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ARTICLE

Quorum sensing controls persistence, resuscitation, and virulence of *Legionella* subpopulations in biofilms

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Abstract

The water-borne bacterium *Legionella pneumophila* is the causative agent of Legionnaires' disease. In the environment, the opportunistic pathogen colonizes different niches, including free-living protozoa and biofilms. The physiological state(s) of sessile *Legionella* in biofilms and their functional consequences are not well understood. Using single-cell techniques and fluorescent growth rate probes as well as promoter reporters, we show here that sessile *L. pneumophila* exhibits phenotypic heterogeneity and adopts growing and nongrowing ("dormant") states in biofilms and microcolonies. Phenotypic heterogeneity is controlled by the *Legionella* quorum sensing (Lqs) system, the transcription factor LvbR, and the temperature. The Lqs system and LvbR determine the ratio between growing and nongrowing sessile subpopulations, as well as the frequency of growth resumption ("resuscitation") and microcolony formation of individual bacteria. Nongrowing *L. pneumophila* cells are metabolically active, express virulence genes and show tolerance toward antibiotics. Therefore, these sessile nongrowers are persisters. Taken together, the Lqs system, LvbR and the temperature control the phenotypic heterogeneity of sessile *L. pneumophila*, and these factors regulate the formation of a distinct subpopulation of nongrowing, antibiotic tolerant, virulent persisters. Hence, the biofilm niche of *L. pneumophila* has a profound impact on the ecology and virulence of this opportunistic pathogen.

Introduction

The Gram-negative bacterium *Legionella pneumophila* ubiquitously occurs in natural as well as anthropogenic water systems and upon inhalation causes a severe pneumonia termed Legionnaires' disease [1, 2]. In the environment, the opportunistic pathogenic bacterium infects and replicates within free-living protozoa, including amoebae and ciliates [3–7]. The mechanism of intracellular replication is evolutionarily conserved, requires the *L. pneumophila* Icm/Dot type IV secretion system (T4SS) and centers on the formation of a unique membrane-bound replication niche, the *Legionella*-containing vacuole (LCV) [8–13]. *L. pneumophila* is a facultative intracellular bacterium, which as an alternative niche also colonizes and forms biofilms in the environment [14–17], as well as under laboratory conditions [18–20].

Environmental bacteria face nutrient shortage and substantial physicochemical fluctuations, which they can respond to by arresting growth [21]. Growth arrest has a profound impact on bacterial physiology and traits, including stress resistance and an augmented tolerance toward antibiotics, called persistence [22, 23]. Pathogenic bacterial persisters are responsible for antibiotic treatment failures in clinical settings and cause relapsing infections [24–26]. However, bacterial persistence predates the clinical use of antibiotics, as *L. pneumophila* reversibly forms highly virulent nonreplicating persisters upon infection of primordial protozoan phagocytes, e.g., *Acanthamoeba castellanii* or *Dictyostelium discoideum* [27].

L. pneumophila adopts a biphasic lifestyle and cycles between a transmissive (growth-arrested, virulent and motile) and a replicative form [28]. The reversible switch between the transmissive and the replicative phase is controlled by the

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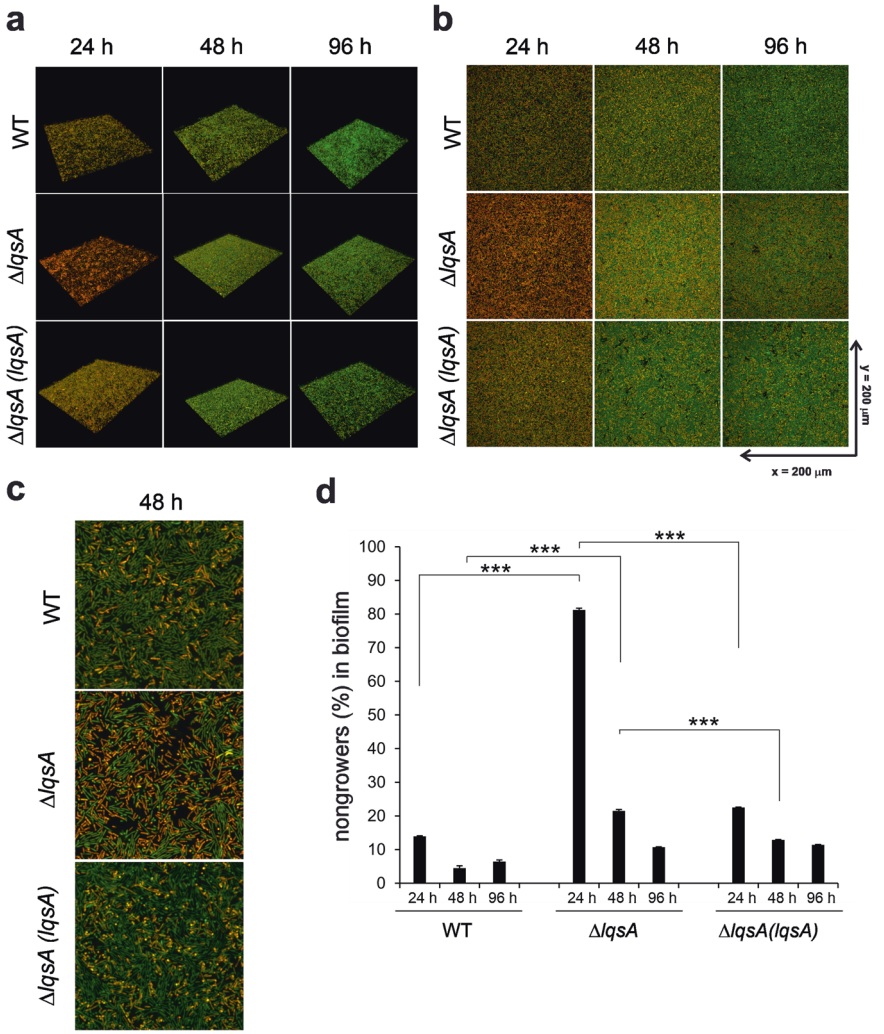
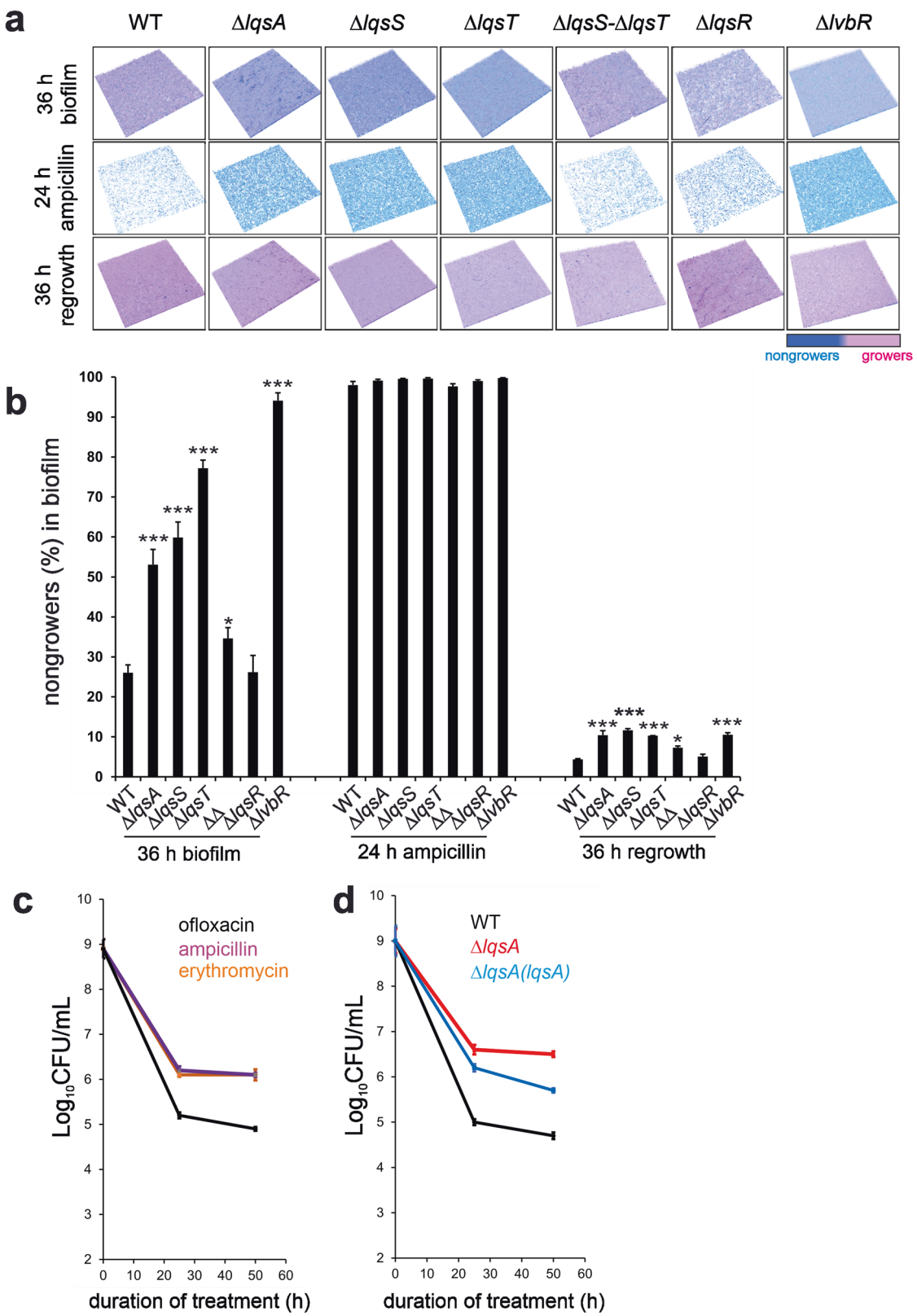


