Supporting Information

Migration of *Acanthamoeba* through *Legionella* biofilms is regulated by the bacterial Lqs-LvbR network, effector proteins and the flagellum

Ramon Hochstrasser[1], Sarah Michaelis[1#], Sabrina Brülisauer[1#], Thomas Sura[2], Mingzhen Fan[1], Sandra Maaß[2], Dörte Becher[2] and Hubert Hilbi[1]*

[1]*Institute of Medical Microbiology, University of Zürich, Gloriastrasse 30, 8006 Zürich, Switzerland.*

[2]*Institute of Microbiology, University of Greifswald, Felix-Hausdorff-Strasse 8, 17489 Greifswald, Germany.*

[#]These authors contributed equally to this work.

Running title: Amoeba migration through *Legionella* biofilms

Key words: Amoeba, *Acanthamoeba*, cell migration, bacterial adherence, biofilm, cell-cell communication, flagellum, *Legionella*, response regulator, sensor kinase, transcription factor, quorum sensing.

Abbreviations: Icm/Dot, intracellular multiplication/defective organelle trafficking; LAI-1, *Legionella* autoinducer-1; LCV, *Legionella*-containing vacuole; Lqs, *Legionella* quorum sensing; LvbR; *Legionella* virulence and biofilm regulator; c-di-GMP, cyclic di-guanosine monophosphate; GFP, green fluorescent protein; T4SS, type IV secretion system.

*Correspondence: E-mail hilbi@imm.uzh.ch,

Tel.: +41 (0)44 634 2650, Fax: +41 (0)44 634 4906

Figure S1
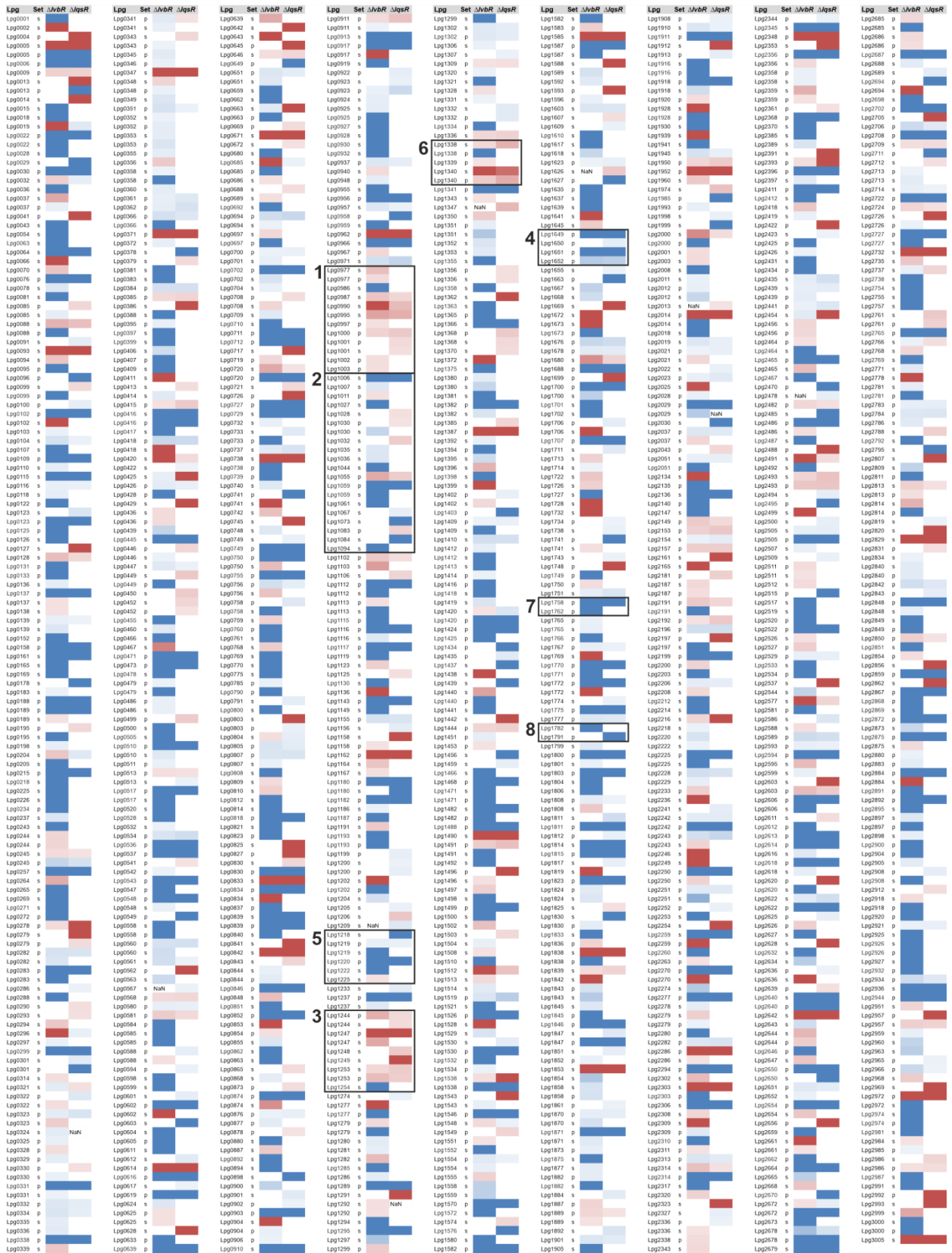
Patterns (boxes):
1 - 133kb genomic region I (Lpg0973-Lpg1005)   3 - Lvh T4SS/Lvr proteins   5-8 - Flagellum/motility
2 - 133kb genomic region II (Lpg1006-Lpg1096)   4 - Inositol catabolism

Fig. S1 (overleaf)**.** Heat map of proteins depleted or enriched in Δ*lvbR* and Δ*lqsR* biofilms compared to JR32.

