Plant pathogenic bacterium can rapidly evolve tolerance to an antimicrobial plant allelochemical

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Running Head: ITC tolerance in *Ralstonia solanacearum*

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Abstract

Crop losses to plant pathogens are a growing threat to global food security and more effective control strategies are urgently required. Biofumigation, an agricultural technique where *Brassica* plant tissues are mulched into soils to release antimicrobial plant allelochemicals called isothiocyanates (ITCs), has been proposed as an environmentally friendly alternative to agrochemicals. While biofumigation has been shown to suppress a range of plant pathogens, its effects on plant pathogenic bacteria remain largely unexplored. Here we used a laboratory model system to compare the efficacy of different types of ITCs against *Ralstonia solanacearum* plant bacterial pathogen. Additionally, we evaluated the potential for ITC-tolerance evolution under high, intermediate and low transfer frequency ITC exposure treatments. We found that allyl-ITC was the most efficient compound at suppressing *R. solanacearum* growth, and its efficacy was not improved when combined with other types of ITCs. Despite consistent pathogen growth suppression, ITC tolerance evolution was observed in the low transfer frequency exposure treatment, leading to cross-tolerance to ampicillin beta-lactam antibiotic. Mechanistically, tolerance was linked to insertion sequence movement at four positions in genes that were potentially associated with stress responses (H-NS histone like protein), cell growth and competitiveness (acyltransferase), iron storage ((2-Fe-2S)-binding protein) and calcium ion sequestration (calcium-binding protein). Interestingly, pathogen adaptation to the growth media also indirectly selected for increased ITC tolerance through potential adaptations linked with metabolism and antibiotic resistance (dehydrogenase-like protein) and transmembrane protein movement (Tat pathway signal protein). Together, our results suggest that *R. solanacearum* can rapidly evolve tolerance to allyl-ITC plant allelochemical which could

31 constrain the long-term efficiency of biofumigation biocontrol and potentially shape

32 pathogen evolution with plants.

33 **Keywords:** Antimicrobial tolerance, Plant allelochemicals, Plant pathogenic bacteria,

34 Biofumigation, Isothiocyanates (ITCs), *Ralstonia solanacearum*

Introduction

Plant pathogens are a growing threat to global food security, accounting for up to 40% of crop losses annually (Savary et al., 2012). The phasing out of environmentally toxic chemical fumigants, such as methyl bromide, has directed attention towards alternative biocontrol strategies (Qin et al., 2004). Plant-derived antimicrobial allelochemicals, such as phenolic acids, terpenes and volatile isothiocyanates (ITCs), are naturally exuded by the roots of legumes (Mondal et al., 2015; Wink, 2013), cereals (Larkin & Halloran, 2015; Mazzola & Gu, 2002) and other crops such as *Brassica* (Kirkegaard et al., 1996; Sarwar et al., 1998). These compounds could potentially be used to control pathogens by biofumigation, which involves mulching plant tissues into soils to release biocidal allelochemicals. While biofumigation has previously been shown to suppress the growth of soil-borne fungal (Angus et al., 1994; Rumberger & Marschner, 2003; Sarwar et al., 1998), nematode (Lord et al., 2011; Ngala et al., 2015) and bacterial pathogens (Hu et al., 2015; Ji et al., 2007), outcomes are still varied, ranging from clear pathogen suppression (Larkin & Griffin, 2007; Matthiessen & Kirkegaard, 2006) to having no effect (Hartz et al., 2005; Kirkegaard et al., 2000; Stirling & Stirling, 2003). A better understanding of the antimicrobial and biocidal effects of plant allelochemicals on pathogens is thus required.

The success of biofumigation is influenced by various factors including soil conditions, the biofumigant plant species, timing of application and the half-life of biocidal compounds (Matthiessen & Kirkegaard, 2006). The biocidal effects of *Brassica*-based biofumigation are believed to result primarily from the release of toxic ITCs from their glucosinolate (GSL) pre-cursors (Gimsing & Kirkegaard, 2009; Lord *et al.,* 2011; Matthiessen & Kirkegaard, 2006). Moreover, other allelochemicals such as dimethyl sulfide and methyl

58 iodide might contribute to the biocidal activity of biofumigant plants (Vervoort et al., 2014;
59 Wang et al., 2009). Even though ITC-liberating GSL levels can potentially reach as high as
60 45.3 mM/m^2 following initial mulching of plant material into the soil (Kirkegaard & Sarwar,
61 1998), their concentrations often decline rapidly due to high volatility, sorption to organic
62 matter, leaching from the soil and microbial degradation (Frick et al., 1998; Gimsing et al.,
63 2007; Hanschen et al., 2015; Matthiessen & Kirkegaard, 2006; Warton et al., 2001). As ITCs
64 often have short half-lives of up to sixty hours (Borek et al., 1995; Gimsing & Kirkegaard,
65 2006), it is important to identify ITCs that are highly effective against pathogens even during
66 short-term exposure.

67 The antimicrobial activity of different types of ITCs can vary depending on their
68 mode of action and the species and genotype of the target pathogen. In the case of
69 bacterial pathogens, several antimicrobial mechanisms have been suggested. For instance,
70 ITCs could damage the outer cell membrane of Gram-negative bacteria leading to changes
71 in cell membrane potential (Sofrata et al., 2011) and leakage of cell metabolites (Lin et al.,
72 2000). Further, it has been suggested that ITCs could bind to bacterial enzymes, such as
73 thioredoxin reductases and acetate kinases and disrupt their tertiary structure and
74 functioning (Luciano & Holley, 2009). It is also possible that some ITCs, such as allyl-ITC,
75 could have multiple targets, making them relatively more toxic to pathogenic bacteria
76 (Luciano & Holley, 2009). However, antimicrobial activity and potential tolerance evolution
77 to ITCs are still poorly understood in plant pathogenic bacteria.

78 Antibiosis is an important mechanism underlying bacterial competition in soils and
79 soil bacteria often produce and are resistant to several antimicrobials, enabling them to
80 outcompete surrounding bacteria for space and nutrients (Hibbing et al., 2010).

81 Antimicrobial tolerance is also important for plant-bacteria interactions, as it can help
82 bacteria to tolerate antimicrobials secreted by plants, such as coumarins, giving them a
83 selective advantage in the plant rhizosphere microbiome (Stringlis et al., 2018). Such
84 tolerance has recently been shown to evolve *de novo* in *Pseudomonas protegens* CHA0
85 bacterium against the antimicrobial scopoletin secreted by *Arabidopsis thaliana* (Li et al.,
86 2020). Prolonged exposure to plant allelochemicals could thus select for more tolerant plant
87 pathogen genotypes also during biofumigation and will likely be affected by the strength
88 and duration of ITC exposure, which is important in determining whether potential
89 tolerance or resistance mutations have enough time to sweep through pathogen
90 populations. If the mutations enabling ITC tolerance are costly, their selective benefit could
91 be further reduced by competition or growth trade-offs, leading to loss of tolerance
92 mutations in the absence of ITCs. While ITC concentrations are known to reach antimicrobial
93 levels during biofumigation in the field (Sarwar et al., 1998), no direct experimental
94 evidence for ITC tolerance evolution in plant pathogenic bacteria exists.

95 To study these questions, we developed a model laboratory system where we tested
96 the growth-inhibiting effects of ITCs produced by Indian mustard (*Brassica juncea*) on
97 *Ralstonia solanacearum* plant pathogenic bacterium, which is the causative agent of
98 bacterial wilt and potato brown rot diseases and a globally important pathogen, affecting
99 over 200 different plant species including various important crops (Elphinstone, 2005;
100 Yabuuchi et al., 1995). The *R. solanacearum* genome is bipartite, consisting of a
101 chromosome and megaplasmid (Salanoubat et al., 2002). Disease control techniques such as
102 crop rotation, the use of clean and certified seeds or resistant plant cultivars, have shown
103 only limited success in controlling *R. solanacearum* (Chellemi et al., 1997; Ciampi-Panno et
104 al., 1989; Ramesh et al., 2009). Indian mustard was chosen as a model biofumigant plant

due to its well-established allelochemical properties (Bending & Lincoln, 1999; Kirkegaard & Matthiessen, 2005; Mazzola et al., 2015; Sarwar et al., 1998), which are predominantly caused by the release of allyl, sec-butyl and 2-phenylethyl ITCs (Bangarwa et al., 2011; Olivier et al., 1999; Yim et al., 2016). As these ITCs might vary in their biocidal activity, we first tested to what extent they suppress *R. solanacearum* growth when applied either alone or in combination at concentrations relevant to field biofumigation (Gimsing et al., 2007; Hanschen et al., 2012; Kirkegaard & Sarwar, 1998; Matthiessen & Kirkegaard, 2006; Rudolph et al., 2015). Subsequently, we explored whether long-term exposure to the most effective ITC type could select for resistant or more ITC-tolerant pathogens in the lab, and if ITC tolerance is associated with competitive costs or cross-tolerance to other antimicrobials. It was found that allyl-ITC was the most suppressive allelochemical. However, long-term exposure selected for ITC-tolerant pathogen mutants that also had increased cross-tolerance to the beta-lactam antibiotic ampicillin. At the molecular level, adaptations were associated with a few parallel mutations and loss of insertion sequences mainly in the megaplasmid. Together these results suggest that while Indian mustard could be used as a biofumigant plant against *R. solanacearum* due to the high antimicrobial activity of allyl-ITC, its long-term efficacy could be constrained by rapid ITC tolerance evolution.

Materials and Methods

(a) Pathogen strain and culture media

We used a *Ralstonia solanacearum* strain (21415687) which was originally isolated from the river Loddon (phylotype II sequevar 1) in the UK as our ancestral pathogen strain (Source: John Elphinstone, Fera Science, 2014). This strain was chosen as river water is the most common environmental source of potato brown rot outbreaks in the UK (Elphinstone *et al*.,

128 1998), and hence highly relevant for UK *R. solanacearum* epidemics. The strain was cultured
129 in CPG broth (1 g casamino acids, 10 g peptone and 5 g glucose per litre of ddH_2O) for 48
130 hours at 28 °C to create cryostocks (20% w/v glycerol) that were preserved at -80 °C. CPG
131 was also used as the main growth media in all experiments except for fitness assays, where
132 lysogeny broth (LB: 10 g tryptone, 5 g yeast, 10 g NaCl per litre of ddH_2O) was also used as a
133 'naïve' growth media to control the effects of *R. solanacearum* adaptation to CPG media
134 during the selection experiment.