Fig. 1 The Lqs system regulates the ratio of nongrowers in *L. pneumophila* biofilms. a–c *L. pneumophila* JR32 or Δ*lqsA* producing Timer (pNP107) or the complemented strain Δ*lqsA*(*lqsA*) producing Timer and LqsA (pNP120) were grown to stationary phase, diluted in AYE broth, and allowed to form biofilms at 25 °C. At the given time points, the Timer color ratio (500 nm/600 nm) was visualized by confocal microscopy. **a** 3D reconstruction ($x = 200$ µm, $y = 200$ µm, $z = 30$ µm), **b** orthogonal view, and **c** magnification are shown. **d** Biofilms formed after 24 h by Timer-producing *L. pneumophila* JR32, Δ*lqsA* or Δ*lqsA*(*lqsA*) were homogenized and analyzed by flow cytometry, and the subpopulation of nongrowing *L. pneumophila* was quantified. Data represent the mean ± SEM of three biological replicates (two-tailed Student's *t* test; $***P < 0.001$). Source data are provided as a Source Data file.

"stringent response" and the second messenger guanosine 3,5-bispyrophosphate (ppGpp) [29, 30], as well as by the *Legionella* quorum sensing (Lqs) system [31, 32]. Components of the Lqs system comprise the autoinducer synthase LqsA, which produces the α-hydroxyketone signaling molecule *Legionella* autoinducer-1 (LAI-1, 3-hydroxypentadecane-4-one) [33], the membrane-bound sensor histidine kinases LqsS [34] and LqsT [35], as well as the prototypic response regulator LqsR [36, 37], which dimerizes upon phosphorylation [38, 39]. While *L. pneumophila* lacking the autoinducer synthase gene *lqsA* (Δ*lqsA*) is barely defective for virulence, the Δ*lqsR* mutant strain shows severe virulence and other phenotypes [32]. The Lqs system is linked to the cyclic-di-GMP signaling network through the transcription factor LvbR [40, 41]. LvbR is a pleiotropic transcription factor, which is negatively regulated by the sensor kinase LqsS, and directly controls the production of proteins involved in c-di-GMP metabolism as well as the architecture of *L. pneumophila* biofilms and pathogen–host cell interactions.

The Lqs system is a major regulator of various *L. pneumophila* traits, including the switch from the -transmissive/virulent to the replicative phase [36], pathogen–host cell interactions [37], bacterial and host cell motility [38, 42], natural competence for DNA uptake [35], as well as extracellular filament formation and expression of a chromosomal "fitness island" [34]. Moreover, the Lqs system and in particular the autoinducer LAI-1 strongly induces the expression of the noncoding small regulatory RNA (sRNA) *6S RNA* [38]. The conserved *6S RNA* is a global regulator of transcription, which in *L. pneumophila* is abundantly produced in the postexponential growth phase and regulates the expression of *icm/dot* T4SS genes, as well as factors implicated in stress response or nutrient acquisition [43, 44].

While intracellular growth of *L. pneumophila* and LCV formation has been intensely studied, the physiology and ecological significance of extracellular, sessile bacteria are poorly understood. In this study, we investigate on a



single-cell level the physiology and biphasic lifestyle of sessile *L. pneumophila*. We show that *L. pneumophila* exhibits phenotypic heterogeneity in biofilms and microcolonies, and—controlled by the Lqs system, the transcription factor LvbR and the temperature—forms subpopulations of growing and nongrowing bacteria.

◀ **Fig. 2 Nongrowing *L. pneumophila* in biofilms are persisters. a** 3D reconstruction of biofilms and **b** quantification of nongrowers by flow cytometry of Timer-producing *L. pneumophila* JR32, Δ*lqsA*, Δ*lqsS*, Δ*lqsT*, Δ*lqsS* Δ*lqsT* (ΔΔ), Δ*lqsR*, or Δ*lvbR* grown for 36 h (green = magenta, red = cyan). After 36 h, biofilms were treated with ampicillin (100 μg mL^{-1}, 24 h), washed and regrown for 36 h. Biofilms formed for 48 h by Timer-producing *L. pneumophila* were treated for the time indicated with erythromycin (60 μg mL^{-1}; orange), ampicillin (100 μg mL^{-1}; magenta), or ofloxacin (30 μg mL^{-1}; black) using **c** the strain JR32, or **d** ofloxacin and the strains JR32 (black), Δ*lqsA* (red), or complemented Δ*lqsA* (cyan). Bacteria were incubated at 25 °C and plated at defined time points to quantify CFU. Data represent the mean ± SEM of three biological replicates (two-tailed Student's *t* test; $*P < 0.05$, $***P < 0.001$).

Nongrowing individuals are metabolically active, highly virulent, and tolerant toward antibiotics. Therefore, these sessile nongrowers are virulent persisters, which likely have a profound impact on the ecology and pathogenicity of *L. pneumophila*.

Results

The Lqs system regulates the ratio of nongrowers in *L. pneumophila* biofilms

L. pneumophila forms biofilms upon undisturbed growth over several days [18, 40]. The physiology and functional features of sessile *L. pneumophila* in biofilms are poorly characterized. To investigate the traits of sessile *L. pneumophila*, we employed the Timer reporter, which allows to monitor the bacterial growth rate and to discriminate growing from nongrowing bacteria [24, 27]. Timer is a stable DsRed variant, which slowly changes its fluorescence from green (500 nm) to red (600 nm), and thus, the fluorescence ratio [500 nm (green)/600 nm (red)] accurately reflects the growth rate of *L. pneumophila* at a single-cell level [27]. Stationary phase Timer-producing *L. pneumophila* uniformly shows the characteristic red fluorescence of nongrowing bacteria (Fig. S1a, b).

To assess growth rate heterogeneity within *L. pneumophila* biofilms and a possible role of the Lqs system for the process, we used Timer-producing *L. pneumophila* wild-type, Δ*lqsA* and a complemented Δ*lqsA* mutant strain (Fig. 1). Stationary phase bacteria were diluted in medium, and biofilm formation as well as bacterial growth rate (Timer color ratio, 500 nm/600 nm) was monitored by confocal microscopy after 24, 48 and 96 h growth at 25 °C. After 24 h, wild-type *L. pneumophila*/Timer formed a biofilm comprising interspersed growing (green) and nongrowing (red/orange) bacteria. The size of the initially substantial nongrowing subpopulation was considerably smaller in biofilms after 48 and 96 h. Interestingly, in biofilms formed by the Δ*lqsA* mutant strain, the portion of nongrowing bacteria was dramatically increased, and this phenotype was reverted upon providing the *lqsA* gene on a plasmid (Fig. 1a–c).

To quantify the percentage of nongrowing sessile *L. pneumophila*, the biofilms formed by Timer-producing wild-type *L. pneumophila*, Δ*lqsA* or complemented Δ*lqsA*, were homogenized by pipetting and analyzed by flow cytometry (Fig. 1d). This approach revealed for wild-type *L. pneumophila* ca. 15% nongrowers in the biofilm after 24 h of growth, which decreased to ca. 5% after 48 h of growth. Strikingly, biofilms formed by Δ*lqsA* mutant bacteria comprised more than 80% nongrowing bacteria after 24 h, which decreased to ca. 20% after 48 h (Fig. 1d). The phenotype was reverted to almost wild-type levels upon providing the *lqsA* gene on a plasmid. Taken together, the findings demonstrate that growing and nongrowing individuals co-exist in *L. pneumophila* biofilms at different ratios over time, and the Lqs system contributes to the regulation of the subpopulation ratio.