Comparative proteomics was performed with biofilms formed by *L. pneumophila* JR32, Δ*lvbR* or Δ*lqsR*. In the heat map all proteins are shown that are depleted or enriched in mutant biofilms (for details see Materials and Methods). Biofilm bacteria were harvested by centrifugation and supernatants (designated "s", extracellular) and cell pellets (designated "p", intracellular) were analyzed separately. The proteins are listed by ascending Lpg numbers. The color code indicates the level of regulation: the darker the color the stronger the corresponding gene is regulated (red: depleted proteins, max. 2^{-5}; blue: accumulated proteins, max. $2^{7.4}$). Boxes highlight regions of interest, which are further discussed. "NaN": not a number.

Fig. S2 (overleaf)**.** Biofilm formation and quantification of *L. pneumophila* strains.

(**A**) Biofilms were initiated with exponential phase *L. pneumophila* Δ*lqsS*, Δ*lqsT* or Δ*lqsS*-Δ*lqsT* mutant strains harboring pNT28 (constitutive production of GFP) in AYE medium within ibiTreat microscopy dishes. Biofilms were grown at 25°C without mechanical disturbance and confocal microcopy images of biofilm architecture were acquired at 4 μm above dish bottom after 1, 2, 3 and 6 days of growth. The images shown are representative