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1 **Identification of Genes Regulating Cell Death in *Staphylococcus aureus***
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20
21 **Abstract**
22
23 *Staphylococcus aureus* is an opportunistic pathogen that causes acute and chronic infections.
24 Due to *S. aureus*' s highly resistant and persistent nature, it is paramount to identify better drug
25 targets in order to eradicate *S. aureus* infections. Despite the efforts in understanding bacterial
26 cell death, the genes and pathways of *S. aureus* cell death remain elusive. Here, we performed a
27 genome-wide screen using a transposon mutant library to study the genetic mechanisms involved
28 in *S. aureus* cell death. Using a precisely controlled heat-ramp and acetic acid exposure assays,
29 mutations in 27 core genes (*hsdR1, hslO, nsaS, sspA, folD, mfd, vraF, kdpB*, USA300HOU_2684,
30 0868, 0369, 0420, 1154, 0142, 0930, 2590, 0997, 2559, 0044, 2004, 1209, 0152, 2455, 0154,
31 2386, 0232, 0350 involved in transporters, transcription, metabolism, peptidases, kinases,
32 transferases, SOS response, nucleic acid and protein synthesis) caused the bacteria to be more
33 death-resistant. In addition, we identified mutations in core 10 genes (*capA, gltT, mnhG1*,
34 USA300HOU_1780, 2496, 0200, 2029, 0336, 0329, 2386, involved in transporters, metabolism,
35 transcription, cell wall synthesis) from heat-ramp and acetic acid that caused the bacteria to be
36 more death-sensitive or with defect in persistence. Interestingly, death-resistant mutants were
37 more virulent than the parental strain USA300 and caused increased mortality in a
38 *Caenorhabditis elegans* infection model. Conversely, death-sensitive mutants were less
39 persistent and formed less persister cells upon exposure to different classes of antibiotics. These
40 findings provide new insights into the mechanisms of *S. aureus* cell death and offer new
41 therapeutic targets for developing more effective treatments caused by *S. aureus.*
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Introduction

S. aureus is a major bacterial pathogen that colonizes on the skin of over one-third of the human population and can cause acute infections such as bacteremia, pneumonia, meningitis and persistent infections such as osteomyelitis, endocarditis, and biofilm infections such as on prosthetic implants [1]. Due to emerging resistance and high risk of nosocomial infections and community-acquired infections, *S. aureus* infections are a major public health concern. To develop better treatments for *S. aureus* and other bacterial infections, understanding how bacterial cells die is crucial.

By definition, bacteria treated with bactericidal antibiotics, such as the β-lactams, quinolones, and aminoglycosides, are killed. Much is known about how the drug makes contact with its target in the bacterial cell. β-lactam antibiotics bind to penicillin binding protein (PBP) disrupting proper cell wall synthesis; quinolones bind to toposismerase or gyrase blocking DNA replication, and aminoglycosides bind to ribosomal proteins resulting in mistranslated proteins [2-4]. Despite having different drug-target interactions, the downstream effects of drug lethality of cidal antibiotics are similar. It has been proposed that bacteria treated with cidal antibiotics have pathways such as SOS response, TCA cycle, ROS formation damaging Fe-S cluster regardless of the drug target [5, 6], however, the ROS theory of cidal antibiotic lethality has been challenged as killing of bacteria can still occur in apparently anaerobic conditions that do not produce ROS [7-9].

Mechanisms pertaining to bacterial cell death were mainly characterized in toxin-antitoxin systems. One of the better characterized Toxin-Antitoxin modules in the context of bacterial cell death is the MazEF module found in *E. coli* and other species such as *Listeria, Enterococcus, Neisseria, Streptococcus* and *Mycobacterium* [10-13]. Upon exposure to stresses such as nutrient depletion, DNA damage, temperature, antibiotics, and oxidative radicals, the MazF antitoxin is degraded and hence, the MazE toxin can degrade cellular mRNA causing cellular shutdown [10, 12]. In particular to *S. aureus*, the CidA and LrgA proteins, which are holin-like molecules with analogous functions to apoptotic regulators of the BCL-2 family in eukaryotes, were proposed to play a role in death and lysis of *S. aureus* [14, 15]. However, the specific process as to how CidA and LrgA regulate cell death is poorly defined.

Although cell death is an important process of a living organism, little is known about the mechanisms. High-throughput screens have been developed to study the cell death mechanism of unicellular eukaryotic organisms such as *S. cerevisiae* upon stress signals from high temperature and acetic acid [16-19], both of which also induce death in bacteria. Here, we performed a high-throughput genetic screen using a transposon mutant library of USA300 to identify genes involved in cell death in *S. aureus* [20]. Under multiple death stimuli, we identified 27 genes whose mutations caused the bacteria to be more death-resistant, while mutations in 10 genes caused the bacteria to be more death-sensitive.

