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3

4 Title:

5 **Pantoea ananatis defeats *Allium* chemical defenses with a plasmid-borne virulence gene cluster**

6

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33 **Abstract**

34 Onion (*Allium. cepa* L.), garlic (*A. sativum* L.), and other members of the *Allium* genus produce volatile  
35 antimicrobial thiosulfinates upon cellular damage. Allicin has been known since the 1950s as the primary  
36 antimicrobial thiosulfinate compound and odorant produced by garlic. However, the roles of endogenous  
37 thiosulfinate production in host-bacterial pathogen interactions have not been described. The bacterial  
38 onion pathogen *Pantoea ananatis*, which lacks both the virulence Type III and Type II Secretion Systems,  
39 induces necrotic symptoms and extensive cell death in onion tissues dependent on a proposed secondary  
40 metabolite synthesis chromosomal gene cluster. We found strong correlation between the genetic  
41 requirements for *P. ananatis* to colonize necrotized onion tissue and its capacity for tolerance to the  
42 thiosulfinate allicin based on the presence of an eleven gene, plasmid-borne, virulence cluster of  
43 sulfur/redox genes. We have designated them ‘*alt*’ genes for allicin tolerance. We show that allicin and  
44 onion thiosulfinates restrict bacterial growth with similar kinetics. The *alt* gene cluster is sufficient to  
45 confer allicin tolerance and protects the glutathione pool during allicin treatment. Independent *alt* genes  
46 make partial phenotypic contributions indicating that they function as a collective cohort to manage thiol  
47 stress. Our work implicates endogenous onion thiosulfinates produced during cellular damage as  
48 mediators of interactions with bacteria. The *P. ananatis*-onion pathosystem can be modeled as a chemical  
49 arms race of pathogen attack, host chemical counter-attack, and pathogen resistance.

50

51 **Significance Statement**

52 Alliums (e.g. onion and garlic), after sustaining cellular damage, produce potent antimicrobial  
53 thiosulfinates that react with cellular thiols. The bacterial onion pathogen *Pantoea ananatis*, which lacks  
54 the virulence Type III and Type II Secretion Systems, induces cell death and necrotic symptoms on  
55 onions. We have identified a plasmid-borne cluster of sulfur/redox virulence genes that 1) are required for  
56 *P. ananatis* to colonize necrotized onion tissue, 2) are sufficient for tolerance to the thiosulfinates, and, 3)  
57 protect the glutathione pool during thiosulfinate treatment. We propose that the thiosulfinate production  
58 potential of *Allium* spp. governs *Allium*-bacterial interaction outcomes and that the *P. ananatis*-onion  
59 pathosystem can be modeled as a chemical arms race of attack and counterattack between the pathogen  
60 and host.

61

62 **Introduction**

63 Plants deploy diverse chemical weaponry to defend themselves from microbial pathogens and  
64 herbivorous pests. Among these forms of chemical defenses, there are several examples of potent reactive  
65 antimicrobials and toxins that are converted into their active forms only after plant tissue damage.  
66 Cellular damage allows preformed precursor substrates and activating enzymes stored in separate sub-

67 cellular compartments to mix and react (1). These damage-driven enzymatic and chemical reactions  
68 allow rapid, potent, and often volatile, reactive chemical responses to tissue damage associated with  
69 herbivory and disease necrotrophy. Charismatic examples include glucosinolates that give brassicaceaus  
70 vegetables their distinct pungency as well as the cyanogenic glycosides that accumulate in rosaceous  
71 seeds (2, 3). Onion (*Allium cepa* L.), garlic (*Allium sativum* L.), and other alliaceous spp. display a classic  
72 example of this form of defense producing an array of reactive organosulfur compounds through a  
73 combination of enzymatic and chemical reactions upon tissue damage (1, 4). These compounds are  
74 associated with the characteristic flavors and odors of onion and garlic and also act as volatile irritants  
75 and antimicrobial compounds (5, 6). The thiosulfinate allicin (diallyl thiosulfinate, structure 3a), produced  
76 by the action of alliinase (EC 4.4.1.4) on alliin (*S*-2-propenyl-L-cysteine sulfoxide, structure 1a), is  
77 responsible for the odor of crushed garlic and is the primary volatile antimicrobial compound in garlic  
78 extract (7, 8). Allicin acts as a thiol toxin, reacting with cellular thiols and producing allyl-mercapto  
79 protein modifications, which can directly inactivate enzymes and cause protein aggregation (9). Allicin  
80 also reacts with reduced glutathione converting it into allylmercaptoglutathione and thereby depleting the  
81 reduced glutathione pool (7, 9). Asymmetric 1-propenyl methyl thiosulfinate (structure 3b) accumulate  
82 after disruption of onion tissue with total thiosulfinate production in onion extracts in the range of 100s of  
83 nmol per gram fresh weight (10). In onion, the combined action of alliinase and a second enzyme,  
84 lachrymatory factor synthase (LFS) converts the majority of isoalliin (*S*-1-propenyl-L-cysteine sulfoxide,  
85 structure 1c) into syn-propanethial-S-oxide (a.k.a. lachrymatory factor, structure 4), the chemical irritant  
86 of onion associated with inducing tears (11). Conversely, garlic, which lacks LFS, accumulates nearly  
87 100-fold higher total thiosulfinate per gram fresh weight than onion (10).

88 Onion center rot, caused by at least four species bacteria in the *Pantoea* genus from the order  
89 Enterobacteriales, is an economically impactful disease of onions that routinely results in significant losses  
90 to yield and marketability (12, 13). In the southeastern United States, onion center rot is primarily caused  
91 by *Pantoea ananatis*. No onion cultivars with resistance to pathogenic *P. ananatis* have yet been  
92 identified. *P. ananatis* invades leaves through wounds and causes blights and wilting of infected leaves.  
93 The pathogen can also invade bulbs through infected foliar tissues. Bulb invasion is associated with  
94 systemic movement of the pathogen from the leaf blade to the corresponding scale in the onion bulb (14).  
95 Preferential feeding by multiple species of thrips insects (*Frankliniella fusca*, and *Thrips tabaci*) around  
96 the onion neck, has been observed to play a role in transmission and dissemination of this pathogen from  
97 epiphytic onion or weed populations into onion foliage (15, 16).

98 *Pantoea ananatis* is a broad-host-range pathogen able to cause disease in diverse monocot hosts  
99 including onion, pineapple, maize, rice, and sudangrass (17). *P. ananatis* is also a rare example of a gram  
100 negative plant pathogen that lacks both a virulence-associated Type III Secretion System (T3SS) used by

101 many plant pathogenic bacteria to deliver immune-dampening effector proteins as well as a Type II  
102 Secretion System (T2SS) commonly used to deliver plant cell wall degrading enzymes associated with  
103 soft rot pathogens (18, 19). This surprising lack of the key pathogenicity factors, typically associated with  
104 gram negative bacterial plant pathogens, has left the primary molecular mechanisms by which *P. ananatis*  
105 causes disease an open question.

106 Recent work by Asselin *et al.* and Takikawa *et al.* identified the horizontally transferred  
107 chromosomal gene cluster HiVir (High Virulence, also known as PASVIL, Pantoea ananatis specific  
108 virulence locus) as a critical pathogenicity factor for *P. ananatis* to produce necrotic symptoms on onion  
109 (20, 21). HiVir is hypothesized to encode a biosynthetic gene cluster for an, as of yet, undescribed  
110 secondary metabolite that may act as a plant toxin. The metabolite synthesized by the HiVir cluster is  
111 predicted to be a phosphonate or phosphinate compound based on the presence of a characteristic *pepM*,  
112 phosphoenolpyruvate mutase gene in the HiVir cluster that is essential for necrosis induction (20).

