Symplasmata are a clonal, conditional, and reversible type of bacterial multicellularity

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Microorganisms are capable of remarkable social behaviours, such as forming transient multicellular assemblages with properties and adaptive abilities exceeding those of individual cells. Here, we report on the formation and structure of genets known as symplasmata produced by *Pantoea eucalypti* bacteria. Each symplasmatum develops clonally and stochastically from a single bacterium into a membrane-delimited, capsule-embedded cluster of progeny cells and with a frequency that depends on temperature, pH, and nutrient availability. Transposon mutagenesis identified several gene products required for symplasmata formation, including master regulator LrhA, replication inhibitor CspD, polysaccharide transporter RfbX3, and autoinducer synthase PhzI. We also show that bacteria inside symplasmata are shaped irregularly with punctuated cell-to-cell contacts, metabolically responsive to environmental stimuli, dispersal-ready, and transcriptionally reprogrammed to anticipate multiple alternative futures in terms of carbon source availability. The structured and conditionable nature of symplasmata offers exciting prospects towards a mechanistic understanding of multicellular behaviours and their ecological significance.

Many examples of multicellularity exist among bacteria1–3, and typically these represent specialized or obligate lifestyles. In this study, we focus on a facultative and transient form of multicellularity observed in bacteria belonging to the genus *Pantoea*. This genus (Family: Enterobacteriaceae) comprises bacteria that can be isolated from a wide variety of environments (soil, water, hospitals) and hosts (insects, animals, plants)4,5. The type species of the genus is *Pantoea agglomerans* (*P. agglomerans*). The epithet ‘agglomerans’ (‘forming into a ball’) dates back to Beijerinck’s original description of the species (as *Bacillus agglomerans*) in 1888 and likely refers to the ability of *P. agglomerans* strains to form multicellular clusters known as symplasmata, also described as zoogloea, sausage forms, caterpillar formations (“raupchenartige Bildungen”), and “Bakterien-verbanden”8–12. The taxonomic history of *Pantoea* isolates is complex4, and in recent years several new *Pantoea* species have been proposed5. As a consequence, many strains previously known as *P. agglomerans* (and capable of forming symplasmata) are now referred to by newer species names, for example *P. eucalypti* 299R (formerly known as *P. agglomerans* 299R and before that *Erwinia herbicola* 299R13–18), *P. vagans* C9-119 (once known as *P. agglomerans* C9-120) and *P. anthophila* Sc1 (previously known as *P. agglomerans* Sc1).

The ecological role and evolutionary beginnings of symplasmata formation in *Pantoea* bacteria remain unclear. There have been reports that these structures may confer tolerance to stresses such as acids, heavy metals and UV21. For plant-colonizing *P. agglomerans* strains, symplasmata have been directly observed on the surfaces of roots and leaves, as well as within plant tissues21–28. Despite the fact that symplasmata have been known for more than a century, our understanding of the genes that underlie their formation is scarce, which limits our ability to formulate and test hypotheses on the ecology and evolution of symplasmata formation. Here, we present the results from a study on symplasmata formation in *P. eucalypti* 299R (*Pe299R*), which is a model strain for the study of bacterial colonization of the phyllosphere, i.e. plant leaf surfaces14,29,30. We offer an in-depth analysis of the formation, structure, and genetics of *Pe299R* symplasmata and discuss in more general terms the formation of symplasmata as a survival strategy in changing and unpredictable environments.

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In our laboratory, we have observed symplasma formation by *P. eucalypti* 299R in liquid cultures, on agar plates, and on the leaf surfaces of green bean plants. Typically, each symplasmatum contained several to hundreds of bacterial cells inside a shared capsule that is up to 20 micrometers thick as visualized by ink counterstaining or transmission electron microscopy (Figs 1 and 2). Capsule material was composed of a glycoconjugate containing sugars (most notably rhamnose, glucose and galactose), uronic acids, glycerol, and inositol (Supplementary Table S1, online). Incubation with metaperiodate, a chemical that digests polysaccharides, removed the capsule, but did not release cells from the clusters (Fig. 1a). Capsular material was also removed during preparation for scanning electron microscopy, leaving a characteristic 'void space' where capsules would be expected (Fig. 2c) and showing the symplasma as spheroids or allantoids with a membrane-like smooth surface (Fig. 2b–d) that proved resistant to treatment with proteinase K (not shown) or staining with a lipophilic fluorescent dye (Supplementary Fig. S1). Occasionally, this membrane ruptured and curled, offering a view of the bacteria inside the symplasmatum (Fig. 2d). Transmission electron microscopy showed the membranes as electron-dense and oftentimes stacked layers (Fig. 2e,f). Bacteria within each symplasmatum displayed an irregular shape and punctuated cell-to-cell contact with neighboring bacteria (Fig. 2f).

