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 \textit{Lp}.

\textit{Legionella spp.} are not known to produce any antibiotics, bacteriocins, or other antibacterial toxins. Bioinformatic surveys of \textit{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 \textit{Lp} success within biofilms. For example, one study of artificial two-species biofilms found that viable \textit{Lp} were able to persist for over two weeks in the presence of several bacterial species (e.g. \textit{Pseudomonas fluorescens}, \textit{Klebsiella pneumoniae}) but not others (e.g. \textit{Pseudomonas aeruginosa}) (Stewart, Muthye, & Cianciotto, 2012). Additionally, \textit{Lp} bacteria are often co-resident with other \textit{Legionella spp.} in man-made structures, with some studies showing that \textit{Lp} proliferation is correlated with a decrease in other \textit{Legionella spp.} populations (Declerck et al., 2007; Pereira, Peplies, Höfle, & Brettar, 2017; Wery et al., 2008). These studies suggest that \textit{Lp} bacteria may compete with other \textit{Legionella spp.} for similar biological niches. The most direct evidence for interbacterial competition comes from Stewart et al., 2011, who found that \textit{Lp} could antagonize the growth of neighboring \textit{Legionella spp.} on the same plate (Stewart, Burnside, & Cianciotto, 2011). This antagonism was apparently specific to \textit{Legionella}, as other bacterial species were unaffected. The molecules mediating this competition have not been identified, although previous work suggested a role for \textit{Lp}'s secreted surfactant, a thin liquid film that facilitates the spread of \textit{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-\textit{Legionella} inhibition.
Here, we use unbiased genetic approaches to find that homogentisic acid (HGA) produced by Lp inhibits the growth of neighboring Legionella spp. We find that HGA 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 although Lp in stationary phase secretes large quantities of this metabolite, low density Lp are highly susceptible to HGA-mediated inhibition. We also identify one gene—lpg1681—that enhances Lp susceptibility to HGA. Based on these findings, we propose that HGA behaves as an unusual public good that may play an important role in structuring Legionella communities.

Results

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

Inspired by previous reports (Stewart et al., 2011), we investigated how 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, ΔbbcB (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 ΔbbcB Lp zone of inhibition (“far”).
growth of neighboring *Legionella micdadei* (*Lm*) plated 1 cm away on solid media, potentially via a secreted inhibitor (Figure 1A). We empirically found that 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. Previous work (Stewart et al., 2011) proposed that inter-*Legionella* inhibition could be caused by *Lp*’s secreted surfactant, which is produced by *Lp* but not *Lm* (Stewart et al., 2009). However, we observed that the zone of inhibition surrounding *Lp* did not always co-occur with the spread of the surfactant front (Supplemental figure 1A), suggesting that *Lp* may secrete a separate, undescribed inhibitor. To test this hypothesis, we deleted a surfactant biosynthesis gene, *bbcB*, from the *Lp* genome (Stewart et al., 2011). The resulting ∆*bbcB* strain did not produce surfactant (Supplemental figure 1B), yet it still inhibited adjacent *Lm*, demonstrating that surfactant production is not required for inter-*Legionella* inhibition (Figure 1A, Supplemental Figure 1C). 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* and those plated outside of the zone of inhibition (Figure 1B). We next asked if surfactant enhanced inhibition by quantifying inhibition from the ∆*bbcB* *Lp* strain on *Lm*. Again, surfactant production had little impact and we observed nearly identical inhibition from both wild type *Lp* and ∆*bbcB* *Lp* (Figure 1C). We therefore conclude that *L. pneumophila* can cause strong growth inhibition of neighboring *Legionella* using an unknown molecule that is distinct from surfactant.

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

To determine which molecule(s) might be responsible for inter-*Legionella* inhibition, we performed an unbiased genetic screen in *Lp*. Briefly, we generated *Lp* mutants using a drug-marked Mariner transposon that randomly and efficiently integrates into the *Legionella* genome (O’Connor, Adepoju, Boyd, & Isberg, 2011). To identify mutants that were defective in producing the inhibitor, we transferred each mutant onto a lawn of *L. micdadei* on low-cysteine plates, and examined the resulting zone of inhibition surrounding each *Lp* mutant (Figure 2A). After screening 2870 clones,
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

![Diagram 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.)

![Diagram 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.)

![Diagram C] (Transposon insertion sites (triangles) identified in the five recovered “no zone” mutants.)

![Diagram D] (HGA-melanin pigment 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.)
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 (Gu, Song, Bonner, Xie, & Jensen, 1998; Steinert et al., 2001) (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 by overexpressing *hpd*, despite the fact that *hpd* overexpression caused a growth defect (Supplemental Figure 2A). In conclusion, all five “no zone” isolates had mutations that disrupted the same metabolic pathway.