Results

Identification of genes important for cell death resistance

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92 To better understand the mechanisms of cell death, we performed a genetic screen using the
93 Nebraska Transposon Mutant Library (NTML) which contains mutations in all the non-essential
94 genes of *S. aureus* USA300, the most common circulating community-acquired MRSA strain in
95 the United States [20]. To design our assay, we utilized the heat-ramp assay that has been used to
96 study cell death programs in yeast [17]. To determine viability, we employed both the traditional
97 agar replica plating for visualization of viable growth on solid media but also stained cells with
98 SYBR Green I/PI, a viability stain that can detect both live and dead cells [21, 22]. Using a *cidA*
99 mutant [15] which has been shown to be death-resistant as a control and the parental strain of
100 USA300 as a death sensitive control, we optimized the condition of our heat-ramp experiment to
101 show the biggest difference between both the death-resistant and death-sensitive phenotypes
102 based on agar plating and the live/dead ratio from viability staining with SYBR Green I/PI.
103
104 For identification of death-resistant mutants, we searched for clones that survive heat-ramp and
105 grow on agar plating (as opposed to USA300 which no longer show colony growth from replica
106 plating) and a live/dead ratio that is higher than our death-resistant mutant, *cidA* control. After
107 the heat-ramp exposure, we identified 74 mutants that were death-resistant. While we cannot
108 pinpoint a specific gene to be the ultimate regulator of cell death, we generated a list of potential
109 regulators of cell death. In order to identify core genes and pathways involved in cell death, we
110 exposed the transposon mutant library to acetic acid stress.
111
112 Acetic acid has been shown to induce cell death in *S. aureus* and in yeast as well [15, 16, 19, 23].
113 Upon treatment with acetic acid (6 mM) overnight, 27 (*hsdR1, hslO, nsaS, sspA, folD, mfd, vraF,*
114 *kdpB*, USA300HOU_2684, 0868, 0369, 0420, 1154, 0142, 0930, 2590, 0997, 2559, 0044, 2004,
115 1209, 0152, 2455, 0154, 2386, 0232, 0350) out of the 74 heat-ramp resistant mutants were also
116 acetic acid resistant (Fig. 1). A majority of the genes identified encode transporters (n=9),
117 involved in transcription (n=4), metabolism (n=3), peptidases (n=2) and phosphatases & kinases
118 (n=2). For genes encoding transferases and proteins involved in stress response, nucleic acid
119 synthesis and protein synthesis, one candidate was found in each category.
120
121 *Death-resistant strains are more virulent in vivo*
122 Upon infection inside a host, in addition to drug stress, the bacteria are exposed to various types
123 of stresses such as oxidative stress especially in the phagosomes of immune cells. We then tested
124 if our death-resistant mutants were more virulent in causing an infection inside the host. After
125 infection of *C. elegans* with the top 4 death-resistant mutants (*folD*, USA300HOU_0997, *sspA*,
126 USA300HOU_0232) and parental strain USA300, we observed that all four mutants significantly
127 decreased the survival of the *C. elegans* and killed the worms faster than USA300 (Fig. 2). By 2
128 days post-infection, the survival of worms infected with our death-resistant mutants had a
129 survival rate of 36% or lower while worms infected with USA300 had a survival of 50%. The
130 most virulent strain was Tn::USA300HOU_0232, a mutation in an iron transporter, as it caused
131 the greatest mortality in the worms, resulting with only 22% survival of the worms by day 2.
132 Our data suggest that bacteria that are more death-resistant could potentially cause more serious
133 infections.
134
135 *Identification of genes important for cell death sensitivity revealed the importance of glutamate*
136 *biosynthesis in cell death*

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137 In order to fully understand the regulatory networks of cell death pathways, it is crucial to
138 examine the genes whose mutations cause the cells to be more sensitive to stress as well. Using
139 the data from our two screens (heat-ramp and acetic acid stress), we adjusted the parameters of
140 data analysis to distinguish mutants that were cell death sensitive. Unlike the cut-offs mentioned
141 previously, cell death sensitive mutants were identified as having no viable growth on agar
142 media and a live/dead ratio that was lower than USA300.
143
144 Our screen revealed that 92 mutants were hypersensitive to the heat-ramp, of which 10 (*capA,*
145 *gltT, mnhG1*, USA300HOU_1780, 2496, 0200, 2029, 0336, 0329, 2386) were also sensitive to
146 acetic acid stress (Fig. 1). Transporters were the more abundant (n=4) followed by genes
147 involved in metabolism (n=2), and lastly with genes involved in transcription (n=1) and cell wall
148 synthesis (n=1). Two of the candidates were hypothetical proteins. Interestingly, two
149 (USA300HOU_2029*, gltT*) of the top four death-sensitive mutants (USA300HOU_0329,
150 USA300HOU_2029, USA300HOU_2386, *gltT*) harbored mutations pertaining to glutamate
151 metabolism.
152
153 *Death-sensitive strains are less persistent in vitro; strains with mutations involved in glutamate*
154 *metabolism are the least persistent*
155 One of the reasons why *S. aureus* can cause persistent and recalcitrant infections is due to its
156 ability to form persister cells. Persisters are dormant cells that are formed during stressed
157 conditions and upon stress removal, the bacteria can revert back to a growing state and
158 consequently, cause a relapse in infection [24]. Bacterial persistence can also be viewed as cells
159 with a strong anti-death program. Given now that we have identified genes whose mutation
160 renders the bacteria death-sensitive, we then wanted to know if these mutations also lead to
161 defective in persistence, forming lower amounts of persister cells. Such mutations could then
162 potentially be drug targets for clearing persistent infections.
163
164 Persisters are induced by treating stationary phase bacteria with high concentrations of
165 bactericidal antibiotics (usually at least 10 X MIC). Cells are then washed to rid of stress and
166 plated on solid medium with no drug for CFU enumeration [25]. We exposed the top 4 death-
167 sensitive mutants to bactericidal antibiotics with different mechanism of actions: gentamicin,
168 meropenem, rifampin, and moxifloxacin. Upon 6-days post exposure of antibiotics, all 4 mutants
169 showed a defect in persistence when exposed to all different classes of antibiotics (Fig. 3). The
170 amount of persisters is dependent on the type of stress which can be seen here since the absolute
171 amount of persisters changes among the drugs tested [24]. However, the overall amount of
172 persister cells formed by the death-sensitive mutants was significantly lower than USA300. The
173 defect in persistence was the most prominent for gentamicin stress. Under gentamicin stress,
174 mutations in *gltT* and USA300HOU_2029, both involved in glutamate metabolism, were
175 completely killed by 4 day-post exposure while USA300 still had over 10^7 CFU/ml. In non-
176 stressed conditions, no growth defects and decrease in viability of cells were observed.
177
178 **Discussion**
179
180 To our knowledge, this is the first comprehensive study to identify genes and pathways that play
181 a role in anti-death and pro-death programs in *S. aureus*. These data suggest that not only are
182 transporters important in regulating cell death pathways in *S. aureus*, but in particular, glutamate