In liquid media, symplasma formation varied with culture conditions. During exponential growth (μ = 0.64 ± 0.02 hr⁻¹) in minimal medium with glucose, at pH 7.2, 30 °C and with shaking, the total number of symplasmata increased exponentially (Fig. 3a). The ratio of symplasmata to solitary cells was relatively constant at about 1 to 200, with symplasma appearing at a rate of 0.003 ± 0.001 hr⁻¹. Similar rates were observed with galactose or fructose as sole carbon source, although fructose led to slightly lower symplasma concentrations (Fig. 3b). Addition of casamino acids (hydrolysate of casein) to the medium significantly reduced symplasma concentration (Fig. 3c). Lowering the pH to 6.0 had little effect. However, at pH 7.8 symplasma formation was almost totally abolished (Fig. 3d). We did not observe symplasmata in cultures grown in rich medium (Lysogeny Broth) or at 37 °C (data not shown). These results confirm that symplasma formation is a conditional phenotype influenced by nutrient and physicochemical factors. Cells inside symplasmata remained responsive to stimulation with chemical signals in the medium (Supplementary Fig. S2) and they were able to replicate upon release from the cluster (Fig. 4). Bacteria typically left symplasmata in rapid bursts and immediately started to multiply (Fig. 4, Supplementary Movie S1), demonstrating the reversible nature of symplasmata and the return of cells to a free-living lifestyle.

The ability to form symplasma was abolished, reduced, or otherwise altered in several transposon insertion mutants of *Pc299R* (Fig. 5, Supplementary Table S2). From a library of 4,981 mutants, we identified three independent transposon insertions in the *lrhA* gene (NCBI gene locus_tag: F385_RS17575) which were linked to complete loss of clustering phenotype, but could be restored by full gene complementation (Supplementary Fig. S3). The *lrhA* gene encodes the LysR-type regulatory protein LrhA. This protein is conserved among *Enterobacteriaceae* as a transcriptional repressor of *flhDC*, which codes for the master regulator of flagellar, motility and chemotaxis genes. Consistent with this, the *lrhA* mutants of *Pc299R* exhibited increased motility on soft agar (Supplementary Fig. S4) and elevated expression of genes belonging to the *flhDC* regulon, including *fli*, *flg*, *che*, *mot*, *fli*, *tsr*, and *aer* genes (see Supplementary Table S3). In *E. coli*, LrhA also stimulates the rate of phase variation to a pilus-mediated adhesive phenotype. In the *Pc299R* *lrhA* mutant, the fimbriae assembly genes *pilB* and *pilC* were indeed downregulated compared to wildtype (Supplementary Table S3). Phase variation is a mechanism that allows bacteria to introduce phenotypic heterogeneity into a population of genotypically identical cells. Such a mechanism may explain the co-existence of solitary cells and symplasmata in culture.

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**Results and Discussion**

**Figure 1. Symplasmata formation by *Pantoea eucalypti* 299R.** (a) Top: phase-contrast image showing a pair of symplasmata amidst single cells. Bottom: counter-staining with ink reveals a capsule surrounding the cells clusters (left) which is gone after one hour incubation with sodium metaperiodate (right). Bars 10 μm. (b) Merged fluorescent image of a mixed culture of *P. eucalypti* cells expressing either GFP (pseudo-colored green) or DsRed (pseudo-colored pink). Each symplasmatum contains cells of one color only. Bar 20 μm. The bacteria shown in panels a and b were taken from liquid cultures growing on M9 plus glucose.
It also has been reported that LrhA exerts quorum sensing-dependent control over capsule production and motility in the plant pathogen *Pantoea stewartii* subsp. *stewartii*. Other genes that were differentially expressed in the *lrhA* mutant (compared to wildtype) were *yfiA* (coding for a ribosome hibernation protein), *spoVR* (sporulation family protein), *pflB* (pyruvate formate lyase), *focA* (formate efflux transporter), *cydAB* (cytochrome d), and *yqhE* (also known as *dkgA*) (Supplementary Table S3). Expression of three of these genes (*pflB*, *focA* and *cydB*) has been reported to increase under anaerobiosis or micro-aerobiosis, which aligns with the notion that cells inside symplasmata experience conditions of reduced oxygen levels.