of at least two independent experiments. Scale bars, 30 μm. (**B**, **C**) Quantification of *L. pneumophila* biofilms grown for 6 days. (**B**) Pseudocolor graph (GFP intensity vs. FSC) depicting the gate for GFP-producing *L. pneumophila* (identified by fluorescence) and the beads population (identified by SSC vs. FSC). (**C**) Means and standard deviation (bacteria/ml) of biological triplicates from 100'000 events of each biofilm suspension (differences between wild-type and mutant *L. pneumophila* are statistically not significant).
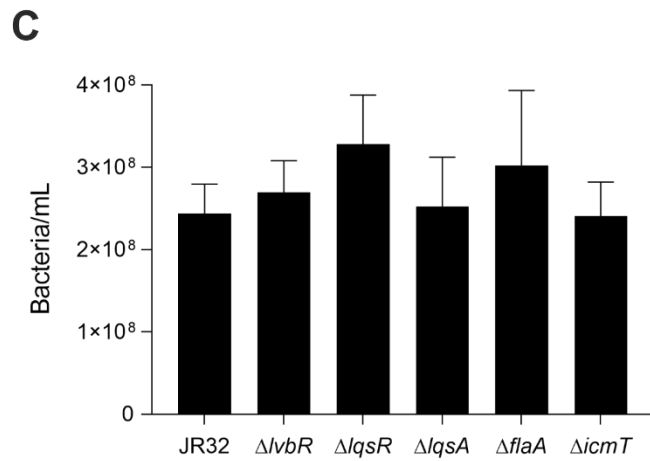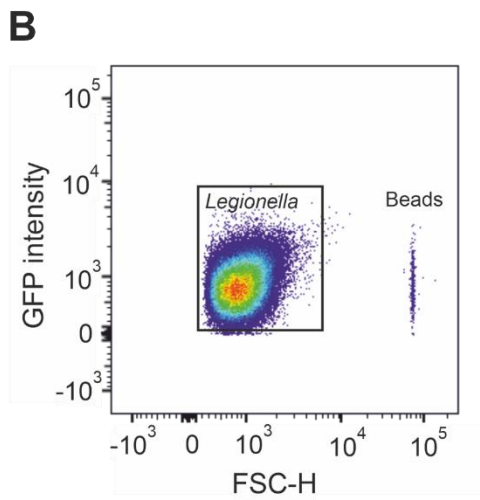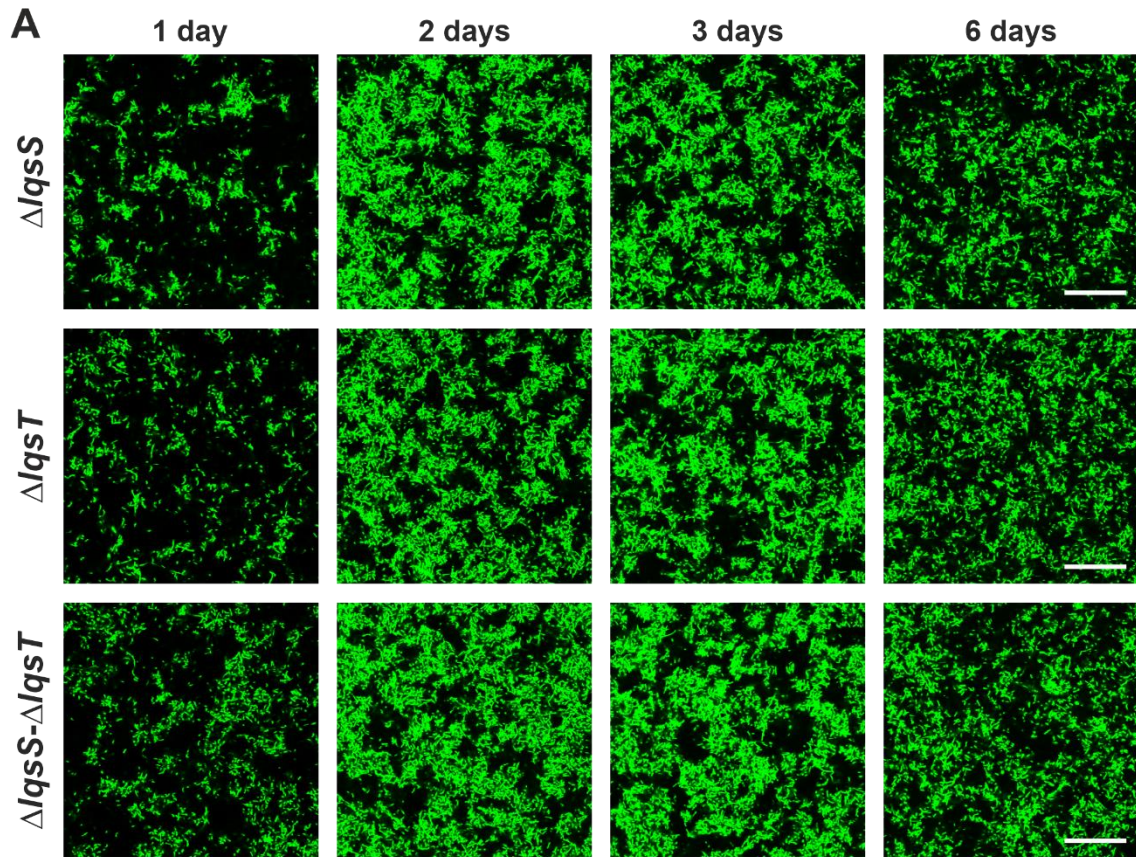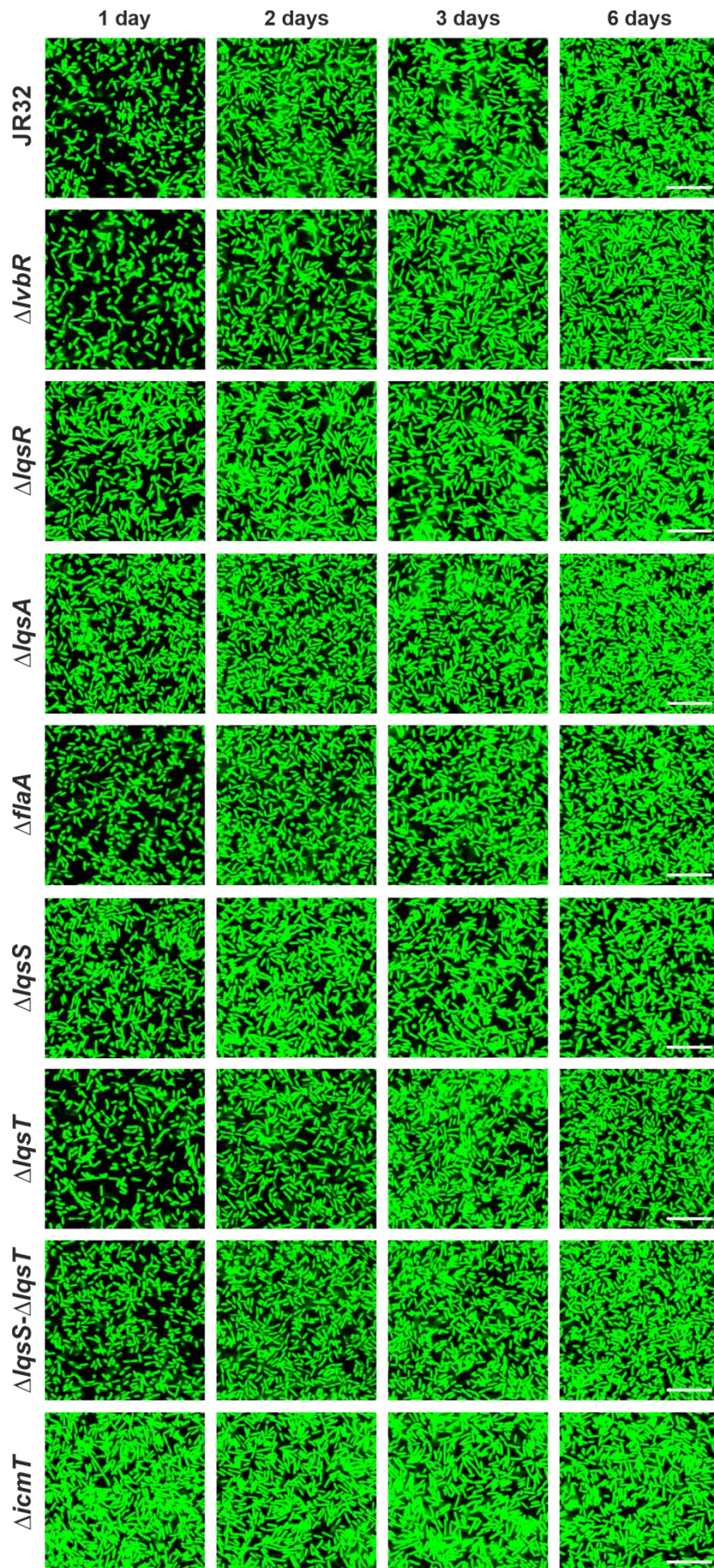
Figure S2

A
| | 1 day | 2 days | 3 days | 6 days |

A

1 day   2 days   3 days   6 days

ΔlqsS

ΔlqsT

ΔlqsS-ΔlqsT

B

GFP intensity

10^5

10^4

10^3

0

-10^3

Legionella   Beads

-10^3 0 10^3 10^4 10^5

FSC-H

C

Bacteria/mL

4×10^8

3×10^8

2×10^8

1×10^8

0

JR32   Δ*lvbR*   Δ*lqsR*   Δ*lqsA*   Δ*flaA*   Δ*icmT*

4

Figure S3

	1 day	2 days	3 days	6 days
JR32				
Δ*lvbR*				
Δ*lqsR*				
Δ*lqsA*				
Δ*flaA*				
Δ*lqsS*				
Δ*lqsT*				
Δ*lqsS*-Δ*lqsT*				
Δ*icmT*				

Fig. S3 (overleaf)**.** Surface adherence of biofilms formed by *L. pneumophila* JR32 and mutant strains.