The HGA-melanin pathway is found in diverse bacteria and eukaryotes (Liu & Nizet, 2009; Nosanchuk & Casadevall, 2003), where it produces homogentisic acid (HGA) from the catabolism of phenylalanine or tyrosine (Fang, Yu, & Vickers, 1989; Steinert et al., 2001) (Figure 2D). Once made, 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, Coon, Quintero, & Weiner, 1995) (Figure 2D). To our knowledge, the HGA-melanin pathway has not previously been implicated in inter-bacterial competition. To test whether intracellular metabolites downstream of HGA are necessary for inhibition, we deleted *hmgA*, the first gene in the pathway that returns HGA into central metabolism. The ∆*hmgA* strain produced a zone of inhibition that was similar or perhaps slightly larger than wild type (Supplemental Figure 2B), suggesting that the intracellular processing of HGA is not important for inhibition. Furthermore, 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 3A). We therefore inferred that synthesis of secreted HGA and/or HGA-melanin is required for *Lp* inhibition of *Lm*.

**HGA inhibits the growth of *Legionella micdadei*, but HGA-melanin pigment does not**
Our genetic studies illustrated the importance of the HGA-melanin pathway in the inhibition of \textit{L. micdadei}. We were somewhat surprised by this result, as prior work has emphasized the beneficial (rather than detrimental) effects of HGA-melanin on \textit{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 \textit{Lp} conditioned media, we never observed any inhibition of \textit{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 \textit{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).
The first metabolite secreted by the HGA-melanin pathway is HGA, which auto-oxidizes and polymerizes to form HGA-melanin (Steinert et al., 2001). In contrast to HGA-melanin, synthetic HGA robustly inhibited Lm growth, both when spotted onto a lawn of Lm and when titrated into AYE rich media (Figure 3B, 3C). Because the inhibitory concentrations of synthetic HGA were relatively high (>50µM), we next asked whether these concentrations are biologically relevant. Compared to other Legionella spp., Lp produces much more pigment (Supplemental Figure 3B), suggesting that it secretes considerably more HGA. To estimate the quantity of secreted HGA, we used a standard curve of synthetic HGA. Following oxidation in AYE rich media for 24 hours, the amount of synthetic HGA-melanin pigment can be measured by OD 400 and compared to the color of oxidized Lp conditioned media to quantify the abundance of HGA (Supplemental Figure 5A). After 48h in culture, we infer that wild type Lp had secreted the equivalent of 1.7mM HGA, while the hyperpigmented ΔhmgA strain secreted about 2.6mM HGA. In contrast, we did not detect any pigment from the non-inhibitory hisC2::Tn strain (Supplemental figure 5B). Thus, HGA can be an abundant secreted metabolite of Lp, with a potential to accumulate to concentrations relevant for inter-Legionella inhibition.

We next asked which molecular features of HGA were relevant for its inhibitory activity. Two HGA-related molecules—2-hydroxyphenylacetic acid and 3-hydroxyphenylacetic acid—differ from HGA by only the removal of a single -OH group. Neither compound inhibited Lm growth at any concentration tested (Supplemental figure 6), suggesting that inhibition of Lm by HGA is relatively specific at the molecular level. We also considered the possibility that HGA as a weak acid could inhibit Lm indirectly by altering the local pH, but we observed that adding HGA at 1mM into AYE media or PBS caused little to no change in pH.

Another well-studied feature of HGA is its redox potential (Eslami, Namazian, & Zare, 2013). If HGA can auto-oxidize, it may cause growth inhibition by oxidizing other nearby molecules, either in the media or on bacterial cells. This scenario is consistent with our observation that oxidized HGA-melanin is inactive (Figure 3B, Supplemental
To test the specific hypothesis that HGA oxidizes and depletes nutrients in the media, we allowed synthetic HGA to oxidize completely for 24h in AYE media before adding Lm (Supplemental Figure 5A). If HGA acts by causing nutrient depletion, we expected that media pre-incubated with HGA would be unable to support normal Lm growth. Contrary to this hypothesis, we observed that Lm grew normally in all conditions and that the pre-oxidation completely abolished synthetic HGA activity (Figure 3D, compare to 3C). We can therefore reject the hypothesis that HGA activity results from indirect nutrient limitation, or any other modifications of the media. Instead, we infer that Lm inhibition results from direct interactions between bacterial cells and either HGA itself or unstable, reactive intermediates produced during HGA oxidation.