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183 metabolism and glutamate transport are important for transformation of a bacteria cell into a both
184 a more death-resistant and a persistent phenotype under stressed conditions. Our findings show
185 that mutations in genes involved in intracellular glutamate level regulation such as
186 USA300HOU_2029 and *gltT* can decrease cell viability and persistence under antibiotic stresses,
187 and environmental stresses such as temperature changes (heat-ramp) and low pH (acetic acid).
188
189 While other studies have searched for cell death genes in *S. aureus* [14, 15, 26, 27], the specific
190 pathway of glutamate metabolism has never been identified as core cell death proteins. Although
191 further research is needed to explore how glutamate metabolism plays a protective role in cell
192 death, hypotheses based on what is currently known about cell death can offer insights into how
193 intracellular glutamate levels could fit in the program of *S. aureus* cell death. Sadykov et al.
194 identified that inactivation of the phosphotransacetylase-acetate kinase (Pta-AckA) pathway
195 which normally generates acetate from acetyl-CoA leads to cell death in *S. aureus* under glucose
196 and oxygen excess [26]. In bacteria, glutamate fermentation can occur via 3-methylaspartate
197 which produces pyruvate followed by acetyl-CoA. Considering that acetate can be produced
198 from acetyl-CoA, our findings may help explain the events that occur upstream of Pta-AckA
199 activation [28]. Additionally, the lethality induced by cidal antibiotics has been shown to be due
200 to ROS generation and radical generation from the Fenton reaction suggesting that death
201 mechanisms result in oxidative responses within the cell [5]. In *Francisella*, glutamate
202 transporter GadC has been shown to neutralize reactive oxygen species [29]. A study performed
203 to evaluate the bactericidal effect of CO-releasing molecules (CO-RMs) showed that CO-RMs
204 stimulated the production of intercellular ROS in the bacteria which was abolished when
205 glutamate was supplemented to the culture [30]. Thus, increased glutamate levels in the stressed
206 cells may protect the cell from ROS-mediated cell death under stress.
207
208 Findings from our previous screens performed to identify genes involved in persistence to
209 rifampicin [25] showed that genes *gltS, gltD, gltA*, all of which are involved in glutamate
210 synthesis, were important. Intriguingly, the protein ArgJ was shown to be a potential core
211 regulator for *S. aureus* persistence in various stresses (different classes of drugs, heat, and low
212 pH) [31]. Glutamate is the substrate for ArgJ (Fig. 4) and since mutants with impaired glutamate
213 biosynthesis and transport showed both death-sensitive and defective persistence phenotypes, it
214 can be speculated that glutamate can be the main driver of anti-death or equivalent to elevated
215 persistence programs where arginine synthesis is a potential downstream effector pathway.
216 Further biochemical studies and metabolomic studies are needed to understand how glutamate
217 and arginine are involved in cell death.
218
219 Our screen also identified mutants whose phenotypes are cell death resistant and when the top
220 four death-resistant mutants were tested in an *in vivo C. elegans* model, we found that they are
221 indeed more virulent than the parental strain USA300 (Fig. 2). The mutant that was the most
222 virulent harbored a mutation in USA300HOU_0232 which encodes an iron transporter. Similarly,
223 the *nsaS* mutant was also identified to be death-resistant from our screens and studies in *S.*
224 *aureus* have shown that NsaS is part of the cell-envelope two-component sensing system[32, 33].
225 In a mutant of *nsaS* in *S. aureus*, there is decreased association with metal ions on the cell
226 surface and as a result, the intracellular concentration of metal ions was reduced [32].
227 Considering how ROS may play a role in cell death [5], a mutated iron transporter may cause the
228 cells to be death-resistant by decreasing the amount of ROS inside the cell [34] as the transporter

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229 limits the amount of iron needed for the Fenton reaction which can damage DNA and cause cell
230 death. USA300HOU_0997 encodes an autolysin family protein that plays a role in peptidoglycan
231 turnover [35]. Gene expression of autolysins have been implicated in increasing cell wall
232 stability under stressed conditions [36]. In vancomycin resistant strains, autolysin genes were
233 downregulated which led to changes in membrane charges and thickness of the cell wall enabling
234 the cell to survive treatment of cell wall inhibitors [37]. It can be speculated here that decreased
235 cell division and peptidoglycan turnover in our USA300HOU_0997 mutant may lead to death
236 resistance. FolD is a bifunctional protein that allows the production of continuous
237 tetrahydrofolate which is a key metabolite for amino acid and nucleic acid biosynthesis [38].
238 The catalytic step of FolD is a checkpoint that regulates folate production [39]. As such, a mutant
239 of *folD* may disturb the negative feedback of folate synthesis and continuously produce
240 important metabolites and nucleotides for continued survival under stressed conditions. For
241 example, MthfR is a Methylenetetrahydrofolate reductase in the same pathway as FolD and has
242 been shown to be important for growth in *S. aureus* [40]. Lastly, the SspA protein encodes for
243 the V8 serine protease that cleaves fibronectin binding proteins. Serine proteases such as SspA
244 are under the *agr* quorum-sensing control operon [41] which coordinates a series of gene
245 expression cascades to withstand stresses such as oxidative stress and the human immune
246 response and facilitate bacterial cell growth and pathogenesis [42, 43]. It has been shown that
247 *agr* negative cells do not undergo cell death as rapidly and were more resistant to cell lysis due to
248 the increased expression of RNAIII [44, 45]. The increased mortality of *C. elegans* infected with
249 the SspA mutant suggests that downregulation of *agr* gene could potentially be involved in
250 causing cell death resistance because *agr*-negative *S. aureus* strains can be found in hosts with
251 chronic infections and cause infections with increased mortality compared to those infected with
252 *agr*-positive strains [46].
253
254 Interestingly, our screen revealed that mutants of *hslO* and *mfd* were more death-resistant which
255 may appear contradictory given that expression of HslO and Mfd are protective for the cell
256 during stress [47, 48]. HslO (or Hsp33) has been shown to be upregulated especially during
257 oxidative stress and that HslO levels were decreased when *S. aureus* cells were transitioning to
258 slow/non-growing status [49]. It is well known that Mfd is involved in prevent DNA damage
259 from oxidative stress, immune response, and drugs [47, 50]. Decreased expression of Mfd in *S.*
260 *aureus* led to decreased biofilm formation [51] and a *mfd* mutant of *C. difficile* has increased
261 toxin production [52]. For *B. subtilis,* Mfd-deficient cells formed less endospores [53] and
262 resulted in a 35-fold overexpression of OhrR, a transcription factor involved in peroxide stress
263 response [54]. Given that Mfd has many nonconical roles, we speculate that under stressed
264 conditions decrease of expression in our *mfd* mutant may allow expression of another
265 transcription factor (such as the OhrR) to activate stress response pathways. It is also possible
266 that the contradictory phenotypes of the *hslO* and *mfd* mutants are due to compensatory
267 mutations in the *hslO* and *mfd* mutants, which are known to occur in loss of function mutants in
268 yeast [55].
269
270 As with any mutant library, secondary mutations may have occurred in some mutants that
271 could affect the phenotypic outcomes. For example, our speculation as to why a *hsdR* mutant is
272 more death-resistant could also be associated to the genetic background of the mutant.
273 Inactivation of HsdR, an endonuclease of the type 1 restriction system in *S. aureus*, allowed *S.*
274 *aureus* to become readily transformable due to decreased cleavage of foreign DNA [56, 57]. In