Nongrowing *L. pneumophila* in biofilms are persisters

Nongrowing bacteria can show increased tolerance toward antibiotics, a phenomenon termed persistence [23–25, 27, 45]. Accordingly, we assessed whether persistence occurs in *L. pneumophila* biofilms and how the formation of sessile *L. pneumophila* persisters is controlled. To this end, stationary phase Timer-producing *L. pneumophila* wild-type or Δ*lqsA*, Δ*lqsS*, Δ*lqsT*, Δ*lqsS*-Δ*lqsT*, or Δ*lqsR* mutants were diluted in medium, and biofilm formation (Timer color ratio, 500 nm/600 nm) was monitored by confocal microscopy after 36 h at 25 °C (Figs. 2a and S2a). At this time point, *L. pneumophila* wild-type and the Δ*lqsS*-Δ*lqsT* as well as the Δ*lqsR* mutant strains formed biofilms comprising mostly growing (green fluorescent = magenta) bacteria. In contrast, the Δ*lqsA*, Δ*lqsS*, and Δ*lqsT* mutant strains formed biofilms with a high ratio of nongrowing (orange/red fluorescent = cyan) *L. pneumophila*.

Furthermore, we tested an *L. pneumophila* strain lacking the transcription factor LvbR, which forms a more "mat"-like biofilm compared to the more patchy biofilm architecture of the parental strain [40]. Interestingly, the Δ*lvbR* mutant strain formed biofilms comprising a largely increased population of nongrowing *L. pneumophila* (Fig. 2a), indicating that the transcription factor also regulates heterogeneity. Taken together, the formation of sessile *L. pneumophila* nongrowers is controlled by the Lqs system as well as by the transcription factor LvbR.

To assess the antibiotic tolerance of the *L. pneumophila lqs* mutant strains and correlate this feature to the growing and nongrowing subpopulations, the biofilms were treated with ampicillin (100× MIC) for 24 h, washed and let regrow

in AYE medium for another 36 h (Figs. 2a and S2a). This approach indicated that ampicillin killed a large portion of wild-type *L. pneumophila* and Δ*lqsS*-Δ*lqsT* as well as Δ*lqsR* mutant bacteria, and affected to a smaller extent Δ*lqsA*, Δ*lqsS*, Δ*lqsT*, and Δ*lvbR* mutant bacteria, which produced a large nongrowing subpopulation in biofilms. The nongrowing bacteria spared by ampicillin regrew upon removal of the antibiotic, and therefore, the nongrowing subpopulation is viable and culturable.

Quantification by flow cytometry validated these findings (Fig. 2b). Biofilms formed by *L. pneumophila* wild-type or the Δ*lqsS*-Δ*lqsT* or Δ*lqsR* mutant strains comprised ca. 25–35% nongrowing bacteria, while biofilms formed by the Δ*lqsA*, Δ*lqsS* or Δ*lqsT* mutant strains comprised ca. 55–80% nongrowing bacteria. Strikingly, biofilms formed by the Δ*lvbR* mutant strain comprised almost only nongrowing bacteria. The treatment with ampicillin (100× MIC) for 24 h left only nongrowing *L. pneumophila*, as documented by a homogenous low Timer color ratio (orange/red fluorescence = cyan). Following the antibiotic treatment, the *L. pneumophila* strains resumed growth, as evidenced by a high Timer color ratio (green fluorescence = magenta), and the ratio of nongrowers formed by the different *L. pneumophila* strains correlated with the ratio observed prior to the antibiotic treatment. Biofilm formation was also assessed by CFU, which indicated that compared to wild-type *L. pneumophila* the Δ*lqsA* mutant produced less biofilm at 36 h (Fig. S2b). Therefore, the fraction of persisters is even higher for the Δ*lqsA* strain when normalized for the number of bacteria present before drug treatment. The final biomass of the biofilms produced by wild-type or *lqs* mutant *L. pneumophila* was comparable.

Antibiotic tolerance and persistence (as opposed to resistance) is typically characterized by biphasic killing curves [45, 46]. To test whether sessile *L. pneumophila* strains show antibiotic tolerance and persistence, stationary phase Timer-producing wild-type *L. pneumophila* was diluted in medium, allowed to form a biofilm, and exposed to antibiotics (Fig. 2c). After 2 days at 25 °C, sessile bacteria were treated with different classes of antibiotics (e.g., ampicillin, ofloxacin, or erythromycin) at a concentration of 100× MIC and plated at given time points to quantify CFU. Under these conditions, biphasic killing kinetics of *L. pneumophila* were observed for all antibiotics tested, consistent with the notion that a subpopulation rapidly died, while another subpopulation persisted for a longer period of time.

In analogous assays, the effect of the Lqs system on the persistence of *L. pneumophila* was tested. To this end, stationary phase Timer-producing wild-type *L. pneumophila*, Δ*lqsA* or complemented Δ*lqsA* was diluted in medium, allowed to form a biofilm and exposed to ofloxacin (Fig. 2d), which among the antibiotics tested most potently killed *L. pneumophila*. The deletion of *lqsA* increased the persistence of *L. pneumophila*, and the complemented strain partially restored the sensitivity to the antibiotic. The degree of persistence was proportional to the size of the nongrowing subpopulation formed by these strains during biofilm formation.

In agreement with a role for the Lqs system in stationary growth phase of *L. pneumophila* (high cell density and LAI-1 concentration), exponentially growing cultures of wild-type or Δ*lqsA* mutant bacteria produced in biofilms a smaller subpopulation of nongrowers (Fig. S3a–c) and displayed a poor persistence toward ofloxacin (Fig. S3d). In summary, the Lqs system and the transcription factor LvbR modulate the ratio between sessile populations of replicating and nonreplicating *L. pneumophila*, the latter of which are persisters that are tolerant toward different classes of antibiotics. Persistence of *L. pneumophila* is proportional to the size of the nongrowing subpopulation.

The Lqs system and LvbR control resuscitation of sessile *L. pneumophila*

Having established that *L. pneumophila* adopts phenotypic heterogeneity in biofilms and forms a subpopulation of nonreplicating persisters, we next analyzed the growth resumption ("resuscitation") of sessile nongrowers. To this end, we developed a protocol to immobilize individual *L. pneumophila* in imaging chambers by agarose embedment [47]. This approach allows assessing traits of sessile *L. pneumophila* and their control on a single-cell level with high spatial resolution (Fig. 3).

Stationary phase Timer-producing *L. pneumophila* wild-type or Δ*lqsA* mutant strains were immobilized in agarose, and microcolony formation was recorded by time lapse confocal microscopy over 40 h (Fig. 3a, Supplementary Movies 1 and 2). After 24 h, ~60% of the red fluorescent growth-arrested wild-type bacteria had resumed growth and formed green fluorescent microcolonies. In contrast, barely any Δ*lqsA* mutant bacteria had resumed growth at this time point, and the mutant phenotype was complemented by plasmid-borne *lqsA* (Fig. 3a, b). The growth resumption frequency (i.e., the resuscitation efficiency) was also much lower for the Δ*lqsS* and Δ*lqsT* mutant strains, while the Δ*lqsR* and Δ*lqsS*-Δ*lqsT* mutants resumed growth at an even higher frequency than the wild-type strain (Fig. 3b). Quantification of the growth resumption frequency revealed that the percentage of Δ*lqsA*, Δ*lqsS*, or Δ*lqsT* mutant bacteria that resumed growth was two to three times lower than that of the wild-type strain, and the growth resumption frequency of the Δ*lqsR* and Δ*lqsS*-Δ*lqsT* mutant strains was 15–20% higher than that of the wild-type strain (Fig. 3c). Judged from the similar overall size of the microcolonies formed by wild-type or *lqs* mutant bacteria (Fig. 3a), the Lqs system controls the growth resumption frequency, but not the growth rate per se. Indeed,