Transposon insertions in the *cspD* gene (locus_tag: F385_RS03285) or in the *rfbX3* gene (locus_tag: F385_RS21230) reduced or altered but did not abolish symplasmata formation (Fig. 5, Supplementary Table S2). In *E. coli*, CspD is a stationary-phase, replication inhibition protein that plays a role in the formation of bacterial persisters, i.e. cells that go quiescent to survive stresses but can regrow under favorable conditions. The *rfbX3* gene codes for a predicted Wzx-like inner-membrane transporter of polysaccharidal O-antigens. Functional restoration of the *rfbX3* mutant required both *rfbX3* and its downstream gene which is predicted to code for a glycosyltransferase (Supplementary Fig. S3). In other mutants, Tn5 insertion sites could be mapped to *phzI* (coding for an N-acyl-homoserine lactone (AHL) synthase; locus_tag: F385_RS04930), *gshA* (gamma-glutamylcysteine synthase, involved in glutathione biosynthesis; locus_tag: F385_RS02625), *yhdP* (sugar transporter; locus_tag: F385_RS20315), and *rodA* (cell-shape determining protein; locus_tag: F385_RS16900). The apparent requirement of *phzI* for symplasmata formation in *P. eucalypti* suggests a role for quorum sensing. AHLs have previously been linked to production of capsular exopolysaccharides in closely related species such as *Pantoea ananatis* and *P. stewartii*, and have recently been shown to play a role in symplasmata formation by *P. agglomerans* YS1927. Complementation of the *phzI* mutant with a full-length *phzI* gene and its native promoter restored symplasmata formation to wildtype levels (Supplementary Fig. S3). We were unable to restore symplasmata formation in the *gshA* and *cspD* mutants by full gene complementation (data not shown).

Using a *gfp* reporter gene, we constructed transcriptional fusions to study the expression of *lrhA*, *rfbX3*, *cspD*, *phzI* and *gshA* in *Pe299R* (Fig. 6). Each reporter fusion was carried on a separate plasmid (p*lrhA*-gfp, p*RfbX3*-gfp, p*CspD*-gfp, p*PhzI*-gfp and p*GshA*-gfp, see Supplementary Table S6), and GFP fluorescence intensity was assessed in individual cells by fluorescence microscopy and flow cytometry. All reporter fusions showed fluorescence intensities above the background signal of the control plasmid (empty vector), with the *lrhA*-gfp fusion producing the highest intensity level (Fig. 6a). This supports the view that in *Pe299R* these symplasmata-associated genes are constitutively expressed during growth in minimal medium with glucose, albeit at levels that vary greatly. Interestingly, the variation in GFP expression among individual bacteria was highest with the *rfbX3*-gfp fusion.

**Figure 2.** Scanning (a–d) and transmission (e–f) electron micrographs of single cells (a) and symplasmata (b–f) formed by *Pantoea eucalypti*. (b,c) Symplasmata at an early developmental stage. White arrowhead indicates an area of exclusion where a capsule would be expected but was removed during preparation for electron microscopy. (d) Close-up of a capsule-stripped mature symplasmum revealing a distinct membrane-like layer keeping together the cells inside. (e) Cross-sectional view of symplasmata with packed cells and capsular matrix. (f) Close-up of a symplasmum cross-section. Arrowhead indicates the membrane-like layer holding the cells together, while white circles point to two of the cell-to-cell contact points. The bacteria shown in panels a–f were taken from liquid cultures growing on M9 plus glucose.
(as shown by the error bars in Fig. 6a), and coincided with the appearance over time of a subpopulation of cells showing elevated GFP expression (Fig. 6b). Using fluorescence microscopy, we showed that GFP expression from the rfbX3-gfp fusion was induced only in cells within Pe299R symplasmata, and not in solitary cells (Fig. 6c,d), which is evidence that rfbX3 is specifically expressed in symplasmata. It also appears that activation of rfbX3 is an important event in the formation of symplasmata and occurs early on in this process (Supplementary Fig. S5). RNA-Seq analysis showed that rfbX3 was downregulated (p-value = 0.024) in the lrhA mutant (which cannot form symplasmata) compared to wildtype. Expression of rfbX3 was observed frequently in stringed groups of 2, 4, 8, or 16 cells, which suggests synchronized cell division during the very early stages of symplasmata formation (Supplementary Fig. S5). Starting at the 8-cell stage, this synchrony appeared to break down and cell packing started to look more irregular, as it does in mature symplasmata. This notion of symplasmata as ‘genets’, i.e. groups of genetically identical individuals, growing together and originating from a single ancestor, is consistent with the observation that in mixed cultures of red and green fluorescent Pe299R, individual clusters never consisted of