135 **(b) Comparing the effects of different types of ITCs for pathogen suppression**

136 To determine antimicrobial activity of ITCs, we first identified concentrations that caused a
137 significant reduction in *R. solanacearum* growth relative to the no-ITC control treatments.
138 To this end, we conducted short-term growth assays where *R. solanacearum* was exposed
139 to allyl, sec-butyl and 2-phenylethyl ITCs at 63, 125, 250, 500, 1000, 2500 and 5000 µM
140 concentrations in CPG media (Suppl. Fig. 2). For this experiment, *R. solanacearum* was
141 revived from cryostocks by growing with shaking (250 rpm) for 48 hours at 28 °C before
142 normalising bacterial density to an optical density (OD) reading of 0.1 (600 nm; Tecan,
143 Sunrise), equalling ~10^7 cells per ml. This method was consistently used to revive and adjust
144 bacterial densities in all growth experiments. *R. solanacearum* was grown in 200 µl CPG
145 media in different ITC concentrations for 148 hours and bacterial densities were measured
146 every 24 hours (OD600 nm). We found that allyl-ITC concentrations as low as 125 µM
147 inhibited *R. solanacearum* growth, while relatively higher concentrations of 500 µM of sec-
148 butyl and 2-phenylethyl ITC were required to inhibit pathogen growth (Suppl. Fig. 2). Based
149 on this data, 500 µM and 1000 µM ITC concentrations were selected because they showed
150 pathogen growth suppression in the case of all measured ITCs (Suppl. Table 1). Furthermore,

151 these concentrations are known to be achievable at least transiently during biofumigation in
152 the field (Gimsing et al., 2007; Hanschen et al., 2012; Kirkegaard & Sarwar, 1998;
153 Matthiessen & Kirkegaard, 2006; Rudolph et al., 2015). To explore the effects of ITCs on
154 pathogen growth alone and in combination, different ITCs were mixed in all possible two-
155 way and three-way combinations using equal concentrations of each ITC within
156 combinations (two-way 50:50%; three-way 33:33:33%) to achieve final low (500 μM) and
157 high (1000 μM) ITC concentrations in 200 μl of CPG media in 96-well microplates.
158 Microplates were cultured at 28 °C (N= 8 for all treatments) and the experiment was run for
159 three days (72 hours), with population density measurements recorded every 24 hours as
160 optical density at 600 nm.

161 (c) Determining pathogen ITC and beta-lactam tolerance evolution in response
162 to repeated allyl-ITC exposure

163 To investigate the potential for ITC tolerance evolution, we set up a 16-day selection
164 experiment where we exposed *R. solanacearum* to 500 μM of allyl-ITC, which has the
165 strongest effect on pathogen growth suppression of all tested ITCs (Fig. 1A; Suppl. Fig. 2).
166 We also manipulated the frequency of ITC exposure using high (1-day), intermediate (2-day)
167 and low (3-day) serial transfer frequency treatments. At each serial transfer, a subset of
168 evolved bacteria (5% of the homogenised bacterial population) was serially transferred to
169 fresh CPG media in the absence (control) and presence of allyl-ITC. ITC treatments thus
170 manipulated both resource renewal and exposure to fresh ITC. The selection experiment
171 was set-up following the same protocols described earlier and following this, separate
172 fitness assays were conducted to directly compare the growth of ancestral and evolved
173 populations (and individual colonies) in the absence and presence of 500 μM allyl-ITC. In

174 addition to testing potential ITC tolerance evolution, we quantified changes in the growth of
175 evolved bacteria in the absence of ITCs to reveal potential adaptations to the CPG growth
176 media. All fitness assays were also repeated in 'naïve' LB media to control the potential
177 effects of pathogen adaptation to the CPG growth media during the selection experiment. In
178 all assays, bacteria were revived and prepared as described earlier, and grown in 96-well
179 microplates in different media (CPG or LB) in the absence or presence of 500 μM allyl-ITC for
180 72 hours. Changes in ITC tolerance were quantified as bacterial growth relative to the
181 ancestral and control treatments based on optical density at 600 nm (48-hour time point).
182 Fitness assays were also conducted for individual bacterial colonies at the final time point
183 where a single ancestral colony and one colony from each replicate selection line per
184 treatment were selected resulting in a total of 49 clones.

185 To explore potential ITC-tolerance mechanisms, we tested if ITC tolerance correlated
186 with tolerance to ampicillin beta-lactam antibiotic (growth assays), which is commonly
187 produced by various soil bacteria (Ranjan et al., 2021). Moreover, we specifically tested for
188 ampicillin tolerance as we identified potential antibiotic-linked insertion sequence
189 movement in our evolved clones, which has previously been shown to confer beta-lactam
190 antibiotic tolerance in clinical settings (Boutoille et al., 2004; Poirel et al., 2003). Ampicillin
191 tolerance was tested using the sequenced isolated clones from the final time point of the
192 selection experiment (intermediate transfer frequency no-ITC, low transfer frequency no-
193 ITC and low transfer frequency ITC exposure treatments) and the ancestral strain (total of 24
194 evolved clones and 8 replicate ancestral clones per treatment). Clones were prepared as
195 described earlier and grown in 96-well microplates in CPG media in the absence or presence
196 of 15 or 30 μg/ml ampicillin. Ampicillin tolerance was quantified as bacterial growth relative
197 to the ancestral clones based on optical density at 600nm (48-hour time point).

198 ### (d) Genome sequencing of evolved bacterial clones

199 A subset of evolved clones was whole genome sequenced to identify potential single
200 nucleotide polymorphisms (SNPs), genomic rearrangements (small insertions and deletions)
201 and potential changes in prophage and insertion sequence movement linked with *R.*
202 *solanacearum* adaptation. Based on phenotypic data, we chose eight clones (1 per replicate
203 selection line) from the low transfer frequency treatments that had evolved in the absence
204 or presence of ITC (16 clones). Moreover, we sequenced the ancestral strain (1 clone) and
205 eight clones from the intermediate transfer frequency no-ITC treatment (8 clones), that
206 showed no evidence of ITC tolerance adaptation (a total of 25 clones), as controls. Genomic
207 DNA was extracted using the Qiagen DNeasy UltraClean Microbial Kit according to the
208 manufacturer's protocol. DNA was quantified using the NanoDrop microvolume
209 spectrophotometer and quality checked by gel electrophoresis imaging. DNA yields of all
210 samples were diluted with EB buffer to 30 ng/µl concentrations and DNA samples were sent
211 to MicrobesNG for sequencing (Illumina 30x coverage; http://www.microbesng.uk).
212 MicrobesNG conducted library preparation using Nextera XT Library Prep Kit (Illumina, San
213 Diego, USA) following the manufacturer's protocol with the following modifications: 2 ng of
214 DNA were used as input, and PCR elongation lasted 1 min. Hamilton Microlab STAR
215 automated liquid handling system was used for DNA quantification and library preparation.
216 Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for
217 Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on the Illumina
218 HiSeq 2500 using a 250 bp paired end protocol. Reads were adapter trimmed using
219 Trimmomatic 0.30 with a sliding window quality cut-off of Q15 (Bolger et al., 2014).
220 Assembly was performed on samples using SPAdes v.3.7 (Bankevich et al., 2012) and contigs
221 were annotated using Prokka v.1.11 (Seemann, 2014). Genomes were analysed using a

222 standard analysis pipeline (Guarischi-Sousa et al., 2016), where reads were first mapped to a
223 high quality and well annotated UY031 reference genome (NCBI accession: NZ_CP012687)
224 which showed 99.95% similarity with our ancestral *R. solanacearum* strain at the
225 chromosome level and 97.87% similarity at the mega-plasmid level. Variant calling was
226 performed using Snippy v.3.2, a rapid haploid variant calling pipeline (Seemann, 2015).
227 When comparing the sequenced genomes, the SNPs identified in both the ancestral strain
228 and the evolved clones were first filtered out as these likely represent pre-existing
229 phylogenetic differences between the reference genome and our ancestral *R. solanacearum*
230 strain. We also compared the control treatment clones isolated from low and intermediate
231 transfer frequency treatments (no ITC exposure) to identify potential mutations linked with
232 CPG media adaptation. The software IMSindel v.1.0.2 (Shigemizu et al., 2018) was used to
233 identify potential intermediate indels with options "—indelsize 10000" and using UY031 as a
234 reference. After running IMSindel, putative indels in all isolates were combined. Putative
235 short indels that were < 50 bp in length were removed. To investigate potential insertion
236 sequences underlying ITC tolerance and media adaptation, insertion sequences were
237 detected in the UY031 with ISEScan v.1.7.2.3; (Xie & Tang, 2017) using default parameters.
238 Potential false positives were determined by blasting insertion sequences against the
239 ISFinder database (https://isfinder.biotoul.fr/) and removing hits with an E-value > e-04.
240 Experimental isolates were then screened for the insertion sequences identified with
241 ISEScan using ISMapper v.2.0; (Hawkey et al., 2015) with default settings. In line with a
242 previous study (Hawkey et al., 2020), ISMapper was run using an IS-removed UY031
243 assembly to improve insertion site precision. The genes flanking putative IS sites were
244 determined by annotating the UY031 assembly using the stand-alone NCBI prokaryotic
245 genome annotation pipeline 2021-07-01.build5508 (Tatusova et al., 2016). Additionally, we

246 determined isolate prophage content and positions to identify potential phenotypic changes
247 via mobile genetic elements. Isolate draft assemblies were generated using Unicycler
248 Illumina-only assembly v.0.4.7 (Wick et al., 2017). Prophages were then identified in draft
249 assemblies using the PHASTER (PHAge Search Tool Enhanced Release) web server (Arndt et
250 al., 2016). Prophage movement was detected by parsing out the 5kb (or to end of contig)
251 flanking regions either side of the prophages in the draft assemblies and mapping them to a
252 closely related complete UY031 genome sequence. Prophage movement was detected if the
253 flanking regions map to different parts of the UY031 genome between isolates. Prophage
254 movement analyses were conducted using custom R and Python scripts available at
255 (https://github.com/SamuelGreenrod/Prophage_movement). All genomes including the
256 ancestral strain have been deposited in the European Nucleotide Archive database under
257 the following accession number: PRJEB42551.

258 **(e) Statistical analysis**

259 Repeated measures ANOVA was performed to analyse all the data with temporal sampling
260 structure and pairwise differences were determined using *post-hoc* t-test with Bonferroni
261 correction. All other statistical analyses (ITC tolerance and cost of tolerance in CPG and LB
262 media and cross-tolerance in ampicillin) were conducted focusing on the 48-hour
263 measurement time point (where ITC was still actively suppressive to *R. solanacearum,* Suppl.
264 Fig. 1) and two-way ANOVA was used to explain variation in bacterial growth between
265 different treatments. Tukey *post-hoc* tests were used to compare differences between
266 subgroups ($p < 0.05$). Where data did not meet the assumptions of a parametric test, non-
267 parametric Kruskal-Wallis test and *post-hoc* Dunn test were used. All statistical analyses and
268 graphs were produced using R (R Foundation for Statistical Computing, R Studio v.3. 5. 1)
269 using ggplot2, tidyverse, ggpubr, lme4, rcompanion and reshape2 packages.

