L. pneumophila deploys a self-active inhibitor for inter-*Legionella* competition

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Abstract

The bacterial pathogen *Legionella pneumophila* alternates between intracellular replication within host eukaryotes and extracellular residence in multi-species biofilms. To persist in the extracellular state, *L. pneumophila* must withstand competition from neighboring bacteria, including other *Legionella*. Here, we find that *L. pneumophila* can exclude other *Legionella* species from its local environment 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* is itself inhibited by HGA secreted from neighboring isogenic strains. This HGA sensitivity is density-dependent and HGA secretion is linked to growth phase, suggesting that production of – and resistance to – this inhibitor are functionally linked. Our genetic approaches further identify *lpg1681* as a gene that modulates *Legionella* susceptibility to HGA. We propose that HGA behaves as an unusual public good, allowing established *Legionella* communities to locally 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 & O'toole, 2000; Foster & Bell, 2012; Ghigo & 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 (Abisado, Benomar, Klaus, Dandekar, & Chandler, 2018; Nadell, Drescher, & Foster, 2016). 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, Fuqua, Parsek, & Peterson, 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 (Declerck, 2010; Declerck, Behets, van Hoef, & Ollevier, 2007; Lau & Ashbolt, 2009; Taylor, Ross, & Bentham, 2013). If *Lp* undergoes this lifecycle within manmade 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 (Fields, Benson, & Besser, 2002; Fraser et al., 1977; McDade et al., 1977). 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 (Hoffmann, Harrison, & Hilbi, 2014; Isberg,

O'Connor, & Heidtman, 2008; Lau & Ashbolt, 2009; Rowbotham, 1980), survival in water under nutrient stress (L. Li, Mendis, Trigui, & Faucher, 2015; Mendis, McBride, & Faucher, 2015), and sensitivity to biocides (Kim, Anderson, Mueller, Gaines, & Kendall, 2002; Lin, Stout, & Yu, 2011). Here, we focus on interbacterial competition as an underappreciated survival challenge for *Lp*.

Legionella spp. are not known to produce any antibiotics, bacteriocins, or other antibacterial toxins. Bioinformatic surveys of *Legionella* genomes have revealed a number of polyketide synthases and other loci that likely produce bioactive metabolites (Johnston, Plumb, Li, Grinstein, & Magarvey, 2016; Tobias et al., 2016), but these have not been shown to exhibit any antimicrobial functions. Nevertheless, there are some hints that interbacterial competition is relevant for *Lp* success within biofilms. For example, one study of artificial two-species biofilms found that viable *Lp* were able to persist for over two weeks in the presence of several bacterial species (e.g. *Pseudomonas fluorescens, Klebsiella pneumoniae*) but not others (e.g. *Pseudomonas aeruginosa*) (Stewart, Muthye, & Cianciotto, 2012). Additionally, *Lp* bacteria are often co-resident with other *Legionella spp.* in man-made structures, with some studies showing that *Lp* proliferation is correlated with a decrease in other *Legionella spp.* populations (Declerck et al., 2007; Pereira, Peplies, Höfle, & Brettar, 2017; Wery et al., 2008). These studies suggest that *Lp* bacteria may compete with other *Legionella spp.* for similar biological niches. The most direct evidence for interbacterial competition comes from Stewart et al., 2011, who found that *Lp* could antagonize the growth of neighboring *Legionella spp.* on the same plate (Stewart, Burnside, & Cianciotto, 2011). This antagonism was apparently specific to *Legionella*, as other bacterial species were unaffected. The molecules mediating this competition have not been identified, although previous work suggested a role for *Lp*'s secreted surfactant, a thin liquid film that facilitates the spread of *Lp* across agar plates (Stewart et al., 2011; Stewart, Rossier, & Cianciotto, 2009). Still, it remained unknown if surfactant played a direct or indirect role in inter-*Legionella* inhibition.

77 Here, we use unbiased genetic approaches to find that homogentisic acid (HGA)
78 produced by *Lp* inhibits the growth of neighboring *Legionella spp*. We find that HGA
79 production co-occurs with surfactant production, but that these are independent,
80 separable phenomena. The redox state of HGA appears to be critical for its activity, as
81 oxidized HGA-melanin pigment is inactive. Unexpectedly, we find that although *Lp* in
82 stationary phase secretes large quantities of this metabolite, low density *Lp* are highly
83 susceptible to HGA-mediated inhibition. We also identify one gene– *lpg1681*– that
84 enhances *Lp* susceptibility to HGA. Based on these findings, we propose that HGA
85 behaves as an unusual public good that may play an important role in structuring
86 *Legionella* communities.
87

88 **Results**

89

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

91 Inspired by previous reports (Stewart et al., 2011), we investigated how
92 *Legionella pneumophila* (*Lp*) engages in inter-*Legionella* competition. *Lp* inhibited the

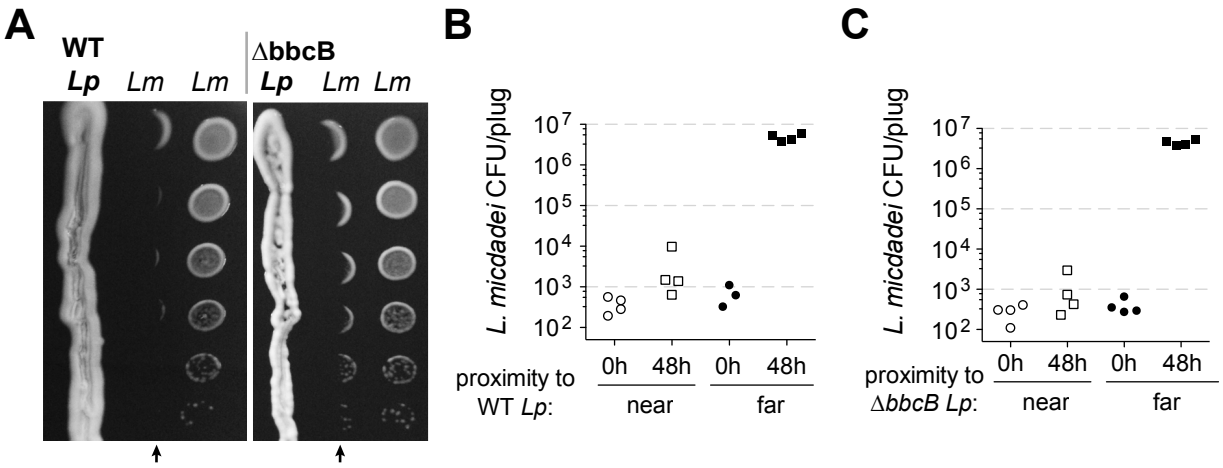

Figure 1: *L. pneumophila (Lp)* produces a secreted inhibitor independent of surfactant. A) When pre-incubated on BCYE charcoal agar plates, *Lp* produces a zone of inhibition, impacting the growth of nearby *L. micdadei (Lm)*. Arrows mark the edge of inhibition fronts. Droplets of *Lm* at different 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 (”near”) or outside of the Δ*bbcB Lp* zone of inhibition (”far”).

93 growth of neighboring *Legionella micdadei* (*Lm*) plated 1 cm away on solid media,
94 potentially via a secreted inhibitor (Figure 1A). We empirically found that this inhibition
95 was most robust when we plated the *Lp* strain on low-cysteine media 3-4 days prior to
96 plating *Lm*, allowing time for the inhibitory molecule to be produced and spread across
97 the plate. Previous work (Stewart et al., 2011) proposed that inter-*Legionella* inhibition
98 could be caused by *Lp*'s secreted surfactant, which is produced by *Lp* but not *Lm*
99 (Stewart et al., 2009). However, we observed that the zone of inhibition surrounding *Lp*
100 did not always co-occur with the spread of the surfactant front (Supplemental figure 1A),
101 suggesting that *Lp* may secrete a separate, undescribed inhibitor. To test this
102 hypothesis, we deleted a surfactant biosynthesis gene, *bbcB*, from the *Lp* genome
103 (Stewart et al., 2011). The resulting Δ*bbcB* strain did not produce surfactant
104 (Supplemental figure 1B), yet it still inhibited adjacent *Lm*, demonstrating that surfactant
105 production is not required for inter-*Legionella* inhibition (Figure 1A, Supplemental Figure
106 1C). To quantify this inhibition, we recovered *Lm* grown at different distances from *Lp*.
107 After 48h incubation, we found a 10,000-fold difference in growth between *Lm*
108 antagonized by *Lp* and those plated outside of the zone of inhibition (Figure 1B). We
109 next asked if surfactant enhanced inhibition by quantifying inhibition from the Δ*bbcB Lp*
110 strain on *Lm*. Again, surfactant production had little impact and we observed nearly
111 identical inhibition from both wild type *Lp* and Δ*bbcB Lp* (Figure 1C). We therefore
112 conclude that *L. pneumophila* can cause strong growth inhibition of neighboring
113 *Legionella* using an unknown molecule that is distinct from surfactant.
114
115 **Transposon screen pinpoints HGA-melanin pathway in inter-*Legionella* inhibition**
116
117 To determine which molecule(s) might be responsible for inter-*Legionella*
118 inhibition, we performed an unbiased genetic screen in *Lp*. Briefly, we generated *Lp*
119 mutants using a drug-marked Mariner transposon that randomly and efficiently
120 integrates into the *Legionella* genome (O'Connor, Adepoju, Boyd, & Isberg, 2011). To
121 identify mutants that were defective in producing the inhibitor, we transferred each
122 mutant onto a lawn of *L. micdadei* on low-cysteine plates, and examined the resulting
123 zone of inhibition surrounding each *Lp* mutant (Figure 2A). After screening 2870 clones,