If the redox state of HGA is critical for inhibition, we reasoned that it should be possible to modulate HGA activity by altering the redox state of the media with reducing agents. We accomplished this by titrating L-cysteine from 25% to 200% of the levels in standard AYE media. In the absence of HGA, these altered cysteine concentrations had little impact on Lm growth (Supplemental Figure 4B). However, lower levels of cysteine greatly sensitized Lm to HGA, while excess cysteine was completely protective (Supplemental Figure 4B). In related experiments, incubation of HGA with reduced glutathione or DTT (dithiothreitol), two other reducing agents, similarly quenched HGA’s inhibitory activity (Supplementary Figure 4C). We conclude that HGA is less potent in rich media because it reacts with and oxidizes the excess cysteine (or other bystander molecules) before it can interact with Lm. Taken together, these results implicate HGA’s oxidizing activity and/or its oxidative intermediates in inter-Legionella inhibition.

*L. pneumophila* can be susceptible to its own inhibitor

Our results so far indicated that, while HGA can be a potent inhibitor, its activity appears to be volatile and capable of reacting with many types of molecules. If *Lp* uses HGA to compete with neighboring *Legionella* spp., we hypothesized that *Lp* would have some form of resistance to its secreted inhibitor. Therefore, we next tested *Lp* susceptibility to inhibition in low-cysteine conditions, as we had previously done for *Lm*. Surprisingly, we found that *Lp* was quite sensitive to inhibition by neighboring *Lp* that
was already growing on the plate (Figure 4A). Indeed, Lp susceptibility closely mirrored the susceptibility of Lm to inhibition (compare to Figure 1A), even though the bacteria secreting the inhibitor were genetically identical to the inhibited Lp. In both cases, we observed a sharp boundary at the edge of the zone of inhibition. In contrast, the “no zone” Lp strain hisC2::Tn did not generate a sharp zone of inhibition against neighboring Lp (Figure 4A), suggesting that the HGA-melanin pathway was responsible for both Lm inhibition and Lp inhibition. To determine whether the susceptibility of Lp to

![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 highly sensitive at lower density (10^7 CFU/mL or lower). Dotted line shows limit of detection.]


HGA was restricted to low-cysteine conditions, we grew *Lp* in the presence of synthetic HGA in rich media. Again, we found that the HGA was inhibitory, causing a growth delay in liquid cultures (Figure 4B). Notably, synthetic HGA was active against *Lp* at the same concentrations that were inhibitory to *Lm* (compare Figure 4B and 3C). These results are consistent with a potential role for HGA in both interspecies and intraspecies *Legionella* inhibition.

We initially found these results to be somewhat perplexing, as we had expected *Lp* to show some resistance to its own inhibitor. Why should *Lp* secrete large quantities of HGA and potentially inhibit its own growth if this process is non-essential (as evidenced by the robust growth of the *hisC2::Tn* mutant)? Although we had already estimated that wild type *Lp* can secrete enough HGA to generate abundant pigment (equivalent to 1.7mM, Supplemental Figure 5B), we did not know if this HGA was secreted all at once, or alternatively if it was released slowly over time. Under the latter scenario, the HGA might oxidize before it accumulated to inhibitory concentrations. To differentiate between these possibilities, we tracked HGA secretion across a growth curve of *Lp* in rich media, using HGA-melanin levels in fully oxidized media to infer the amount of secreted HGA. It has long been known that *Lp* produces abundant HGA-melanin pigment in stationary phase, when the bacteria are undergoing very slow or no growth (Figure 4C) (Berg, Hoff, Roberts, & Matin, 1985; Pine, George, Reeves, & Harrell, 1979; Wiater, Sadosky, & Shuman, 1994). By comparing to a synthetic HGA standard curve (Supplemental Figure 5), we estimate that *Lp* produces a burst of HGA in stationary phase, secreting over 250µM within 5 hours (Figure 4C). HGA secretion then continues after the population has ceased growing. These quantities of HGA should be more than enough to be inhibitory to *Lp* (Figure 4B).

Based on the timing of HGA secretion, we hypothesized that *Lp* might be more resistant to this inhibitor when at high density and/or when the cells are not actively growing. To test this hypothesis, we washed and diluted stationary-phase *Lp* in nutrient-free PBS with 125µM HGA and measured viability by CFUs at 0 and 24 hours. No measurable darkening of the HGA was detected in this assay, suggesting that the
oxidation and de-activation of HGA was considerably slowed in low-nutrient conditions. We found that Lp incubated at high density with HGA (above 10^8 CFU/mL) were largely protected from inhibition (Figure 4D). In contrast, when we diluted the same culture to lower density (below 10^7 CFU/mL), bacteria were extremely sensitive to HGA, with at least a 100,000-fold reduction in CFUs. Similarly, HGA did not inhibit Lp growth rich media when bacteria were inoculated at high density (Supplemental figure 7). Based on the substantial, density-dependent difference in HGA susceptibility, we wondered if Lp might regulate HGA resistance via quorum sensing. However, when we deleted lqsR, the putative quorum sensing response regulator from Lp (Tiaden et al., 2007), this had no detectable impact on HGA susceptibility (Supplemental figure 7). Therefore, the density-dependent susceptibility of Lp to HGA must be independent of this pathway.

