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Density-dependent resistance protects *Legionella pneumophila* from its own antimicrobial metabolite, HGA

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Abstract

To persist in the extracellular state, the bacterial pathogen *Legionella pneumophila* must withstand competition from neighboring bacteria. Here, we find that *L. pneumophila* can antagonize the growth of neighboring *Legionella* species using a secreted inhibitor: HGA (homogentisic acid), the unstable, redox-active precursor molecule to *L. pneumophila*'s brown-black pigment. Unexpectedly, we find that *L. pneumophila* can itself be inhibited by HGA secreted from neighboring, isogenic strains. Our genetic approaches further identify *lpg1681* as a gene that modulates *L. pneumophila* susceptibility to HGA. We find that *L. pneumophila* sensitivity to HGA is density-dependent and cell intrinsic. This resistance is not mediated by the stringent response nor the previously described *Legionella* quorum-sensing pathway. Instead, we find that *L. pneumophila* cells secrete HGA only when they are conditionally HGA-resistant, which allows these bacteria to produce a potentially self-toxic molecule while restricting the opportunity for self-harm. We speculate that established *Legionella* communities may deploy molecules such as HGA as an unusual public good that can protect against invasion by low-density competitors.

Introduction

Inter-bacterial conflict is ubiquitous in nature, particularly in the dense and highly competitive microenvironments of biofilms (Davey and O'toole 2000; Foster and Bell 2012; Ghigo and Rendueles 2015). In these settings, bacteria must battle for space and nutrients while evading antagonism by neighboring cells. One strategy for managing these environments is for bacteria to cooperate with their kin cells, sharing secreted molecules as public goods (Nadell, Drescher, and Foster 2016; Abisado et al. 2018). However, these public goods are vulnerable to exploitation by other species or by 'cheater' bacterial strains that benefit from public goods but do not contribute to their production. For this reason, many bacteria participate in both cooperative and antagonistic behaviors to survive in multispecies biofilms. Bacterial antagonistic factors can range from small molecules to large proteins, delivered directly or by diffusion, and can either act on a broad spectrum of bacterial taxa or narrowly target only a few species. Although narrowly targeted mechanisms may seem to be of less utility than those that enable antagonism against diverse bacterial competitors, targeted strategies can be critical for bacterial success because they tend to mediate competition between closely-related organisms that are most likely to overlap in their requirements for restricted nutrients and niches (Hibbing et al. 2010).

The bacterium *Legionella pneumophila* (*Lp*) naturally inhabits nutrient-poor aquatic environments where it undergoes a bi-phasic lifestyle, alternating between replication in host eukaryotes and residence in multi-species biofilms (Lau and Ashbolt 2009; Declerck et al. 2007; Declerck 2010; Taylor, Ross, and Bentham 2013). If *Lp* undergoes this lifecycle within man-made structures such as cooling towers or showers, the bacterium can become aerosolized and cause outbreaks of a severe, pneumonia-like disease in humans, called Legionnaires' disease (Fraser et al. 1977; McDade et al. 1977; Fields, Benson, and Besser 2002). Because of the serious consequences of *Lp* colonization, the persistence and growth of *Legionella* in aquatic environments has been the subject of numerous studies. These studies have examined replication within protozoan hosts (Rowbotham 1980; Isberg, O'Connor, and Heidtman 2008; Lau and Ashbolt 2009; Hoffmann, Harrison, and Hilbi 2014), survival in water under nutrient

48 stress (Li et al. 2015; Mendis, McBride, and Faucher 2015), and sensitivity to biocides
49 (Kim et al. 2002; Lin, Stout, and Yu 2011). Here, we focus on interbacterial competition
50 as an underappreciated survival challenge for *Lp*.
51
52 *Legionella spp.* are not known to produce any antibiotics, bacteriocins, or other
53 antibacterial toxins. Bioinformatic surveys of *Legionella* genomes have revealed a
54 number of polyketide synthases and other loci that likely produce bioactive metabolites
55 (Johnston et al. 2016; Tobias et al. 2016), but these have not been shown to exhibit any
56 antimicrobial functions. Nevertheless, there are some hints that interbacterial
57 competition is relevant for *Lp* success within biofilms. For example, one study of artificial
58 two-species biofilms found that viable *Lp* were able to persist for over two weeks in the
59 presence of several bacterial species (e.g. *Pseudomonas fluorescens, Klebsiella*
60 *pneumoniae*) but not others (e.g. *Pseudomonas aeruginosa*) (Stewart, Muthye, and
61 Cianciotto 2012). Additionally, *Lp* bacteria are often co-resident with other *Legionella*
62 *spp.* in man-made structures, with some studies showing that *Lp* proliferation is
63 correlated with a decrease in other *Legionella spp.* populations (Wery et al. 2008;
64 Pereira et al. 2017; Declerck et al. 2007). These studies suggest that *Lp* bacteria may
65 compete with other *Legionella spp.* for similar biological niches.
66
67 The most direct evidence for interbacterial competition comes from Stewart et al.,
68 2011, who found that *Lp* could antagonize the growth of neighboring *Legionella spp.* on
69 the same plate (Stewart, Burnside, and Cianciotto 2011). The molecules mediating this
70 competition have not been identified, although previous work suggested a role for *Lp*'s
71 secreted surfactant, a thin liquid film that facilitates the spread of *Lp* across agar plates
72 (Stewart, Rossier, and Cianciotto 2009; Stewart, Burnside, and Cianciotto 2011). Still, it
73 remained unknown if surfactant played a direct or indirect role in inter-*Legionella*
74 inhibition.
75
76 Here, we use unbiased genetic approaches to find that homogentisic acid (HGA)
77 produced by *Lp* inhibits the growth of neighboring *Legionella spp*. We find that HGA
78 production co-occurs with surfactant production, but that these are independent,

separable phenomena. The redox state of HGA appears to be critical for its activity, as oxidized HGA-melanin pigment is inactive. Unexpectedly, we find that *Lp* itself is susceptible to HGA inhibition. We also identify one gene– *lpg1681*– that enhances *Lp* susceptibility to HGA. However, we find that *Lp* cells can be resistant to HGA at high-density, which is also when they secrete large amounts of HGA. This high-density resistance is cell intrinsic and appears to be independent of growth phase, the stringent response or the previously described quorum-sensing pathway in *Legionella*. Based on these findings, we propose that HGA has the potential to play an important role in structuring *Legionella* communities.

Results

L. pneumophila inhibits *L. micdadei* via an unknown, secreted inhibitor

Inspired by previous reports (Stewart, Burnside, and Cianciotto 2011), we investigated how *Legionella pneumophila* (*Lp*) engages in inter-*Legionella* competition. We found that *Lp* inhibited the growth of neighboring *Legionella micdadei* (*Lm*) plated 1 cm away on solid media, suggesting that it produces a secreted inhibitor (Figure 1A). This inhibition was most robust when we plated the *Lp* strain on low-cysteine media 3-4 days prior to plating *Lm*, allowing time for the inhibitory molecule to be produced and spread across the plate. To quantify this inhibition, we recovered *Lm* grown at different distances from *Lp*. After 48h incubation, we found a 10,000-fold difference in growth between *Lm* antagonized by *Lp* versus *Lm* plated outside of the zone of inhibition (Figure 1B).

Previous studies (Stewart, Burnside, and Cianciotto 2011) had proposed that inter-*Legionella* inhibition could be caused by *Lp*'s secreted surfactant, which is produced by *Lp* but not *Lm* (Stewart, Rossier, and Cianciotto 2009). We tested this hypothesis by deleting a surfactant biosynthesis gene, *bbcB*, from the *Lp* genome (Stewart, Burnside, and Cianciotto 2011). The resulting Δ*bbcB* strain did not produce surfactant (Supplemental figure 1B), yet it still inhibited adjacent *Lm* (Figure 1A, Supplemental Figure 1C). When quantified, we observed nearly identical inhibition from both wild type *Lp* and Δ*bbcB Lp* (Figure 1C) indicating that the surfactant did not enhance inhibition levels. Furthermore, we observed that the zone of inhibition

110 surrounding wildtype *Lp* did not always co-occur with the spread of the surfactant front
111 (Supplemental figure 1A). From these results, we conclude that *L. pneumophila* can
112 cause strong growth inhibition of neighboring *Legionella* using an unknown molecule

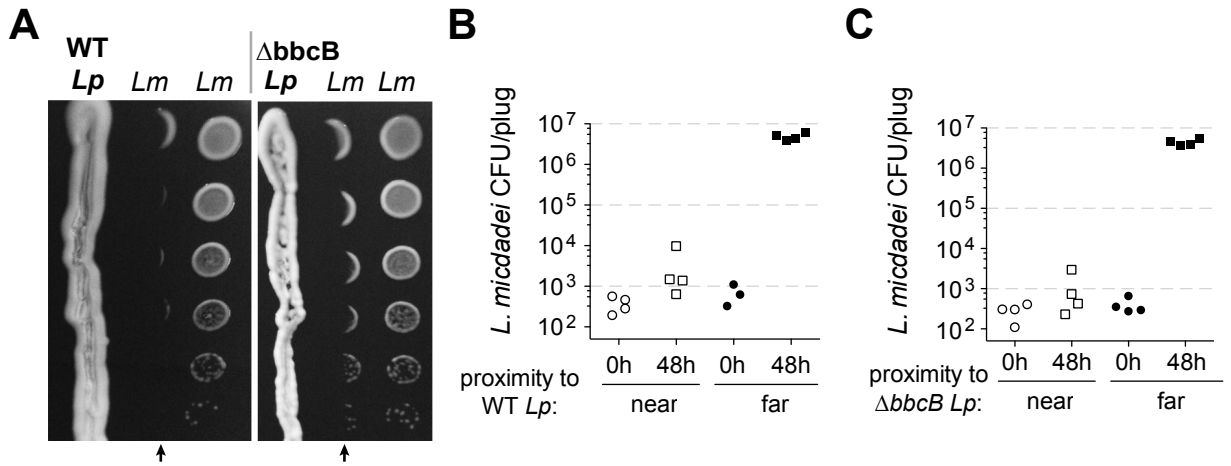

Figure 1: *L. pneumophila (Lp)* produces a secreted inhibitor independent of surfactant. A) When pre-incubated on BCYE charcoal agar plates, *Lp* produces a zone of inhibition, impacting the growth of nearby *L. micdadei (Lm)*. Arrows mark the edge of inhibition fronts. Droplets of *Lm* at different dilutions were added to the plate 3 days after streaking *Lp*. Outside of the zone of inhibition, *Lm* grows in a circle where spotted on the plate, while inhibition either prevents growth completely or results in a crescent of growth away from *Lp*. WT *Lp* (left panel) generates a similar zone of inhibition as a surfactant-null mutant, Δ*bbcB* (right panel). B) Quantification of *Lm* growth within (”near”) or outside of (”far”) the wild type *Lp* zone of inhibition. C) Quantification of *Lm* growth within or outside of the Δ*bbcB Lp* zone of inhibition. In B and C bacteria were sampled and removed from the plate in a “plug” of fixed area before plating for viable CFUs.

113 that is distinct from surfactant.
114

115 **Transposon screen pinpoints HGA-melanin pathway in inter-*Legionella* inhibition**

116 To determine which molecule(s) might be responsible for inter-*Legionella*
117 inhibition, we performed an unbiased genetic screen in *Lp*. We generated *Lp* mutants
118 using a drug-marked Mariner transposon that randomly and efficiently integrates into
119 the *Legionella* genome (O'Connor et al. 2011). To identify mutants that were defective in
120 producing the inhibitor, we transferred each mutant onto a lawn of *L. micdadei* on low-
121 cysteine plates and examined the resulting zone of inhibition surrounding each *Lp*
122 mutant (Figure 2A). After screening 2870 clones, we isolated 19 mutants that produced
123 a smaller zone of inhibition than wild type *Lp*, as well as 5 mutants that showed a
124 complete loss of inhibition (Figure 2B, Supplemental Table 1). We refer to these as
125 “small zone” and “no zone” mutants, respectively. Among the “small zone” mutants,
126 some had defects in surfactant spreading on plates, while others enhanced surfactant

127 spread (Supplemental figure 2A), further distinguishing inter-bacterial inhibition from
128 surfactant secretion.

