 3 fold. Even at very low concentrations, for example 10 μM NaSH, there was still a noticeable increase in circulate intermediate formation. A similar situation was seen with the donor Na_2S. At 10 μM there was a 2.5 fold increase in circular intermediate production (Fig. 3b), even though growth of those cells was little affected (Fig. 1b). There was a concentration dependent increase in this affect up to 1 mM after which the production of the intermediate was reduced.

AP39 was found to have similar effects on circular intermediate production after 2 h (Fig. 3c). A nearly 2-fold increase was seen on treatment with 10 μM AP39, while a concentration dependent affect was seen up to 500 μM. As with the other H_2S donors, higher concentrations saw a sharp reduction in the presence of the circular intermediate.

With H_2S donors such as NaSH or AP39 there was little effect on cell growth (except at the highest concentrations used) up to 6 h of treatment. Therefore, the *xerC* expression and production of circular intermediates assays were repeated after 5 h of H_2S donor treatments.

Fig. 1 Growth of *Pseudomonas syringae* pv. *phaseolicola (Pph)* 1302A in the presence of H_2S donors. *Pph* 1302A was grown overnight in LB broth and diluted to 8×10^8 CFU/ml (OD_{600} 1.0). Cells (1 ml) were sub-cultured into 9 ml fresh LB broth and the optical density measured and recorded. Cultures were incubated at 25 °C with shaking (10 xg). After 1 h the H_2S donors were added: **a**; sodium hydrosulfide (NaSH), **b**; sodium sulfide (Na_2S) **c**; AP39, at the concentrations indicated. Optical densities were recorded hourly for 8 h. *n* = 3
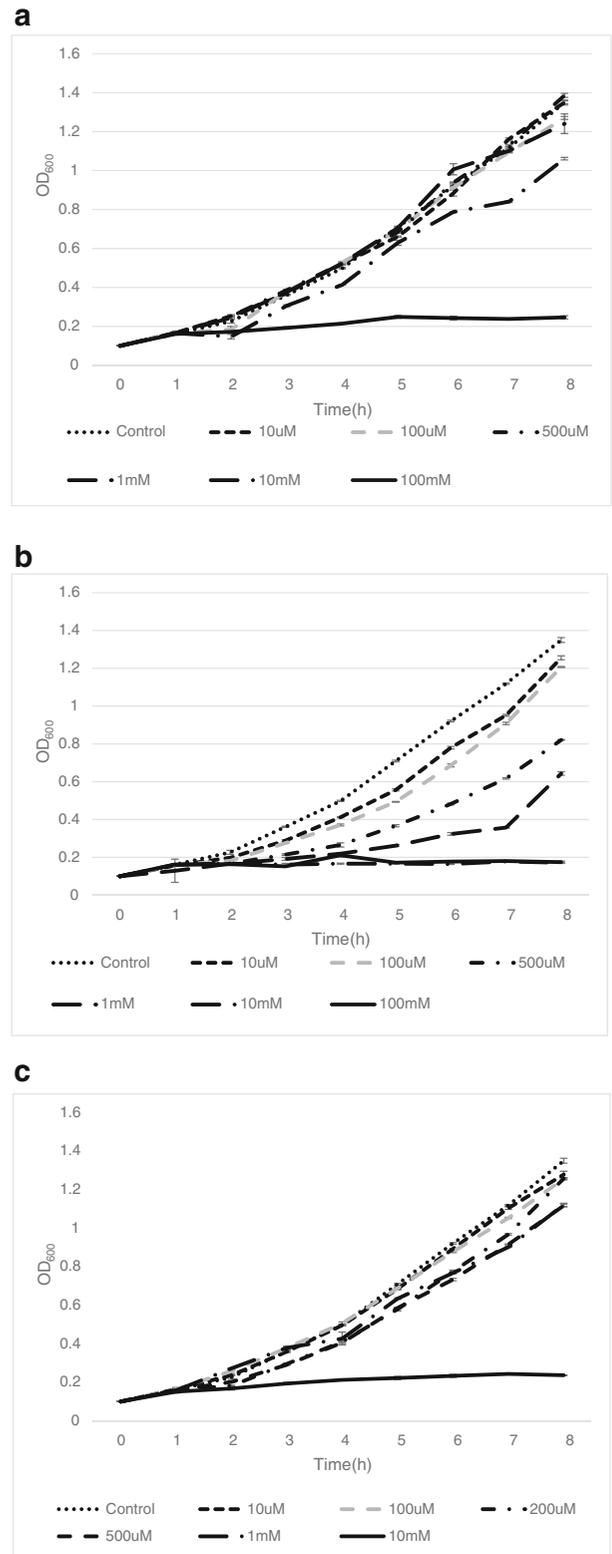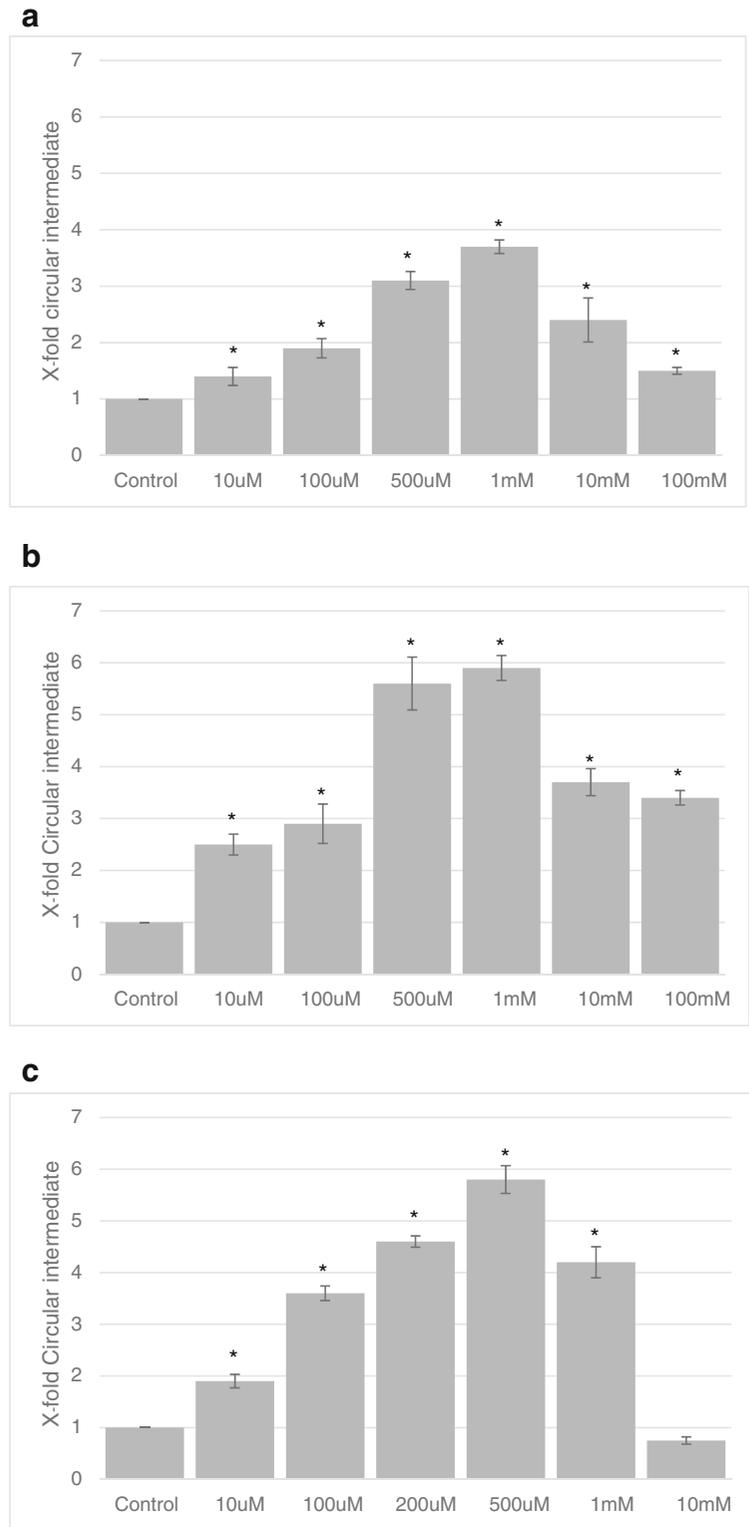