Figure 3. Concentration of Pantoea eucalypti symplasmata in liquid cultures. (a) Total number of symplasmata against total number of bacterial cells in minimal medium with 0.4% glucose. Diamonds, squares and triangles represent three biological replicate experiments. (b) Symplasmata concentration in mid-log phase cultures with different carbon sources (0.4%). Error bar is one standard deviation calculated from triplicate measurements. (c) Symplasmata concentration after 24 hours of incubation in minimal medium with 0.4% glucose in the presence (M9GCA) or absence (M9G) of 0.2% casamino acids. Data shown represent the mean of independent biological replicates in flasks (M9G: 8; M9GCA: 5). Error bars represent one standard deviation. (d) Symplasmata concentration after 24 hours of incubation in M9G set with various pH (results from one experiment are shown).
differently colored cells (Fig. 1b), confirming the hypothesis of Achouak et al.\textsuperscript{22} that each symplasmatum is clonal, i.e. derived from a single bacterial progenitor.

The transcriptional profile of cells within symplasmata was markedly different from that of solitary cells, as revealed by reporter gene fusions (Fig. 6) and RNA-Seq analysis (Supplementary Fig. S6, Supplementary Table S4). Cells in symplasmata showed 343 differentially expressed genes (p-value < 0.05) compared to single cells, which corresponded to 8% of \textit{Pe}299R genes, of which half were upregulated and half were downregulated. Many of the genes that were upregulated (≥5-fold, p-value < 0.05) code for structural or regulatory proteins involved in the uptake or degradation of carbon substrates, including galactose \textit{(mglB, mglA, mglD)}, galactofuranose/pyranose \textit{(ytfQ)}, rhamnose \textit{(rhaT, rhaS)}, arabinose \textit{(araC)}, ribose \textit{(rbsK)}, maltose \textit{(malE)}, glucuronate/galactonate \textit{(uxuA)}, glycerol 3-phosphate \textit{(glpT)}, glycerol \textit{(glpF)}, inositol \textit{(ioiD, ioiI)}, and fatty acids \textit{(fadL, fadI, fadD)}. Interestingly, many of these compounds were also present in the symplasmatal capsule material (Supplementary Table S1), and most of the cognate genes are predicted to be regulated by the cAMP receptor protein (CRP)\textsuperscript{44,45}. In addition to genes involved in carbon uptake and metabolism, upregulated genes in symplasmata included \textit{cspD} and other stationary phase-inducible, CRP-dependent\textsuperscript{46} genes (e.g. \textit{csiE, csiA}), as well as \textit{ompR1}, which encodes a putative response regulator of unknown function that has 97% amino acid similarity with OmpR1 in \textit{Pantoea vagans} C9-1. We did not observe significant differences in the expression of other symplasmata-associated genes, differently colored cells (Fig. 1b), confirming the hypothesis of Achouak et al.\textsuperscript{22} that each symplasmatum is clonal, i.e. derived from a single bacterial progenitor.

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including rfbX3. This could indicate that rfbX3 expression is transient and mostly required during the early stages of symplasmata formation, as previously noted. Finally, the chemotaxis genes cheRBYZ were slightly downregulated in symplasmata compared to single cells, whereas they were highly upregulated in the lrhA mutant compared to wildtype (Supplementary Table S3 and S4).