Biofilms were initiated with exponential phase *L. pneumophila* JR32, Δ*lvbR*, Δ*lqsR*, Δ*lqsA*, Δ*lqsS*, Δ*lqsT*, Δ*lqsS*-Δ*lqsT*, Δ*flaA* or Δ*icmT* mutant strains harboring pNT28 (constitutive production of GFP) in AYE medium within ibiTreat microscopy dishes. Biofilms were grown at 25°C without mechanical disturbance and confocal microcopy images of biofilm attachments were acquired at the dish bottom (0 µm) after 1, 2, 3 and 6 days of growth. The images shown are representative of at least two independent experiments. Scale bars, 10 µm.

**Fig. S4** (overleaf)**.** *L. pneumophila* cluster formation on *A. castellanii* in JR32 or mutant strain biofilms.

Biofilms of (**A**) *L. pneumophila* JR32, Δ*lvbR*, Δ*lqsR*, Δ*lqsA*, Δ*flaA* or Δ*icmT* mutant strains harboring pNT28 (constitutive production of GFP), or (**B**, **C**) strain JR32 harboring pNT28 were grown for 6 days in AYE medium at 25°C, and *A. castellanii* was added to preformed biofilms. Bacterial adherence and cluster formation were monitored by confocal microscopy (**A**, **B**) ca. 4 µm above or (**C**) at the dish bottom ("clusters": arrow heads, "trails": arrows). (**D**, **E**) Biofilms of *L. pneumophila* JR32 or Δ*flaA* mutant strains constitutively producing GFP (pNT28) or GFP and FlaA under control of its promoter (Δ*flaA*/+*flaA*, pSB001) were grown for 6 days in AYE medium at 25°C, and *A. castellanii* was added to preformed biofilms. Confocal microscopy images of (**D**) overview and (**E**) zoom-in of *L. pneumophila* adherence and cluster formation on amoebae. The images shown are representative of three independent experiments. Scale bars, 30 µm.
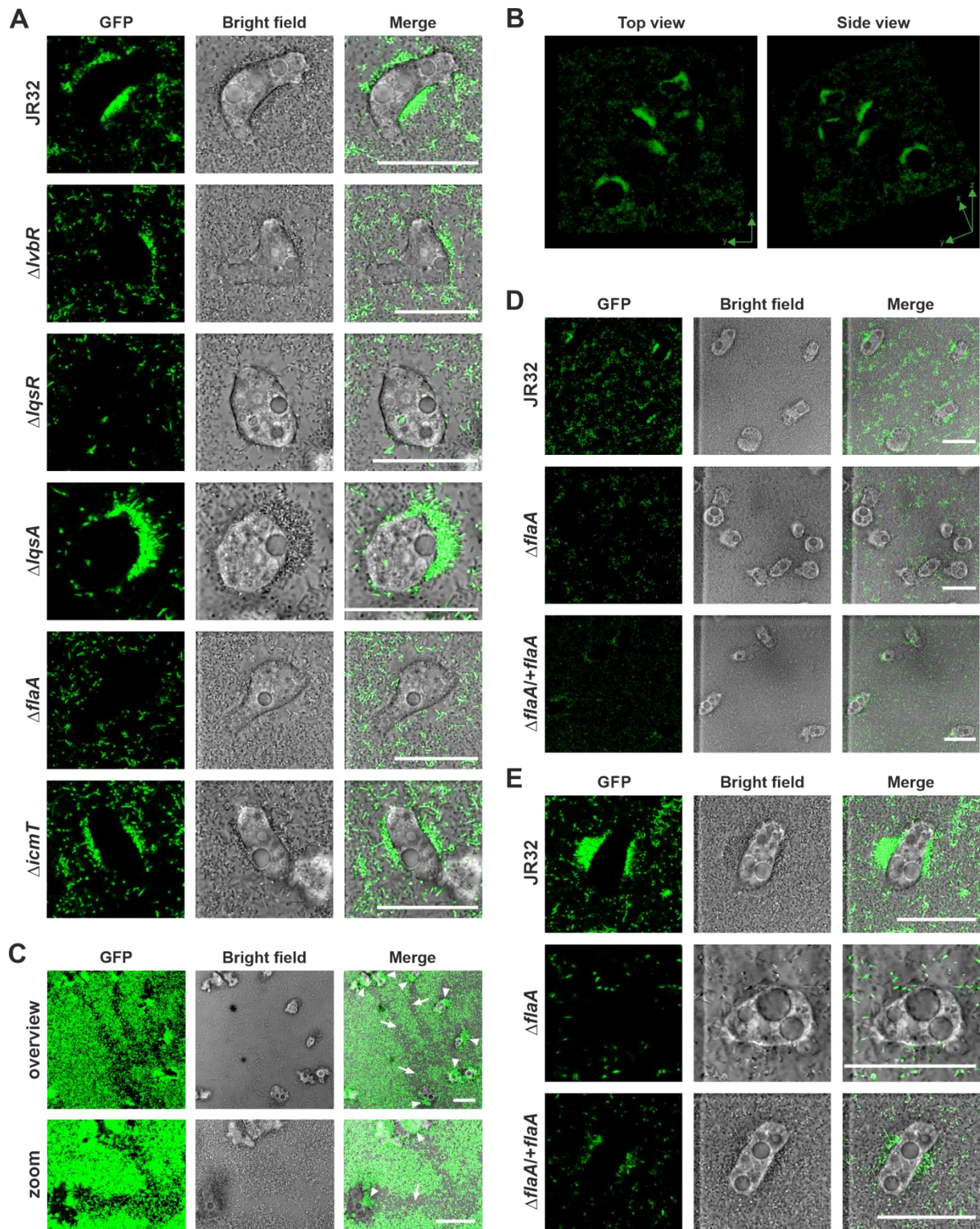
Figure S4

