Dialkylresorcinols as bacterial signaling molecules

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It is well recognized that bacteria communicate via small diffusible molecules, a process termed quorum sensing. The best understood quorum sensing systems are those that use acylated homoserine lactones (AHLs) for communication. The prototype of those systems consists of a LuxI-like AHL synthase and a cognate LuxR receptor that detects the signal. However, many proteobacteria possess LuxR receptors, yet lack any LuxI-type synthase, and thus these receptors are referred to as LuxR orphans or solos. In addition to the well-known AHLs, little is known about the signaling molecules that are sensed by LuxR solos. Here, we describe a novel cell–cell communication system in the insect and human pathogen *Photorhabdus asymbiotica*. We identified the LuxR homolog PauR to sense dialkylresorcinols (DARs) and cyclohexanediones (CHDs) instead of AHLs as signals. The DarABC synthesis pathway produces the molecules, and the entire system emerged as important for virulence. Moreover, we have analyzed more than 90 different *Photorhabdus* strains by HPLC/MS and showed that these DARs and CHDs are specific to the human pathogen *P. asymbiotica*. On the basis of genomic evidence, 116 other bacterial species are putative DAR producers, among them many human pathogens. Therefore, we discuss the possibility of DARs as novel and widespread bacterial signaling molecules and show that bacterial cell–cell communication goes far beyond AHL signaling in nature.

quorum sensing | cell–cell communication | LuxR solos | *Photorhabdus* | pathogenic bacteria

Bacterial communication via small diffusible molecules to mediate group-coordinated behavior, referred to as quorum sensing, is well recognized. The prototypical quorum sensing system of Gram-negative bacteria consists of a LuxI-like autoinducer synthase that produces acyl-homoserine lactones (AHLs) as signals and a LuxR-type receptor that detects the AHLs to control expression of specific genes (1). Usually, *luxI*/*luxR* pairs are genetically clustered; however, there are examples in which the *luxI*/*luxR* functional pairs are distantly located on the bacterial chromosome or on plasmids (2). The AHLs are synthesized by LuxI and are sensed by the cognate LuxR-type receptor when exceeding a threshold concentration. On AHL binding, LuxR binds to the promoter/operator regions of the target genes/operons, resulting in changes in gene expression in response to the number of cells, and has been shown to play an important role in virulence of animal and human pathogens (1). As *luxI* is usually included among the target genes, setting up a positive feedback loop between signal input and output, AHLs are also designated as autoinducers.

In addition to this prototypical arrangement, many proteobacteria have LuxR homologs with no cognate LuxI autoinducer synthase or possess additional LuxR homologs in addition to a functional LuxI/LuxR quorum sensing system (3). Those LuxR homologs are designated as LuxR orphans (4) or LuxR solos (2). LuxR solos have been found in AHL-producing as well as in non-AHL-producing bacteria. They might allow bacteria to respond to endogenous as well as exogenous signals produced by their neighbors, exemplified by SdiA of *Escherichia coli* and *Salmonella enterica*, detecting AHLs produced by other bacteria (5). LuxR-type proteins commonly have a modular domain organization consisting of a conserved C-terminal DNA-binding domain and an N-terminal signal-binding domain, which typically is an AHL domain in AHL sensors and is important for ligand binding (6). Recently, we described that the LuxR solo PluR of the insect pathogen *Photorhabdus luminescens* detects α-pyrones named photopyrones (PPYs) instead of AHLs as signals. The PPYs are produced by a ketosynthase-like enzyme named photopyrone synthase PpyS (7). Therefore, PluR was the first example of a LuxR solo detecting a non-AHL endogenous signal. It regulates expression of the *pcfABCDEF* operon, resulting in the production of *Photorhabdus* clumping factor (PCF) that contributes to the pathogenicity of the bacteria (7). Furthermore, the three known *Photorhabdus* species harbor an exceptionally high number of LuxR-type receptors potentially sensing as-yet-unknown signaling molecules and enable the study of their role in cell–cell communication (8,9). However, much of the existing data on quorum sensing in Gram-negative bacteria rely on AHL signaling, whereas our study expands the diversity of signaling molecules particularly used for cell–cell communication in animal and human pathogenic bacteria.

Results

The DarABC/PauR Pair Represents a Novel Quorum Sensing Circuit. The insect and human pathogen *P. asymbiotica* harbors a *pcf* locus that is highly homologous to that of the closely related insect pathogen *P. luminescens*. It consists of the *pcfABCDEF*

Significance

Bacteria can communicate with each other by small diffusible molecules, a process termed quorum sensing. Many bacteria use acylated homoserine lactones (AHLs) as signals, which are sensed by so-called LuxR-type receptors. With the photopyrones from the insect pathogenic bacterium *Photorhabdus luminescens*, we recently identified the first quorum sensing molecules different from AHLs that are sensed by a LuxR-type receptor. Here we describe the second novel quorum sensing molecule sensed by a LuxR-type receptor of *Photorhabdus* species, PauR of the human pathogen *Photorhabdus asymbiotica*. We demonstrate that *P. asymbiotica* communicates via dialkylresorcinols (DARs) and cyclohexanediones (CHDs). As the synthesis pathway is widespread, and often present in human pathogens, we discuss DARs and CHDs as novel and widespread signaling molecules.