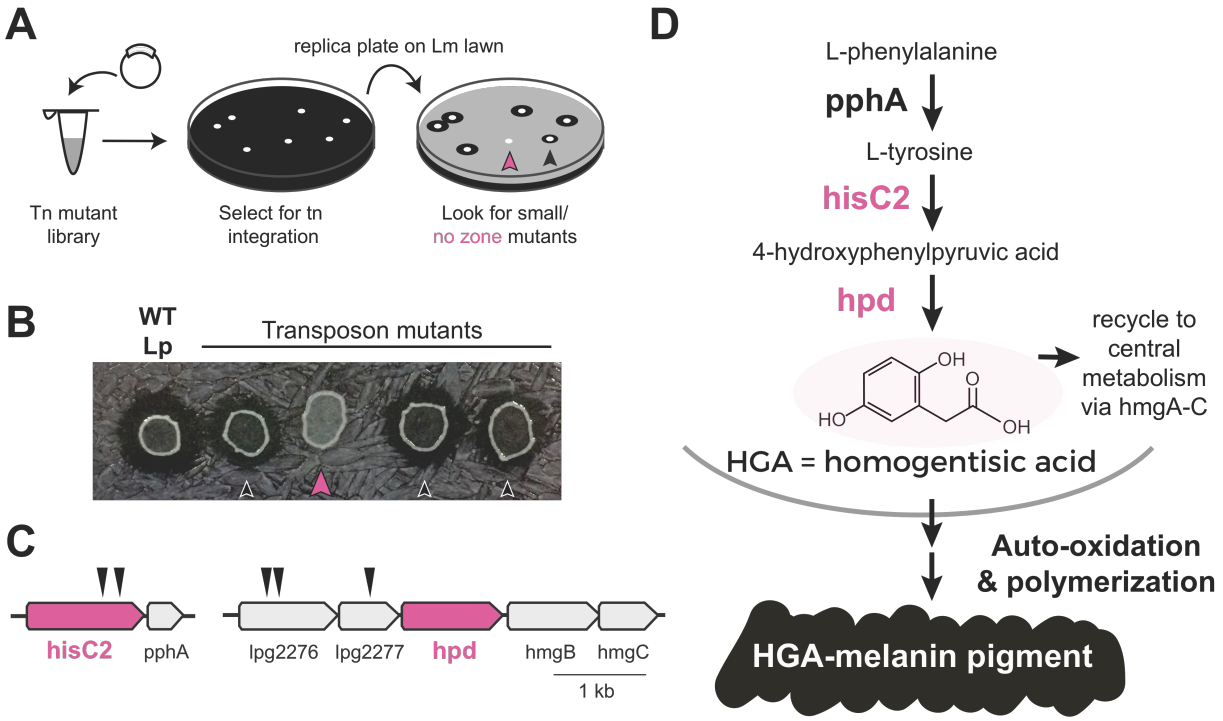

Figure 2: Transposon mutagenesis screen implicates HGA-melanin pathway in production of inhibitor. **A)** Screen for mutant *L. pneumophila* (*Lp*) that do not inhibit *L. micdadei* (*Lm*). Following electroporation of a Mariner-containing plasmid, *Lp* mutants were selected for transposon integration. 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.

we isolated 19 mutants that produced a smaller zone of inhibition than wild type *Lp*, as well as 5 mutants that showed a complete loss of inhibition (Figure 2B, Supplemental Table 1). We refer to these as "small zone" and "no zone" mutants, respectively.

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

138 two uncharacterized genes, *lpg2276* and *lpg2277* (Figure 2C). These two genes lie
139 immediately upstream of *hpd* (*lpg2278*), which is known to act with *hisC2* in the HGA-
140 melanin pathway (Gu, Song, Bonner, Xie, & Jensen, 1998; Steinert et al., 2001) (Figure
141 2D). Because transposon insertions at the beginning of an operon can disrupt the
142 expression of downstream genes via polar effects, we hypothesized that the insertions
143 we recovered in *lpg2276* and *lpg2277* altered inter-*Legionella* inhibition via disruption of
144 *hpd* expression. Indeed, we were able to complement insertions in both genes by
145 overexpressing *hpd*, despite the fact that *hpd* overexpression caused a growth defect
146 (Supplemental Figure 2A). In conclusion, all five "no zone" isolates had mutations that
147 disrupted the same metabolic pathway.
148
149 The HGA-melanin pathway is found in diverse bacteria and eukaryotes (Liu &
150 Nizet, 2009; Nosanchuk & Casadevall, 2003), where it produces homogentisic acid
151 (HGA) from the catabolism of phenylalanine or tyrosine (Fang, Yu, & Vickers, 1989;
152 Steinert et al., 2001) (Figure 2D). Once made, HGA can either be further metabolized
153 and recycled within the cell via HmgA-C, or it can be secreted outside of the cell, where
154 it auto-oxidizes and polymerizes to form a black-brown pigment called HGA-melanin, or
155 pyomelanin (Kotob, Coon, Quintero, & Weiner, 1995) (Figure 2D). To our knowledge,
156 the HGA-melanin pathway has not previously been implicated in inter-bacterial
157 competition. To test whether intracellular metabolites downstream of HGA are
158 necessary for inhibition, we deleted *hmgA*, the first gene in the pathway that returns
159 HGA into central metabolism. The Δ*hmgA* strain produced a zone of inhibition that was
160 similar or perhaps slightly larger than wild type (Supplemental Figure 2B), suggesting
161 that the intracellular processing of HGA is not important for inhibition. Furthermore, we
162 observed defects in HGA-melanin pigmentation in all of the "no zone" mutants as well
163 as some of the "small zone" mutants (Supplemental Figure 3A). We therefore inferred
164 that synthesis of secreted HGA and/or HGA-melanin is required for *Lp* inhibition of *Lm.*
165
166 **HGA inhibits the growth of *Legionella micdadei*, but HGA-melanin pigment does**
167 **not**

Our genetic studies illustrated the importance of the HGA-melanin pathway in the inhibition of *L. micdadei*. We were somewhat surprised by this result, as prior work has emphasized the beneficial (rather than detrimental) effects of HGA-melanin on *Legionella* growth, such as improved iron scavenging (Chatfield & Cianciotto, 2007) and protection from light (Steinert, Engelhard, Flügel, Wintermeyer, & Hacker, 1995). We therefore asked whether the active inhibitor produced by the pathway was HGA-melanin, or alternatively if it could be a transient precursor molecule (Figure 3A). In repeated experiments testing the activity of HGA-melanin pigment from *Lp* conditioned media, we never observed any inhibition of *Lm*. We hypothesized that perhaps the pigment secreted into rich media was too dilute to be active, or alternatively that other nutrients in the media might interfere with inhibition. To address these concerns, we isolated a crude extract of HGA-melanin from *Lp* conditioned media via acid precipitation (as in (Chatfield & Cianciotto, 2007)), washing and concentrating the pigment approximately 10-fold. In multiple assays, the concentrated pigment also showed no inhibitory activity (Figure 3B, Supplemental Figure 4A).