Figure S5
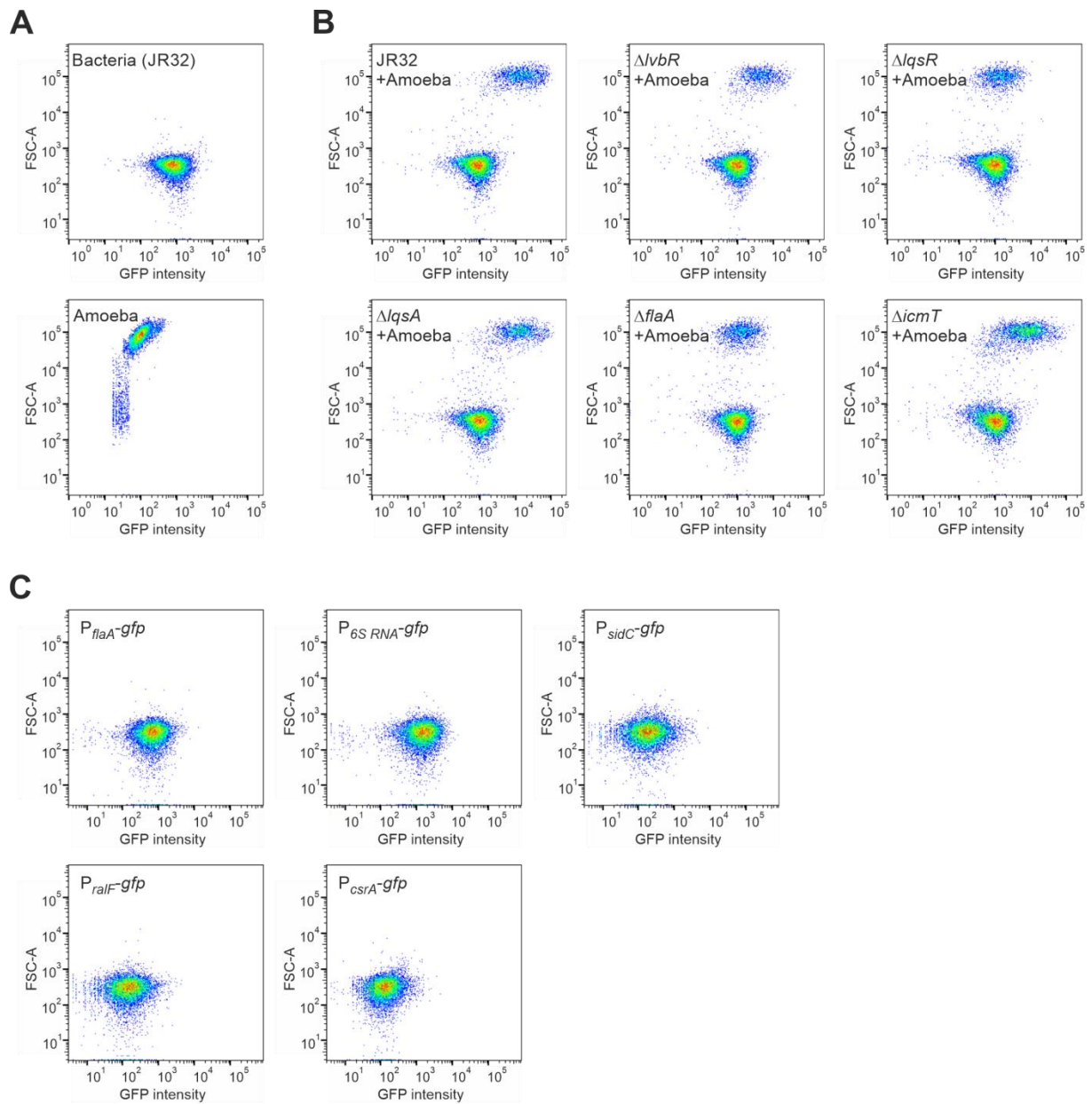
Fig. S5. Quantification of amoeba-associated *L. pneumophila* clusters by flow cytometry. Quantification by flow cytometry (forward scatter area, FSC-A, vs. fluorescence intensity) using FlowJo software of (**A**) *L. pneumophila* strain JR32 or *A. castellanii*, (**B**) amoebae-associated or non-associated, GFP-positive *L. pneumophila* (JR32, $\Delta lvbR$, $\Delta lqsR$, $\Delta lqsA$, $\Delta flaA$ or $\Delta icmT$ harboring pNT28) (protocol #1, see Material and Methods), or (**C**) non-associated, GFP-positive *L. pneumophila* JR32 harboring promoter expression reporters (P$_{flaA}$, P$_{6SRNA}$, P$_{sidC}$, P$_{sidF}$, or P$_{csrA}$) (protocol #2, see Material and Methods).