Fig. 2 *xerC* integrase expression in *Pseudomonas syringae* pv. *phaseolicola (Pph)* 1302A treated with H$_2$S donors. *Pph* 1302A was treated with H$_2$S donors for two hours at the concentrations indicated, after which the expression of *xerC* was assayed using qPCR. Results are displayed as X-fold expression. All data were standardized by simultaneous qPCR analysis of *acpP* expression and error bars represent standard error of the mean of three experimental replicates. *above bars indicate a significant difference compared to the control at $p < 0.05$ assessed by ANOVA and Student's t-test. **a**; sodium hydrosulfide (NaSH), **b**; sodium sulfide (Na$_2$S) **c**; AP39. $n = 3$
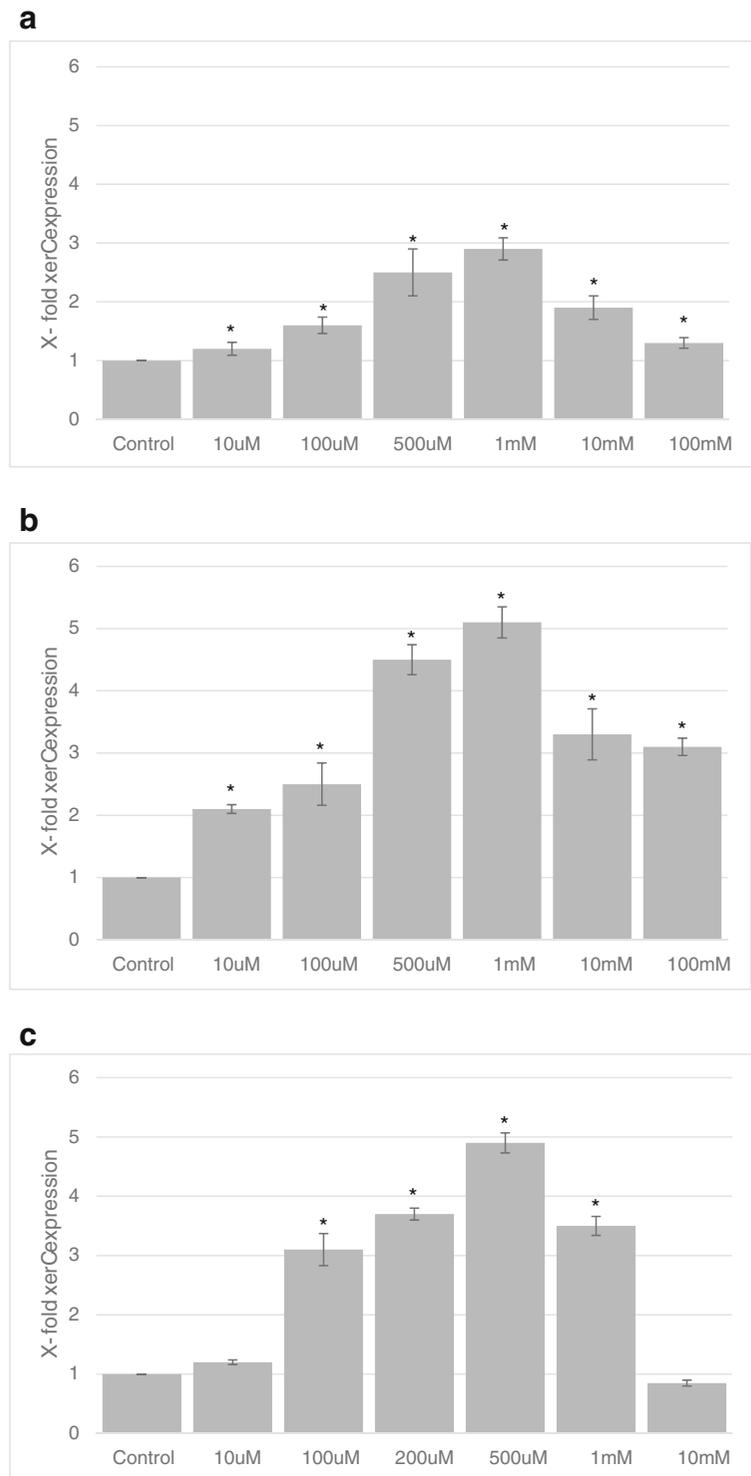
With AP39, there was very little increased effect of the longer treatments. The maximal levels of *xerC* expression (Fig. 4a) and circular intermediate production (Fig. 4b) were still seen with 500 μM and higher