113 Upon induction of HiVir-dependent necrosis, it is expected that onion tissues would become a  
114 noxious and challenging environment for microbial colonization due to the endogenous production of  
115 reactive sulfur antimicrobial compounds. In prior comparative genomics analysis of *P. ananatis*, we  
116 identified four clusters of plasmid borne genes, OVRA-D, that correlated with onion virulence (19).  
117 Using mutational analysis, we identified an eleven gene sub-cluster within OVRA that is critical for  
118 colonization of onions during disease as well as growth in necrotized onion bulb tissue and onion extracts.  
119 This cluster is comprised of genes with annotations associated with sulfur metabolism and redox and  
120 confers tolerance to the thiosulfinate allicin. We determined that onion extract contains thiosulfinates at  
121 concentrations sufficient to restrict bacterial growth and restricts *Pantoea* growth with similar kinetics to  
122 allicin. We designated these genes *alt* for allicin tolerance. Expression of the *alt* cluster conferred  
123 virulence capacity and allicin tolerance to a natural *P. ananatis* isolate lacking *alt* genes and also  
124 conferred allicin tolerance to *E. coli*. We observed that the *alt* genes likely encode an additive cohort of  
125 protective enzymes to manage cellular thiol stress with multiple genes independently conferring partial  
126 phenotypes. These results demonstrate that onion thiosulfinates play an important role in biotic  
127 interactions with bacteria and that *P. ananatis* pathogenic interactions with onion can be modeled as a  
128 chemical arms race based on pathogen attack, host counterattack, and specialized pathogen adaptations  
129 for chemical defense.

130

131 **Results:**

132 **Plasmid-borne OVRA genes promote onion scale colonization and facilitate growth in onion  
133 extract.**

134                   Onion pathogenic *P. ananatis* causes tissue necrosis on onions in a cultivar-independent manner  
135 (19, 20, 22, 23). Recently, Asselin *et al.*, identified the proposed biosynthetic gene cluster HiVir carrying  
136 a *pepM* gene essential for the production of both onion leaf and bulb necrosis (20). We previously  
137 developed an assay for screening *P. ananatis* pathogenetic potential based on the development of a  
138 clearing zone around the inoculation site of red onion scales (19). The red scale clearing phenotype  
139 required the HiVir *pepM* gene similar to other necrosis phenotypes (Fig 1A, 1B). We determined that the  
140 clearing zone in red onion scales is associated with extensive onion cell death although plant cell walls  
141 appear to be left intact. This observation was based on apparent plasmolysis and the staining of nuclei by  
142 propidium iodide, indicating loss of plasma membrane integrity, which was consistent with the lack of  
143 staining with the vital stain fluorescein diacetate (24, 25). This is in stark contrast to observations of cells  
144 from non-cleared tissue in the same scale samples (Fig. 1C).

145                   In our previous comparative genomics analysis we identified 57 genes in four contiguous blocks  
146 on a 161-KB megaplasmid that were strongly correlated with *P. ananatis* virulence on onion (NCBI  
147 accession CP020945.2) (Fig. 2A) (19). We sought to determine whether these plasmid-borne OVR  
148 (Onion Virulence Regions) genes contributed to *P. ananatis*-mediated onion center rot. Using allelic  
149 exchange, we generated deletion mutants of the OVRA, OVRB, OVRC, and OVRD clusters in *P.*  
150 *ananatis* PNA 97-1R (WT) (SI Appendix, Materials and Methods). We observed that the OVRA cluster  
151 deletion strain both produced smaller clearing zones in a red scale necrosis assay (Fig. 2B and 2C) and  
152 reached two log-fold lower bacterial load in onion scale tissue compared to the WT strain and other OVR  
153 deletions (Fig. 2C). As PNA 97-1R WT was able to grow to high loads in the dead onion tissue of scale  
154 clearing zones, we posited that bacterial growth in clarified, filter sterilized red onion juice (red onion  
155 extract: ROE) would be effective proxy for extreme onion tissue damage and that monitoring growth  
156 capacity of *P. ananatis* in ROE would mimic colonization potential in dead onion tissue. We monitored  
157 bacterial growth in ROE as the change in OD<sub>600</sub> over time. PNA 97-1R was unable to grow in full  
158 strength ROE. However, in half strength ROE, WT,  $\Delta$ OVRB,  $\Delta$ OVRC, and  $\Delta$ OVRD strains grew well  
159 while the  $\Delta$ OVRA strain had a dramatic growth defect (Fig. 2D). We considered two hypotheses for why  
160 a  $\Delta$ OVRA strain lost the capacity for efficient growth in ROE and necrotized onion tissue 1)  $\Delta$ OVRA lost  
161 the ability to utilize key nutrients from host tissue or, 2)  $\Delta$ OVRA lost the ability to tolerate onion  
162 inhibitory factor(s). To test the nutritional model we assessed bacterial growth in a 1:1 mixture of ROE  
163 and LB media. The  $\Delta$ OVRA strain still displayed a strong growth defect in ROE:LB (Fig. 2D). The  
164 reduced growth of a  $\Delta$ OVRA strain under nutritionally replete conditions indicated that the onion  
165 inhibitory factor model was more probable. We observed similar growth patterns in ROE and ROE:LB by  
166 natural variant *Pantoea* isolates based on the presence or absence of OVRA genes (Fig. S1).

167 **The 11 gene *alt* sub-cluster in OVRA confers tolerance to allicin, protects the glutathione pool  
168 during allicin treatment, and promotes onion bulb colonization.**

169       Based on the hypothesis that onion inhibitory factors could be onion thiosulfinate, or possibly  
170 another sulfur compound, we focused on the eleven contiguous OVRA genes annotated for functions  
171 related to sulfur metabolism and redox (Fig. 3A). Based on various gene annotation pipelines, these 11  
172 genes encode a TetR-family repressor, four reductases including a glutathione disulfide reductase, two  
173 potential peroxidases, a thioredoxin and a thioredoxin-like gene, a carbon sulfur lyase and a cysteine-  
174 transporter family protein (Table S1). We used allelic exchange to delete the 11 gene sulfur  
175 metabolism/redox cluster from OVRA. Using zone of inhibition assays as well as liquid media growth  
176 assays, we observed that the OVRA sub-deletion had increased sensitivity to the thiosulfinate allicin (Fig.  
177 3B, Fig S2A) as well as to garlic extract (Fig. S2B). Thus, we chose to name this 11 gene region the *alt*  
178 cluster for allicin tolerance. We had noted previously that the sequenced *Enterobacter cloaceae* isolate  
179 EcWSU1, which is also an onion bulb pathogen, carries cluster of plasmid borne genes similar to the  
180 *Pantoea alt* cluster (Fig. S3) (19). Similar patterns of allicin and garlic extract sensitivity were observed  
181 in natural variant *Pantoea* isolates based on the presence or absence of OVRA genes or the *alt*-like cluster  
182 in *Enterobacter* isolates (Fig. S4). In addition, three chromosomal clusters conferring allicin tolerance via  
183 heterologous expression were recently described from the non-pathogenic garlic saprophyte *Pseudomonas*  
184 *fluorescens* PfAR-1 (26). These clusters from PfAR-1 share similar gene content to the *alt* cluster  
185 although not fine scale synteny (26). We observed that a  $\Delta$ OVRB/C/D mutant showed similar levels of  
186 allicin tolerance to WT PNA 97-1R (Fig. 3B). However, we also observed that the  $\Delta$ OVRA/B/C/D  
187 mutant had higher allicin sensitivity than the  $\Delta$ alt mutant. (Fig. 3B). This indicates that some OVR genes  
188 outside 11 gene *alt* gene cluster contribute to full allicin tolerance in PNA 97-1R.

189       Depletion of the glutathione pool through direct reaction between allicin and reduced glutathione  
190 is proposed as a major component of allicin's antibacterial activity (9). We determined the percentage of  
191 total glutathione after 1 h of allicin treatment according to the procedure described by Müller *et al.* 2016  
192 (9). PNA 97-1R WT maintained a higher percentage of glutathione compared to non-treated cells than a  
193  $\Delta$ alt strain indicating that the presence of the *alt* cluster counters allicin-mediated depletion of the  
194 glutathione pool (Fig. 3C).

195       We measured the total thiosulfinate content of full strength ROE to be  $243 \mu\text{M} \pm 16$  (n=8) using a  
196 4-mercaptopuridine (4-MP) spectrophotometric assay (27). This is well above the allicin MIC values  
197 previously reported for *E. coli* ( $141.75 \mu\text{M} \pm 10$ ) and many other bacteria (7, 9). We also determined that  
198 allicin and onion thiosulfinate have similar capacity to delay the growth of PNA 97-1R and that the  $\Delta$ alt  
199 mutant displayed consistently increased growth delay compared to WT PNA 97-1R across a range of

200 allicin and onion thiosulfinate concentrations indicating similar antibacterial efficacy (Fig. 3D-E). We  
201 calculated the thiosulfinate MIC values of PNA 97-1R WT and  $\Delta alt$  strains to be  $125\pm5$   $\mu\text{M}$  and  $80\pm5$   
202  $\mu\text{M}$  respectively, based on unchanged  $\text{OD}_{600}$  at 24 hours in liquid LB incubated at  $28^\circ\text{C}$ .