8

Figure S6
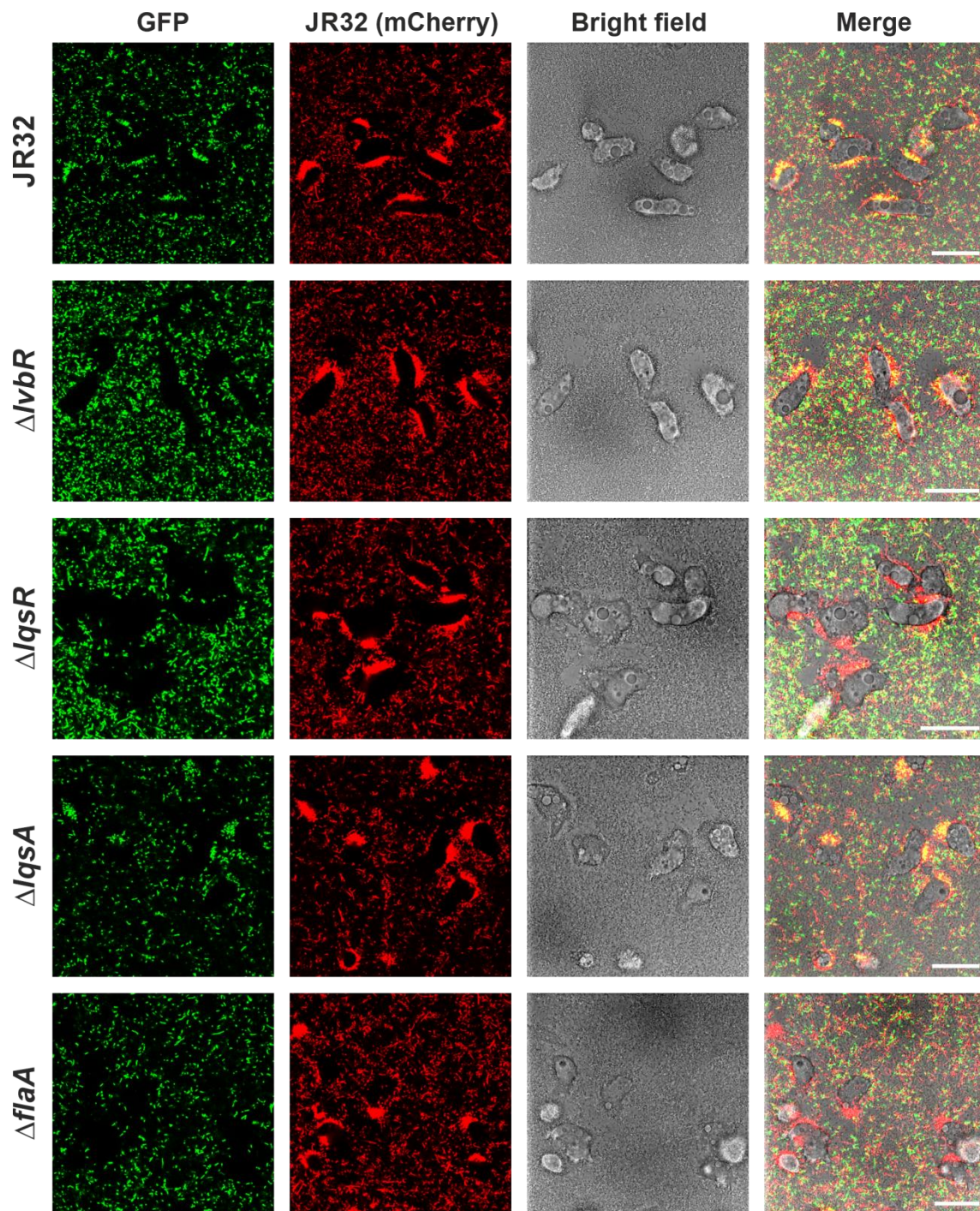
Fig. S6. Competition of mCherry-producing *L. pneumophila* JR32 and GFP-producing mutant strains for cluster formation on amoebae.