While symplasmata formation by Pe299R on leaf surfaces was observed in a lab-controlled environment (not shown), we do not know whether this phenotype is expressed on plant leaves in nature. However, symplasmata formation was common among Pantoea strains (P. agglomerans and P. anthophila) isolated freshly from the leaf surfaces of field-grown and harvest-ready lettuce (Supplementary Table S5), so we can rule out that symplasmata formation is a trait resulting from the domestication of model bacteria like Pe299R. Reports of symplasmata formation in natural environments by other authors also support this view. Moreover, we observed symplasmata in lab cultures of the fireblight-control strain P. vagans (formerly agglomerans) C9-119,47.

The data we present here are consistent with a model of symplasmata formation in which a small subpopulation of solitary cells enters a developmental program that involves the ‘staying-together’ of each cell and its subsequent offspring. The precise mechanism that underlies this transition to multicellularity cannot presently be inferred, but it is responsive to environmental clues (e.g., pH, nutrients) and possibly entails LrhA-dependent, pilus-mediated aggregation. Part of the symplasmatal developmental program in P. eucalypti involves activation of rfbX3 and its downstream gene to generate an extracellular capsule that covers the cells within each symplasmatum. The glycosyl residues found in the extracellular polymeric substances produced by Pe299R share similarities with the chemical composition of stewartan and amylovoran, two exopolysaccharides produced by Pantoea and Erwinia species. Inside the capsule, Pe299R cells continue to multiply and eventually are starved for glucose and primed for utilization of other carbon sources, including those that are stored in the capsule. In E. coli, glucose-limiting conditions are known to induce the expression of multiple genes for the uptake of alternative carbon sources, offering the cells a fitness advantage upon presentation of those sources. Symplasmata formation might thus represent a possible bet-hedging strategy in which a subset of the population undergoes a multicellular-staged transcriptional reprogramming that prepares cells for multiple alternative futures. Such a strategy is likely to be advantageous to P. eucalypti in its native habitats, including the plant leaf surface, where nutritional conditions fluctuate both spatially and temporally. Achouak et al. showed that P. agglomerans NO30 forms symplasmata upon contact with the roots of its host plant (rice), but not wheat (non-host), which

Figure 6. Expression of symplasmata-associated genes in Pantoea eucalypti as measured by GFP reporter fusions. (a,b) Intensity of GFP fluorescence measured by flow cytometry in individual Pe299R bacteria carrying a reporter plasmid. (a) Histograms show the mean GFP fluorescence intensity (FL1-A) in individual bacteria after 8 hours of growth in M9G liquid cultures, expressed as arbitrary units and calculated from a sample of 10,000 cells; bars show results from two independent experiments; error bar indicates 1 SD. (b) Count histograms of Pe299R bacteria carrying the pRfbX3-gfp plasmid; we show the distribution of 100,000 cells after 0, 1, 5, 4, 6 and 8 hours of incubation. (c,d) Micrographs of Pe299R (pRfbX3-gfp) after 21 hours of growth on the surface of M9G agarose. A symplasmatum surrounded by single cells is shown. Phase contrast (c) and GFP fluorescence with pseudo-color green (d) are shown. Bar is 5 μm.
suggests that symplasmata formation can be modulated by plant interactions. It was suggested that the symplasmatal capsule improves attachment of *P. agglomerans* to rice roots\(^2\). We were able to corroborate this for *P. eucalypti* by showing that symplasmata were harder to remove from surfaces than solitary cells (Supplementary Fig. S7). Recently, it was shown that cells inside of symplasmata were more resistant to stress than solitary cells\(^21\). Taken together, these observations by us and those of others suggest that symplasmata may play different, but not mutually exclusive roles in the ecology of *P. eucalypti* and *P. agglomerans* in their native habitats.

We have described in this study the specific characteristics of symplasmata: clonal, conditional, reversible, but also stochastic and probably adaptive. In our view, this makes symplasmata formation an attractive phenotype to advance the study of bacterial multicellularity. The highly structured nature of symplasmata (i.e. cells on the inside, capsular material on the outside, separated by a membrane-like entity) fits the definition of patterned multicellularity\(^2\), with the membrane providing a clear boundary to what is ‘self’ and what is not\(^2\). The organization of cells within each symplasmatum also hints at a basic level of coordination. The possible involvement of bacterial cell-to-cell junctions in this coordination will require further scrutiny, particularly in light of the predicted advantages of resource exchange between cells inside of a cluster\(^25\). Our careful characterization of *P. eucalypti* symplasmata offers new insights into a century-old observation, and paves the way for exciting new studies on bacterial multicellularity and its ecological role in environmental habitats.