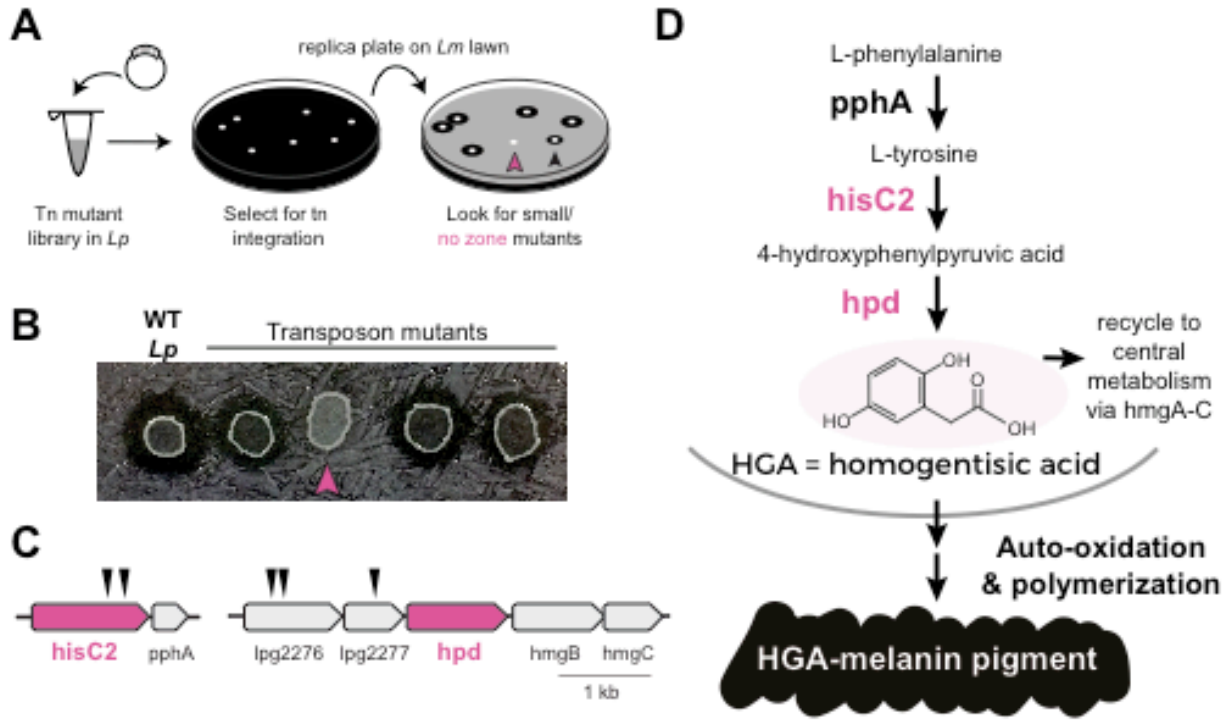

Figure 2: Transposon mutagenesis screen implicates HGA-melanin pathway in production of inhibitor. **A)** Screen for mutant *L. pneumophila* (*Lp*) that do not inhibit *L. micdadei* (*Lm*). Following electroporation of a Mariner-transposon-containing plasmid, *Lp* mutants were selected for transposon integration. Colonies were patched or replica plated onto a lawn of *Lm*. Mutants of interest generated a zone of inhibition that was reduced (black arrowhead) or absent (pink arrowhead) compared to WT *Lp*. **B)** Selected transposon mutants produce abnormal zones of inhibition when grown on a lawn of *Lm*. Pink arrowhead indicates the *hisC2*::Tn "no zone" mutant. **C)** Transposon insertion sites (triangles) identified in the five recovered "no zone" mutants. **D)** HGA-melanin synthesis pathway. HGA is exported from the cell where it auto-oxidizes and polymerizes to form HGA-melanin. Genes indicated in pink were validated by complementation to have essential roles in *Lm* inhibition.

129

130 We focused on the "no zone" mutants, as these had the strongest defects in
131 inhibition. These 5 mutants carried transposon insertions in two separate operons
132 (Figure 2C). The first operon had two insertions in the *hisC2* gene (*lpg1998)*, which
133 breaks down tyrosine as part of the HGA-melanin metabolic pathway (Figure 2D). Its
134 downstream gene, *pphA*, converts phenylalanine to tyrosine in the same pathway. To
135 validate the role of *hisC2* in inhibition, we overexpressed this gene in the *hisC2*
136 transposon mutant background and found that *hisC2* alone was sufficient to
137 complement the mutant phenotype (Supplemental Figure 2B). Having confirmed the
138 role of *hisC2*, we turned to the second operon, where we had recovered transposon
139 insertions in two uncharacterized genes, *lpg2276* and *lpg2277* (Figure 2C). These two

genes lie immediately upstream of *hpd* (*lpg2278*), which is known to act with *hisC2* in the HGA-melanin pathway (Steinert et al. 2001; Gu et al. 1998) (Figure 2D). Because transposon insertions at the beginning of an operon can disrupt the expression of downstream genes via polar effects, we hypothesized that the insertions we recovered in *lpg2276* and *lpg2277* altered inter-*Legionella* inhibition via disruption of *hpd* expression. Indeed, we were able to complement insertions in both genes, which had yielded 'no zone' mutants, by overexpressing *hpd,* despite the fact that *hpd* overexpression caused a growth defect (Supplemental Figure 2B). In conclusion, all five "no zone" isolates had mutations that disrupted the same metabolic pathway involved in the production of HGA-melanin. Consistent with these findings, we observed defects in HGA-melanin pigmentation in all of the "no zone" mutants as well as some of the "small zone" mutants (Supplemental Figure 2E).

The HGA-melanin pathway is found in diverse eukaryotes and bacteria (Nosanchuk and Casadevall 2003; Liu and Nizet 2009) including *L. pneumophila* (Supplemental figure 2D). This pathway produces homogentisic acid (HGA) from the catabolism of phenylalanine or tyrosine (Fang, Yu, and Vickers 1989; Steinert et al. 2001) (Figure 2D). HGA can either be further metabolized and recycled within the cell via HmgA-C, or it can be secreted outside of the cell, where it auto-oxidizes and polymerizes to form a black-brown pigment called HGA-melanin, or pyomelanin (Kotob et al. 1995) (Figure 2D). To test whether intracellular metabolites downstream of HGA are necessary for inhibition, we deleted *hmgA*, the first gene in the pathway that recycles HGA back into central metabolism. We found that the Δ*hmgA* strain produced a zone of inhibition that was similar or slightly larger than wild type (Supplemental Figure 2C). We therefore inferred that synthesis of secreted HGA and/or HGA-melanin, but not its recycling and intracellular processing, is required for *Lp* inhibition of *Lm.*

HGA inhibits the growth of *Legionella micdadei*, but HGA-melanin does not

To our knowledge, the HGA-melanin pathway has not previously been implicated in inter-bacterial competition. To the contrary, prior work has emphasized the beneficial (rather than detrimental) effects of HGA-melanin on *Legionella* growth, by providing

171 improved iron scavenging (Chatfield and Cianciotto 2007) and protection from light
172 (Steinert et al. 1995). We therefore asked whether the active inhibitor produced by the
173 pathway was HGA-
174 melanin, or
175 alternatively a
176 precursor molecule
177 (Figure 3A). We
178 tested the potential
179 inhibitory activity of
180 HGA-melanin
181 pigment from *Lp*
182 conditioned media
183 in multiple
184 experiments;
185 however, we never
186 observed any
187 inhibition of *Lm*. We
188 wished to rule out
189 the possibility that
190 the pigment
191 secreted into rich
192 media was too dilute
193 to be active, or that
194 other nutrients in the
195 media might
196 interfere with
197 inhibition. We,
198 therefore, isolated a
199 crude extract of
200 HGA-melanin from
201 *Lp* conditioned

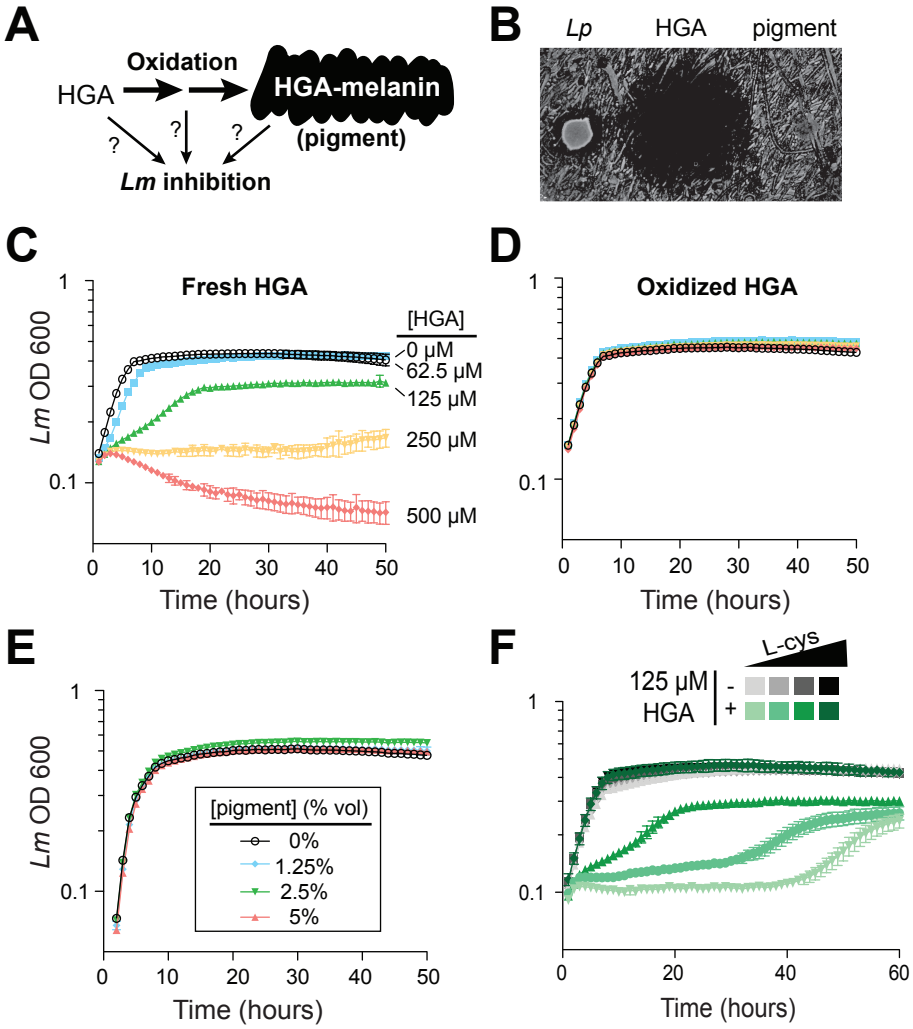

Figure 3: Synthetic HGA inhibits *Legionella micdadei* growth, depending on its oxidation state. **A)** We tested whether inter-bacterial inhibition is caused by HGA, HGA-melanin, or an oxidative intermediate. **B)** Zones of inhibition on a lawn of *Lm* generated from live *Lp* bacteria, synthetic 50mM HGA, or concentrated pigment extract. HGA prevents *Lm* growth in a large region (central dark circle) but pigment does not. **C)** Growth inhibition of *Lm* from increasing concentrations of synthetic HGA in rich AYE media. **D)** Pre-oxidation of synthetic HGA in AYE media for 24h eliminates its inhibitory activity, resulting in normal *Lm* growth. Concentrations of HGA colored as in panel C. **E**) Addition of oxidized HGA-melanin pigment from *L. pneumophila* has little impact on *Lm* growth in AYE liquid media. **F**) In the absence of HGA (gray symbols), titration of L-cysteine (L-cys) from 25%-200% of standard AYE media has little impact on *Lm* growth. However, HGA activity is enhanced in low-cysteine media and decreased in high-cysteine media (green symbols). All error bars show standard deviations among 3-4 replicates.