Taken together, these results show that Lp can be highly susceptible to inhibition by adjacent, genetically identical bacteria via HGA. Similar to our findings in Lm, the potency of HGA-mediated inhibition is exacerbated in low nutrient conditions. We also observe that Lp secretes abundant HGA when in stationary phase and at high density. Because Lp at high density also appear to be protected from HGA, this strategy may restrict the potential for self-harm.

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

Our results suggested that Lp bacteria are sensitive to HGA at low density but resistant at high density. We next investigated the genetic basis of HGA susceptibility and resistance in Lp. First, we hypothesized that the HmgA-C proteins, which break down intracellular HGA and recycle it back into central metabolism, might also process and deactivate extracellular HGA (Rodriguez-Rojas et al., 2009) (Figure 2D). In growth curves of the ΔhmgA mutant with increasing concentrations of synthetic HGA, we found that its response to HGA was nearly identical to that of wild type Lp (Supplemental Figure 8). These results suggest that the intracellular recycling pathway does not play an appreciable role in Lp responses to HGA.
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 \textit{Lp} and \textit{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 \textit{Lp} plate selected with HGA, whereas only 3-5 colonies grew on \textit{Lm} plates selected with HGA, or on low-cysteine plates of \textit{Lp} or \textit{Lm} without HGA. Based on these results, we focused on HGA-selected mutants of \textit{Lp}. After retesting the phenotypes of spontaneous mutants on HGA + low-cysteine plates, we recovered 29 \textit{Lp} strains that grew significantly better than wild type \textit{Lp} (Figure 5A). In addition, all 29 strains grew

![Figure 5](image)

\textbf{Figure 5:} \textit{L. pneumophila} susceptibility to HGA is modulated by \textit{lpg1681}. **A** Growth of HGA-selected spontaneous mutants (*) compared to wild type \textit{Lp}. All isolates grew better than wild type in selection conditions (HGA + low cysteine), as well in low cysteine conditions. **B** Predicted structure of \textit{lpg1681}-containing operon in \textit{Lp} strains. \textit{lpg1681} is a hypothetical gene that lies downstream of \textit{lpg1682}, a predicted oxidoreductase/dehydrogenase, and upstream of \textit{dsbD2}, a thiol:disulfide interchange protein. **C** In rich media, a spontaneous \textit{lpg1681} mutant (\textit{lpg1681*}) and the \textit{lpg1681} deletion strain (\textit{Δlpg1681}) are less sensitive to growth inhibition by HGA than wild type \textit{Lp}, as seen by a shorter growth delay at each concentration of HGA. Overexpression of \textit{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.
better than wild type *Lp* on low-cysteine plates without HGA, raising the possibility that HGA susceptibility is related to cysteine metabolism and/or that the strains had adapted to low-cysteine conditions.

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                                                  |

* = introduction of a stop codon

To determine the underlying genetic basis of these phenotypes, we sequenced the genomes of all 29 strains plus our wild type strain of *Lp* to a median depth of 118x and identified mutations genome-wide. Each mutant strain carried 1 to 3 unique point mutations relative to the starting strain, and most of these mutations were found in a few shared loci (Table 1). Nineteen of the twenty-nine strains carried mutations in translation-related machinery, 5 had missense mutations in *secY* or *secD* (*lpg0349* and *lpg2001*), members of the translocon complex that moves polypeptides across the cytosolic membrane, 1 had a mutation in *aceE* pyruvate dehydrogenase (*lpg1504*), and 4 had mutations in a hypothetical gene, *lpg1681* (Figure 5B). We suspect that the translation-related mutations had highly pleiotropic effects on protein expression by disrupting elongation factor P function and thereby enhancing ribosome stalling at polyproline tracts (Doerfel et al., 2013; Marman, Mey, & Payne, 2014; Navarre et al., 2010; Yanagisawa, Sumida, Ishii, Takemoto, & Yokoyama, 2010). Based on the
frequency of polyprolines in the *Lp* proteome, disruptions to elongation factor P function have the potential to impact the expression of about 33% of *Lp* proteins. The phenotypes observed in these mutants could therefore result from either a large scale shift in *Lp* gene expression, or from the altered expression of specific susceptibility genes.