203 We further monitored the contribution of *alt* to virulence based on systemic colonization of intact  
204 onion plants. This was conducted by tracking the capacity of Tn7Lux-labeled auto-bioluminescent PNA  
205 97-1R derivative strains to systemically colonize onion bulbs 20 days after mechanical inoculation. The  
206 inoculations were conducted by piercing the onion neck just above the bulb shoulder. This form of  
207 inoculation is expected to mimic natural infection by simulating thrips-mediated feeding site preference  
208 and *Pantoea* transmission at the onion neck (16). The PNA 97-1R  $\Delta alt$  strain showed a dramatic loss of  
209 auto-bioluminescence in the bulb at 20 DPI indicating reduced bacterial colonization (Fig. 4A). We  
210 observed a similar loss to bulb-associated autobioluminescence with a  $\Delta pepM$  HiVir cluster mutant  
211 indicating that both loci are independently important for onion bulb colonization by PNA 97-1R (Fig.  
212 4A). Similar patterns of auto-bioluminescence were seen during direct scale inoculation with  $\Delta alt$   
213 displaying the lowest bioluminescence. The  $\Delta pepM$  strain, which critically does not induce necrosis  
214 associated with thiosulfinate release, showed higher scale colonization than the  $\Delta alt$  strain, but limited to  
215 the zone around the inoculation puncture site. In contrast, the  $\Delta alt \Delta pepM$  strain showed less colonization  
216 than a  $\Delta pepM$  strain presumably due to increased sensitivity to the thiosulfinate release associated with  
217 the inoculation puncture (Fig. 4B).

218 **The *alt* cluster genes are sufficient for allicin tolerance and likely function as a cohort to tolerate  
219 thiol stress and promote onion scale colonization.**

220 We generated a series of nested complementation constructs carrying either the entire 11-gene *alt* cluster  
221 or sub-regions in the pBBR1-derivative plasmid pBS46 to determine their phenotypic contributions (Fig.  
222 5A)(28). Complementation of PNA 97-1R  $\Delta alt$  with the full *alt* cluster clone (*altB-J*) fully complemented  
223 onion scale colonization measured by both bacterial load and autobioluminescence, growth in ROE, and  
224 allicin tolerance based on both zone of inhibition and dilution plate assays (Fig. 5B-E, Fig. S5, S7-S10).  
225 Using the nested complementation constructs, the *alt* cluster sub-region *altB-G* displayed near wild type  
226 phenotypes in all assays. Sub-clusters *altB-A* and *altD-G* consistently showed partial complementation of  
227 *alt* phenotypes while *altH-altJ* showed minor independent phenotypic complementation in some assays.  
228 As these three *alt* sub-clusters share no genes in common, this supports a model of independent additive  
229 contributions of these genes to overall allicin tolerance and onion colonization phenotypes. The *altH-altI*  
230 genes, annotated as a carbon-sulfur lyase and cysteine-family-exporter genes, were not able to  
231 complement  $\Delta alt$  phenotypes independently. The genes *altA* and *altR* have distant similarity to the *nemA*  
232 reductase and *nemR* TetR-family repressor genes of *E. coli* respectively that respond to bleach and N-

233 ethylmaleimide and confer increased tolerance to these reactive compounds (29). A  $\Delta altR$  mutant  
234 displayed reduced growth lag in ROE while  $altR$  overexpression complementation conversely displayed  
235 increased growth lag (Fig. S6). This is consistent with AltR serving to repress the ROE growth phenotype  
236 of the  $alt$  cluster.

237 We expressed  $altB-J$  in the *P. ananatis* isolate PNA 02-18, which naturally lacks the OVR genes  
238 but possesses the HiVir gene cluster. The  $altB-J$  full cluster expression clone conferred multiple gain of  
239 function phenotypes on PNA 02-18 allowing the strain to colonize onion scales, grow well on ROE, and  
240 display increased tolerance to allicin (Fig. 6A-D, Fig. S5, S11). Transformation of  $altB-J$  into PNA 02-18  
241 produced phenotypes consistent with complementation of the PNA 97-1R  $\Delta alt$  strain. Similarly,  
242 heterologous expression of  $altB-J$  conferred increased allicin tolerance to *E. coli* DH5 $\alpha$  indicating the  $alt$   
243 genes are sufficient to confer allicin tolerance to bacteria outside of *Pantoea* (Fig. 6D-E, Fig. S12).

244

## 245 **Discussion**

246 We observed a high degree of correlation between the genetic requirements for *P. ananatis* strains to  
247 colonize necrotized onion bulb tissue, grow in ROE, and their capacity for thiosulfinate tolerance. This  
248 suggests that tissue damage-induced endogenous production of thiosulfinate in onion exerts an  
249 antimicrobial effect on non-adapted bacterial strains and that thiosulfinate play an important role in  
250 biotic interactions between the onion host and bacterial pathogens.

251 The total thiosulfinate potential by *Alliums* may be an important factor both for disease outcomes and  
252 *Allium*-biotic interactions in general. Onion pathogenic *Burkholderia* have been shown to be sensitive to  
253 thiosulfinate *in vitro* as have many other plant pathogenic microbes (30, 31). In garlic, the level of host  
254 resistance to the chive gnat (*Bradysia odoriphaga*) was shown to correlate with cultivar-level variations in  
255 thiosulfinate production potential (32). *P. ananatis* routinely causes economically impactful outbreaks of  
256 center rot disease in sweet onions, which accumulate lower amounts of S-alk(en)yl-L-Cys sulfoxide  
257 precursor compounds (structures 1b, 1c) than other onions and thus, have comparatively less capacity for  
258 thiosulfinate production (5, 33). The presence of the  $alt$ -like cluster in onion-virulent *Enterobacter*  
259 *cloacea* EcWSU1 supports the idea that  $alt$  genes are adaptive for colonization of onion bulb tissue during  
260 disease. Interestingly, while there are many bacterial pathogens that cause disease in onion bulbs, there  
261 are comparatively few bacterial diseases of garlic bulbs (34). *Pseudomonas salomonii*, has been reported  
262 to cause “café au lait” disease on garlic leaves and carries gene clusters for allicin tolerance similar to  
263 those recently described in *P. fluorescens* PfAR-1 (26, 35). Garlic leaves have less allicin production  
264 potential than garlic bulbs (36, 37). The garlic saprophytic *P. fluorescens* strain PfAR-1 was found to

265 carry three, nearly identical, chromosomal loci capable of conferring allicin tolerance with high  
266 specificity when heterologously expressed in *E. coli* or *Pseudomonas syringae* (26). However, while  
267 *PfAR-1* was isolated from garlic bulb, it is not a garlic pathogen and does not cause disease-associated  
268 necrosis on garlic. We speculate that three chromosomal allicin tolerance clusters of *PfAR-1* are  
269 potentially adaptive for stable saprophytic colonization of the garlic bulb niche. We presume that extreme  
270 thiosulfinate tolerance measures may be required to tolerate the potentially high thiosulfinate levels  
271 released via even minor coincidental wounding events over the life of the garlic bulb association.

272 The silencing of LFS has been used in laboratory settings to create “tearless” onions (5, 38). The lack of  
273 LFS activity in these lines drives increased flux of isoalliin into the dramatically increased production of  
274 1-propenyl-based thiosulfinate after tissue damage (5, 38). Consistent with the capacity of garlic, which  
275 lacks an LFS-like enzyme, to produce nearly 100 fold more thiosulfinate than onion per g fresh weight,  
276 and the paucity of garlic bulb infecting bacterial pathogens in general, we hypothesize that these LFS-  
277 silenced onion lines should display increased resistance to *P. ananatis* infection by overwhelming the  
278 pathogen’s capacity for thiosulfinate tolerance. Conversely, onions with reduced alliinase activity rather  
279 than LFS activity would be expected to produce less thiosulfinate and have increased susceptibility to *P.*  
280 *ananatis* including naturally *alt*-lacking but HiVir containing isolates.