202 media via acid precipitation (as in (Chatfield and Cianciotto 2007)), washed and
203 concentrated the pigment approximately 10-fold and repeated the assay; the
204 concentrated pigment also showed no inhibitory activity (Figure 3B, 3E).
205
206 The first metabolite secreted by the HGA-melanin pathway is HGA. We tested
207 whether HGA could behave as an inhibitor even though HGA-melanin could not.
208 Indeed, we found that synthetic HGA robustly inhibited *Lm* growth, both when spotted
209 onto a lawn of *Lm* and when titrated into AYE rich media (Figure 3B, 3C), We found that
210 inhibition of *Lm* by HGA is relatively specific at the molecular level; neither 2-
211 hydroxyphenylacetic acid nor 3-hydroxyphenylacetic acid, two HGA-related molecules
212 that differ from HGA by only a single -OH group, were able to inhibit *Lm* growth at any
213 concentration tested (Supplemental figure 3A).
214
215 Because HGA, but not HGA-melanin can inhibit *Lm* growth (Figure 3B, 3E), we
216 inferred that the oxidative state of HGA might be important to its inhibitory activity. HGA
217 is a reactive molecule, which auto-oxidizes (Eslami, Namazian, and Zare 2013) and
218 polymerizes to form HGA-melanin through a series of non-enzymatic steps that are not
219 genetically encoded (Steinert et al. 2001) and are therefore undetectable by our genetic
220 screen. Given its auto-oxidative potential, we next tested whether HGA might cause
221 growth inhibition by oxidizing other nearby molecules, either in the media or on bacterial
222 cells. We allowed synthetic HGA to oxidize completely for 24h in AYE media before
223 adding *Lm* (Supplemental Figure 4D). We found that pre-oxidation completely abolished
224 synthetic HGA activity, even at very high HGA concentrations (Figure 3D, compare to
225 3C). This experiment also ruled out the possibility that HGA acts by causing nutrient
226 depletion or other modifications of the media, since media pre-incubated with HGA for
227 24h is still able to support normal *Lm* growth. Instead, we infer that *Lm* inhibition results
228 from direct interactions between bacterial cells and either HGA itself or unstable,
229 reactive intermediates produced during HGA oxidation.
230
231 Small, reactive, quinone-like molecules similar to HGA are known to react with
232 oxygen to produce H_2O_2, which is broadly toxic to bacteria (Hassan and Fridovich

233 1980). In such cases, extracellular catalase has been shown to protect bacteria against
234 the toxic effects of H_2O_2 (Hassan and Fridovich 1980; Imlay 2013). To test if HGA
235 toxicity occurs via a similar mechanism as H_2O_2, we asked if extracellular catalase was
236 sufficient to protect *Lm* from HGA-mediated toxicity. Even at very high catalase
237 concentrations, we found that catalase provided no protection from HGA (Supplemental
238 Figure 3B), ruling out the production of extracellular H_2O_2 as a potential mechanism of
239 action for HGA-mediated inhibition. We also considered the possibility that HGA as a
240 weak acid could inhibit *Lm* indirectly by altering the local pH, but we observed that
241 adding HGA at 1mM into AYE media or PBS caused little to no change in pH.
242
243 Given that the redox state of HGA is critical for inhibition, we reasoned that it
244 should be possible to modulate HGA activity by altering the redox state of the media
245 using reducing agents. We accomplished this by titrating L-cysteine from 25% to 200%
246 of the levels in standard AYE media. In the absence of HGA, these altered cysteine
247 concentrations had little impact on *Lm* growth (Figure 3F). However, lower cysteine
248 concentrations greatly sensitized *Lm* to HGA, while excess cysteine was completely
249 protective (Figure 3F). These findings may help partially explain why HGA's inhibitory
250 activity on *Legionella* has not been previously detected, as *Legionella* species are
251 typically studied in cysteine-rich media. We found that synthetic HGA is readily able to
252 react with cysteine in vitro (Supplemental Figure 3D) presumably impacting the
253 oxidation state of HGA. Moreover, incubation of HGA with two other reducing agents,
254 DTT (dithiothreitol) or reduced glutathione, similarly quenched HGA's inhibitory activity
255 (Supplemental Figure 3E). From these experiments, we conclude that HGA is less
256 potent in rich media because it reacts with excess cysteine (or other bystander
257 molecules) before it can interact with *Lm*. In sum, these results implicate the reactive
258 activity of HGA and/or its transient, oxidative intermediates in inter-*Legionella* inhibition.
259
260 In these experiments, synthetic HGA was a robust inhibitor of *Lm.* However, this
261 inhibition required relatively high concentrations of HGA (>50μM). We next quantified
262 the amount of HGA secreted by *Lp* to determine if these levels were biologically
263 relevant. Compared to other *Legionella spp.*, *Lp* produces much more pigment

264 (Supplemental Figure 2D), suggesting that it secretes considerably more HGA. To
265 estimate the quantity of secreted HGA, we created a standard curve of synthetic HGA in
266 AYE rich media. We allowed the HGA added to completely oxidize to HGA-melanin,
267 which can be measured by OD 400 readings. In this way, we can use the pigment levels
268 after oxidation as a reliable measure of total HGA that was produced by a given time-
269 point (Supplemental Figure 4D). Using this calibration, we estimated that wild type *Lp*
270 had secreted the equivalent of 1.7mM HGA after 48h of culture, whereas the
271 hyperpigmented Δ*hmgA* strain secreted about 2.6mM HGA. Thus, the levels of HGA
272 produced by *Lp* are considerably higher than the inhibitory concentrations of synthetic
273 HGA used in our assays (50-500 μM), at least under lab conditions. In contrast, we did
274 not detect any pigment from the non-inhibitory *hisC2*::Tn strain (Supplemental figure
275 4E). From these experiments, we conclude that HGA is an abundant, secreted, redox-
276 active metabolite of *Lp*, which can accumulate in concentrations that are relevant for
277 inter-*Legionella* inhibition.
278
279 ***L. pneumophila* can be susceptible to its own inhibitor**
280 Our results so far indicated that HGA can be a potent, redox-active inhibitor of
281 *Lm*, which is volatile and capable of reacting with many types of thiol-containing
282 molecules. If *Lp* uses HGA to compete with neighboring *Legionella spp.*, we anticipated
283 that that *Lp* would have evolved some form of resistance to its own secreted inhibitor.
284 Therefore, we next tested *Lp* susceptibility to inhibition in low-cysteine conditions, as we
285 had previously done for *Lm*. Surprisingly, we found that *Lp* was quite sensitive to
286 inhibition by neighboring *Lp* that was already growing on the plate (Figure 4A). Indeed,
287 *Lp* susceptibility closely mirrored the susceptibility of *Lm* to inhibition (compare to Figure
288 1A), even though the bacterial cells secreting the inhibitor were genetically identical to
289 the inhibited *Lp*. In both cases, we observed a sharp boundary at the edge of the zone
290 of inhibition. In contrast, the “no zone” *Lp* strain *hisC2*::Tn did not generate a sharp zone
291 of inhibition against neighboring *Lp* (Figure 4A), suggesting that the HGA-melanin
292 pathway was responsible for both *Lm* and *Lp* inhibition. Furthermore, we found that
293 synthetic HGA was able to inhibit *Lp* in liquid cultures (Figure 4B) at the same
294 concentrations that were inhibitory to *Lm* (compare Figure 4B and 3C).

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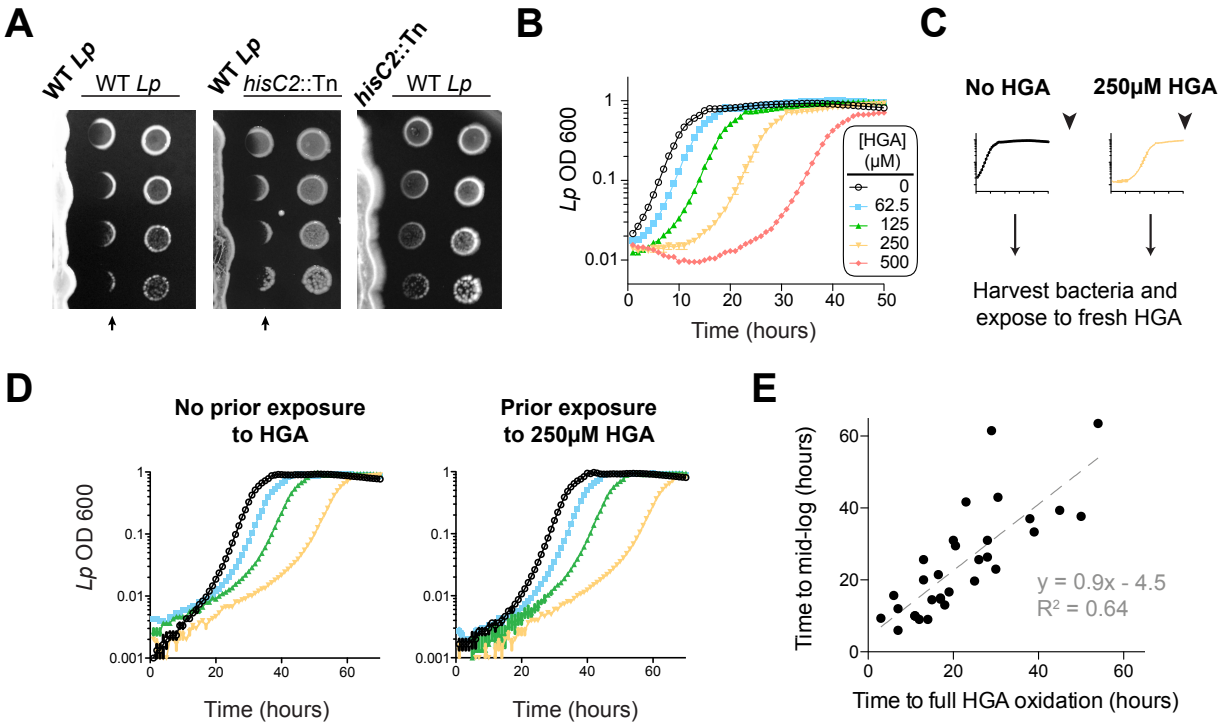

Figure 4: *L. pneumophila* is susceptible inhibition by HGA. **A**) When pre-incubated on agar plates, *Lp* produces a zone of inhibition (arrows), preventing the growth of genetically-identical *Lp* plated 3 days later. The "no zone" mutant *hisC2*::Tn does not produce a sharp front of inhibition, implicating HGA. **B**) Increasing concentrations of synthetic HGA inhibit the growth of *Lp*, causing a growth delay in rich media. Error bars showing standard deviation are small and mostly obscured by the symbols. **C**) To test if Lp population recovery at late time points following HGA exposure is due to the outgrowth of HGA-resistant mutants, we grew *Lp* with or without HGA and sampled bacteria at the end of the experiment (arrowhead) that were unexposed to HGA or were exposed to 250μM HGA. These were used to inoculate media +/- fresh HGA. **D)** Prior HGA exposure did not lead to subsequent resistance. **E)** The time for *Lp* to grow in the presence of HGA is correlated with the time for synthetic HGA to oxidize at each concentration, suggesting that *Lp* delays growth until HGA has sufficiently oxidized to lose inhibitory activity. Plot shows data combined across 8 experiments. Linear fit curve and equation are shown.

296 However, our comparisons between *Lm* and *Lp* revealed one important
297 difference in their response to HGA inhibition. Unlike *Lm,* the *Lp* cultures exposed to
298 HGA exhibited a population rebound after a dose-dependent growth delay, measured
299 by both OD600 and plating for viable CFUs (Figure 4B, Supplemental Figure 4C). This
300 rebound response was shared between the KS79 lab strain and the Philadelphia-1
301 original *Lp* strain (Supplemental Figure 4A). We hypothesized that the *Lp* rebound
302 response following HGA inhibition occurs because of selection and outgrowth of HGA-
303 resistant mutants following exposure. To test this possibility, we collected 'post-rebound'
304 stationary phase *Lp* previously exposed to 250μM HGA and compared their subsequent
305 HGA sensitivity to unexposed *Lp* (Figure 4C-D). We found that both cultures showed
306 nearly identical susceptibility to HGA inhibition, suggesting that *Lp* population rebounds
307 are not driven by genetic adaptation.