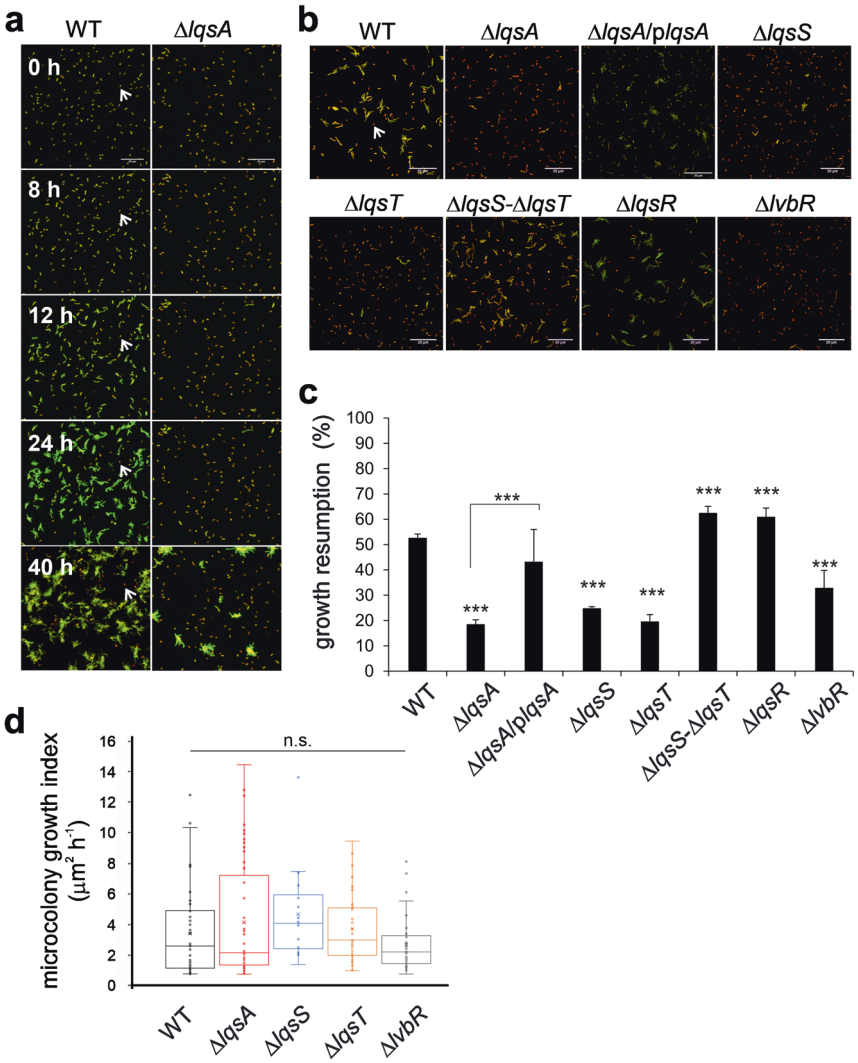


Fig. 3 The Lqs system and LvbR control resuscitation of sessile *L. pneumophila*. **a** Stationary phase Timer-producing *L. pneumophila* wild-type (WT) or Δ*lqsA* were immobilized in AYE/0.5% agarose, and microcolony formation was recorded by time lapse microscopy over 40 h. Arrow heads indicate individuals that did not resume growth. Scale bars, 20 µm. **b** Fluorescence micrographs of microcolonies (scale bars, 20 µm), and **c** quantification of growth resumption of Timer-producing *L. pneumophila* wild-type (WT), Δ*lqsA*, Δ*lqsA*/p*lqsA*, Δ*lqsS*, Δ*lqsT*, Δ*lqsS*-Δ*lqsT*, or Δ*lqsR* immobilized in AYE/0.5% agarose for 24 h. Data represent the mean ± SEM of three biological replicates (two-tailed Student's *t* test; $***P < 0.001$). **d** Stationary phase Timer-producing *L. pneumophila* wild-type (WT) or isogenic mutant strains were immobilized in AYE/0.5% agarose, and let form microcolonies for 40 h. Microcolony growth was analyzed using ImageJ (particle analysis, segmentation and area measurement), and the microcolony growth index was defined as [microcolony area (µm^2)/time (h)]. Growth index differences between WT ($n = 50$), Δ*lqsA* ($n = 50$), Δ*lqsS* ($n = 18$), Δ*lqsT* ($n = 32$), or Δ*lvbR* ($n = 38$) were statistically not significant (n.s.).

quantification of the microcolony growth revealed that there were no significant differences in growth between wild-type *L. pneumophila* and the mutant strains, which show reduced growth resumption frequency (Δ*lqsA*, Δ*lqsS*, Δ*lqsT*, or Δ*lvbR*) (Fig. 3d).

If growing bacteria were used as inoculum, the frequency of microcolony formation of wild-type and Δ*lqsA* mutant bacteria was similar (Fig. S4a, b). This result is in agreement with the notion that the Lqs system is operative only in stationary phase (high cell density and LAI-1 concentration). Taken together, stationary phase *L. pneumophila* shows an inherent heterogeneity to resuscitate and resume sessile growth, which is controlled by the Lqs system.

Finally, compared to wild-type *L. pneumophila*, ca. 50% fewer Δ*lvbR* mutant bacteria resumed growth under these conditions, which is similar to the Δ*lqsA*, Δ*lqsS*, and Δ*lqsT* strains and contrasts the Δ*lqsR* strain (Fig. 3b, c). Hence, the transcription factor LvbR is a positive regulator of growth resumption, while the response regulator LqsR is a negative regulator of the process. In summary, the Lqs system and the transcription factor LvbR contribute to control growth resumption ("resuscitation") of sessile *L. pneumophila*.

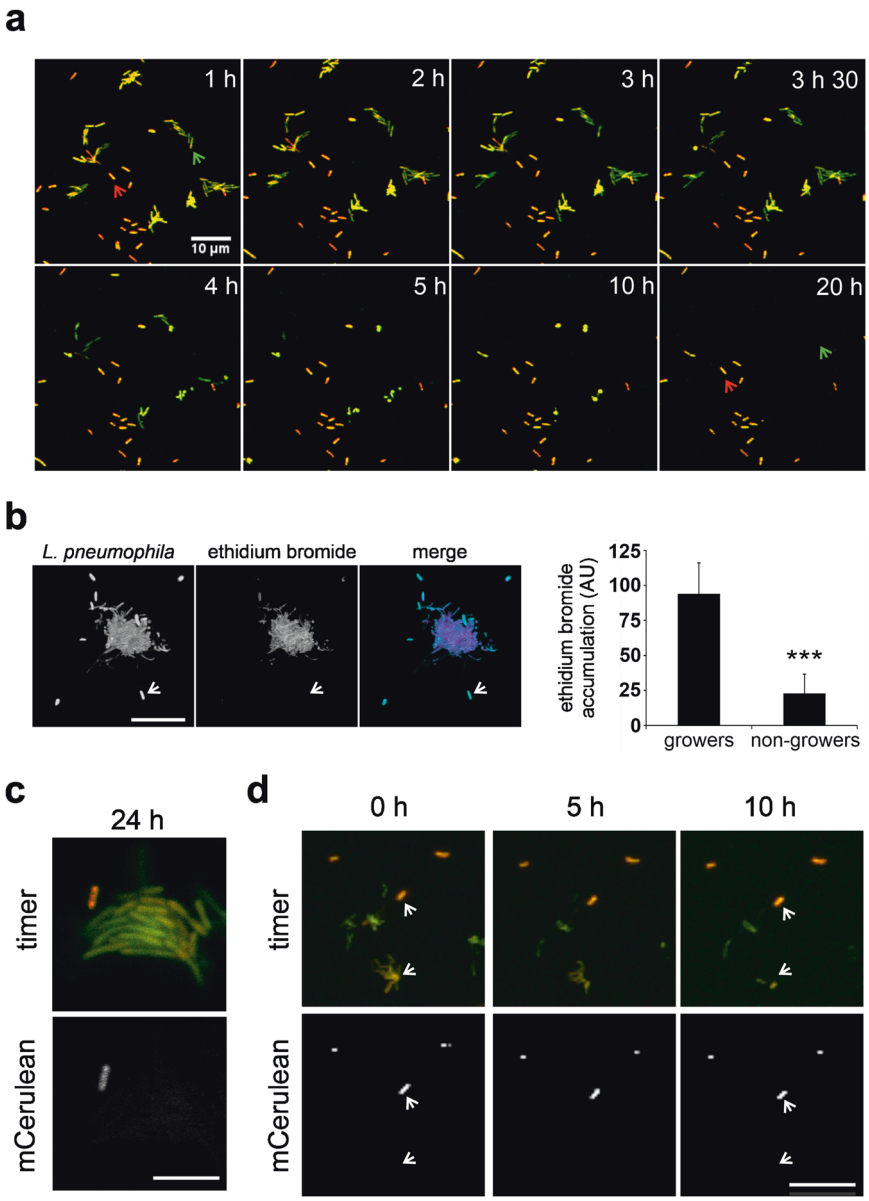


Fig. 4 Sessile *L. pneumophila* nongrowers are antibiotic tolerant and virulent. Timer-producing *L. pneumophila* JR32 was immobilized in AYE/0.5% agarose and allowed to form microcolonies for 24 h. **a** Ampicillin (100 µg mL^{-1}) was added, and bacterial killing was monitored by confocal microscopy for up to 20 h. Arrow heads indicate an antibiotic susceptible, growing microcolony (green), and an antibiotic tolerant nongrowing individual (red). **b** Ethidium bromide was added (1 µg mL^{-1}), and accumulation of the fluorescent compound was monitored by confocal microscopy after 1 h. Arrow heads indicate a nongrower that did not accumulate ethidium bromide. Data represent the mean ± SEM of three biological replicates (two-tailed Student's *t* test; ***$P < 0.001$). **c**, **d** Timer-producing *L. pneumophila* JR32 (P_{tac}-*timer*-P_{sidC}-*mCerulean*) was immobilized in AYE/0.5% agarose and allowed to form microcolonies. After 24 h, the bacteria were assessed by confocal microscopy for mCerulean production (expression of the virulence gene *sidC*) (**c**) in absence of antibiotics or (**d**) upon treatment with ampicillin (100 µg mL^{-1}; 10 h). Arrow heads indicate a growing microcolony (green) or a nongrowing individual (red), which are negative or positive for mCerulean (*sidC* expression), respectively. Scale bars, 10 µm (**a**, **b**, **d**) and 5 µm (**c**).