Given that all the HGA-selected mutants grew better than wild type *Lp* on both low-cysteine plates and on low-cysteine plates with HGA overlays, we asked whether the mutants had specific resistance to HGA, if they had primarily adapted to low-cysteine conditions, or if they had pleiotropic mutations that broadly impacted cell growth. Growth curves of representative HGA-selected mutants with or without HGA in cysteine-rich AYE media showed that *lpg0288* and *aceE* mutants exhibited some growth defects in cysteine-rich media, consistent with potential pleiotropic impacts on the cell (Supplemental Figure 8E). Mutants in *lpg1681*, *secD*, and *secY* had no growth defects and also showed a reproducible decrease in HGA susceptibility (Figure 5C, Supplemental Figure 8E). Of these, *lpg1681* mutants showed the largest improvement in growth in the presence of HGA.

The hypothetical gene *lpg1681* is predicted to encode a small, 105 amino acid protein with no predicted domains apart from two transmembrane helices. This gene is adjacent in the genome to *lpg1682*, a predicted oxidoreductase/dehydrogenase, and *lpg1680*, the thiol:disulfide exchange protein DsbD2 (Figure 5B). Functional studies of DsbD2 (aka DiSulfide Bond reductase D2) have demonstrated that it interacts with thioredoxin to regulate disulfide bond remodeling in the periplasm (Inaba, 2009; Kpadeh, Day, Mills, & Hoffman, 2015). If *lpg1681* has a redox function related to its neighboring genes, we expected its syntenic locus to be conserved across bacterial strains and species. We found that the *lpg1680-1682* locus is present and conserved among over 500 sequenced *Lp* strains currently in NCBI databases. Outside of *L. pneumophila*, *lpg1681* is mostly restricted to the *Legionella* genus, present in about half of the currently sequenced species (Burstein et al., 2016) (Supplemental Figure 9). A homolog of *lpg1681* is also found in the draft genome of *Piscirickettsia litoralis*, a gamma
proteobacterium and fish pathogen (Wan et al., 2016). In all cases, *lpg1681* resides upstream of *dsbD2*, suggesting a functional link between these proteins (Supplemental Figure 9).

Because *lpg1681* appears to reside within an operon with redox-related genes and HGA activity is linked to its redox potential, we viewed *lpg1681* as a promising candidate for a gene involved in HGA susceptibility. We constructed *lpg1681* overexpression and deletion strains in *Lp* and tested the susceptibility of these strains to HGA. Similar to the spontaneous mutants we recovered, the Δ*lpg1681* strain was more resistant to HGA in rich media (Figure 5C). Conversely, overexpression of *lpg1681* increased *Lp* sensitivity, resulting in longer growth delays than wild type at high concentrations of HGA. We therefore conclude that wild type *lpg1681* expression sensitizes *Lp* to inhibition by extracellular HGA.

**Discussion**

In this study, we identify HGA as a mediator of inter-*Legionella* inhibition. Furthermore, we find that inter-*Legionella* inhibition and surfactant production are separate, but co-occurring, phenomena. Although surfactant is not required for inhibition, we hypothesize that surfactant may enhance the spread of HGA and/or its reactive intermediates away from the secreting *Lp* bacteria, which may explain previous results (Stewart et al., 2011).

The HGA-melanin pathway is well-studied and widespread among bacteria and eukaryotes, so we were surprised to find a previously undescribed role for HGA in interbacterial competition. Somewhat paradoxically, HGA-melanin production in bacteria has previously been implicated both in the production of and protection from reactive oxygen species (production: (Noorian et al., 2017); protection: (Keith, Killip, He, Moran, & Valvano, 2007; Orlandi, Bolognese, Chiodaroli, Tolker-Nielsen, & Barbieri, 2015)). Based on our results, we hypothesize that HGA may be responsible for oxidative damage, while the HGA-melanin pigment serves other, beneficial roles. The redox-
based inhibition that we observe for HGA is analogous to that of pyocyanin from *Pseudomonas aeruginosa* and similar phenazine pigments, which are also produced at high cell density (Baron, Terranova, & Rowe, 1989; Hassan & Fridovich, 1980). One major difference is that pyocyanin impacts an extremely broad range of species, including diverse bacteria and eukaryotes (Baron et al., 1989; Kerr et al., 1999; Noto, Burns, Beavers, & Skaar, 2017). In contrast, previous work found that *Lp* secretions can inhibit *Legionella spp.*, but have little impact on other bacterial taxa (Stewart et al., 2011). We speculate that the observed specificity arises simply because *Legionella spp.* are quite sensitive to oxidative stress relative to other bacteria (Domingue, Tyndall, Mayberry, & Pancorbo, 1988; Hoffman, Pine, & Bell, 1983). Nevertheless, because the ecological niche of *Lp* appears to overlap with other *Legionella* strains and species (Pereira et al., 2017; Wery et al., 2008), HGA-mediated inter-*Legionella* inhibition has a strong potential to impact the success of *Lp* in both natural and manmade environments.