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275 our *hsdR* mutant, it could be possible that secondary mutations or genetic modifications have
276 occurred in the genome that caused the death-resistant phenotype. While we performed whole
277 genome sequencing on our mutant clones to confirm the location of the transposon, the
278 possibility of secondary mutations playing a role in the phenotypes observed here have yet to be
279 explored. However, we performed the screen twice followed by individual confirmation. The
280 same subculture from the same stock was also used each time to ensure reproducibility among
281 all replicates. Meanwhile, even if there is a secondary mutation, the decreased expression of
282 the specific genes identified here due to the transposon insertion would still be a valid part of
283 the mechanisms of cell death.
284
285 Our work adapted assays that are used in the yeast community to identify genes involved in yeast
286 cell death [16-19]. In the high-throughput screen consisting of yeast mutants and even in yeast
287 strains of different backgrounds, categories of genes that were redundant among the studies
288 include carbohydrate metabolism, transcription factors, and ion transport [18, 58, 59].
289 Interestingly, amino acid transport was the most significantly enriched term for genes involved in
290 positive regulation of acetic acid-induced death; two of the identified transporters were involved
291 in metabolism of glutamate (GDH1 and GDH2) [18]. A similar approach of a "heat-ramp" stress
292 was used in one study on *B. subtilis* using a water bath [60]. Their study revealed that heat shock
293 proteins, sporulation, competence, and carbon metabolism were important. While we did not
294 identify similar genes, carbon metabolism was identified in both our screens. Heat shock proteins
295 were heavily enriched in their study but not ours which can be attributed to the candidates in our
296 library which only contain mutants of non-essential genes and only 2 candidates out of 1,952
297 were heat shock proteins [61].
298
299 Despite the significant findings of this study, there are some limitations. First, while we
300 generated a list of the mutants that are death-sensitive and death-resistant to both heat ramp
301 and acetic acid stress, the screens performed here are only a snapshot of what is happening in
302 the cell. The phenotypes seen here were determined by the condition of our assays and can be
303 affected by the level of stress (e.g. concentration of the acetic acid, ramping time and
304 temperature for the heat ramp) [17]. Although we included both a wild type USA300 strain
305 and a CidA mutant, a known death-resistant mutant [15, 62], to optimize our conditions, it is
306 important to recognize that our screen may not be comprehensive in finding all the mediators
307 of cell death. While our goal was more conservative and was to search for core regulators, the
308 significant role of other genes and pathways involved in cell death that were not classified as
309 core regulators should not be undermined. Our screen only explored cell death at the DNA
310 level and further protein studies would provide more comprehensive insight into the effects of
311 gene transposon insertions in regulating cell death. Even though this mutant library contained
312 all the non-essential genes in USA300, we cannot overrule the fact that cell death is an
313 important program for any living organism and cell death regulators may be essential genes
314 which are not included in our library.
315
316 Given that we know the killing activity of antibiotics extend beyond the drug-target interactions
317 [5], understanding the effector proteins and downstream events of bacterial cell death can help
318 provide novel drug treatment approaches for bacterial infections. For example, cell death can be
319 artificially induced by disruption of the negative regulator of cell death in a similar fashion to
320 how apoptotic pathways are exploited to treat cancer cells. Recently, it was shown that

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extracellular death peptides from both *E. coli* and *P. aeruginosa* can induce toxic endoribonucleolytic activities of MazEF in *M. tuberculosis* suggesting promising therapeutic outcomes upon manipulation of the cell death mediators in bacteria [63]. Similarly, since ROS formation serves an important role in the lethality of cidal antibiotics, a drug combination with metabolic perturbations may enhance killing of currently used antibiotics. For example, causing defects in peroxide-detoxifying enzymes have been shown to increase antimicrobial lethality [64]. On the other hand, it is of concern that more than half of the population in the US consumes nutritional supplements such as vitamins which are antioxidants [65] and the potential antagonistic effects of an antioxidant diet in a patient taking antibiotics will require further investigation.

In conclusion, we report the molecular basis of cell death in *S. aureus*. To our knowledge, this is the first report on identification of *S. aureus* cell death genes from a whole genome perspective. Our extensive screen also offers insights to common core mechanisms that are relevant to not only cell death but bacterial persistence, a phenomenon that's at the core of recalcitrant infections and biofilm formations. Our studies provide insights to possible new drugable targets, biomarkers for recalcitrant infections for diagnostic purposes and novel vaccine targets for prevention of bacterial infections. The similarity in functional groups found between our study and other yeast studies suggest that our work also sheds light into cell death pathways of eukaryotic systems such as pathogenic fungi and cancer stem cells.