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Data deposition: All DNA and protein sequences from *P. asymbiotica* PB68.1 and *P. asymbiotica* ATCC 43949 have been deposited in the GenBank database, www.ncbi.nlm.nih.gov/genbank/ [accession nos. KP258225 (ATCC 43949, PauR), KP258226 (ATCC 43949, PCF operon), KP258227 (ATCC 43949, DarABC), KP258228 (PB68.1, PauR), KP258229 (PB68.1, PCF operon), KP258230 (PB68.1, DarABC)].

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(*pau_04068-pau_04063*) operon and a neighboring gene encoding a LuxR solo we named PauR (Pau_04062). PauR shares 81% identity and 93% similarity with the LuxR solo PluR of *P. luminescens* (Fig. 1*A*). However, as a cognate signal synthase has been identified for PluR (PpyS) (7), it is disputable whether PluR can be still designated as a LuxR "solo." Therefore, we designate PluR, as well as PauR, not as LuxR solos at this stage

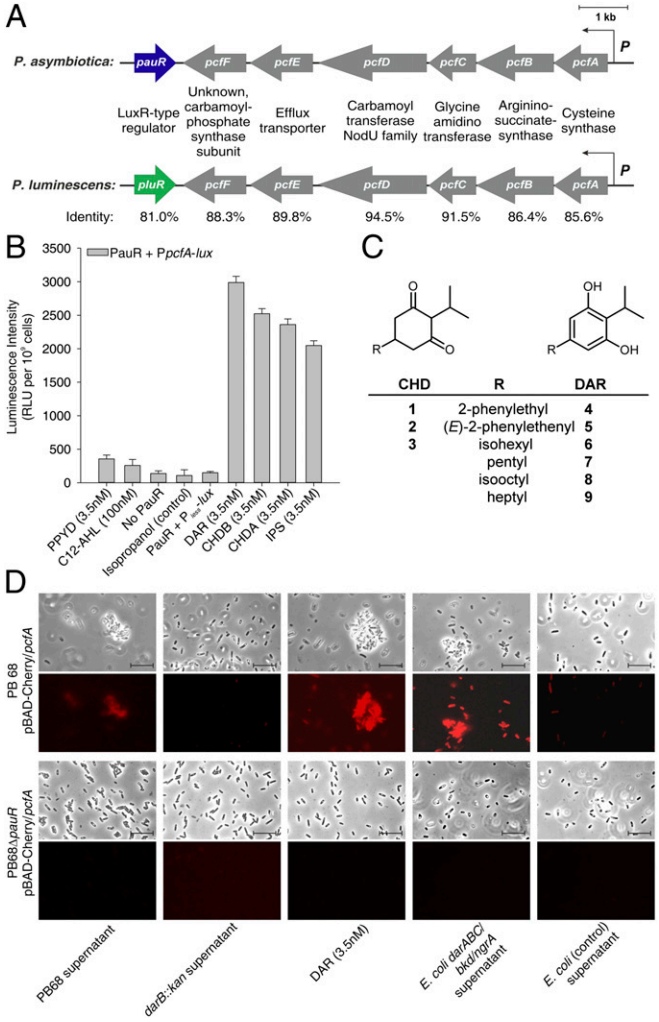

Fig. 1. The LuxR solo PauR specifically activates expression of the *pcfABCDEF* operon on induction with dialkylresorcinols. (*A*) Schematic presentation of the *pcf/pauR and pcf/pluR* locus of *P. asymbiotica* and *P. luminescens*, respectively. Identities of the respective protein sequences were generated by the ExPASy LALIGN Tool (embnet.vital-it.ch/software/LALIGN_form.html). (*B*) PauR specifically senses 2,5-dialkylresorcinol (DAR) and not photopyrones (PPYD) or acyl-homoserinelactones (C12-AHL). *E. coli* LMG194 strain harboring a P_{pcfA}-*luxCDABE* (P_{pcfA}-*lux*) fusion, as well as pBAD-*pauR*, were cultivated and exposed to different signaling molecules, PPYD 3.5 nM, 100 nM C12-AHL, isopropanol, 3.5 nM DAR (**6**), 3.5 nM CHDB (**3**), 3.5 nM CHDA (**1**), and 3.5 nM IPS (**5**), respectively. As controls, cells with no PauR or cells harboring a *luxCDABE* operon without a promoter (P_{less}) were used. Error bars represent SD of at least three independently performed experiments. RLU, relative light units. (*C*) Structures of known CHDs and DARs identified in *Photorhabdus* strains. (*D*) *P. asymbiotica* strain PB68.1 carrying plasmid pBAD-Cherry/*pcfA* from late stationary growth phase (P_{pcfA} promoter activity is almost off) was exposed to different extracts (PB68.1 supernatant, PB68.1 *darB::kan* supernatant, *E. coli* LMG194 expressing *darABC/bkd/ngrA*, and *E. coli* LMG194 harboring empty plasmids) or pure DAR (**6**) and then analyzed for fluorescence as well as cell clumping in the microscope. The figure represents one characteristic of at least three independently performed experiments.