Sessile *L. pneumophila* nongrowers are antibiotic tolerant and virulent

Using the agarose-embedment microcolony assay, we next assessed antibiotic tolerance of sessile *L. pneumophila* subpopulations. To this end, stationary phase wild-type bacteria producing Timer were immobilized in agarose, let form microcolonies for 24 h and treated with ampicillin. The killing of *L. pneumophila* was monitored by confocal microscopy for up to 20 h (Fig. 4a, Supplementary Movie 3). Under these conditions, the green fluorescent growing bacteria were quantitatively killed and only red fluorescent nongrowing bacteria survived. Hence, the nongrowing sessile bacteria are persisters.

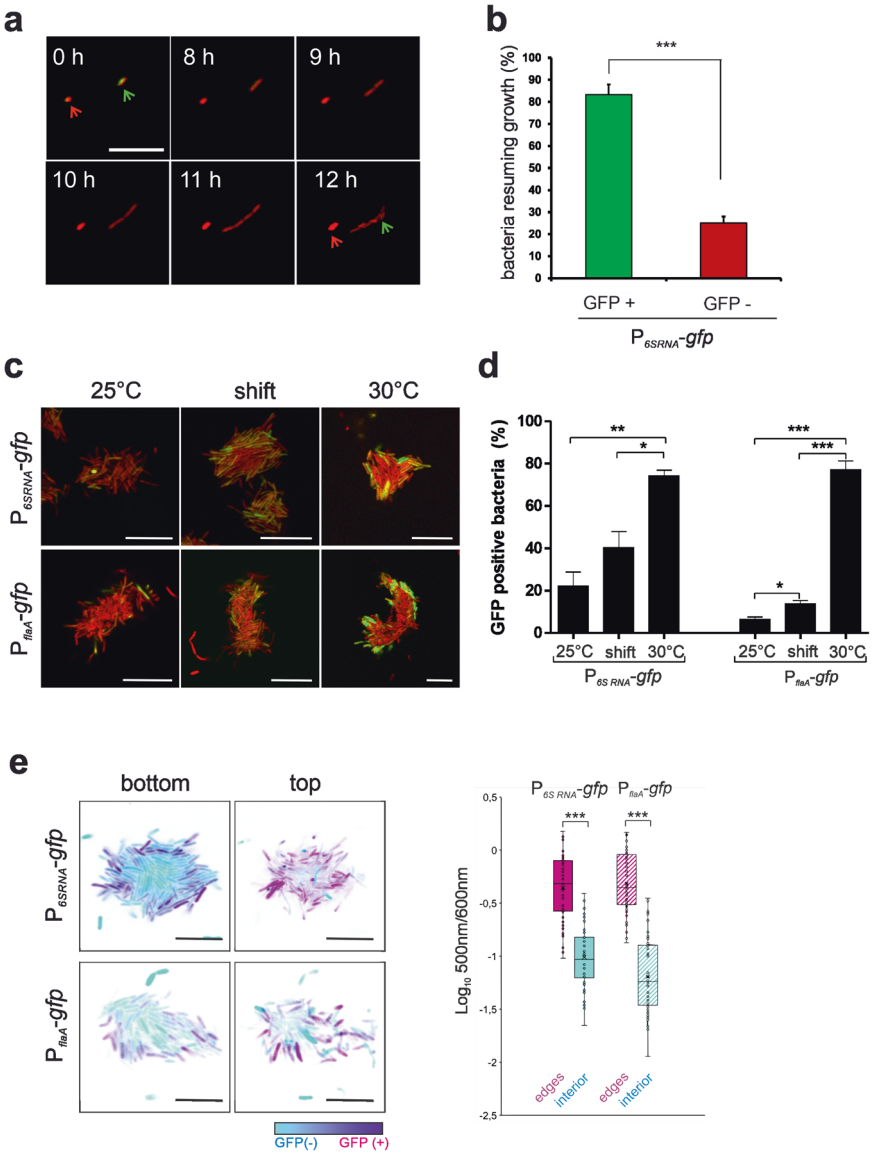


Fig. 5 Sessile *L. pneumophila* shows temperature-dependent phenotypic heterogeneity. a, **b** Stationary phase mCherry-producing *L. pneumophila* JR32 (P_{tac}-*mCherry*-P_{6SRNA}-*gfp*) was immobilized in AYE/0.5% agarose, and the percentage of sessile bacteria resuming growth was visualized and quantified by time lapse microscopy. Arrow heads indicate GFP-positive and GFP-negative bacteria expressing *6S RNA* reporter or not, which resume growth or not, respectively. Scale bar, 10 µm. Data represent the mean ± SEM of three biological replicates (two-tailed Student's *t* test; $***P < 0.001$). **c** Stationary phase mCherry-producing *L. pneumophila* JR32 harboring P_{tac}-*mCherry*-P_{6SRNA}-*gfp* (upper panel) or P_{tac}-*mCherry*-P_{flaA}-*gfp* (lower panel) was embedded in AYE/0.5% agarose and allowed to form microcolonies at 25 °C (72 h), at 25 °C (48 h) followed by a shift to 30 °C for 24 h, or at 30 °C (72 h). Scale bars, 10 µm. **d** Biofilms were formed with stationary phase mCherry-producing *L. pneumophila* JR32 harboring P_{tac}-*mCherry*-P_{6SRNA}-*gfp* or P_{tac}-*mCherry*-P_{flaA}-*gfp* at 25 °C (72 h), at 25 °C (48 h) followed by a shift to 30 °C for 24 h, or at 30 °C (72 h). Bacteria were fixed with 4% PFA, and analyzed by flow cytometry and FlowJo. Data represent the mean ± SEM of three biological replicates (two-tailed Student's *t* test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). **e** Microcolony formation of *L. pneumophila* JR32 harboring P_{tac}-*mCherry*-P_{6SRNA}-*gfp* or P_{tac}-*mCherry*-P_{flaA}-*gfp* at 30 °C (72 h) was analyzed by confocal microcopy and 3D reconstruction (left panels; scale bars, 10 µm), and reporter (GFP/green = magenta, mCherry/red = cyan) fluorescence ratio $\log_{10}$ 500 nm/600 nm (right panels; mean and SEM, two-tailed Student's *t* test, $***P < 0.001$).

Possibly, the nongrowers show increased persistence upon treatment with antibiotics due to decreased antibiotic uptake or increased efflux. To assess drug accumulation by sessile bacteria, agarose-embedded wild-type *L. pneumophila*/Timer grown for 24 h was treated with ethidium bromide, and the retention of the fluorescent compound was assessed by confocal microscopy (Fig. 4b). One hour after treatment, almost all growing bacteria accumulated and retained ethidium bromide, while only a minor portion (ca. 25%) of the nongrowers did so. This result is in agreement

with the notion that decreased drug uptake or increased efflux contributes to antibiotic tolerance of nongrowing *L. pneumophila*.

Next, we assessed the expression of the gene encoding the Icm/Dot substrate SidC by sessile *L. pneumophila* as a proxy for bacterial virulence. Using agarose-embedded *L. pneumophila* wild-type harboring the fluorescent reporter P_{tac}-*timer*-P_{sidC}-*mCerulean* [27], we detected *sidC* expression exclusively in nongrowing but not in growing bacteria (Fig. 4c). This result indicates that sessile stationary phase *L. pneumophila* individuals that do not resume growth express the virulence gene *sidC*, and consequently, are likely virulent. In agreement with this notion, 24 h biofilms originating from stationary phase wild-type *L. pneumophila* harboring the reporter P_{tac}-*timer*-P_{sidC}-*mCerulean* comprised a much larger subpopulation of nongrowers expressing *sidC* as compared to biofilms formed by exponentially growing individuals (Fig. S5). Treatment of the *L. pneumophila* P_{tac}-*timer*-P_{sidC}-*mCerulean* reporter strain with ampicillin selectively killed the growing bacteria, and only the nongrowing, *sidC*-expressing bacteria survived (Fig. 4d, Supplementary Movies 4 and 5). Taken together, these findings indicate that the antibiotic treatment enriches virulent bacteria and *L. pneumophila* nongrowers are virulent persisters.