Biofilm formation was initiated with a 1:1 ratio of exponential phase mCherry-producing *L. pneumophila* JR32 (pNP102) and GFP-producing JR32 or mutant strains (pNT28). Biofilms were grown in AYE medium within ibiTreat microscopy dishes at 25°C for 6 days. *A. castellanii* were added to preformed biofilms, and amoebae with adherent bacterial clusters were monitored by confocal microcopy above the dish bottom. The images shown are representative of at least two independent experiments. Scale bars, 30 μm.
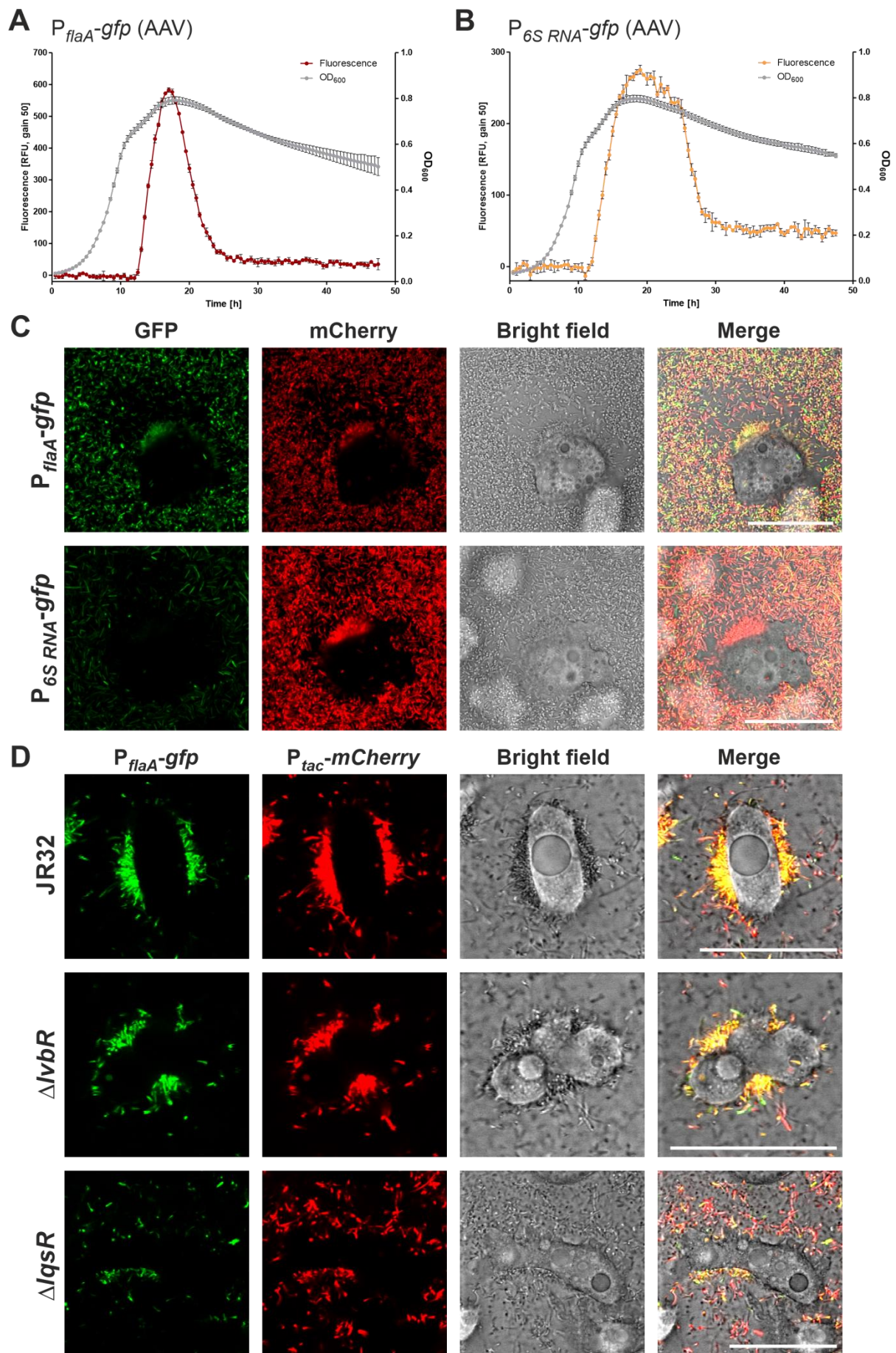
Figure S7
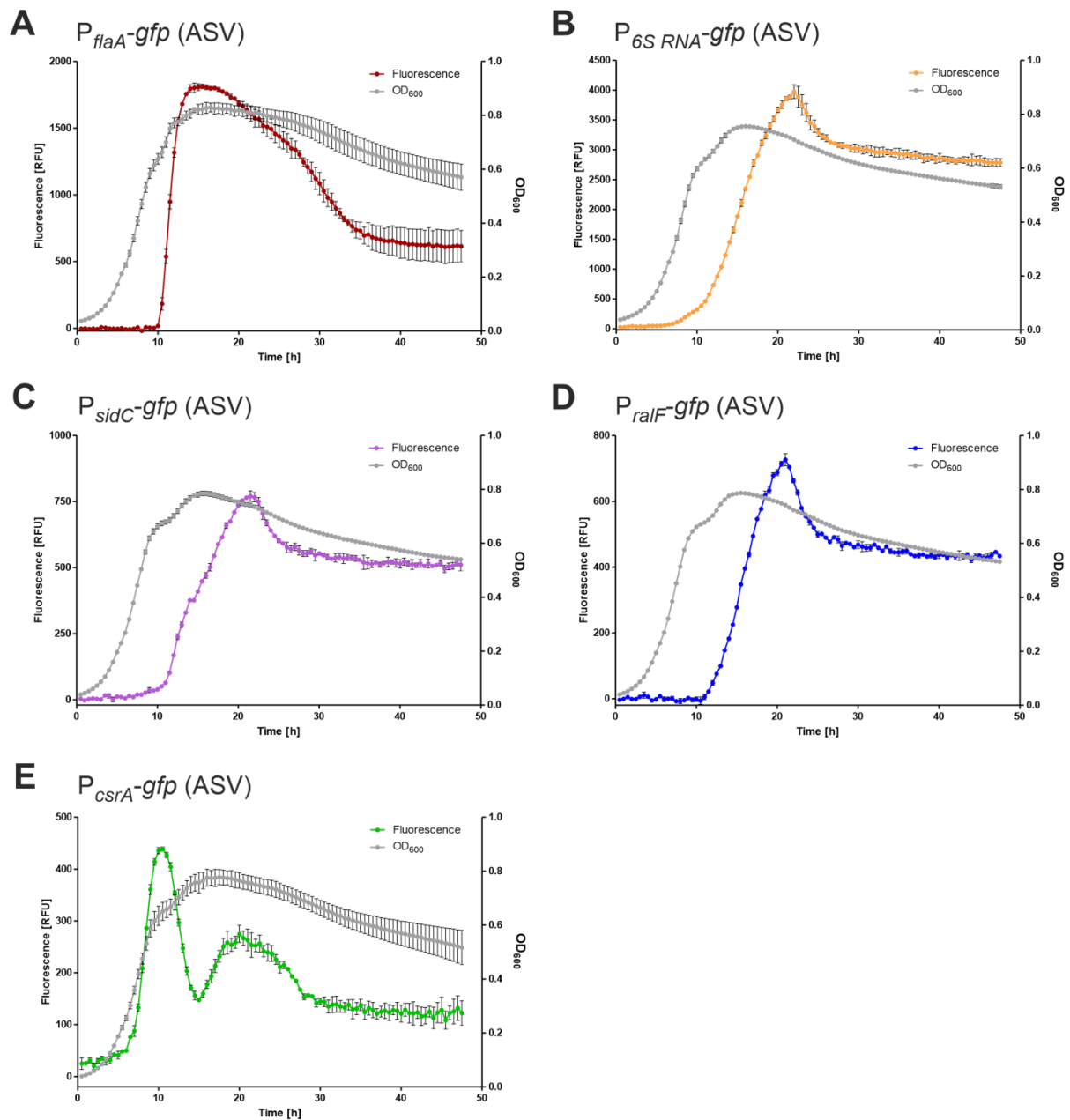
Fig. S7. Gene expression kinetics of *L. pneumophila* JR32 in AYE broth using single promoter reporters.