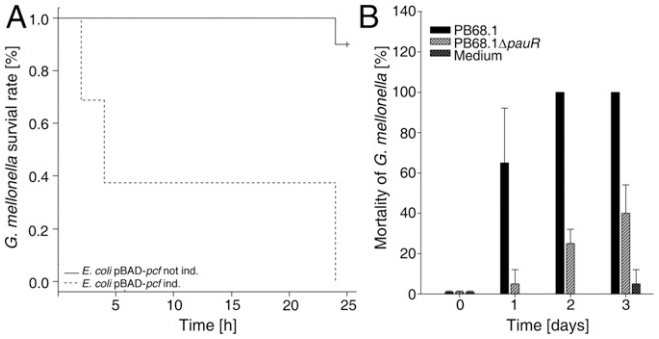

Fig. 2. The DarABC/PauR quorum sensing circuit is important for pathogenicity. (*A*) Pathogenicity of *E. coli* harboring plasmid pBAD-*pcfABCDEF*, induced with 0.2% (wt/vol) arabinose or not induced (control), against *G. mellonella*. Ten larvae were infected, and the number of dead animals was recorded at the points after infection, as indicated. The portion of surviving animals was plotted versus the time according to the logrank method (31); $P = 8.5 \times 10^{-6}$. (*B*) Pathogenicity of *P. asymbiotica* PB68.1 and PB68.1 Δ*pauR* against *G. mellonella*. Cells were diluted in CASO medium, and ~100 cells were injected in a volume of 10 μL into a *G. mellonella* last instar larva. Ten larvae were infected, and the number of dead animals was recorded at the points after infection, as indicated. The experiment was performed three times, and error bars represent the SDs.

but, instead, simply as LuxR-type receptors. Interestingly, the *P. asymbiotica* genome does not encode a LuxI synthase and also lacks a *ppyS* gene. Hence, *P. asymbiotica* produces neither any AHLs nor PPYs. To test whether PauR can nevertheless sense exogenous PPYs or AHLs, we used an *E. coli* reporter strain similar to that which was already successfully used for PPY sensor PluR (7), which carried promoter fusion of P_{pcfA} of *P. asymbiotica* with *luxCDABE* and PauR. Neither PPYs nor AHLs significantly induced PauR-mediated reporter gene expression (Fig. 1*B* and *SI Appendix*, Fig. S1). All *Photorhabdus* strains produce the dialkylresorcinol isopropylstilbene (compound **5**) (Fig. 1*C*), which is important for nematode development (10) and acts as antibiotic (11). However, HPLC/MS analysis of several different *Photorhabdus* strains revealed a specific subset of dialkylresorcinols (DARs) (compounds **4** and **6**–**9**; Fig. 1*C*) and their biochemical precursors cyclohexanediones (CHDs; compounds **1**–**3**; Fig. 1*C*), which are exclusively produced in *P. asymbiotica* (*SI Appendix*, Fig. S2) and synthesized by a pathway encoded by the *darABC* operon (10, 12). Analysis of the previously isolated main derivatives **1**, **3**, **5**, and **6** showed that all compounds were identified as specific inducers of PauR-mediated reporter gene activity (Fig. 1*B*). To elucidate whether DarABC/PauR is a cell–cell communication system, we used a *P. asymbiotica* P_{pcfA}-*mCherry* reporter strain and investigated growth phase-dependent reporter gene activity. P_{pcfA} activity was detectable in the early exponential growth phase and showed a significantly lower level in the late stationary growth phase (*SI Appendix*, Fig. S3). Furthermore, although *P. asymbiotica* is described as nonsusceptible to genetic manipulation in the literature (13), we were able to generate a Δ*pauR* deletion mutant in *P. asymbiotica* to test whether P_{pcfA} activation is PauR-dependent, which was indeed the case (Fig. 1*D* and *SI Appendix*, Fig. S3). Neither fluorescence nor cell clumping was observed when conditioned medium of a *P. asymbiotica darB::kan* strain was used, proving that the *darABC* operon is essential for PauR-dependent *pcfABCDEF* activation (Fig. 1*D* and *SI Appendix*, Fig. S4). In addition, heterologous overproduction of the *darABC* operon in an *E. coli* strain harboring the *bkd* operon and *ngrA* resulted in production of DAR and CHD, which were released into the supernatant. This *E. coli* culture fluid then induced fluorescence and cell clumping in the *P. asymbiotica* P_{pcfA}-*mCherry* reporter strain (Fig. 1*D*), revealing that DarABC/PauR constitutes a cell–cell communication circuit. This was not the case when using the

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Δ*pauR* P_{pcfA}-*mCherry* strain as reporter (Fig. 1*D*), demonstrating that PauR is essential as a DAR/CHD signal receptor. Detailed analysis of selected DARs and CHDs to induce P_{pcfA} activity revealed compound **6** (DAR) to be the most specific signal for PauR activation with a concentration as low as 3.5 nM (Fig. 1*D* and *SI Appendix*, Fig. S5).