Sessile *L. pneumophila* shows temperature-dependent phenotypic heterogeneity

In order to further assess physiological traits of sessile *L. pneumophila* and their control, we constructed transcriptional reporters for the sRNA *6S RNA* [43], or the *flaA* gene encoding the major flagellum constituent, flagellin [48]. The *6S RNA* and the *flaA* gene serve as proxies for stress response or stationary phase motility, respectively, and are positively regulated by the Lqs system and synthetic LAI-1 [38].

L. pneumophila constitutively producing mCherry and an unstable GFP variant under control of the P_{6SRNA} promoter (P_{tac}-*mCherry*-P_{6SRNA}-*gfp*) or the P_{flaA} promoter (P_{tac}-*mCherry*-P_{flaA}-*gfp*) [27] were grown in AYE medium to stationary phase, and the expression of the reporter constructs was assessed by confocal fluorescence microscopy and flow cytometry (Fig. S1a, b). Under these conditions, all bacteria expressed the P_{flaA} reporter, while only approximately half of the bacteria expressed the P_{6SRNA} reporter.

Using the agarose-embedment microcolony assay, we assessed the growth of sessile *L. pneumophila* bacteria expressing the P_{6SRNA} reporter or not. To this end, stationary phase agarose-embedded bacteria were grown for 12 h, and single-cell bacterial growth was monitored by fluorescence microscopy (Fig. 5a, Supplementary Movie 6). During this period, ~50% bacteria started to divide, while the others remained growth-arrested. Interestingly, more than 80% of the *L. pneumophila* expressing the P_{6SRNA} reporter resumed growth, while only about 25% of the bacteria not expressing the P_{6SRNA} reporter divided (Fig. 5b). Taken together, sessile *L. pneumophila* immobilized on a glass surface shows phenotypic heterogeneity, where the expression of the *6S RNA* reporter is positively correlated with the frequency of growth resumption.

To further assess the traits of sessile *L. pneumophila*, we let microcolony formation proceed at 25 °C or 30 °C for a prolonged time (up to 72 h) and analyzed the temporal and spatial expression of the *6S RNA* reporter by confocal microscopy (Figs. 5c and S6a). Approximately 75% of *L. pneumophila* harboring a plasmid containing P_{tac}-*mCherry*-P_{6SRNA}-*gfp* expressed the *6S RNA* reporter at 30 °C, but only ca. 20% were GFP-positive at 25 °C (Figs. 5d and S6a). The expression of the *6S RNA* reporter was also induced by a shift to 30 °C for 24 h after growth at 25 °C for 48 h (Figs. 5c, d and S6b). *L. pneumophila* bacteria expressing the *6S RNA* reporter localized preferentially and statistically significantly to the boundaries of the microcolonies (Fig. 5c, e, Supplementary Movie 7).

The temperature dependency of gene expression was even more striking for the *flaA* reporter, since ~80% of *L. pneumophila* harboring a plasmid containing P_{tac}-*mCherry*-P_{flaA}-*gfp* expressed the *flaA* reporter at 30 °C, but less than 10% were GFP-positive at 25 °C (Fig. 5c, d, Supplementary Movie 8). The spatial distribution of the P_{flaA} reporter-expressing *L. pneumophila* subpopulation was similar to the one observed for *6S RNA* expression (Fig. 5c, e). In summary, the expression of the *6S RNA* reporter and even more so, the *flaA* reporter, is temperature dependent and significantly upregulated at 30 °C. Moreover, the *6S RNA* or *flaA* reporters are expressed by spatially distinct subpopulations of sessile *L. pneumophila* in microcolonies.

The Lqs system and LvbR control heterogeneous gene expression in *L. pneumophila* microcolonies

The Lqs system regulates *6S RNA* and *flaA* in *L. pneumophila* on a population level in medium [38]. We wondered whether and how quorum sensing regulates *6S RNA* expression and its spatial distribution in *L. pneumophila* on a single-cell level in microcolonies. To this end, agarose-embedded *L. pneumophila* wild-type or the isogenic mutant strains Δ*lqsA*, Δ*lqsS*, Δ*lqsT*, Δ*lqsS*-Δ*lqsT*, or Δ*lqsR* harboring a plasmid containing P_{tac}-*mCherry*-P_{6SRNA}-*gfp* were allowed to form microcolonies for 72 h at 25 or 30 °C.

Only a few wild-type *L. pneumophila* expressed *6S RNA* in the microcolonies at 25 °C as described above (Figs. 5c, d and S6a). At 30 °C, however, a much larger subpopulation of wild-type *L. pneumophila* and a similar percentage of