281 The ability of independent *alt* genes to confer partial phenotypic complementation and the functional  
282 predictions of the *alt* genes supports the model that the *alt* cluster encodes an additive cohort of proteins  
283 that collectively and cooperatively manage the impacts of cellular thiol stress as opposed to either direct  
284 inactivation or exclusion of thiosulfinate. Genetic dissection of the *PfAR-1* allicin tolerance clusters also  
285 demonstrated a cohort effect for allicin tolerance. The *PfAR-1* genes, *dsbA*, *trx*, and *aphD*, made the  
286 largest single gene contributions to allicin tolerance based on Tn insertional mutations and single gene  
287 overexpression tests (26). PNA 97-1R *alt* genes with similar annotations, *altC*, *altD*, and *altE*  
288 respectively, are all included on the *altB-G* sub-cluster clone, which conferred near wild type levels of  
289 complementation. The distant similarity of AltR to NemR could indicate a possible mode of action for  
290 AltR response to thiol stress. NemR carries a redox-sensitive cysteine residue critical for response to  
291 bleach and N-ethylmalimide and release of NemR from DNA to de-repress transcription of the NemA  
292 reductase (29). We hypothesize that thiosulfinate reaction with AltR cysteine residues may similarly  
293 allow AltR to respond to thiol stress, release from DNA and thereby de-repress expression of *alt* genes.  
294 Determining the roles played by specific Alt proteins in thiol stress perception and tolerance will be a  
295 fruitful area for future study.

296 In *P. ananatis*, the production of necrotic symptoms in onion, mediated by the HiVir chromosomal  
297 cluster, and colonization of necrotic onion tissue, mediated by the *alt* cluster, are genetically separable.

298 The HiVir chromosomal cluster shows clear signs of horizontal gene transfer, while the plasmid-borne *alt*  
299 cluster is flanked by a recombinase-like gene (19, 20). Our disease model predicts that *P. ananatis*  
300 isolates lacking HiVir but possessing the *alt* cluster could benefit from co-associations with HiVir+,  
301 necrosis-inducing isolates as social cheaters. *P. ananatis* is a rare example of an aggressive gram negative  
302 plant pathogen that requires neither a T3SS to deliver virulence effectors to the plant cytosol nor a T2SS  
303 to deliver plant cell wall degrading enzymes to cause plant disease. This is unlike other well characterized  
304 examples of plant pathogenic *Pantoea*, *P. agglomerans* pvs. *betae* and *gypsophila* and *P. stewartii* subsp.  
305 *stewartii* that are dependent on a Hrp1-class T3SS for plant pathogenicity (39). Another characterized  
306 example of a Hrp-independent gram negative phytopathogen is *Xanthomonas albilineans* for which the  
307 phytotoxin albidicin, a DNA gyrase inhibitor synthesized by a hybrid PKS/NRPS pathway, is required to  
308 cause leaf scald disease of sugar cane (40, 41). Similarly, the ability of *P. ananatis* to cause necrosis on  
309 onion is dependent on the HiVir pathogenicity gene cluster, a non-PKS/NRPS gene cluster proposed to  
310 drive the synthesis of an, as of yet, undiscovered phosphonate secondary metabolite potentially  
311 functioning as a phytotoxin (20).

312 The pathogenic lifestyle of *P. ananatis* on onion more closely resembles that of some necrotrophic plant  
313 pathogens such as *Alternaria* or *Fusarium* than other described bacterial plant pathogens. These  
314 fungal pathogens produce phytotoxins as important virulence factors and proliferate on dead plant tissue  
315 (42, 43). In the case of *Fusarium oxysporum* f. sp. *lycopersici*, pathogen tomatinase enzyme-mediated  
316 resistance to antimicrobial tomatine saponins is critical for full virulence on tomato (44). Similarly, the *P.*  
317 *ananatis* *alt* genes confer tolerance to thiosulfinate and are required for colonization of necrotized onion  
318 tissue. We propose that a chemical arms race model provides a good framework for understanding *P.*  
319 *ananatis* disease on onion. *Pantoea* HiVir induces cell death in onion cells, potentially through synthesis  
320 of an undescribed phosphonate phytotoxin. Onion cell death coincides with post mortem generation of  
321 thiosulfinate which can restrict the growth of a non-adapted *P. ananatis*. The presence of the *alt* genes in  
322 *P. ananatis* confers tolerance to damage-induced onion chemical defenses and allows proliferation of  
323 adapted bacterial strains. This chemical arms race model for disease and defense in the *P. ananatis* onion  
324 pathosystem provides an interesting evolutionary contrast to the plant immune receptor/virulence protein  
325 effector arms race model that underlies many plant-pathogen interactions.

326

## 327 **Materials and Methods**

### 328 **Bacterial strains and culture conditions.**

329 Overnight (O/N) cultures of *E. coli*, *Pantoea* spp., and *Enterobacter cloacae* were routinely cultured from  
330 single clones recovered on LB parent plates and were grown in 5 mL of LB media in 14 mL glass culture  
331 tubes at 28°C (*Pantoea*) or 37°C (*E. coli* and *E. cloacae*) with 200 rpm shaking (Table S6).

332 **Plasmid constructs and generation of mutants.**

333 Plasmids were typically constructed via Gateway cloning of PCR products, overlap-extension joined PCR  
334 fragments or synthesized DNA fragments. Deletion mutants were generated via allelic exchange using the  
335 pR6KT2G vector (*SI Appendix, Materials and Methods*, Table. S2-S5). Tn7 transposants were generated  
336 essentially as described in Choi *et al.* 2008 (45). Expression plasmids were introduced via  
337 electrotransformation.

338 **Foliar necrosis assay**

339 Onion seedlings (cv. 'Century', 12L:12D, six-weeks) were inoculated by depositing a 10 µl of bacterial  
340 suspension ( $OD_{600} 0.3 \approx 1 \times 10^8$  CFU/mL; dH<sub>2</sub>O) 1 cm from the central leaf apex on a wound created with  
341 sterile scissors. Leaves were evaluated 3 days post inoculation (DPI).

342 **Red onion scale necrosis assay**

343 Consumer produce red onions were cut to approximately 3 cm wide scales, sterilized in a 3% household  
344 bleach solution for 1 m, promptly removed and rinsed in dH<sub>2</sub>O and dried. Scales were placed in a potting  
345 tray (27 × 52 cm) with pre-moistened paper towels (90 mL dH<sub>2</sub>O). Individual onion scales were wounded  
346 cleanly through the scale with a sterile 20 µl pipette tip and inoculated with 10 µl of bacterial O/N LB  
347 culture. Sterile deionized water was used as a negative control. The tray was covered with a plastic  
348 humidity dome and incubated at RT for 72 h. Following incubation, lesion sizes were measured by  
349 recording the diameter and small squares (0.06-0.08 g) of tissue were excised from a region 1 cm from the  
350 inoculation wound. Tissues were weighed and placed in plastic maceration tubes with beads, beat with a  
351 GenoGrinder SPEX SamplePrep 2010, ten-fold serially diluted with sterile dH<sub>2</sub>O and plated on rifampicin  
352 amended LB plates to determine the colony forming units per gram of onion tissue.

353 **Confocal imaging and staining.**

354 Scales were inoculated as described above. 100-125 mm<sup>2</sup> sections were cut with a razor from the  
355 underside of onion scales and peeled at one corner with tweezers to minimize mechanical damage to other  
356 cells within the sample. Peeled samples were stained in fluorescein diacetate (FDA; 2 µg/mL) and  
357 propidium iodide (PI; 10µg/ml) at room temperature for 15 m in dark conditions as previously described  
358 (25). Stained samples were mounted on a slide in water under a coverslip for live-cell imaging. Confocal  
359 microscopy was performed with a Zeiss LSM 880 confocal microscope using the 10x objective.

360 Fluorescein was excited using 488 nm laser and emission collected between 508 and 535 nm. Z-stack  
361 imaging was used to image cells at multiple focal planes. PI was excited using a 543 nm laser and  
362 emission collected with 615-700 nm. Images were processed using the Zen software.