Analysis of the cell density-dependent promoter activities of *pauR*, *pcfA*, and *darA* in the wild-type and the Δ*pauR* strain (*SI Appendix*, Fig. S6) revealed an increase of P_{pauR} activity in wild-type background in the exponential growth phase to reach a maximum in the stationary growth phase. Furthermore, a positive feedback regulation via PauR could be observed, indicating that DarABC/PauR constitutes a quorum sensing system, as P_{pauR} activity was dramatically decreased in the Δ*pauR* background. P_{pcfA} activity was maximal during the exponential growth phase and had a somewhat lower level in the stationary growth phase, which is comparable to the time-course of P_{pauR} activity. In contrast, P_{darA} activity remained basal in both wild-type and Δ*pauR* cells. Reconstitution of the *pcf* operon, PauR, and the DAR biosynthesis in *E. coli* confirmed the role of all parts of this signaling circuit (*SI Appendix*, Fig. S7). Furthermore, the toxicity of *E. coli* overproducing PCF against the greater waxmoth *Galleria mellonella* revealed that PCF is also a virulence factor in *P. asymbiotica*, comparable with the *P. luminescens* PCF homolog (7) (Fig. 2*A*). Moreover, we observed that *P. asymbiotica* Δ*pauR* was less virulent towards *G. mellonella* (Fig. 2*B*). As we did not observe reduced virulence of the *P. luminescens* Δ*pluR* strain compared with the wild-type strain (7), we conclude that PauR-mediated cell clumping might have a greater effect on insect pathogenicity in *P. asymbiotica* than PCF in *P. luminescens*.

Conserved Amino Acids in the Signal-Binding Domain of PauR Are Crucial for DAR Sensing. In general, the sequence identity of LuxR family members is quite low (18–25%), and as a consequence, the signal-binding domains of LuxR-type receptors (PFAM03472) are not highly conserved (Fig. 3*A*). Only six amino acids are classified as highly conserved in the AHL-binding domain of AHL sensors, which are Trp57, Tyr61, Asp70, Pro71, Trp85, and Gly113 (positions with respect to the AHL sensor TraR) (4, 14). The amino acids Trp57, Tyr61, and Asp70 (positions with respect to TraR) interact with the oxygen of the amide group and the carbonyl-oxygen of the lactone, as well as the amide group-nitrogen, respectively (15, 16). The observation that many LuxR homologs such as LuxR solos have substitutions of one or more of these six conserved amino acids originally prompted the idea that these proteins may sense signals other than AHLs, as recently demonstrated for PluR (7). In agreement with this observation, a sequence alignment of PauR and PluR against other receptors with homology to LuxR revealed that only two of six conserved amino acids (Tyr66, Asp75) are conserved in both proteins (Fig. 3*A*). As the third amino acid (Trp57 in TraR) that is involved in the AHL recognition is replaced with Thr in PluR, as well as PauR (position 62 in PluR and PauR, respectively), binding of a different signal molecule than AHL in both receptors seemed likely. To investigate a possible binding mode of DAR (**6**) in the active site of PauR, we generated a homology model of PauR based on the crystal structure of QscR from *P. aeruginosa* (17), which showed the highest sequence identity of available crystal structures to PauR (30.3%) (*SI Appendix*, Fig. S8). Docking experiments with this model using DAR (**6**) as ligand revealed a hydrogen bond formed between the DAR–hydroxy group and Asp75, as well as an arene–arene interaction between Thr62 and Tyr66 and the DAR aromatic ring (Fig. 3*B*). Replacement of Thr62 as well as Asp75 against Ala in PauR in fact reduced sensitivity of DAR (**6**) down to ~50%, respectively. Moreover, replacement of Tyr66 against Ala caused complete loss of DAR (**6**) sensing (Fig. 3*C*), supporting the predictions of the in silico docking experiments. The loss in PauR binding to DAR (**6**) could also be demonstrated in silico, using the built-in residue scan function of Molecular Operating Environment (MOE) 2012.10 (*SI Appendix*, Table S4).