Methods

Culture Media, Antibiotics, and Chemicals

Meropenem, moxifloxacin, rifampicin, gentamicin, and erythromycin were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Stock solutions were prepared in the laboratory, filter-sterilized and used at indicated concentrations. Bacterial strains used in this study include USA300 and the Nebraska-Transposon Mutant Library (NTML) [20]. *S. aureus* strains were cultured in tryptic soy broth (TSB) and tryptic soy agar (TSA) with the appropriate antibiotics and growth conditions. Transposon-insertion mutants grew in erythromycin (50 μg/ml), the antibiotic selective marker.

Genetic Screen to identify cell death mutants

For the heat-ramp, we performed the assay as described [17]. Briefly, we normalized the concentration of the bacteria to OD600= 0.5 using PBS as the diluent. Then, we placed the samples in the thermocycler with a protocol following: 30°C for 1 minute, ramp from 30 ^{o}C to 62 ^{o}C with a step-interval of 0.5 ^{o}C per 30 seconds. For acid stress, acetic acid (6 mM) were added into stationary phase cultures and incubated overnight. To enumerate for cell counts, the mutant library was replica transferred to TSA plates to score for mutants that failed to grow after stress. For viability staining, SYBR Green I/PI staining was performed as described [21, 22]. SYBR Green I (10,000× stock, Invitrogen) was mixed with PI (20 mM, Sigma) in distilled H_2O with a ratio of 1:3 (SYBR Green I to PI) in 100 ul distilled H2O and stained for 30 minutes in room temperature. Prior to the heat-ramp, the SYBR Green I/PI dye was added to the bacteria as the heat-ramp impaired the uptake of the dye. For the acetic acid stress, the dyes were added at the end of the exposure [17]. The green and red fluorescence intensity was detected using a Synergy H1 microplate reader by BioTek Instruments (VT, USA)

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367 at excitation wavelength of 485 nm and 538 nm and 612 nm for green and red emission,
368 respectively. The live/dead ratio was calculated by dividing the green/red fluorescence.
369
370 *Persister Assays*
371 Selected drugs were added to overnight stationary phase cultures for 6 days. At the selected
372 time points, bacterial cultures were washed with 1 X PBS to remove stress, serially diluted,
373 and plated onto TSA with no drugs for cell enumeration [25].
374
375 *Nematode-killing Assay*
376 *C. elegans* N2 Bristol worms (Caenorhabditis Genetics Center) were synchronized to the same
377 growth stage by treatment with alkaline hypochlorite solution as described [66]. Worms of adult
378 stage were washed and suspended in bleaching solution with 5% hypochlorite for 9 minutes to
379 lyse all the adult stages but keeping the eggs intact. Bleach was removed by centrifugation at
380 1,500 rpm for 1 minute and washed three times with M9 buffer. The pellet was incubated in M9
381 buffer at 20 ^{o}C with gentle agitation and proper aeration. L4 stage adult worms were obtained
382 after 48 hours at 20 ^{o}C. For each assay, 20-30 worms were added to liquid M9 buffer
383 supplemented with 5-Fluoro-2'-deoxyuridine (10 μM) to inhibit progeny formation. *S. aureus*
384 (10^{6} CFU) that were grown overnight at 30 ^{o}C in TSB containing the appropriate antibiotics as
385 needed were added into the buffer containing the worms. The samples were scored for live
386 and dead worms every 24 hours.
387
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392
393
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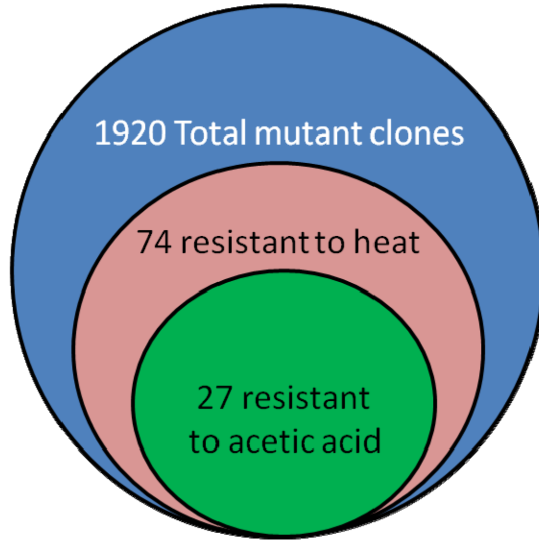