363 **Preparation of allicin and *alliaceous* extracts**

364 Allicin was synthesized using the protocol of Albrecht *et al.*, 2017 with some modifications (46). 15  $\mu$ L of  
365 diallyl disulfide 96% , 25  $\mu$ L of glacial acetic acid, and 15  $\mu$ L of 30%  $H_2O_2$  were added to a 200 $\mu$ L PCR  
366 tube and agitated for 4 h at 28°C. The reaction was quenched in 2mL of methanol. Fresh *alliaceous*  
367 extracts were prepared using the Breville Juice Fountain Elite or a kitchen blender. Solid debris were  
368 removed by straining subsequent macerates through cheese cloth and filter paper. Fine particulates were  
369 pelleted by centrifugation (10,000 g, 1.5 h, 4°C). Semi-clarified extracts were sterilized with a Nalgene  
370 0.2 micron vacuum filter sterilization unit. All extracts were used within the week of preparation and  
371 stored at -20°C. Thiosulfinate concentrations were quantified using the 4-mercaptopypyridine (4-MP)  
372 spectrophotometric assay (27).

373 **Liquid growth assays**

374 Growth assays were conducted using 100-well honeycomb plates with the Bioscreen C system (Lab  
375 Systems Helsinki, Finland). The instrument was run for 48 h with low agitation at 28°C. Each well had  
376 400  $\mu$ L: 360  $\mu$ L of the respective growth media and 40 $\mu$ L of an  $OD_{600} = 0.3$  bacterial suspension in sterile  
377 d $H_2O$ , with a minimum of 3 well replicates. ROE was diluted with sterile water ( $\approx 100 \mu$ M thiosulfinate).  
378 ROE:LB media consisted of LB with an equal volume of ROE ( $\approx 80 \mu$ M thiosulfinate). Absorbance  
379 values were recorded every hour. Raw absorbance readings were normalized by subtracting the initial  
380 absorbance readings from subsequent hourly readings.

381 **Zone of inhibition assay**

382 Styrene petri plates (100  $\times$  15 mm) with 20 mL LB were spread with a bacterial suspension ( $OD_{600} 0.3 \approx$   
383  $1 \times 10^8$  CFU/mL; d $H_2O$ ) using a sterile cotton swab. 0.125 cm $^2$  agar plugs were removed from the plates  
384 with a biopsy punch to create up to three wells per plate. 50  $\mu$ L of either garlic extract or allicin stock  
385 solution were added to the wells. Plates were incubated for 24 h at RT and evaluated for a zone of  
386 inhibition (cm $^2$ ) by measuring the radius, calculating the inhibition, area and subtracting the well area.

387 **Serial dilution allicin sensitivity plates**

388 The allicin stock solution was added to molten LB cooled to 55°C in sterile conical vial tubes and poured  
389 into square plates to set, achieving relative allicin concentrations of 70  $\mu$ M for *Pantoea* and 140  $\mu$ M for

390 *E. coli*. The O/N cultures of strains to be tested underwent ten-fold series dilutions and 10 uL volumes of  
391 each dilution were plated on the LB control and allicin-amended LB plates. The plates were incubated  
392 overnight (28°C *Pantoea*, 37°C *E. coli*) and imaged the following morning.

393 **Sweet onion systemic infection**

394 Sterile wooden toothpicks were soaked in a bacterial suspension ( $OD_{600} 0.3 \approx 1 \times 10^8$  CFU/mL; dH<sub>2</sub>O) and  
395 were inserted horizontally through the entire neck of five-month-old the onion plants (cv. Sapelo Sweet)  
396 just below the leaf fan and above the shoulder. Toothpicks were left in the plants. At 20 d the onions were  
397 harvested for imaging. Onions were removed from soil (which was sterilized following the experiment  
398 and discarded), rinsed with water, and cut twice horizontally at the center of the bulb to produce a 1.5 cm  
399 section of the center of the onion. The remaining portion of the top of the bulb had foliage removed and  
400 was cut vertically. Sliced onions were imaged with a color camera followed by long exposure imaging  
401 with the analyticJena UVP Chemstudio.

402 **Quantification of glutathione**

403 Glutathione quantification was conducted essentially as described by Müller *et al.* 2016 using a  
404 Glutathione (GSH) Colorimetric Detection Kit (Arbor Assays) (9). Optical readings were conducted with  
405 Tecan Spectra Rainbow spectrophotometer in 96 well styrene plates and the glutathione concentration of  
406 the samples were determined according to the manufacturers recommendations.

407 **False color luminescence images**

408 Luminescence false color images were merged in ImageJ to create compound images of luminescence  
409 and brightfield captures. The TIFF file of the long exposure capture (luminescence) and brightfield  
410 capture were opened with ImageJ Fiji release (<https://imagej.net/Citing>). The images were merged  
411 (image→ mergechannels→ [select blue for brightfield and yellow for luminescence]). The output file was  
412 saved in PNG format for publication images.

413

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537

## 538 **Figure Legends**

539 Fig 1. The HiVir *pepM* gene is required for inducing cell death in red onion scales. (A)  $\Delta$ *pepM* mutant in  
540 PNA 97-1R background does not cause foliar lesions on onion blades. Images were taken 3 DPI on 6-  
541 week-old onion seedlings (cv. 'century'). Images are not to scale. (B)  $\Delta$ *pepM* does not induce red onion  
542 scale clearing. Images were taken 3 DPI and are not to scale. (C) Confocal images of onion epidermal  
543 cells in non-cleared and cleared regions stained to determine cellular integrity. BF(grayscale), bright field;  
544 PI (red), propidium iodide; FDA(green), fluorescein diacetate. Bar = 100  $\mu$ m.

545 Fig 2. Plasmid-borne OVRA genes promote onion scale colonization and facilitate growth in onion  
546 extract. (A) Linear graphical representation of the Onion Virulence Region (OVR) gene clusters on the  
547 pOVR mega-plasmid of *P. ananatis* PNA 97-1R (NCBI accession CP020945.2). (B) Representative  
548 bacterial lesions produced on red onion scales. Image was taken three days post inoculation. (C) PNA 97-  
549 1R  $\Delta$ OVRA produces smaller lesions (top,  $N=24$ ) and reaches lower bacterial loads in onion scale tissue  
550 (bottom,  $N=12$ ). The data from three independent replicates is presented (one-way ANOVA followed by  
551 Tukey's post-test,  $p<0.001$ , letters represent significant dif). Error bars represent  $\pm$ SD.  $\log_{10}$  (cfu/g),  
552 colony-forming units per gram of onion scale tissue. (D) PNA 97-1R  $\Delta$ OVRA growth (change in  $OD_{600}$ ,  
553 Bisocreen C) in liquid culture is inhibited in an aqueous red onion extract (ROE) and delayed when  
554 supplemented with LB. This experiment was repeated three times with similar results ( $N=6$ ). Error bars  
555 represent  $\pm$ SEM.

556 Fig 3. The 11 gene alt sub-cluster in OVRA is critical for tolerance to allicin and onion thiosulfinates. (A)  
557 Linear graphical representation of the OVR gene clusters of PNA 97-1R mega-plasmid expanded to  
558 present 11 genes in the *alt* cluster. Genes are color coded to proposed function. (B) Allicin inhibition area  
559 of PNA 97-1R mutants. PNA 97-1R  $\Delta alt$  and quadruple mutant PNA 97-1R  $\Delta$ OVRA/B/C/D have  
560 increased susceptibility to allicin compared to PNA 97-1R WT and triple mutant PNA 97-1R  
561  $\Delta$ OVRB/C/D. This experiment was repeated three times with similar results ( $N=6$ , one-way ANOVA  
562 followed by Tukey's post-test,  $p<0.001$ , letters represent significant dif). Error bars represent  $\pm$ SD.  
563 Representative images of clearing zones are included above respective bars. (C) The percent change in  
564 total glutathione comparing untreated to 1 h allicin stress treatments. PNA 97-1  $\Delta alt$  has significantly  
565 lower levels of glutathione as a percent of its paired untreated sample compared to PNA 97-1R WT. The  
566 data from four independent replicates is presented ( $N=4$ , t-test). Error bars represent  $\pm$ SE. (D) WT and  
567  $\Delta alt$  48 h thiosulfinate growth response curves. A dilution series of allicin in LB was generated and the  
568 thiosulfinate content was measured (4-MP assay) and is presented on the x-axis. The time point at which  
569 the culture reached half max  $OD_{600}$  (Bioscreen C) during 48 h was is presented on the y-axis (yellow  
570 circle). A linear regression of the allicin growth response (solid black line, [WT]  $y=0.162x+3.64$ , [ $\Delta alt$ ]  
571  $y=0.23x+5.08$ ) was plotted along with 95% confidence intervals (dotted red line). The thiosulfinate  
572 concentration and growth response of a ROE dilution series in LB is plotted over the allicin response  
573 curve (pink triangle). This experiment was repeated three times. Each point represents an independent  
574 bisocreen well. (E) Simplified thiosulfinates reaction pathways from garlic and onion adapted from  
575 Lanzotti et al. 2006 and Borlinghaus et al. 2019 (26, 47). LFS = Lachrymatory Factor Synthase.