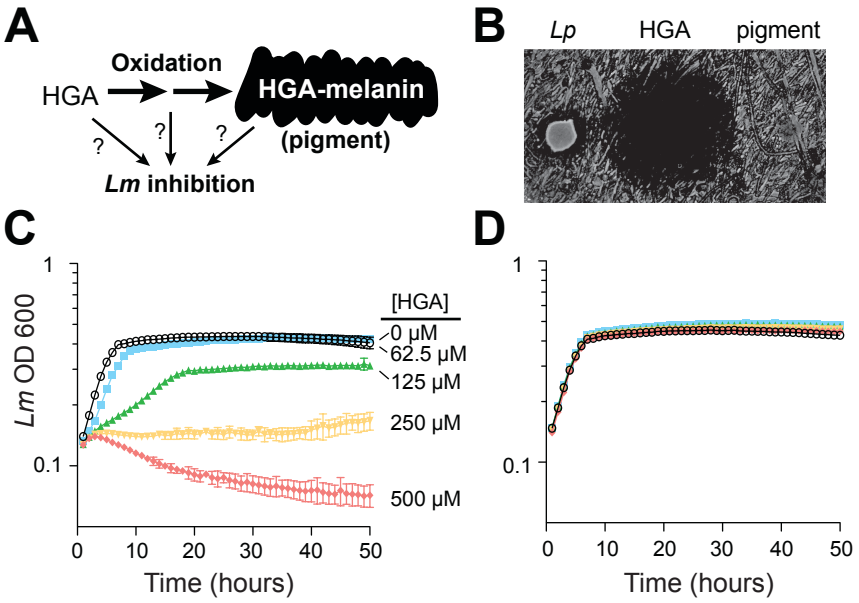

Figure 3: Synthetic HGA inhibits *Legionella micdadei* growth, depending on its oxidation state. **A)** We tested whether inter-bacterial inhibition is caused by HGA, HGA-melanin, or an oxidative intermediate. **B)** Zones of inhibition on a lawn of *Lm* generated from live *Lp* bacteria, synthetic 50mM 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 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. Error bars show standard deviations among 3-4 replicates.

199 The first metabolite secreted by the HGA-melanin pathway is HGA, which auto-
200 oxidizes and polymerizes to form HGA-melanin (Steinert et al., 2001). In contrast to
201 HGA-melanin, synthetic HGA robustly inhibited *Lm* growth, both when spotted onto a
202 lawn of *Lm* and when titrated into AYE rich media (Figure 3B, 3C). Because the
203 inhibitory concentrations of synthetic HGA were relatively high (>50μM), we next asked
204 whether these concentrations are biologically relevant. Compared to other *Legionella*
205 *spp.*, *Lp* produces much more pigment (Supplemental Figure 3B), suggesting that it
206 secretes considerably more HGA. To estimate the quantity of secreted HGA, we used a
207 standard curve of synthetic HGA. Following oxidation in AYE rich media for 24 hours,
208 the amount of synthetic HGA-melanin pigment can be measured by OD 400 and
209 compared to the color of oxidized *Lp* conditioned media to quantify the abundance of
210 HGA (Supplemental Figure 5A). After 48h in culture, we infer that wild type *Lp* had
211 secreted the equivalent of 1.7mM HGA, while the hyperpigmented Δ*hmgA* strain
212 secreted about 2.6mM HGA. In contrast, we did not detect any pigment from the non-
213 inhibitory *hisC2*::Tn strain (Supplemental figure 5B). Thus, HGA can be an abundant
214 secreted metabolite of *Lp*, with a potential to accumulate to concentrations relevant for
215 inter-*Legionella* inhibition.
216
217 We next asked which molecular features of HGA were relevant for its inhibitory
218 activity. Two HGA-related molecules— 2-hydroxyphenylacetic acid and 3-
219 hydroxyphenylacetic acid— differ from HGA by only the removal of a single -OH group.
220 Neither compound inhibited *Lm* growth at any concentration tested (Supplemental figure
221 6), suggesting that inhibition of *Lm* by HGA is relatively specific at the molecular level.
222 We also considered the possibility that HGA as a weak acid could inhibit *Lm* indirectly
223 by altering the local pH, but we observed that adding HGA at 1mM into AYE media or
224 PBS caused little to no change in pH.
225
226 Another well-studied feature of HGA is its redox potential (Eslami, Namazian, &
227 Zare, 2013). If HGA can auto-oxidize, it may cause growth inhibition by oxidizing other
228 nearby molecules, either in the media or on bacterial cells. This scenario is consistent
229 with our observation that oxidized HGA-melanin is inactive (Figure 3B, Supplemental

230 Figure 4A). To test the specific hypothesis that HGA oxidizes and depletes nutrients in
231 the media, we allowed synthetic HGA to oxidize completely for 24h in AYE media before
232 adding *Lm* (Supplemental Figure 5A). If HGA acts by causing nutrient depletion, we
233 expected that media pre-incubated with HGA would be unable to support normal *Lm*
234 growth. Contrary to this hypothesis, we observed that *Lm* grew normally in all conditions
235 and that the pre-oxidation completely abolished synthetic HGA activity (Figure 3D,
236 compare to 3C). We can therefore reject the hypothesis that HGA activity results from
237 indirect nutrient limitation, or any other modifications of the media. Instead, we infer that
238 *Lm* inhibition results from direct interactions between bacterial cells and either HGA
239 itself or unstable, reactive intermediates produced during HGA oxidation.
240
241 If the redox state of HGA is critical for inhibition, we reasoned that it should be
242 possible to modulate HGA activity by altering the redox state of the media with reducing
243 agents. We accomplished this by titrating L-cysteine from 25% to 200% of the levels in
244 standard AYE media. In the absence of HGA, these altered cysteine concentrations had
245 little impact on *Lm* growth (Supplemental Figure 4B). However, lower levels of cysteine
246 greatly sensitized *Lm* to HGA, while excess cysteine was completely protective
247 (Supplemental Figure 4B). In related experiments, incubation of HGA with reduced
248 glutathione or DTT (dithiothreitol), two other reducing agents, similarly quenched HGA's
249 inhibitory activity (Supplementary Figure 4C). We conclude that HGA is less potent in
250 rich media because it reacts with and oxidizes the excess cysteine (or other bystander
251 molecules) before it can interact with *Lm*. Taken together, these results implicate HGA's
252 oxidizing activity and/or its oxidative intermediates in inter-*Legionella* inhibition.
253
254 ***L. pneumophila* can be susceptible to its own inhibitor**
255
256 Our results so far indicated that, while HGA can be a potent inhibitor, its activity
257 appears to be volatile and capable of reacting with many types of molecules. If *Lp* uses
258 HGA to compete with neighboring *Legionella spp.*, we hypothesized that *Lp* would have
259 some form of resistance to its secreted inhibitor. Therefore, we next tested *Lp*
260 susceptibility to inhibition in low-cysteine conditions, as we had previously done for *Lm*.
261 Surprisingly, we found that *Lp* was quite sensitive to inhibition by neighboring *Lp* that

262 was already growing on the plate (Figure 4A). Indeed, *Lp* susceptibility closely mirrored
263 the susceptibility of *Lm* to inhibition (compare to Figure 1A), even though the bacteria
264 secreting the inhibitor were genetically identical to the inhibited *Lp*. In both cases, we
265 observed a sharp boundary at the edge of the zone of inhibition. In contrast, the "no
266 zone" *Lp* strain *hisC2*::Tn did not generate a sharp zone of inhibition against
267 neighboring *Lp* (Figure 4A), suggesting that the HGA-melanin pathway was responsible
268 for both *Lm* inhibition and *Lp* inhibition. To determine whether the susceptibility of *Lp* to