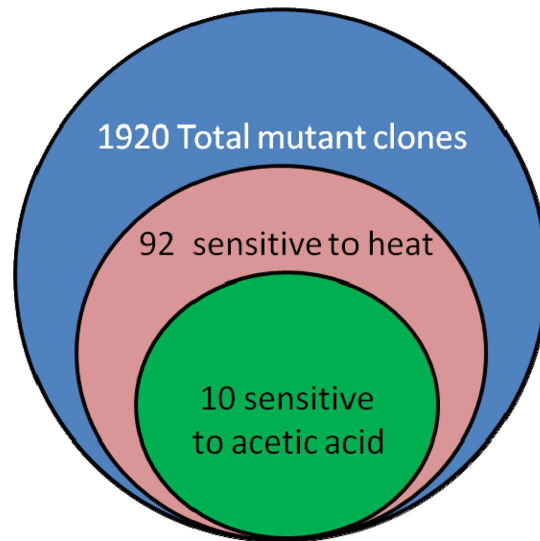

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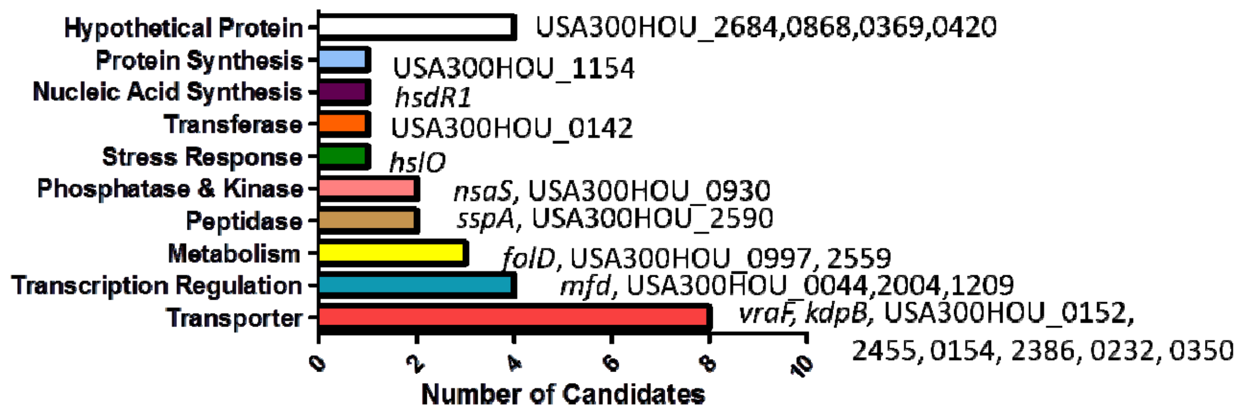

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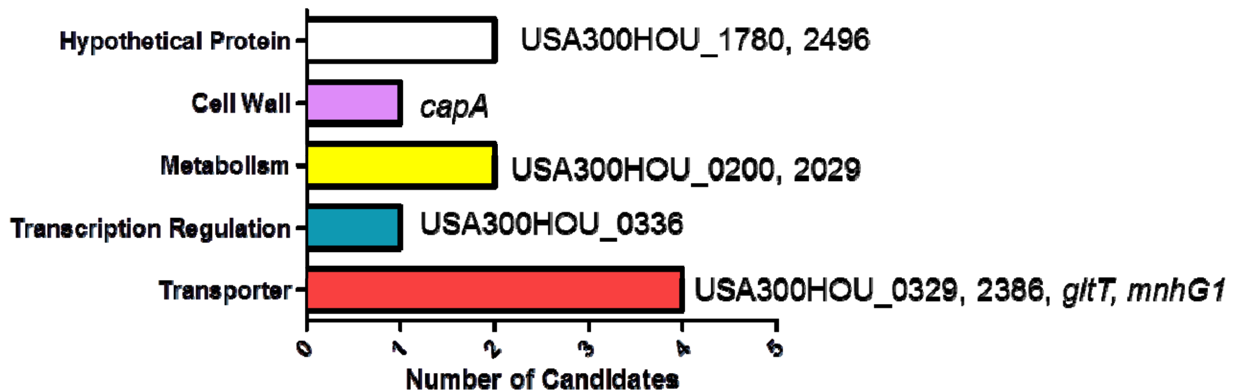

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421 **Figure 1.** Identification of genes involved in causing bacterial cell death-resistance and cell
422 death-sensitivity. (A) Summary of the number of candidates identified from the Nebraska
423 Transposon Mutant Library (NTML) as being more death-resistant (A) and death-sensitive (B) in
424 heat-ramp and acetic acid stress. The breakdown of the respective categories of genes whose
425 mutations cause death-resistance (C) and death-sensitivity (D) to both stresses.
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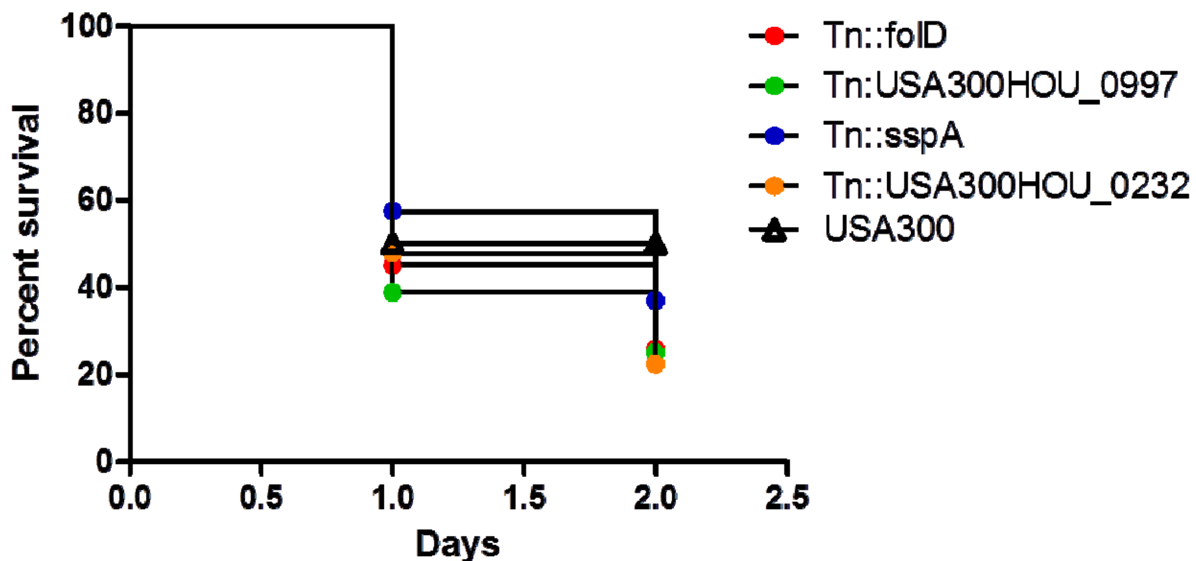

435
436 **Figure 2.** Strains that are more death-resistant caused more virulent infections in *C. elegans*.
437 *C. elegans* (n=20-30) infected (10^6 CFU) with mutants showing resistance to cell death showed
438 increased mortality than *C. elegans* infected with parental strain USA300.