Results

(a) Only allyl-ITC suppressed pathogen growth irrespective of the presence of other ITCs

We first determined the effects of different ITCs on *R. solanacearum* growth alone and in combination. Overall, there was a significant reduction in *R. solanacearum* densities in the presence of ITCs (ITC presence: $F_{1, 120}$= 6.33, p< 0.01; Tukey: p< 0.05; Fig. 1B). However, this effect was mainly driven by the allyl-ITC, which significantly reduced bacterial densities compared to the no-ITC control treatment (ITC type: $F_{7, 114}$= 49.45, p< 0.001; Tukey: p< 0.05), while other ITCs had no significant effect on the pathogen (p> 0.05; Fig. 1B). Increasing the ITC concentration from low to high (500 to 1000 μM) had no effect on inhibitory activity in either single or combination ITC treatments (ITC concentration in single ITC treatment: $F_{1, 43}$= 2.0, p= 0.17; combination ITC treatment: $F_{1, 59}$= 0.68, p= 0.41; Fig. 1B). However, a significant interaction between ITC type and ITC concentration in both single and combination treatments was found (ITC concentration × ITC type in single ITC treatment: $F_{2, 39}$= 4.67, p< 0.05; in combination ITC treatment: $F_{3, 53}$= 4.94, p< 0.01; Fig. 1B), which was driven by the increased inhibitory activity of allyl-ITC at high concentration (Tukey: p< 0.05). As a result, ITC combinations were less inhibitory than single ITC treatments (Number of ITCs: $F_{2, 103}$= 3.82, p<0.05; Fig. 1B), which was due to reduced allyl-ITC concentration in combination treatments (total ITC concentrations were kept the same between treatments). Similarly, ITC combinations that included allyl-ITC significantly reduced bacterial densities relative to the control treatment (Allyl-ITC presence: $F_{1, 57}$= 36.21, p< 0.001; Fig. 1B), and the presence of allyl-ITC had a clearer effect at the high ITC concentration (Allyl-ITC presence × ITC concentration: $F_{1, 57}$= 7.51, p< 0.01; Fig. 1B). Together

293 these results suggest that allyl-ITC was the most inhibitory compound and its antimicrobial
294 activity was not enhanced by the presence of other ITCs.

295 (b) Pathogen growth was more clearly suppressed in high and intermediate
296 ITC exposure treatments during an experimental evolution experiment

297 To study the evolutionary effects of ITCs, we exposed the ancestral *R. solanacearum* strain
298 to allyl-ITC at the low concentration (500 μM) and manipulated the frequency of exposure
299 to ITC by transferring a subset of evolved bacterial population to fresh ITC-media mixture
300 everyday (high), every second day (intermediate) and every third day (low) for a total of 16
301 days. As a result, this manipulation also affected the resource renewal rate. Overall, bacteria
302 reached the highest population densities in the low transfer frequency treatments and the
303 second highest in the intermediate transfer frequency treatments (Transfer frequency: $F_{2,}$
304 $_{45}$= 4.66, $p< 0.001$; $p< 0.05$ for pairwise comparison; Fig. 2). While allyl-ITC exposure
305 significantly reduced bacterial densities in all ITC-containing treatments (ITC presence: $F_{1, 46}$=
306 30.68, $p< 0.001$; Fig. 2), bacterial growth was least affected in the low transfer frequency
307 treatment (ITC presence × Transfer frequency: $F_{2, 42}$= 4.36, $p< 0.05$; $p< 0.001$ for all pairwise
308 comparisons; Fig. 2). The inhibitory activity of allyl-ITC also varied over time: while relatively
309 more constant suppression was observed in the high and intermediate transfer frequency
310 treatments, pathogen growth suppression became clear in the low transfer frequency
311 treatment only towards the end of the selection experiment potentially due to media
312 growth adaptation in the no-ITC control treatment (Time × Transfer frequency × ITC
313 presence: $F_{2, 673}$= 7.33, $p< 0.001$; Fig. 2). Together these results suggest that the long-term
314 ITC activity varied temporally and depended on the ITC exposure and serial transfer
315 frequency.

316 ### (c) ITC tolerance evolution was observed only in the low transfer frequency
317 ITC exposure treatment

318 Fitness assays were conducted at the end of the selection experiment to compare the
319 growth of the ancestral strain and evolved populations from different treatments in the
320 presence and absence of allyl-ITC (experimental concentration: 500 μM). The ancestral
321 strain reached lower densities in the presence of ITC compared to evolved populations
322 regardless of the ITC treatment they had evolved in during the selection experiment
323 (Evolutionary history: $F_{2, 45}$= 5.39, p< 0.01; Tukey: p< 0.05; Fig. 3A). However, ITC tolerance
324 was mainly observed in the low transfer frequency ITC exposure treatment, while
325 populations that had evolved in the high or intermediate transfer frequency treatments did
326 not significantly differ from the ancestral strain (Transfer frequency within ITC-exposed
327 populations: $F_{2, 19}$= 24.72, p< 0.001; Tukey: p< 0.05; Fig. 3A). Surprisingly, even the control
328 populations that had evolved in the absence of ITCs in the low transfer frequency treatment
329 showed an increase in ITC tolerance (p< 0.05; Fig. 3A). One potential explanation for this is
330 that these populations adapted to grow better in CPG media, which could have helped to
331 compensate for the mortality imposed by allyl-ITC during the fitness assays. To test this, we
332 compared the growth of ancestral and evolved populations in the absence of allyl-ITC in the
333 CPG media (Fig. 3B). We found that all control populations showed improved growth in the
334 CPG media compared to ITC-exposed populations regardless of the transfer frequency
335 treatment (Evolutionary history: $F_{1, 40}$= 20.00, p< 0.001; Transfer frequency: $F_{2, 40}$= 2.66, p=
336 0.08, in all pairwise comparisons, Tukey: p< 0.05; Fig. 3B). In contrast, none of the ITC-
337 exposed populations showed improved growth in CPG media relative to the ancestral strain
338 (Tukey: p< 0.05; Fig. 3B), which suggests that ITC exposure constrained *R. solanacearum*
339 adaptation to the growth media.

340 To disentangle the effects due to adaptation to the media and allyl-ITC, we repeated
341 fitness assays in 'naïve' LB growth media which the bacteria had not adapted to. ITC
342 tolerance was observed only when bacterial populations had previously been exposed to
343 allyl-ITC (Evolutionary history: $F_{2, 49}$= 18.82, p< 0.001; Tukey: p< 0.05; Fig. 3C), and this effect
344 was driven by adaptation in the low transfer frequency ITC exposure treatment (no ITC
345 tolerance was observed in the high and intermediate transfer frequency treatment; Transfer
346 frequency: $F_{2, 49}$= 4.37, p< 0.01; Tukey: p< 0.05; Fig. 3C). Crucially, CPG-adapted control
347 populations showed no signs of ITC tolerance, but instead, suffered reduced growth in LB
348 media relative to the ancestral strain and ITC-exposed populations (Evolutionary history: $F_{2,}$
349 $_{49}$= 94.89, p< 0.001; Fig. 3D), which was clearest in the low transfer frequency exposure
350 treatment (Evolutionary history × Transfer frequency: $F_{2, 49}$= 23.17, p< 0.001; Fig. 3D).

351 We further validated our population level fitness results using individual clones (one
352 randomly chosen clone per replicate population per treatment). In line with previous
353 findings, ITC-exposed clones showed increased ITC tolerance compared to the control and
354 ancestral bacterium in the LB media (Evolutionary history: $F_{2, 49}$= 14.20, p< 0.001; Fig. 4A),
355 and tolerance evolution was the greatest in the low transfer frequency ITC exposure
356 treatment (Transfer frequency: $F_{2, 49}$= 11.15, p< 0.001; Tukey: p< 0.05; Evolutionary history ×
357 Transfer frequency: $F_{2, 49}$= 3.04, p< 0.05; Fig. 4A). Together, our results suggest that ITC
358 tolerance, which evolved in the low transfer frequency ITC exposure treatment was robust
359 and independent of the growth media it was quantified in. Moreover, while all control
360 populations adapted to grow better in the CPG media, this adaptation had a positive effect
361 on ITC tolerance only when quantified in CPG media and when the clones had evolved in the
362 low transfer frequency treatment.

363 ### (d) Evolution of ITC-tolerance confers cross-tolerance to ampicillin beta-
364 lactam antibiotic

365 We also tested if exposure to allyl-ITC could have led to cross-tolerance to other
366 antimicrobials such as the beta-lactam antibiotic ampicillin. Overall, both low (15 μg/ml) and
367 high (30 μg/ml) ampicillin concentrations had negative effects on *R. solanacearum* growth
368 relative to the no-ampicillin control treatment (Ampicillin concentration: $F_{2, 93}$= 50.12, p<
369 0.001; Tukey: p< 0.05; high concentration was relatively more inhibitory, Suppl. Fig. 3).
370 However, the evolved clones from the low transfer frequency ITC exposure treatment
371 reached significantly higher bacterial densities than the ancestral strain (Evolutionary
372 history: $F_{3, 92}$= 3.51, p< 0.05; Tukey: p< 0.05; Suppl. Fig. 3), while evolved clones derived from
373 low and intermediate transfer frequency control treatments (no prior ITC exposure) did not
374 differ from the ancestral strain (Tukey: p> 0.05; Suppl. Fig. 3). Ampicillin tolerance was only
375 observed in the high ampicillin concentration (High ampicillin concentration: $F_{3, 28}$= 8.22, p<
376 0.001; Suppl. Fig. 3C; Low ampicillin concentration: $F_{3, 28}$= 1.551, p= 0.223; Suppl. Fig. 3B).
377 Together these results suggest that ITC tolerance conferred cross-tolerance to ampicillin for
378 clones that had evolved in the low transfer frequency ITC exposure treatment.

379 ### (e) Media adaptation and ITC tolerance are linked to a few mutations and loss
380 of insertion sequences

381 A subset of clones which were phenotyped regarding ITC and ampicillin tolerance were
382 selected for genome sequencing (N=25). All isolated colonies showed ancestral, fluid colony
383 morphotype with no evidence for spontaneous evolution of small colony types as observed
384 previously (Khokhani et al., 2017; Perrier et al., 2019). Specifically, we focused on comparing
385 parallel small mutations and indels, intermediate indels (>50 bp) and prophage and

386 insertion sequence (IS) movement between populations that had evolved in the absence
387 and presence of ITC in the low transfer frequency treatments (evidence of ITC tolerance
388 evolution) with ancestral and control populations from the intermediate transfer frequency
389 treatment (no ITC tolerance evolution observed). Potential genetic changes were
390 investigated in both the chromosome and megaplasmid of the bipartite genome.