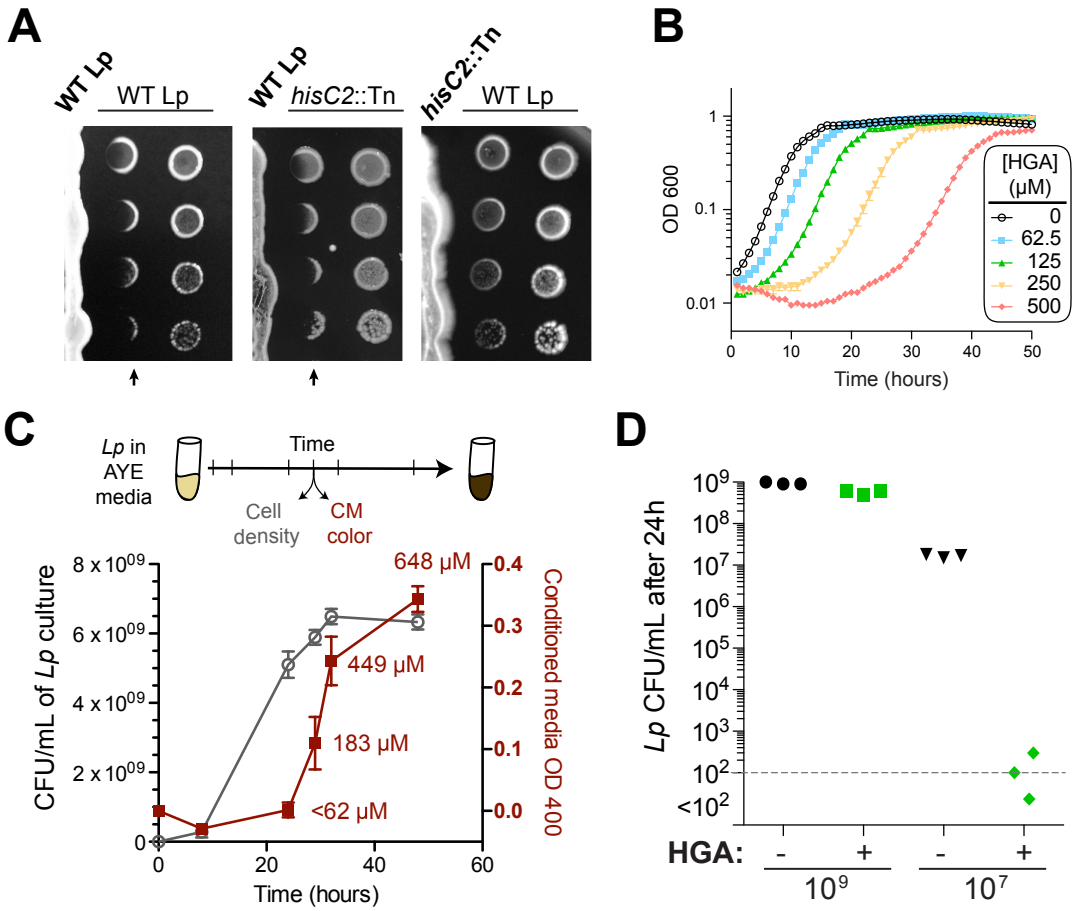

Figure 4: *L. pneumophila* is susceptible self-inhibition by HGA. **A**) When pre-incubated on agar plates, *Lp* produces a zone of inhibition (arrows), impacting the growth of *Lp* plated 3 days later. The "no zone" mutant *hisC2*::Tn does not produce a sharp front of inhibition. **B**) Increasing concentrations of synthetic HGA inhibit the growth of *Lp*, causing a growth delay. Error bars showing standard deviation are small and mostly obscured by the symbols. **C**) Timing of HGA-melanin pigment accumulation in AYE rich media, measured by OD400 after allowing for full oxidation (red boxes, right y-axis) across a growth curve of *Lp* (gray circles, left y-axis). Abundant HGA is secreted into conditioned media (CM) in early stationary phase and continues after cells cease growth. Estimates of secreted HGA concentration (red) are based on a standard curve of synthetic HGA. Error bars show standard deviations from 4 replicates. **D**) Reduction in viable CFUs following 24h incubation with of *Lp* without (-) or with (+) 125μM HGA in nutrient-free PBS. When incubated at high density (10^9 CFU/mL), bacteria are resistant to HGA, while they are higly sensitive at lower density (10^7 CFU/mL or lower). Dotted line shows limit of detection.

269 HGA was restricted to low-cysteine conditions, we grew *Lp* in the presence of synthetic
270 HGA in rich media. Again, we found that the HGA was inhibitory, causing a growth
271 delay in liquid cultures (Figure 4B). Notably, synthetic HGA was active against *Lp* at the
272 same concentrations that were inhibitory to *Lm* (compare Figure 4B and 3C). These
273 results are consistent with a potential role for HGA in both interspecies and intraspecies
274 *Legionella* inhibition.
275
276 We initially found these results to be somewhat perplexing, as we had expected
277 *Lp* to show some resistance to its own inhibitor. Why should *Lp* secrete large quantities
278 of HGA and potentially inhibit its own growth if this process is non-essential (as
279 evidenced by the robust growth of the *hisC2*::Tn mutant)? Although we had already
280 estimated that wild type *Lp* can secrete enough HGA to generate abundant pigment
281 (equivalent to 1.7mM, Supplemental Figure 5B), we did not know if this HGA was
282 secreted all at once, or alternatively if it was released slowly over time. Under the latter
283 scenario, the HGA might oxidize before it accumulated to inhibitory concentrations. To
284 differentiate between these possibilities, we tracked HGA secretion across a growth
285 curve of *Lp* in rich media, using HGA-melanin levels in fully oxidized media to infer the
286 amount of secreted HGA. It has long been known that *Lp* produces abundant HGA-
287 melanin pigment in stationary phase, when the bacteria are undergoing very slow or no
288 growth (Figure 4C) (Berg, Hoff, Roberts, & Matin, 1985; Pine, George, Reeves, &
289 Harrell, 1979; Wiater, Sadosky, & Shuman, 1994). By comparing to a synthetic HGA
290 standard curve (Supplemental Figure 5), we estimate that *Lp* produces a burst of HGA
291 in stationary phase, secreting over 250μM within 5 hours (Figure 4C). HGA secretion
292 then continues after the population has ceased growing. These quantities of HGA
293 should be more than enough to be inhibitory to *Lp* (Figure 4B).
294
295 Based on the timing of HGA secretion, we hypothesized that *Lp* might be more
296 resistant to this inhibitor when at high density and/or when the cells are not actively
297 growing. To test this hypothesis, we washed and diluted stationary-phase *Lp* in nutrient-
298 free PBS with 125μM HGA and measured viability by CFUs at 0 and 24 hours. No
299 measurable darkening of the HGA was detected in this assay, suggesting that the

300 oxidation and de-activation of HGA was considerably slowed in low-nutrient conditions.
301 We found that *Lp* incubated at high density with HGA (above 10^8 CFU/mL) were
302 largely protected from inhibition (Figure 4D). In contrast, when we diluted the same
303 culture to lower density (below 10^7 CFU/mL), bacteria were extremely sensitive to
304 HGA, with at least a 100,000-fold reduction in CFUs. Similarly, HGA did not inhibit *Lp*
305 growth rich media when bacteria were inoculated at high density (Supplemental figure
306 7). Based on the substantial, density-dependent difference in HGA susceptibility, we
307 wondered if *Lp* might regulate HGA resistance via quorum sensing. However, when we
308 deleted *lqsR*, the putative quorum sensing response regulator from *Lp* (Tiaden et al.,
309 2007), this had no detectable impact on HGA susceptibility (Supplemental figure 7).
310 Therefore, the density-dependent susceptibility of *Lp* to HGA must be independent of
311 this pathway.
312
313 Taken together, these results show that *Lp* can be highly susceptible to inhibition
314 by adjacent, genetically identical bacteria via HGA. Similar to our findings in *Lm*, the
315 potency of HGA-mediated inhibition is exacerbated in low nutrient conditions. We also
316 observe that *Lp* secretes abundant HGA when in stationary phase and at high density.
317 Because *Lp* at high density also appear to be protected from HGA, this strategy may
318 restrict the potential for self-harm.
319
320 **Non-essential gene *lpg1681* sensitizes *L. pneumophila* to HGA**
321
322 Our results suggested that *Lp* bacteria are sensitive to HGA at low density but
323 resistant at high density. We next investigated the genetic basis of HGA susceptibility
324 and resistance in *Lp*. First, we hypothesized that the HmgA-C proteins, which break
325 down intracellular HGA and recycle it back into central metabolism, might also process
326 and deactivate extracellular HGA (Rodriguez-Rojas et al., 2009) (Figure 2D). In growth
327 curves of the Δ*hmgA* mutant with increasing concentrations of synthetic HGA, we found
328 that its response to HGA was nearly identical to that of wild type *Lp* (Supplemental
329 Figure 8). These results suggest that the intracellular recycling pathway does not play
330 an appreciable role in *Lp* responses to HGA.
331

Having excluded the obvious candidate pathway for HGA resistance, we pursued an unbiased genetic approach. Because HGA is strongly inhibitory to low density bacteria, we performed a selection for spontaneous, HGA-resistant mutants 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, Supplemental Figure 4), we mixed the bacteria with 1mM HGA in water agar and poured overlays onto low-cysteine BCYE plates. After six days, an average of 53 colonies had grown up on each *Lp* plate selected with HGA, whereas only 3-5 colonies grew on *Lm* plates selected with HGA, or on low-cysteine plates of *Lp* or *Lm* without HGA. Based on these results, we focused on HGA-selected mutants of *Lp*. After retesting the phenotypes of spontaneous mutants on HGA + low-cysteine plates, we recovered 29 *Lp* strains that grew significantly better than wild type *Lp* (Figure 5A). In addition, all 29 strains grew