Another major difference between phenazines and HGA is that phenazines have not been reported to be self-active, because the phenazine-producing bacteria are protected by their antioxidant defenses (Hassett, Charniga, Bean, Ohman, & Cohen, 1992). However, self-active effects can be difficult to observe, particularly if bacteria at high cell density are resistant. In our hands, intraspecies inhibition by HGA was apparent only in assays competing high-density populations with low-density, invading competitors. Furthermore, we note that the inhibitory activity of molecules like HGA is transient and is quenched by the cysteine-rich media typically used to grow *Legionella* in the lab (Figure 3D, Supplemental Figure 4). This may explain why HGA-mediated inhibition of *Legionella* has not been previously reported. Still, our complementary inhibition assays performed in rich media, low-cysteine media, and nutrient-free media suggest that HGA inhibition can occur across environmental settings, and only becomes more potent as conditions approach the nutrient-limited, slow growth conditions that *Legionella* likely experience within biofilms.
In many modes of interbacterial competition, organisms that produce toxic molecules also possess resistance genes to avoid self-targeting. It was therefore initially perplexing that \textit{Lp} can produce a self-active inhibitor, particularly given that genes for HGA production and the HGA-sensitizing gene \textit{lpg1681} are both non-essential under laboratory conditions. One possible explanation is that the benefits of HGA or HGA-melanin for \textit{Lp} outweigh the self-harm of HGA. These benefits could include antagonism against other \textit{Legionella} as demonstrated here, or previously described benefits of HGA-melanin, including UV protection and iron reduction (Chatfield & Cianciotto, 2007; Steinert et al., 1995). Antagonism against eukaryotic cells may be another benefit; the \textit{hpd} gene in the \textit{Lp} HGA synthesis pathway was originally named \textit{ll}y, or legiolysin, because expression of the gene in \textit{E. coli} caused erythrocyte lysis on blood agar plates (Wintermeyer et al., 1994). Finally, the redox activity of HGA may play a beneficial role in \textit{Legionella} gene expression and/or anaerobic respiration within biofilms, as is the case for pyocyanin and other phenazines in \textit{Pseudomonas} (Mavrodi et al., 2013).

Beyond these benefits, the context and timing of HGA secretion may constrain the potential for self-harm. In laboratory conditions, HGA secretion occurs primarily in late stationary phase, when cells are at high density (~10^9 CFU/mL) (Figure 4C). Because \textit{Lp} at high density are also more resistant to HGA (Figure 4D), \textit{Lp} self-protection apparently involves a definition of “self” based on growth phase, density, and/or regulatory state, rather than one based on the presence of genetically-encoded resistance genes. Consistent with this idea, our selection scheme to identify genes involved in HGA resistance (Figure 5) recovered mostly pleiotropic defects in translation, in addition to \textit{lpg1681}, which enhances \textit{Lp} susceptibility to HGA (Table 2). It may therefore be difficult for \textit{Lp} to evolve resistance to HGA at low cell densities without altering other important cellular functions.
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.
Materials and Methods

Bacterial strains and growth conditions The bacterial strains and plasmids used for this study are listed in Table 3. As our wild type Legionella pneumophila (Lp) strain, we used KS79, which was derived from JR32 and ultimately from isolate Philadelphia-1 (de Felipe et al., 2008; Rao, Benhabib, & Ensminger, 2013; Sadosky, Wiater, & Shuman, 1993). Compared to JR32, the KS79 strain has a comR deletion to enable genetic manipulation (de Felipe et al., 2008). We used Legionella micdadei (Lm) tatlock as our susceptible strain (Garrity, Brown, & Vickers, 1980). Liquid cultures of Legionella were grown shaking in AYE liquid media at 37˚C (De Jesús, O'Connor, & Isberg, 2013). To manipulate the redox state of AYE, we altered the amount of cysteine added to the media from 0.4 g/L in standard AYE to 0.1, 0.2, and 0.8 g/L. On solid media, Legionella were grown either on BCYE agar plates either containing the standard cysteine concentration (0.4g/L) or in “low cysteine” conditions (0.05g/L) (Feeley et al., 1979; Solomon & Isberg, 2000). E. coli strains used for cloning were grown in LB media.

Where indicated, antibiotics were used at the following concentrations in solid and liquid media, respectively; chloramphenicol (5 µg/mL and 2.5 µg/mL), kanamycin (40 µg/mL) and ampicillin (50 µg/mL and 25 µg/mL). For counter-selection steps while generating deletion strains, 5% sucrose was added to BCYE plates. For top agar and overlay experiments, we used 0.7% agar dissolved in water, which was kept liquid at 50˚C before pouring over BCYE plates.