**Methods**

**Bacterial strains, culture conditions, and plasmid constructs.** Bacterial strains and plasmids used in this study are listed in Supplementary Table S6. Rifampicin (Rif)-resistant *Pantoea eucalypti* strain 299R\(^{26}\) (abbreviated here as Pe299R) and its kanamycin (Km)-resistant plasmid- or Tn5-derivatives (see below) were routinely grown in Lysogeny Broth (LB) with shaking at 275 rpm and 30 °C, or on LB agar plates at 28 °C. Final concentrations of Rif and Km were 20 and 50 μg per ml, respectively. Defined medium was M9\(^{56}\) supplemented with 0.4% glucose, without (M9G) or with (M9GCA) 0.2% casamino acids (casein hydrolysate) (Fischer Bioreagents, Fair Lawn, USA). Strains Pe299R (pFRU48) and Pe299R (pFRU97) constitutively express green and red fluorescent protein, respectively\(^29\), and were used to demonstrate the clonal nature of symplasmata as described in the Supplementary Methods.

**Transposon mutagenesis, mutant analysis, and reporter gene fusions.** A library of Pe299R transposon mutants was generated with the EZTn5 transposome system (Epicentre, Madison, USA). Individual colonies were screened by low-magnification microscopy for impaired symplasmata formation on M9 glucose agar plates (see Supplementary Methods online for details). For selected mutants, transposon-flanking DNA was sequenced, mapped to the *P. eucalypti* genome (Genbank accession ANKX00000000), and used for gene complementation assays, as described in Supplementary Methods. For several candidate genes, we constructed transcriptional reporter fusions by cloning DNA fragments encompassing ~100 bp downstream of the start codon as well as the presumed operator/promoter region (300–600 bp upstream of the start codon). We used the following primer pairs for the genes lrhA, rfbX3, cpsD, gshA, and phzI, respectively (amplicon size indicated in brackets): lrhA-for2/lrhA-rev2 (670 bp); pst-for2/pst-rev2 (710 bp); cpsD-for1/cspD-rev2 (640 bp); yqAB-for1/gshA-rev1 (569 bp); phzR-for2/phzI-rev1 (443 bp) (Supplementary Table S7). Amplicons were digested with restriction enzymes EcoRI (or SacI, or BamHI) and XhoI and inserted into vector pPROBE'-gfp[tagless] or pPROBE'-gfp[AAV] (Table S5) cut with enzymes EcoRI (or SacI, or BamHI) and SaII. The ligation product, which confers resistance to kanamycin, was introduced into *E. coli* TOP10 cells by heat-shock, and the transformants were selected on LB Km agar plates. Plasmids were extracted and transformed into Pe299R by electroporation. For reporter analyses, Pe299R carrying the empty vector pPROBE'-gfp or one of the reporter plasmids (Supplementary Table S6) was grown overnight in 3 ml LB Km. Five ml of fresh LB with antibiotics was inoculated with 1/200 of the o/n culture and incubated at 30 °C with shaking until cultures reached mid-exponential phase (2–3 hours). Cells were harvested at 2,500 g for 10 min, washed twice with M9, and diluted in M9 to an OD\(_{600}\) of 0.2. In a 250 ml-steel flask, 20 ml of M9G Km was inoculated with 200 μl of this cell suspension and incubated at 30 °C with shaking. After 0, 90, 180, 240, 360 and 480 min, the flask was sampled to measure GFP fluorescence in individual bacteria (see Supplementary Methods online for details).

**Quantification of symplasmata concentration.** From overnight cultures of Pe299R and mutants grown in LB medium with appropriate antibiotics, 25 μl was inoculated into 5 ml of fresh LB and incubated for 3 hours at 30 °C with shaking. Cells were harvested by centrifugation at 2,500 g for 10 min, washed twice with M9 devoid of carbon source and resuspended in the same medium to an OD\(_{600}\) of 0.2. From this bacterial suspension, 400 μl of this cell suspension was added to 20 ml of M9G in a 250 ml-flask, and incubated at 30 °C with shaking. After various periods of incubation, a sample from the culture was mixed 1:40 with 50 mM sodium acetate at pH 5.2 in the presence or absence of 10 mM sodium metaperiodate (Sigma-Aldrich). After one hour of incubation at 37 °C, the clusters were analyzed by mixing with Indian ink and microscopy, as described above.