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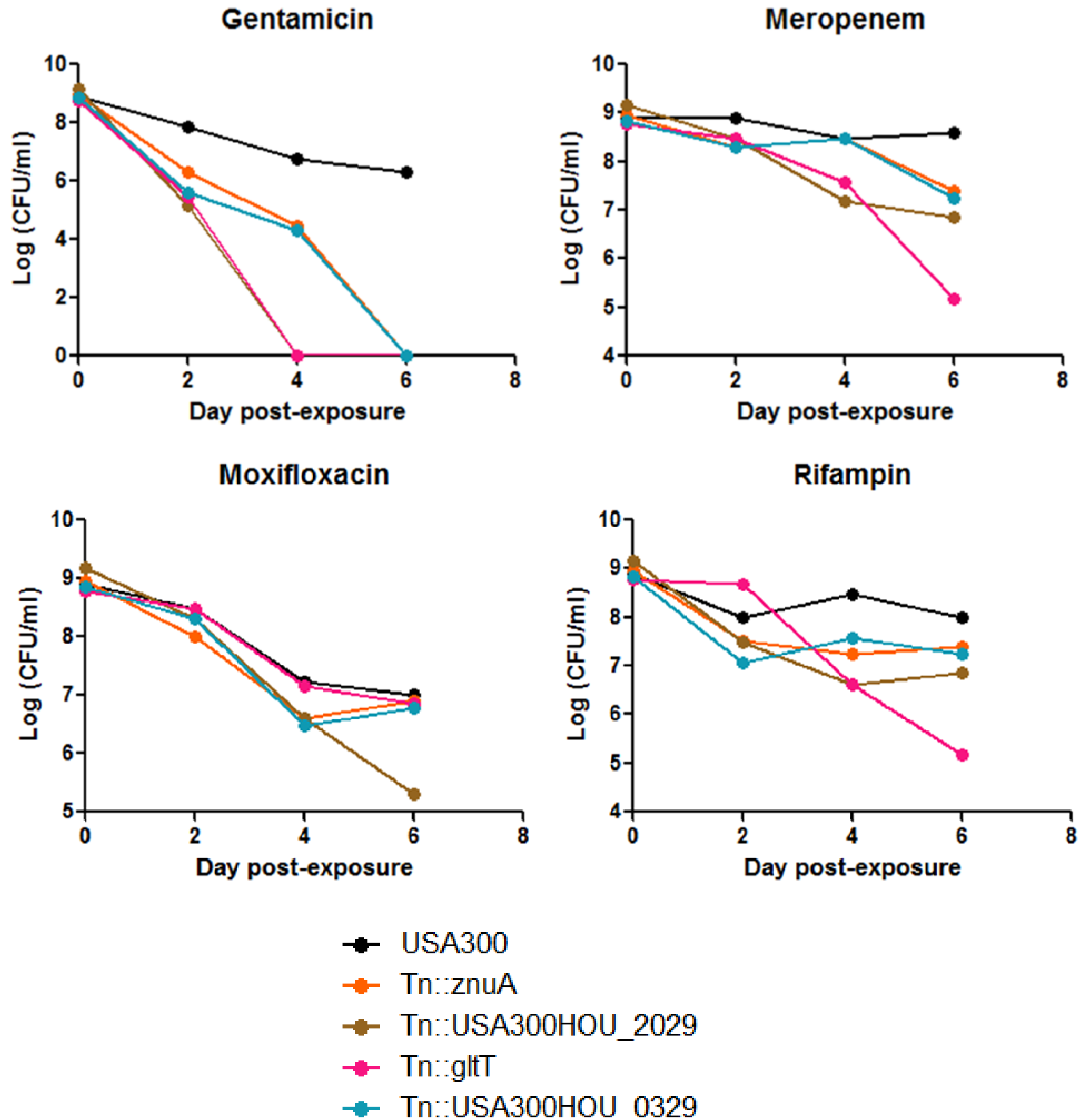

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441 **Figure 3.** Strains that are more death-sensitive also show decreased persistence to bactericidal
442 antibiotics. Mutants that were more cell-death sensitive showed defective persistence to
443 gentamicin (60 μg/ml), meropenem (20 μg/ml), moxifloxacin (40 μg/ml), and rifampin (2 μg/ml)
444 upon prolonged drug exposure up to 6 days.
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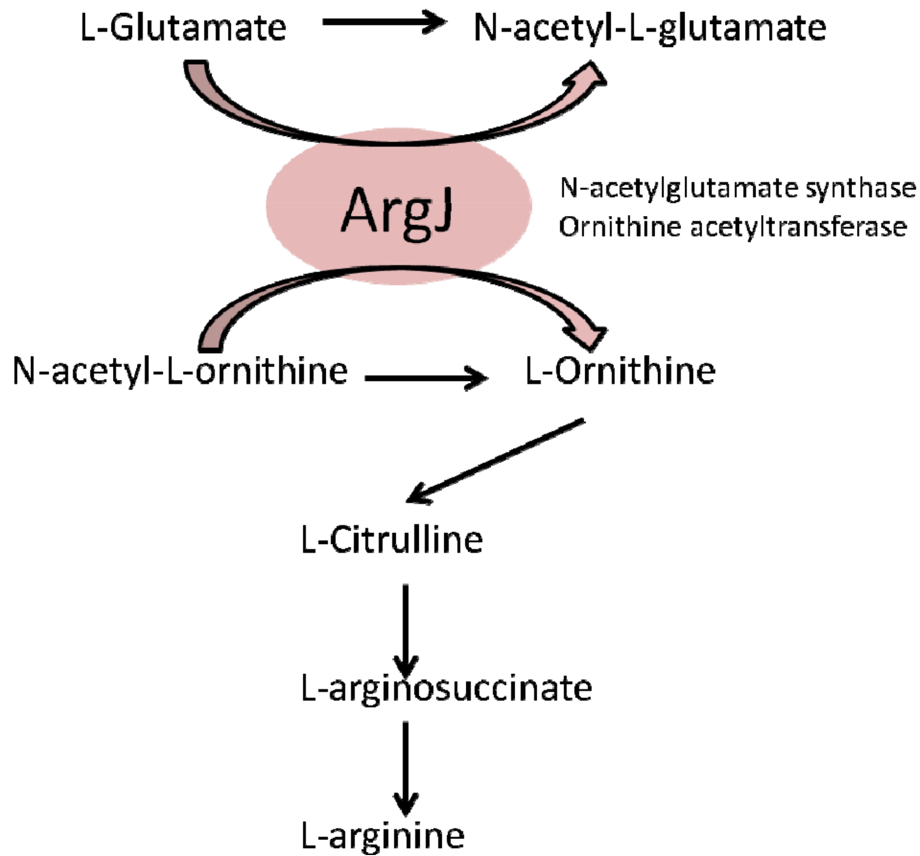