391 Only a few mutations were observed in 1 to 6 different genes, which was expected
392 considering the relatively short duration of the selection experiment (16 days). Of these
393 mutations, 8 were non-synonymous and 4 synonymous (Table 1). Some mutations were
394 observed across all treatments, indicative of adaptation to the culture media or other
395 experimental conditions. For example, parallel non-synonymous mutations in *hisH1* gene
396 controlling imidazole glycerol phosphate synthase were observed in 6/8 to 8/8 replicate
397 clones in all treatments (Table 1; Fig. 5). Similarly, non-synonymous mutations in
398 serine/threonine protein kinase genes (between 5/8 to 8/8 replicate clones) and
399 synonymous mutations in putative deoxyribonuclease *RhsC* gene (between 1/8 to 5/8
400 replicate clones) were found across all treatments (Table 1; Fig. 5). A single clone that had
401 evolved in the absence of allyl-ITC in the intermediate transfer frequency treatment had a
402 unique non-synonymous mutation in the gene encoding the putative HTH-type
403 transcriptional regulator *DmlR* and another clone originating from this treatment had a
404 mutation in the IS5/IS1182 family transposase encoding gene (Table 1; Fig. 5). Additionally,
405 we observed mutations exclusively in the low transfer control clones in genes encoding the
406 dehydrogenase-like uncharacterised protein (3/8 replicate clones) and Tat pathway signal
407 protein (2/8 replicate clones; Table 1; Fig. 5), which may explain ITC tolerance via media
408 adaptation. However, no clear parallel mutations exclusive to the low frequency ITC-
409 exposed populations were found.

410 In terms of putative intermediate indels (>50 bp), we identified 122 and 116 indel
411 sites in the chromosome (Chr) and megaplasmid (MP), respectively. Almost all of these were
412 insertions (Chr, 119/122; MP, 113/116) and the majority were singletons (Chr, 101/122; MP,
413 95/116) or doubletons (Chr, 14/122; MP, 13/116). The number of intermediate indels did
414 not differ between evolutionary treatments either in the chromosome (Kruskal-Wallis: x^2=
415 3.65; df= 2; p= 0.161) or megaplasmid (Kruskal-Wallis: x^2= 3.46; df= 2; p= 0.178). As a result,
416 this genetic variation was likely non-adaptive and driven by random drift.

417 To identify other potential molecular mechanisms, variation in prophages and
418 insertion sequences (ISs) was investigated. Two prophages were found in all sequenced
419 isolates: Inoviridae prophage ϕRS551 and a novel, unclassified prophage (Table S2).
420 Prophage genome positions were almost identical between all sequenced isolates (Table
421 S2). Therefore, no evidence for systematic prophage movement was observed in the
422 evolved isolates relative to the ancestral strain. In contrast, ISs appeared to be highly mobile
423 regarding 15 variable positions in the chromosome and 15 variable positions in the
424 megaplasmid (Suppl. Fig. 5). In most variable positions (7 in the chromosome and 9 in the
425 megaplasmid), the gain or loss of ISs was infrequent, occurring in up to three clones per
426 treatment (Suppl. Fig. 5), which is indicative of non-adaptive, random IS movement.
427 However, the remaining IS positions showed higher frequency of gain or loss, indicating of
428 potentially adaptive IS movement which was also in some cases treatment-specific. For
429 example, an IS element in position 2302900 on the chromosome absent in the ancestral
430 strain was observed in 2 clones in the intermediate transfer frequency control and 2 low
431 transfer ITC treatment clones, while it was gained by 5 clones in low transfer control
432 treatment. The IS element in this position was found to be close to the start codon (~50 bp)
433 of an acyltransferase. In two of the low transfer control clones, the IS was found to disrupt

434 the gene (Fig. 5), potentially knocking out acyltransferase gene expression after inserting
435 into this position. Moreover, three IS elements in the megaplasmid were almost exclusively
436 lost in the low transfer frequency treatment (Fig. 5). In one of the positions (209500), the IS
437 disrupted a putative calcium-binding protein in the intermediate transfer control clones but
438 was absent in 4/8 low transfer control and 4/8 low transfer ITC treatment clones. In the
439 other two positions (243500 and 253900), the ISs were intergenic (positioned 450 bp and
440 104 bp (243500) and 301 bp and 46 bp (253900) from their left- and right-flanking genes;
441 Fig. 5). The right-flanking genes closest to the ISs included a (2Fe-2S)-binding protein
442 (243500) and an H-NS histone family protein (253900), whilst the left-flanking genes
443 included the type III effector *HopG1* (243500) and an unknown hypothetical protein
444 (253900). The frequency of IS absence in these positions (243500 and 253900) differed
445 between low transfer treatments. Specifically, in position 243500, the IS was absent in 7/8
446 low transfer control and 5/8 low transfer ITC treatment clones. Meanwhile, in position
447 253900, the IS was absent in 4/8 low transfer control and 6/8 low transfer ITC treatment
448 clones. However, despite these patterns, the extent of IS loss did not differ statistically
449 between low transfer control and ITC-exposed clones when analysed individually (Mann-
450 Whitney: 209500: w= 32, n1= 8, n2= 8, p= 1; 243500: w= 40, n1= 8, n2= 8, p= 0.29; 253900:
451 w= 24, n1= 8, n2= 8, p= 0.35) or in combination (Mann-Whitney: w= 32, n1= 8, n2= 8, p= 1).
452 Together, these results suggest that media adaptation and ITC tolerance was potentially
453 driven by parallel mutations in a few genes and more frequent loss of IS elements in the low
454 transfer frequency treatments.

455 # Discussion

456 Here we studied the effects of *Brassica*-derived ITC allelochemicals for the suppression and
457 tolerance evolution of plant pathogenic *R. solanacearum* bacterium in a model
458 biofumigation experiment. We found that only allyl-ITC suppressed *R. solanacearum*
459 growth, while no reduction in pathogen densities were observed when sec-butyl and 2-
460 phenylethyl ITCs were applied alone or in combination. By using experimental evolution, we
461 further showed that long-term allyl-ITC exposure selected for ITC tolerance in the low
462 transfer frequency ITC exposure treatment and was associated with cross-tolerance to
463 ampicillin. At the genetic level, tolerance evolution was associated with the loss of IS
464 elements. Together, our results suggest that allyl-ITC derived from Indian mustard is
465 effective at suppressing the growth of the *R. solanacearum* pathogen *in vitro*. However,
466 prolonged exposure could select for increased ITC tolerance, potentially reducing the
467 efficiency of ITC-based biocontrol.

468 Only allyl-ITC suppressed pathogen growth and its effects were not enhanced by the
469 presence of other ITCs. This contradicts previous studies which demonstrated *R.*
470 *solanacearum* sensitivity to 2-phenylethyl ITC at concentrations as low as 330 μM (Smith &
471 Kirkegaard, 2002). However, in the previous experiment *R. solanacearum* was exposed to 2-
472 phenylethyl ITC in agar instead of liquid media, which has been shown to increase the
473 toxicity of ITCs (Sarwar et al., 1998). Moreover, it is possible that different *R. solanacearum*
474 strains respond differently to ITCs, which could also explain discrepancy between ours and
475 other studies. While the suppressive effects of sec-butyl ITC have previously been
476 demonstrated against dust mites (Yun et al., 2012) and fungi (Bainard et al., 2009), no
477 antimicrobial activity has been observed in bacteria. Variation in the antimicrobial activity of

478 ITCs could be explained by differences in chemical side-chain structure and molecular
479 weight which govern ITC volatility and hydrophobicity (Sarwar et al., 1998). Previous studies
480 have shown greater pathogen suppression by ITCs with aliphatic compared to aromatic
481 sidechains in fungal pathogens (Kurt et al., 2011; Sarwar et al., 1998), insect pests
482 (Matthiessen & Shackleton, 2005), and weeds (Vaughn et al., n.d.). With bacteria, the
483 toxicity of allyl-ITC could be attributed to its high volatility, very short R-side chains and high
484 reactivity (Kirkegaard & Sarwar, 1998; Manici et al., 1997; Neubauer et al., 2014). These
485 properties could enable rapid diffusion through the liquid media before ITC is lost in the
486 gaseous phase (Wang et al., 2009). This is supported by a study by Sarwar *et al.* (Sarwar et
487 al., 1998), where a droplet of aliphatic allyl-ITC was shown to volatilise at room temperature
488 in 5 minutes, whilst aromatic 2-phenylethyl ITC remained in the liquid for over 72 hours.
489 Together, our result suggests that high volatility and reactivity could be important
490 properties determining the antibacterial effects of ITCs.

491 The evolution of ITC tolerance was mainly observed in the low transfer frequency ITC
492 exposure treatment. However, we also found that low transfer frequency control
493 populations showed improved tolerance measured in CPG media even though they had not
494 been exposed to allyl-ITC during the experiment. As all treatments were kept separate from
495 each other using tightly sealed bags, this effect is unlikely explained by 'cross selection' due
496 to ITC volatilisation. Alternatively, ITC tolerance evolution could have been linked to certain
497 metabolic adaptations in this transfer frequency treatment. In support of this, we found that
498 evolved control bacterial populations showed improved growth in the CPG media relative to
499 ancestral and ITC-exposed populations, indicative of media adaptation. While similar media
500 adaptations were observed in all control treatment populations, it is not clear why ITC
501 tolerance did not evolve under one- and two-day transfer frequency treatments. One

502 potential explanation for this could be growth-dependent effects on mutation rates. For
503 example, prior studies have shown that bacterial mutation rates can be elevated at
504 stationary phase (Loewe et al., 2003; Navarro Llorens et al., 2010), which could have
505 promoted ITC tolerance and media adaptation in the low transfer frequency treatment
506 where bacteria had spent the relatively longest time at stationary phase (Suppl. Fig. 1).
507 Alternatively, stationary phase growth conditions could have triggered expression of stress
508 tolerance genes, enabling selection for mutants with relatively higher ITC tolerance (Navarro
509 Llorens et al., 2010). For example, expression of *RpoS* sigma factor in *P. aeruginosa* has
510 previously been linked to elevated antibiotic resistance and biofilm formation at stationary
511 phase (Murakami et al., 2005; Olsen, 2015). While more work is needed to elucidate these
512 mechanisms, it is likely that the periodic 3-day growth cycle was important for driving ITC
513 tolerance evolution in our experimental conditions. Interestingly, the ITC tolerance that
514 evolved in the absence of allyl-ITC exposure was specific to CPG media and disappeared
515 when measured in 'naïve' LB media. This result suggests that ITC tolerance observed in
516 control populations was likely driven by adaptation to CPG growth media. Such adaptation
517 may have helped to offset the suppressive effects of allyl-ITC by boosting pathogen growth
518 to compensate increased mortality. Alternatively, it is possible that the glucose availability
519 in the CPG media indirectly favoured the evolution of ITC tolerance via metabolic
520 adaptations, which has previously been shown to occur both in the absence (Knöppel et al.,
521 2017) and presence of clinical antibiotics (Zampieri et al., 2017). Together, our results
522 suggest that prior exposure to allyl-ITC was required for the evolution of robust ITC
523 tolerance, which was independent of the growth media.