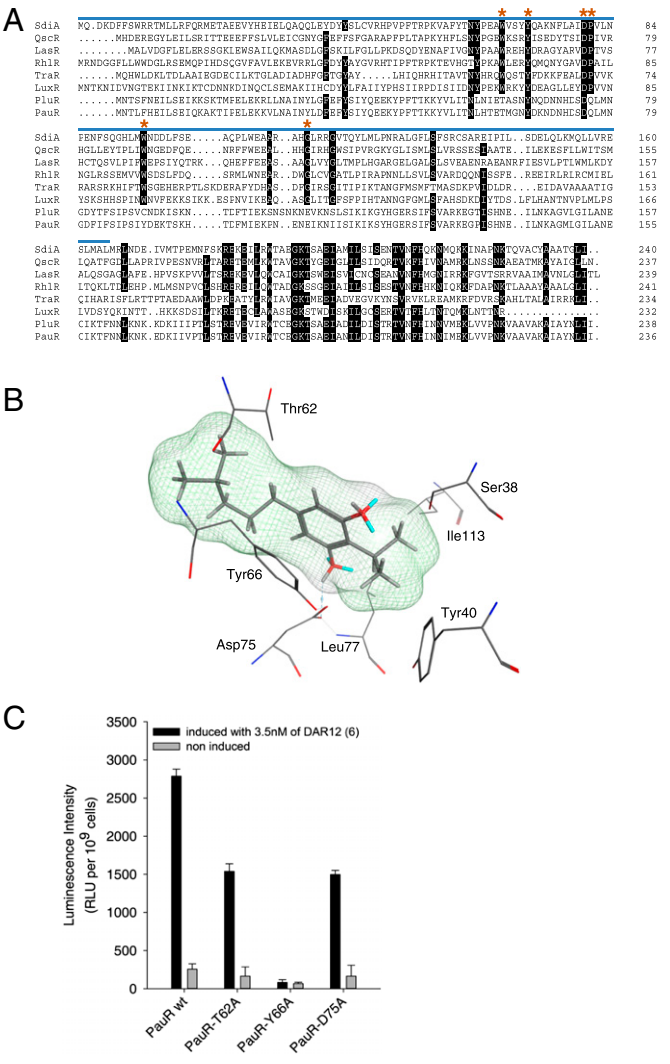

Fig. 3. Dialkylresorcinol binding by PauR. (*A*) Alignment of SdiA of *S. enterica* var. Typhimurium; QscR, LasR, and RhlR of *P. aeruginosa*; TraR of *Agrobacterium tumefaciens*; LuxR of *Vibrio fischeri*; PluR of *P. luminescens*; and PauR of *P. asymbiotica*. Highly conserved amino acids (75–100%) are highlighted in black. The AHL-domain ("autoind_bind," PFAM03472) is marked by the blue line. Amino acids involved in AHL-binding (Trp57, Tyr61, Asp70, Pro71, Trp85, and Gly113; positions numbered as in TraR) (4, 14, 19) are labeled with red asterisks. (*B*) A detailed view of the proposed PauR-binding pocket and the intermolecular interactions between the docked ligand **6** (DAR) and PauR. The cavity of the ligand is shown in a line representation, and possible hydrogen bonds are shown as dashed lines. (*C*) Influence of the amino acid replacements Thr62, Tyr66, and Asp75 against Ala in the signaling domain of PauR on sensing of DAR (**6**). *E. coli* LMG194 strains harboring a P_{pcfA}-*luxCDABE* (P_{pcfA}-*lux*) fusion and producing different PauR derivatives (PauR wild-type, PauR-T62A, PauR-Y66A, or PauR-D75A, respectively) were cultivated (non-induced) before 3.5 nM of **6** was added (induced). As controls, cells with no PauR or cells harboring a *luxCDABE* operon without a promoter (P_{less}) were used as shown in Fig. 1*B*. Error bars represent SD of at least three independently performed experiments. RLU, relative light units.

DAR-Dependent Quorum Sensing Systems in Other Pathogenic Bacteria. In this context, it is worth mentioning that the *darABC* operon responsible for the biosynthesis of DARs and CHDs has been detected in 116 bacteria (*SI Appendix*, Fig. S9 and *SI Appendix*, Table S5), several of which are pathogenic to humans, animals, or

plants (12). Whereas *darABC* forms an operon in *Photorhabdus* strains, in most bacteria, *darC* is separate from *darAB*, and in approximately one-third of the analyzed bacteria, *darA* and *darB* are also separated from each other (Fig. 4*A*). *Porphyromonas gingivalis* ATCC 33277 is the only organism encoding DarB but not DarA, pointing to the exclusive presence of CHDs, but not DARs, in this strain. A detailed search of all *darB*-containing genomes for *luxI* and *luxR* homologs revealed that 46% of the bacteria encoding *darABC* also encode *luxR* homologs; however, LuxI homologs are rare in these bacteria and are mainly found in pseudomonads. In addition, 48% of bacterial genomes analyzed encode neither any LuxI nor a LuxR homolog, although possessing *darB*, suggesting a role for CHDs and DARs in these organisms other than acting as a quorum sensing signal in combination with LuxR-type receptors (Fig. 4*B* and *SI Appendix*, Fig. S10 and Table S7). Because LuxR-type receptors are not highly conserved (4), it is impossible to define whether a PauR solo, that is a LuxR homolog sensing DARs/CHDs in bacteria that do not harbor DarABC, exists.

Discussion

Here we identified a novel quorum sensing circuit that uses DARs/CHDs as signaling molecules. Thus, our findings reveal novel bacterial signaling molecules that are sensed by a LuxR homologous receptor. The LuxR-homologous receptor PauR does not sense AHLs or PPYs but, instead, DARs and CHDs, which are endogenously produced in a specific subset by the insect and human pathogenic bacterium *P. asymbiotica*. Furthermore, PauR most specifically detects DAR, and on ligand binding, the expression of the adjacent *pcf* operon is activated, leading to cell clumping. PCF-derived cell clumping then contributes to the high pathogenicity of *P. asymbiotica* toward insect larvae (Fig. 5). Indeed, quorum sensing-regulated processes are often linked with pathogenicity; for example, in *Streptococcus pneumoniae* or *Vibrio cholerae*, via regulating the expression of virulence factors at high cell density (1).