576 Fig 4. Onion bulb colonization of Tn7Lux labeled mutants 20 DPI. (A) Onions were inoculated at the  
577 neck with Tn7Lux labeled mutants 20 days prior to sampling. Onions were sliced longitudinal (top row)  
578 and transverse at bulb midline (bottom row). Bioluminescence was captured with 2 min exposure  
579 (yellow) and merged with the brightfield image (blue). Full color images were also taken of the same  
580 samples. Images are representative of three independent experiments and are not to scale. (B) Red onion  
581 scale colonization of Tn7Lux labeled mutants 3 DPI. Image signals are presented as described previously.  
582 Images are representative of three independent experiments and are not to scale.

583 Fig 5. Nested complementation of the *alt* gene cluster. (A) Graphical representation of nested  
584 complementation clones spanning different regions of the *alt* cluster. Regions were cloned into the  
585 expression vector pBS46 and transformed into the PNA 97-1R  $\Delta alt$  background. Colors indicate regions  
586 cloned and correspond to panels B and D. (B) Complementation clone bacterial load (top,  $N=12$ ) and  
587 allicin tolerance (bottom,  $N=9$ ) in the  $\Delta alt$  background (ev = empty vector). The data from three  
588 independent replicates is presented. (one-way ANOVA followed by Tukey's post-test,  $p<0.001$ , letters

589 represent significant dif). Error bars represent  $\pm$ SD.  $\text{Log}_{10}$  cfu/g, colony-forming units per gram of onion  
590 scale tissue. (C) Scale colonization of Tn7Lux labeled complementation clones 3 DPI. Bioluminescent  
591 strain signals were captured with 2 min exposure (yellow) and merged with the brightfield image (blue).  
592 Images are representative of three independent experiments. (D) Complementation constructs rescue  
593 growth in ROE (change in  $\text{OD}_{600}$ ). This experiment was repeated three times with similar results ( $N=4$ ).  
594 Error bars represent  $\pm$ SE. (E) Complementation clone ten-fold serial dilution of  $\text{OD}_{600}$  0.3 on LB  
595 rifampicin and LB allicin amended plates Each row is a cropped from a larger photo representing one of  
596 three independent experimental replicates conducted in duplets. Original uncropped images (Fig S7-S10).

597 Fig 6. Complementation of *alt* associated phenotypes in heterologous backgrounds. (A) Bacterial load  
598 (top,  $N=12$ ) and allicin tolerance (bottom,  $N=9$ ) of heterologous expression clones in the PNA 02-18  
599 naturally OVR-lacking background. The data from three independent replicates is presented. (one-way  
600 ANOVA followed by Tukey's post-test,  $p<0.001$ , letters represent significant dif). Error bars represent  
601  $\pm$ SD.  $\text{Log}_{10}$  cfu/g, colony-forming units per gram of onion scale tissue. (B) Onion scale colonization of  
602 Tn7Lux labeled heterologous expression clones 3 DPI. Luminescent strain signals were captured with 2  
603 min exposure (yellow) and merged with the brightfield image (blue). Images are representative of three  
604 independent experiments. (C) Bioscreen growth of PNA 02-18 *alt* expression clones in ROE over 48h.  
605 Error bars represent  $\pm$ SE. This experiment was repeated three times with similar results ( $N=4$ ). (D)  
606 Allicin tolerance of *E. coli* DH5 $\alpha$  heterologous expression clones. The data from three independent  
607 replicates is presented ( $N=9$ , one-way ANOVA followed by Tukey's post-test,  $p<0.001$ , letters represent  
608 significant dif). (E) Heterologous expression of *altB-J* in *P. ananatis* PNA 02-18 and *E. coli* DH5 $\alpha$  in ten-  
609 fold serial dilution of  $\text{OD}_{600}$  0.3 on LB and LB allicin amended plates. Each row is a cropped from a  
610 larger photo representing one of three independent experimental replicates conducted in duplets. Original  
611 uncropped images (Fig. S11, S12).

612 Fig S1. Growth of natural variant *Pantoea* isolates in aqueous red onion extract (ROE) and ROE  
613 amended with LB. (A) Growth of isolates (change in  $\text{OD}_{600}$ ) in ROE. (B) Growth of isolates in ROE:LB.  
614 This experiment was repeated three times with similar results ( $N=6$ ). Error bars represent  $\pm$ SE.

615 Fig S2. Growth of PNA 97-1R  $\Delta alt$  in LB broth amended with allicin. Bioscreen growth of strains  
616 (change in  $\text{OD}_{600}$ ), PNA 97-1R WT and PNA 97-1R  $\Delta alt$ . (A) synthesized allicin amended LB. (B) garlic  
617 extract amended LB. Allicin concentration determined with 4-MP assay. This experiment was repeated  
618 three times with similar results ( $N=4$ ). Error bars represent  $\pm$ SE.

619 Fig S3. PNA 97-1R *alt* cluster shares homology with a plasmid-borne gene cluster of onion rot pathogen  
620 *Enterobacter cloacae* EcWSU1. Percent amino acid identity is depicted above homologous EcWSU1  
621 genes. NCBI locus numbers are included for EcWSU1 genes.

622 Fig S4. Natural variants of *P. ananatis* and *Enterobacter cloacae* with *alt*-like genes have increased  
623 tolerance to allicin and garlic extracts. Area of inhibition for garlic extract (top) and allicin (bottom) is  
624 represented. Representative zones of inhibition for allicin above corresponding bars. The data from one of  
625 three independent replicates is presented ( $N=6$ , one-way ANOVA followed by Tukey's post-test) ( $N=6$ , t-  
626 test, \*\*\* $p<0.0001$ ). Error bars represent  $\pm$ SD.

627 Fig S5. Calculated total lesion luminescence (CTLL) of complementation constructs in WT and  
628 heterologous *Pantoea* background. Values were quantified by defining the lesion area in stacked images  
629 and quantifying relative pixel intensity in imageJ. CTLL= integrated density – (area of lesion X mean  
630 luminescence of background readings, ctrl). (A) CTLL of PNA 97-1R nested complementation constructs.  
631 The data from six independent replicates is presented ( $N=18$ , one-way ANOVA followed by Tukey's  
632 post-test). (B) CTLL of PNA 02-18 expressing *altB-J*. The data from three independent replicates is  
633 presented ( $N=12$ , one-way ANOVA followed by Tukey's post-test).

634 Fig S6. Growth curve of *altR* mutants in LB:ROE. Bioscreen growth of isolates (change in  $OD_{600}$ ), ev =  
635 empty vector. This experiment was repeated three times with similar results ( $N=6$ ). Error bars represent  
636  $\pm$ SE.

637 Fig. S7. WT,  $\Delta alt$ , *altH-J* ten-fold serial dilution on LB rifampicin and LB allicin amended plates. Fig. 5  
638 cropped panels highlighted in red.

639 Fig S8. WT,  $\Delta alt$ , *altH-J* ten-fold serial dilution on LB rifampicin and LB allicin amended plates. Fig. 5  
640 cropped panels highlighted in red.

641 Fig S9. WT,  $\Delta alt$ , *altH-J* ten-fold serial dilution on LB rifampicin and LB allicin amended plates. Fig. 5  
642 cropped panels highlighted in red.