**Chemical treatment of symplasmata.** After centrifugation of an overnight culture of Pe299R in M9G at 2,500 g for 5 min, the top of the cell pellet (enriched for symplasmata) was collected by pipetting and mixed with 50 mM sodium acetate at pH 5.2 in the presence or absence of 10 mM sodium metaperiodate (Sigma-Aldrich). After one hour of incubation at 37 °C, the clusters were analyzed by mixing with Indian ink and microscopy, as described above.
**Electron microscopy.** For scanning electron microscopy, 1 mL of an overnight M9G culture of Pe299R was harvested by centrifugation at 2,500 g for 5 min. After removal of the supernatant by pipetting, Karnovsky fixative was added (a volume approximately the size of the pellet) and the sample was incubated overnight at 4 °C. Further sample preparation was done by the Electron Microscopy Laboratory of the Department of Pathology and Laboratory Medicine, School of Medicine, University of California at Davis. In short, the bacterial pellet was rinsed in 0.1 M Sørensen’s buffer prior to application to coverslips coated with polylysine. After one hour of settling, cells were rinsed again with buffer, then dehydrated in increasing concentrations of ethanol. Coverslips were dried in a Tousimis 931 GL critical point dryer (Tousimis Research Corporation, Rockville, USA), mounted on specimen support stubs using carbon tape, and gold-coated using a Pelco SC-7 sputter coater (Ted Pella, Inc., Redding, USA). Samples were examined using a XL30 TMP microscope (Philips, Eindhoven, Netherlands). For transmission electron microscopy, 20 mL of an overnight M9G culture of Pe299R was passed three times through the same nylon net filter with an 11 μm pore size (Millipore, Hayward, USA). The filter was transferred to a 15 mL Falcon tube containing 2 mL of PBS, and the bacteria were resuspended by vortexing. This suspension was centrifuged at 2,500 g for 5 min, supernatant was removed, 100 μl of Karnovsky fixative was added, and cells were stored at 4 °C until further processing by the Electron Microscopy Laboratory. Briefly, the pellet was rinsed in 0.1 M phosphate buffer, then fixed with a solution of 1% osmium in phosphate buffer for one hour. The sample was dehydrated by serial incubation in solutions with increasing concentration of acetone. Resin was infiltrated in the sample and polymerized at 70 °C for 90 min. Ultrathin sections were cut using an ultramicrotome, prepared and analyzed with a CM120 electron microscope (Philips) and a MegaScan 795 camera (Gatan, Pleasanton, USA).

**RNA extractions and RNA-Seq analysis.** Pe299R wildtype and lrhA::Tn5 strains were grown overnight in 5 mL of LB Rif at 30 °C with shaking. Five mL of fresh LB with the same antibiotic were inoculated with 1/200 of preculture and incubated under the same conditions. Bacterial cultures were harvested in mid-log phase after ~3 hours of incubation, by centrifugation at 2,500 g for 10 min. Supernatant was discarded, the pellet rinsed twice in 5 mL of M9GCA in a 250 mL glass flask, then resuspended in the same medium and diluted to an OD600 of 0.2. Twenty mL of M9GCA in a 250 mL glass flask were inoculated with 100 μl of bacterial suspension and incubated as previously. Once OD600 reached ~0.5 (~8 hours of incubation), cultures were passed three times through the same nylon net filter with an 11 μm pore size (Millipore). A 2 mL filtrate sample (corresponding to ~5 × 10^8 bacteria) was mixed well with 4 mL of RNA Protect solution (Qiagen) and kept at room temperature until further use, but for no longer than 30 min. In parallel, four 40 mL aliquots of M9G in 500 mL glass flasks were inoculated with 200 μl of overnight M9G culture of Pe299R and incubated under the same conditions. After ~13–15 hours of incubation, cultures were pooled together as one sample and polymerized at 70 °C for 90 min. Ultrathin sections were cut using an ultramicrotome, prepared and analyzed with a CM120 electron microscope (Philips) and a MegaScan 795 camera (Gatan, Pleasanton, USA).

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Author Contributions
R.T. and J.H.J.L. conceived the study and wrote the manuscript. R.T. performed the majority of experiments and analyzed the data.

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