PauR shows the typical domain structure of LuxR family proteins, consisting of a C-terminal DNA-binding domain and an N-terminal predicted AHL-binding domain. Recently, we have identified α-pyrones named PPYs as bacterial signaling molecules in *P. luminescens* used for quorum sensing, which are sensed by the LuxR homolog PluR (7). These two regulators, PauR and PluR, are highly homologous to each other; however, sensing different signaling molecules allows a species-specific cell–cell communication. *P. asymbiotica* is the only *Photorhabdus* species that is not only pathogenic toward insects but also toward humans. The use of DARs instead of PPYs as quorum sensing molecules might therefore be an important evolutionary step from invertebrate to human pathogenicity. Using quorum sensing molecules different from AHLs could be deeply rooted in the N-terminal signal-binding domain of PauR and PluR. In general, six conserved amino acids in signal-binding domain of AHL-sensing LuxR-type receptors are important for AHL binding. However, PauR and PluR only share two of them (Tyr66 and Asp75), which emerged as important for ligand sensing in all LuxR-type receptors (7, 9). Other than these subtle differences, PauR and PluR are very closely related to AHL-sensing LuxRs (*SI Appendix*, Fig. S11). In addition, the PauR-specific amino acid Thr62 in the signal-binding domain also emerged as important for DAR sensing. In addition to these, several other amino acids have been identified that shape the ligand pocket and are therefore crucial for signal-binding in AHL sensors (18). Moreover, a subfamily of LuxR-type proteins from plant-associated bacteria harbor substitutions within the six conserved amino acid motif in the signal-binding domain, suggesting the detection of plant-derived signal molecules, rather than AHLs (4, 19). Recently, these have been assumed to be part of a novel interkingdom signaling circuit and to be important for communication between plants and both pathogens and beneficial bacteria and might have undergone coevolution with the related host plant (20). This lends support to the idea that LuxR-homologous receptors have evolved to their distinct signaling molecules that are sensed for the adaptation to specific needs in different environments.

Unfortunately, the subtle differences of LuxR protein sequences, resulting in high specificities in ligand binding (Fig. 3*A* and *SI Appendix*, Fig. S11), hampers the correlation between DAR or CHD ligand specificity of PauR homologs identified in

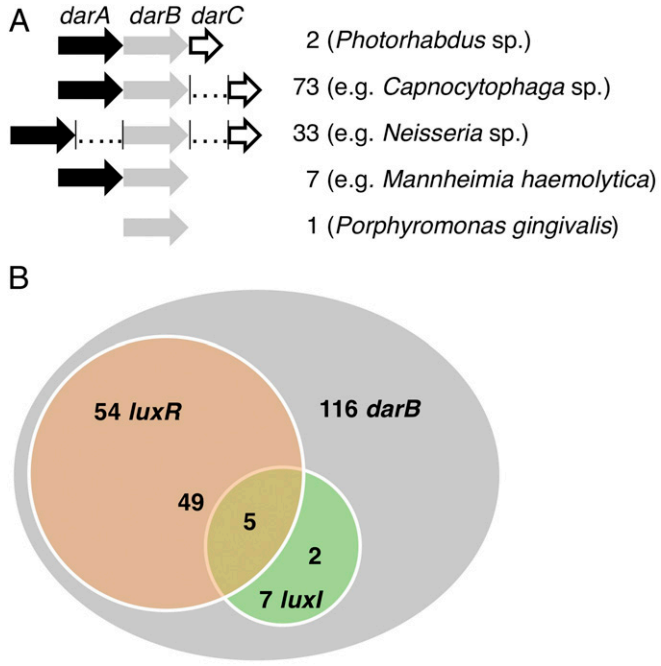

Fig. 4. Structure and assembly of *darB* containing operons (*A*) and correlation between *darB*, *luxR*, and *luxI* in *darB* encoding bacterial genomes (*B*).

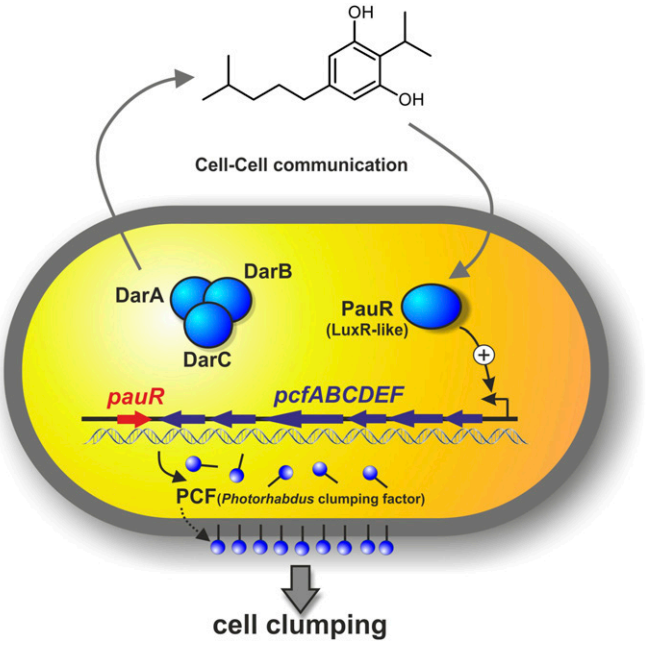

Fig. 5. Model of the DarABC/PauR mediated quorum sensing system in *P. asymbiotica*, using dialkylresorcinol **6** as a signaling molecule.

P. asymbiotica and other strains just based on protein sequences (*SI Appendix*, Fig. S11). Nevertheless, the high abundance of LuxR but low abundance of LuxI homologs in these bacteria (Fig. 4*B*) might indicate that the DarABC-derived compounds are indeed also used as signals in these bacteria. As among these bacteria are several important human, animal, and plant pathogens such as *Neisseria*, *Capnocytophaga*, or *Flavobacterium*, the role of these compounds for pathogenicity has to be explored in the future. CHD derivatives are known as natural products for 10 y (21) and have originally been described as pollinator attractant of *Chiloglottis* orchids as well as sex pheromones of its pollinator, the thynnine wasp *Neozeleboria cryptoides* (22). DAR derivatives have been described as antibiotics (10, 23), cytotoxins (24), free radical scavengers (25), and growth-stimulating factors (26) and can also be part of flexirubins and related pigments (12, 27, 28), which might be involved in protection against lipid peroxidation and photooxidative damage (29). Thus, especially in bacteria without LuxR homologs, DARs and CHDs might have additional functions other than acting as signals. Recently, a LuxI solo named SscI has been identified in the marine sponge symbiont *Ruegeria* sp. (30), revealing that similar to DARs, AHLs might fulfill functions other than acting as quorum sensing signal in those bacteria.