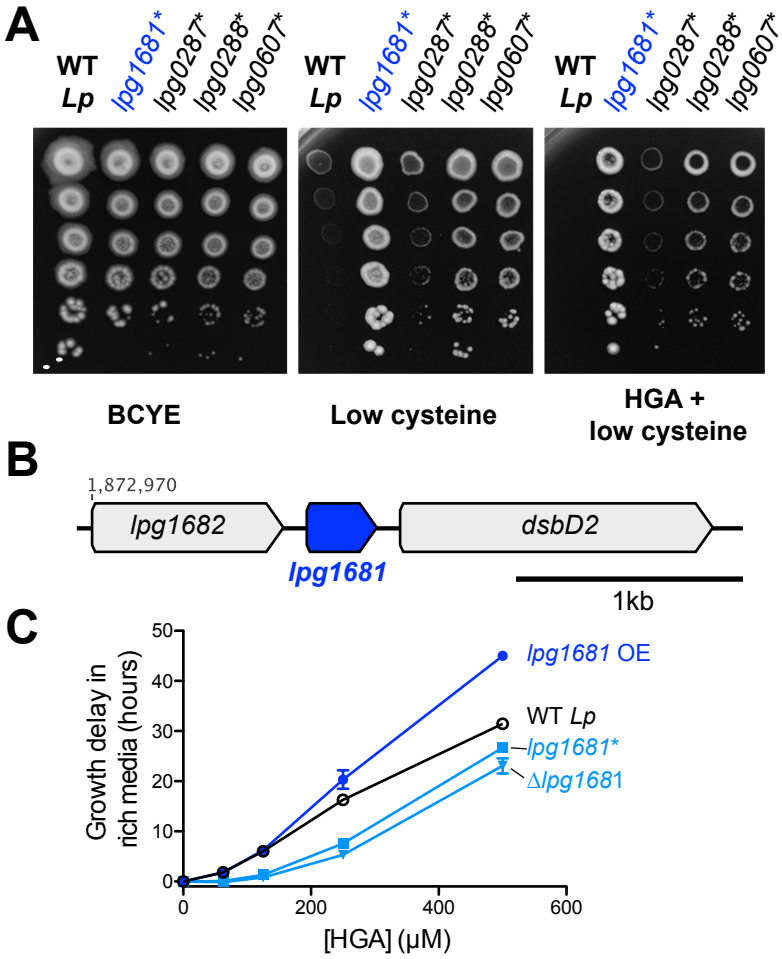

Figure 5: *L. pneumophila* susceptiblity to HGA is modulated by *lpg1681*. **A**) Growth of HGA-selected spontaneous mutants (*) compared to wild type *Lp.* All isolates grew better than wild type in selection conditions (HGA + low cysteine), as well in low cysteine conditions. **B)** Predicted structure of *lpg1681*-containing operon 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. **C**) 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.

363 better than wild type *Lp* on low-cysteine plates without HGA, raising the possibility that
364 HGA susceptibility is related to cysteine metabolism and/or that the strains had adapted
365 to low-cysteine conditions.
366
367 **Table 1**: Genes Mutated in HGA-Selected *L. pneumophila*

Mutated locus	Function or Product	# Spontaneous Mutants w/ this mutation	Mutations Recovered
lpg1681	Hypothetical Protein	4	R49K, R49S, R49G, T50K
lpg0288	YjeK, 2,3-beta-lysine aminomutase	11	W8S, Q9*, L26G, K39*, A50P, R101L, D196G, H215R, Q243K, I250F, Q294*
lpg0607	PoxA/YjeA/GenX, Elongation factor P beta-lysine transferrase	5	W100*, Q184L, A218V, Q258*, 1 bp deletion in S156
lpg0325	RpS7 ribosomal 30S protein	1	G100D
lpg0336	RplP 50s ribosomal protein	1	G88R
lpg0287	Elongation factor P	1	Stop>Q (70 AA Extension)
lpg0349	SecY	3	N118K, Q132R, R369L
lpg2001	SecD	2	V238F, A277G
lpg1504	AceE pyruvate dehydrogenase	1	P272C

368 * =introduction of a stop codon
369 To determine the underlying genetic basis of these phenotypes, we sequenced
370 the genomes of all 29 strains plus our wild type strain of *Lp* to a median depth of 118x
371 and identified mutations genome-wide. Each mutant strain carried 1 to 3 unique point
372 mutations relative to the starting strain, and most of these mutations were found in a few
373 shared loci (Table 1). Nineteen of the twenty-nine strains carried mutations in
374 translation-related machinery, 5 had missense mutations in *secY* or *secD* (*lpg0349* and
375 *lpg2001*), members of the translocon complex that moves polypeptides across the
376 cytosolic membrane, 1 had a mutation in *aceE* pyruvate dehydrogenase (*lpg1504*), and
377 4 had mutations in a hypothetical gene, *lpg1681* (Figure 5B). We suspect that the
378 translation-related mutations had highly pleiotropic effects on protein expression by
379 disrupting elongation factor P function and thereby enhancing ribosome stalling at
380 polyproline tracts (Doerfel et al., 2013; Marman, Mey, & Payne, 2014; Navarre et al.,
381 2010; Yanagisawa, Sumida, Ishii, Takemoto, & Yokoyama, 2010). Based on the

382 frequency of polyprolines in the *Lp* proteome, disruptions to elongation factor P function
383 have the potential to impact the expression of about 33% of *Lp* proteins. The
384 phenotypes observed in these mutants could therefore result from either a large scale
385 shift in *Lp* gene expression, or from the altered expression of specific susceptibility
386 genes.
387
388 Given that all the HGA-selected mutants grew better than wild type *Lp* on both
389 low-cysteine plates and on low-cysteine plates with HGA overlays, we asked whether
390 the mutants had specific resistance to HGA, if they had primarily adapted to low-
391 cysteine conditions, or if they had pleiotropic mutations that broadly impacted cell
392 growth. Growth curves of representative HGA-selected mutants with or without HGA in
393 cysteine-rich AYE media showed that *lpg0288* and *aceE* mutants exhibited some
394 growth defects in cysteine-rich media, consistent with potential pleiotropic impacts on
395 the cell (Supplemental Figure 8E). Mutants in *lpg1681*, *secD*, and *secY* had no growth
396 defects and also showed a reproducible decrease in HGA susceptibility (Figure 5C,
397 Supplemental Figure 8E). Of these, *lpg1681* mutants showed the largest improvement
398 in growth in the presence of HGA.
399
400 The hypothetical gene *lpg1681* is predicted to encode a small, 105 amino acid
401 protein with no predicted domains apart from two transmembrane helices. This gene is
402 adjacent in the genome to *lpg1682*, a predicted oxidoreductase/dehydrogenase, and
403 *lpg1680*, the thiol:disulfide exchange protein DsbD2 (Figure 5B). Functional studies of
404 DsbD2 (aka DiSulfide Bond reductase D2) have demonstrated that it interacts with
405 thioredoxin to regulate disulfide bond remodeling in the periplasm (Inaba, 2009; Kpadeh,
406 Day, Mills, & Hoffman, 2015). If *lpg1681* has a redox function related to its neighboring
407 genes, we expected its syntenic locus to be conserved across bacterial strains and
408 species. We found that the *lpg1680-1682* locus is present and conserved among over
409 500 sequenced *Lp* strains currently in NCBI databases. Outside of *L. pneumophila*,
410 *lpg1681* is mostly restricted to the *Legionella* genus, present in about half of the
411 currently sequenced species (Burstein et al., 2016) (Supplemental Figure 9). A homolog
412 of *lpg1681* is also found in the draft genome of *Piscirickettsia litoralis*, a gamma