524 At the genetic level, ITC tolerance was not associated with any clear parallel mutations
525 or indels in the low transfer frequency treatments. Three clones from the low transfer

526 frequency control treatment had unique mutations in a gene encoding a dehydrogenase-like
527 uncharacterised protein. Dehydrogenase genes have previously been associated with both
528 metabolism and antibiotic resistance (Marshall et al., 1999). For instance, in *Escherichia coli*,
529 a mutation in a glucose dehydrogenase gene has been shown to function in
530 lipopolysaccharide modification and calanic acid biosynthesis, which enabled resistance to
531 polymyxin and other antimicrobial peptides (Lacour et al., 2008; Rodionova et al., 2020),
532 and may have contributed to ITC tolerance in these clones. Additionally, two clones from
533 the low transfer control treatment had mutations in a gene encoding a Tat pathway signal
534 protein which is involved in protein translocation across membranes (Palmer et al., 2005),
535 and may have enabled improved growth in the CPG media. Three clones from the
536 intermediate transfer frequency treatment had unique mutations in a gene encoding a
537 probable transcription regulator protein. While there is little information available regarding
538 this gene, it is located beside the IS2 transposase *TnpB* gene, potentially affecting its
539 regulation in DNA replication, recombination and repair activity (Pasternak et al., 2013).
540 Instead of treatment-specific parallel mutations, certain mutations were found across all
541 treatments. For example, mutations in genes encoding putative serine/threonine protein
542 kinases, amino acid biosynthesis (*hisH1* gene) and DNA replication, recombination and
543 repair (putative *RhsC* gene) were common for clones isolated from all treatments.
544 Mutations observed in serine/threonine protein kinase genes could have potentially
545 affected ITC tolerance if these enzymes were targeted by the ITCs as has been shown before
546 in the fungus *Alternaria brassicicola* (Calmes et al., 2015), and bacterial pathogen *E. coli*
547 (Luciano & Holley, 2009). However, as these mutations were not specific to ITC-treatment
548 clones, they were probably associated with bacterial growth and metabolism.

549 In *R. solanacearum*, insertion sequences (ISs) have been shown to affect host
550 virulence and phenotypic plasticity by inserting into and disrupting type III effectors and
551 global virulence regulators (Gonçalves et al., 2020; Jeong & Timmis, 2000). Therefore, we
552 investigated whether IS movement may be the cause of *R. solanacearum* ITC tolerance
553 adaptation. We identified one IS position in the chromosome and three positions in the
554 megaplasmid which showed treatment specific patterns. The gain of IS at position 2302900
555 was primarily observed with low transfer control isolates and was situated either ~50 bp
556 from the start codon or inside of a putative acyltransferase. Acyltransferases have a broad
557 range of functions including lipid storage (Ohlrogge & Browse, 1995), phospholipid
558 biosynthesis (Li et al., 2017), and the production of toxins (Greene et al., 2015) and
559 antibiotics (Kozakai et al., 2020). Whilst many of these functions are critical to cell growth,
560 some such as the production of toxins would be redundant when grown in media.
561 Therefore, gene disruption by ISs in the low transfer control may increase fitness by allowing
562 energy and nutrients to be re-directed towards promoting cell growth and competitivity,
563 potentially at the expense of reduced virulence *in planta*. We also found loss of two ISs in
564 the intergenic region of the megaplasmid in the low transfer control and ITC treatments.
565 While these were intergenic, they were close (~50-100 bp) to the start codons of their right
566 flanking genes and could have affected gene expression. In position 243500, the IS was
567 situated close to a (2Fe-2S)-binding protein gene. Iron-sulfur clusters have been implicated
568 in cellular metabolism, protein structural stabilisation, iron storage, and the regulation of
569 gene expression (Johnson et al., 2005). In the other position (253900), the IS was situated
570 close to an H-NS histone like protein gene and while non-significant, was lost more
571 frequently across low transfer ITC treatment clones (6/8) than low transfer control isolates
572 (4/8). H-NS histone like proteins are transcriptional repressors generally involved in

573 adaptation to environmental challenges like temperature stress and osmolarity gradients
574 (Atlung & Ingmer, 1997). Further, H-NS histone like proteins have been shown to stabilise
575 the sigma factor *RpoS* (Hommais et al., 2001) which acts as a master regulator of the
576 bacterial stress response. Whilst the H-NS histone-like protein could affect ITC tolerance by
577 mediating the bacterial stress response, the impact of the (2Fe-2S)-binding protein is less
578 clear. Notably, in *Campylobacter jejuni*, genes containing iron-sulfur clusters have been
579 found to be upregulated in response to ITCs, potentially due to their susceptibility to
580 oxidative stress caused by ITC exposure (Dufour et al., 2013). Therefore, by altering the
581 expression of the (2Fe-2S)-binding protein, IS loss could increase the pool of cellular iron-
582 sulfur cluster proteins and compensate for losses caused by ITC oxidative stress. In the final
583 megaplasmid IS position (209500), we identified a loss of IS from a calcium-binding protein
584 gene, which had likely disrupted gene expression or protein function in this gene with the
585 ancestral strain. In human breast cancer cells, ITCs, including phenethyl- (Tusskorn et al.,
586 2013) and allyl-ITC (Bo et al., 2016) have been found to induce mitochondrial calcium ion
587 mobilisation resulting in cytotoxicity through a reduction in mitochondrial membrane
588 potential. Whilst further work is required to determine the causes of ITC cytotoxicity in *R.*
589 *solanacearum*, upregulation of calcium-binding protein gene expression could have
590 increased ITC tolerance by facilitating the sequestration of free calcium ions. However, like
591 other genetic changes, loss of this IS did not occur statistically more often in the presence of
592 ITC selection. As a result, specific genetic mechanisms underlying ITC tolerance remain
593 elusive.

594 In conclusion, our findings demonstrate that allyl-ITC could potentially be used to
595 suppress the growth of *R. solanacearum* plant pathogen. However, repeated ITC exposure

could select for mutants with increased ITC tolerance, potentially weakening the long-term efficiency of ITCs and biofumigation. Future work should focus on validating these findings in more complex natural environments. For example, it is currently not clear if *R. solanacearum* ITC tolerance evolves in the plant rhizosphere in the presence of other microbes that could constrain mutation supply rate via resource and direct competition. Moreover, different resistance mechanisms could be selected depending on soil physiochemical properties and nutrient and plant root exudate availability, while it is not clear if the ITC concentrations used in this experiment are achievable through biofumigation and whether they might have negative effects on beneficial soil microbes. More efficient ITC application could be attained by drilling the biofumigant plants into fields at the time of flowering when GSL levels are highest using finely chopped plant material, which maximises cell disruption and ITC release to the soil (Back et al., 2019). In addition, the efficacy of *Brassica*-based biofumigation could potentially be improved by using plant cultivars with elevated levels of sinigrin, the GSL precursor to allyl-ITC. Comprehensive *in vivo* work is thus required to validate the potential of allyl-ITC for *R. solanacearum* biocontrol in the field. It would also be interesting to study if ITC tolerance leads to life-history traits in *R. solanacearum,* potentially affecting its virulence or competitiveness in the rhizosphere.

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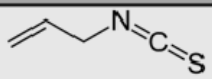
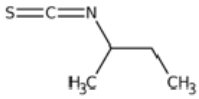
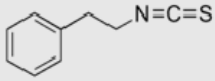
Tables

Location	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Chromosome	Plasmid	Plasmid	Plasmid
Position	293900	294413	585646	1257034	1258240	2302809	2830656	2874048	3123064	44512	105610	124931
Type	snp	snp	ins	snp	snp	snp	ins	ins	ins	del (27bp)	del (1bp)	snp
Ref	G	C	T	G	C	C	A	C	A	TCGTGAGCGGCA AGCCGGCACATCG CAA	TG	G
Alt	A	T	TCGTGCTG	C	G	T	ACAGCAACGG	CGGGCACT	AC	T	T	A
Effect	Synonymous variant	Synonymous variant	Frameshift variant	Missense variant	Missense variant	Synonymous variant	Conservative inframe insertion	Frameshift variant	Conservative inframe insertion	Frameshift variant	Synonymous variant	Missense variant
Locus tag	RSUY_02640	RSUY_02640	RSUY_05230	RSUY_11710	RS_RS11675	RSUY_21390	RSUY_26530	ATK36_5281	BSE24067_05643	NA8A_21102	RSIPO_03141	RSUY_33140
Product	Putative deoxyribonuclease RhsC	Putative deoxyribonuclease RhsC	Imidazole glycerol phosphate synthase subunit HisH1	HTH-type transcriptional regulator DmlR	Probable transcription regulator protein (86.1% similarity)	F-box domain-containing protein (83.3% similarity)	Putative serine/threonine protein kinase (84.6% similarity)	Dehydrogenase-like uncharacterised protein (90.9% similarity)	Tat pathway signal protein (90.9% similarity)	DNA-3-methyladenine glycosidase II (100% similarity)	Hypothetical transmembrane protein (100% similarity)	IS5/IS1182 family transposase (100% similarity)
Int No ITC 1												
Int No ITC 2												
Int No ITC 3												
Int No ITC 4												
Int No ITC 5												
Int No ITC 6												
Int No ITC 7												
Int No ITC 8												
Low No ITC 1												
Low No ITC 2												
Low No ITC 3												
Low No ITC 4												
Low No ITC 5												
Low No ITC 6												
Low No ITC 7												
Low No ITC 8												
Low ITC 1												
Low ITC 2												
Low ITC 3												
Low ITC 4												
Low ITC 5												
Low ITC 6												
Low ITC 7												
Low ITC 8												

Table 1. Mutated *Ralstonia solanacearum* genes and annotated gene functions observed in intermediate and low transfer frequency control (no-ITC), and ITC-exposed low transfer frequency treatments. Gene function predictions were derived based on BLAST using UNIPROT and percentage (%) sequence similarity is included for putative (hypothetical) proteins. Filled cells denote for the presence of mutations in given clones and white cells denote for the absence of given mutations. Replicates are named by treatments, IntNoITC= Intermediate transfer frequency, no ITC; LowNoITC= Low transfer frequency, no ITC; LowITC= Low transfer frequency, ITC.