308

309 We therefore considered an alternate possibility that *Lp* populations exposed to
310 HGA remain static until HGA levels fall below inhibitory concentrations, reflecting the
311 auto-oxidation and loss of HGA activity over time. This possibility was supported by
312 CFU measurements, which showed that HGA is bacteriostatic but not bacteriocidal
313 against *Lp* during the growth delay (Supplemental Figure 4C). Furthermore, we
314 observed a strong, linear correlation between the time required to fully oxidize a given
315 concentration of HGA and the length of the growth delay induced by *Lp* (Figure 4E).
316 Based on our multiple observations, we favor the parsimonious conclusions that
317 synthetic HGA causes initial, bacteriostatic inhibition of *Lp* until it has been sufficiently
318 oxidized and thereby inactivated, enabling *Lp* growth. We note that the liquid culture
319 assays (Figure 4B) differ from the co-plating assays, in which we did not observe *Lp*
320 rebound (Figure 4A). In the latter case, we presume that bacteria continually secrete
321 fresh HGA to replace the oxidized HGA over time. Thus, our results confirm a surprising
322 role for HGA in both interspecies and intraspecies *Legionella* inhibition.
323

324 **Non-essential gene *lpg1681* sensitizes *L. pneumophila* to HGA**

325 We next investigated the molecular basis of *L. pneumophila* susceptibility and
326 resistance to HGA using bacterial genetics. First, we tested the role of the HmgA-C
327 proteins, which break down intracellular HGA and recycle it back into central
328 metabolism. We hypothesized that HmgA-C proteins might also be able to deactivate
329 extracellular HGA (Rodriguez-Rojas et al. 2009) (Figure 2D). Contrary to this
330 hypothesis, we found that the growth response of the Δ*hmgA* mutant to increasing
331 concentrations of synthetic HGA was nearly identical to that of wild type *Lp*
332 (Supplemental Figure 4B). These results suggest that the intracellular recycling pathway
333 does not play an appreciable role in *Lp* resistance to extracellular HGA.
334

335 Having excluded the obvious candidate pathway for *Lp* resistance to HGA, we
336 pursued an unbiased forward genetics approach. Because HGA is strongly inhibitory to
337 low density bacteria, we performed a selection for spontaneous, HGA-resistant mutants
338 of *Lp* and *Lm* using a high HGA concentration that normally prevents almost all growth

for both species. To prevent HGA from reacting with media components and becoming inactive (as in Figure 3D, 3F), we mixed the bacteria with 1mM HGA in agar overlays poured onto low-cysteine BCYE plates (Figure 5A). After six days, an average of 53 colonies had grown up on each *Lp* plate under HGA selection, whereas only 3-5 colonies grew on *Lm* plates exposed to with HGA. We also found only 3-5 colonies of either *Lm* or *Lp* exposed to low-cysteine plates without HGA. Based on these results, we focused on HGA-selected mutants of *Lp*. We retested the phenotypes of spontaneous mutants on HGA + low-cysteine plates and recovered 29 *Lp* strains that consistently grew better than wild type *Lp* (Figure 5B). Notably, all recovered mutants had a decrease in HGA sensitivity relative to wild type, but none were completely resistant.

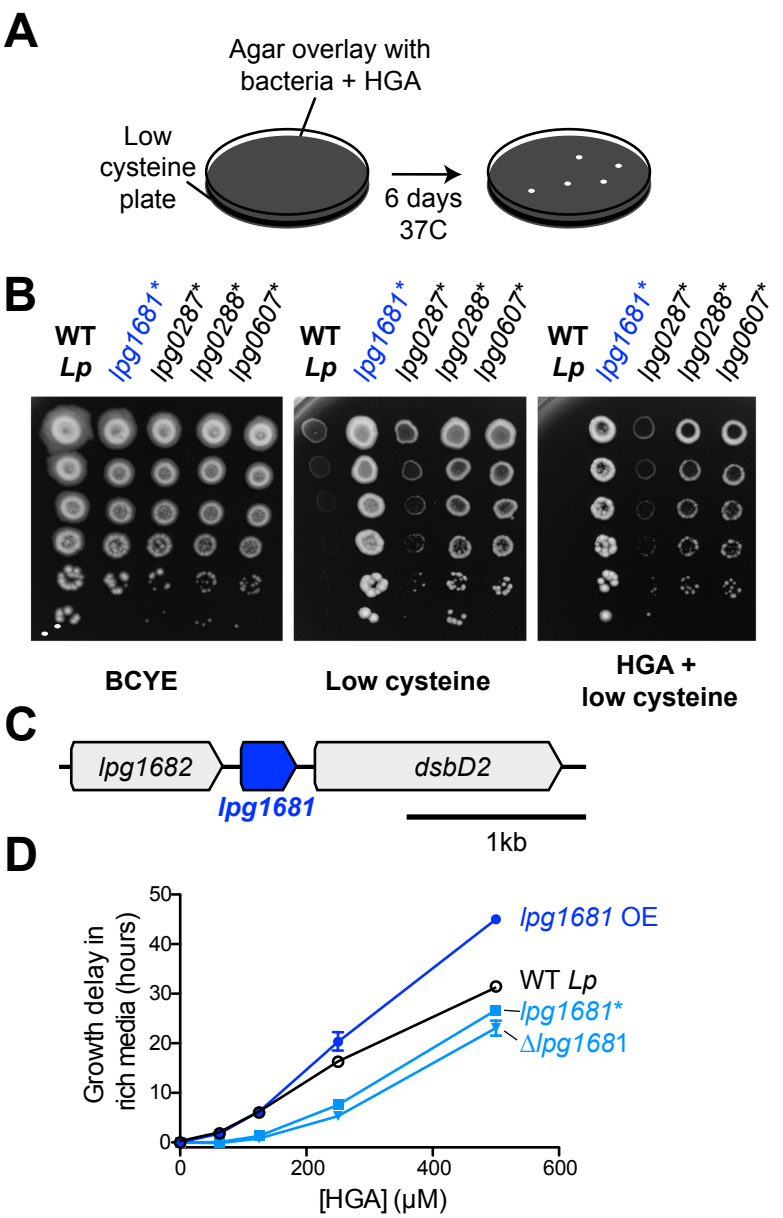

Figure 5: *L. pneumophila* susceptiblity to HGA is modulated by *lpg1681*. **A**) Scheme to select for *Lp* spontaneous HGA-resistant mutants. **B)** Growth of HGA-selected mutants (*) compared to wild type *Lp.* All isolates grew better than wild type in selection conditions (HGA + low cysteine), as well in low cysteine conditions. **C)** Syntenic region of *lpg1681* in *Lp* strains. *lpg1681* is a hypothetical gene that lies downstream of *lpg1682*, a predicted oxidoreductase/dehydrogenase, and upstream of *dsbD2*, a thiol:disulfide interchange protein. **D**) In rich media, a spontaneous *lpg1681* mutant (*lpg1681**) and the *lpg1681* deletion strain (Δ *lpg1681*) are less sensitive to growth inhibition by HGA than wild type *Lp,* as seen by a shorter growth delay at each concentration of HGA. Overexpression of *lpg1681* (OE) heightens sensitivity to HGA (longer growth delay). Graphs here summarize experiments similar to those in Fig 4B. See Supplemental Figure 8 for full data.

370

371 To determine the underlying genetic basis of these phenotypes, we sequenced
372 the genomes of all 29 strains plus the starting, wildtype strain of *Lp* to a median depth of
373 118x and identified mutations genome-wide. Each mutant strain carried 1 to 3 unique
374 point mutations relative to the starting strain, but most of these mutations were found
375 only in a few shared loci (Table 1). The most abundant category of mutants was genes
376 related to translation. 19 of 29 resistant *Lp* strains carried mutations in translation-
377 related machinery, of which 17 carried mutations in elongation factor P (*lpg0287*) or
378 enzymes responsible for adding post-translational modifications to elongation factor P
379 (*lpg0607* and *lpg0288*). Elongation factor P acts to re-start stalled ribosomes at
380 polyproline tracts, and its post-translational modifications are essential for these
381 functions (Yanagisawa et al. 2010; Navarre et al. 2010; Doerfel et al. 2013; Marman,
382 Mey, and Payne 2014). Based on the frequency of polyprolines in the *Lp* proteome,
383 disruptions to elongation factor P function have the potential to impact the expression of
384 about 33% of *Lp* proteins. The HGA resistance phenotypes we observed in these 17 *Lp*
385 mutants could therefore result from either a large-scale shift in gene expression, or from
386 the altered expression of specific susceptibility genes.

387

388 Elongation factor P disruption has also been previously linked to the activation of
389 the stringent response pathway (Nam et al. 2016), which is important for coordinating a
390 variety of stress responses in *Legionella* including oxidative stress (Molofsky and
391 Swanson 2004; Oliva, Sahr, and Buchrieser 2018). We therefore tested if the stringent
392 response pathway is involved in regulating HGA susceptibility or resistance. We
393 assayed HGA susceptibility in mutant *Lp* strains with disruptions to *rpoS*, *letA*, *relA*, and
394 *spoT*, which act early in the stringent response pathway to regulate and/or respond to
395 the levels of the alarmone ppGpp (Supplemental Figure 7C) (Bachman and Swanson
396 2001; Hammer, Tateda, and Swanson 2002; Dalebroux, Edwards, and Swanson 2009).
397 We found that the HGA susceptibility of all these mutants was similar to that of wild
398 type; furthermore, complementation with these genes did not alter HGA susceptibility
399 (Supplemental Figure 7D). Thus, we find no evidence of a role for the stringent
400 response in HGA susceptibility or resistance. Instead, we propose that disruptions to

401 elongation factor P result in pleiotropic translation defects that together lead to HGA
402 resistance via still-unknown mechanisms. In addition to the translation-related mutants,
403 we found 5 missense mutations in *secY* or *secD* (*lpg0349* and *lpg2001*), members of
404 the Sec secretion apparatus that moves polypeptides across the cytosolic membrane; 1
405 mutation in *aceE* pyruvate dehydrogenase (*lpg1504*); and 4 mutations in a hypothetical
406 gene, *lpg1681* (Table 1, Figure 5C). The Sec apparatus is involved in the secretion of
407 many substrates and mutations to this machinery could also lead to extensive
408 pleiotropic defects.
409
410 Instead, we focused on the relatively uncharacterized hypothetical gene *lpg1681*,
411 which encodes a small, 105 amino acid protein with no predicted domains apart from
412 two transmembrane helices. This gene is adjacent in the genome to *lpg1682*, which
413 encodes for a predicted oxidoreductase/dehydrogenase, and *lpg1680*, which encodes
414 for the thiol:disulfide exchange protein DsbD2 (Figure 5C, Supplemental Figure 6).
415 Functional studies of DsbD2 (aka DiSulfide Bond reductase D2) have demonstrated that
416 it interacts with thioredoxin to regulate disulfide bond remodeling in the periplasm (Inaba
417 2009; Kpadeh et al. 2015). If *lpg1681* has a redox function related to its neighboring
418 genes, we expected its syntenic locus to be conserved across bacterial strains and
419 species. Consistent with this prediction, we found that the *lpg1680-1682* locus is
420 present and conserved among over 500 sequenced *Lp* strains currently in NCBI
421 databases. Outside *L. pneumophila*, *lpg1681* is mostly restricted to the *Legionella*
422 genus, present in about half of the currently sequenced species (Burstein et al. 2016)
423 (Supplemental Figure 6). A homolog of *lpg1681* is also found in the draft genome of
424 *Piscirickettsia litoralis*, a gamma proteobacterium and fish pathogen (Wan et al. 2016).
425 In all cases, *lpg1681* resides upstream of *dsbD2*, suggesting a functional link between
426 these proteins (Supplemental Figure 6) and implicating *lpg1681* in a role in redox
427 homeostasis. We, therefore, viewed *lpg1681* as a promising candidate for a gene
428 involved in HGA susceptibility.
429
430 We constructed *lpg1681* overexpression and deletion strains in *Lp* and tested the
431 susceptibility of these strains to HGA. Similar to the spontaneous mutants we