In summary, our finding adds a new chapter to bacterial cell–cell communication and shows that signaling via LuxR family regulators extends beyond AHL-derived quorum sensing. The fact that at least the biosynthesis gene clusters for DAR and CHD biosynthesis is widespread in different bacterial taxa (*SI Appendix*, Fig. S10), including several pathogenic species, might point to a wide distribution of these compounds, or even the underlying signaling circuits.

Materials and Methods

Strains and Plasmids. All strains used in this study are listed in *SI Appendix*, Table S1, the plasmids in *SI Appendix*, Table S2, and oligonucleotides in *SI Appendix*, Table S3. For details of strain and plasmid construction see *SI Appendix*.

Materials. All AHLs used in this study were purchased in reagent grade from Fluka (Deisenhofen). Kanamycin, ampicillin, gentamycin sulfate, and L-arabinose were obtained in reagent grade from Roth (Karlsruhe), and chloramphenicol was from Sigma-Aldrich (Deisenhofen). Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was purchased from AppliChem (Darmstadt). All other materials were reagent-grade and were obtained from commercial sources.

Promoter Activity Analyses. Fluorescence of *P. asymbiotica* mCherry reporter strains was investigated with a fluorescence microscope (Leica), using an excitation wavelength of 546 nm and a 605-nm suppression filter with 75-nm bandwidth, and quantified in an Infinite 500 Plate reader (Tecan) with an excitation wavelength of 560 nm (20-nm bandwidth) and an emission wavelength of 610 nm (20-nm bandwidth). The integration time was set to 20 μs, and the number of measurements was 10 for measurement of fluorescence and for optical density. Raw fluorescence data were normalized with the optical density (OD_{600}) of the respective culture. A colony of the respective *P. asymbiotica* strain (*pcfA*-promoter activity is turned off) carrying the pBAD-Cherry-*pcfA* reporter plasmid was suspended in 100 μL *P. asymbiotica* culture supernatant or Casein-Soya-Peptone (CASO) medium, respectively, with equal optical densities. HPLC-derived samples of culture supernatants were added to cells resuspended in CASO medium in a ratio of 1:100. Purified DARs/CHDs were added in concentrations of 3.5–350 nM. Cells were then incubated aerobically at 37 °C for 60 min before fluorescence was analyzed. Time courses of *pcfA*, *pauR*, or *darA* promoter activities were performed by cultivating *P. asymbiotica* PB68.1 or *P. asymbiotica* PB68.1 Δ*pauR* carrying plasmid pBAD-Cherry/*pcfA*, pBAD-Cherry/*pauR*, or pBAD-Cherry/*darA*, respectively, in 24-well micotiter plates at 37 °C and 130 × *g* in an Infinite 500 Reader (Tecan, Austria) for 50 h. Every 30 min, fluorescence and OD_{600} was determined and normalized as described earlier.

Specificity of PauR Sensing Toward Different Signaling Molecules. To quantify the specificity of the sensing of PauR toward distinct signaling molecules, *E. coli* LMG194 was transformed with plasmid pBAD24 containing *pauR* and a reporter plasmid carrying a *pcfA-luxCDABE* promoter fusion. The ability of PauR to activate *pcfA* promoter activity in the presence of different signaling molecules was measured via *luxCDABE* expression, and therefore luminescence as readout. Briefly, *E. coli* LMG194 carrying plasmids pBAD24-*pauR* and pBBR1-MCS5-TT-RBS-P*pcfA-lux* was cultivated overnight in M9 minimal medium at 37 °C. As controls, *E. coli* LMG194 carrying plasmids pBAD24-*pauR* and pBBR1-MCS5-TT-RBS-*lux* (promoter-less) and pBAD24 (empty plasmid) and pBBR1-MCS5-TT-RBS-P*pcfA-lux*, respectively, was used and cultivated overnight in M9 minimal medium at 37 °C. The overnight cultures were adjusted to an OD_{620} of 0.05 and then aerobically cultivated in 96-well plates at 37 °C. At an OD_{620} of 0.1, 3.5 nM of **6** (DAR) and 3.5 nM of photopyrone D (PPYD) or different AHLs in concentrations of 1, 10, and 100 nM (*N*-butyryl-DL-homoserinelactone, *N*-butyryl-DL-homocysteinthiolactone, N -3-oxo-hexanoyl-DL-homoserinelactone, *N*-octanoyl-DL-homoserinelactone, *N*-decanoyl-DL-homoserinelactone, *N*-dodecanoyl-DL-homoserinelactone, and *N*-tetradecanoyl-DL-homoserinelactone) was added. Isopropanol, used as solvent for PPYD, and ethylacetate, used as solvent for AHLs, were used as negative controls and added to the *E. coli* LMG194 cells harboring a P_{pcfA}-*luxCDABE* (P_{pcfA}-*lux*) fusion, as well as pBAD-*pauR*. Every hour, the OD_{620}, as well as the luminescence, was monitored in a Sunrise plate reader (Tecan) and a Centro luminometer (Berthold Technologies), respectively.