413 proteobacterium and fish pathogen (Wan et al., 2016). In all cases, *lpg1681* resides
414 upstream of *dsbD2*, suggesting a functional link between these proteins (Supplemental
415 Figure 9).
416
417 Because *lpg1681* appears to reside within an operon with redox-related genes
418 and HGA activity is linked to its redox potential, we viewed *lpg1681* as a promising
419 candidate for a gene involved in HGA susceptibility. We constructed *lpg1681*
420 overexpression and deletion strains in *Lp* and tested the susceptibility of these strains to
421 HGA. Similar to the spontaneous mutants we recovered, the Δ*lpg1681* strain was more
422 resistant to HGA in rich media (Figure 5C). Conversely, overexpression of *lpg1681*
423 increased *Lp* sensitivity, resulting in longer growth delays than wild type at high
424 concentrations of HGA. We therefore conclude that wild type *lpg1681* expression
425 sensitizes *Lp* to inhibition by extracellular HGA.
426
427

428 **Discussion**

429 In this study, we identify HGA as a mediator of inter-*Legionella* inhibition.
430 Furthermore, we find that inter-*Legionella* inhibition and surfactant production are
431 separate, but co-occurring, phenomena. Although surfactant is not required for
432 inhibition, we hypothesize that surfactant may enhance the spread of HGA and/or its
433 reactive intermediates away from the secreting *Lp* bacteria, which may explain previous
434 results (Stewart et al., 2011).
435
436 The HGA-melanin pathway is well-studied and widespread among bacteria and
437 eukaryotes, so we were surprised to find a previously undescribed role for HGA in
438 interbacterial competition. Somewhat paradoxically, HGA-melanin production in bacteria
439 has previously been implicated both in the production of and protection from reactive
440 oxygen species (production: (Noorian et al., 2017); protection: (Keith, Killip, He, Moran,
441 & Valvano, 2007; Orlandi, Bolognese, Chiodaroli, Tolker-Nielsen, & Barbieri, 2015)).
442 Based on our results, we hypothesize that HGA may be responsible for oxidative
443 damage, while the HGA-melanin pigment serves other, beneficial roles. The redox-

444 based inhibition that we observe for HGA is analogous to that of pyocyanin from
445 *Pseudomonas aeruginosa* and similar phenazine pigments, which are also produced at
446 high cell density (Baron, Terranova, & Rowe, 1989; Hassan & Fridovich, 1980). One
447 major difference is that pyocyanin impacts an extremely broad range of species,
448 including diverse bacteria and eukaryotes (Baron et al., 1989; Kerr et al., 1999; Noto,
449 Burns, Beavers, & Skaar, 2017). In contrast, previous work found that *Lp* secretions can
450 inhibit *Legionella spp.,* but have little impact on other bacterial taxa (Stewart et al.,
451 2011). We speculate that the observed specificity arises simply because *Legionella spp.*
452 are quite sensitive to oxidative stress relative to other bacteria (Domingue, Tyndall,
453 Mayberry, & Pancorbo, 1988; Hoffman, Pine, & Bell, 1983). Nevertheless, because the
454 ecological niche of *Lp* appears to overlap with other *Legionella* strains and species
455 (Pereira et al., 2017; Wery et al., 2008), HGA-mediated inter-*Legionella* inhibition has a
456 strong potential to impact the success of *Lp* in both natural and manmade
457 environments.
458
459 Another major difference between phenazines and HGA is that phenazines have
460 not been reported to be self-active, because the phenazine-producing bacteria are
461 protected by their antioxidant defenses (Hassett, Charniga, Bean, Ohman, & Cohen,
462 1992). However, self-active effects can be difficult to observe, particularly if bacteria at
463 high cell density are resistant. In our hands, intraspecies inhibition by HGA was
464 apparent only in assays competing high-density populations with low-density, invading
465 competitors. Furthermore, we note that the inhibitory activity of molecules like HGA is
466 transient and is quenched by the cysteine-rich media typically used to grow *Legionella*
467 in the lab (Figure 3D, Supplemental Figure 4). This may explain why HGA-mediated
468 inhibition of *Legionella* has not been previously reported. Still, our complementary
469 inhibition assays performed in rich media, low-cysteine media, and nutrient-free media
470 suggest that HGA inhibition can occur across environmental settings, and only becomes
471 more potent as conditions approach the nutrient-limited, slow growth conditions that
472 *Legionella* likely experience within biofilms.
473

474 In many modes of interbacterial competition, organisms that produce toxic
475 molecules also possess resistance genes to avoid self-targeting. It was therefore initially
476 perplexing that *Lp* can produce a self-active inhibitor, particularly given that genes for
477 HGA production and the HGA-sensitizing gene *lpg1681* are both non-essential under
478 laboratory conditions. One possible explanation is that the benefits of HGA or HGA-
479 melanin for *Lp* outweigh the self-harm of HGA. These benefits could include antagonism
480 against other *Legionella* as demonstrated here, or previously described benefits of
481 HGA-melanin, including UV protection and iron reduction (Chatfield & Cianciotto, 2007;
482 Steinert et al., 1995). Antagonism against eukaryotic cells may be another benefit; the
483 *hpd* gene in the *Lp* HGA synthesis pathway was originally named *lly,* or legiolysin,
484 because expression of the gene in *E. coli* caused erythrocyte lysis on blood agar plates
485 (Wintermeyer et al., 1994). Finally, the redox activity of HGA may play a beneficial role
486 in *Legionella* gene expression and/or anaerobic respiration within biofilms, as is the
487 case for pyocyanin and other phenazines in *Pseudomonas* (Mavrodi et al., 2013).
488
489 Beyond these benefits, the context and timing of HGA secretion may constrain
490 the potential for self-harm. In laboratory conditions, HGA secretion occurs primarily in
491 late stationary phase, when cells are at high density (~10^9 CFU/mL) (Figure 4C).
492 Because *Lp* at high density are also more resistant to HGA (Figure 4D), *Lp* self-
493 protection apparently involves a definition of “self” based on growth phase, density,
494 and/or regulatory state, rather than one based on the presence of genetically-encoded
495 resistance genes. Consistent with this idea, our selection scheme to identify genes
496 involved in HGA resistance (Figure 5) recovered mostly pleiotropic defects in
497 translation, in addition to *lpg1681*, which enhances *Lp* susceptibility to HGA (Table 2). It
498 may therefore be difficult for *Lp* to evolve resistance to HGA at low cell densities without
499 altering other important cellular functions.
500

The features of *Lp* sensitivity and resistance to HGA that we have uncovered (production of a self-toxic compound, resistance to that compound based on regulatory state, and barriers to evolving resistance) have interesting implications for *Legionella* sociomicrobiology. In biofilms, bacterial populations can cooperate in the production of shared, secreted public goods (Nadell et al., 2016; Steenackers, Parijs, Foster, & Vanderleyden, 2016). For *Lp*, such public goods include the production of siderophores, surfactant, and quorum sensing signals (Liles, Scheel, & Cianciotto, 2000; Spirig et al., 2008; Stewart et al., 2011). However, such public goods can be vulnerable to cheating if other bacteria exploit these extracellular resources. In this context, HGA is an unusual public good (Figure 6). Because high-density, established bacterial communities are largely resistant to inhibition, they can use HGA to protect against low-density, invading *Legionella* with little harm to themselves. Due to these dynamics, we propose that HGA can act as a niche-protective public good. Moreover, because HGA activity can be felt well outside the spread of other *Lp* public goods such as surfactant (Supplemental Figure 1), HGA may simultaneously protect a number of other public goods from being exploited by invading bacteria. Across many bacterial species, the production and use of public goods is regulated by quorum sensing (Abisado et al., 2018). Although our results suggest that the known quorum sensing pathway in *Lp* is not involved in HGA production or resistance (Supplemental Figure 7), future studies on the regulation of HGA resistance may identify additional molecular pathways that are critical to the social behaviors of *Legionella* in bacterial communities.

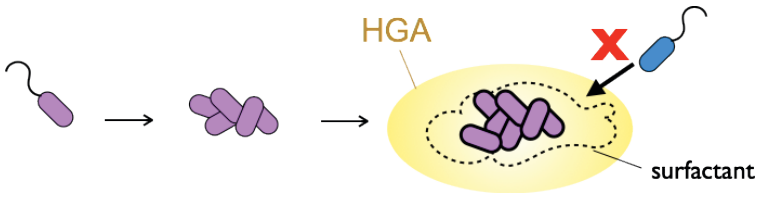

Figure 6: Model for HGA activity in biofilms. *Lp* (purple) colonizes a surface and grows to form a microcolony. Once cells are at high density, they secrete abundant HGA (yellow). Through unknown mechanisms, high density *Lp* are resistant to HGA's effects (bold outline), while low density *Lp* or other *Legionella* species (blue) are inhibited by HGA and cannot invade the microcolony's niche. The territorial protection via HGA allows for the established *Lp* community to secrete other public goods such as surfactant, which are protected from exploitation by other bacteria.