Gene deletions and complementations Genomic knockouts in L. pneumophila were generated as previously described (Wiater et al., 1994). Briefly, we used an allelic exchange plasmid (pLAW344) harboring chloramphenicol and ampicillin selection cassettes and the counter-selection marker SacB, which confers sensitivity to sucrose. Into this plasmid, we cloned ~1kb regions upstream and downstream of the gene of interest to enable homologous recombination. Following electroporation and selection on chloramphenicol, we used colony PCR to verify insertion of the plasmid into the chromosome, before counter-selection on sucrose media. From the resulting colonies, we performed PCR and Sanger sequencing to verify clean gene deletion. For
complementation, the coding region of a candidate gene was cloned into a plasmid (pMMB207c) following a ptac promoter (J. Chen et al., 2004). To induce gene expression, strains carrying pMMB207c-derived plasmids were exposed to 1mM IPTG. All constructs were assembled using Gibson cloning (NEB Catalog #E2621).

**Inhibition assays on agar plates** To visualize inhibition between neighboring *Legionella* on solid media, a streak of approximately 5 x 10^6 CFU of the inhibitory strain of *Lp* was plated across the center of a low-cysteine BCYE plate. After 3 days growth at 37°C, dilutions of susceptible *Lp* or *Lm* were plated as 10µL spots approximately 1 cm and >2 cm from the central line. Once spots were dry, plates were then incubated for an additional 3 days before scoring for inhibition. This assay was also used to quantify the bactericidal inhibition of *Lm*, with slight modifications. Here, all *Lm* was plated in 20µL spots at 10^6 CFU/mL. The time of plating susceptible *Lm* was treated as t=0. Once spots were dry, plugs were extracted from within the *Lm* spots using the narrow end of a Pasteur pipette. These plugs were transferred into media, vortexed, and plated to quantify CFU. This procedure was repeated after 48h at 37°C to compare *Lm* viability and growth within (“near”, Figure 1B-C) or outside (“far”) of the zone of inhibition.

For inhibition assays on bacterial lawns, we plated 10µL drops of either live *Lp* or chemical compounds on top of a lawn of 5 x 10^7 CFU *Lm* on low-cysteine BCYE, and assessed growth of the lawn after 3 days at 37°C. Synthetic HGA (Sigma: #H0751) was dissolved in water at a concentration of 100 mM and filter sterilized before use. HGA-related compounds, 2-hydroxyphenylacetic acid (Sigma: #H49804) and 3-hydroxyphenylacetic acid (Sigma: #H49901), were prepared in the same way. To test the impact of DTT (Sigma: #43819) and glutathione (oxidized: Sigma #G4376 , reduced: Sigma #G6529) on HGA-mediated inhibition, filter-sterilized solutions dissolved in water were mixed in equimolar ratios with HGA, and incubated shaking at room temperature for 1 hour before spotting onto bacterial lawns. HGA-melanin pigment was prepared from *Lp* conditioned media as previously described (Zheng, Chatfield, Liles, & Cianciotto, 2013) from KS79, the unpigmented *hisC2::Tn* mutant, and the hyperpigmented ∆hmGA mutant. Briefly, conditioned media was collected and sterile
filtered from 100 mL cultures of *Lp* in AYE media grown shaking at 37°C for 3 days. The conditioned media was acidified to a pH of 1.5 and transferred to 4°C for 2 hours to precipitate. Precipitated pigment was collected by centrifugation at 4000 x g for 15 minutes and then washed with 10 mM HCl. Pelleted pigment was then returned to neutral pH and resuspended in PBS at 10X before testing.

**Transposon mutagenesis screen** For random transposon insertion mutagenesis, we used a Mariner transposon from the pTO100 plasmid (O’Connor et al., 2011). We electroporated this plasmid into the KS79 strain and allowed cells to recover at 37°C for 5 hours. To select for cells with integrated transposons, cultures were plated on BCYE/Kan/sucrose plates and incubated at 37°C for 3 days before screening individual mutant colonies.