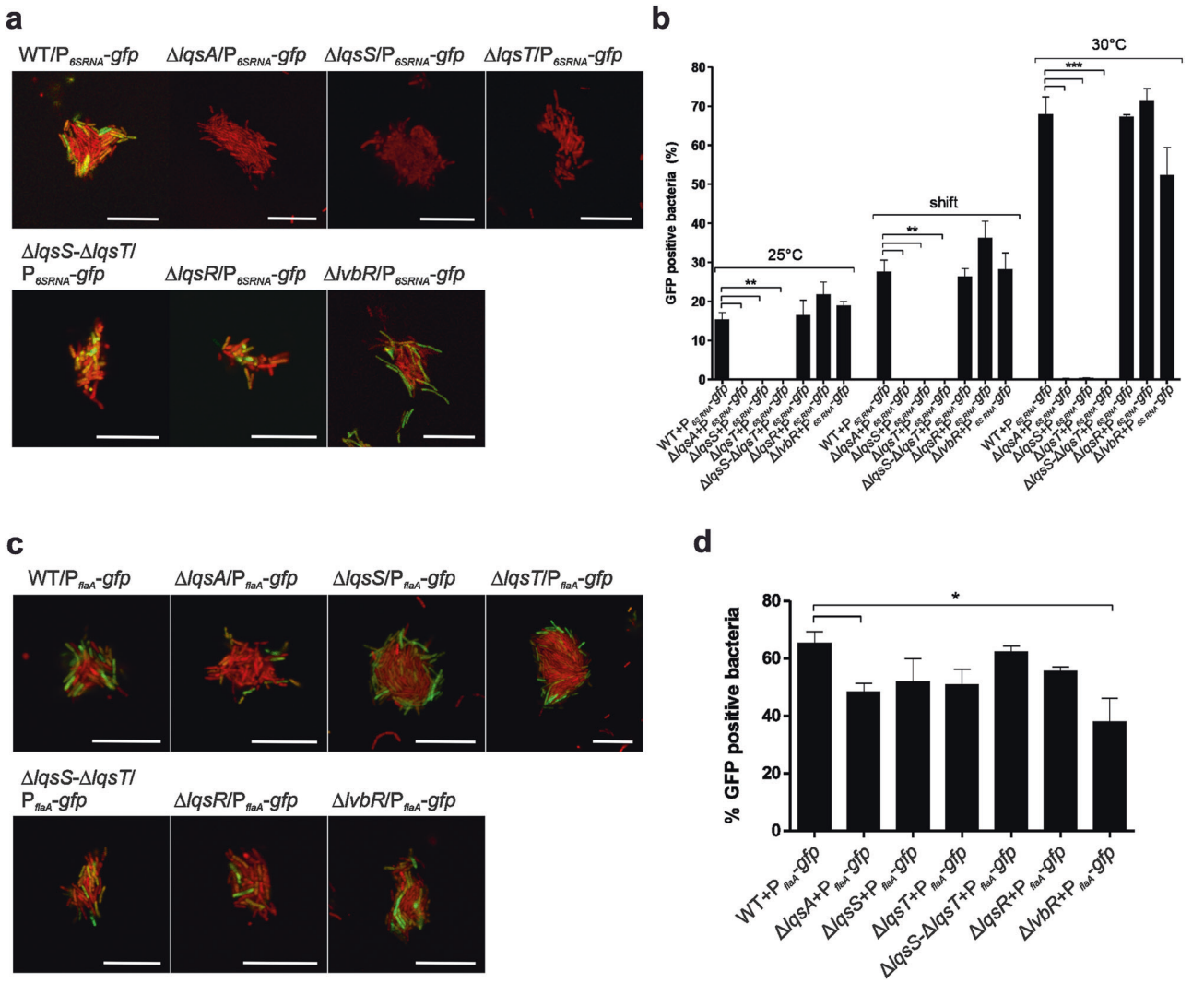


Fig. 6 Heterogeneous gene expression in *L. pneumophila* microcolonies is controlled by quorum sensing. Stationary phase mCherry-producing *L. pneumophila* JR32 or Δ*lqsA*, Δ*lqsS*, Δ*lqsT*, Δ*lqsS*-Δ*lqsT*, Δ*lqsR*, or Δ*lvbR* harboring **a** P_{tac}-*mCherry*-P_{6SRNA}-*gfp* or **c** P_{tac}-*mCherry*-P_{flaA}-*gfp* was embedded in AYE/0.5% agarose and allowed to form microcolonies at 30 °C (72 h). Microcolony formation was analyzed by confocal microcopy and 3D reconstruction. Scale bars, 10 µm. Quantification by flow cytometry of mCherry-producing, *6S RNA*-expressing *L. pneumophila* JR32 or Δ*lqsA*, Δ*lqsS*, Δ*lqsT*, Δ*lqsS*-Δ*lqsT*, Δ*lqsR*, or Δ*lvbR* harboring **b** P_{tac}-*mCherry*-P_{6SRNA}-*gfp* grown at 25 °C (72 h), 30 °C (72 h), or 25 °C (72 h) followed by a shift to 30 °C for another 24 h, or **d** P_{tac}-*mCherry*-P_{flaA}-*gfp* grown at 30 °C (72 h) and fixed with 4% PFA. Data represent the mean ± SEM of three biological replicates (two-tailed Student's *t* test; $*P < 0.05$, $**P < 0.01$).

Δ*lqsR* and Δ*lqsS*-Δ*lqsT* mutant bacteria expressed *6S RNA*, in particular at the microcolony boundaries (Figs. 6a and S6c). In contrast, microcolonies formed by the Δ*lqsA*, Δ*lqsS* or Δ*lqsT* mutant strains did not express *6S RNA* at any temperature, indicating that the Lqs system indeed tightly controls the expression of *6S RNA* in sessile *L. pneumophila*. Similar results were obtained upon shifting the growth temperature from 25 to 30 °C for 24 h (Fig. S6b). The quantification of *6S RNA* expression in sessile *L. pneumophila* revealed an ~4-fold increase of *6S RNA*-positive wild-type or Δ*lqsR* and Δ*lqsS*-Δ*lqsT* mutant *L. pneumophila* from growth at the nonpermissive temperature (25 °C) to growth at the permissive temperature (30 °C), while there was no *6S RNA* expression at all in the Δ*lqsA*, Δ*lqsS* or Δ*lqsT* mutants (Fig. 6b).

The transcription factor LvbR regulates the formation of persisters in biofilms (Fig. 2) and growth resumption of sessile *L. pneumophila* (Fig. 3). Accordingly, we also tested a role for the transcription factor in heterogeneous expression of *6S RNA* in microcolonies. At the permissive temperature (30 °C), a subpopulation of Δ*lvbR* mutant bacteria indeed expressed *6S RNA*, in particular at the microcolony boundaries (Figs. 6a and S6c). The quantification of *6S RNA* expression in sessile Δ*lvbR* mutant *L. pneumophila*

revealed ~20% of *6S RNA*-positive bacteria at the non-permissive temperature (25 °C), 30% upon shifting the temperature from 25 to 30 °C for 24 h, and ca. 50% *6S RNA*-positive bacteria grown for 72 h at the permissive temperature (30 °C) (Fig. 6b). Compared to wild-type *L. pneumophila*, ~25% fewer Δ*lvbR* mutant expressed *6S RNA* at 30 ºC, indicating that the LvbR transcription factor regulates *6S RNA* expression. Taken together, these results demonstrate that the Lqs system and to a lesser extent LvbR positively regulate heterogeneous *6S RNA* expression in sessile *L. pneumophila*.

We also assessed the role of quorum sensing for *flaA* expression in *L. pneumophila* microcolonies. *L. pneumophila* wild-type and the above mutant strains harboring a plasmid containing P_{tac}-*mCherry*-P_{flaA}-*gfp* were allowed to form microcolonies at 30 °C for 72 h (Figs. 6c and S6c). Microcolonies formed by *L. pneumophila* wild-type, *lqs* mutant or *lvbR* mutant strains produced a subpopulation of bacteria that expressed *flaA*. The expression pattern was similar to the expression of *6S RNA*, with GFP-positive bacteria mainly at the boundaries of the microcolonies. The quantification of *flaA* expression in sessile *L. pneumophila* revealed ~65% *flaA*-positive wild-type *L. pneumophila* and ca. 25 or 40% fewer Δ*lqsA* or Δ*lvbR* mutant bacteria, respectively (Fig. 6d). The expression of *flaA* by the other *lqs* mutant strains was also slightly, but statistically not significantly, reduced. Taken together, the Lqs system (in particular *lqsA*) and LvbR regulate the fraction and spatial distribution of sessile P_{flaA}-expressing *L. pneumophila* in microcolonies.

In stationary phase *L. pneumophila 6S RNA* is upregulated [43], and the *6S RNA* reporter is positively regulated by the Lqs system (Fig. 6a, b). To test whether *6S RNA* is not only a manifestation of phenotypic heterogeneity, but also a regulator of the process, we assessed biofilm formation of a strain lacking *6S RNA*. Timer-producing *L. pneumophila* Δ*6S RNA* mutant bacteria or the parental strain Δ*comR* [43] were grown to stationary phase, diluted in fresh medium and let form a biofilm for 24 h. The quantification by flow cytometry revealed a ratio of ca. 10% nongrowing *L. pneumophila* in biofilms formed either by the Δ*6S RNA* mutant or the parental strain (Fig. S7a). In addition, both strains showed similar growth resumption frequency after immobilization in agarose (Fig. S7b). Hence, the *6S RNA* does neither regulate the formation of nongrowers in *L. pneumophila* biofilms nor the growth resumption heterogeneity.

In summary, the Lqs system and the transcription factor LvbR determine the fraction and spatial distribution of the *L. pneumophila* subpopulation, which in microcolonies expresses *6S RNA* and *flaA*. While the Lqs-dependent upregulation of the *6S RNA* is a feature of phenotypic heterogeneity, the *6S RNA* itself does not control the formation of biofilms or phenotypic heterogeneity of sessile *L. pneumophila*.

Discussion

In this study, we assessed traits of sessile *L. pneumophila* and their control on a single-cell level in biofilms and microcolonies. We revealed that sessile *L. pneumophila* exhibits phenotypic heterogeneity and forms growing and nongrowing subpopulations. Nongrowing sessile *L. pneumophila* are metabolically active and virulent persisters. *L. pneumophila* nongrowers might contribute as a "dormant" form to the long-term survival of the bacteria in the environment, and therefore, this feature of the opportunistic pathogen is likely of broad ecological significance.

In most ecosystems, nongrowing bacterial cells dominate. In clinical settings these bacteria are a major cause of chronic or relapsing infections due to their intrinsic capacity to be resuscitated in susceptible hosts and due to their notorious antibiotic tolerance [49–51]. Prominent examples of environmentally transmitted opportunistic pathogens, which adopt a nongrowing ("dormant" or "viable but non culturable") state, include *Vibrio cholerae*, *Mycobacterium tuberculosis*, and *Pseudomonas aeruginosa*, the causative agents of cholera, tuberculosis, and pneumonia, respectively [52–54].

A sessile lifestyle on abiotic or biotic (biofilm) surfaces is arguably the most predominant form of bacterial life in the environment. Here, we provide evidence that upon sessile growth in biofilms or on abiotic surfaces *L. pneumophila* forms a population of nongrowing, virulent persisters (Fig. 4). *L. pneumophila* is a ubiquitous environmental bacterium and upon inhalation might become an "accidental" pathogen. Hence, the virulent, antibiotic tolerant form of *L. pneumophila* is likely important in clinical settings. In fact, these nongrowing bacteria might be the cause for severe, relapsing and antibiotic-nonresponsive forms of Legionnaires' disease. Accordingly, the biofilm niche comprising different *L. pneumophila* subpopulations has a profound impact on the pathogenesis of the opportunistic pathogen.

In the environment, the subpopulation of sessile nongrowing virulent persisters may efficiently infect protozoan predators, naturally present in microbial communities. Functionally distinct subpopulations of sessile *L. pneumophila* may therefore adopt a bet-hedging strategy [55], which prepares for either extracellular growth (surface colonization) or intracellular growth. Thus, overall the sessile *L. pneumophila* population has the potential to colonize a range of vastly different environmental niches.