Fig. 3 Circular intermediate production in *Pseudomonas syringae* pv. *phaseolicola (Pph)* 1302A treated with H_2S donors. *Pph* 1302A was treated with H_2S donors for two hours at the concentrations indicated, after which the formation of circular intermediate was assayed using qPCR. Results are displayed as X-fold expression. All data were standardized by simultaneous qPCR analysis of *acpP* expression and error bars represent standard error of the mean of three experimental replicates. *above bars indicate a significant difference compared to the control at $p < 0.05$ assessed by ANOVA and Student's t-test. **a**; sodium hydrosulfide (NaSH), b; sodium sulfide (Na_2S) C; AP39. $n = 3$

concentrations had less of an effect. Similar data were obtained with NaSH and Na_2S as donor molecules (data not shown), indicating that longer exposure to the H_2S had little further effect.

Fig. 4 *xerC* expression and circular intermediate production in *Pseudomonas syringae* pv. *phaseolicola (Pph)* 1302A following 5 h treatment with AP39. *Pph* 1302A was treated with the H$_2$S donor AP39 for five hours at the concentrations indicated, after which the *xerC* expression (**a**) and circular intermediate (**b**) were assayed using qPCR. Results are displayed as X-fold expression. All data were standardized by simultaneous qPCR analysis of *acpP* expression and error bars represent standard error of the mean of three experimental replicates. $n = 3$. *above bars indicate a significant difference compared to the control at $p < 0.05$ assessed by ANOVA and Student's t-test
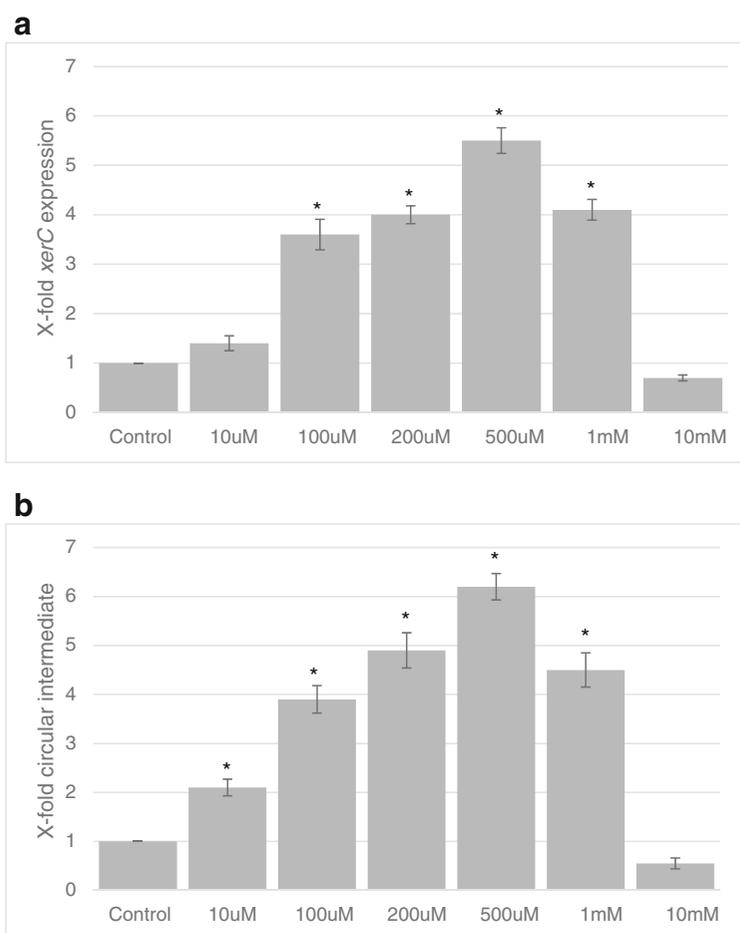
Discussion

Plant pathogens will be successful at causing disease if they can invade the tissues of the plant and overcome the plant's defences. Bacteria such as *Pph* produce effector molecules that may be recognised by the plant, often resulting in a defence known as the HR (Bashir et al. 2013). However, bacterial genomes are dynamic and change in response to different environments. For example, exposure of *Pph* to the HR of the plant can trigger the loss of a large region of the bacterial genome known as PPHGI-1 (Pitman et al. 2005). This GI carries an effector gene that triggers the HR but forms a circular intermediate that can be lost from the bacterial cell and therefore allow the pathogen to cause disease. Therefore, the genomic content of the bacteria is fluid, allowing these pathogens to lose or gain DNA and the functionality that that may carry.

The elements of the plant defences which may trigger the loss of GIs from bacteria are not well understood. However, it is known that plants will produce a range of reactive molecules when challenged with a pathogen. These include reactive oxygen species (ROS: O'Brien et al. 2012) such as hydrogen peroxide, nitric oxide (NO: Bellin et al. 2013) and hydrogen sulfide (H$_2$S: Calderwood and Kopriva 2014). Such molecules are used as signalling components in plants, often interacting together resulting in a coordinated response (Hancock and Whiteman 2014, 2015). Such signalling may lead to the initiation of the HR in plants and so the cessation of the pathogen progression into the plant. It can be seen therefore that invading plant pathogens are going to be subjected to the presence of a range of toxic compounds. However, such compounds may not just be plant derived. Bacteria too can produce H$_2$S (Clarke 1953). Therefore, it is important to understand the effects of such compounds on plant pathogens.