432 recovered, we found that the Δ*lpg1681* strain was more resistant to synthetic HGA in
433 rich media (Figure 5D, Supplemental Figure 5). Conversely, overexpression of *lpg1681*
434 increased *Lp* sensitivity, resulting in longer growth delays than wild type at high
435 concentrations of HGA. We therefore conclude that wild type *lpg1681* expression
436 sensitizes *Lp* to inhibition by extracellular HGA. Given its genetic linkage with *DsbD*, our
437 findings further suggest that alteration of disulfide bond regulation in the periplasm
438 might constitute one means to mitigate HGA susceptibility.
439
440 ***L. pneumophila* susceptibility to HGA is density-dependent**
441 Our unbiased genetic screen for *Lp* resistance to HGA only revealed mutants
442 that were partially resistant to HGA. These mutants had a smaller growth delay than
443 wild type at a given HGA concentration, but all remained qualitatively susceptible to
444 inhibition. These results suggest that the genetic routes for *Lp* to completely escape
445 from HGA-mediated inhibition are limited. Yet, *Lp* secretes abundant HGA into its local
446 environment, despite the fact that HGA secretion is not required for *Lp* growth or
447 metabolism (as seen by the robust growth of the *hisC2::Tn* mutants, Figures 2 and 4).
448 Thus, our findings do not provide an adequate explanation for the paradox of how *Lp*
449 cells can secrete a toxic compound to which they apparently carry no heritable
450 resistance.
451
452 We therefore considered a distinct mechanism by which *Lp* might avoid self-
453 inhibition: *Lp* might produce and secrete HGA only during conditions to when it is not
454 susceptible to HGA. To address this possibility, we investigated when and where *Lp*
455 secretes HGA. We tracked HGA secretion across a growth curve of *Lp* in rich media,
456 using our previously described assay of using a standard curve of synthetic HGA to
457 estimate HGA levels. It has long been known that *Lp* produces abundant HGA-melanin
458 pigment in stationary phase, when the bacteria are undergoing very slow or no growth
459 (Pine et al. 1979; Berg et al. 1985; Wiater, Sadosky, and Shuman 1994). By comparing
460 to a synthetic HGA standard curve (Supplemental Figure 4F), we estimate that *Lp*
461 produces a burst of HGA in stationary phase, secreting over 250μM within 5 hours
462 (Figure 6A). HGA secretion then continues after the population has ceased growing.

463 These quantities of HGA are more than enough to be inhibitory to *Lp* growth (Figure
464 4B).
465
466 For *Lp* to avoid self-inhibition from HGA, we hypothesized that *Lp* might be
467 resistant to this inhibitor at high density and/or when the cells are in a stationary phase
468 of growth. We therefore investigated if cell density or growth phase impacted HGA
469 susceptibility (Figure 6B). For *Lp* exposed to HGA in rich media, we found that the
470 growth phase of bacteria used to inoculate the experiment had little impact on HGA
471 susceptibility (Figure 6C). In contrast, when cells were inoculated at high density
472 (10^9/mL instead of 10^8/mL), they were resistant even to high concentrations of HGA
473 (Figure 6C), suggesting that cell density might be linked to HGA resistance. However,
474 because both cell density and growth phase are changing over time during our assays
475 in rich media, we cannot fully separate their contributions to HGA-resistance in *Lp*. We
476 therefore created a new assay to assess HGA susceptibility. We exposed *Lp* bacteria to
477 HGA at different dilutions in nutrient-free PBS, which ensured that the bacteria did not
478 grow or change cell density during the course of the experiment. After 24 hours
479 exposure to 125μM HGA, we assessed bacterial viability by plating for viable CFUs
480 (Figure 6D). No measurable darkening of the HGA was detected in this assay,
481 suggesting that the oxidation and de-activation of HGA was considerably slowed in low-
482 nutrient conditions. We found that *Lp* bacteria incubated at high density with HGA
483 (above 10^8 CFU/mL) were largely protected from inhibition (Figure 6D). However, at
484 lower density (10^7 CFU/mL), *Lp* bacteria were extremely sensitive to HGA, with at
485 least a 10^6-fold reduction in CFUs, to below our limit of detection. As in the rich media
486 assay, this density-dependent resistance to HGA was not altered by inoculum growth
487 phase (Figure
488 6D).

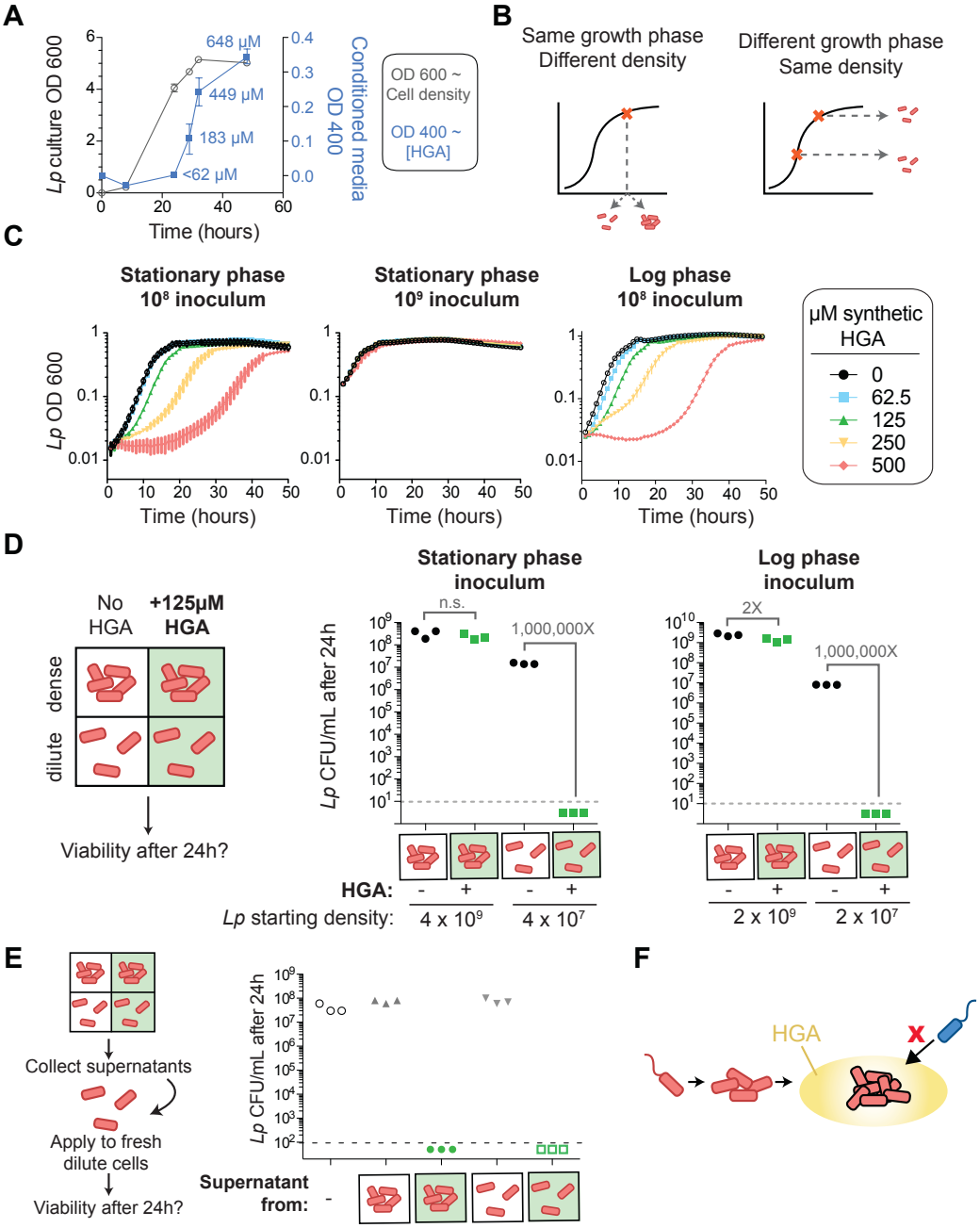

Figure 6: HGA susceptibility in *Lp* is linked to cell density, independent of inoculum growth phase**. A)** Timing of HGA secretion by *Lp* in rich media, measured by OD400 of conditioned media (CM) after allowing for full oxidation (blue boxes, right y-axis). A matched growth curve of *Lp* is presented for comparison (gray circles, left y-axis). Abundant HGA is secreted in during stationary phase. Estimates of secreted HGA concentration (blue) are based on a standard curve of synthetic HGA. Error bars show standard deviations. **B)** Schematic showing how experiments controlled for inoculum density vs. growth phase. To test the impact of cell density, a single culture was diulted to multiple densities at the start of the experiment. To test the impact of growth phase, *Lp* was sampled at multiple stages of growth and diluted to the same CFU/mL at the start of the experiment. **C**) *Lp* growth is inhibited by HGA when cells are inoculated at relatively low density (10^8 CFU/mL), but HGA is ineffective in inhibiting growth when cells are inoculated at high density (10^9 CFU/mL). Growth phase of *Lp* inoculum has little impact on HGA susceptibility. **D**) Viable CFUs following 24h incubation of *Lp* with or without 125µM HGA in nutrient-free PBS. When incubated at high density, bacteria are mostly resistant to HGA, while they are higly sensitive at lower density. Dotted line shows limit of detection. Brackets indicate the fold change in viable CFUs due to HGA exposure **E**) High-density cells do not inactivate extracellular HGA. Supernatants from *Lp* +/- HGA at two densities were collected and applied to dilute cells for 24h. Viable CFUs were counted following supernatant exposure. HGA in all supernatants remained active against low density *Lp*. **F)** Model for HGA activity. *Lp* (orange) colonizes a surface and grows to form a microcolony. Once cells are at high density, they secrete abundant HGA (yellow). Through unknown mechanisms, high density *Lp* are resistant to HGA's effects (black outline), while low density *Lp* or other *Legionella* species (blue) are inhibited by HGA and cannot invade the microcolony's niche.