Nongrowing forms of environmental bacteria not only ensure long-term survival, but also need to retain the capacity to resume growth and be resuscitated. Resuscitation can happen at the onset of favorable conditions or by stochastic re-initiation of growth. Indeed nongrowing individual bacteria have been shown to stochastically re-initiate proliferation at a relatively low rate, regardless of the environmental conditions [56, 57]. Here, we show that the growth resumption frequency of sessile *L. pneumophila* under favorable conditions (rich medium) is genetically controlled. The Lqs system positively regulates growth resumption, and stationary phase Δ*lqsA*, Δ*lqsS* or Δ*lqsT* mutant strains resumed growth on a abiotic surface with a two to three times lower frequency compared to the parental strain (Fig. 3). Similarly, *L. pneumophila* lacking the transcription factor LvbR showed an impaired growth resumption frequency, and thus, LvbR also positively regulates the process. The mechanism underlying growth resumption frequency is unclear. The regulators might indeed control a switch from the nongrowing to the growing state. Alternatively, the regulators might control cell survival during stationary phase stress, allowing efficient growth resumption upon the encounter of more favorable conditions.

Quorum sensing is a major regulator of phenotypic heterogeneity of sessile *L. pneumophila*. The Lqs system controls the ratio between nongrowing and growing *L. pneumophila* in biofilms (Figs. 1 and 2), growth resumption (resuscitation) of sessile bacteria forming microcolonies (Fig. 3), antibiotic tolerance of *L. pneumophila* in biofilms (Figs. 2 and S2), and microcolonies (Fig. 4), as well as heterogeneous gene expression (Figs. 5 and 6). Phenotypic heterogeneity was primarily observed with stationary phase bacteria, and exponentially growing *L. pneumophila* cultures produced a much smaller subpopulation of nongrowers and antibiotic tolerant bacteria in biofilms (Fig. S3). These observations are in agreement with a role for the Lqs system in stationary growth phase (high cell density and LAI-1 concentration). Given that *L. pneumophila* predominantly exists in a nongrowing form in the environment, the Lqs system, which is operative in stationary phase, is likely also relevant for the control of phenotypic variation under natural conditions. Interestingly, the Lqs system negatively regulates the occurrence of nongrowing *L. pneumophila* in biofilms (Figs. 1 and 2a) and positively regulates the occurrence of nongrowers in phagocytes [27]. Hence, quorum sensing oppositely regulates the ratio between nongrowing and growing *L. pneumophila* in sessile or intracellular niches.

In addition to the Lqs system, the transcription factor LvbR controls phenotypic heterogeneity and the emergence of nongrowing, virulent *L. pneumophila* persisters (Figs. 2 and 3). LvbR is a pleiotropic regulator, which determines various *L. pneumophila* traits, such as biofilm architecture, natural competence for DNA uptake, and pathogen–host cell interactions [40, 41]. The transcription factor directly binds to the promoter of *hnox1/lpg1056*, which is divergently transcribed from the *lvbR* gene. Hnox1 is a nitric oxide sensor, which inhibits the diguanylate cyclase activity of Lpg1057 and reduces c-di-GMP production [58]. Thus, by negatively regulating the inhibitor Hnox1, LvbR positively regulates the diguanylate cyclase activity of Lpg1057 and, consequently, c-di-GMP levels are increased. It remains to be determined, whether LvbR controls phenotypic heterogeneity of *L. pneumophila* through the c-di-GMP network, and/or whether other regulatory circuits are involved.

Phenotypic heterogeneity of sessile *L. pneumophila* is not only controlled by endogenous circuits (Lqs, LvbR), but also by external cues such as the temperature. Compared to 25 °C the transcriptional reporters for *6S RNA* or *flaA* expression were upregulated at 30 ºC ca. 4- or 8-fold, respectively, and a shift from 25 to 30 °C for 24 h already led to an induction of the reporters (Figs. 5, 6, and S6). Temperature is an important environmental cue sensed by many bacteria to adapt their behavior to changing environmental conditions [59]. We reveal in this study that *L. pneumophila* senses and responds to small changes in the environmental temperature. To our knowledge, this is also the first description of a thermo-regulated sRNA in *L. pneumophila*.

At the permissive temperature of 30 °C, the Lqs system and LvbR positively regulate *6S RNA* expression. The Lqs system apparently overruled the regulation by temperature, since the Δ*lqsA*, Δ*lqsS*, or Δ*lqsT* mutant strains failed to express *6S RNA* at any of the temperatures tested. Moreover, neither the Δ*lqsS*-Δ*lqsT* double sensor kinase mutant nor the Δ*lqsR* response regulator mutant seemed to be involved in the regulation of *6S RNA* expression (Fig. 6). These observations might be explained by reciprocal (and thus neutralizing) regulatory roles of the LqsS and LqsT sensor histidine kinases [35], and the presence of (a) regulatory element(s) other than the response regulator LqsR implicated in *6S RNA* expression downstream of the sensor kinases.

The expression of *6S RNA* as well as of *flaA* occurred with pronounced spatial heterogeneity, and the genes were preferentially expressed at the microcolony boundaries (Figs. 5 and 6). The functional significance of this heterogeneous expression pattern is unclear. The *6S RNA* and *flaA* genes serve as proxies for stationary phase stress response or motility, respectively. Perhaps, peripheral bacteria that are preferentially exposed to stressors and predators might selectively upregulate stress response and virulence pathways. To facilitate the spread of sessile *L. pneumophila*, peripheral bacteria might preferentially become motile,

since they are less firmly embedded in a biofilm (or microcolony). Alternatively, the spatial heterogeneity of P_{6SRNA} and P_{flaA} reporter induction might be the result of stochastic gene expression, which broadens the bacterial response repertoire in the context of a bet-hedging strategy [55].

The spatial heterogeneity of *6S RNA* and *flaA* reporter expression is controlled by the Lqs system (Fig. 6). The Lqs system and the cognate signaling molecule LAI-1 are required (but might not be sufficient) to regulate gene expression. Accordingly, the *6S RNA* and *flaA* promoter activities and corresponding expression reporters might function as (indirect) LAI-1 biosensors. It is tempting to speculate that the bacterial cells in a *L. pneumophila* microcolony or biofilm respond in a heterogeneous manner to LAI-1, and/or that the production of LAI-1 proceeds with spatial heterogeneity. However, other factor(s) likely (co-) determine the pattern of spatial heterogeneity of gene expression in sessile *L. pneumophila*.

In the current study, we identified endogenous genetic (*lqs*, *lvbR*) and exogenous physical (temperature) determinants of phenotypic heterogeneity of sessile *L. pneumophila*. The consequences of phenotypic heterogeneity are distinct subpopulations of growing and nongrowing bacteria, the latter of which are virulent and antibiotic tolerant persisters. This work paves the way for future studies addressing mechanistic aspects of cues and consequences of the phenotypic heterogeneity of sessile *L. pneumophila* in biofilms and on abiotic surfaces.

Methods

For details see Supplementary Information.

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table S1. The Timer reporter is a stable DsRed variant, which slowly changes its fluorescence from green (500 nm) to red (600 nm) [60]. Accordingly, the Timer fluorescence ratio [500 nm (green)/600 nm (red)] reflects the growth rate of *L. pneumophila* at a single-cell level [27]. The dual fluorescence reporters (P_{tac}-*mCherry*-P_{flaA}-*gfp*, P_{tac}-*mCherry*-P_{6SRNA}-*gfp*) allow an assessment of *L. pneumophila* promoter activity.

Biofilm and microcolony analysis by confocal microscopy and flow cytometry

For biofilm formation, planktonic exponential or stationary phase grown *L. pneumophila* strains were diluted in fresh AYE/chloramphenicol at an OD_{600} of 0.1, placed in a multiwell plate or an ibidi imaging chamber, and incubated at 25 or 30 °C for the indicated time while avoiding mechanical disturbance [18, 40]. The formation and analysis of microcolonies is detailed in [47].

Biofilms and microcolonies were analyzed by confocal laser scanning microscopy (Leica TCS SP8 X CLSM). Flow cytometry was performed with a FACS-Fortessa II using homogenized and fixed biofilm samples. The gating for the Timer reporter was performed as described [27].

Biphasic kill curves

L. pneumophila biofilms grown for 48 h were treated or not with different classes of antibiotics at >100× MIC (ampicillin 100 µg mL^{-1}, erythromycin 60 µg mL^{-1}, or ofloxacin 30 µg mL^{-1}) at 25 °C, washed three times, diluted, and plated on CYE agar plates to quantify CFU.

Data availability

All data is available in the main text or the Supplementary Material and provided as source data files.

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Author contributions NP conceived the study with input from HH. NP designed the experiments. NP and BS performed the experiments. NP and HH wrote the paper with input from BS.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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