531 **Materials and Methods**

532

533 **Bacterial strains and growth conditions** The bacterial strains and plasmids used for
534 this study are listed in Table 3. As our wild type *Legionella pneumophila (Lp)* strain, we
535 used KS79, which was derived from JR32 and ultimately from isolate Philadelphia-1 (de
536 Felipe et al., 2008; Rao, Benhabib, & Ensminger, 2013; Sadosky, Wiater, & Shuman,
537 1993). Compared to JR32, the KS79 strain has a *comR* deletion to enable genetic
538 manipulation (de Felipe et al., 2008). We used *Legionella micdadei (Lm)* tatlock as our
539 susceptible strain (Garrity, Brown, & Vickers, 1980). Liquid cultures of *Legionella* were
540 grown shaking in AYE liquid media at 37˚C (De Jesús, O'Connor, & Isberg, 2013). To
541 manipulate the redox state of AYE, we altered the amount of cysteine added to the
542 media from 0.4 g/L in standard AYE to 0.1, 0.2, and 0.8 g/L. On solid media, *Legionella*
543 were grown either on BCYE agar plates either containing the standard cysteine
544 concentration (0.4g/L) or in “low cysteine” conditions (0.05g/L) (Feeley et al., 1979;
545 Solomon & Isberg, 2000). *E. coli* strains used for cloning were grown in LB media.
546 Where indicated, antibiotics were used at the following concentrations in solid and liquid
547 media, respectively; chloramphenicol (5 µg/mL and 2.5 µg/mL), kanamycin (40 µg/mL)
548 and ampicillin (50 µg/mL and 25 µg/mL). For counter-selection steps while generating
549 deletion strains, 5% sucrose was added to BCYE plates. For top agar and overlay
550 experiments, we used 0.7% agar dissolved in water, which was kept liquid at 50˚C
551 before pouring over BCYE plates.

552

553 **Gene deletions and complementations** Genomic knockouts in *L. pneumophila* were
554 generated as previously described (Wiater et al., 1994). Briefly, we used an allelic
555 exchange plasmid (pLAW344) harboring chloramphenicol and ampicillin selection
556 cassettes and the counter-selection marker SacB, which confers sensitivity to sucrose.
557 Into this plasmid, we cloned ~1kb regions upstream and downstream of the gene of
558 interest to enable homologous recombination. Following electroporation and selection
559 on chloramphenicol, we used colony PCR to verify insertion of the plasmid into the
560 chromosome, before counter-selection on sucrose media. From the resulting colonies,
561 we performed PCR and Sanger sequencing to verify clean gene deletion. For

562 complementation, the coding region of a candidate gene was cloned into a plasmid
563 (pMMB207c) following a ptac promoter (J. Chen et al., 2004). To induce gene
564 expression, strains carrying pMMB207c-derived plasmids were exposed to 1mM IPTG.
565 All constructs were assembled using Gibson cloning (NEB Catalog #E2621).
566
567 **Inhibition assays on agar plates** To visualize inhibition between neighboring
568 *Legionella* on solid media, a streak of approximately 5 x 10^6 CFU of the inhibitory
569 strain of *Lp* was plated across the center of a low-cysteine BCYE plate. After 3 days
570 growth at 37˚C, dilutions of susceptible *Lp* or *Lm* were plated as 10µLspots
571 approximately 1 cm and >2 cm from the central line. Once spots were dry, plates were
572 then incubated for an additional 3 days before scoring for inhibition. This assay was also
573 used to quantify the bactericidal inhibition of *Lm*, with slight modifications. Here, all *Lm*
574 was plated in 20µLspots at 10^6 CFU/mL. The time of plating susceptible *Lm* was
575 treated as t=0. Once spots were dry, plugs were extracted from within the *Lm* spots
576 using the narrow end of a Pasteur pipette. These plugs were transferred into media,
577 vortexed, and plated to quantify CFU. This procedure was repeated after 48h at 37˚C to
578 compare *Lm* viability and growth within ("near", Figure 1B-C) or outside ("far") of the
579 zone of inhibition.
580 For inhibition assays on bacterial lawns, we plated 10µL drops of either live *Lp* or
581 chemical compounds on top of a lawn of 5 x 10^7 CFU *Lm* on low-cysteine BCYE, and
582 assessed growth of the lawn after 3 days at 37˚C. Synthetic HGA (Sigma: #H0751) was
583 dissolved in water at a concentration of 100 mM and filter sterilized before use. HGA-
584 related compounds, 2-hydroxyphenylacetic acid (Sigma: #H49804) and 3-
585 hydroxyphenylacetic acid (Sigma: #H49901), were prepared in the same way. To test
586 the impact of DTT (Sigma: #43819) and glutathione (oxidized: Sigma #G4376 , reduced:
587 Sigma #G6529) on HGA-mediated inhibition, filter-sterilized solutions dissolved in water
588 were mixed in equimolar ratios with HGA, and incubated shaking at room temperature
589 for 1 hour before spotting onto bacterial lawns. HGA-melanin pigment was prepared
590 from *Lp* conditioned media as previously described (Zheng, Chatfield, Liles, &
591 Cianciotto, 2013) from KS79, the unpigmented *hisC2*::Tn mutant, and the
592 hyperpigmented Δ*hmgA* mutant. Briefly, conditioned media was collected and sterile

593 filtered from 100 mL cultures of *Lp* in AYE media grown shaking at 37˚C for 3 days. The
594 conditioned media was acidified to a pH of 1.5 and transferred to 4˚C for 2 hours to
595 precipitate. Precipitated pigment was collected by centrifugation at 4000 x g for 15
596 minutes and then washed with 10 mM HCl. Pelleted pigment was then returned to
597 neutral pH and resuspended in PBS at 10X before testing.
598
599 **Transposon mutagenesis screen** For random transposon insertion mutagenesis, we
600 used a Mariner transposon from the pTO100 plasmid (O'Connor et al., 2011). We
601 electroporated this plasmid into the KS79 strain and allowed cells to recover at 37˚C for
602 5 hours. To select for cells with integrated transposons, cultures were plated on
603 BCYE/Kan/sucrose plates and incubated at 37˚C for 3 days before screening individual
604 mutant colonies.
605 To identify transposon mutants with defects in *Lm* inhibition, we transferred each
606 *Lp* mutant onto a low-cysteine plate with a lawn of 5 x 10^7 CFU *Lm* and visually
607 screened for those with either small zones of inhibition or no zone of inhibition. Mutants
608 were added on top of the *Lm,* either by replica plating with sterile Whatman paper
609 (Whatman: #1001150) or by transferring with a sterile toothpick. Plates were then
610 incubated at 37˚C for 3 days and scored. All putative mutants underwent clonal re-
611 isolation, were diluted to OD 600 of 0.1, and spotted on *Lm* lawns to retest their
612 phenotypes. To map the sites of transposon integration, we used arbitrary PCR as
613 described in (T. Chen, Yong, Dong, & Duncan, 1999), with primers redesigned to work
614 with the pTO100 transposon (Table 3). Briefly, this protocol involved two PCR steps to
615 amplify the DNA flanking the transposon. The first step used low annealing
616 temperatures to allow the arb1 randomized primer to bind many sites in the flanking
617 DNA while the pTO100_F or pTO100_R primer annealed within the transposon,
618 generating multiple products that overlapped the flanking DNA. These products were
619 amplified in the second step PCR using the arb2 and pTO100_Rd2 primers, and we
620 used the pTO100_Rd2 primer for Sanger sequencing. PCR programs and conditions
621 were as in (T. Chen et al., 1999).
622