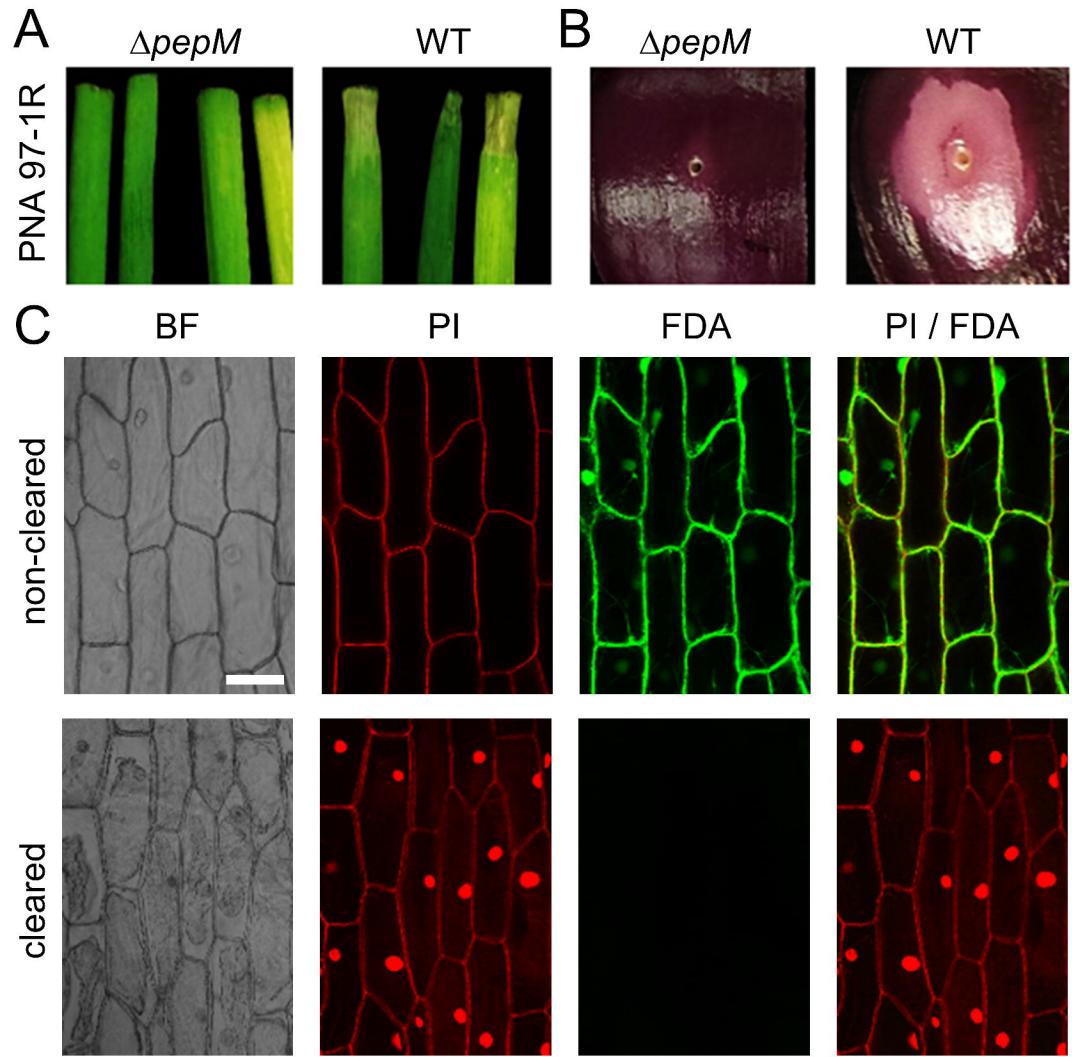
643 Fig S10. WT,  $\Delta alt$ , *altH-J* ten-fold serial dilution on LB rifampicin and LB allicin amended plates. Fig. 5  
644 cropped panels highlighted in red.

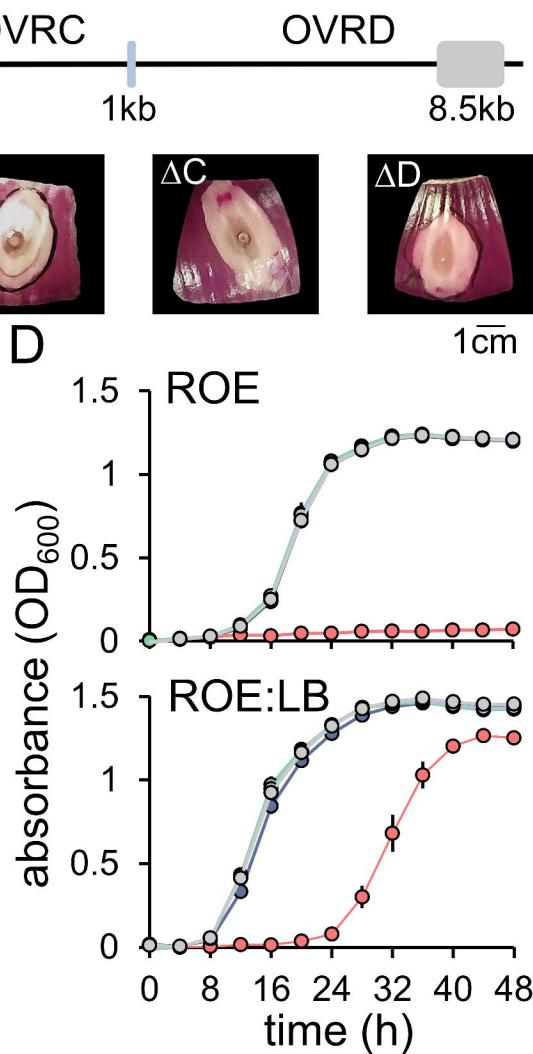
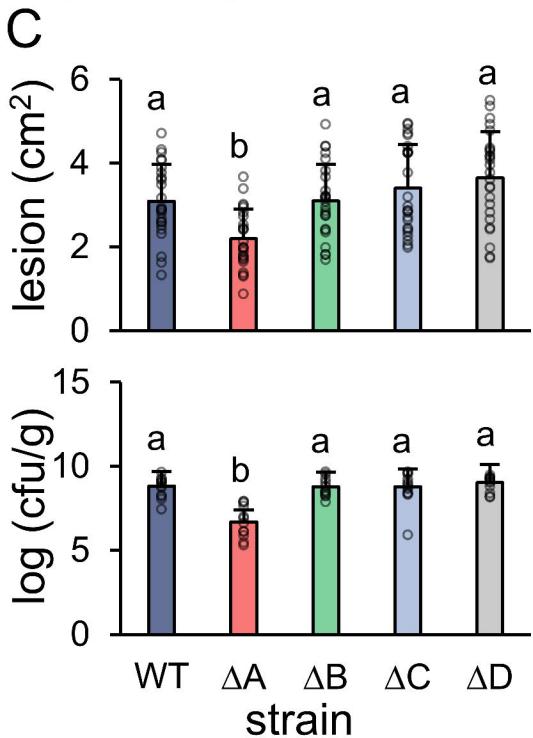
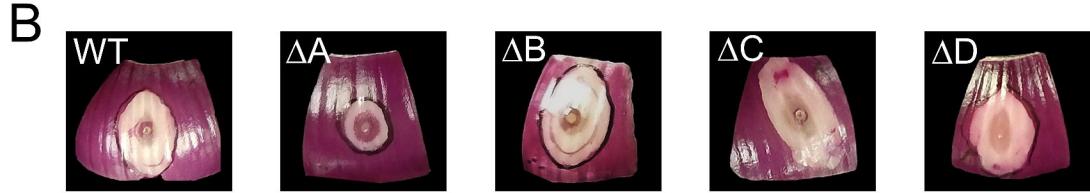
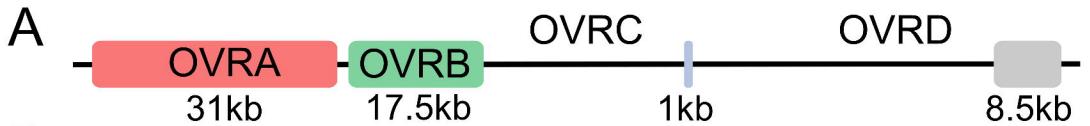
645 Fig S11. *P. ananatis* PNA 02-18 WT, ev, and *altB-J* ten-fold serial dilution on LB and LB allicin  
646 amended plates. Fig. 6 cropped panels highlighted in red.

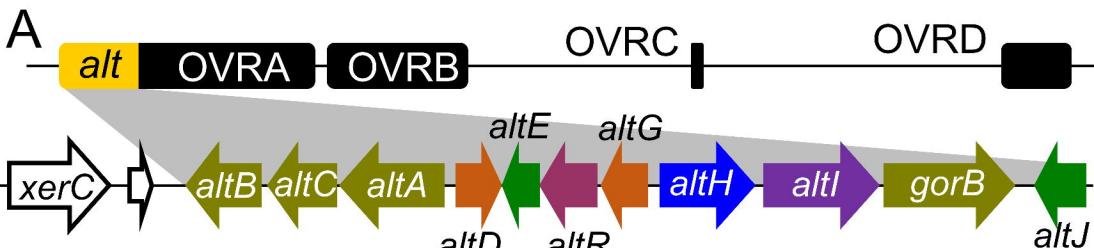
647 Fig S12. *E. coli* DH5 $\alpha$  WT, ev, and *altB-J* ten-fold serial dilution on LB and LB allicin amended plates.  
648 Fig. 6 cropped panels highlighted in red.