L. pneumophila JR32 harboring (**A**) P_{flaA}-*gfp* (pCM009), (**B**) P_{6SRNA}-*gfp* (pRH049), (**C**) P_{sidC}-*gfp* (pRH035), (**D**) P_{ralF}-*gfp* (pRH032) or (**E**) P_{csrA}-*gfp* (pRH031) reporter constructs were grown at 37°C in AYE medium in microplates while orbitally shaking. GFP fluorescence (relative fluorescence units, RFU) at a gain of 50 and optical density at 600 nm (OD_{600}) were monitored over time using a microplate reader. The kinetics of the GFP fluorescence or OD_{600} values are shown and depicted by the left or right y-axis, respectively. The data are means and standard deviations of technical triplicates and representative of three independent measurements.

Figure S8

11

Fig. S8 (overleaf)**.** Gene expression kinetics of *L. pneumophila* JR32 in AYE broth and in amoeba-adherent bacteria in biofilms using dual promoter reporters.

L. pneumophila JR32 harboring (**A**) P_{flaA}-*gfp* (pSN7) or (**B**) P_{6SRNA}-*gfp* (pSN25) reporter constructs were grown at 37°C in AYE medium within microplates while orbitally shaking. GFP fluorescence (relative fluorescence units, RFU) at a gain of 50 and optical density at 600 nm (OD_{600}) were monitored over time using a microplate reader. The kinetics of the GFP fluorescence (left y-axis) or OD_{600} (right y-axis) values are shown. The data are means and standard deviations of technical triplicates and representative of three independent measurements. (**C**, **D**) Biofilms of *L. pneumophila* JR32, Δ*lvbR* or Δ*lqsR* harboring dual reporter plasmids expressing P_{tac}-*mcherry* (constitutive) and P_{flaA}-*gfp* (pSN7) or P_{6SRNA}-*gfp* (pSN25) were grown in AYE medium for 6 days. *A. castellanii* was added to preformed biofilms, and confocal microcopy images of amoebae with adherent bacterial clusters were acquired close to the bottom of microscopy dishes. The images shown are representative of 2-3 independent experiments. Scale bars, 30 μm.

Supplementary Table

Table S1. Oligonucleotides used in this study.

Oligonucleotide	Sequence 5' - 3' [a]	Comments
oRH040	<u>CTTCCAGATGTATGCTCTTCT</u>TTATGAGGCTGAGTTTTTAG	P*lvbR*-*lvbR* (re) (into *Bsp*QI-site)
oRH041	<u>CTTGCTGGCGTTCGGG</u>GCGTGCTGATTGGTCC	P*lvbR*-*lvbR* (fo) (into *Bsp*QI-site)
oRH128	<u>GGAAACAGAATTCGAGCT</u>CGTCTGGGATTTATAAAGATAAATTGC	P*csrA* (fo) (into *Sac*I- and *Xba*I-site)
oRH129	<u>CATATGTATATCTCCTTCTTAAATCTAG</u>AGACTAAAATCTCCCTAACATAGC	P*csrA* (re) (into *Sac*I- and *Xba*I-site)
oRH174	<u>TGATCCGTCGATCGCCCGGG</u>GATCCCTATCGACCTAACAAAGATAATACAGATTGCG	P*flaA*-*flaA* (fo) (into *Bam*HI-site)
oRH175	<u>CCGCATTACGCGTCTCGAG</u>GATCCACCAGAACATTTAACCCATAAACCTTCC	P*flaA*-*flaA* (re) (into *Bam*HI-site)
oRH180	<u>CCGGGCATATGATCGATG</u>GATCCGGTATTCATCTTCCTCAAACG	P*ralF* (fo) (into *Bam*HI- and *Bgl*II-site)
oRH182	<u>CCGGGCATATGATCGATG</u>GATCCGTCTGGGATTTATAAAGATAAATTGC	P*csrA* (fo) (into *Bam*HI- and *Bgl*II-site)
oRH210	<u>GTGAAAAGTTCTTCTCCTTTAGATCTTTTC</u>ATGACTAAAATCTCCCTAACATAGC	P*csrA* (re) (into *Bam*HI- and *Bgl*II-site)
oRH211	<u>GTGAAAAGTTCTTCTCCTTTAGATCTTTTC</u>ATAATCTGCTCCTTTAGTCTGAG	P*ralF* (re) (into *Bam*HI- and *Bgl*II-site)
oRH215	<u>GGAAACAGAATTCGAGCT</u>CCATGGCCTGGCTCCTCTC	P*6SRNA* (fo) (into *Sac*I- and *Xba*I-site)
oRH216	<u>CATATGTATATCTCCTTCTTAAATCTAG</u>ACTGGCCTCCAAAATTGTACTGTTG	P*6SRNA* (re) (into *Sac*I- and *Xba*I-site)
oRH217	<u>CCGGGCATATGATCGATG</u>GATCCCGGGAACTCAATAAATCAAC	P*sidC* (fo) (into *Bam*HI- and *Bgl*II-site)
oRH218	<u>GTGAAAAGTTCTTCTCCTTTA</u>GATCTTTTCATTTTCTAGTGTCTTGTTTATAAG	P*sidC* (re) (into *Bam*HI- and *Bgl*II-site)

[a] Regions overlapping with destination vector are underlined.