Figures and figure legends

A

Isothiocyanate	IUPAC name	Molar mass (g mol^{-1})	Structure
Allyl	3-isothiocyanato-1-propene	99.15	
Sec-Butyl	2-isothiocyanatobutane	115.19	
2-Phenylethyl (PEITC)	(2-Isothiocyanatoethyl) benzene	163.24	

B

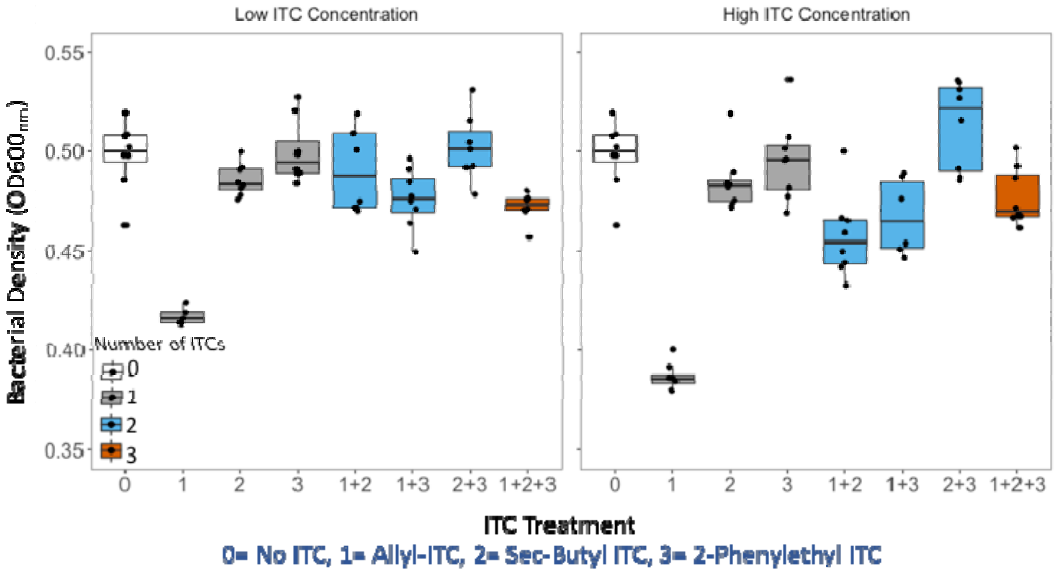
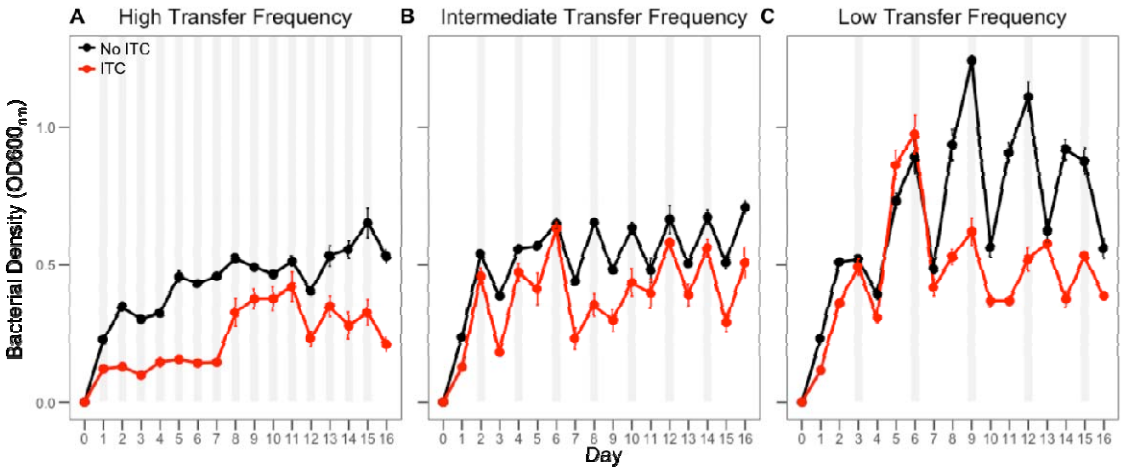


Figure 1. The antimicrobial activity of different ITCs against *Ralstonia solanacearum* pathogen when applied alone and in combination. The chemical properties of the three different ITCs predominantly released from Indian mustard biofumigant plant (*Brassica juncea*) (A), and their effects on *R. solanacearum* growth after 48h exposure when applied alone and in combination in liquid microcosms at low (500 µM) and high (1000 µM) concentrations (B). In (B) boxplot colours represent different ITC treatments that are labelled on X-axes as follows: (0): no-ITC (control); (1) allyl-ITC; (2) sec-butyl ITC and (3) 2-phenylethyl ITC. Individual data points show bacterial densities for each technical replicate

(N=8). The boxplots show the minimum, maximum, interquartile range and the median (black line).



998

999 **Figure 2. *Ralstonia solanacearum* density dynamics ($OD600_{nm}$) during the evolution**

1000 **experiment in the absence and presence of allyl-ITC in high, intermediate and low transfer**

1001 **frequency treatments.** In all panels, black and red lines correspond to *R. solanacearum*

1002 densities in the absence and presence of 500 µM allyl-ITC, respectively. Panels A-C

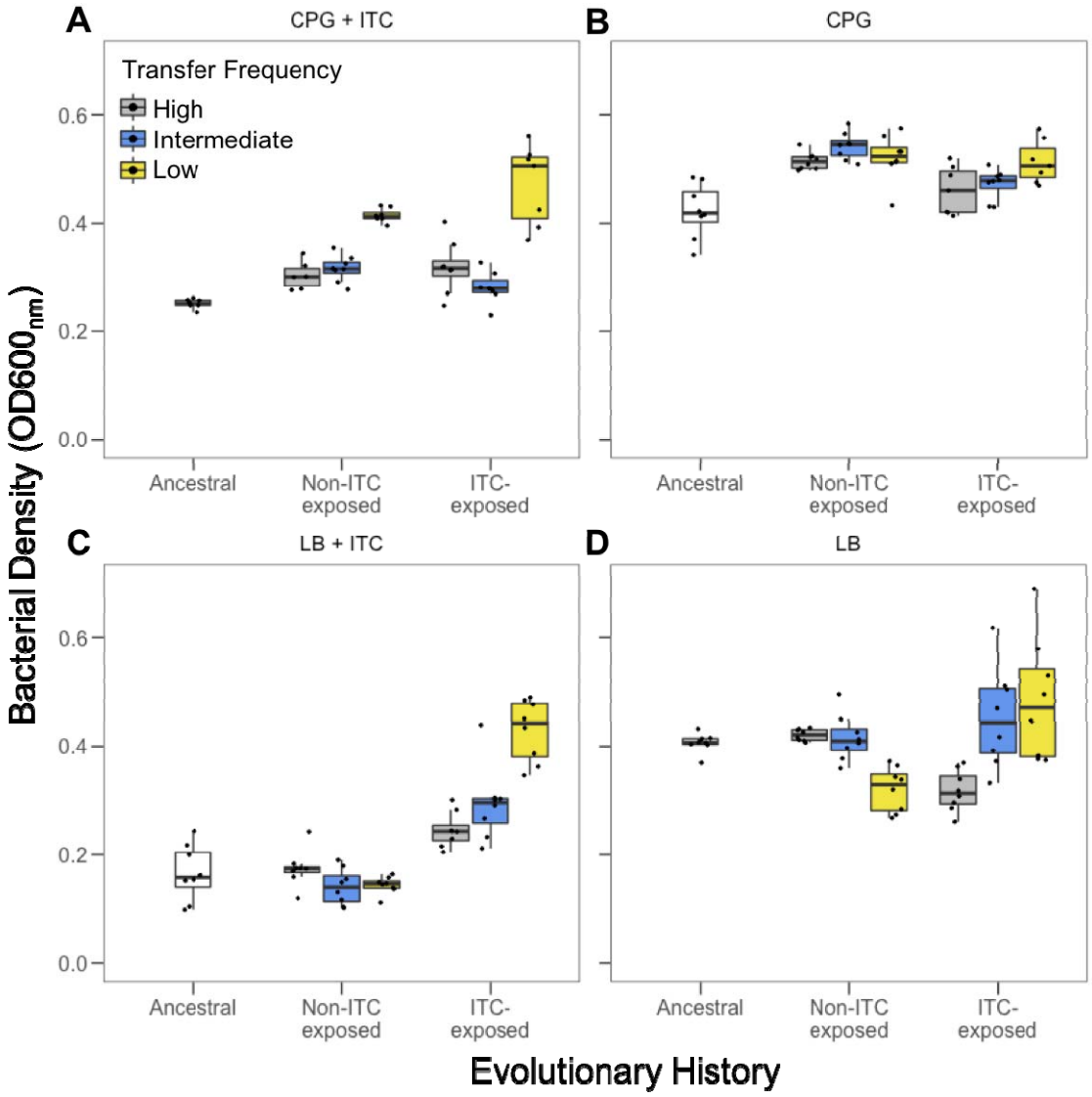
1003 correspond to high (1-day), intermediate (2-day) and low (3-day) transfer frequency

1004 treatments, respectively. Grey shaded areas indicate the time point of serial transfers, while

1005 optical density reads were taken at 24-hour intervals in all treatments. Each time point

1006 shows the mean of eight biological replicates and bars show ±1 error of mean.

1007



1008

1009 **Figure 3. Comparison of *Ralstonia solanacearum* ITC tolerance between the ancestral**

1010 **clone and evolved populations from high, intermediate and low transfer frequency**

1011 **treatments at the end of the evolution experiment in CPG and LB media.** ITC tolerance was

1012 determined as *R. solanacearum* growth ($OD600_{nm}$) after 48h exposure to 500 µM allyl-ITC in

1013 CPG (A) and LB (C) media. Growth was also measured in the absence of allyl-ITC in both CPG

1014 (B) and LB (D) media. High (1-day), intermediate (2-day) and low (3-day) transfer frequency

1015 treatments are shown in grey, blue and yellow boxplots, respectively, and boxplots show

1016 the minimum, maximum, interquartile range and the median (black line). Individual data

1017 points show bacterial densities for each biological replicate population (N=8).

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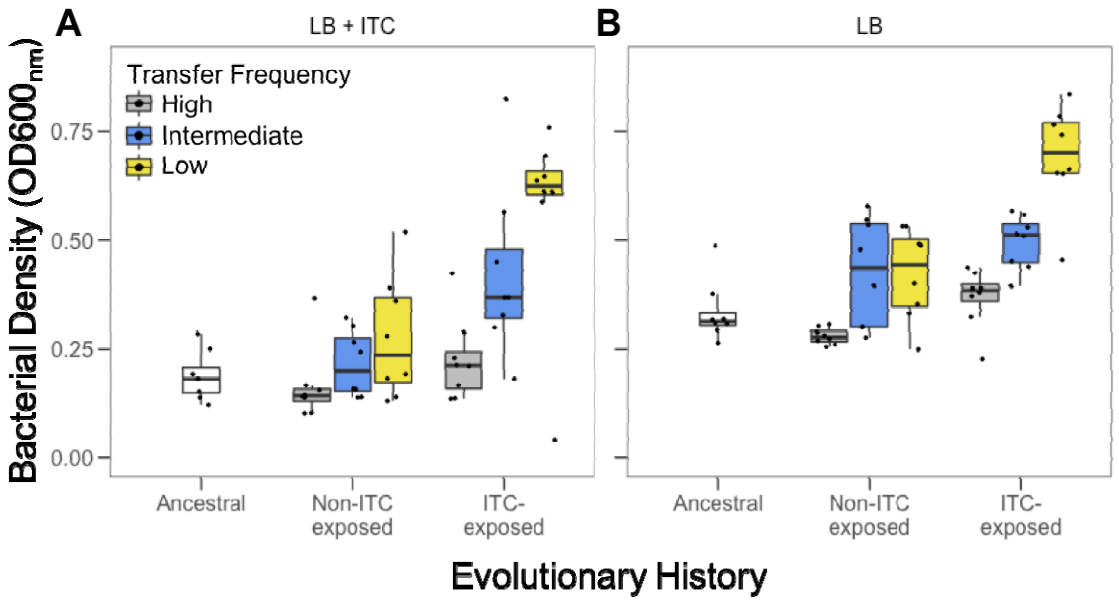

1019

1020 **Figure 4. Comparison of *Ralstonia solanacearum* ITC tolerance between the ancestral and**

1021 **evolved clones from high, intermediate and low transfer frequency treatments at the end**

1022 **of the evolution experiment in LB media.** ITC tolerance was determined as *R. solanacearum*

1023 growth ($OD600_{nm}$) after 48h exposure to 500 µM allyl-ITC in LB media (A). Growth was also

1024 measured in the absence of allyl-ITC (B). High (1-day), intermediate (2-day) and low (3-day)

1025 frequency treatments are shown in grey, blue and yellow, respectively, and boxplots show

1026 the minimum, maximum, interquartile range and the median (black line). Individual data

1027 points show bacterial densities for each biological replicate population (N=8).