489

490 We reasoned that the quorum sensing pathway was most likely to control the
491 substantial, density-dependent difference in HGA susceptibility. We therefore
492 investigated if HGA resistance depended on the previously described *Lp* quorum
493 sensing response regulator, *lqsR* (Tiaden et al. 2007). We found that deleting *lqsR* had
494 no detectable impact on HGA susceptibility or resistance (Supplemental figure 7B).
495 Therefore, the density-dependent susceptibility of *Lp* to HGA must be independent of
496 the *lqsR* pathway.
497
498 We next investigated the basis of high-density resistance to HGA by *Lp* bacteria.
499 We considered the hypotheses that high-density cells could alter the activity of
500 extracellular HGA, either through the secretion of inactivating compounds or through
501 bulk, non-specific binding of HGA to bacterial biomass, leading to a reduction in its
502 effective concentration. To test both hypotheses, we recovered the supernatants from
503 high-density and low-density bacteria that had been incubated with or without HGA, and
504 applied these supernatants to fresh, low-density *Lp* to test their HGA activity (Figure
505 6E). We found that the supernatants from HGA-exposed *Lp* remained fully inhibitory,
506 even after 24h incubation with high-density bacteria. Furthermore, we found that adding
507 heat-killed *Lp* bacterial cells to low-density viable *Lp* bacteria did not enhance the
508 latter's resistance to HGA inhibition (Supplemental Figure 7A). Therefore, we conclude
509 that HGA susceptibility appears to be density-dependent and yet cell-intrinsic. Because
510 *Lp* bacteria at high density both secrete and are protected from HGA, this strategy of
511 secreting HGA only when *Lp* cells are conditionally HGA-resistant may allow *Lp* to
512 produce a broadly active inhibitor while restricting the potential for self-harm.
513
514
515 **Discussion**

516 The HGA-melanin pathway is well-studied and widespread among bacteria and
517 eukaryotes. In this study, we identify HGA as a mediator of inter-*Legionella* inhibition,
518 both between *Legionella* species and even between genetically identical populations of
519 *L. pneumophila*. To our knowledge, this is the first time that HGA has been described to
520 have antimicrobial activity. One reason HGA-mediated inhibition may not have been

521 previously documented is that HGA is a redox-active, unstable molecule with transient
522 activity. Our study finds that synthetic HGA can auto-oxidize over the course of an
523 experiment to form inactive HGA-melanin (Figure 3, Supplemental Figure 4D), allowing
524 exposed *Lp* populations to rebound following initial inhibition (Figure 4B). Intriguingly,
525 although *Lp* populations recover upon HGA oxidation, *Lm* populations do not,
526 suggesting that HGA may cause more harm to *Lm* cells. We find that the quenching of
527 HGA's inhibitory activity occurs even more rapidly in the cysteine-rich microbial media
528 typically used to grow *Legionella* in the lab (Supplemental Figure 4D). Conversely, we
529 find that HGA becomes more potent in low-cysteine media or in PBS (Figures 3F, 6D),
530 where oxidation of HGA into HGA-melanin occurs more slowly; such nutrient-poor
531 conditions may better replicate the nutrient-poor (oligotrophic) aquatic environments of
532 *Legionella's* natural habitat (Atlas 1999; Boamah et al. 2017).
533
534 Although dense, stationary-phase cultures of *L. pneumophila* secrete abundant
535 HGA (Figure 6A, Supplemental Figure 2D), we also find that these bacteria do not
536 possess heritable resistance to HGA (Figure 4B-D) and are highly susceptible to HGA
537 inhibition at low cell density. However, they exhibit conditional, density-dependent, cell
538 intrinsic resistance to HGA at high cell density (Figure 6). This lack of heritable
539 resistance makes HGA-mediated inhibition different from classical antibiotics or toxins,
540 which typically are produced by bacteria that also express resistance genes or
541 antitoxins. HGA inhibition is also distinct from that caused by toxic metabolic by-
542 products in two important ways. First, HGA production is not required for efficient growth
543 or metabolism in *Legionella* species (Supplemental Figure 2D, and see growth of
544 *hisC2*::Tn mutant in Figure 4A). Second, high-density populations of *L. pneumophila*
545 that produce HGA are themselves protected from by HGA inhibition (Figure 6). Only
546 neighboring, low-density *Legionella* are strongly inhibited (Figure 4A, 6D). The strong
547 density-dependence of *Legionella*'s susceptibility to HGA may be another reason that it
548 has been previously undiscovered despite intense study of these bacteria.
549
550 HGA-mediated inhibition of *Legionella* is reminiscent of the antimicrobial activities
551 of phenazines, another class of small aromatic molecules such as pyocyanin from

552 *Pseudomonas aeruginosa*. Both types of molecules are redox-active (Hassan and
553 Fridovich 1980), are produced at high cell density (Hassan and Fridovich 1980; Baron,
554 Terranova, and Rowe 1989), are able to chemically react with thiol groups (Cheluvappa
555 et al. 2008; Heine et al. 2016), and result in the production of a colored pigment (Price-
556 Whelan, Dietrich, and Newman 2006). Phenazine inhibitory activity typically comes from
557 redox cycling and the production of reactive oxygen species, including H_2O_2 (Hassan
558 and Fridovich 1980; Cheluvappa et al. 2008). Oddly, HGA-melanin production has
559 previously been implicated both in the production of (Noorian et al. 2017) and protection
560 from (Keith et al. 2007; Orlandi et al. 2015) reactive oxygen species. The catalase
561 experiments presented here have ruled out the production of extracellular H_2O_2 as a
562 possible mechanism explaining HGA inhibition (Supplemental Figure 3B-C). Instead,
563 based on association of *lpg1681* and *DsbD2* with reduced HGA sensitivity (Figure 5),
564 the ability of diverse thiols to quench HGA's activity (Supplemental Figure 3E), and
565 precedents from phenazines (Heine et al. 2016), we propose that HGA (and/or its
566 transient, oxidative intermediates) may be toxic by forming adducts on cysteine residues
567 or otherwise disrupting disulfide bonding. Alternatively, HGA inhibition could occur via
568 the production of other reactive oxygen species, including potentially the generation of
569 intracellular superoxide and/or H_2O_2 (Hassan and Fridovich 1979), which would not be
570 affected by catalase treatment. As both of these mechanisms should be inhibitory to a
571 number of bacterial taxa, it will be interesting to broadly survey HGA susceptibility
572 outside of *Legionella*.
573
574 The density-dependence of *Lp*'s resistance to HGA is unusual and worthy of
575 future study. Because high-density cells do not inactivate or bind up extracellular HGA
576 (Figure 6E) and because heat-killed cells cannot protect live, low-density *Lp* from
577 inhibition (Supplemental Figure 7A), we infer that resistance is cell-intrinsic, resulting
578 from differing physiology and/or gene expression between high- and low-density cells.
579 Two pathways that commonly regulate such defenses include the stringent response
580 pathway, which becomes active under nutrient limitation and stress, and quorum
581 sensing pathways, which become active at high cell density (Bachman and Swanson
582 2001; Hammer, Tateda, and Swanson 2002; Dalebroux, Edwards, and Swanson 2009;

583 Hochstrasser and Hilbi 2017). Although our experiments disrupting these pathways
584 suggest that neither pathway contributes to density-dependent susceptibility or
585 resistance (Supplemental Figure 7B-D), we note that quorum sensing in *Legionella*
586 remains understudied, and such pathways vary considerably across bacterial taxa
587 (Hochstrasser and Hilbi 2017; Miller and Bassler 2001). Future work using unbiased
588 approaches to investigate the regulation of HGA susceptibility may be able to uncover
589 additional density-sensing pathways, possibly including an undescribed mode of
590 quorum sensing in *Legionella*.
591
592 *L. pneumophila* is often co-isolated with other *Legionella* species, which likely
593 compete for similar ecological niches (Wery et al. 2008; Pereira et al. 2017). HGA-
594 mediated inter-*Legionella* inhibition therefore has a strong potential to impact the
595 success of *Lp* in both natural and manmade environments. Because high-density,
596 established *Lp* bacterial communities are largely resistant to HGA inhibition, these
597 communities might use HGA to protect against low-density, invading *Legionella*
598 competitors with little harm to themselves (Figure 6F). In this model, motile *Lp* can
599 disperse, colonize a new surface, and grow into a microcolony using the locally
600 available nutrients. In these early stages, no HGA is produced. After the *Lp* population
601 grows up and crosses a certain cell density threshold, the cells become HGA-resistant
602 through a cell-intrinsic mechanism. When this dense population enters stationary phase,
603 it also begins to secrete abundant HGA into the local environment. This secreted HGA
604 has minimal impact on the resistant, producer cells. However, it can inhibit the growth of
605 nearby, low-density *Legionella*, whether the neighboring cells are other *Legionella*
606 species or even genetically-identical *Lp*. Given these dynamics observed in the lab, we
607 speculate that HGA and other such inhibitors may be deployed as a bacterial niche-
608 protective strategy.
609
610 **Materials and Methods**
611
612 **Bacterial strains and growth conditions** The bacterial strains and plasmids used for
613 this study are listed in Supplemental Table 2. As our wild type *Legionella pneumophila*

614 *(Lp)* strain, we used KS79, which was derived from JR32 and ultimately from isolate
615 Philadelphia-1 (de Felipe et al. 2008; Sadosky, Wiater, and Shuman 1993; Rao,
616 Benhabib, and Ensminger 2013). Compared to JR32, the KS79 strain has a *comR*
617 deletion to enable genetic manipulation (de Felipe et al. 2008). We used *Legionella*
618 *micdadei (Lm)* tatlock as our susceptible strain (Garrity, Brown, and Vickers 1980;
619 Hébert, Steigerwalt, and Brenner 1980). Liquid cultures of *Legionella* were grown
620 shaking in AYE rich liquid media at 37˚C (De Jesús, O'Connor, and Isberg 2013).
621 Unless otherwise indicated, experiments were inoculated with stationary phase
622 *Legionella*, grown from a single colony in AYE for 16-18 hours as described, to a
623 density of 3 - 4 x 10^9 CFU/mL. For experiments with log phase *Lp*, an overnight culture
624 was diluted into fresh AYE at 1:8 ratio (to a density of 4 - 5 x 10^8 CFU/mL) and allowed
625 to grow to a density of 10^9 CFU/mL before setting up the experiment.
626
627 To manipulate the redox state of AYE, we altered the amount of cysteine added to the
628 media from 0.4 g/L in standard AYE to 0.1, 0.2, and 0.8 g/L. On solid media, *Legionella*
629 were grown either on BCYE agar plates either containing the standard cysteine
630 concentration (0.4g/L) or in “low cysteine” conditions (0.05g/L) (Feeley et al. 1979;
631 Solomon and Isberg 2000). *E. coli* strains used for cloning were grown in LB media.
632 Where indicated, antibiotics were used at the following concentrations in solid and liquid
633 media, respectively; chloramphenicol (5 µg/mL and 2.5 µg/mL), kanamycin (40 µg/mL)
634 and ampicillin (50 µg/mL and 25 µg/mL). For counter-selection steps while generating
635 deletion strains, 5% sucrose was added to BCYE plates. For agar overlay experiments,
636 we used 0.7% agar dissolved in water, which was kept liquid at 50˚C before pouring
637 over low cysteine BCYE plates.
638
639 **Gene deletions and complementations** Genomic knockouts in *L. pneumophila* were
640 generated as previously described (Wiater, Sadosky, and Shuman 1994). Briefly, we
641 used an allelic exchange plasmid (pLAW344) harboring chloramphenicol and ampicillin
642 selection cassettes and the counter-selection marker SacB, which confers sensitivity to
643 sucrose. Into this plasmid, we cloned ~1kb regions upstream and downstream of the
644 gene of interest to enable homologous recombination. Following electroporation and

645 selection on chloramphenicol, we used colony PCR to verify insertion of the plasmid into
646 the chromosome, before counter-selection on sucrose media. From the resulting
647 colonies, we performed PCR and Sanger sequencing to verify clean gene deletion. For
648 complementation, the coding region of a candidate gene was cloned into a plasmid
649 (pMMB207c) following a ptac promoter (J. Chen et al. 2004). To induce gene
650 expression, strains carrying pMMB207c-derived plasmids were exposed to 1mM IPTG.
651 All constructs were assembled using Gibson cloning (NEB Catalog #E2621).
652
653 **Inhibition assays on agar plates** To visualize inhibition between neighboring
654 *Legionella* on solid media, a streak of approximately 5 x 10^6 CFU of the inhibitory
655 strain of *Lp* was plated across the center of a low cysteine BCYE plate. After 3 days
656 growth at 37˚C, dilutions of susceptible *Lp* or *Lm* were plated as 10µLspots
657 approximately 1 cm and >2 cm from the central line. Once spots were dry, plates were
658 then incubated for an additional 3 days before scoring for inhibition. This assay was also
659 used to quantify the bactericidal inhibition of *Lm*, with slight modifications. Here, all *Lm*
660 was plated in 20µL spots at 10^6 CFU/mL. The time of plating susceptible *Lm* was
661 treated as t=0. Once spots were dry, plugs were extracted from within the *Lm* spots
662 using the narrow end of a Pasteur pipette. These plugs were transferred into media,
663 vortexed, and plated to quantify CFU. This procedure was repeated after 48h at 37˚C to
664 compare *Lm* viability and growth within ("near", Figure 1B-C) or outside ("far") of the
665 zone of inhibition.
666
667 For inhibition assays on bacterial lawns, we plated 10µL drops of either live *Lp* or
668 chemical compounds on top of a lawn of 5 x 10^7 CFU *Lm* on low cysteine BCYE, and
669 assessed growth of the lawn after 3 days at 37˚C. Synthetic HGA (Sigma: #H0751) was
670 dissolved in water at a concentration of 100 mM and filter sterilized before use. To limit
671 the potential for HGA oxidation prior to use, 100mM aliquots prepared in water were
672 stored frozen at -20C and discarded after 1-2 weeks. HGA-related compounds, 2-
673 hydroxyphenylacetic acid (Sigma: #H49804) and 3-hydroxyphenylacetic acid (Sigma:
674 #H49901), were prepared in the same way. To test the impact of DTT (Sigma: #43819)
675 and glutathione (oxidized: Sigma #G4376 , reduced: Sigma #G6529) on HGA-mediated