In the current study, the effects of a range of H_2S donor molecules were tested to assess their effects on the growth of a well-studied plant pathogen, *Pph* 1302A (Arnold et al. 2011). The donors here were NaSH, Na_2S and AP39. The characteristics of the release of the H_2S from these compounds needs to be considered. Both NaSH and Na_2S are relatively unstable and will release H_2S very quickly; it is an instant pH-dependent dissociation. Furthermore, H_2S is only slightly soluble in water and will rapidly enter the gas phase and be lost to the atmosphere. Therefore to overcome this other compounds have been developed that release H_2S over a more physiological time period. One such compound is GYY4137 (Li et al. 2013) which releases H_2S much more slowly over a longer period of time and has been shown to have effects in both animals (Li et al. 2013) and plants (Lisjak et al. 2010). A more recent development is a H_2S releasing compound designed to be targeted to the mitochondria of eukaryotic cells, that is, AP39. However, with the similarities between mitochondria and bacteria (Gray 2012) it is conceivable that AP39 would have effects in bacteria mediated by its capacity to release H_2S.

Interestingly there was little effect of NaSH or AP39 on the growth of *Pph* unless treated at very high concentrations, at which point both appeared to be toxic. However, some bacteria, for example *E. coli*, have been seen to be resistant to H_2S (Forte et al. 2016), seemingly being able to maintain respiration through the use of a cytochrome oxidase *bd* complex. Na_2S had more of an effect on *Pph* 1302A at lower concentrations although its kinetics of release of H_2S would be expected to be the same as that of NaSH.

On the other hand, it may be expected that low concentrations of H_2S would promote growth, although here no evidence of that is seen (Fig. 1). H_2S is a source of sulfur for many organisms while it has been shown that many bacteria can partake in sulfur metabolism (Friedrich et al. 2001), including Pseudomonads (Friedrich and Mitrenga 1981). For mitochondria it has also been shown that H_2S can be a source of reducing equivalents (Bouillaud et al. 2013). Therefore increased growth at low, sub-lethal concentrations of H_2S may be possible in a range of organisms, both prokaryote and eukaryote.

It is now well established that bacteria such as *Pph* can lose GIs and other mobile elements if exposed to the plant environment during the HR. PPHGI-1 is lost from *Pph*1302A for example (Pitman et al. 2005), with the conditions to which the bacteria are exposed during the HR appearing to favour those individuals which lack the avirulence gene *avrPphB* (Pitman et al. 2005; Arnold et al. 2007; Lovell et al. 2011). Loss of PPHGI is proceeded by excision of the island from the chromosome and the formation of a circular intermediate. Excision, as measured by *xerC* expression and circular intermediate formation can be assayed by PCR-based methods or by the use of fluorescent tags (Godfrey et al. 2010). Here, all the H_2S donors used led to an increase in *xerC* expression and the formation of the circular intermediate indicative of the sulfide gas mediating island excision. Effects were seen at concentrations of H_2S donors, not affecting bacterial growth although higher concentrations of H_2S did show reduced GI loss. Interestingly, longer exposure (5 h) to the H_2S donors did not have a larger effect. This may be due the labile nature of the donors and loss of H_2S to the atmosphere during the assay. The effects of the H_2S appear to have driven the GI excision to start, but continued exposure to H_2S may not be required for the process to be taken to completion. Therefore, *in planta*, bacteria exposed to H_2S generated during the HR are also likely to be driven to undergo such genetic changes.

In conclusion, exposure of plant pathogenic bacteria such as *Pph* to H_2S, to which such bacteria may be exposed during the process of plant invasion, will not have their growth restricted. However, they may be driven to undergo genetic modifications which will increase their propensity to cause disease in the plant. Therefore understanding the role of triggers, such as H_2S, to bacterial genetic changes is important to ascertain and may be a way to modulate plant-pathogen interactions in the future. Lastly, GIs in pathogens are not restricted to plants, but are also found in animal pathogens (Wei and Guo 2011). Furthermore, H_2S has been implicated in the mechanisms by which bacteria can be resistant to antibiotics (Shatalin et al. 2011). Therefore a full understanding of how bacteria respond to, and have effects mediated by, H_2S is important to obtain and may have future implications for diseases in both animals and plants.

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