Cell Clumping Assay. To visualize cell clumping, the bacteria were analyzed by phase contrast microscopy. Briefly, *E. coli* LMG194 carrying plasmid pBAD24, pBAD-*pcfABCDEF*, or pBAD-*pcfABCDEF/pauR*, respectively, was cultivated overnight in M9 minimal medium, adjusted to an OD_{620} of 0.05, and then further cultivated. Expression of the *pcfABCDEF* operon was then induced by addition of 0.2% (wt/vol) arabinose or via the native promoter with 3.5 nM of **6** (DAR) at an OD_{620} of 0.5. After 1 h of further incubation at 37 °C under shaking with 200 × *g* in a Thermomixer (Eppendorf), 20 μL of each sample was analyzed for cell clumping in a DCI microscope (Zeiss), using phase contrast.

Effects of Amino Acid Replacements in PauR on DAR Sensing. To quantify the influence of amino acid replacements in the signaling domain of PauR, *E. coli* LMG194 was cultivated with plasmid pBAD24 containing *pauR* or *pauR* derivatives and a reporter plasmid carrying a *pcfA-luxCDABE* promoter fusion. The ability of PauR and its derivatives to activate *pcfA* promoter activity in the presence of **6** (DAR) was measured via *luxCDABE* expression, and therefore luminescence as readout. Briefly, *E. coli* LMG194 carrying plasmids pBAD24-*pauR* and pBBR1-MCS5-TT-RBS-P*pcfA-lux*, pBAD-*pauR*-T62A and pBBR1-MCS5-TT-RBS-P*pcfA-lux*, pBAD-*pauR*-Y66A and pBBR1-MCS5-TT-RBS-P*pcfA-lux*, or pBAD-*pauR*-D75A and pBBR1-MCS5-TT-RBS-P*pcfA-lux*, respectively, was cultivated overnight in M9 minimal medium. As controls, *E. coli* LMG194 carrying plasmids pBAD24-*pauR* and pBBR1-MCS5-TT-RBS-*lux* (promoter-less) and pBAD24 (empty plasmid) and pBBR1-MCS5-TT-RBS-P*pcfA-lux*, respectively, were used and cultivated over night in M9 minimal medium. The overnight cultures were adjusted to an OD_{620} of 0.05 and were then aerobically cultivated in 96-well plates at 37 °C. At an OD_{620} of 0.1, 3.5 nM of **6** (DAR) was added and the OD_{620} as well as the luminescence were monitored in a Sunrise plate reader (Tecan) and a Centro luminometer (Berthold Technologies), respectively.

Pathogenicity Bioassays. *G. mellonella* larvae (Terraristika Express) were surface sterilized in a 70% (vol/vol) ethanol bath followed by washing with sterile water and then incubated on ice for 10 min to reduce movements. Larvae were infected with cell suspensions by injection of 10 μL cell suspensions containing ∼100 *P. asymbiotica* cells or 4,000 *E. coli* cells, respectively, s.c., using a sterilized microsyringe (Hamilton 1702 RN, 25 μL), and incubated at room temperature. Mortality rate was determined by counting dead and alive animals after points indicated. Survival rate of *E. coli*-infected larvae was evaluated according to the log rank test (31).

Molecular Modeling of PauR and Docking. The protein sequence of PauR was loaded into the MOE 2012.10. Then a BLAST search was performed to find an appropriate template crystal structure (32). For homology modeling, the crystal structure coordinates of QscR cocrystalized with *N*-3-oxo-dodecanoyl-L-homoserine lactone from *Pseudomonas aeruginosa* (PDB ID code 3SZT) was used. The sequence identity of PauR with its reference structure QscR was 30.3%. To avoid deletions or insertions in conserved regions, the alignment was inspected and corrected manually if necessary. A series of 10 models was constructed with MOE, using a Boltzmann-weighted randomized procedure combined with specialized logic for the handling of sequence insertions and deletions (33, 34). The model with the best packing quality function was selected for full-energy minimization. MOE packing score for PauR was 2.2882798, using Merck Molecular Force Field 99 × (MMFF99X). The stereo-

chemical qualities of the model were assessed using Ramachandran plot: 4.2% outliner, 7.2% allowed, and 88.6% core.

Protein-ligand docking calculations were carried out using the program GOLD (version 5.1) (35), using the empirical scoring function for advanced protein-ligand docking CHEMPLP (36). The binding site of PauR was centered at D75, and the default docking parameters were used.

The virtual mutagenesis of PauR was carried out with the built-in residue scan function of MOE 2013.0802. The effect of the introduced mutations is then predicted by MOE, using the GBVI/WSA dG (37) scoring function to estimate the loss of affinity of the protein–ligand complex. The protein stability is predicted by MOE, using an energy equation to predict the change of stability of the wild-type and the mutant. To evaluate the residue scan function, the procedure was performed with the available crystal structure of QscR bound to *N*-3-oxo-dodecanoyl-homoserine lactone and the experimentally measured loss of affinity (17). The scores for ligand affinity and protein stability for the QscR derivatives are shown in *SI Appendix*, Table S4.

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