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449 **Figure 4.** Proposed model of cell-death pathway through glutamate and arginine metabolism
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470
471 **Table 1.** Top 4 genes whose mutations result in resistance to cell death in both heat-ramp and
472 acetic acid stress

Accession Number	Gene Name (if applicable)	Function	KEGG Ontology
USA300HOU_1008	*folD*	Methylenetetrahydrofolate dehydrogenase; methenyltetrahydrofolate cyclohydrolase	Metabolism of cofactors and vitamins; One carbon pool by folate
USA300HOU_0997		Bifunctional N-acetylmuramoyl-L-alanine amidase/mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase	Metabolism
USA300HOU_0996	*sspA*	Glutamyl endopeptidase	Cellular community prokaryotes; Quorum sensing; Serine Peptidases of chymotrypsin family
USA300HOU_0232		Iron ABC transporter membrane binding protein	Environmental Information Processing; Membrane transport; ABC transporters; Mineral and organic ion transporters; Iron transporter

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489 **Table 2.** Top 4 genes whose mutations result in sensitivity to cell death in both heat-ramp and
490 acetic acid stress

Accession Number	Gene name (if applicable)	Function	KEGG Ontology
USA300HOU_0329		ABC transporter-ATP binding protein	Protein families: Signaling and cellular processes; Transporters
USA300HOU_2029		Amidohydrolase	Metabolism: Amino acid metabolism; Alanine, aspartate and glutamate metabolism
USA300HOU_2386	*znuA*	Zinc transport system substrate-binding protein	Environmental Information Processing; Membrane transport; Metallic cation, iron-siderophore and vitamin B12 transporters; Zinc transporter
USA300HOU_2366	*gltT, gltP*	Proton glutamate symport protein	Protein families: Signaling and cellular processes; Electrochemical potential-driven transporters

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509 **Supplementary Table 1.** Genes whose mutations resulted in cell death resistance to both heat-
510 ramp stress and acetic acid stress

	Gene name (if applicable)	Function	Accessory Number
Transporters			
	vraF	ABC transporter ATP-binding protein	USA300HOU_0682
	kdpB	Potassium-transporting ATPase subunit B	USA300HOU_2071
		ABC transporter ATP-binding protein	USA300HOU_0152
		Oligopeptide ABC transporter ATP-binding protein	USA300HOU_2455
		ABC transporter ATP-binding protein	USA300HOU_0154
		Iron ABC transporter membrane binding protein	USA300HOU_0232
		PTS system ascorbate-specific transporter subunit IIC	USA300HOU_0350
		ABC transporter ATP-binding protein	USA300HOU_2386
Transcription Regulators			
	mfd	Transcription-repair coupling factor	USA300HOU_0497
		Transcription regulator	USA300HOU_0044
		Transcription regulator	USA300HOU_2004
		GntR family transcription regulator	USA300HOU_1209
Peptidases			
	sspA	Glutamyl endopeptidase	USA300HOU_0996
		Peptidase	USA300HOU_2590
Metabolism			
	folD	Methylenetetrahydrofolate dehydrogenase; Methenyltetrahydrofolate cyclohydrolase	USA300HOU_1008
		Bifunctional N-acetylmuramoyl-L-alanine amidase, Mannosyl-glycoprotein endo-beta-N-acetylglucosaminidase	USA300HOU_0997
		Phytoene dehydrogenase	USA300HOU_2559
Phosphatases & Kinases			
	nsaS	Nisin susceptibility-associated sensor histidine kinase	USA300HOU_2623
		HAD family phosphatase	USA300HOU_0930
Transferases			
		Glycosyltransferase	USA300HOU_0142

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Stress Response			
	hslO	Hsp33-like chaperonin	USA300HOU_0506
Nucleic Acid Synthesis			
	hsdR1	Type I site-specific deoxyribonuclease restriction subunit	USA300HOU_0033
Protein Synthesis			
	rsmB	rRNA (cytosine-5-)-methyltransferase	USA300HOU_1154
		Acetyltransferases [Translation, ribosomal structure and biogenesis]	USA300HOU_2684
Hypothetical Proteins			
			USA300HOU_0868
			USA300HOU_0369
			USA300HOU_0420

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539 **Supplementary Table 2.** Genes whose mutations resulted in more cell death after heat-ramp
540 and acetic acid stress

	Gene name (if applicable)	Function	Accessory Number
Transporters			
	gltT	Proton glutamate symport protein	USA300HOU_2366
	mnhG1	Monovalent cation antiporter subunit G	USA300HOU_0649
		ABC transporter ATP-binding protein	USA300HOU_0329
		ABC transporter ATP-binding protein	USA300HOU_2386
Metabolism			
		Isochorismatase	USA300HOU_0200
		Amidohydrolase	USA300HOU_2029
Transcription			
		Transcription regulator	USA300HOU_0336
Cell wall			
	capA	Capsular polysaccharide biosynthesis protein	USA300HOU_2664
Hypothetical Proteins			
			USA300HOU_1780
			USA300HOU_2496

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