676 inhibition, filter-sterilized solutions dissolved in water were mixed in equimolar ratios
677 with HGA, and incubated shaking at room temperature for 1 hour before spotting onto
678 bacterial lawns. HGA-melanin pigment was prepared from *Lp* conditioned media as
679 previously described (Zheng et al. 2013) from KS79, the unpigmented *hisC2*::Tn
680 mutant, and the hyperpigmented Δ*hmgA* mutant. Briefly, conditioned media was
681 collected and sterile filtered from 100 mL cultures of *Lp* in AYE media grown shaking at
682 37˚C for 3 days. The conditioned media was acidified to a pH of 1.5 and transferred to
683 4˚C for 2 hours to precipitate. Precipitated pigment was collected by centrifugation at
684 4000 x g for 15 minutes and then washed with 10 mM HCl. Pelleted pigment was then
685 returned to neutral pH and resuspended in PBS at 10X before testing.
686
687 **Transposon mutagenesis screen** For random transposon insertion mutagenesis, we
688 used a Mariner transposon from the pTO100 plasmid (O'Connor et al. 2011). We
689 electroporated this plasmid into the KS79 strain and allowed cells to recover at 37˚C for
690 5 hours. To select for cells with integrated transposons, cultures were plated on
691 BCYE/Kan/sucrose plates and incubated at 37˚C for 3 days before screening individual
692 mutant colonies.
693
694 To identify transposon mutants with defects in *Lm* inhibition, we transferred each
695 *Lp* mutant onto a low cysteine plate with a lawn of 5 x 10^7 CFU *Lm* and visually
696 screened for those with either small zones of inhibition or no zone of inhibition. This
697 transfer of *Lp* mutants was achieved either by replica plating using sterile Whatman
698 paper (Whatman: #1001150) or by manual transfer with a sterile toothpick. Plates were
699 then incubated at 37˚C for 3 days and scored. All putative mutants underwent clonal re-
700 isolation, were diluted to OD 600 of 0.1, and spotted on fresh *Lm* lawns to retest their
701 phenotypes. To map the sites of transposon integration, we used arbitrary PCR as
702 described in (T. Chen et al. 1999), with primers redesigned to work with the pTO100
703 transposon (Table 3). Briefly, this protocol involved two PCR steps to amplify the DNA
704 flanking the transposon. The first step used low annealing temperatures to allow the
705 arb1 randomized primer to bind many sites in the flanking DNA while the pTO100_F or
706 pTO100_R primer annealed within the transposon, generating multiple products that

707 overlapped the flanking DNA. These products were amplified in the second step PCR
708 using the arb2 and pTO100_Rd2 primers, and we used the pTO100_Rd2 primer for
709 Sanger sequencing. PCR programs and conditions were as in (T. Chen et al. 1999).
710

711 **HGA inhibition assays in AYE rich media** For rich media assays (e.g. Figures 3C-F,
712 4B, 4D), overnight cultures of *Legionella* were diluted to 10^8 CFU/mL in AYE, mixed
713 with synthetic HGA (at 0, 62.5, 125, 250, or 500μM final) or with isolated pigment in 96
714 well plates, and grown shaking at 425 cpm at 37˚C. The cytation 3 imaging reader
715 (BioTek™ CYT3MV) was used to monitor growth by OD 600 measurements. Because
716 oxidized pigment from synthetic HGA is detected at OD 600 as well, each experiment
717 included bacteria-free control wells containing media and each concentration of HGA.
718 To correct OD 600 readings for pigment development, at each time point we subtracted
719 the control well reading from bacterial wells that received the same concentration of
720 synthetic HGA.
721

722 For experiments with HGA "pre-oxidation" (Figure 3D), we diluted HGA in AYE
723 media and incubated this solution shaking at 37˚C for 24 hours in the plate reader
724 before adding *Lm* bacteria. Complete oxidation of HGA during the 24 hours was
725 monitored using OD400 to track the accumulation of HGA-melanin pigment
726 (Supplemental figure 4). To test if extracellular catalase could protect from HGA
727 inhibition (Supplemental figure 3B), we incubated *Lm* at 10^7 CFU/mL with or without
728 125μM HGA and either 0, 1, 10, or 100 U/mL of bovine catalase (Sigma #C30). As a
729 control to ensure that the catalase was active, we incubated the bacteria as above with
730 catalase and 2mM H_2O_2 (Sigma #88597).
731

732 In *Lp*, HGA inhibition in AYE rich media resulted in a growth delay, similar to an
733 extended lag phase (Figure 4B). To determine if this delay was due to genetic
734 adaptation, we sampled *Lp* after 70 hours growth with 250 μM HGA or without HGA
735 (Figure 4C-D). These bacteria were washed once and resuspended in fresh AYE,
736 before being diluted back to 10^8 CFU/mL and then exposed to fresh, synthetic HGA as
737 above. To assess the correlation between HGA oxidation and the length of the *Lp*

738 growth delay (Figure 4E), we pooled data from 8 experiments on different days that
739 measured wild type *Lp* (KS79) exposed to a range of HGA concentrations in AYE. We
740 considered the "Time to HGA saturation" as the length of time required for a given
741 concentration of HGA to stop forming additional HGA-melanin, measured as the time
742 until OD400 readings increased by less than or equal to 0.001 units per hour. The "Time
743 to mid-log" was measured as the time when *Lp* exposed to that concentration of HGA
744 had grown to an OD600 of 0.1. To compare sensitivity to HGA among *Lp* strains, we
745 calculated the lag phase from the growth curve of each well using the GrowthRates
746 program (Hall et al. 2014). We excluded a small number of samples where the growth
747 curve was not well fit (R<0.99), and then for each strain used the difference in lag time
748 between the samples with and without HGA to calculate the growth delay due to HGA
749 (Figures 5C).
750
751 **HGA inhibition assays in PBS** While we were able to manipulate inoculum growth
752 phase and cell density in the AYE assays, during these experiments the bacteria altered
753 their density and growth phase as they grew in rich media. To separate the impacts of
754 cell density and growth phase on HGA susceptibility, we used a complementary assay
755 in which we evaluated *Legionella* viability when exposed to HGA in nutrient-free PBS at
756 different cell densities. This design ensured that the bacteria maintained a constant cell
757 density throughout the course of the experiment. Stationary phase cultures were
758 washed once and re-suspended in PBS. We diluted these bacteria to estimated starting
759 concentrations of 10^9, 10^8, and 10^7 CFU/mL and plated for CFU at t=0. We
760 distributed the remaining bacteria into 96 well plates with or without 125 μM HGA.
761 Plates were incubated shaking in a plate reader at 425 cpm at 37˚C for 24 hours before
762 plating to quantify CFU on BCYE plates. CFU were counted after 3-4 days growth at
763 37˚C.
764
765 To determine if high density bacteria were protected via mass action effects that
766 diluted out the amount of HGA per cell through binding of bulk material, we asked if the
767 addition of dense, heat-killed bacteria could protect low-density *Lp* from HGA
768 (Supplemental figure 7A). To prepare high-density heat-killed bacteria, an overnight

769 culture of *Lp* was washed once in PBS, resuspended to 2 x 10^9 CFU/mL, and
770 incubated at 100-110 ˚C for 60 minutes. After heating, this sample was diluted 1:2 and
771 mixed with 10^7 CFU/mL live *Lp* in PBS +/- 125μM HGA to assess protection. As a
772 control, 10^9 and 10^7 CFU/mL live *Lp* with or without HGA were tested simultaneously.
773 Cells were incubated and plated as above to assess viability. To determine if high
774 density bacteria were protected via HGA degradation, we tested if the supernatants
775 from HGA-exposed, high-density bacteria retained the potency to inhibit low-density
776 bacteria (Figure 6E). To generate supernatants, we set up 2mL samples containing
777 10^8 or 10^7 CFU/mL of *Lp* in PBS +/- 125μM HGA and incubated them shaking at
778 37˚C for 20 hours. After plating aliquots for viable CFU, we pelleted the remaining
779 bacteria and sterile filtered 1mL of each supernatant through a 0.2μm filter. Each
780 supernatant was tested in triplicate, incubated with fresh *Lp* at 10^7 CFU/mL in a 96
781 well plate as above for 24 hours before plating for CFU. As a control, 10^7 CFU/mL live
782 *Lp* were incubated in PBS alone.
783
784 **Quantification of HGA's ability to react with cysteine** HGA is known to be a redox-
785 active molecule, with a redox potential of +0.636V (Eslami, Namazian, and Zare 2013).
786 As this measurement can be altered by pH and temperature, we assessed the ability for
787 HGA to oxidize cysteine in our experimental conditions using Ellman's reagent (also
788 known as 5,5'-dithiobis-(2-nitrobenzoic acid), DTNB, Invitrogen #D8451). Ellman's
789 reagent reacts in the presence of reduced thiol groups on L-cysteine to form a yellow
790 color, which can be read as 412nm absorbance. When thiol groups are oxidized, the
791 Ellman's reagent is colorless. We used the ability for HGA to decrease the amount of
792 reduced cysteine as a proxy for its oxidizing ability. Stock solutions of both 100mM HGA
793 and 1.5mM L-cysteine (Sigma #C6852) were prepared fresh in PBS at the start of the
794 experiment. Different concentrations of HGA (from 125μM to 8mM) were incubated in
795 triplicate, shaking at 25ºC with 1.5mM cysteine in PBS for 16 hours. These conditions
796 were compared to a standard curve of cysteine from 0-2mM, which were incubated in
797 parallel with the experimental samples to account for cysteine oxidation over time. To
798 quantify the remaining free thiol groups, 180uL of 0.08 mg/mL Ellman's reagent was
799 mixed with 17.65uL of each experimental or standard sample in a 96 well plate,

800 incubated for 3 hours at 25ºC, and read for 412nm absorbance. The “decrease in
801 reduced cysteine” was calculated as the difference between the initial and final
802 measured cysteine concentrations, based on the standard curve conversion.
803
804 **Estimation of amount of HGA secreted by *Lp*** HGA-melanin is a black-brown pigment
805 that is easily detected at OD 400. We took advantage of this coloration to estimate the
806 amount of HGA that had been secreted by *Lp* by comparing the color of conditioned
807 media to a standard curve of oxidized synthetic HGA. To isolate conditioned media from
808 pigment mutant strains, cultures of KS79, Δ*hmgA*, and HisC::Tn were inoculated with
809 fresh colonies from a BCYE plate into 5 mL AYE and were grown shaking at 37˚C for 48
810 hours. We then collected conditioned media by pelleting the bacteria and passing the
811 supernatant through a 0.2μm filter. To harvest conditioned media for a time course,
812 cultures of *Lp* were inoculated into 5 ml AYE and grown shaking at 37˚C. After 15, 20,
813 24, 39, 44, and 48 hours, we measured the OD 600 of the culture and collected
814 conditioned media. To create a standard curve, we diluted synthetic HGA into AYE at
815 the following concentrations: 62.5 μM, 125 μM, 250 μM, 500 μM, and 1 mM. The
816 conditioned media and standard curve samples were incubated in a 96 well plate in a
817 plate reader shaking at 37˚C for 24 hours to allow the HGA to oxidize. We used OD 400
818 data from the 24 hour time point to generate a standard curve for each HGA
819 concentration and calculated a line of best fit using linear regression. This equation was
820 used to estimate the amount of secreted HGA that corresponded to the OD 400 of each
821 conditioned media sample. (Supplemental Figure 4).
822