1028

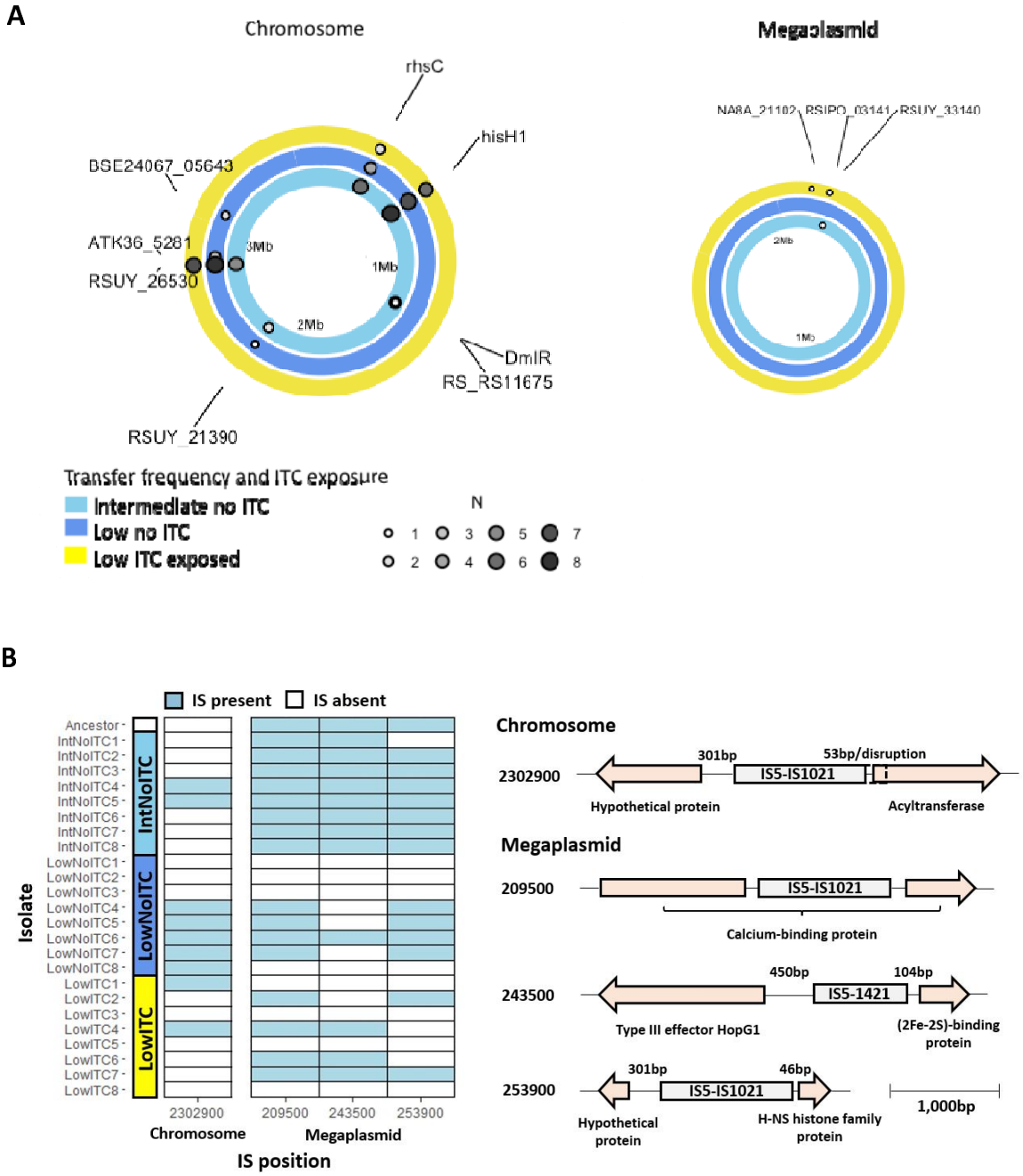

Figure 5. Mutations (A) and insertion sequences (IS; B) associated with evolved *Ralstonia solanacearum* clones. Each ring in panel A represents the *R. solanacearum* genome (Chromosome on the left and Megaplasmid on the right). Rings are grouped by the sequenced treatments) in different colours (see key) and dots represent mutations at different loci. Dots are sized and coloured by the number of replicates that had the same mutations (N=8) in the indicated locus. Labels show the gene name, when named, or the

1037 numbered locus tag. Distance marker is shown as Mb within each ring. In panel B, tile plot
1038 shows presence (filled tiles) and absence (unfilled tiles) of insertion sequences (ISs) in each
1039 isolate. The X-axis of the tile plot shows the IS position rounded to the nearest 100 bp. Gene
1040 schematics on the right show insertion sequence at each position and nearby genes. Gene
1041 annotation and distance between insertion sequence and genes are shown, with gene size
1042 and distance proportional to the scale bar (bottom right).

Supplementary Materials

Supplementary Table 1. The mean density reduction (%) of *Ralstonia solanacearum* bacterium when exposed to 500 or 1000 µM allyl, sec-butyl and 2-phenylethyl ITCs in CPG growth media after 24, 48 or 72 hours relative to when grown in the absence of ITCs. This table is based on the same data presented in Supplementary Fig. 2.

ITC Type and Concentration (µM)	Time (h)	Bacterial density reduction (%) compared to control
Allyl-ITC, 500	24	66
	48	54
	72	27
Allyl-ITC, 1000	24	66
	48	47
	72	41
Sec-Butyl ITC, 500	24	33
	48	26
	72	9
Sec-Butyl ITC, 1000	24	30
	48	27
	72	8
2-Phenylethyl ITC, 500	24	39
	48	13
	72	10
2-Phenylethyl ITC, 1000	24	38
	48	18
	72	13

1049 **Supplementary Table 2. Prophage information of ancestral and experimental isolate assemblies as determined using flanking regions**

1050 **mapped to UY031.** Replicates are named by treatments, IntNoITC= Intermediate transfer frequency, no ITC; LowNoITC= Low transfer

1051 frequency, no ITC; LowITC= Low transfer frequency, ITC.

1052

Clone	Prophage	Left flank UY031 position	Right flank UY031 position	Length (kb)	GC content (%)	Total proteins #
UY031	Unclassified A	-	-	37	62.76	44
	RS551	-	-	13.4	61.24	16
	PHAGE_Vibrio_VHML_NC_004456	-	-	18.5	64.64	29
Ancestor	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237639	13.1	58.58	17
IntNoITC1	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	17
IntNoITC2	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	17

IntNoITC3	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	43
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	17
IntNoITC4	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	43
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	18
IntNoITC5	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	18
IntNoITC6	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	43
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	18
IntNoITC7	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	43
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1218233-1223232	13.1	58.58	17
IntNoITC8	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	41
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	17
LowNoITC1	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42

	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	17
LowNoITC2	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	18
LowNoITC3	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	41
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	18
LowNoITC4	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	43
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	17
LowNoITC5	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	43
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236385-1237640	13.1	58.58	18
LowNoITC6	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	41
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237640	13.1	58.58	18
LowNoITC7	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	41
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LowNoITC8	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
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LowITC1	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	43
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237640	13.1	58.58	17
LowITC2	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	43
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237640	13.1	58.58	17
LowITC3	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237640	13.1	58.58	18
LowITC4	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237639	13.1	58.58	17
LowITC5	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	41
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237639	13.1	58.58	18
LowITC6	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42

	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237639	13.1	58.58	17
LowITC7	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	41
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237639	13.1	58.58	18
LowITC8	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	41
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237639	13.1	58.58	18

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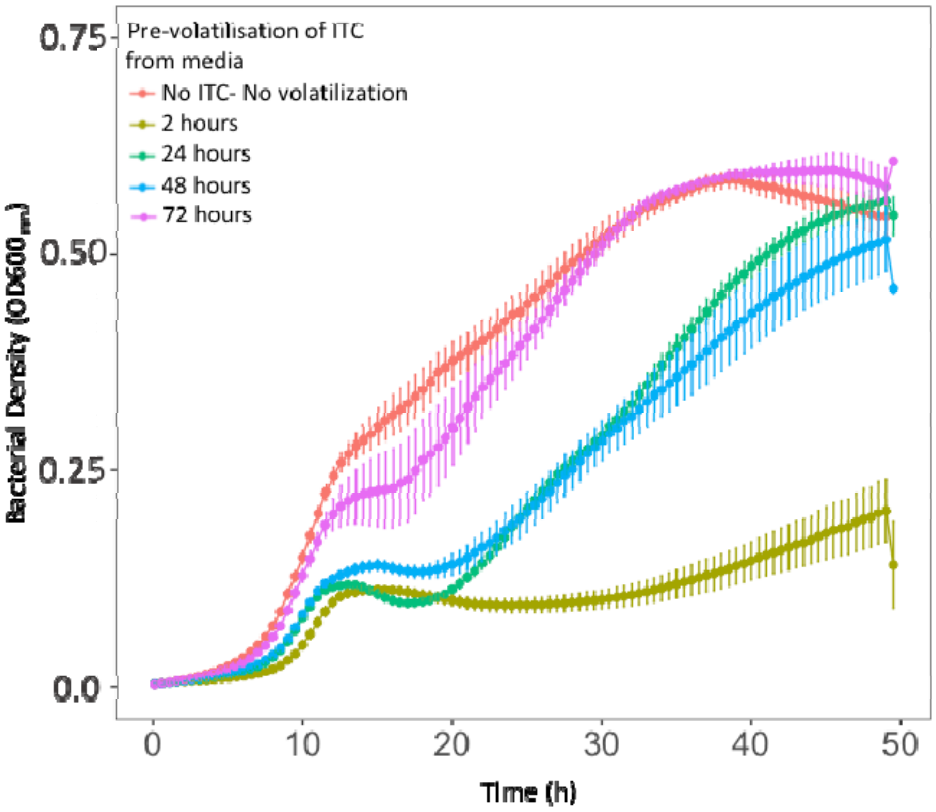