To identify transposon mutants with defects in *Lm* inhibition, we transferred each *Lp* mutant onto a low-cysteine plate with a lawn of 5 x 10^7 CFU *Lm* and visually screened for those with either small zones of inhibition or no zone of inhibition. Mutants were added on top of the *Lm*, either by replica plating with sterile Whatman paper (Whatman: #1001150) or by transferring with a sterile toothpick. Plates were then incubated at 37°C for 3 days and scored. All putative mutants underwent clonal re-isolation, were diluted to OD 600 of 0.1, and spotted on *Lm* lawns to retest their phenotypes. To map the sites of transposon integration, we used arbitrary PCR as described in (T. Chen, Yong, Dong, & Duncan, 1999), with primers redesigned to work with the pTO100 transposon (Table 3). Briefly, this protocol involved two PCR steps to amplify the DNA flanking the transposon. The first step used low annealing temperatures to allow the arb1 randomized primer to bind many sites in the flanking DNA while the pTO100_F or pTO100_R primer annealed within the transposon, generating multiple products that overlapped the flanking DNA. These products were amplified in the second step PCR using the arb2 and pTO100_Rd2 primers, and we used the pTO100_Rd2 primer for Sanger sequencing. PCR programs and conditions were as in (T. Chen et al., 1999).
**HGA inhibition assays in liquid cultures** For rich media assays (e.g. Figures 3C, 3D, 4B), overnight cultures of *Legionella* were diluted to $10^8$ CFU/mL in AYE, mixed with synthetic HGA or pigment in 96 well plates, and grown shaking at 425 cpm at 37˚C. The cytation 3 imaging reader (BioTek™ CYT3MV) was used to monitor growth by OD 600 measurements. Because oxidized pigment from synthetic HGA is detected at OD 600, each experiment included bacteria-free control wells containing media and each concentration of HGA. To correct OD 600 readings for pigment development, at each time point we subtracted the control well reading from bacterial wells that received the same concentration of synthetic HGA. For experiments with HGA “pre-oxidation” (Figure 3D), we diluted HGA in AYE media and incubated this solution shaking at 37˚C for 24 hours in the plate reader before adding bacteria. Complete oxidation of HGA during the 24 hours was monitored using OD400 to track the accumulation of HGA-melanin pigment (Supplemental figure 5). In *Lp*, HGA inhibition in AYE rich media resulted in a growth delay, similar to an extended lag phase (Figure 4B). To compare sensitivity to HGA among *Lp* strains, we calculated the lag phase from the growth curve of each well using the GrowthRates program (Hall, Acar, Nandipati, & Barlow, 2014). We excluded a small number of samples where the growth curve was not well fit (R<0.99), and then for each strain used the difference in lag time between the samples with and without HGA to calculate the growth delay due to HGA (Figures 5C).

As a complementary assay, we evaluated *Legionella* viability when exposed to HGA in nutrient-free PBS at different cell densities. Stationary phase cultures were washed once and re-suspended in PBS. We diluted these bacteria to estimated starting concentrations of $10^9$, $10^8$, and $10^7$ CFU/mL and plated for CFU at t=0. We distributed the remaining bacteria into 96 well plates with or without 125 µM HGA. Plates were incubated shaking in a plate reader at 425 cpm at 37˚C for 24 hours before plating to quantify CFU on BCYE plates. CFU were counted after 3-4 days growth at 37˚C.

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

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

Twenty-nine isolates were more resistant to HGA than wild type *Lp* upon retesting. We sequenced and analyzed genomic DNA from these isolates and a matched wild type strain as follows. DNA was prepared from each strain using a
Purelink genomic DNA mini kit (Invitrogen, #K1820). DNA concentrations were quantified using Qubit and normalized to 0.5 ng/μL. Barcoded libraries were prepared using tagmentation according to Baym et al. 2015 (Baym et al., 2015), analyzed with QuantIT DNA quantification, pooled, and sequenced with 50 bp paired-end reads on an Illumina HiSeq 2500 machine. Reads were trimmed for quality and to remove Nextera indices with Trimmomatic (Bolger, Lohse, & Usadel, 2014) and mapped to the Philadelphia-1 genome (Chien et al., 2004) using Bowtie2 with default parameters (Langmead, Trapnell, Pop, & Salzberg, 2009). Coverage plots were generated for each strain using bamcoverage (Ramírez et al., 2016) and visually examined for evidence of large genomic deletions and amplifications. None were observed, apart from a prophage that was present in the reference genome but missing from all sequenced strains, including our wild type KS79 strain. Variants were detected for each mutant using Naive Variant Caller (Blankenberg D, et al. In preparation). Those variants that were detected in mutant strains but not the wild type strain were considered as putative causative mutations. For each of these mutations, we inspected the mapped reads and excluded faulty variant calls that either were adjacent to coverage gaps or that did not appear to be fixed in the clonal mutant and/or wild type sequences, likely due to errors in read mapping. After this manual filtering, 1-3 well-supported mutations remained for each mutant genome. Nine of the mutants had undergone selection on a different day from the other mutants: in addition to various unshared mutations, these nine strains each carried exactly the same missense mutation in rplX, which we disregarded as a background mutation that likely arose before selection. Following this exclusion, each mutant carried only a single well-supported mutation in a coding region. Most often this coding mutation was the only mutation we detected, although one mutant carried two additional intergenic point mutations. The coding mutations were point mutations or small deletions that resulted in non-synonymous changes, frame shifts, or gene extensions. Across different mutants, the mutations we uncovered were repeatedly found in the same, few loci (Table 1).

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