623 **HGA inhibition assays in liquid cultures** For rich media assays (e.g. Figures 3C, 3D,
624 4B), overnight cultures of *Legionella* were diluted to 10^8 CFU/mL in AYE, mixed with
625 synthetic HGA or pigment in 96 well plates, and grown shaking at 425 cpm at 37˚C. The
626 cytation 3 imaging reader (BioTek™ CYT3MV) was used to monitor growth by OD 600
627 measurements. Because oxidized pigment from synthetic HGA is detected at OD 600,
628 each experiment included bacteria-free control wells containing media and each
629 concentration of HGA. To correct OD 600 readings for pigment development, at each
630 time point we subtracted the control well reading from bacterial wells that received the
631 same concentration of synthetic HGA. For experiments with HGA "pre-oxidation" (Figure
632 3D), we diluted HGA in AYE media and incubated this solution shaking at 37˚C for 24
633 hours in the plate reader before adding bacteria. Complete oxidation of HGA during the
634 24 hours was monitored using OD400 to track the accumulation of HGA-melanin
635 pigment (Supplemental figure 5). In *Lp*, HGA inhibition in AYE rich media resulted in a
636 growth delay, similar to an extended lag phase (Figure 4B). To compare sensitivity to
637 HGA among *Lp* strains, we calculated the lag phase from the growth curve of each well
638 using the GrowthRates program (Hall, Acar, Nandipati, & Barlow, 2014). We excluded a
639 small number of samples where the growth curve was not well fit (R<0.99), and then for
640 each strain used the difference in lag time between the samples with and without HGA
641 to calculate the growth delay due to HGA (Figures 5C).
642
643 As a complementary assay, we evaluated *Legionella* viability when exposed to
644 HGA in nutrient-free PBS at different cell densities. Stationary phase cultures were
645 washed once and re-suspended in PBS. We diluted these bacteria to estimated starting
646 concentrations of 10^9, 10^8, and 10^7 CFU/mL and plated for CFU at t=0. We
647 distributed the remaining bacteria into 96 well plates with or without 125 μM HGA.
648 Plates were incubated shaking in a plate reader at 425 cpm at 37˚C for 24 hours before
649 plating to quantify CFU on BCYE plates. CFU were counted after 3-4 days growth at
650 37˚C.
651
652 **Estimation of amount of HGA secreted by *Lp*** HGA-melanin is a black-brown pigment
653 that is easily detected at OD 400. We took advantage of this coloration to estimate the

654 amount of HGA that had been secreted by *Lp* by comparing the color of conditioned
655 media to a standard curve of oxidized synthetic HGA. To isolate conditioned media from
656 pigment mutant strains, cultures of KS79, Δ*hmgA*, and HisC::Tn were inoculated with
657 fresh colonies from a BCYE plate into 5 mL AYE and were grown shaking at 37˚C for 48
658 hours. We then collected conditioned media by pelleting the bacteria and passing the
659 supernatant through a 0.2μm filter. To harvest conditioned media for a time course,
660 cultures of *Lp* were inoculated into 5 ml AYE and grown shaking at 37˚C. After 15, 20,
661 24, 39, 44, and 48 hours, we measured the OD 600 of the culture and collected
662 conditioned media. To create a standard curve, we diluted synthetic HGA into AYE at
663 the following concentrations: 62.5 μM, 125 μM, 250 μM, 500 μM, and 1 mM. The
664 conditioned media and standard curve samples were incubated in a 96 well plate in a
665 plate reader shaking at 37˚C for 24 hours to allow the HGA to oxidize. We used OD 400
666 data from the 24 hour time point to generate a standard curve for each HGA
667 concentration and calculated a line of best fit using linear regression. This equation was
668 used to estimate the amount of secreted HGA that corresponded to the OD 400 of each
669 conditioned media sample. (Supplemental Figure 5).
670

671 **HGA-resistant mutants** Because the inhibitory activity of HGA is quenched through
672 interactions with rich media (Supplemental Figure 4), it was not possible to select for
673 HGA-resistant mutants by mixing HGA into BCYE agar. Instead, to reduce the potential
674 for HGA to react with media components while allowing sufficient access to nutrients for
675 mutant cells to grow, we selected for HGA-resistant mutants by mixing 4 x 10 ^7 CFU
676 *Legionella* with 1mM HGA in 4mL of 0.7% water agar and pouring this solution as an
677 overlay on a low-cysteine BCYE plate. Plates were incubated at 37˚C for 6 days, before
678 candidate resistant colonies were picked and clonally isolated. The HGA resistance and
679 growth of each isolate was re-tested on overlays with or without 1mM HGA on both
680 regular and low-cysteine BCYE.
681

682 Twenty-nine isolates were more resistant to HGA than wild type *Lp* upon
683 retesting. We sequenced and analyzed genomic DNA from these isolates and a
684 matched wild type strain as follows. DNA was prepared from each strain using a

685 Purelink genomic DNA mini kit (Invitrogen, #K1820). DNA concentrations were
686 quantified using Qubit and normalized to 0.5 ng/uL. Barcoded libraries were prepared
687 using tagmentation according to Baym et al. 2015 (Baym et al., 2015), analyzed with
688 QuantIT DNA quantification, pooled, and sequenced with 50 bp paired-end reads on an
689 Illumina HiSeq 2500 machine. Reads were trimmed for quality and to remove Nextera
690 indices with Trimmomatic (Bolger, Lohse, & Usadel, 2014) and mapped to the
691 Philadelphia-1 genome (Chien et al., 2004) using Bowtie2 with default parameters
692 (Langmead, Trapnell, Pop, & Salzberg, 2009). Coverage plots were generated for each
693 strain using bamcoverage (Ramírez et al., 2016) and visually examined for evidence of
694 large genomic deletions and amplifications. None were observed, apart from a
695 prophage that was present in the reference genome but missing from all sequenced
696 strains, including our wild type KS79 strain. Variants were detected for each mutant
697 using Naive Variant Caller (Blankenberg D, et al. In preparation). Those variants that
698 were detected in mutant strains but not the wild type strain were considered as putative
699 causative mutations. For each of these mutations, we inspected the mapped reads and
700 excluded faulty variant calls that either were adjacent to coverage gaps or that did not
701 appear to be fixed in the clonal mutant and/or wild type sequences, likely due to errors
702 in read mapping. After this manual filtering, 1-3 well-supported mutations remained for
703 each mutant genome. Nine of the mutants had undergone selection on a different day
704 from the other mutants: in addition to various unshared mutations, these nine strains
705 each carried exactly the same missense mutation in *rplX*, which we disregarded as a
706 background mutation that likely arose before selection. Following this exclusion, each
707 mutant carried only a single well-supported mutation in a coding region. Most often this
708 coding mutation was the only mutation we detected, although one mutant carried two
709 additional intergenic point mutations. The coding mutations were point mutations or
710 small deletions that resulted in non-synonymous changes, frame shifts, or gene
711 extensions. Across different mutants, the mutations we uncovered were repeatedly
712 found in the same, few loci (Table 1).
713
714 **Evolution of *lpg1681*** The genes in the HGA-melanin synthesis pathway are highly
715 conserved in diverse bacteria and across the *Legionella* genus, with all genes present in

716 all 41 currently sequenced *Legionella spp.* genomes (Burstein et al., 2016). In contrast,
717 we were able to identify *lpg1681* in only 30 *Legionella spp.* genomes, as well as a single
718 draft genome outside the *Legionella* genus– *Piscirickettsia litoralis*, an intracellular fish
719 pathogen (Wan et al., 2016). Across the *lpg1681*-containing genomes, there is evidence
720 for extensive recombination of the flanking loci, yet *lpg1681* is always found upstream of
721 *dsbD2*. We identified most of these homologs of *lpg1681* using a jackhmmr search
722 (Finn et al., 2015), followed by cross-referencing the homologs with the *Legionella*
723 orthology groups defined by Burstein et al., 2016. From this starting set, additional
724 *lpg1681* orthologs were identified in unannotated, intergenic regions by searching for
725 >200bp open reading frames upstream of *dsbD* orthologs, and confirming the homology
726 of these regions using MAFFT alignments (Katoh, Misawa, Kuma, & Miyata, 2002).
727 Through this method, we located *lpg1681* in all currently sequenced *Legionella*
728 genomes that contain an annotated *dsbD2* gene, with the exception of *L. shakespearei.*
729 We categorized the *lpg1681*-containing loci into those with similar synteny, based on
730 the orthology group annotations in Burstein et al. 2016. We colored and provided names
731 for the neighboring genes in Supplemental figure 9 if they had a homolog in the *L.*
732 *pneumophila* Philadelphia-1 genome that was not annotated as a hypothetical gene. To
733 assess the conservation of the *lpg1680-lpg1682* among *L. pneumophila* strains, we
734 used blastn in the NCBI nr and wgs databases with the full *lpg1680-lpg1682* genomic
735 DNA sequence as a query. We found that the full region was conserved with few
736 mutations across 501 currently sequenced *L. pneumophila* strains.

Acknowledgements

We thank Howard Shuman for providing *Legionella* strains as well as the pLAW344 and pMMB207c plasmids. The pTO100 plasmids carrying the transposon were kindly provided by Tamara O'Connor (O'Connor et al., 2011). We also thank Michelle Hays, Kevin Forsberg, Alistair Russell, Janet Young, and Howard Shuman for providing feedback on the manuscript.

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