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1055

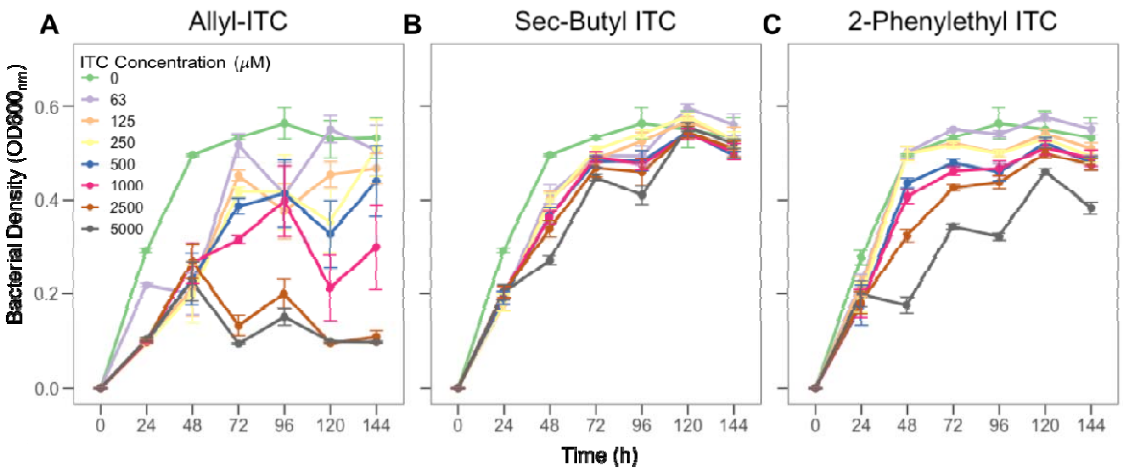
1056 **Supplementary Figure 1. The effect of allyl-ITC pre-volatilisation for antibacterial activity**

1057 **against *Ralstonia solanacearum*.** *R. solanacearum* bacterial growth was measured in CPG

1058 media supplemented with 0 (No allyl-ITC) or 500 µM of allyl-ITC that had been allowed to

1059 volatilise for 2, 24, 48 or 72 hours (see key). All data points show the mean of eight technical

1060 replicates and bars show ±1 standard error of the mean (SEM).



1061

1062 **Supplementary Figure 2. Effects of allyl, sec-butyl and 2-phenylethyl ITCs on *Ralstonia***

1063 ***solanacearum* growth at different ITC concentrations.** In all panels, *R. solanacearum*

1064 bacterial densities are shown on the Y-axis as optical density ($OD600_{nm}$), measured at 24-

1065 hour intervals (X-axis). In all panels, different line colours refer to different ITC

1066 concentrations (see key in A). All data points show the mean of eight technical replicates

1067 and bars show ±1 standard error of the mean (SEM).

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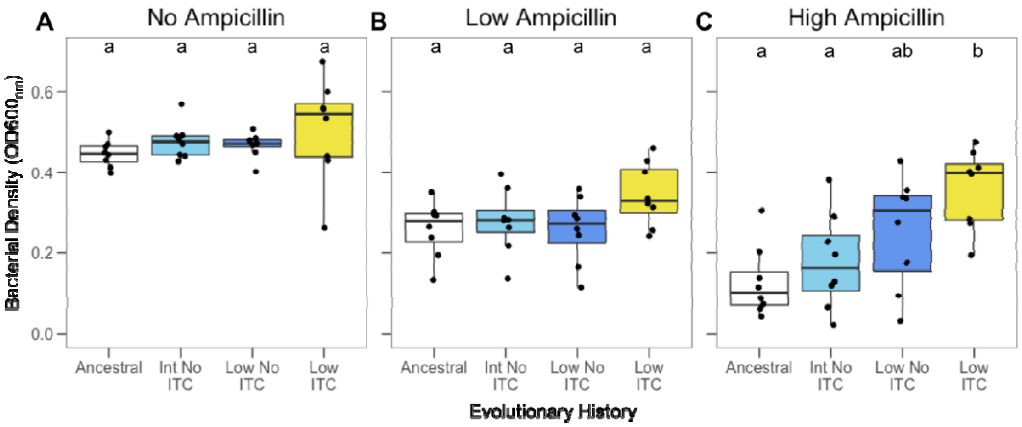
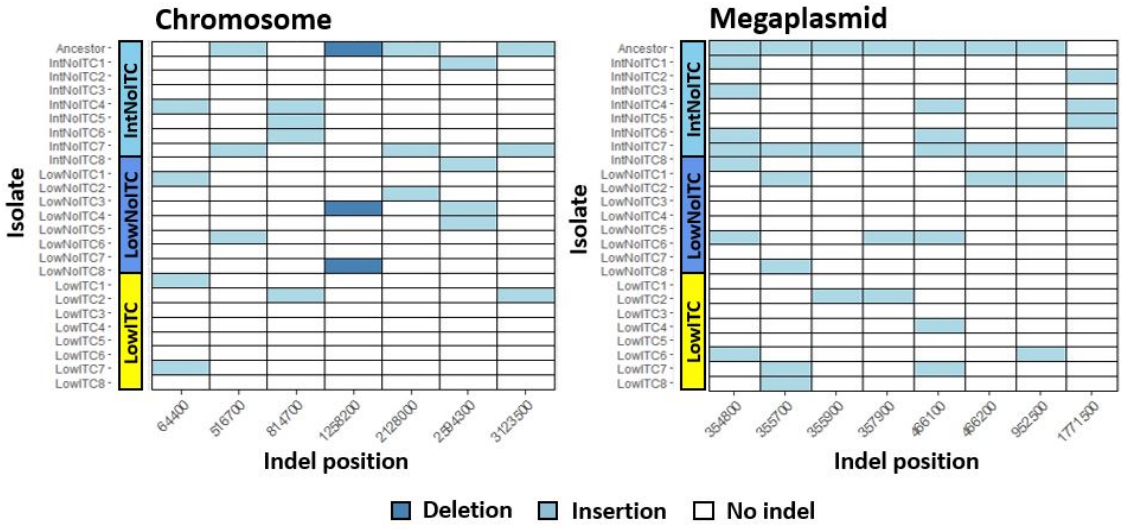


Supplementary Figure 3. *Ralstonia solanacearum* tolerance to ampicillin beta-lactam antibiotic. Ampicillin tolerance was measured as the growth of ancestral and evolved *R. solanacearum* clones isolated from intermediate (Int) and low transfer frequency (Low) control treatments (no-ITC) and ITC-exposed low transfer frequency treatment in the absence (A) and presence (B-C) of ampicillin (15 and 30 µg/ml concentrations). Boxplots show the minimum, maximum, interquartile range and the median (black line) after 48 hours. Individual data points show bacterial densities for each biological replicate clone (N= 8). Different small case letters above boxplots indicate significant pairwise differences (Tukey: $p < 0.05$) between treatments within each panel.



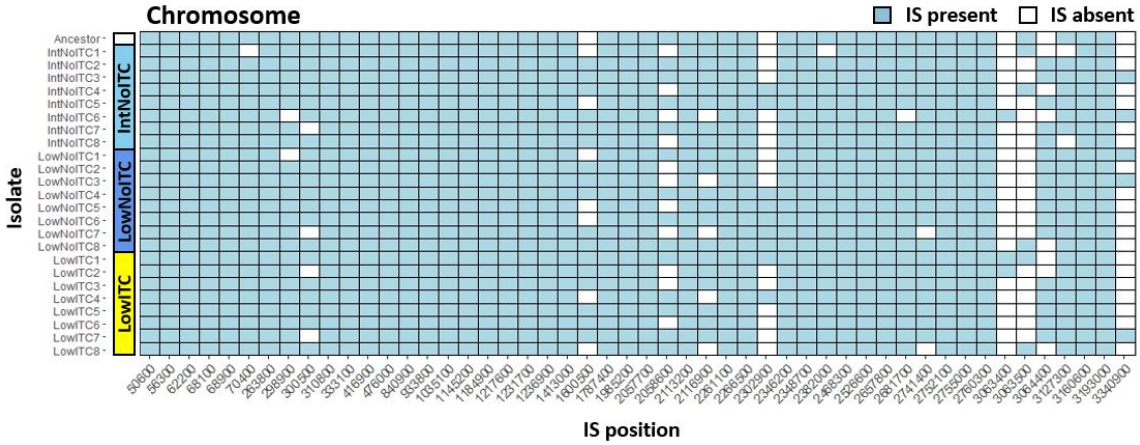
1079

1080 **Supplementary Figure 4. Presence and absence of intermediate indels found in more than**

1081 **two isolates in the chromosome and megaplasmid.** The X-axis shows the indel position

1082 rounded to the nearest 100bp. The Y-axis shows isolates grouped as shown in Figure 5.

1083



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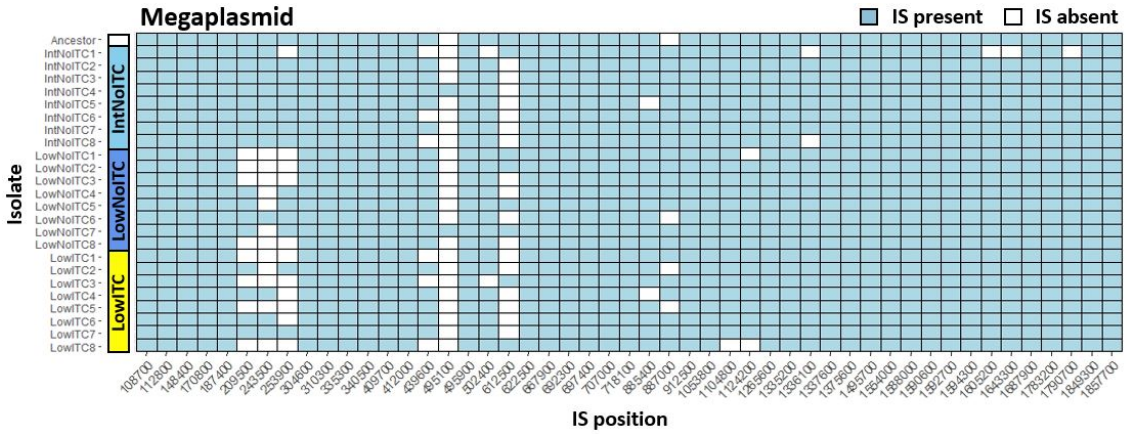

1085

1086 **Supplementary Figure 5. Presence and absence of insertion sequences in the chromosome**

1087 **and megaplasmid.** The X and Y-axes show the insertion sequence position and experimental

1088 isolate, respectively, as outlined in Figure 5.

1089