823 **HGA-resistant mutants** Because the inhibitory activity of HGA is quenched through
824 interactions with cysteine in rich media (e.g. Figure 3F), it was not possible to select for
825 HGA-resistant mutants by mixing HGA into BCYE agar. Instead, to reduce the potential
826 for HGA to react with media components while allowing sufficient access to nutrients for
827 mutant cells to grow, we selected for HGA-resistant mutants by mixing 4 x 10^7 CFU
828 *Legionella* with 1mM HGA in 4mL of 0.7% molten agar and pouring this solution as an
829 overlay on a low cysteine BCYE plate. Plates were incubated at 37˚C for 6 days, before
830 candidate resistant colonies were picked and clonally isolated. The HGA resistance and

831 growth of each isolate was re-tested on overlays with or without 1mM HGA on both
832 regular and low cysteine BCYE.
833
834 Twenty-nine isolates were more resistant to HGA than wild type *Lp* upon
835 retesting. We sequenced and analyzed genomic DNA from these isolates and a
836 matched wild type strain as follows. DNA was prepared from each strain using a
837 Purelink genomic DNA mini kit (Invitrogen, #K1820). DNA concentrations were
838 quantified using Qubit and normalized to 0.5 ng/uL. Barcoded libraries were prepared
839 using tagmentation according to Baym et al. 2015 (Baym et al. 2015), analyzed with
840 QuantIT DNA quantification, pooled, and sequenced with 50 bp paired-end reads on an
841 Illumina HiSeq 2500 machine. Reads were trimmed for quality and to remove Nextera
842 indices with Trimmomatic (Bolger, Lohse, and Usadel 2014) and mapped to the
843 Philadelphia-1 genome (Chien et al. 2004) using Bowtie2 with default parameters
844 (Langmead et al. 2009). Coverage plots were generated for each strain using
845 bamcoverage (Ramírez et al. 2016) and manually examined for evidence of large
846 genomic deletions and amplifications. None were observed, apart from a prophage that
847 was present in the reference genome but missing from all sequenced strains, including
848 our wild type KS79 strain. Variants were detected for each mutant using Naive Variant
849 Caller (Blankenberg D, et al. In preparation). Those variants that were detected in
850 mutant strains but not the wild type strain were considered as putative causative
851 mutations. For each of these mutations, we inspected the mapped reads and excluded
852 faulty variant calls that either were adjacent to coverage gaps or that did not appear to
853 be fixed in the clonal mutant and/or wild type sequences, likely due to errors in read
854 mapping. After this manual filtering, 1-3 well-supported mutations remained for each
855 mutant genome. Nine of the mutants were isolated on a different day from the other
856 mutants: in addition to various unshared mutations, these nine strains each carried
857 exactly the same missense mutation in *rplX*, which we disregarded as a background
858 mutation that likely arose before selection. Following this exclusion, each mutant carried
859 only a single well-supported mutation in a coding region. Most often this coding
860 mutation was the only mutation we detected, although one mutant carried two additional
861 intergenic point mutations. The coding mutations were point mutations or small

862 deletions that resulted in non-synonymous changes, frame shifts, or gene extensions.
863 Across different mutants, the mutations we uncovered were repeatedly found in the
864 same, few loci (Table 1).
865
866 **Evolution of *lpg1681*** The genes in the HGA-melanin synthesis pathway are highly
867 conserved in diverse bacteria and across the *Legionella* genus, with all genes present in
868 all 41 currently sequenced *Legionella spp.* genomes (Burstein et al. 2016). In contrast,
869 we were able to identify *lpg1681* in only 30 *Legionella spp.* genomes, as well as a single
870 draft genome outside the *Legionella* genus– *Piscirickettsia litoralis*, an intracellular fish
871 pathogen (Wan et al. 2016). Across the *lpg1681*-containing genomes, there is evidence
872 for extensive recombination of the flanking loci, yet *lpg1681* is always found upstream of
873 *dsbD2*. We identified most of these homologs of *lpg1681* using a jackhmmr search
874 (Finn et al. 2015), followed by cross-referencing the homologs with the *Legionella*
875 orthology groups defined by Burstein et al., 2016. From this starting set, additional
876 *lpg1681* orthologs were identified in unannotated, intergenic regions by searching for
877 >200bp open reading frames upstream of *dsbD* orthologs, and confirming the homology
878 of these regions using MAFFT alignments (Katoh et al. 2002). Through this method, we
879 located *lpg1681* in all currently sequenced *Legionella* genomes that contain an
880 annotated *dsbD2* gene, with the exception of *L. shakespearei.* We categorized the
881 *lpg1681*-containing loci into those with similar synteny, based on the orthology group
882 annotations in Burstein et al. 2016. We colored and provided names for the neighboring
883 genes in Supplemental figure 6 if they had a homolog in the *L. pneumophila*
884 Philadelphia-1 genome that was not annotated as a hypothetical gene. To assess the
885 conservation of the *lpg1680-lpg1682* among *L. pneumophila* strains, we used blastn in
886 the NCBI nr and wgs databases with the full *lpg1680-lpg1682* genomic DNA sequence
887 as a query. We found that the full region was conserved with few mutations across 501
888 currently sequenced *L. pneumophila* strains.

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Supplemental Figure and Table legends

Supplemental Figure 1. Separation of surfactant and antimicrobial phenotypes.
A) *Lm* inhibition sometimes co-occurs with contact with WT *Lp's* secreted surfactant (left), but sometimes is observable outside of the surfactant front (right). The leading edge of the surfactant front (arrowheads) is faintly visible when light is reflected off adjacent regions of the

plate ("light"). Each image is a composite of two fields of view, with inverted colors to enable visualization of the surfactant front. B) Lack of surfactant production from *Lp* with a deletion of *bbcB*, evident by a defect in spreading on BCYE plates. C) The *bbcB* mutant can inhibit the growth of neighboring *Lm*, despite lacking surfactant secretion.

Supplemental figure 2. Genetic validation linking inhibition-defective mutants to the HGA-melanin pathway. A) Inter-bacterial inhibition does not correlate with surface spreading. For example, two mutants with transposon insertions in *lpg1874* (general secretion system protein L) and *lpg1875* (general secretion system protein M) share "small zone" inhibition phenotypes, yet show opposite spreading phenotypes on BCYE. B) Overexpression of either *hisC2* or *hpd* from a plasmid was sufficient to complement the "no zone" phenotype in all recovered mutants. B) Unlike mutations to *hisC2*, deletion of the *hmgA* gene does not disrupt *Lm* inhibition, showing that the intracellular recycling of HGA is not required for inhibitor production. Colors in images from B and C were inverted to facilitate visualization of the zone of inhibition. D) Pigmentation of AYE media following 48 hours growth of various *Legionella* species. While multiple species produce some pigment, many are less pigmented than *L. pneumophila*. The *L. micdadei* susceptible strain does not secrete detectable pigment. E) After 48 hours growth in AYE, none of the "no zone" mutants (represented here by *hisC2*::Tn) produce pigment. A subset of "small zone" mutants also have pigmentation defects (e.g. *lpg2872*::Tn and *lpg1875*::Tn), further implicating the HGA-melanin pathway in inhibition.

Supplemental Figure 3. Impacts of chemical compounds on HGA-mediated inhibition of *Legionella*. A) HGA inhibits *Lm* growth but HGA-related compounds do not. Chemical names and structures are shown above the corresponding plates. On each plate, different concentrations of each compound were spotted onto a lawn of *Lm* in 10μL droplets, arranged as indicated at the right. B) Addition of catalase does not rescue *Lm* susceptibility to 250μM HGA. C) Control experiment showing catalase is active, based on its ability to protect *Lp* from H_2O_2. D) Quantification of HGA's ability to oxidize cysteine. Data points are in purple, with a linear line of best fit. A 1:1 line is shown in gray, for reference. E) The potency of HGA is decreased when pre-incubated with reducing agents. 100mM HGA was mixed with dithiothreitol (DTT), oxidized glutathione (Ox. Glut), or reduced glutathione (R. Glut) for 15 minutes prior to spotting 10μL onto a lawn of *Lm* and allowing to grow for 3 days. Key below each image indicates where each solution was added.

Supplemental Figure 4. HGA-induced growth delays and quantification of HGA production. A-B) Growth curves of *Lp* strains upon exposure to HGA. For each experiment, we provide a matched wild type (KS79) control for comparison. A) Clinical isolate Philadelphia-1 and lab strain KS79 exhibited nearly identical growth curves in the presence of HGA. B) The Δ*hmgA* deletion strain responded to HGA similarly to wild type KS79, showing that HmgA-C do not play a significant role in HGA susceptibility. C) Viable CFU counts of KS79 *Lp* exposed to HGA. Even at high HGA concentrations, in rich media HGA is bacteriostatic at early time points, followed by population recovery. D-F) Using standard curves of synthetic HGA to estimate the amount of HGA secreted by *Lp*. D) OD400 was used to track the oxidation of synthetic HGA in AYE media. The 24 hour timepoint (arrow) was used for the standard curve and all experiments estimating HGA concentrations, as the HGA had completed oxidation by this point. E) Standard curve showing OD400 of oxidized synthetic HGA used to estimate the amount of HGA secreted into *Lp* conditioned media after 48 hours growth. The equation for the linear regression is shown along with a table with the OD400 readings and estimated HGA concentrations for each sample. F) Similar standard curve used for time course experiment of HGA secretion in wild type *Lp* in Figure 6. Standard curves were generated for each experiment independently, in parallel with experimental samples.

Supplemental Figure 5. Growth curves of *lpg1681 Lp* strains upon exposure to HGA. For each experiment we provide a matched wild type (KS79) control for comparison. The spontaneous *lpg1681* mutant and the deletion mutant both had less severe growth delays from HGA than wild type. Conversely, the *lpg1681* overexpression strain was sensitized to HGA, with longer growth delays than wild type. Dashed gray line shows time for the 250 μM HGA condition to reach an OD of 0.1, to facilitate comparisons.

Supplemental Figure 6. Evolution of genes in the *lpg1681* locus among A) *Legionella* species and B) the fish pathogen *Piscirickettsia*. In the species that carry hypothetical gene *lpg1681*, it always resides upstream of the thiol:disulfide interchange gene *dsbD2*, despite extensive turnover of neighboring genes. Species separated by a "/" have similar syntenic loci. Annotated genes are colored, with redox-related genes in shades of blue. Hypothetical genes are white. Gray shading indicates gene homology among the species.

Supplemental Figure 7. Density-dependent HGA resistance acts independently of bulk cellular material, the *lqsR* quorum sensing pathway, and the stringent response pathway. A) The presence of dense heat-killed bacteria is not sufficient to protect dilute, live *Lp* cells from HGA inhibition. B) The susceptibility of wild type *Lp* (KS79) is nearly identical to that of *Lp* with a deletion of *lqsR*, the proposed quorum sensing response regulator in *L. pneumophila*. C) Schematic illustrating part of the stringent response pathway in *L. pneumophila*. In low nutrient conditions, RelA and SpoT generate the alarmone ppGpp, which activates a variety of downstream stress responses via the LetA/LetS two component system and the RpoS sigma factor. D) *L. pneumophila* stringent response mutants show similar, density-dependent susceptibility to HGA as wild type. Complementation of *relAspoT, rpoS,* and *letA* mutants by plasmid expression (with pSpoT, pRpoS, and pLetA respectively) similar had little effect on HGA inhibition.

Supplemental table 1: Transposon mutants of *L. pneumophila* with defects in *L. micdadei* inhibition. *The transposon insertions were mapped to a window that overlapped with both genes noted.

Supplemental table 2: Strains and plasmids

Supplemental table 3: Primers used for cloning