649

650



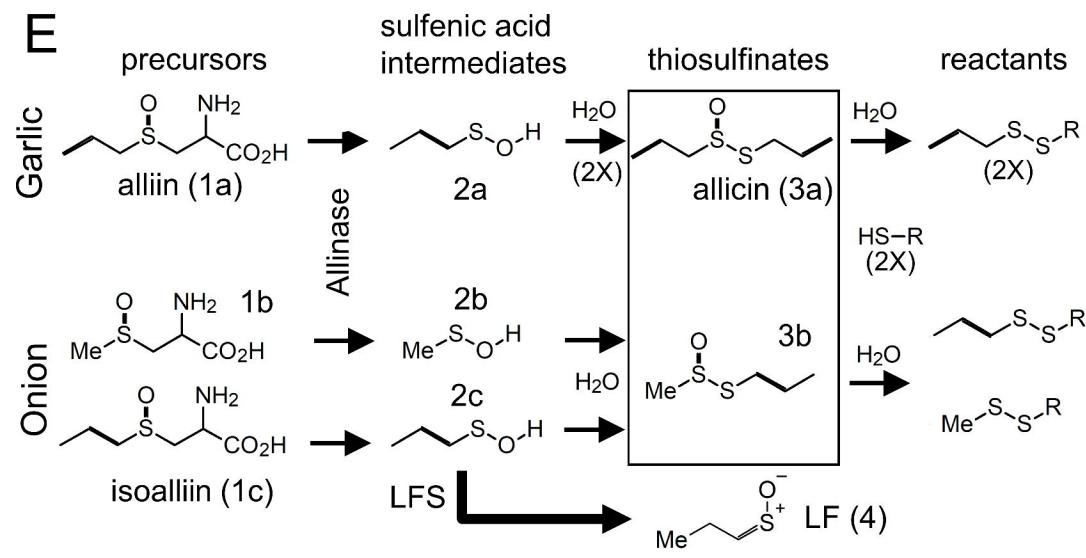
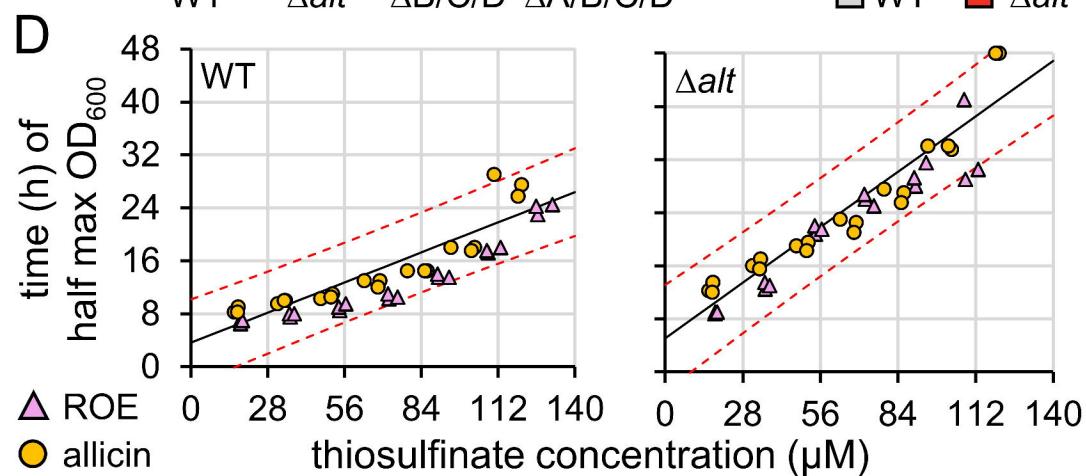
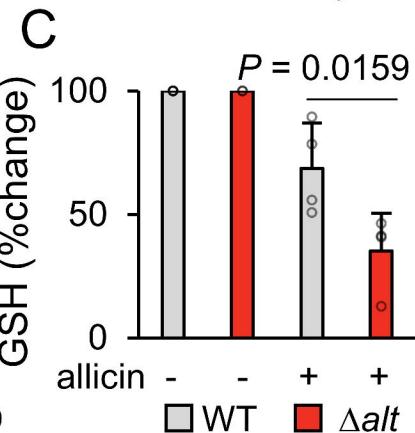
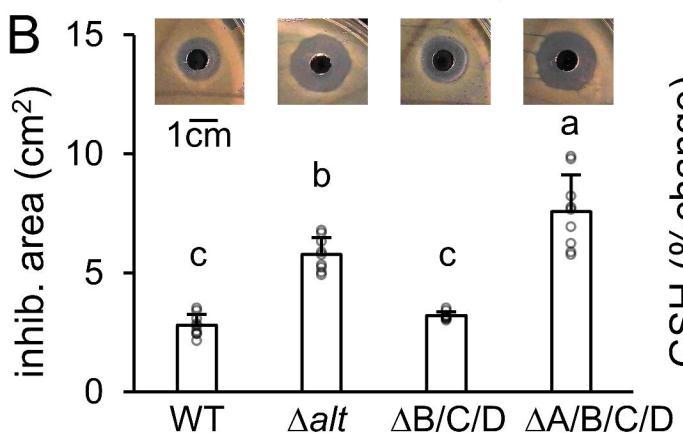


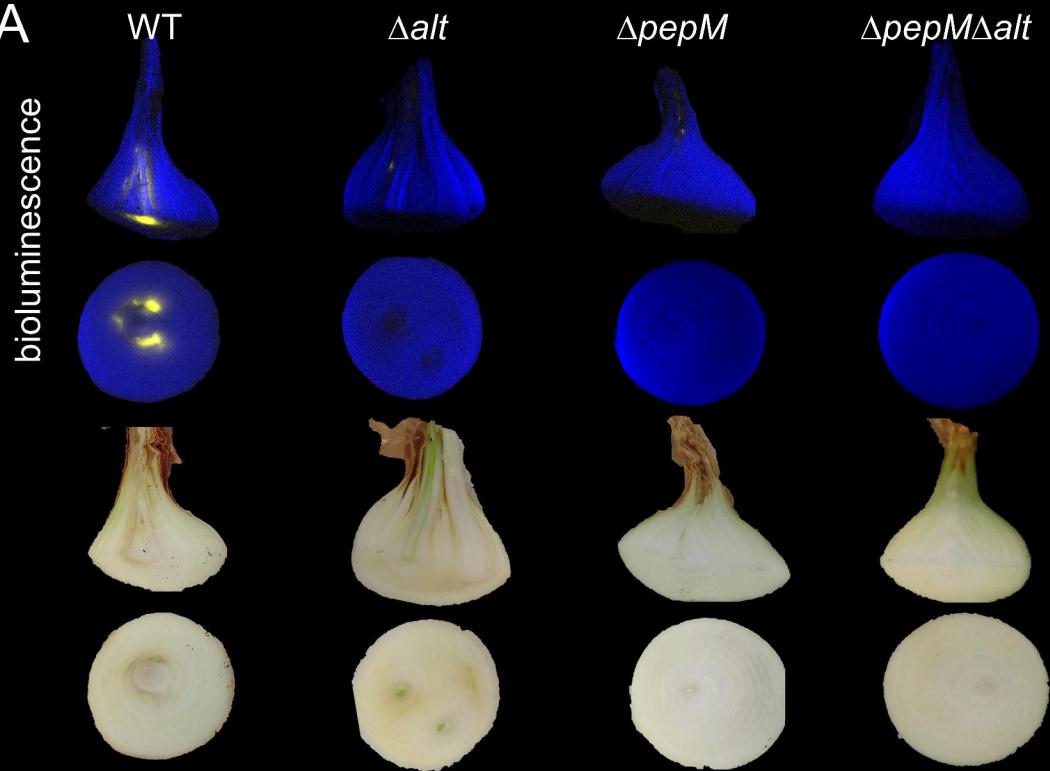


█ thiol/oxidoreductase  
█ thioredoxin-/like

█ peroxidase  
█ repressor

█ cysteine exporter  
█ carbon-sulfur lyase



**A****B**